Microbiota composition and intestinal integrity remain unaltered after the inclusion of hydrolysed *Nannochloropsis gaditana* in *Sparus aurata* diet

I. M. Cerezo-Ortega1,5, D. E. Di Zeo-Sánchez2,5, J. García-Márquez2, I. Ruiz-Jarabo2,3, M. I. Sáez-Casado1, M. C. Balebona1, M. A. Moriñigo1 & S. T. Tapia-Paniagua1*

The use of lysed microalgae in the diet of carnivorous fish can increase the bioavailability of proteins and bioactive compounds, such as unsaturated fatty acids or vitamins in the digestive tract. These are essential molecules for the proper physiological development of fish in aquaculture. However, some antinutritional components and other undesirable molecules can be released from an excess of microalgae supplied, compromising the integrity of the intestine. The inclusion of small amounts of hydrolized microalgae in the fish diet can be a good strategy to avoid negative effects, improving the availability of beneficial compounds. *Nannochloropsis gaditana* is an interesting microalgae as it contains nutraceuticals. Previous studies reported beneficial effects after its inclusion in the diet of *Sparus aurata*, a widely cultured species in Europe and in all Mediterranean countries. However, administration of raw microalgae can produce intestinal inflammation, increased intestinal permeability, bacterial translocation and disturbance of digestion and absorption processes. The aim of this study was to evaluate changes in the intestinal microbiota and barrier stability of *S. aurata* fed with low inclusion (5%) hydrolysed *N. gaditana*. Intestinal microbiota was analyzed using Illumina MiSeq technology and libraries were constructed using variable regions V3–V4 of 16S rDNA molecules. Analysis were based in the identification, quantification and comparison of sequences. The predictive intestinal microbial functionality was analyzed with PICRUSt software. The results determined that the intestinal microbiota bacterial composition and the predictive intestinal microbiota functionality did not change statistically after the inclusion of *N. gaditana* on the diet. The study of gene expression showed that genes involved in intestinal permeability and integrity were not altered in fish treated with the experimental diet. The potential functionality and bacterial taxonomic composition of the intestinal microbiota, and the expression of integrity and permeability genes in the intestine of the carnivorous fish *S. aurata* were not affected by the inclusion of hydrolysed 5% *N. gaditana* microalgae.

**Abbreviations**

OTU  Operational taxonomic unit  
PCA  Principal Component Analysis  
KEGG  Kyoto encyclopedia of genes and genomes  
STAMP  Statistical Analysis of Metagenomics Profiles  
qPCR  Quantitative PCR  
SCFA  Short-chain fatty acid

1Department of Microbiology, Faculty of Sciences, CEI-MAR-International Campus of Excellence in Marine Science, University of Málaga, Málaga, Spain. 2Department of Biology, Faculty of Marine and Environmental Sciences, CEI-MAR-International Campus of Excellence in Marine Science, University of Cádiz, Cádiz, Spain. 3Department of Animal Physiology, Faculty of Biological Sciences, University Complutense, Madrid, Spain. 4Department of Biology and Geology, CEI-MAR-International Campus of Excellence in Marine Science, University of Almería, Almería, Spain. 5These authors contributed equally: I. Cerezo and D. E. Di Zeo-Sánchez. *email: stapia@uma.es
Research and improvement of commercial diets are crucial in aquaculture. The search for new sustainable products and the need to provide a better health status in aquaculture organisms are environmental priorities. In order to ensure an optimal development or produce a beneficial effect on organisms, new functional diets are currently being developed in the aquaculture industry.

Some strategies have been focused on the administration of probiotics, prebiotics, symbiotics, or its products, postbiotics, and bioactive compounds. The bioactive compounds are substances of natural origin with a high biological value, commonly found in terrestrial plants and algae because they are rich in proteins, essential amino acids, and polyunsaturated fatty acids. Nutraceutical compounds usually exert immunomodulatory and growth-promoting effects, reasons why their use has been proposed as a strong preventive strategy against opportunistic infections in the aquaculture industry and to improve cultured fish production.

Various genera of microalgae are also important sources of bioactive compounds, such as sterols, proteins, enzymes, pigments, or vitamins. One example is *Nannochloropsis*, which is a genus of robust, oleaginous microalgae that is considered a optimal candidate for commercial application. Specifically, *N. gaditana* (Lubian, 1982) is a promising alga for use in aquaculture. *N. gaditana* has a great photosynthetic efficiency and accumulates high quantities of carbon dioxide in the form of the lipids, such as omega-3 fatty acids (EPA, C20:5n-3), eicosapentaenoic acid (EPA, C22:6n-3), and docosahexaenoic acid (DHA, C22:6n-3). Also, it is a great pigment source, and contains aminoacidic and antioxidant compounds. Its production is not expensive, and it is used to feed fish, especially in their larval stages.

However, the presence of a cell wall with cellulose can reduce in vivo bioavailability of nutrients and other intracellular compounds when the algae is incorporated to the feed. This inaccessibility may also hide the expected positive results in the organisms. Four types of treatments to disrupt the algae cell wall to release their intracellular components have been described: enzymatic, chemical, physical, and mechanical. Each procedure provides different accessibility and modifications of the cellular components. Despite physical procedures are usually preferred as they provide greater digestibility while keeping the composition unaltered, enzymatic methods using enzymes with cellulase capacity have been successfully tested in culture fish and need to be considered. In particular, enzymatic hydrolysis of microalgae cell wall may be a promising category since this process occurs under mild temperatures and reduce the possibilities of inhibiting by-products release. However, the enzymatic hydrolysis process of the cell wall involves the risk of releasing intracellular substances that may cause undesirable physiological alterations to the fish. The liberation of molecules from cellular lysis may affect the intestinal microbiota composition and functionality or increase inflammation, intestinal permeability, or bacterial translocation in the intestine. For example, some microalgal species contain lectins, which are considered anti-nutritional elements. However, in gilthead seabream (*Sparus aurata*) juveniles fed with diets supplemented with *Arthrospira* enzymatic hydrolysates (2 and 4%), highlighted beneficial effects by describing increased activities of digestive enzymes such as trypsin, leucine aminopeptidase, or alkaline phosphatase, improving digestive processes and intestinal absorption capacity, and low inclusion of *N. gaditana* in *S. aurata* showed to be sufficient to obtain optimum growth without substantially increasing the cost of the feed.

The mucus layer and the intracellular structures known as tight junctions play a major role in regulating the paracellular passage and permeabilization of luminal elements, such as nutrients, toxic molecules or infectious agents across the intestinal barrier. In fish, the permeability and integrity of this barrier is particularly affected by stress, changes in the microbiota, the presence of pathogens, changes in salinity, and diet. However, the effects of algae hydrolysates on the taxonomic composition and functionality of intestinal microbiota and the stability of intestinal barrier have not yet been assayed. Those two aspects are implicated in digestion and absorption process, and closely related to the healthy status of fish. For these reasons, in this work, the analysis of the taxonomic composition and potential functionality of intestinal microbiota, and the measurement of the expression of genes related to the intestinal barrier stability in *S. aurata* fed with low inclusion of hydrolyzed *N. gaditana* have been carried out.

**Results**

**Sequencing data analysis.** Raw read sequences of the 16S rRNA gene from *S. aurata* gut microbiota in this study are publicly available in the NCBI SRA depository within BioProject PRJNA700500, with BioSample accession numbers SAMN17721432-SAMN17721459.

Illumina Miseq sequencing yielded a total of 22,605,600 reads. After bioinformatic processing, a total of 74,453,78 ± 9905.93 readings per sample were obtained and classified into 261 OTUs. The data size was normalized to 53,370 reads, which corresponds to the minimum number of reads obtained in all the samples. The sequences were filtered through the rarefaction curves at 53,370 readings, thus leaving 245 OTUs. Sequence coverage was ≥ 99% in all cases (Fig. 1).

The medium number of OTUs, and Chao1, Shannon and Simpson indexes were calculated for each sample of experimental groups (Table 1). The alfa diversity values in the two experimental groups and sections assayed did not show significant differences between the treatment Control-A and FH-A or Control-P and FH-P, where “A” means “anterior section” and “P” “posterior section” of intestine.

**Taxonomic composition of the intestinal microbiota.** The predominant *Phylum* detected in all fish fed with two diets were Proteobacteria, followed by Firmicutes, Cyanobacteria and Actinobacteria, and although the quantity of them were slightly different in function of the diet administrated, no significant differences were found among diets or intestinal sections (Fig. 2).

The bacterial families and group found in the anterior section of specimens were heterogeneous in all the groups. In this section of the intestine, Enterobacteriaceae, Pseudomonadaceae, Sphingomonadaceae, Propionibacteriaceae, Fusobacteriaceae and Actinomycetales, Vibrionales, and Bacilli, which were not identify to family...
level, its average values increase in FH-A compared to Control-A, but not significantly. *Clostridiaceae*, *Gemelaceae*, *Carnobacteriaceae* and *Tissierellaceae* were not present in the treatment FH-A, but they are present in Control A. *Fusobacteriaceae* was no present in Control-A but it appears in FH-A (Fig. 3A). In the posterior section, *Moraxellaceae*, *Vibrionaceae*, *Actinomycetales*, and *Bacilli_unclassified* increased, but *Enterobacteriaceae and Pseudomonadaceae* decreased in FH-P. *Carnobacteriaceae* family and *Clostridiales* order disappeared in FH-P treatment. Statistically significant differences were not detected in any case and data have been represented to show the variation between treatments (Fig. 3B).

Some genera were detected between diets As an example, in the anterior section some genera like *Clostridiales* or *Anaerococcus* are observable in Control-A but not in FH-A (Fig. 4A). In the posterior section, *Enhydrobacter*,

Figure 1. Rarefaction curves for each fish. Sequences were standardized to equal sample sizes for direct comparison.

| Ingredients (g/kg DM) | Control diet | FH diet |
|----------------------|--------------|---------|
| Fishmeal LP^a         | 200          | 200     |
| N. gaditana meal      | –            | 50      |
| Squid meal^b          | 20           | 20      |
| Fish protein hydrolysate^c | 10      | 10   |
| Krill meal^b          | 20           | 20      |
| Gluten meal^b         | 150          | 150     |
| Soybean protein concentrate^d | 366     | 335     |
| Fish oil              | 114          | 105     |
| Soybean lecithin      | 10           | 10      |
| Maltodextrin          | 42           | 32      |
| Lisin                 | 12           | 12      |
| Metionin              | 5            | 5       |
| Choline chloride^e     | 5            | 5       |
| Vitamin and mineral premix | 20      | 20     |
| Guar gum^f             | 10           | 10      |
| Alginates^g            | 10           | 10      |
| Crude protein (%)      | 47.7 ± 1.5   | 46.6 ± 0.8 |
| Crude lipid (%)        | 17.2 ± 0.5   | 16.5 ± 0.0 |
| Ash (%)                | 5.3 ± 0.3    | 5.4 ± 0.1 |

Table 1. Ingredient composition of experimental diets used in the feeding trial. FH diet contains 5% hydrolysed raw algal biomass. ^a Norsildemel (Bergen, Norway). ^b Lifebioencapsulation (Almería, Spain). ^c (81% crude protein, 8.8% crude lipid) Sopropeche (France). ^d (65% crude protein, 8% crude lipid) DSM (France). ^e Sigma-Aldrich (Madrid, Spain).
Actinomycetales or Bacilli unclassified increased their average values in the FH-P treatment, but Vibrio and Sphingomonas decreased in this group (Fig. 4B).

Principal coordinates analysis (PCoA) scores are plotted based on the relative abundance of OTUs of intestinal microbiota from analyzed specimens. Each point represents a single sample, and the distance between points represents how compositionally different the samples are from one another. The points did not show a clear differences in the microbial community composition between control and FH diet (Fig. 5).

**Predictive study about the intestinal microbiota functionality.** The functional study executed by PICRUSt software presented seven principal levels (metabolism, genetic information processing, environmental information processing, cellular processes, organismal systems, human diseases and drug development), but only three of them related to intestinal functions have been independently studied (metabolism, cellular processes and environmental information processing). Results obtained show that these functions were not affected by FH diet.

The three functions analyzed (metabolism, cellular processes and environmental information processing) did not change between treatments in the KEGG 1 category, although the processes related to environmental information processing and cellular processes were increased lightly in FH-A (Fig. 6A) and FH-P (Fig. 6B) treatments, but without statistical differences.

In KEGG 2 category, functions had similar values between treatments, except in the carbohydrates and amino acid metabolism that increased in FH-A (Fig. 6A) and FH-P (Fig. 6B), but statistical differences were not found.

Statistical differences were not found between intestinal sections in any studied KEGG 3 category.

**Gene expression.** In general, the FH group data showed less dispersion in the gene expression data than the control individuals. In addition, a trend was observed on down-regulation of the genes involved in the intestinal permeability, especially in the posterior section, and in the pro-inflammatory response in both intestine sections (p > 0.05) (Fig. 7A–M). Non-significant slightly up-regulation was also observed in the genes pept1 and muc2 related to nutrition and intestine protection, respectively, in the anterior section in fish receiving the FH diet (p > 0.05) (Fig. 7D,N). However, no significant differences were observed in the relative expression of the genes analyzed in the intestinal samples of fish fed with control or FH diets (Fig. 7).
Figure 3. Average values of bacterial family groups obtained in the intestinal microbiota samples in anterior (A) and posterior (P) sections (A,B, respectively) of S. aurata. Control (C-number) and N. gaditana hydrolyzed (FH-number) diets. ETC: families represented < 1%.
Figure 4. Average values of bacterial genera groups obtained in the intestinal microbiota samples in posterior (P) and anterior (A) sections (A, B, respectively). Control (C-number) and N. gaditana hydrolyzed (FH-number) diets. ETC: genera represented < 1%.
Discussion
The present study highlights that the carnivorous fish *S. aurata* fed with 5% of hydrolyzed microalgae *N. gaditana* did not modify its intestinal microbiota after 12 weeks. This could result in an advance in the formulation of fish diets as microalgae are important sources of essential compounds and beneficial nutriceuticals.

In the present study, the tendency of more homogeneous data (less values of variance) and medium values of diversity index between the anterior and posterior sections have been observed when the fish are fed with the FH diet. Reference41 concluded that changes in diversity are due to an adaptation of the microbiota to digest and assimilate the ingredients added, such as cell walls, polysaccharides and lipids, which affect or modify colonization by minority groups. Avoiding loss of diversity is considered as a positive aspect that protects intestinal mucose and gives protection against diseases42. Likewise, authors have established that microbial diversity is considered a biomarker for fish health and an image of a good metabolic capacity43 and recently44, affirm that *S. aurata* have a plastic microbiota which effectively adapts to the metabolic challenges induced by dietary changes. The same authors have proposed studies focused on how these microbial changes correlate with health, growth, and disease resilience.

Previous studies carried out by Ref.26 about the effect of *N. gaditana* on seabream's intestinal microbiota have not shown alterations in intestinal morphology and function with the same percentage of inclusion as in the present study, but the duration of the trial was limited to 1 month and *N. gaditana*’s entire cells were used. The phylum Actinobacteria, Firmicutes and Proteobacteria are part of the normal microbiota in *S. aurata*45–47. Some groups such as Micrococcaceae and Ralstonia present antimicrobial activity, biosynthesis of bioactive compounds and can produce beneficial secondary metabolites for the host48, and they have been detected in the intestinal microbiota of the FH group. Micrococcaceae family contain enzyme producing bacteria capable of producing amylases, cellulases, proteases, lipases, phytases, tannases, xylanase and chitinase49. These enzymes can contribute to the digestion and assimilation of algal products. According to Ref. 50 members of Actinobacteria group are butyrate-producing bacteria. Butyrate is a short-chain fatty acid (SCFA) with important and demonstrated beneficial effects, also in fish such as *S. aurata*51. In this study Actinobacteria have been detected but the fine representation of all the members of this group may be minor to 1% and they are incluyed in ETC group (0.1% Streptococcus, or 0.6% Actinomyces, among others).

In the posterior section, FH group showed a low increase in the presence of Actinobacteria and a decrease in Cyanobacteria, but not statistically significant. Cyanobacteria in general produces lipopolysaccharides that are inflammatory agents and gastrointestinal irritants52 and causes damage in the host by facilitating the colonization of other dangerous bacteria53,54.

Clostridiaceae is a family that appeared in FH-P group. Those group is related to dysbiosis in human55 but in other animals such as pigs, Clostridiaceae has been related to improvements in feed efficiency and growth performance, being also considered beneficial to fish44. FH-A group showed Bacilli and Actinomycetales. Those groups are present when the diet is enriched in vegetals56.

Some genera were detected in the present study were significantly different between samples. In the anterior and posterior sections it was observable genera like Clostridiales in Control-A but was not present in FH-A. In the posterior section, Enhydrobacter appearedin the treatment FH-P. Enhydrobacter is present in the microbiota of fish such as Dicentrarchus labrax and Salmo trutta57. It is known that this genus utilizes certain amino acids such as l-arginine, l-serine and l-alanine58, while some species present cellulase activity59,60. This fact may be of interest as cellulases hydrolyze the cellular wall of *N. gaditana*, allowing the fish to absorb the intracellular compounds of this algae. Thus, the intestinal microbiota of *S. aurata* is able to adapt to dietary changes, as proposed before44.

Figure 5. Principal principal coordinate analysis (PCoA) of the similarity of microbiota (OTUs) in anterior and posterior sections (A,B, respectively) of each individual of *S. aurata* fed with a Control or *N. gaditana* hydrolyzed (FH) diets.
By a taxonomical description of the bacterial groups present in the intestinal microbiota the information about their function at the gut is unknown. The disponibility of a predictive software allows us to relate the sequences obtained with the OTUs and the predominant functions going on at the intestine. In this study, no differences were found in any of the seven principal functional pathways. The three functional pathways analyzed, metabolism, cellular processes and environmental information processing represented 63.12 ± 0.83% of total functional enrichment per sample. Additionally, KEGG 2 and 3 levels were analyzed but none of them were statistically different between treatments with the exception of a slight increasing trend detected in the

Figure 6. PICRUSt predictions of the functional composition of S. aurata gut microbiota. (A) Represents KEGG pathway at level 1,2 and 3, in anterior section and (B) in posterior section. Blue and yellow bars make reference to Control and FH diets, respectively.

By a taxonomical description of the bacterial groups present in the intestinal microbiota the information about their function at the gut is unknown. The disponibility of a predictive software allows us to relate the sequences obtained with the OTUs and the predominant functions going on at the intestine. In this study, no differences were found in any of the seven principal functional pathways. The three functional pathways analyzed, metabolism, cellular processes and environmental information processing represented 63.12 ± 0.83% of total functional enrichment per sample. Additionally, KEGG 2 and 3 levels were analyzed but none of them were statistically different between treatments with the exception of a slight increasing trend detected in the
Figure 7. Boxplots for relative gene expression in the intestine of gilthead seabream fed with the experimental diet (FH) and the control group (C). (A) necrosis tumoral factor α (tnf-α); (B) cyclooxygenase 2 (cox2); (C) intestine mucin (imuc); (D) mucin 2 (muc2); (E) cadherin 1 (cdh1); (F) cadherin 17 (cdh17); (G) claudin 12 (cldn12); (H) claudin 15 (cldn15); (I) zona-occludens 1 (zoi1); (J) ocludin (ocun); (K) vimentin (vim); (L) integrin 6-β (itgb6); (M) tubulin (tub) and N, peptide transporter 1 (pept1). Bars represent relative gene expression (mean + standard error) for each group, in the intestinal anterior section (A) and the posterior section (P). The crosses correspond to the means and the horizontal bars to the median. Points above or below the upper and lower limits are considered outliers. Differences between experimental groups in each section were considered at p < 0.05.
FH treatment (but not statistically significative). It is important to point out that the inclusion of hydrolyzed *N. gaditana* does not increase the presence of functions related to bacterial toxin synthesis. The rest of the functions...
pept1 gene expression between the groups analyzed. However, both Control and FH groups showed higher expression levels of pept1 in the anterior intestine section compared to the posterior section. Additionally, a slight up-regulation of pept1 was observed in the FH group compared to the control group in the anterior intestine section. Higher pept1 expression in the anterior section is consistent with studies indicating that the proximal intestine is the area of highest expression of this protein in fish, specifically at the absorptive epithelial cells along the mucosal folds. The trend indicating a slight up-regulation of pept1 in the FH group may suggest improved nutrient absorption. In higher vertebrates, more weight gain and increased expression of pept1 in the intestine were found in response to a high-quality soybean protein diet. Reference found that a diet containing 15% of vegetable proteins (green pea protein concentrate) induced significantly lower levels of pept1 transcripts and were associated with lower growth in gilthead seabream. Gilthead seabream fed on a strict vegetable protein diet also showed lower expression levels of pept1, and has been related to a lower digestibility and small peptide transport. Taking pept1 gene expression as a marker of the dietary protein quality and absorption efficiency, our results suggest that the substitution of 5% of N. gaditana in the diet is probably not affecting the nutrient intake in gilthead seabream, or that the high dispersion between individuals may be affecting the significance of the results and, therefore, hiding possible beneficial effects.

Mucins contribute to protect the intestine epithelium against a broad spectrum of damages. Enhanced gut mucin production has been related to reduced bacterial translocation in fish fed with a diet containing yeast-derived oligosaccharides. In this study, the inclusion of N. gaditana did not affect the mucins related genes (muc2 and imuc) between the Control and FH groups.

In summary, the inclusion of 5% N. gaditana microalgae subjected to enzymatic hydrolysis in the commercial diet does not alter the composition and functionality of the intestinal microbiota, nor the expression of integrity and permeability genes in the intestine of the carnivorous fish S. aurata.

Materials and methods

Feed preparation. The N. gaditana biomass was produced in closed tubular photobioreactors in La Estación Experimental de las Palmerillas (Fundación Cajamar, Almería, Spain) as reported by Ref. Briefly, N. gaditana sludge containing up to 150 g L⁻¹ of raw microalgae biomass was hydrolyzed using commercial enzymes with cellulase activity (Viscozyme) under controlled conditions (pH 5.0 and 50 °C under continuous stirring) for 4 h, providing 2% (w/w) enzyme. The experimental aquafeed was elaborated at the CEIA3-Universidad de Almería facilities (Servicio de Prenios Experimentales, http://www.ua.es/stecnicos_spe) including 5% of the hydrolyzed algae (FH diet). The microalgae concentrations employed were based on previous studies. The solids were mixed in a kneader and, subsequently, the resulting mixture was extruded to obtain granules with the desired diameter (2–3 mm). Feed was stored at −20 °C until use. Control feed composition is showed in Table 1.

Experiment design and sampling. The feeding assay was carried out at Servicios Centrales de Investigación en Cultivos Marinos (SCI-CM, CASEM, University of Cádiz, Puerto Real, Cádiz, Spain; Facilities for Breeding, Supplying and Users of Experimental Animals; Spanish Operational Code REGA ES11028000312). In the trial, juveniles of gilthead seabream of approximately 17.08 ± 0.38 g and 10.2 ± 0.1 cm fork length were represented in the same quantity. The hydrolyzed cells do not affect negatively the bacterial functions, studied by predictive form.

In other studies with S. aurata juveniles, it was observed that low dietary supplementation with Arthrosira hydrolysates (2 and 4%) had no effects on the intestinal mucosa. Similar results were found by Saenz et al. (personal communication/unpublished results) in S. aurata within the present study. In this sense, the results reported by a visual analysis of images obtained by transmission electron microscopy (TEM) and scanning electron microscopy (SEM) have not revealed signs of damage or irritation in the intestinal microvilli. In this regard, N. gaditana in the diet of S. aurata does not alter neither the intestinal microbiota composition nor its functions, significantly. Our in vivo results support previous information described in vitro by Ref., where N. gaditana added to S. aurata intestinal extract lead to an improvement in the digestion and assimilation of nutrients.

The inclusion of 5% N. gaditana in the diet has not produced any significant alteration in the expression of genes involved in the enterocytes cell-to-cell adhesion, the cytoskeleton and the epidermal cell-base membrane adhesion (cdh1, cdh17, cdh12, cdhn15, vim, itgb6, ocln, tub, and zol) and, therefore, it is very likely that the integrity and permeability of the analyzed fish remain unaltered. This information is further supported by the absence of changes in the expression of markers commonly used to measure pro-inflammatory processes (inf-a and cox2), which have been associated with increased permeability and intestinal disruption in several studies. The absence of statistically significant differences in our study could also be due to a high dispersion between individuals. However, a tendency on a down-regulation of the tight-junction genes analysed was observed in the FH group. Experimental diets that have shown to impair fish growth have also been related to a reduction in the expression of genes involved in maintaining tight junctions whereas their upregulation has been associated with a healthier intestinal barrier. Contradictorily, a gene up-regulation of tight junctions in response to various experimental diets has also been related to nutritional, osmoregulatory and physiological alterations, thus indicating an undesirable state in gilthead seabream. Therefore, further analysis of immune system-related genes, as well as histological studies are necessary to confirm the actual status of the gut integrity and permeability.

The peptide transporter 1 (PepT1) is a low-affinity/high-capacity nutrient transporter that mediates the uptake of dipeptides and tripeptides from diet, which are essential for fish to sustain development, growth and metabolism. In our study, no statistically significant difference was observed in pept1 gene expression between the groups analyzed. However, both Control and FH groups showed higher expression levels of pept1 in the anterior intestine section compared to the posterior section. Additionally, a slight up-regulation of pept1 was observed in the FH group compared to the control group in the anterior intestine section. Higher pept1 expression in the anterior section is consistent with studies indicating that the proximal intestine is the area of highest expression of this protein in fish, specifically at the absorptive epithelial cells along the mucosal folds. The trend indicating a slight up-regulation of pept1 in the FH group may suggest improved nutrient absorption. In higher vertebrates, more weight gain and increased expression of pept1 in the intestine were found in response to a high-quality soybean protein diet. Reference found that a diet containing 15% of vegetable proteins (green pea protein concentrate) induced significantly lower levels of pept1 transcripts and were associated with lower growth in gilthead seabream. Gilthead seabream fed on a strict vegetable protein diet also showed lower expression levels of pept1, and has been related to a lower digestibility and small peptide transport. Taking pept1 gene expression as a marker of the dietary protein quality and absorption efficiency, our results suggest that the substitution of 5% of N. gaditana in the diet is probably not affecting the nutrient intake in gilthead seabream, or that the high dispersion between individuals may be affecting the significance of the results and, therefore, hiding possible beneficial effects.

Mucins contribute to protect the intestine epithelium against a broad spectrum of damages. Enhanced gut mucin production has been related to reduced bacterial translocation in fish fed with a diet containing yeast-derived oligosaccharides. In this study, the inclusion of N. gaditana did not affect the mucins related genes (muc2 and imuc) between the Control and FH groups.

In summary, the inclusion of 5% N. gaditana microalgae subjected to enzymatic hydrolysis in the commercial diet does not alter the composition and functionality of the intestinal microbiota, nor the expression of integrity and permeability genes in the intestine of the carnivorous fish S. aurata.
(mean ± SEM) were randomly distributed in 6 tanks of 80 L capacity (N = 15 fish per tank). The experiment was divided into two different fed conditions, one receiving a commercial diet (control group) and the other receiving a diet supplemented with 5% of fresh hydrolyzed *N. gaditana* paste (FH group) for 86 days. At the end of the growth assay, the animals (fasted for 12 h) were netted and deeply anesthetized with 2-phenoxethanol (1 mL/L, Sigma–Aldrich 77699), and intestinal samples from seven fish per treatment (three fish from one tank, and two fish from the other two tanks) were extracted. Then, each trawl was divided into two major sections, anterior and posterior sections, and kept at −80 °C until use. Remaining fish from each tank were employed in a parallel study (unpublished results).

**DNA extraction and sequencing by Illumina Miseq technology.** DNA of both intestinal sections were extracted according to the protocol described by Ref. [72] based on saline precipitation, with modifications [73]. Briefly, the samples were mixed with 300 μL of resuspension buffer (0.1 M Tris–HCl, 0.01 M NaCl, 0.1 M EDTA, pH 8) and 300 μL of lysis buffer (0.1 M Tris–HCl, 0.1 M EDTA, 0.01 M NaCl, 1% SDS, pH 8.0), gently inverting the tube to mix thoroughly. The samples were treated with 32 μL NaCl 6 M and proteinase K (150 μg/mL) at 55 °C for 2 h, and lysozyme (10 mg/mL) at room temperature. Next, 6 M NaCl was added to reach a final concentration of 1.5 M. The solution was chilled on ice for 10 min followed by centrifugation at 13,000 rpm for 3 min. The clear supernatant containing genomic DNA was transferred to another tube containing an equal volume of isopropanol. The tubes were inverted gently several times. The DNA was pelleted by centrifugation at 13,000 rpm for 3 min. The DNA pellet was then washed in 70% ethanol. The dried DNA pellet was resuspended in 100 μL of TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) and stored at 4 °C. DNA quality and integrity were visualized by electrophoresis in 1% agarose gels, stained with GelRed Nucleic Acid Stain 20000x (InTRON Biotechnology, Seoul, Korea). Concentration was determined using Qubit 2.0 fluorometer (Thermo Fisher Scientific, Germany). DNA was stored at −20 °C for further processing and 30 ng were used for subsequent analyses.

Libraries were constructed by the Ultrasquencing Service of the Bioinnovation Center (University of Malaga, Spain) using the Illumina MiSeq Platform. Sequencing was carried out using forward (5′ TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTATGGAAGAGG GAC TATCCTAATCC-3′) and reverse (5′ GTCTCGTGGGCTCAGGA GATGTGTAATAGACAGAGAGACTACHVGGGTATCTAATCC-3′) primers targeting the V3–V4 variable regions of the 16S rDNA gene.

A workflow based on the MOTHER program (version 1.39.5) was used to remove Illumina adapter sequences and demultiplexing. The reads were filtered excluding reads < 80 bp or > 2000 bp long. Besides, the singleton sequences and the chimeras were discarded by UCHIME version 4.2 (https://drive5.com/uchime). Non-specific PCR amplicons were eliminated. The remaining representative, non-chimeric sequences were then subjected to taxonomic assignment against the Greengenes 16S database (May 2013), with 97% 16S similarity as the cutoff. PCR amplicons were eliminated. The remaining representative, non-chimeric sequences were then subjected to taxonomic assignment against the Greengenes 16S database (May 2013), with 97% 16S similarity as the cutoff. Non-parametric Kruskal–Wallis statistical test was performed. The differences were considered statistically significant assuming p < 0.05. Finally, a multivariate analysis of OTU data was performed via Principal Coordinate Analysis (PCoA) of OTU profiles using Bray Curtis metric to represent differences and clustered into Operational Taxonomic Units (OTUs).

After generating the taxonomic profile of microbiome samples, a comparison of taxa present in the samples was carried out. The data size was normalized to the minimum number of reads obtained in all the samples. All statistical analyses were performed using statistical software R and a web tool MicrobiomeAnalyst [4]. To determine the level of sequencing depth, rarefaction curves were obtained by plotting the number of observed OTUs against the number of sequences and Good’s coverage coefficient calculated. Alpha diversity was estimated based on Shannon, Chao1, and Simpson indexes to assay taxonomic and phylogenetic structure diversity, respectively. The results are presented at phylum, family and genus taxonomic levels. The group with relative abundance less than 1% have been considered “ETC” according to Ref. [75] where’s taxa constituting ≥ 1% of the total number of cells and ‘rare phylotypes’ as taxa constituting ≤ 0.1% of the total number of cells. This limit is defined based on the levels that can be detected with PCR-dependent techniques [76]. We have used 1% because this is the limit of detection in molecular techniques such as DGGE, which we have used in the past to study intestinal microbiota [77–79]. Non-parametric Kruskal–Wallis statistical test was performed. The differences were considered statistically significant assuming p < 0.05. Finally, a multivariate analysis of OTU data was performed via Principal Coordinate Analysis (PCoA) of OTU profiles using Bray Curtis metric to represent differences between microbiota of each group. PCoA are plotted against each other to summarize the microbial community compositional differences between samples.

**Functional profiling of microbial communities.** Predictive microbiota functions were made using PICRUSt (version 1.1.3), a tool designed to infer metagenomes information from 16S rRNA amplicon sequencing data [40]. The resulting metagenomics data were entered into the Greengenes database (version 13.5), and the metagenome prediction of bacterial communities was conducted using the calculated dataset after normalizing for DNAr16S copy number. Nearest Sequenced Taxon Index (NSTI) scores for evaluating the accuracy of predicted metagenomes were categorized with the Kyoto encyclopedia of genes and genomes (KEGG) pathways database [41]. Bacterial functional profiles until KEGG modules level 3 were compared, and the STAMP (Statistical Analysis of Metagenomis Profiles) was used to analyze the differential abundance of modules by intestinal sections and diet using ANOVA multiple-comparison test with post hoc Tukey–Kramer (p < 0.05).

**RNA isolation and quantitative PCR (qPCR).** In order to assess the gene expression throughout the intestinal tract, RNA was isolated from each intestine section, following TRIsure™ (Bioline, England) manufacturer instructions. RNA quantity was determined in Qubit (Thermo Scientific) and reverse transcription was performed using First Strand cDNA Synthesis Kit (Thermo Scientific) with 500 ng of total RNA. One microliter of each cDNA was employed as the template in the qPCRs to analyze each gene transcription. Specific primers were used for the quantification of the relative gene expression genes involved in intestinal permeability and
Statistical analysis was performed using XLSTAT v2014.5.03 software (Addinsoft, New York, NY, USA). Results are shown as means of fold change ($2^{(\Delta \Delta Cq)}$) ± standard deviation (SD). The normality and homogeneity of the data were previously evaluated using the Shapiro–Wilk and Levene tests, respectively. The existence of statistically significant differences in the RT-qPCR values between the control and FH groups was determined by One-Way Analysis of Variance (ANOVA), applying the Tukey post-hoc test. When normality of the data could not be assumed, non-normal data were logarithmic transformed, and the non-parametric Kruskal–Wallis test was performed. The differences were considered statistically significant assuming $p < 0.05$.

**Ethics approval.** Fish were kept and handled following the guidelines for experimental procedures in animal research from the Ethics and Animal Welfare Committee of the University of Cadiz, according to the Spanish (RD53/2013) and European Union (2012/707/EU) legislation. The Ethical Committee from the Autonomous Andalusian Government approved the experiments (Junta de Andalucía reference number 01/04/2019/047).

**Declaration ARRIVAL 2.0 protocol.** This study is reported in accordance with ARRIVE guidelines (https://arriveguidelines.org).

**Consent for publication.** All the authors read and agree to the content of this paper and its publication.

**Data availability**

Raw read sequences of the 16S rRNA gene from *S. aurata* gut microbiota in this study are publicly available in the NCBI SRA depository within BioProject PRJNA700500, with BioSample accession numbers SAMN17721432-SAMN17721459.

Received: 21 March 2021; Accepted: 30 August 2021
Published online: 21 September 2021

**References**

1. Thilsted, S. H. et al. Sustaining healthy diets: The role of capture fisheries and aquaculture for improving nutrition in the post-2015 era. *Food Policy* **61**, 126–131 (2016).
2. Won, S. et al. Evaluation of dietary natural mineral materials as an antibiotic replacer on growth performance, non-specific immune responses and disease resistance in rainbow trout, *Onchorhyncus mykiss*. *Aquac. Res.* **48**, 4735–4747 (2017).
3. Gaber, M. M. The effects of plant-protein-based diets supplemented with yucca on growth, digestibility, and chemical composition of Nile Tilapia (*Oreochromis niloticus*, L.) fingerlings. *J. World Aquac. Soc.* **37**, 74–81 (2006).
4. Bae, J. et al. Evaluation of seven different functional feed additives in a low fish meal diet for olive flounder, *Paralichthys olivaceus*. *Aquaculture* **525**, 735333 (2020).
5. Amenyogbe, E. et al. The exploitation of probiotics, prebiotics and synbiotics in aquaculture: Present study, limitations and future directions: A review. *Aquac. Int.* **28**, 1017–1041 (2020).

|          | OTUs        | Chao 1     | Shannon   | Simpson   | Good coverage (%) |
|----------|-------------|------------|-----------|-----------|-------------------|
| Control-A| 52.57 ± 11.82 | 70.50 ± 19.28 | 2.75 ± 0.26 | 0.89 ± 0.02 | 99.98 ± 0.005     |
| Control-P| 48.57 ± 3.95  | 68.15 ± 18.13 | 2.47 ± 0.25 | 0.80 ± 0.03 | 99.99 ± 0.001     |
| FH-A     | 48.29 ± 5.79  | 58.75 ± 11.85 | 2.12 ± 0.45 | 0.87 ± 0.11 | 99.98 ± 0.007     |
| FH-P     | 47.14 ± 5.25  | 65.75 ± 13.74 | 2.54 ± 0.21 | 0.88 ± 0.02 | 99.99 ± 0.004     |

**Table 2.** Number of OTUs and alpha diversity indexes of bacterial communities in the anterior (A) and posterior (P) intestinal sections of *S. aurata* fed with control (Control) and *N. gaditana* (FH) diets. Mean values and standard deviation were represented.
6. Beltrán, J. M. G., Espinosa, C., Guardiola, F. A. & Esteban, M. A. In vitro effects of Origanum vulgare leaf extracts on gilthead seabream (Sparus aurata L.) leucocytes, cytotoxic, bactericidal and antioxidant activities. Fish Shellfish Immunol. 79, 1–10 (2018).

7. Gisbert, E. et al. Olive oil bioactive compounds increase body weight, and improve gut health and integrity in gilthead seabream (Sparus aurata). Br. J. Nutr. 117, 351–363 (2017).

8. Silveira Júnior, A. M., Faustino, S. M. M. & Cunha, A. C. Bioprospection of biocompounds and dietary supplements of microalgae with immunostimulating activity: A comprehensive review. PeerJ 7, e7685 (2019).

9. Bullón, C., Volpatti, D. & Galeotti, M. Current research on the use of plant-derived products in farmed fish. Aquac. Res. 46, 513–551 (2015).

10. Almendinger, M. et al. Characterization of selected microalgae and cyanobacteria as sources of compounds with antioxidant capacity. Algal Res. 53, 102168 (2021).

11. Michalak, Ł. & Chojnacka, K. Algae as production systems of bioactive compounds. Eng. Life Sci. 15, 160–176 (2015).

12. Plaza, M. et al. Screening for bioactive compounds from algae. J. Pharnm. Biomed. Anal. 51, 450–455 (2010).

13. Chakrabarty, S. B., Horn, P. & Hancz, C. Application of phytochemicals as growth-promoters and endocrine modulators in fish culture. Rev. Aquac. 6, 1–19 (2014).

14. Chi, C. et al. Immunomodulatory effects of a bioactive compound isolated from Drypoтирis crassirhizoma on the Grass Carp. Clinophytophysicodend idella. J. Immunol. Res. 2016, 1–10 (2016).

15. Hoseinifar, S. H. In vitro effects of Origanum vulgare leaf extracts on gilthead seabream (Sparus aurata) fed on Gracilaria gracilis powder. Fish Shellfish Immunol. 83, 232–237 (2018).

16. Elumalai, P. et al. Characterization of selected microalgae and cyanobacteria as sources of compounds with antioxidant capacity. PeerJ 7, e7685 (2019).

17. Xu, S. et al. Evaluation of dried seaweed Gracilaria lemaneiformis as an ingredient in diets for teleost fish Siganus canaliculatus. Aquac. Int. 19, 1067–1018 (2011).

18. Xuan, X., Wen, X., Li, S., Zhu, D. & Li, Y. Potential use of macro-algae Gracilaria lemaneiformis in diets for the black sea bream, Acanthoptus schlegeli, juvenile. Aquaculture 412–413, 167–172 (2013).

19. Younis, E.-S.M., Al-Quffail, A. S., Abdel-Warith, A.-W.A. & Al-Hafedh, Y. S. Effect of dietary fish meal replacement by red algae, Gracilaria arciata, on growth performance and body composition of Nile tilapia Oreochromis niloticus. Saudi J. Biol. Sci. 25, 198–203 (2018).

20. Li, J. et al. Characterization of lipopolysaccharides and liposomes of Nannochloropsis reveals the mechanisms of oil synthesis in microalgae. Plant Cell 26, 1645–1665 (2014).

21. Ryckebosch, E. et al. Nutritional evaluation of microalgae oils rich in omega-3 long chain polyunsaturated fatty acids as an alternative for fish oil. Food Chem. 160, 393–400 (2014).

22. Camacho-Rodríguez, J. et al. A quantitative study of eicosapentaenoic acid (EPA) production by Nannochloropsis gaditana for aquaculture as a function of dilution rate, temperature and average irradiance. Appl. Microbiol. Biotechnol. 98, 2429–2440 (2014).

23. Bigogno, C. Lipid and fatty acid composition of the green oleaginous alga Parachlorella incisa, the richest plant source of arachidonic acid. Phytochemistry 60, 497–503 (2002).

24. Lubían, L. M. et al. Nannochloropsis (Eustigmatophyceae) as source of commercially valuable pigments. J. Appl. Phycol. 12, 249–255 (2000).

25. Millao, S. & Uguique, E. Antioxidant activity of supercritical extracts from Nannochloropsis gaditana: Correlation with its content of carotenoids and tocopherols. J. Fluids 111, 143–150 (2016).

26. Jorge, S. S. et al. Short-term supplementation of gilthead seabream (Sparus aurata) diets with Nannochloropsis gaditana modulates intestinal microbiota without affecting intestinal morphology and function. Aquac. Nutr. 25, 1388–1398 (2019).

27. Li, Q., Zhou, Z., Zhang, D., Wang, Z. & Cong, W. Lipid extraction from Nannochloropsis gaditana Disruption of lipid and its effect on fatty acid composition. J. Supercrit. Fluids 19–24 (2001).

28. Matos, A. P. et al. Disruption of Nannochloropsis gaditana (Eustigmatophyceae) rigid cell wall by non-thermal plasma prior to lipid and its effect on fatty acid composition. Braz. J. Chem. Eng. 36, 1419–1428 (2019).

29. Lee, A. K., Lewis, D. M. & Ashman, P. J. Disruption of microalgal cells for the extraction of lipids for biofuels: Processes and specific energy requirements. Biomas Bioenergy 46, 89–101 (2012).

30. Mendes-Pinto, M. M., Raposo, M. F. J., Bowen, J., Young, A. J. & Morais, R. Evaluation of different cell disruption processes on encysted cells of Haematococcus pluvialis: Effects of astatinum recovery and implications for bio-availability. J. Appl. Phycol. 13, 19–24 (2001).

31. Yap, B. H., Dumsday, G. J., Scales, P. J. & Martin, G. J. O. Energy evaluation of algal cell disruption by high pressure homogenisation. Bioreour. Technol. 184, 280–285 (2015).

32. Vizcaino, A. J., Sáez, M. I., Martínez, T. F., Acén, F. G. & Alarcón, F. I. Differential hydrolysis of proteins of four microalgae by the digestive enzymes of gilthead sea bream and Senegalese sole. Algal Res. 37, 145–153 (2019).

33. Mahdy, A., Mendez, L., Blanco, S., Ballesteros, M. & González-Fernández, C. Protease cell wall degradation of Chlorella vulgaris: Effect on methane production. Bioreour. Technol. 171, 421–427 (2014).

34. Zhang, J.-X. et al. Soybean β-conglycinin induces inflammation and oxidation and causes dysfunction of intestinal digestion and absorption in fish. PLoS One 8, e58115 (2013).

35. Torrecillas, S. et al. Dietary phytoerythrin and galactomannan oligosaccharides in low fish meal and fish oil-based diets for European sea bass (Dicentrarchus labrax) juveniles: Effects on gut health and implications on in vivo gut bacterial translocation. PLoS One 14, e0222063 (2019).

36. Alarcón, F. Effect of inhibitors present in protein sources on digestive proteases of juvenile sea bream (Sparus aurata). Aquat. Living Resour. 12, 233–238 (1999).

37. Sathasivam, R., Radhakrishnan, R., Hashem, A. & Abd Allah, E. F. Microalgae metabolites: A rich source for food and medicine. Saudi J. Biol. Sci. 26, 709–722 (2019).

38. Galafat, A. et al. Evaluation of Arthrosira sp. enzyme hydrolysat e as dietary additive in gilthead seabream (Sparus aurata) juveniles. J. Appl. Phycol. 32, 3089–3100 (2020).

39. Ayala, M. D. et al. Influence of low dietary inclusion of the microalga Nannochloropsis gaditana (Lubian 1982) on performance, fish morphology, and muscle growth in juvenile Gilthead Seabream (Sparus aurata). Animals 10, 2270 (2020).

40. Sundh, H. & Sundell, K. S. Microalgal Health in Aquaculture Environmental Impacts on Fish MSC. Microalg. Health in Aquaculture (Elsevier Inc., 2015). https://doi.org/10.1016/B978-0-12-417186-2-00007-8

41. Nayak, S., Al Ashhab, A., Zilberg, D. & Khozin-Goldberg, I. Dietary supplementation with omega-6 LC-PUFA-rich microalgae regulates mucosal immune response and promotes microbial diversity in the Zebrafish Gut. Biology 9, 119 (2020).

42. Nayak, S., Khozin-Goldberg, I., Cohen, G. & Zilberg, D. Dietary supplementation with ω6 LC-PUFA-rich algae modulates zebrafish immune function and improves resistance to streptococcal infection. Front. Immunol. 9, 1960 (2018).

43. De Schryver, P. & Vadstein, O. Ecological theory as a foundation to control pathogenic invasion in aquaculture. JMSF 8, 2360–2368 (2014).

44. Piazzon, M. C. et al. Genetic selection for growth drives differences in intestinal microbiota composition and parasite disease resistance in gilthead sea bream. Microbiome 8, 168 (2020).
Scientific Reports

45. Estruch, G. et al. Impact of fishmeal replacement in diets for gilthead sea bream (Sparus aurata) on the gastrointestinal microbiota determined by pyrosequencing the 16S rRNA gene. PLoS One 10, e0136389 (2015).

46. Fonseca, E., Cerqueira, R. & Fuentes, J. Impact of ocean acidification on the intestinal microbiota of the marine sea bream (Sparus aurata L.). Front. Physiol. 10, 1446 (2019).

47. Tapia-Paniagua, S. T. et al. The effect of spray-dried porcine plasma on gilthead seabream (Sparus aurata) intestinal microbiota. Aquac. Nutr. 26, 801–811 (2020).

48. Jami, M., Ghanbari, M., Kneifel, W. & Domig, K. J. Phylogenetic diversity and biological activity of culturable Actinobacteria isolated from freshwater fish gut microbiota. Microbiol. Res. 175, 6–15 (2015).

49. Ray, A. K., Ghosh, K. & Ringø, E. Enzyme-producing bacteria isolated from fish gut: A review. Aquac. Nutr. 18, 465–492 (2012).

50. Parada Venegas, D. et al. Short chain fatty acids (SCFAs)-mediated gut epithelial and immune regulation and its relevance for inflammatory bowel diseases. Front. Immunol. 10, 277 (2019).

51. Estensoro, I. et al. Dietary butyrate helps to restore the intestinal status of a marine teleost (Sparus aurata) fed extreme diets low in fish meal and fish oil. PLoS One 11, e0166564 (2016).

52. Toxic Cyanobacteria in Water: A Guide to Their Public Health Consequences, Monitoring, and Management. (E & FN Spon, 1999).

53. Suo, Y. et al. Response of gut health and microbiota to sulfide exposure in Pacific white shrimp Litopenaeus vannamei. Fish Shellfish Immunol. 63, 87–96 (2017).

54. Zanchett, G. & Oliveira-Filho, E. Cyanobacteria and cyanotoxins: From impacts on aquatic ecosystems and human health to anticarcinogenic effects. Toxins 5, 1896–1917 (2013).

55. Le Bastard, Q. et al. Systematic review: Human gut dysbiosis induced by non-antibiotic prescription medications. Aliment Pharmacol. Ther. 47, 332–345 (2018).

56. Gilbert, E. R., Li, H., Emmerson, D. A., Webb, K. E. & Wong, E. A. Dietary protein quality and feed restriction influence abundance of nutrient transporter mRNA in the small intestine of broiler chicks. J. Nutr. 138, 262–271 (2008).

57. Estruch, G. et al. Long-term feeding with high plant protein based diets in gilthead seabream (Sparus aurata, L.) leads to changes in the inflammatory and immune related gene expression at intestinal level. BMC Vet. Res. 14, 1–15 (2018).

58. Menegol, T. et al. Mixed trophic production of polyunsaturated fatty acids and carotenoids by the microalga Nannochloropsis gaditana. J. Appl. Phycol. 31, 2823–2832 (2019).

59. Martinez, G., Shaw, E. M., Carrillo, M. & Zanuy, S. Protein salting-out method applied to genomic DNA isolation from fish whole blood. Biotechniques 50, 238–239 (1998).

60. Dharwal, A. et al. MicrobiomeAnalyst: A web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. Nucleic Acids Res. 45, W180–W188 (2017).

61. Pedrós-Aló, C. Marine microbial diversity: Can it be determined? Trends Microbiol. 14, 257–263 (2006).

62. Parada Venegas, D. et al. Dietary impacts on the intestinal microbiota of a marine teleost (Sparus aurata) fed extreme diets low in fish meal and fish oil. PLoS One 11, e0166564 (2016).

63. Toxic Cyanobacteria in Water: A Guide to Their Public Health Consequences, Monitoring, and Management. (E & FN Spon, 1999).

64. Egerton, S., Culloty, S., Whooley, J., Stanton, C. & Ross, R. P. The gut microbiota of marine fish. Front. Microbiol. 6, 1046 (2015).

65. Sandhya, S. Role of the normal gut microbiota. J. Appl. Microbiol. 121, 209–221 (2014).

66. Tapia-Paniagua, S. T. et al. Intestinal microbiota diversity of the flat fish Solea senegalensis (Kaup, 1858) following probiotic administration. Microb. Ecol. 60, 310–319 (2010).

67. Cerezo, R., Meseguer, I. & Esteban, M. A. Effects of dietary inulin, Bacillus subtilis and microalgae on intestinal gene expression in gilthead seabream (Sparus aurata L.). Fish Shellfish Immunol. 34, 843–848 (2013).

68. Wang, J., Wu, X., Lu, R., Meng, X. & Nie, G. Peptide transporter 1 (PepT1) expression in fish: A review. Aquac. Fish. 2, 193–206 (2017).

69. Cerezuela, R., Meseguer, J. & Esteban, M. A. Effects of dietary inulin, Bacillus subtilis and microalgae on intestinal gene expression in gilthead seabream (Sparus aurata L.). Fish Shellfish Immunol. 34, 843–848 (2013).

70. Wang, J., Wu, X., Lu, R., Meng, X. & Nie, G. Peptide transporter 1 (PepT1) expression in fish: A review. Aquac. Fish. 2, 193–206 (2017).

71. Dharwal, A. et al. MicrobiomeAnalyst: A web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. Nucleic Acids Res. 45, W180–W188 (2017).

72. Pedrós-Aló, C. Marine microbial diversity: Can it be determined? Trends Microbiol. 14, 257–263 (2006).

73. Parada Venegas, D. et al. Dietary impacts on the intestinal microbiota of a marine teleost (Sparus aurata) fed extreme diets low in fish meal and fish oil. PLoS One 11, e0166564 (2016).

74. Toxic Cyanobacteria in Water: A Guide to Their Public Health Consequences, Monitoring, and Management. (E & FN Spon, 1999).

75. Kanehisa, M. KEGG: Kyoto encyclopedia of genes and genomes. Nucleic Acids Res. 28, 27–30 (2000).

76. Langille, M. G. I. et al. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nat. Biotechnol. 31, 814–821 (2013).

77. Távara-Paniagua, S. T. et al. The treatment with the probiotic Shewanella putrefaciens Pdp11 of specimens of Solea senegalensis exposed to high stocking densities to enhance their resistance to disease. Fish Shellfish Immunol. 41, 209–221 (2014).

78. Tapia-Paniagua, S. T. et al. Dietary administration of the probiotic S. putrefaciens Pdp11: Effects on the intestinal microbiota and immune-related gene expression of farmed Solea senegalensis treated with oxytetracycline. Fish Shellfish Immunol. 46, 449–458 (2015).

79. Vidal, S. et al. Effects on intestinal microbiota and immune genes of Solea senegalensis after suspension of the administration of Shewanella putrefaciens Pdp11. Fish Shellfish Immunol. 58, 274–283 (2016).

80. Langille, M. G. I. et al. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nat. Biotechnol. 31, 814–821 (2013).

81. Kanehisa, M. KEGG: Kyoto encyclopedia of genes and genomes. Nucleic Acids Res. 28, 27–30 (2000).

Acknowledgements

The authors are thankful to Rocío Bautista Moreno, as Bioinformatics Analyst in the Bioinformatics Unit at Supercomputing and Bioinnovation Center, University of Malaga, for her valuable assistance.
Author contributions
All authors conducted the research, analysed the data, and reviewed the manuscript and approved the final version.

Funding
This project has been funded for the CEIMAR foundation through the "Proyecto de Jóvenes Investigadores (CEIJ-C05.3)" and with the support of spin-off LifeBioencapsulation S.L.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-021-98087-5.

Correspondence and requests for materials should be addressed to S.T.T.-P.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2021