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# Impact of high dietary plant protein with or without marine ingredients in gut mucosa proteome of gilthead seabream (*Sparus aurata*, L.)


## Abstract

The digestive tract, particularly the intestine, represents one of the main sites of interactions with the environment, playing the gut mucosa a crucial role in the digestion and absorption of nutrients, and in the immune defence. Previous researches have proven that the fishmeal replacement by plant sources could have an impact on the intestinal status at both digestive and immune level, compromising relevant productive parameters, such as feed efficiency, growth or survival. In order to evaluate the long-term impact of total fishmeal replacement on intestinal mucosa, the gut mucosa proteome was analysed in fish fed with a fishmeal-based diet, against plant protein-based diets with or without alternative marine sources inclusion. Total fishmeal replacement without marine ingredients inclusion, reported a negative impact in growth and biometric parameters, further an altered gut mucosa proteome. However, the inclusion of a low percentage of marine ingredients in plant protein-based diets was able to maintain the growth, biometrics parameters and gut mucosa proteome with similar values to FM group.

A total fishmeal replacement induced a big set of underrepresented proteins in relation to several biological processes such as intracellular transport, assembly of cellular macrocomplex, protein localization and protein catabolism, as well as several molecular functions, mainly related with binding to different molecules and the maintenance of the cytoskeleton structure. The set of downregulated proteins also included molecules which have a crucial role in the maintenance of the normal function of the enterocytes, and

therefore, of the epithelium, including permeability, immune and inflammatory response regulation and nutritional absorption. Possibly, the amino acid imbalance presented in VM diet, in a long-term feeding, may be the main reason of these alterations, which can be prevented by the inclusion of 15% of alternative marine sources.

### Significance

Long-term feeding with plant protein based diets may be considered as a stress factor and lead to a negative impact on digestive and immune system mechanisms at the gut, that can become apparent in a reduced fish performance. The need for fishmeal replacement by alternative ingredients such as plant sources to ensure the sustainability of the aquaculture sector has led the research assessing the intestinal status of fish to be of increasing importance. This scientific work provides further knowledge about the proteins and biologic processes altered in the gut in response to plant protein based diets, suggesting the loss of part of gut mucosa functionality. Nevertheless, the inclusion of alternative marine ingredients was able to reverse these negative effects, showing as a feasible option to develop sustainable aquafeeds.

### Keywords

gilthead seabream; plant sources; gut mucosa; alternative marine ingredients ; proteome; label-free LC-MS/MS assay

## Introduction

Reducing fishmeal and fish oil content, or their total replacement, in aquafeeds is becoming necessary for ensuring aquaculture sustainability, being particularly relevant in the production of carnivorous fish. As one of the most important Mediterranean aquaculture species, the gilthead sea bream (*Sparus aurata*, L.) has received great

attention, and several studies have been focused on defining new feeds and alternative protein sources [1–5].

Plant based meals are likely the most commonly protein source used as alternative to fishmeal and marine origin by-products, and its inclusion in aquafeeds has been successfully achieved, even with a total replacement [6,7]. Nevertheless, previous studies have demonstrated that the use of plant based sources could affect not only fish growth, but also digestive capacity or immune status [8,9]. In this sense, the presence of anti-nutritional factors and the imbalances in essential amino acids, *inter alia*, may be responsible for the appearance of multiple changes at the gut level [10].

The gut mucosa, a layer which consist of the intestinal epithelium and the underlying connective tissue or lamina propria, plays an important role in digestion, absorption and metabolism of dietary nutrients, ion regulation and immune defence [11]. The gastrointestinal tract is continuously exposed to the presence of water, ions, dietary nutrients and different bacteria, being the main entrance of pathogens in fish [12]. Mucus covering the mucosa is the first line of defence, containing a set of biologically active components (antibacterial peptides, lysozymes, complement proteins, lectins and humoral antibodies) and preventing the colonization of pathogenic agents [13]. Nevertheless, gut epithelia, formed by intestinal epithelial cells, is the highly selective barrier against commensal and potentially pathogenic luminal microbes [14], being a protective barrier but also an interactive layer that regulates the fluxes of solutes, nutrients, antigens and immune-related molecules between the lumen and lamina propria [15]. In this regard, epithelial cells contribute to the innate and adaptive response through the interaction with immune cells from lamina propria [16]. Hence, the effects of fishmeal replacement on intestinal mucosa function and structure could be a determinant parameter for the progression of fish performance.

Omics technologies have been used in aquaculture during the last decade [17], providing relevant physiological information [18,19], which can be missed by transcriptomics [20]. Two-dimensional gel electrophoresis has been the most used technique in quantitative

proteomics studies in aquaculture up to today. However, gel-free strategies such as liquid chromatography (LC) coupled to MS (LC-MS) have become the most-widely used technology for high-throughput proteomic studies of biological tissues and other complex mixtures, allowing to analyse simultaneously a large number of protein [21,22].

The study of the gut mucosa using omics could help for a better understanding of the relation between nutritional changes and fish performance, as well as of its role in intestinal health, contributing to the feasibility of incorporating high dietary levels of plant proteins in aquafeeds for carnivorous species. In this regard, proteomic studies have been carried out in numerous species [23], such as Atlantic salmon (*Salmo salar*) [24,25], rainbow trout (*Oncorhynchus mykiss*) [26–29], Atlantic cod [30], common carp [31,32], zebrafish (*Danio rerio*) [33], Nile tilapia (*Oreochromis niloticus*) [34,35] and also in gilthead seabream [36–38]. However, most of these works focused on the response of the liver metabolism to dietary changes and few authors have studied the gut response to changes in dietary composition or feeding pattern [26,27,39,40]. Along these lines, changes in the protein composition, including enzymes and serum albumin, in the pyloric caecea of rainbow trout in response to different alternative protein sources have been evaluated [39]. Impact of short-term starvation in the proteome of the gut epithelia in this species has been also assessed [40], reporting an increase of the lymphocytes cytotoxicity, a reduction of the permeability to macromolecules and a negative impact on the inhibition on serine protease-induced stress and bacterial infection. Furthermore, alterations in lipid and energy metabolic activity in the intestine of Atlantic salmon in response to fish oil replacement have been also observed in previous research [25].

Proteome alterations on gut, skin or even plasma proteome caused by high dietary plant protein inclusion have been reverted thanks to some dietary additives, such as *Candida utilis* yeast [41] or sodium butyrate [38], in turns improving the fish performance and the immune status [42,43]. In this sense, marine alternative ingredients with potential functional properties, such as krill and squid meal, can be an interesting option to improve plant-based diets for carnivorous fish, reducing the inclusion of synthetic dietary

supplements such as synthetic amino acids or minerals, and providing an optimal nutrient efficiency and economic profit ratio [44].

To sum up, the aim of the current study was to assess the differences in proteome gut mucosa of gilthead seabream in response to complete replacement of fishmeal by plant protein sources with or without marine alternative ingredients (squid and krill meal) in aquafeeds for gilthead seabream.

# Methods

## Ethics approval

The experimental protocol was reviewed and approved by the Committee of Ethics and Animal Welfare of the Universitat Politècnica de València (UPV), following the Spanish Royal Decree 53/2013 and the European Directive 2010/63/UE on protection of animals used for scientific purposes and complies with ARRIVE guidelines.

## Experimental setup

The growth assay was conducted in 9 cylindrical tanks (1750 L) using a marine water recirculating system (75 $m^3$ capacity), which includes a rotary mechanical filter and a gravity biofilter (6 $m^3$ capacity), at the Universitat Politècnica de València (UPV). All tanks were equipped with aeration, and the water was heated with a heat pump installed in the system. During the experiment, water parameters were as follows: 23±1.5 ºC, 30±1.7 g $L^{-1}$ salinity, 6±0.5 mg $O_2$ $L^{-1}$, and 7.5 pH. All tanks had similar lighting conditions, with a natural photoperiod (from January to August).

## Fish and acclimatisation

Gilthead seabream came from the fish farm BERSOLAZ (Bersolaz Spain, S.L.U, Culmarex Group), located in Port de Sagunt (Valencia, Spain). After their arrival at the

136 facilities at the UPV, fish were acclimated to laboratory conditions for two weeks, being
137 fed daily by hand, to apparent satiation, three times per day (8:00, 13:00 and 18:00) with
138 a standard commercial diet (proximate composition: 55.2% crude protein (CP), 18.3%
139 crude lipid (CL), 11.6% carbohydrate, 9.4% ash (A) and 6.4% moisture) which was also
140 provided by Bersolaz S.L.U. Fish were weighed before starting the growth assay (initial
141 weight = 12±1.9 g) and then randomly distributed into the nine experimental tanks (40
142 fish per tank).
143

144 ## Diets

145 Experimental diets were manufactured as pellets by cooking-extrusion using a semi-
146 industrial twin-screw extruder (CLEXTRAL BC-45, Firminy, St Etienne, France) located
147 at the UPV, with 100 rpm screw speed, 110 ºC, 40 atm pressure and 2-4 mm diameter
148 pellets as processing conditions.
149 Three different diets were assayed in triplicates tanks: the FM diet, a fishmeal based
150 control diet, in which fishmeal (59%) was the main source of protein; the VM diet, a plant-
151 meal based diet in which the whole protein content was of plant origin, and the VM+ diet,
152 a plant-meal based diet including 10% squid meal and 5% krill meal. Squid and krill were
153 obtained as by-products from the companies Max Nollert (Utrecht, Netherlands) and
154 Ludan Renewable Energy (Valencia, Spain), respectively, thereby containing a 15%
155 level of marine-origin protein. VM and VM+ were supplemented with different synthetic
156 crystalline amino acids in order to achieve optimal amino acid requirements reported for
157 gilthead seabream juveniles [45]. Ingredients and proximate composition of the
158 experimental diets are shown in Table 1.
159

160 **Table 1.** Ingredients and proximal composition of diets tested in the growth assay

| | FM | VM | VM+ |
|---|---|---|---|
| **Ingredients (g 100g$^{-1}$)** | | | |
| Fishmeal | 58.9 | | |
| Wheat meal | 26.0 | | |
| Wheat gluten | | 29.5 | 22.2 |

| | | | |
|---|---|---|---|
| Faba bean meal | | 4.1 | 4.0 |
| Soybean meal | | 18.2 | 16.0 |
| Pea meal | | 4.1 | 4.0 |
| Sunflower meal | | 15.8 | 16.0 |
| Krill meal | | | 5.0 |
| Squid meal | | | 10.0 |
| Fish oil | 3.81 | 9.0 | 7.75 |
| Soybean oil | 9.29 | 9.0 | 7.75 |
| Soy Lecithin | 1.0 | 1.0 | 1.0 |
| Vitamin-mineral mix* | 1.0 | 1.0 | 1.0 |
| Calcium phosphate | | 3.8 | 3.8 |
| Arginine | | 0.5 | |
| Lysine | | 1.0 | 1.0 |
| Methionine | | 0.7 | 0.5 |
| Taurine | | 2.0 | |
| Threonine | | 0.3 | |
| **Proximate composition (% dry weight)** | | | |
| Dry matter | 89.5 | 88.4 | 89.5 |
| Crude protein | 44.2 | 45.0 | 44.6 |
| Ash | 9.9 | 6.6 | 7.2 |
| Crude lipid | 18.3 | 18.3 | 18.8 |
| CHO | 27.6 | 30.1 | 29.9 |
| Gross Energy (MJ $g^{-1}$) | 22.7 | 23.3 | 23.4 |
| **Digestible values (% dry weight)**** | | | |
| Protein | 42.7 | 41.9 | 42.1 |
| Lipid | 18.1 | 17.8 | 18.3 |
| CHO | 24.2 | 22.1 | 22.6 |
| Energy (MJ $g^{-1}$) | 21.4 | 20.1 | 20.8 |

*Vitamin and mineral mix (values are g $kg^{-1}$ except those in parenthesis): 25; choline, 10; DL-atocopherol, 5; ascorbic acid, 5; $(PO_4)_2Ca_3$, 5; retinol acetate, 1 000 000 (IU $kg^{-1}$); calcipherol, 500 (IU $kg^{-1}$); DL-a-tocopherol, 10; menadione sodium bisulphite, 0.8; thiamine hydrochloride, 2.3; riboflavin, 2.3; pyridoxine hydrochloride, 15; cyanocobalamine, 25; nicotinamide, 15; pantothenic acid, 6; folic acid, 0.65; biotin, 0.07; ascorbic acid, 75; inositol, 15; betaine, 100; polypeptides 12. DM, dry matter; CP, crude protein; A, ashes; CL, crude lipid; CHO, carbohydrates (calculated by difference: CHO= 100-CP-CL-A)

**Digestible values have been estimated using the apparent digestibility coefficients obtained from a previous digestibility trial [44].

## Macronutrients and amino acids analysis

Chemical analyses of ingredients were determined prior to diet formulation. Ingredients were analysed according to AOAC (1990) procedures: dry matter (DM) by heating at 105 °C to constant weight, ash (A) by incineration at 550 °C to constant weight), crude protein (CP), N × 6.25, by the Kjeldahl method after an acid digestion (Kjeltec 2300 Auto Analyser, Tecator Höganas, Sweden), crude lipid (CL) by methyl-ether extraction (Soxtec 1043 extraction unit, Tecator). All analyses were performed in triplicate. Diets were also assayed using the same procedures. Proximate composition is also showed in Table 1.

Amino acids of raw materials and experimental diets were also analysed, through a Waters HPLC system (Waters 474, Waters, Milford, MA, USA) consisting of two pumps (Model 515, Waters), an auto sampler (Model 717, Waters), a fluorescence detector (Model 474, Waters) and a temperature control module. Aminobutyric acid was added as an internal standard pattern before hydrolysation. The amino acids were derivatised with AQC (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate). Methionine and cysteine were determined separately as methionine sulphone and cysteic acid after oxidation with performic acid. Amino acids were separated with a C-18 reverse-phase column Waters Acc. Tag (150 mm × 3.9 mm), and then transformed into methionine and cysteine. Digestible essential and non-essential amino acids content of different diets (Table 2) were obtained from individual amino acids coefficients from a previous trial [44].

**Table 2.** Dietary ($^{C}AA$) and Digestible ($^{D}AA$) essential amino acids and non-essential amino acids (NEAA) expressed in g·100g$^{-1}$ of dry matter.

| | FM | | VM | | VM+ | | Optimum* |
|---|---|---|---|---|---|---|---|
| **EAA (g·100g$^{-1}$)** | **$^{C}AA$** | **$^{D}AA$** | **$^{C}AA$** | **$^{D}AA$** | **$^{C}AA$** | **$^{D}AA$** | |
| Arginine | 3.39 | 3.26 | 3.30 | 2.94 | 3.58 | 3.33 | 2.50 |
| Histidine | 1.00 | 0.96 | 0.82 | 0.77 | 0.81 | 0.77 | 0.85 |
| Isoleucine | 1.47 | 1.42 | 1.17 | 1.08 | 1.08 | 1.03 | 1.15 |
| Leucine | 3.24 | 3.12 | 2.98 | 2.77 | 2.45 | 2.36 | 2.24 |
| Lysine | 3.68 | 3.60 | 2.26 | 2.12 | 2.38 | 2.32 | 2.31 |
| Methionine | 1.16 | 1.13 | 1.06 | 1.02 | 1.05 | 1.02 | 1.17 |
| Phe+Tyr** | 3.14 | 3.05 | 3.00 | 2.84 | 2.79 | 2.69 | 2.59 |
| Threonine | 1.98 | 1.90 | 1.44 | 1.32 | 1.28 | 1.20 | 1.34 |
| Valine | 2.01 | 1.93 | 1.47 | 1.34 | 1.32 | 1.25 | 1.44 |
| **NEAA (g·100g$^{-1}$)** | | | | | | | |
| Alanine | 2.96 | 2.84 | 1.62 | 1.47 | 1.53 | 1.46 | |
| Aspartate | 4.43 | 4.09 | 3.09 | 2.72 | 3.04 | 2.80 | |
| Cysteine | 0.38 | 0.35 | 0.56 | 0.50 | 0.64 | 0.58 | |
| Glutamine | 2.99 | 2.90 | 1.90 | 1.82 | 2.11 | 2.06 | |
| Glycine | 8.11 | 7.52 | 13.03 | 11.45 | 11.26 | 10.36 | |
| Proline | 2.38 | 2.29 | 3.72 | 3.53 | 3.32 | 3.22 | |
| Serine | 1.90 | 1.81 | 1.90 | 1.77 | 1.76 | 1.68 | |
| Tyrosine | 1.34 | 1.31 | 1.13 | 1.07 | 1.03 | 0.99 | |

***Optimum essential amino acid profile recommended for gilthead sea bream juveniles [45]

**Phe+Tyr, Phenylalanine + Tyrosine

## Growth assay

The trial lasted 156 days. Fish were observed daily in tanks in order to check their health status. At the end of the experiment, all fish were weighed individually, in order to evaluate fish growth and determine growth parameters. Three fish per tank were sacrificed by cold shock after anaesthesia using clove oil (87% eugenol, Guinama ®, Valencia, Spain) dissolved in water (1 mg / 100 mL of saltwater), in order to minimize the suffering of fish. During the experiment, fish were fed by hand to apparent satiation, three times per day during the first 60 days (8:00, 13:00 and 18:00) and twice per day (9:00 and 14:00) from then up to the end of the experiment. Food managers distributed the pellets slowly, allowing all fish to eat, in a weekly regime of six day of feeding and one of fasting. Temperature, pH, oxygen, ammonia, nitrite and nitrate concentrations were monitored throughout the growth assay.

## Growth and nutritional parameters and biometric measurements

Final weight (FW), specific growth rate (SGR), survival, feed intake (FI) and feed conversion ratio (FCR) were determined using the tank as experimental unit. Condition factor (CF), viscerosomatic index (VSI), hepatosomatic index (HSI) and mesenteric fat index (MFI) were obtained at the beginning and end of the growth assay, using three fish per tank, nine per treatment. The weight (GW) and the length of the gut tract (GL) were also measured.

## Statistics

Growth, nutritive and biometric indices were analysed through an analysis of variance using the statistical package Statgraphics ® Plus 5.1 (Statistical Graphics Corp., Rockville, MO, USA), with a Newman-Keuls test for the comparison of the means and a level of significance of $p<0.05$. Data expressed as percentages were arcsine-

transformed prior to analysis, and data were checked for normality (Shapiro-Wilk test), independence and homogeneity of variances (Levene's test) to satisfy the assumptions of ANOVA.

## Proteomics

### Sampling

At the end of the growth trial, three fish per tank (9 fish per diet), were slaughtered on ice after euthanizing with clove oil and dissected in order to obtain the gastrointestinal tract. Fish were fasted for 24 hours before sampling.

After discarding the stomach and pyloric caeca, the first intestinal third of the gut (foregut) was removed, sliced longitudinally and washed with phosphate buffered saline solution to remove digesta. Intestinal mucosa was scraped using sterilized large scalpel blades, stored in Eppendorf tubes, flash frozen in liquid nitrogen and stored at -80º C.

### Tissue extraction and protein precipitation

Gut mucosal scrapings from one fish per tank (three per diet) were placed in 8M urea (Malinckrodt AR®, LabGuard®) in homogenization tubes ($^{RT}$Precellys® Ceramic Bead Tube, 1.4 mm / 0.5 mL tubes) and then ground using the homogenizer PrecellysTM Control Device (Bertin Technologies), with the following conditions: 6,500 m/s and 3 rounds of 20 s. Tubes were centrifuged (14,000 rpm, 4º C, 15 min) and supernatants transferred to new Eppendorf tubes.

Tissue extracts were subjected to cold acetone precipitation: cold acetone (Acetone HPLC grade, Fisher Chemical) was added to samples in a proportion of 5:1 (5 ml cold acetone: 1 mL sample), tubes were incubated overnight at -20ºC and then centrifuged at 14,000 rpm and 4ºC during 10 min. Supernatants were discarded and pellets were dried, resuspended in 8M urea, shaken for two hours at room temperature using a vortex and then centrifuged (14,000 rpm, room temperature, 5 min). Supernatants were

collected; protein concentration was determined using a BCA assay kit (ThermoScientific, Meridian Rd., Rockford, IL, USA) and samples stored at -20ºC.

**Denaturation, reduction, alkylation and digestion**

A volume of sample with a protein amount of 50 µg was subjected to simultaneous denaturation and reduction, using DL-dithiothreitol (Sigma) in 25 mM ammonium bicarbonate (Sigma-Aldrich) Buffer (ABC), in a final concentration of 10 mM, and incubating at 60º C and 750 rpm agitation for 60 min. Iodoacetamide (Sigma) in 25 mM ABC Buffer was used for alkylation, in a final concentration of 20 mM, and vortexed at room temperature for 60 min in dark conditions. 25 mM ABC buffer was added to samples after alkylation to reduce the urea concentration, in a proportion of 5:1 (5 mL 25 mM ABC buffer: 1 mL sample). A trypsin/lysine-C enzyme mix (Trypsin/Lys-C mix mass spec grade, Promega) was used for the digestion. Enzyme pellets were resuspended with 25 mM ABC buffer and 3.2 µg of enzyme mix were added per sample. $CaCl_2$ solution was added to samples in a final concentration of 1 mM just before starting incubation at 37ºC and 500 rpm agitation for 3 hours. An additional 1.6 µg of enzyme mix was added to samples and these were incubated again in the same conditions overnight.

**C18 column purification**

Digested peptides were purified using C18 columns (MicroSpin Column 96/pk, $C_{18}$ Silica, 5-200 µL loading, 5-60 µg capacity, The Nest Group, Inc.). Columns were previously conditioned with 200 µL 100% acetonitrile (ACN) twice and 200 µL 3% ACN 0.1% formic acid (FA) (Fluka Analytical) twice, by centrifuge at 2000 rpm at room temperature for 2 min. Elutions were discarded. Samples were loaded onto columns and centrifuged with the same conditions, and the elution was reloaded and centrifuged again. Columns were washed four times with 200 µL 3% ACN 0.1% FA. Finally, columns were placed in new clean tubes and eluted twice with 100 µL 60% ACN 0.1 % FA. Samples were dried using a vacuum centrifuge and stored at -20 ºC.

**LC-MS/MS load**

Samples were resuspended in 50 µL 3% ACN 0.1% FA, vortexed for 30 min at room temperature and centrifuged (14000 rpm, room temperature, 10 min). Peptide concentration was determined using the BCA assay kit and 3% ACN 0.1% FA was added to each sample to achieve a final concentration of 0.1 µg/µL.

**LC-MS/MS assay**

The samples were analyzed using the Dionex UltiMate 3000 RSLC Nano System coupled to the Q Exactive™ HF Hybrid Quadrupole-Orbitrap MS (Thermo Scientific, Waltham, MA, USA). Peptides (10 µl) were loaded onto a trap column (20 µm x 350 mm) and washed using a flow rate of 5 µl/minute with 2% ACN 0.01% FA. The trap column was then switched in-line with the analytical column after 5 minutes. Peptides were separated using a reverse phase Acclaim PepMap RSLC C18 (75 µm x 15 cm) analytical column using a 120 minute method at a flow rate of 300 nL/min. Mobile phase A consisted of 0.01% and a mobile phase B consisted of 80% ACN 0.01 % FA. The linear gradient started at 5% B and reached 30% B in 80 min, 45% B in 91 min, and 100% B in 93 min. The column was held at 100% B for the next 5 min before being brought back to 5% B and held for 20 minutes. Sample was injected into the QE-HF through the Nanospray Flex™ Ion Source fitted with an emission tip from Thermo Scientific. Data acquisition was performed monitoring the top 20 precursors at 120,000 resolution with an injection time of 100 millisec.

**Data Analysis**

The freely available MaxQuant software package (version 1.5.5.1, Max Planck Institute of Biochemistry) was used for the analysis of mass-spectrometric data set. Only 896 protein sequences are registered in the UniProt database for gilthead seabream species (updated to November 5, 2019), and 3159 if a higher taxon as ‘Sparidae’ was considered.

In order to perform an efficient protein identification, the UniProt database for the teleost fish zebrafish (Danio rerio; 59217 sequences, updated to November 5, 2019), which genome sequence is available [18], was used for the mass spec file analysis. A Danio rerio proteome is available in UniProt. (UP000000437), which has 46.847 sequences, including 3.138 revised sequences (Swiss Prot), and the used database included the *Danio rerio* proteome sequences and other revised and non-revised sequences to a total of 59.217 when the analysis was performed.

The search parameters were: first search peptide tolerance: 20 ppm, main search peptide tolerance: 4.5 ppm, other instrument group-specific parameters by default. The enzymes considered were trypsin and LysC, with 2 Max. missed cleavages. Oxidation of methionine residues (variable) and carbamidomethylation of cysteine residues (fixed) were included as modifications. Sequences and identification of global parameters were used by default, with a False Discovery Ratio of 0.01. Match between runs was considered, with a Match time window of 1 min and an Alignment Time Window of 20 min. Label-free quantification (LFQ) was used to obtain the normalized LFQ intensity. LFQ intensity values were obtained from the MaxLFQ algorithms, included in the MaxQuant software packages. These algorithms were developed in order to achieve a highest accuracy of quantification in label-free LC-MS/MS assays without “house-hold proteins”, extracting the maximum ratio information from peptide signals in a given number of samples [46]

Contaminants and reverse proteins were removed from the analysis. Only proteins with at least 2 MS/MS counts, and a minimum of two different peptides used for identification (with the UniProt database) were considered for quantitative analysis. InfernoRDN application (Pacific Northwest National Laboratory), which provides an easy-to-use to R (version 3.4.0) for proteomic data analysis, was used to analyse the analysis of variance (ANOVA) and principal component analysis (PCA). Only proteins with values of intensity in all the samples were considered for the ANOVA. ANOVA and subsequent analyses

were performed using only the protein sets obtained from the intensity data analysis. This decision is further addressed later in the 'Results' and 'Discussion' section.

After ANOVA analysis, proteins with a p-value<0.05 were subjected to 2-groups cross comparison. Proteins with an average fold change (FC) ≥2 or ≤0.5, or with a t-test<0.05 (and a FC ≥1.5 or ≤0.75) were selected for the functional analysis of the different comparisons (FM vs VM, and FM vs VM+).

Cluster analysis and heatmap plot were generated using the ClustVis software [47]. The hierarchical clustering of samples was performed using the "average linkage" as agglomeration method and "Euclidean" as distance metric.

**Reproducibility validation**

Two samples were run per triplicate in the Q-Exactive in order to validate the reproducibility of the assay. Data was analysed using the same database and search parameters, but match between runs was not considered. Contaminants and reverse proteins were removed, and peptides and proteins only present in one or two runs were considered when MS/MS counts ≥ 6. Taking as basis the identified peptides, the variation coefficient of intensity values for each peptide was reported. If we consider the identified proteins, the variation coefficients of both intensity and LFQ intensity values were determined. In both cases, the median of each set of variation coefficients were determined.

**Functional annotation**

An Enrichment Analysis (two-tailed Fisher's Exact Test) was performed, using Blast2GO software (version 2.8.0), for each comparison [48]. The test list included the differentially expressed proteins after the fold-change and t-test filtering, and the reference list (or background) was obtained from the UniProt *Danio rerio* database, containing the different Gene Ontology™ terms (GO terms) associated to all the proteins of this database. Enriched GO terms were filtered according to p-value (<0.05) and FDR (<0.05)

and classified in the three GO annotation domains: biological process, cell component and molecular function.

### KEGG pathways and Protein clustering

The Database for Annotation, Visualization and Integrated Discovery (DAVID, version 6.8) was used for the KEGG annotation [49,50]. The list of differentially expressed used as input in the Blast2GO analysis was also used for the DAVID functional annotation using the *Danio rerio* sequences included in its repository. Pathways with a p-value<0.05 were considered as significantly affected, and the proteins included in each one were clustered and their interaction determined using String 11.0 version software. The Markov Cluster Algorithm (MCL) was used to determine the possible protein clusters with an inflation parameter of 1.8.

## Results

### Growth, nutritive and biometric assessment

Fish weight (FW), specific growth rate (SGR), condition factor (CF), hepatosomatic index (HSI), visceral fat index (VFI) and gut weight (GW) were affected by the dietary composition (Table 3). FM and VM+ group reported mostly the highest values in the fish growth, while VM group registered the lowest indices. On the other hand, in biometric parameters, VM+ group showed intermedia results between FM and VM, except for the CF. No differences were found in survival neither in the nutritive parameters.

**Table 3**. Growth, nutritive and biometric indices of fish before and after the growth period in the different experimental groups

| | I | FM | VM | VM+ |
|---|---|---|---|---|
| **FW** | 12.0±1.93 | 178.7±11.33$^{a}$ | 129.7±11.33$^{b}$ | 183.8±11.33$^{a}$ |

| | | | | |
|---|---|---|---|---|
| **SGR** | | 1.76±0.06[a] | 1.48±0.06[b] | 1.75±0.06[a] |
| **Survival** | | 90.8±0.06 | 94.2±0.06 | 94.2±0.06 |
| **FI** | | 3.38±0.18 | 3.12±0.18 | 2.96±0.18 |
| **FCR** | | 1.93±0.26 | 1.82±0.26 | 1.99±0.26 |
| **CF** | 1.25±0.09 | 2.15±129[a] | 1.90±0.129[ab] | 1.73±0.129[b] |
| **VSI** | 11.0±1.44 | 7.19±0.553 | 7.14±0.553 | 7.94±0.553 |
| **HSI** | 1.15±0.160 | 1.41±0.108[a] | 0.91±0.108[c] | 1.13±0.108[b] |
| **MFI** | 0.67±0.606 | 1.79±0.331[a] | 0.95±0.331[b] | 1.23±0.331[ab] |
| **GW** | 0.21±0.039 | 3.62±0.256[a] | 2.65±0.256[b] | 3.67±0.256[a] |
| **GL** | 7.4±1.89 | 11.1±1.32 | 10.7±1.32 | 14.3±5.1.32 |

FW, fish weight; SGR, specific growth rate; FI, feed intake ratio; FCR, feed conversion ratio; CF, condition factor; VSI, viscerosomatic index; HSI, hepatosomatic index; MFI, mesenteric fat index; GW, gut weight; GL, gut length; I, initial FW (g); SGR (%·day$^{-1}$) = 100 × Ln (final fish weight (g) /initial fish weight (g)) / days; Survival (%) = 100 × (final number of fish / initial number of fish); FI (g 100 g fish$^{-1}$day$^{-1}$) = 100 × feed consumption (g) / average biomass (g) × days; FCR (g feed g$^{-1}$ fish$^{-1}$) = feed offered (g) / weight gain (g). CF (g cm$^{-3}$) = 100 × total weight (g)/ total length (cm)$^{3}$; VSI (%) = 100 × visceral weight (g) / total weight (g); HSI (%) = 100 × liver weight (g) / total weight (g); MFI (%) = 100 × mesenteric fat weight (g) / total weight (g); GW (g); GL (cm).

Data from growth and nutrient parameters are the means of 3 tank (n=3) and of 3 fish per tank (n=9) for biometric parameters; data in the same row with different superscripts indicates statistical differences at P<0.05. Newman-Keuls test was applied for the comparison of the means.

## Proteomic profile

### LC-MS/MS assay

S1 and S2 (Supplementary Data) includes all the combined information about the identified peptides and S3 contains the information on the identified proteins reconstructed from the set of peptides.

1355 proteins were identified after the MaxQuant assay. After removing contaminants and reverse sequences, the list reduced to 1328 proteins. 754 (56.78%) of them were found in all the samples. A summary of the proteins identified in the different groups and

individual samples is shown in Table 4. Samples from the VM group, especially sample VM2 (63.6%), reported lower percentages of identifications in comparison to the total amount. In consequence, the represented protein population in VM group was lower (776) than the other groups, FM and VM+ (1163 and 1174, respectively). Under these circumstances, LFQ intensity was discarded for subsequent analyses. The prerequisite to apply MaxLFQ algorithms is to have a dominant population of proteins that change minimally between experimental conditions [46] what was not accomplished in VM group. Therefore, henceforth, only the intensity data set were used for the differential analysis. This decision is further explained in the Discussion section.

**Table 4.** Number of proteins identified in the different runs and experimental groups

| | **FM** | | | **VM** | | | **VM+** | | |
|---|---|---|---|---|---|---|---|---|---|
| | **FM1** | **FM2** | **FM3** | **VM1** | **VM2** | **VM3** | **VM+1** | **VM+2** | **VM+3** |
| **Identifications** | 1233 (92.8%) | 1225 (92.2%) | 1244 (93.7%) | 1068 (80.4%) | 845 (63.6%) | 1219 (91.8%) | 1247 (93.9%) | 1229 (92.6%) | 1257 (94.7%) |
| **Represented in the group*** | 1291 (97.2%) | | | 1279 (96.3%) | | | 1299 (97.8%) | | |
| **Represented in all samples** | 1163 (87.6%) | | | 776 (58.4%) | | | 1174 (88.4%) | | |

*Proteins represented in the group were identified in at least one run of the group

## Reproducibility validation

S4 (Supplementary Data) summarizes the reproducibility assessment of the LC-MS/MS assay. Percentage of peptides and proteins that display intensity values in the three runs of both sets of runs (R1 and R2) is around the 65 % and the 80%, respectively. However, these percentages increase to 100% (75% when we work with LFQ Intensity) after removing the low abundant peptides (or proteins) from the analysis, confirming a high reproducibility in qualitative terms.

After filtering, the percentage of peptides showing a variation coefficient in intensity below 20% was 78% for R1 and 79% for R2. Regarding the proteins, this percentage ranged from 66% to 69%, if the intensity values were considered, and from 72% to 75%

when LFQ intensity data set was used. The medians of the variation coefficients for both sets of runs were around the 13% and the 8%, working with Intensity and LFQ Intensity, respectively. These values are common and highly acceptable for a LC-MS/MS assay.

**Quantitative analysis**

The quantitative analysis was performed after removing in each sample those proteins with a total MS/MS counts<2 and global intensity ≠ 0. After filtering, the global set of identified proteins in seabream gut mucosa proteome was 1265 (Supplementary Data S5). After PCA, considering intensity data (Figure 1), the samples belonging to FM and VM+ grouped closer and separately to VM samples.

If a PCA (Figure 2A) and Heatmap analysis (Figure 2B) is performed taking as basis the differential expressed proteins among groups (FM vs VM vs VM+), each experimental group showed a differential distribution. The heatmap plot confirmed the variability observed in the PCA distribution, being classified each experimental group in a different hierarchical branch. The set of differentially expressed proteins, including their individual and average intensity values, fold change and t-test values, for each comparison, is reported in the Supplementary Data (S6).

Comparing groups in pairs (Table 5), FM and VM+ groups showed a generalized upregulation of the whole-set of proteins compared to VM. This up-regulation was even more pronounced in VM+ group. The list including the significantly over- or under-represented proteins for each comparison, and the proteins exclusively found in specific group are shown in Supplementary Data (S7).

**Table 5**. Comparisons between experimental groups after filtering based on the fold change and the t-test

| | **Intensity** | |
|---|---|---|
| | Overexpressed (Present only) in the first group | Overexpressed (Present only) in the second group |

| | | |
|---|---|---|
| **FM vs VM** | 193 (12) | 0 (1) |
| **FM vs VM+** | 9 (1) | 33 (1) |
| **VM+ vs VM** | 216 (11) | 0 (0) |

For each comparison, the proteins over-expressed or present only in the first group were under-expressed or absent in the second group, and vice versa.

In order to evaluate if differential expressed proteins were common or specific of each comparison, Venn diagrams were created (Figure 3B). The gene name of the proteins that were shared in each comparison is detailed in table of the Figure 3 A. Most of the differentially expressed proteins of FM *vs* VM and VM+ *vs* VM comparisons were shared, in fact no specifically differential protein were registered in the FM vs VM+ comparison, reinforcing the idea that both groups have a very similar gut mucosa proteome. As a consequence, taking account the similarity between FM and VM+, the functional annotation and the KEGG Pathway analysis were performed only for FM vs VM comparison, FM as control group and VM as most differential group. This decision is further considered in the Discussion section.

**Functional annotation**

A total of 199 protein IDs, from the set of differentially expressed proteins in the comparison FM *versus* VM (206 proteins), were recognized by Blast2GO and submitted to the Enrichment Analysis. All the enriched GO terms delivered by Blast2GO, and classified in three different domains of the Gene Ontology annotation (the biological processes in which the protein is involved, the molecular function of the protein, and its location in the cell), can be observed with their p-value in the Figure 4. A detail of the output delivered by Blast2GO can be found in Table S8 (Supplementary Data). Furthermore, the gene name and gene description of the proteins included in each enriched GO terms is also detailed in Table S9 (Supplementary Data).

Among the biological processes affected, intracellular transport processes, mediated or not by vesicles, the assembly of cellular macrocomplex, as the phagolysosome,

localization of protein and other macromolecules, protein catabolism and one carbon metabolic processes mediated by tetrahydrofolate were the most common. Regarding the cell components, membrane proteins, including Golgi and the endomembrane system, were most affected by dietary treatment, but several cytosolic proteins were also altered. Most of the proteins are constituents of the cytoskeleton, vesicles and different intracellular protein complexes as the proteasome, the Arp2/3 protein complex, ribonucleoprotein complexes, the eukaryotic translation initiation factor 3 complex or the glycerol-3-phosphate dehydrogenase complex. Finally, several molecular functions were enriched according to Blast2GO analysis: binding to nucleotides, small molecules, ions, carbohydrate derivatives, cofactors and cytoskeleton proteins, nucleoside-triphosphatase and hydrolase activity, catalytic activity, structural molecule activity, receptor activity and structural constituent of cytoskeleton, among others.

**KEGG pathways**

From the list of 206 differentially expressed proteins, 202 IDs (1 was redundant, and 3 were not recognised) were identified by DAVID. The 58.4% (118) presented KEGG annotation and 5 KEGG pathways were significantly affected (p-value<0.05) (Table 6). Moreover, a cluster analysis was carried out to better identify link proteins between KEGG pathways and between the proteins included in each KEGG pathway. No relevant clusters were obtained between the different pathways, but significant interaction score (>0.400) were found in the proteins belong to phagosome and proteasome KEGG pathways (Supplementary data S10). Three clusters were obtained for phagosome KEGG pathway composed by actin, tubulin and dynein proteins; ATPase and vesicles-associated proteins. Only one cluster was obtained for proteasome KEGG pathway, leading by proteasome subunits.

**Table 6.** Affected KEGG pathways according to DAVID analysis, including the number of proteins altered in each pathway

| KEGG Pathway | Nº of proteins | UniProt Accesion | Gene Name | %* | PValue |
|---|---|---|---|---|---|
| Phagosome | 14 | Q6NY92, Q8JHG2, B2GPU0, A7MCK9, Q90ZM2, A8WG05, Q7SX58, Q2LEK1, O42271, A0A0R4IG84, Q6PC95, Q6NWJ5, Q6IQK3, Q2YDQ3 | actb2, atp6v0a1a, atp6v1c1a, atp6v1h, coro1a, dync1h1, sec22bb, sec61al1, tuba1b, tuba8l2, tuba8l4, vamp3, zgc:123298 | 11.9 | 0.00002 |
| Proteasome | 7 | F1QY43, F1QGH9, Q6NYV1, Q7ZYX7, Q6IQH4, Q9PUC4, Q7ZUJ8 | psmb3, psmd1, psmd11b, psmd3, psmd7, psmd8, psme2 | 5.9 | 0.00107 |
| Salmonella infection | 8 | Q7ZUQ0, Q6P2T5, Q6NZZ2, A8WG05, Q7ZUQ1, Q2LEK1, Q9DGR5, Q6PE28 | actb2, arpc1a, arpc2, arpc4, cdc42l, dync1h1, mapk1, rhogd | 6.8 | 0.00440 |
| Regulation of actin cytoskeleton | 13 | E7FBD5, Q7ZUQ0, Q6P2T5, Q6NZZ2, A8WG05, Q7ZUQ1, Q9DGR5, Q7ZWC7, B3DKN5, Q6NSN6, A0A0R4IZT6, Q9DGQ5, Q4V9A9 | LOC573682, actb2, arpc1a, arpc2, arpc4, cdc42l, iqgap1, mapk1, msna, mylka, ppp1caa, ppp1cc, scinla | 11.0 | 0.01318 |
| Endocytosis | 13 | E9QEB6, Q66HW2, A0A0R4IYC4, Q7ZUQ0, Q6P2T5, Q6NZZ2, I3IT87, Q7ZUQ1, F1R966, E9QBV1, A0A0R4I9G6, Q6IQ70, Q1ED30 | arf2b, ehd1b, rab11a, arpc1a, arpc2, arpc4, ap2m1b, cdc42l, cltca, cltcb, gbf1, tsg101a, vps35 | 6.4 | 0.03138 |

| | | | | | |
|---|---|---|---|---|---|
| Amino sugar and nucleotide sugar metabolism | 5 | Q803Z1, Q90XP7, E7F1A0, Q8QFU2, Q3S343 | chs1, gfpt2, gpia, uap1, ugdh | 4.2 | 0.03177 |

* Percentage of proteins altered in the specific KEGG pathway from the total of proteins with KEGG annotation

# Discussion

## Biometric Parameters, Growth and Survival

The present assay reported significant differences in weight, specific growth rate and other biometric indices, indicating a negative effect of total fishmeal replacement on growth performance of gilthead seabream. In previous trials, total fishmeal replacement has been successfully achieved for gilthead seabream without affecting fish growth [44]. Differences with present results could be explained by the initial fish weight (around 12 g in the present trial compared to more than 100 g in the previous assays), since lower levels of fishmeal replacement can be reached during the first stages of growth [1]. On the other hand, the inclusion of alternative marine sources at 15% level in a plant based diet reversed the negative effect on fish growth, registering a similar fish growth to fishmeal based feeds (FM group) in agreement with previous studies [7,51] . Therefore, in the current study, we confirm that a low dietary inclusion of marine alternative ingredients in plant based diets can be more economical and environmentally sustainable option than only fishmeal or plant based diets.

Regarding biometric parameters, previous findings in Atlantic salmon (*Salmo salar* ) [52] and other salmonids species [53] reported a positive strong correlation between condition factor and total lipid content (in mesenteric fat found in the abdominal cavity), suggesting higher fat synthesis and deposition [54] just as it is observed study in fish fed FM diet in the current trial that is according with the major digestible energy of this diet regarding the other two. The mesenteric fat weight reduction observed VM could be carried out in declines as fat reserves used to maintain metabolic function [55] and the

lower digestible fat than FM diet. Early studies in fish fed plant-protein-based diets have found out an opposite trend between the mesenteric adipose tissue and hepatic lipoprotein lipase expression, reflecting a reduced fatty acid uptake [56].

## Proteome Analysis

MaxLFQ algorithms were used in order to achieve the highest quantification accuracy in the proteomic assay. Nevertheless, their application requires the existence of a dominant population of proteins that change minimally between experimental conditions[46]. In the present work, a big set of proteins was observed to be under expressed in the VM group in comparison to FM and VM+ samples, affecting LFQ Intensity data and hiding the potential interesting differences between dietary groups. Considering samples preparation was carried out in parallel in unique experimental condition, and samples were loaded randomly in the LC-MS/MS after peptide quantification, similar LC-MS/MS performances in quantitative terms were expected. Since reproducibility assessment showed also a good correlation when Intensity data was considered, the analysis was only carried out with the outputs of the Intensity data.

Taking account these considerations, a high impact of total fishmeal replacement was observed in gut mucosa proteome, with approximately 20% of identified proteins significantly underrepresented in the VM respect to FM gut mucosa. By contrast, VM+ diet was able to recover this down-regulation, with similar gut proteome profile to FM group. In fact, when the comparison groups in pairs was performed, most differentially expressed proteins of FM *vs* VM and VM+ *vs* VM were shared. Therefore, according the similar growth, biometrics parameters and gut mucosa proteome obtained FM and VM+ groups at the end of the trial, the functional analysis will be focused only on FM and VM comparison.

Plant protein sources can lead to an impact on a great variety of biological processes and metabolic pathways in the gut mucosa [57], which could affect fish performance[38]. Although most of the knowledge about protein function was obtained from human

558 studies, thus its application to fish physiology has to be considered with caution [57],
559 present results seem to point to long-term feeding with a complete plant based diet may
560 cause a loss of part of the mucosa functionality.
561 Most of the downregulated proteins identified in VM group were related with transport of
562 molecules, cell communication, cell metabolism, structural functions and assembly of
563 protein complexes as the phagosome or the proteasome, necessary for the normal
564 function of the enterocytes and therefore, of the epithelium.
565
566 **Effects on the enterocytes functionality**
567 The homeostatic balance between epithelial cell proliferation and apoptosis is essential
568 for the maintenance of the epithelial function, including regulation of epithelial
569 permeability, the inflammatory response or the absorption of nutrients [58]. Apoptosis
570 plays a central role in epithelial organization and cell turnover, and defects on apoptotic
571 pathways in enterocytes have been related to villus atrophy, epithelial hyperplasia or loss
572 of normal absorptive function [59]. The 26S proteasome (psmd1, psmd11b, psmd3,
573 psmd7, psmd8), a key multiprotein complex in cell proteostatic mechanisms [60], and
574 other proteasome subunits (psmb3, psme2), were underexpressed proteins in group VM
575 (Table S7 and S10), what has been correlated with a major apoptosis [61,62]. Other
576 proteins related with apoptotic regulation, the regulation of cell polarization, the migration
577 of cells and the maintenance of a homeostatic state, such as villin-like [59], the gelsolin
578 [59], the Ap1m2 [63], the annexins A1 [64] and A2 [65] or the AP2-complex [66] were
579 also downregulated in this group (Table S7).
580 In rainbow trout liver, a downregulation effect on the proteasome pathway in response
581 to starvation was reported [26] and pathways involved in cellular protein degradation
582 seem to be sensitive to plant protein inclusion [27], while the partial replacement of
583 fishmeal by soybean meal induced inflammation, cellular repair and apoptosis in the
584 distal intestine of Atlantic salmon [67]. These results suggest that the proteasome
585 pathway could play a protective role in the epithelial cells, and it can be regulated by the

586 dietary composition and energy level. In this regard, the impact on the regulation of the
587 apoptosis mechanisms reported in the present work might be explained by dietary
588 factors.
589 Furthermore, several proteins involved in protein synthesis and metabolism, such as
590 translation-related proteins (eif3bb, eif3c, eif3eb, eif3l, eif4e1c) and the mentioned
591 apoptotic-related proteins, were also under-expressed in gut mucosa of fish fed VM diet
592 (Table S7). Therefore, an unbalanced or deficient protein turnover between protein
593 synthesis and degradation into amino acids could take place, leading ultimately to not
594 satisfy the necessary renewal of proteins in the cell. Indeed, higher rates of cell renewal
595 have been related to inflammatory responses [68] in order to maintain the population of
596 functional enterocytes, so a reduced cell recovery rate could be associated with the loss
597 of the epithelium properties [67].
598 On the other hand, intracellular transport processes, especially protein transport and
599 Golgi vesicle-mediated transport GO terms, were altered in the VM group (Table S8 and
600 S9). Golgi complex is the main organelle involved in protein transport and plays a crucial
601 role in the maintenance of homeostasis in polarized cells such as the enterocytes [69].
602 In this regard, proteins such as clathrin (cltca, cltcb), coatomer protein and adaptor-
603 related protein complex, which are related to intracellular protein transport, were under-
604 regulated in the VM group (Table S7).
605 Finally, one-carbon metabolism GO term, which supports amino acid metabolism,
606 nucleotide biosynthesis and redox defence, among several physiological processes [70],
607 and the carbohydrate derivatives metabolism GO term, which is also related with many
608 cellular functions, were also altered (Table S8 and S9).
609
610 **Effect on epithelial permeability, immune response and inflammatory activity**
611 The downregulation of proteins such as actin cytoskeleton-related proteins (arpc1a,
612 arpc4, actr3, actb2; Table S7) and myosin-related proteins (mylka, myo1cb, myo1b,
613 myh9a, myo6b; Table S7) could be related to a lack of capacity to regulate the

permeability of the intestinal barrier, which is necessary for an inflammatory and immune response against luminal environmental changes. The actin cytoskeleton dynamics seem to be regulated by the phosphorylation of the myosin light chain [71] and also by several different cytoskeletal, scaffolding, signalling and polarity proteins [72]. It is anchored to epithelial tight junctions between the enterocytes, which play an important role in the regulation of epithelial barrier permeability by luminal and tissue stimuli and in the selective exchange of molecules between the intestinal lumen and lamina propria [72, 73], being a crucial structure for the intestinal status. Thus, disruptions on this regulation mechanism can lead to inflammatory reactions and affected immune states [15, 74] and also to malabsorption of nutrients [75] .

Since intestinal barrier is continuously exposed to commensal bacteria and dietary nutrients, these can have an influence in the pathways related to the presence and localization of tight junction proteins[76]. An increase of transepithelial uptake capacity, perhaps caused by an increased permeability, in response to saponins, which are present in soy, has been reported in different species, including fish [77]. Nevertheless, an impact on gene expression of tight junction proteins has been observed in fish fed high soy dietary levels [78], which suggested the tightening of the tight junctions, maybe in response to antinutrients. Therefore, further research is needed in order to go into the exact role in permeability regulation of the different proteins.

The intestinal epithelium is involved in modulation of the gastrointestinal microbiota through the activation of inflammatory responses [79,80] or by immunotolerance development to luminal microbiota [81]. Bacterial translocation through the epithelial barrier can take place following the paracellullar route, between adjacent epithelial cells [82] or through the enterocytes [81] by the formation of phagosomes [13]. Phagocytosis has been also described in macrophages/monocytes and neutrophils during the innate immune response [13]. Thus, besides the effect on epithelial permeability, the alterations in phagocytic processes observed in the present work (Supplementary data S10) could be also linked to a lack of capacity in the gut mucosa of initiate an inflammatory process,

642 exert an effective innate immune response and develop an inmunotolerance to
643 commensal bacteria. A remarkable impact on the gut microbiota composition of the
644 gilthead seabream has been reported when fishmeal was completely replaced by plant
645 sources [83], and the differences in the gut bacterial community could be explained by
646 an immune dysregulation. In this regard, the underregulation in the VM group of proteins
647 related to the modulation of inflammatory and immune reactions, such as the leukotriene
648 A-hydrolase [84], the annexins (anxa11a, anxa11b, anxa4; Table S7) [64], Meprin A [85]
649 or the angiotensin converting enzyme (ace, ace2; Table S7) [86] may also be related.
650 Extensive research has been carried out regarding to the impact of including plant protein
651 sources in the diet on the inflammatory and immune response of fish, also in gilthead
652 seabream [8,87,88]. The level of fishmeal replacement, as well as the duration of the
653 dietary treatment, seems to be decisive in the trigger of an immunostimulated or
654 immunosuppressed status [8,89]. In this respect, the suppression of innate immune
655 capacity by high levels of inclusion of plant proteins has been previously observed in
656 rainbow trout [90], but also in gilthead seabream [8]. The long-term decrease in the
657 plasma complement level after feeding with a fishmeal replacement above 75% level has
658 been described [8], suggesting a possible immunosuppression. Moreover, a long-term
659 immunosuppression at the gut mucosa level, based on gene expression, was suggested
660 in fish fed using only plant protein sources [91]. Fish fed the VM diet, in contrast to fish
661 fed the VM+ diet, could be unable to meet the energy and resources requirements to
662 sustain an inflammatory response during all the growth assay due to nutritional dietary
663 deficiencies, decreasing the efficiency of local immune mechanisms and leading
664 ultimately to a chronic immune suppression [89], exhaustion, weakness [91]. On the
665 other hand, a transcriptomic modulation induced by dietary decrease of fishmeal and fish
666 oil was also observed [92], reporting an upregulated amount of inflammatory markers
667 with higher grade of leucocyte infiltration in the submucosa, especially in the anterior
668 intestine, and changes in other genes related with cell differentiation and proliferation,
669 antioxidant defence, immunity, epithelial architecture and permeability and mucus

production. At the histological level, the inclusion of plant ingredients has revealed changes potentially related to intestinal inflammation, based on the number of goblet cells, the level of infiltration of leukocytes, the grade of supranuclear vacuolization and the submucosa thickness [8,9,44,88], although major histopathological signs were not reported.

The changes observed in gut mucosa proteome in gilthead seabream could lead to an increased susceptibility to pathogens and a partial loss of intestinal functions [38]. In this sense, downregulation mucins gains relevance, since alters the mucus composition layer and therefore the epithelium protection, join the downregulation of proteins related to digestion [42].

**Effects on nutritional absorption/secretion**

The role of the gut mucosa on the absorption of nutrients could be also negatively affected by the total fishmeal replacement. The structural modifications in the gut epithelia described in seabream in response to plant protein inclusion in diets [8,9,93] could modulate nutrient transport, since transporters are immersed in the lipid membrane of the enterocytes. Moreover, the impact of plant sources on the digestive protease balance [9], the brush border enzyme activities [94] or the asynchronous utilization of amino acids from different origins [95] can lead to a lower luminal nutrient availability [96]. In fact, nutrient absorption in gilthead seabream is affected by the use of high levels of plant sources [44]. In the present work, it has been described altered proteins and Go terms related to metabolism, protein transport and the maintenance of enterocyte structure in VM group, which could have had a consequence on the nutrient assimilation performance [76], decreasing fish growth.

**Deficiencies of plant protein based diets**

The slight differences in diet formulation might explain the differences observed in gut proteome between the VM and VM+ groups. The VM+ diet contains a low amount of

698 synthetic amino acids respect to VM diet in turns to improve the essential amino acid
699 bioavailability, as was reported in previous trials [44]. Estruch et al. [44] observed an
700 increase of ammonia excretion in VM group in comparison with VM+ and FM groups
701 what suggests a lower-level protein synthesis due to an imbalance of ingested amino
702 acids, a higher catabolism level of amino acids and, ultimately, a lower growth.
703 Therefore, VM diet may be consider a deficient diet from a nutritional point of view, no
704 covering the minimum energy requirements. Moreover, diet AA imbalances in VM diet
705 can lead ultimately to immune dysfunctions, as already observed in previous
706 experiments [8,91]. Therefore, long-term feeding with nutritionally deficient diets could
707 be considered as a chronic stress, what that entails a high energy expenditure and
708 metabolic activity [89] and affecting the immune status [97]. Since immune mechanisms
709 require a continuous energy availability, they will face a lack of resources at a long-term,
710 which can be ultimately lead to a higher mortality [44]. Moreover, the allocation of most
711 of the energy expenditure to the maintenance of an effective immune response during
712 the trial could also explain differences in proteome observed in the present trial.
713 Finally, krill meal also provides in plant-based diets a high amount of phospholipids in
714 diets, ensuring the storage of energy, that can be mobilized for transport to tissues,
715 particularly important to overcome stressful conditions [98]. Besides, chitin, which is
716 present in the krill meal at 4%, could act on the seabream immune status , as noted in
717 previous experiments [91]. Fish fed winter diets containing 5% of krill exhibit higher
718 number of proteins upregulated in plasma regarding the immune system and cell
719 protection mechanisms than fish fed high dietary plant proteins level [99]. Nonetheless,
720 further research is necessary in order to better understand how small dietary changes
721 can have such high effects on the immune status of the fish that manifest, ultimately, in
722 fish growth, feed conversion ratio and survival, which are the most important parameters
723 from the productivity point of view.
724

725 ## Conclusions

In the present study, a long-term downregulation of proteins involved in epithelial permeability, inflammatory response and enterocyte homeostasis (including cell apoptosis, metabolism and protein transport) was observed in the gut mucosa of gilthead seabream with the complete replacement of fishmeal by plant ingredients, leading to poor growth and nutritive performance. This outcome suggests a possible suppression of the function of the gut epithelia over the long-term, which could be reversed with the inclusion of low amounts of alternative marine ingredients in plant based diets. Therefore, the inclusion of marine alternative ingredients in plant based diets has been demonstrated as more economical and environmentally sustainable option than 100% fishmeal or plant based diets.

# Declarations

## CRediT author statement

**Guillem Estruch**: Writing – Original Draft, investigation, formal analysis. **Silvia Martínez-Llorens**: Conceptualization, Methodology. **Ana Tomás-Vidal:** Investigation, Methodology. **Raquel Monge-Ortiz**: Investigation. **Miguel Jover-Cerdá**: Visualization. **Paul B. Brown**: Resources, Data Curation. **David S. Peñaranda**: Writing – Review & Editing, Supervision.


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## Consent for publication

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## Competing interests

The authors declare that they have no competing interests.

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## Figure captions

**Figure 1:** PCA three-dimensional plot, considering all the proteins identified in the MaxQuant assay

Percentages represent the variability of data sets which is explained by the different Principal Components

**Figure 2** PCA three-dimensional plot and heatmap plot of the proteins differentially expressed between groups

A) PCA plot considering intensity data. B) HeatMap plot considering intensity data.

Percentages in (A) represent the variability of data sets which is explained by the different Principal Components. HeatMap were constructed based on hierarchical clustering of samples using the average linkage as agglomeration method with Euclidean distances.

**Figure 3** Table of the common differentially expressed protein sets in the three two-groups comparison and their graphical representation using Venn diagrams.

A) Gene name of the common differentially expressed protein in the two groups comparison. B) Venn diagrams with the percentage and number of the common differentially expressed proteins in the three two-groups comparison.

Percentages are referred to the total number of differentially expressed proteins in each approach

**Figure 4** Go term enrichment analysis of differentially expressed proteins in the three GO annotation domains: Biological processes, Cell components and Molecular functions

An Enrichment analysis (two-tailed Fisher's Exact Test) of the GO terms for the three categories was performed using Blast2GO software (version 2.8.0) for the differential proteins obtained from FM vs VM comparison. The significance of the analysis (p-value)

is indicated besides of each bar, and the X axis indicates the number of proteins included in each GO term.

# Supplementary Material

**S1**. Peptides information (Raw data)

**S2**. Identified pepides (Raw data)

**S3**. Identified proteins (Raw data)

**S4.** Reproducibility assessment of the LC-MS/MS assay

**S5.** Proteins identified after filtering

**S6.** Proteins differentially expressed according to ANOVA analysis

**S7.** Proteins differentially expressed between experimental groups

**S8.** Enriched GO terms from the Blast2GO analysis

**S9**. Proteins in Enriched GO terms from the Blast2GO analysis

**S10.** Phagosome and Proteosome KEGG pathway and String analyses. A) Phagosome KEGG pathway; B) String analysis of altered proteins involved in Phagosome KEGG pathway; C) Proteosome KEGG pathway; D) String analysis of altered proteins involved in Proteosome KEGG pathway. Red stars indicated the altered proteins in each pathway