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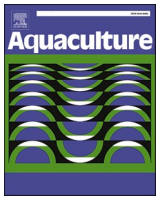

A novel fish meal-free diet formulation supports proper growth and does not impair intestinal parasite susceptibility in gilthead sea bream (*Sparus aurata*) with a reshape of gut microbiota and tissue-specific gene expression patterns

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ABSTRACT

The exponential growth of the aquaculture sector requires the development of sustainable aquafeeds with less dependence on marine products. The maximized replacement of fish meal (FM) and fish oil (FO) with plant ingredients has been extensively studied in the economically important species gilthead sea bream (*Sparus aurata*). Recently, major progress has been done with other alternative raw materials, though some non-pathological inflammatory response persisted with feed formulations that increased the circularity of resource utilization. In the present study, we evaluated the effects on growth performance, gene expression, intestinal microbiota and disease resistance of a FM-free diet (NoPAP SANA), based on plant ingredients, aquaculture by-products, algae oil, insect meal and bacterial fermentation biomasses as main dietary oil and protein sources, and supplemented with a commercially available health-promoting feed additive (SANACORE®GM). Juveniles of 21 g initial body weight were fed control or NoPAP SANA diets for 34 days, and head kidney, liver and posterior intestine were collected for gene expression analyses using customized PCR-arrays. Each tissue-specific PCR covered 96 genes in total and included markers of growth performance, lipid and energy metabolism, antioxidant defence, immune system, and intestinal function and integrity. From the same fish, the adherent bacteria of the posterior intestine were studied by Illumina sequencing of the V3-V4 region of the 16S rRNA. The remaining fish were challenged with the intestinal parasite *Enteromyxum leei* for 78 days and sampled for parasite diagnosis. Both control and NoPAP SANA fish grew efficiently considering gilthead sea bream standards. Before parasite challenge, the NoPAP SANA group showed differential expression of 17, 2 and 4 genes in liver, head kidney and posterior intestine, respectively. The intestinal bacterial composition showed no major differences in diversity or at the phylum level. However, 29 abundant OTUs significantly changed with the diet. From these, 10 OTUs were significantly correlated with differentially expressed genes in the different target tissues. Inferred metagenome analyses revealed that the altered microbiota with NoPAP SANA diet could account for changes in 15 metabolic pathways. The intensity and prevalence of infection after the parasite challenge did not significantly vary between dietary treatments, and infected fish from both groups showed similar disease outcome. Altogether, these results indicate that the NoPAP SANA diet promoted optimal growth and a healthy condition in gilthead sea bream without affecting susceptibility against the tested intestinal parasite, as often observed with alternative diets following current industry formulations.

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1. Introduction

The global growth of aquaculture production has increased the demand of fish meal (FM) and fish oil (FO) for feeding omnivorous and carnivorous fish species, but this high dependence on fisheries resources is environmentally and ecologically unsustainable. Thus, extensive research is directed towards the search of new raw materials and fish feed formulations (Turchini et al., 2019). The most obvious alternative are plant ingredients, and a high and combined replacement of FM/FO is feasible in salmonids and commonly farmed marine fish without detrimental effects on growth performance (Benedito-Palos et al., 2016; Egerton et al., 2020; Perera et al., 2019; Simó-Mirabet et al., 2018; Torrecillas et al., 2017). However, the immune response is highly influenced by the nutritional background (Oliva-Teles, 2012), and the use of plant-based diets is often accompanied by intestinal infiltration of inflammatory cells and impaired disease outcomes (Estensoro et al., 2016; Piazzon et al., 2017; Romarheim et al., 2013; Urán et al., 2008). This drawback effect is remediated, at least partially, by means of different dietary supplementation strategies. For instance, fish protein hydrolysates mitigated most of the detrimental effects of the soya-induced enteritis in salmon (Egerton et al., 2020). Likewise, in gilthead sea bream, dietary sodium butyrate helped to ameliorate the inflammation, disease susceptibility and intestinal dysbiosis caused by plant-based diets (Estensoro et al., 2016; Piazzon et al., 2017). However, extensive research in novel fish feed formulations that adhere to the circularity principles, maximise resource efficiency and contribute towards zero waste in the agro-food value chain and feed cost-effectiveness is needed (Campos et al., 2020). In that sense, aquaculture by-products, insect meal products and single-cell biomasses/oils are among the most promising alternatives for aquafeed production.

Certainly, the short production times, the small land requirements, and the independence of weather conditions makes the production of microbial biomass specially interesting over other animal or vegetal protein sources (García-Garibay et al., 2003). Their use as replacers of marine feedstuffs has been tested with variable success in fish and shrimp, depending on the species, the level of replacement, and the source and nature of the microbial biomass (Davies and Wareham, 1988; Delamare-Debouteville et al., 2019; Hardy et al., 2018). In particular, in gilthead sea bream, a commercialized product (LSAqua SusPro) of bacterial single cell proteins and processed animal proteins (PAP) highly supported an efficient growth performance, and interestingly, the reshaping of gut microbiota might have contributed to mitigate the pro-inflammatory signs of fish fed low FM diets with a high inclusion level of plant ingredients (Solé-Jiménez et al., 2021).

Insect meal products constitute another promising alternative for FM replacement, as they are beneficial not only for growth purposes, but also as a functional ingredient boosting the immune system and protecting against pathogenic microflora (Mousavi et al., 2020; Nogales-Mérida et al., 2019). However, the nutritional profile, and microbial load and biodiversity of insect meals is largely dependent on insect species, stage of development and rearing conditions (Nogales-Mérida et al., 2019; Osimani et al., 2017), making comparisons among insect meal products and feeding trials sometimes difficult. The use of defatted black soldier (*Hermetia illucens*) meal to replace FM at levels above 18% in turbot (*Psetta maxima*) and 35% in meagre (*Argyrosomus regius*) did not affect the feed conversion ratio, but resulted in an impaired growth condition (Guerreiro et al., 2021; Kroeckel et al., 2012). Contrarily, higher levels of replacement using black soldier meal and defatted *Tenebrio molitor* did not compromise gut health and growth performance at short-term in European sea bass (*Dicentrarchus labrax*) or gilthead sea bream (Basto et al., 2021; Magalhães et al., 2017; Panteli et al., 2021). Recent studies also described how different probiotics, food additives or insect meal preparations reshape the intestinal microbiota of European sea bass, gilthead sea bream or rainbow trout (*Oncorhynchus mykiss*), eliciting species-specific responses of microbial structure and functional dynamics (Antonopoulou et al., 2019; Bruni et al., 2018; Huyben et al., 2019; Moroni et al., 2021; Naya-Català et al., 2021b; Panteli et al., 2021; Terova et al., 2019). Furthermore, the gut microbiota-diet-inflammation trialogue has been addressed in gilthead sea bream within the GAIN EU project, correlating changes in diet composition and gut microbiota with host transcriptomics at both the local (anterior intestine) and systemic (liver and head kidney) level, using alternative formulations including a concept (NoPAP) based on plant ingredients, aquaculture by-products, insect meal and bacterial fermentation biomasses (Naya-Català et al., 2021a). Such approach served to confirm and extend the notion that NoPAP-based diets are specially promising to mitigate the inflammatory drawback effects of low FM/FO diets in marine farmed fish. To go further, we tested herein a novel aquafeed formulation containing a gut health modulator, protein and lipid aquaculture by-products, black soldier meal and microbial biomass as the main feed ingredients in a FM-free diet. The functional validation was made on the basis of measurements of growth performance, tissue-specific gene expression patterns (liver, head kidney, posterior intestine), intestinal microbiota composition and parasite disease outcome in fish challenged by effluent exposure with the myxozoan *Enteromyxum leei,* an enteric parasite of high economic importance for sparid farmed fish (Palenzuela, 2006).

2. Materials and methods

2.1. Ethics statement

All procedures were carried out according to Institute of Aquaculture Torre de la Sal (IATS, CSIC) and CSIC Review Boards, European (2010/63/EU) animal directives and Spanish laws (Royal Decree RD53/2013) on the handling of experimental animals.

2.2. Animals

Gilthead sea bream juveniles were purchased from a Mediterranean hatchery (Piscimar, Burriana, Spain) and adapted for two months to the indoor experimental facilities of IATS, CSIC under natural photoperiod and temperature conditions (40°5′N; 0°10′E). Seawater was pumped ashore (open system), oxygen content was always above 85% saturation, and unionized ammonium remained below 0.01 mg/L. During the experimental period (May 2020-August 2020), water temperature increased from 18 °C in May to 24 °C in June, and 28 °C in August.

2.3. Diets

Two extruded isoproteic (47%) and isolipidic (17%) diets (2 and 3 mm pellet size) were formulated and produced by Sparos Lda (Olhão, Portugal), following advanced industry practices (Table 1). In the control (CTRL) diet, the inclusion level of poultry meal, FM foodstuffs and plant protein ingredients was 10%, 20% and 54.1%, respectively. The experimental diet (NoPAP SANA) was formulated to be FM-free diet, containing 3% of FM hydrolysed aquaculture by-products, 10% of insect meal, 15% of microbial biomass generated by fermentation of *Corynebacterium glutamicum* and *Methylococcus capsulatus,* and a reduced supply of plant protein sources (38%). FO (4.9%) and vegetable oil (7.9%) were used as the main dietary lipid sources in the CTRL diet, whereas the NoPAP SANA diet contained FO (2.5%), salmon oil by-products (9.6%) and algae oil (1%) to provide 2% of EPA + DHA. Additionally, the NoPAP SANA diet was conveniently supplemented with monocalcium phosphate, L-tryptophan, DL-methionine, and a gut health factor SANACORE®GM (0.5%) from Adisseo (Dendermonde, Belgium).

2.4. Experimental setup

The experimental setup included two phases: the initial feeding period (pre-challenge period) and the parasite challenge period. The pre-challenge period started in May 2020. Fish of 21.3 g (± 0.21 SEM) were randomly distributed in two 2500 L tanks of 160 fish each in an

Table 1
Ingredients and chemical composition of control and experimental diets.

Ingredients	CTRL (%)	NoPAP SANA (%)
Fish meal Super Prime	10	
Fish meal by-products	7	
Fish protein hydrolysate	3	
Fish protein hydrolysate aquaculture by product		3
Poultry meal	10	
Insect meal (Black soldier fly)		10
Fermentation biomass (*Corynebacterium glutamicum*)		5
Fermentation biomass (*Methylococcus capsulatus*)		10
Soy protein concentrate	6	4.5
Pea protein concentrate		6.1
Wheat gluten	4	3
Corn gluten meal	10	7.5
Soybean meal 48	12.5	
Rapeseed meal	7	
Sunflower meal 40	5	20
Wheat meal	10.61	
Whole peas		6.12
Pea starch (raw)		3.6
Vitamin & Min Premix (1)[1]	1	
Vitamin & Min Premix (2)[2]		1
GAIN Macroalgae SHP[3]		2.5
GAIN Macroalgae SHP Se-rich[4]		0.1
GAIN Microalgae WUR Se-rich[5]		0.2
Vitamin E50	0.03	0.03
Betaine HCl	0.1	0.1
Antioxidant[6]	0.2	0.2
Sodium propionate	0.08	0.08
Monoammonium phosphate	0.55	2.6
L-Lysine HCl 99%		0.3
L-Threonine		0.05
L-Tryptophan	0.06	0.1
DL-Methionine	0.05	0.3
Yttrium oxide	0.02	0.02
Fish oil	4.9	2.5
Salmon oil by product		9.6
Algae oil (Veramaris)		1
Rapeseed oil	7.9	
SANACORE[7]		0.5
Composition analysis (% dry matter)		
Protein	50.5	49.6
Lipid	17	17.6
Ash	8.3	7.2
Total P	1.6	1.8
EPA + DHA	1.9	2.0
Energy (MJ/kg dry matter)	22.3	22.6

[1] Vitamin & Mineral Premix with I and Se: Vitamins (IU or mg/kg diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 20,000 IU; DL-cholecalciferol, 2000 IU; thiamine, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotinic acid, 200 mg; folic acid, 15 mg; ascorbic acid, 1000 mg; inositol, 500 mg; biotin, 3 mg; calcium pantothenate, 100 mg; choline chloride, 1000 mg, betaine, 500 mg. Minerals (g or mg/kg diet): cobalt carbonate, 0.65 mg; copper sulphate, 9 mg; ferric sulphate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulphate, 7.5 mg; sodium chloride, 400 mg; calcium carbonate, 1.86 g; excipient wheat middlings.

[2] Vitamin & Mineral Premix witout I and Se.

[3] Macroalgae source of iodine and other minerals developed by Salten HavbrukPark (SHP) composed by a mixture of *Laminaria digitata*, *L. hyperborea* and *Saccharina latissima* species.

[4] Macroalgae source of Se developed by SHP composed by a mixture of *Alaria esculenta*, *Ascophyllum nodosum*, *Fucus serratus*, *Fucus vesiculosus*, *Himanthalia elongata*, *L. digitata*, *Mastocarpus stellatus*, *Palmaria palmata*, *Pelvetia canaliculata*, *Porphyra spp*, *Saccharina latissima*, *Ulva lactuca*, and *Vertebrata lanosa* species.

[5] Macroalgae source of Se developed by Wageningen University & Research (WUR) from the *Nannochloropsis oceanica* sp.

[6] VERDILOX™ IP GT Dry, Kemin, Belgium.

[7] SANACORE is a broad-spectrum, health-promoting additive consisting of a mixture of organic acids, inactivated yeast and yeast extracts (*Saccharomyces cerevisiae*) with herbal extracts on a mineral carrier.

open flow-through seawater system under natural photoperiod and temperature. All fish were tagged into the dorsal skeletal muscle with passive integrated transponders (PIT) (ID-100A 1.25 Nano Transponder, Trovan), and were individually weighed and measured at the beginning and end of the experimental periods, using a FR-200 Fish Reader W (Trovan, Madrid, Spain) for data capture and pre-processing. The feeding period lasted 34 days (May 2020–June 2020) and fish were fed by means of automatic feeders once daily (12:00 am), six days per week, near to visual satiety with CTRL or NoPAP SANA diet for the entire duration of the pre-challenge period. Normal fish feeding behaviour was assessed routinely by camera monitoring, and the amount of feed given was registered daily.

For the parasite challenge, 105 fish belonging to each dietary group, with a mean body weight of 43 g were transported to the pathology facilities at IATS, CSIC and distributed in six 500 L tanks (35 fish/tank, 3 tanks/diet) with open flow and natural photoperiod and temperature. The feeding regime during the challenge period was by hand at visual satiety. Two replicated tanks per diet were challenged by effluent exposure with the intestinal parasite *Enteromyxum leei*, as previously described (Estensoro et al., 2010; Estensoro et al., 2011). Briefly, for each diet, two recipient or challenged tanks (R: 70 fish/diet, 35 fish/tank) were set to receive exclusively the effluent water from a donor tank containing 30 heavily infected fish (mean body weight 140 g, mean *Enteromyxum leei* Ct value 27, prevalence of infection 100%). The effluent exposure was maintained throughout the experiment. Fish in the two remaining tanks constituted the control non-challenged fish (C: 35 fish/diet), as stated in Supplementary Fig. 1. At 40 days post-exposure (dpe) fish were anesthetized, individually weighted and measured, and non-lethally sampled for parasite diagnosis. The non-lethal sample consisted of a probe of the rectal mucosa with a cotton swab. Then a diagnostic PCR was carried out with specific primers for *E. leei* 18S rRNA gene, as previously described (Piazzon et al., 2017) in order to determine infection establishment and prevalence. In light of this result, regarding the parasite dynamics already described in this infection model (Estensoro et al., 2011), the final lethal sampling was performed at 78 dpe.

2.5. Sample collection

At the end of the pre-challenge period, after a 48 h fasting period, nine fish/diet were anesthetized with 0.1 g/L of tricaine-methasulfonate (MS-222, Sigma-Aldrich), and then sacrificed by severing the spinal cord immediately downstream of the nape with a knife. The intestine (excluding the pyloric caeca) of each fish was dissected out, weighed and measured aseptically to calculate the intestine weight index (IWI) and intestine length index (ILI). Then, head kidney, liver and posterior intestine tissue portions (~0.4 cm) were placed in RNA later for subsequent molecular analyses. The remaining posterior intestine was opened and washed with sterile Hank's balanced salt solution before collecting the autochthonous (adherent) intestinal bacteria by scraping intestinal mucosa with the blunt end of a clean scalpel. In this study, posterior intestine was chosen because it is the main target tissue for the parasite used in the infection trial (Estensoro et al., 2014). Autochthonous microbiota was targeted as this population is the one that remains for long periods in the host and has more impact on host physiology than allochthonous populations. Then, mucus samples were transferred to a sterile Eppendorf tube, and put on ice until subsequent DNA extraction for microbiota analysis.

At the end of the parasite challenge (78 dpe), all fish were sacrificed, weighted and measured, and the entire intestine of 25 fish/tank (50 fish/diet in R groups and 25 fish/diet in C groups) was collected for molecular diagnosis, as previously described (Palenzuela et al., 2020).

2.6. Gene expression analysis

Total RNA from liver, head kidney and posterior intestine was

extracted using a MagMax-96 total RNA isolation kit (Life Technologies, Carlsbad, CA, USA). The RNA yield was higher than 3.5 μg with absorbance measures (A260/280) of 1.9–2.1. Synthesis of cDNA was performed with the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA) using random decamers and 500 ng of total RNA in a final volume of 100 μL. Reverse transcription (RT) reactions were incubated 10 min at 25 °C and 2 h at 37 °C. Negative control reactions were run without the enzyme. As reported in previous studies (Estensoro et al., 2016; Naya-Català et al., 2021a), three customized PCR-array layouts were designed for the simultaneous profiling of a tissue-specific gene panel (Supplementary Table 1). Liver layout contained 44 genes, including markers of GH/IGF axis (9), lipid metabolism (15) energy/oxidative metabolism (10), and antioxidant defence (10). Head kidney layout comprised 29 selected genes, including markers of cytokines and chemokine-related proteins (11), immunoglobulins (2), acute phase response proteins and proteolytic activity (4), T-cell and monocyte/macrophage markers (7), and pattern recognition receptors (PRRs, 5). Intestine layout involved 44 selected genes, including markers of epithelial integrity (11), nutrient transport (4), mucins (2), cytokines and chemokine-related proteins (13), immunoglobulins (2), T cell and monocyte/macrophage markers (4), and PRRs (8). For each array, qPCR reactions were performed using an iCycler IQ Real-Time Detection System (Bio-Rad, Hercules, CA, USA). Diluted RT reactions (×6) were used for qPCR assays in a 25 μL volume in combination with a SYBR Green Master Mix (Bio-Rad, Hercules, CA, USA) and specific primers at a final concentration of 0.9 μM (Supplementary Table 2). The program used for PCR amplification included an initial denaturation step at 95 °C for 3 min, followed by 40 cycles of denaturation for 15 s at 95 °C and annealing/extension for 60 s at 60 °C. All the pipetting operations were made by means of an EpMotion 5070 Liquid Handling Robot (Eppendorf, Hamburg, Germany) to improve data reproducibility. The efficiency of PCRs (> 92%) was checked, and the specificity of reactions was verified by analysis of melting curves (ramping rates of 0.5 °C/10 s over a temperature range of 55–95 °C), and linearity of serial dilutions of RT reactions ($r^2 > 0.98$). Fluorescence data acquired during the extension phase were normalized by the delta-delta Ct method (Livak and Schmittgen, 2001), using beta-actin as housekeeping gene due to its stability in different experimental conditions (average Ct between experimental groups varied less than 0.2).

2.7. DNA extraction from mucus samples

PI mucus samples (200 μL) from 9 fish/group were treated with 250 μg/mL of lysozyme (Sigma) for 15 min at 37 °C. Then, DNA was extracted using the High Pure PCR Template Preparation Kit (Roche) following the manufacturer's instructions. DNA concentration, quality and purity were measured using a Nanodrop 2000c (Thermo Scientific) and agarose gel electrophoresis (1% *w/v* in Tris-EDTA buffer). The DNA yield was enough to perform sequencing of each sample, without the need of pooling, allowing to determine individual variations of microbial populations. DNA was stored at −20 °C until sequencing.

2.8. Illumina MiSseq sequencing and bioinformatic analysis

The V3-V4 region of the 16S rRNA gene (reference nucleotide interval 341–805 nt) was sequenced using the Illumina MiSeq system (2 × 300 paired-end run) at the Genomics Unit from the Madrid Science Park Foundation (FPCM). The details on the PCR and sequencing of amplicons are described elsewhere (Piazzon et al., 2019). Raw sequence data from this experiment were uploaded to the Sequence Read Archive (SRA) under Bioproject accession number PRJNA750446 (BioSample accession numbers: SAMN20458383–8400). Raw forward and reverse reads were quality filtered using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and pre-processed using Prinseq (Schmieder and Edwards, 2011). Terminal N bases were trimmed in both ends and sequences with >5% of total N bases were discarded. Reads that were < 150 bp long, with Phred quality score < 28 in both of the sequence ends and with a Phred average quality score < 26 were excluded. Then, forward and reverse reads were merged using fastq-join (Aronesty, 2013).

Bacteria taxonomy assignment was performed using the Ribosomal Database Project (RDP) release 18 as a reference database (Cole et al., 2014). Reads were aligned with a custom-made pipeline using VSEARCH and BLAST (Altschul et al., 1990; Rognes et al., 2016). Alignment was performed establishing high stringency filters (≥ 90% sequence identity, ≥ 90% query coverage). Taxonomic assignment results were filtered and data were summarized in an operational taxonomic units (OTUs) table. Sample depths were normalized by total sum scaling and then made proportional to the total sequencing depth, following the recommendations previously described (McKnight et al., 2019).

2.9. Inferred metagenome and pathway analysis

Piphillin was used to normalize the amplicon data by 16S rRNA gene copy number and to infer metagenomic contents (Iwai et al., 2016). This analysis was performed with the OTUs significantly driving the separation by diet in the PLS-DA analysis (described in the Statistics section). For the analysis, a sequence identity cut-off of 97% was used, and the inferred metagenomic functions were assigned using the Kyoto Encyclopaedia of Genes and Genomes database (KEGG, Oct 2018 Release). Raw KEGG pathway output from Piphillin was analysed with the R Bioconductor package DESeq2 using default parameters, after flooring fractional counts to the nearest integer (Bledsoe et al., 2016; Love et al., 2014; Piazzon et al., 2020).

2.10. Statistics

Data on growth and gene expression were analysed by Student *t*-test or one-way ANOVA followed by a Student-Newman-Keuls post hoc test using SigmaPlot v14 (Systat Software Inc., San Jose, CA, United States). When the test of normality or equal variance failed, Kruskal-Wallis ANOVA on ranks followed by Dunn's test was applied instead. The significance level was set at $P < 0.05$. For microbiota analyses, rarefaction curves (plotting the number of observed taxonomic assignations against the number of sequences), species richness estimates and alpha diversity indexes were obtained using the R package phyloseq (McMurdie and Holmes, 2013). Differences in species richness, diversity indexes and phylum abundance were determined by Kruskal-Wallis test using the Dunn's post-test, with a significance threshold of $P < 0.05$. Beta diversity across groups was tested with permutational multivariate analysis of variance (PERMANOVA) using the non-parametric method *adonis* from the R package Vegan with 10,000 random permutations. To study the separation among groups, supervised partial least-squares discriminant analysis (PLS-DA) and hierarchical clustering of samples were sequentially applied using EZinfo v3.0 (Umetrics, Umea, Sweden) and R package ggplot2, respectively. The contribution of the different genes to the group separation was determined by the minimum variable importance in the projection (VIP) values achieving the complete clustering of the conditions with a VIP value of 1. Hotelling's T^2 statistic was calculated by the multivariate software package EZinfo v3.0. The quality of the PLS-DA model was evaluated by the parameters R2Y (cum) and Q2 (cum), which indicate the fit and prediction ability, respectively. To assess whether the supervised model was being over-fitted, a validation test consisting on 500 random permutations was performed using SIMCA-P+ (v11.0, Umetrics). For the OTU-gene correlations, the expression values of the differentially expressed genes ($P < 0.05$) from the three tissues and the normalized counts values from the OTUs driving the separation in the PLS-DA model (VIP ≥ 1) for each individual fish were used (18 samples in total, 9 CTRL and 9 NoPAP SANA). The Spearman rank correlation coefficients and the corresponding *P* values were calculated with the *cor.test* function from the *corrplot* R package

(Wei and Simko, 2021) with two-sided alternative hypothesis. Significant gene-OTU correlations were accepted at a $P < 0.001$ and visualized with *corrplot* package. A correlation network was built using Cytoscape v3.8.2 (Smoot et al., 2011).

For the parasite challenge, parasitological variables studied were prevalence of infection (percentage of infected fish in a sampled group) and intensity of infection (median Ct values of fish that were PCR positive for the parasite). Each individual was treated as a replicate and each group included all the fish (replicate tanks were not treated individually, as no tank effect was detected). Chi-square test of independence was used to assess differences in prevalence of infection. Kruskal-Wallis test (Dunn's post-test) was used to determine differences in intensity values. Statistical significance was considered at $P < 0.05$. To evaluate the recovery trend observed between the intermediate (40 dpe) and final samplings (78 dpe) the individual ΔCt values (Cts at 78 dpe – Ct at 40 dpe) were plotted against the Ct values at 40 dpe and the regression lines for each diet group were calculated.

3. Results

3.1. Growth performance

Data on growth performance before the parasite challenge are reported in Table 2. After 34 days of feeding, final body weight and standard length were not significantly altered by the dietary intervention. However, condition factor was significantly lower in NoPAP SANA fish (2.61) than in CTRL fish (2.66). The same trend was found for the specific growth rates (SGR), which decreased slightly from 0.0233 day^{-1} in CTRL fish to 0.0227 day^{-1} in NoPAP SANA fish (less than 3% reduction). The same slight impairment applied to total feed intake (g diet per fish) throughout the dietary preconditioning period. Regarding biometric indexes, HSI, MFI and ILI were not significantly altered by the dietary treatment. However, the IWI was significantly higher in NoPAP SANA fish (4.65%) than in CTRL fish (4.15%).

Table 2
Effects of dietary treatment on growth performance of gilthead sea bream juveniles, before the parasite challenge. Data on body weight, body length, condition factor and specific growth rate are the mean ± SEM of 160 fish from each dietary group. Data on feed intake and feed conversion ratio are the value of the whole tank. Biometrics of liver, mesenteric fat and intestine are the mean ± SEM of 12 fish per group. *P* values of t-test are indicated, and statistically significant differences are marked in bold font with asterisks (* < 0.05, *** < 0.001).

	CTRL	NoPAP SANA	*P* value
Initial body weight (g)	21.30 ± 0.28	21.32 ± 0.30	0.977
Final body weight (g)	43.92 ± 0.53	43.02 ± 0.50	0.218
Final standard length (cm)	118.07 ± 0.48	118.01 ± 0.47	0.928
Final condition factor[1]	2.66 ± 0.02	2.61 ± 0.01	**0.018** *
Feed intake (g DM/fish)[2]	22.76	21.71	
FCR[3]	0.93	0.97	
SGR (%)[4]	0.0233 ± 0.0002	0.0227 ± 0.0002	**0.039** *
Liver (g)	0.62 ± 0.02	0.61 ± 0.02	0.781
Mesenteric fat (g)	0.68 ± 0.08	0.62 ± 0.06	0.562
Intestine weight (g)	1.81 ± 0.09	2.00 ± 0.09	0.126
Intestine length (cm)	10.85 ± 0.45	11.26 ± 0.55	0.571
HSI (%)[5]	1.44 ± 0.04	1.44 ± 0.07	0.990
MFI (%)[6]	1.54 ± 0.15	1.43 ± 0.13	0.590
IWI (%)[7]	4.15 ± 0.08	4.65 ± 0.11	**<0.001** ***
ILI (%)[8]	92.65 ± 3.78	94.40 ± 4.47	0.767

[1] Condition factor = 100 × fish weight (g)/fish length (cm)^3.
[2] Feed intake = dry feed weight (g)/fish.
[3] Feed conversion ratio = dry feed intake /wet weight gain.
[4] Specific growth rate = 100 × (ln final body weigh – ln initial body weight)/days.
[5] Hepatosomatic index = 100 × (liver weight/fish weight).
[6] Mesenteric fat index = 100 × (mesenteric fat weight/fish weight).
[7] Intestinal weight index = 100 × (intestine weight/fish weight).
[8] Intestinal lenght index = 100 × (intestine length/fish length).

3.2. Gene expression profiling

Before the parasite challenge, the expression of selected genes was determined in liver, head kidney and posterior intestine. All genes included in the PCR-arrays were found at detectable levels in the three analysed tissues (Supplementary Tables 3-5). As summarized in Fig. 1, 17 out of 44 genes were differentially expressed (DE) in liver, being the expression of markers of GH/IGF axis (*igf1)*, lipid metabolism (*elovl6*, *fads2*, *scd1a*, *hl*, *pla2g6*, *cyp7a1*, *pparβ* and *pparγ*), energy/oxidative metabolism (*h-fabp*, *nd5*, *coxii*) and antioxidant defence (*ucp1, gpx4*, *prdx5*, *cu-zn-sod/sod1, mn-sod/sod2*) significantly down-regulated in fish fed the NoPAP SANA diet. In head kidney, 2 out of 29 genes were affected by the NoPAP SANA diet. In this case, the gene expression of interleukin 8 (*il8*) and toll-like receptor 2 (*tlr2*) was up-regulated in fish fed the NoPAP SANA diet. In intestine, 4 out of 44 genes were down-regulated in fish fed the NoPAP SANA diet. These genes were markers of epithelial integrity (*cdh17*), mucus production (*muc2*), cytokine production (*il12β*) and cellular specificity (*cd8β*).

3.3. Alpha diversity and microbial composition

Illumina sequencing of the 18 analysed PI samples yielded 4,068,405 high quality and merged reads, with a mean of 226,022 reads per sample (Supplementary Table 6). The reads were assigned to 1712 OTUs at 97% identity threshold. Up to 91% of the OTUs were classified at the level of genus, family (96%), order (97%), class (99%) and phylum (99%). Rarefaction analysis showed curves that approximated saturation (horizontal asymptote), thus a good coverage of the bacterial community was achieved and the number of sequences for analysis was considered appropriate (Supplementary Fig. 2).

No significant differences were found in diversity indexes (Shannon and Simpson), but a significantly higher richness (Observed and ACE values, $P < 0.05$) was found in NoPAP SANA fish (Table 3). At the phylum level (Fig. 2), Proteobacteria, Firmicutes and Actinobacteria constituted close to 90% of the total bacterial population with no significant changes between dietary groups. Bacteroidetes, other important phylum in this species, significantly decreased in NoPAP SANA fish (4.3%) when compared to CTRL fish (6.1%). Fusobacteria, usually found in low proportion in the intestine of gilthead sea bream (0.1% in control fish), significantly increased up to 2.6% in the NoPAP SANA group.

3.4. Microbiota discriminant analysis

PERMANOVA test did not show statistically significant differences in bacterial composition when comparing animals fed different diets ($P = 0.066$, $F = 1.1692$, $R^2 = 0.0681$). However, to study and validate in more detail the microbiota differences among groups, a PLS-DA model (R2Y = 99%, Q2 = 55%) with three components was constructed and statistically validated (Fig. 3A, Supplementary Fig. 3). The first two components explained more than 95% of total variance, clearly separating CTRL fish from NoPAP SANA fish along the x-axis (component 1, 88.79%). To determine which groups of bacteria were driving these separations at a high level of confidence, the minimum VIP value driving the correct separation of groups in the model was determined throughout a heatmap representation (Fig. 3B). Such approach disclosed 160 discriminant OTUs (VIP ≥ 1), which can be accessed in Supplementary Table 7.

Fig. 4 shows the list of most abundant bacteria (at least 1% in one of the groups; 29 OTUs) that exclusively drove the separation by dietary groups. For these abundant discriminant bacteria, 17 OTUs were increasing in fish fed the NoPAP SANA diet, highlighting *Photobacterium damselae* and several Alphaproteobacteria (*Sphingomonas*, *Bradyrhizobium* and *Brevundimonas*) and Actinobacteria (*Rothia*, *Actinomyces* and *Micrococcus*). On the other hand, 12 OTUs decreased in the NoPAP SANA group, including *Vibrio alginolyticus*, *Phenylobacterium* and *Propionibacterium*, which represented a high proportion in the NoPAP SANA

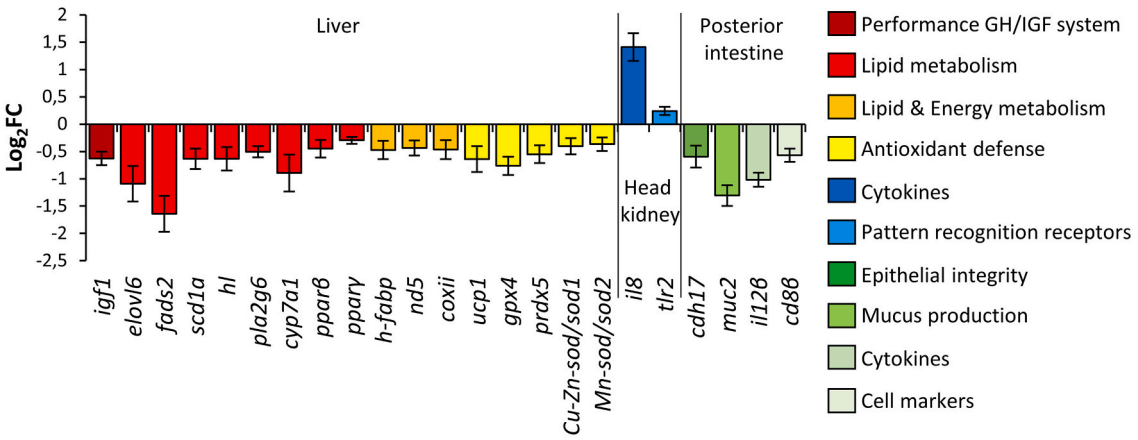

Fig. 1. Mean ± SEM of the Log_2 fold changes of the differentially expressed genes from the PCR-array panels run in liver, head kidney and spleen (*t*-test, $P < 0.05$) when comparing NoPAP SANA vs. CTRL group. Different colours represent different associated functions depicted in the legend on the right.

Table 3
Richness (Observed and ACE) and diversity (Shannon and Simpson) indexes. Data are represented as the mean ± SEM ($n = 9$). *P* values of Kruskall-Wallis test are indicated, and statistically significant differences are marked in bold font with asterisks (* < 0.05).

	CTRL	NoPAP SANA	*P* value
Observed	207.56 ± 55.98	257.89 ± 52.34	**0.042***
ACE	306.76 ± 128.94	375.06 ± 81.57	**0.017***
Shannon	2.15 ± 0.74	2.42 ± 0.39	0.626
Simpson	0.76 ± 0.24	0.85 ± 0.07	0.758

group and were almost absent in the CTRL group.

3.5. Correlations between host gene expression and changes in gut microbiome

Using Spearman correlations, a total of 19 significant unique DE gene-OTU correlations were found ($P < 0.001$), with a magnitude of correlation between −0.81 and 0.80 (Fig. 5). Interestingly, most taxa found here were abundant (> 1%) in at least one of the groups. In liver, 12 out of 17 DE genes presented a correlation with a total of 6 OTUs. The taxa with the higher number of interactions was *Pseudoxanthomonas*, with a total of 7 positive correlations with *fads2*, *scd1a*, *hl*, *pparβ*, *gpx1*, *prdx5* and *mn-sod/sod2*. In head kidney, the two DE genes correlated with four OTUs, *il8* with Caulobacteraceae and *Actinomyces*, and *tlr2* with Bacteria and *Cupriavidus*. In posterior intestine, only a negative interaction between *muc2* and *Tepidiphilus* was found.

3.6. Inferred metagenome and pathway analysis

In an attempt to evaluate the biological significance of the microbiota changes driven by NoPAP SANA, pathway analysis was performed using the inferred metagenomes of the discriminant OTUs (VIP ≥ 1). The results showed that 15 pathways could significantly differ between the NoPAP SANA group and the CTRL fish (Fig. 6). Among these, the sphingolipid signalling pathway was the only underrepresented pathway in NoPAP SANA group. On the contrary, calcium signalling pathway, flavone and flavonol biosynthesis, biosynthesis of type II polyketide products, bile secretion, fatty acid elongation and glycan degradation were the most overrepresented inferred pathways.

3.7. Parasite challenge

At the beginning of the experimental infection (0 days post-exposure = 0 dpe), fish fed the CTRL and NoPAP SANA diets were selected to avoid initial differences in weight, length and CF (Fig. 7 A-C). At 40 dpe, the prevalence of infection was 45.7% and 66.6% for CTRL and NoPAP SANA fish, respectively. This difference was statistically significant ($P = 0.01821$). In agreement with the infection status, all recipient (R) groups showed significantly lower growth parameters than the control non-challenged groups (C), with no differences between diets. SGR for the

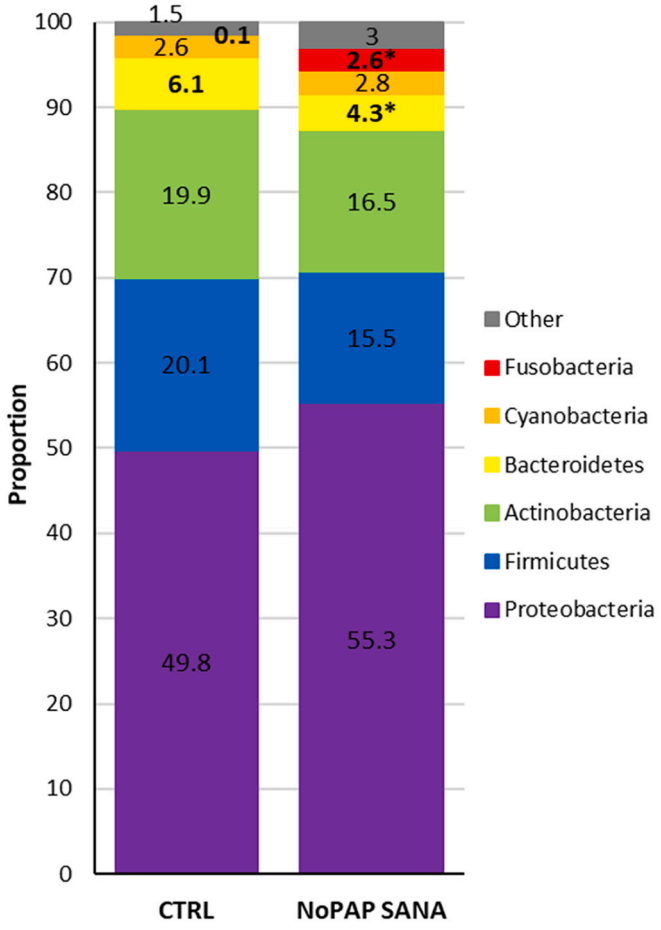

Fig. 2. Relative abundance of bacterial phyla in the two diet groups. Only the phyla that are present in at least 1% in one of the groups are represented. Bold letters with asterisks indicate significant differences between CTRL and NoPAP SANA groups (Kruskal-Wallis + Holm-Sidak tests, $P < 0.05$).

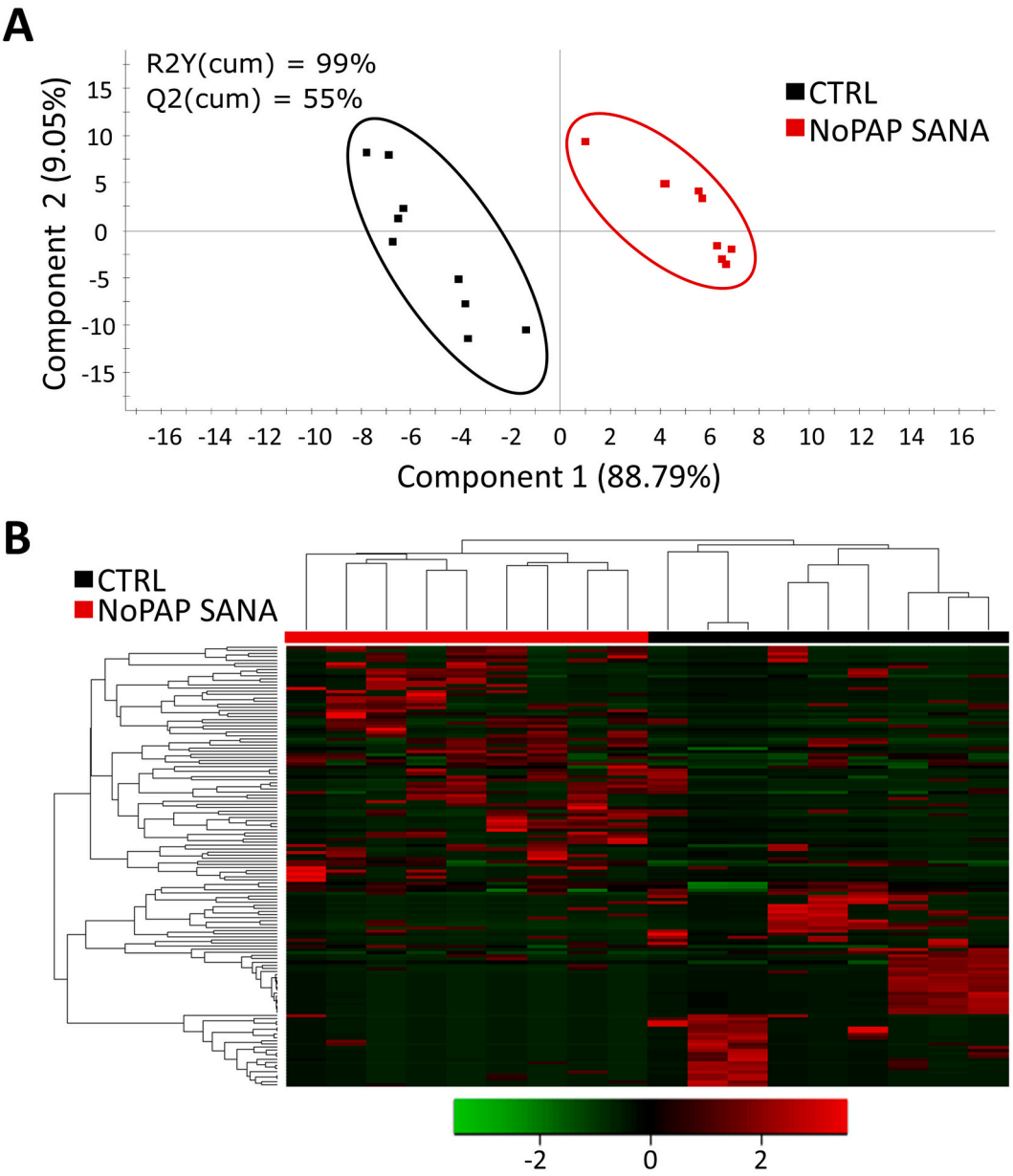

Fig. 3. Two-dimensional PLS—DA score plot (A) constructed using the variable diet representing the distribution of the samples between the first two components in the model. The goodness of fit and validation by permutation test can be found in Supplementary Fig. 3. (B) abundance distribution (*Z*-score) heatmap of the OTUs identified to drive the separation by diet (VIP > 1).

whole challenge period was significantly lower in R groups, with no differences between diets (Fig. 7 D). When evaluating the SGR decrease by periods, SGR from 0 to 40 dpe in both R groups decreased in more than −50% in relation to their corresponding non-challenged groups (−53.99% and − 53.52% for CTRL and NoPAP SANA groups, respectively). FCR for the whole challenge period in C groups was close to 1 in both diets, but increased (worsened) similarly in both R groups (Fig. 7 D). At 78 dpe, CTRL and NoPAP SANA groups had a prevalence of infection of 55.4% and 68.6%, respectively (with no significance, $P = 0.2236$), and differences in the growth parameters followed the same trend as in the intermediate sampling, except for the CF of R groups that recovered to control values (Fig. 7 C). This ulterior amelioration of the disease signs was also reflected in the SGR of R groups in the 40–78 dpe period, which was much less decreased than in the previous period (−9.15% in NoPAP-SANA and 14.59% in CTRL diets). This biometric recovery was coincident with a decrease in the intensity of infection between the two sampling points, being the median Ct values significantly higher at 78 dpe than at 40 dpe, with no differences between diets (Fig. 7 E). In fact, Fig. 7 F shows how individual fish from both groups had a similar decreasing rate of infection intensity.

4. Discussion

Traditionally, FM replacement in marine fish feeds has been approached using plant proteins, but the current trend is shifting towards the substitution of FM and plant proteins with other alternative and more sustainable raw materials, towards the principles of circular economy and zero waste in combination with stricter criteria of animal health and welfare. In that sense, the supplementation of a well-balanced FM-free diet with broken cells of the microalgae *Phaeodactylum tricornutum* supported optimal growth in gilthead sea bream juveniles with a potential stimulatory effect that might be relevant as a prophylactic measure before a predictable stressful event (Reis et al., 2021). Similarly, in rainbow trout, the inclusion of black soldier fly and

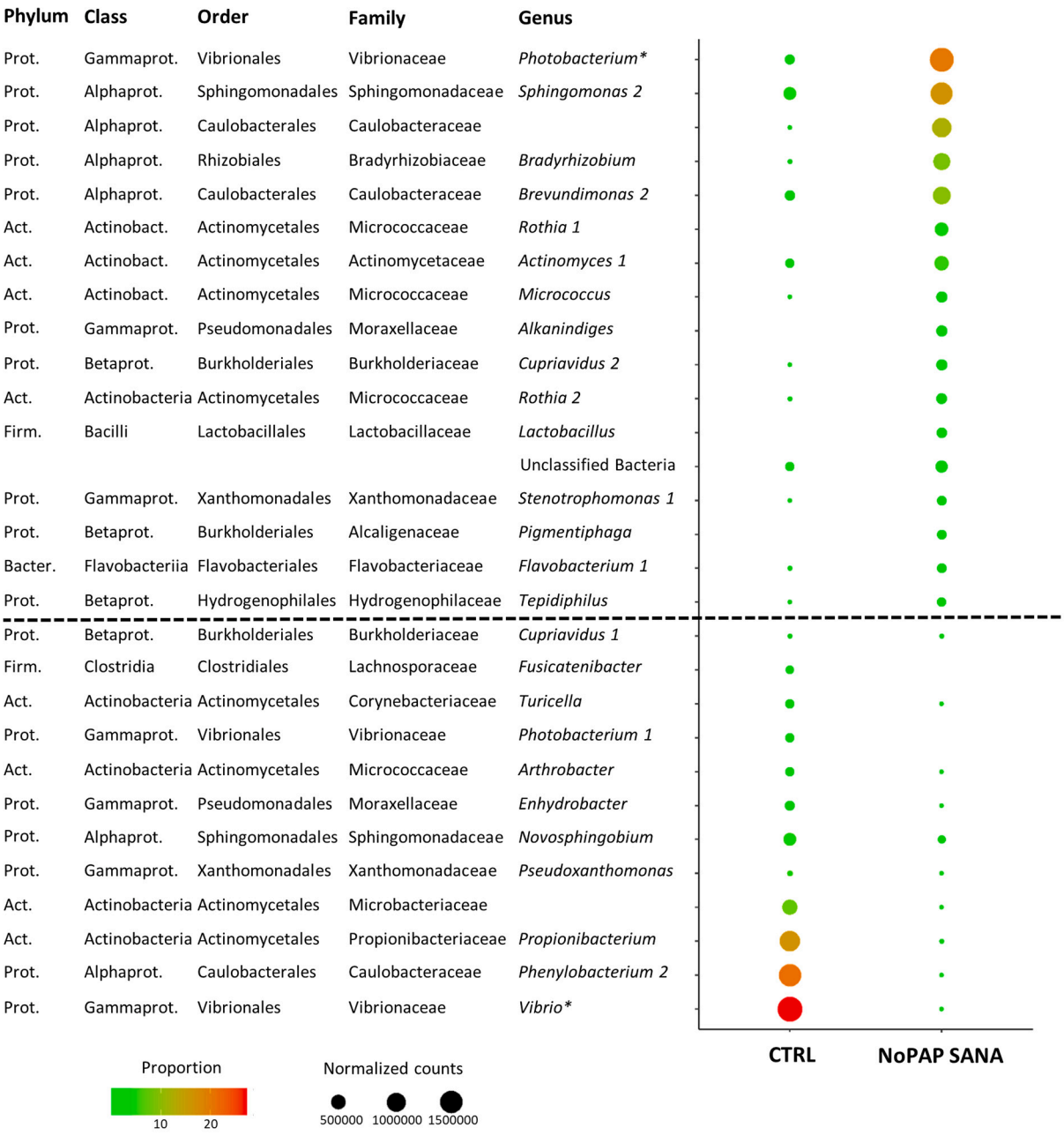

Fig. 4. Dot plot map depicting the most abundant (> 1% in at least one group) discriminant OTUs that drive the separation by diet. The size of the dots represents the mean normalized counts in each group. The colour scale represents the mean abundance in percentage of each OTU in each group. Asterisks (*) indicate that reads corresponding to these OTUs were mainly assigned to *Photobacterium damselae* and *Vibrio alginolyticus*.

poultry meal in a FM-free plant-based diet improved growth, gut barrier function and inflammation induced by a plant-based diet (Gaudioso et al., 2021). Potential benefits of new fish feed formulations also arise from recent studies in gilthead sea bream, linking changes in diet composition, growth, host transcriptomics and gut microbiota (Naya-Català et al., 2021a). The same methodological approach was considered herein for a better-balanced diet formulation containing a gut health modulator, protein and lipid aquaculture by-products, black soldier meal and microbial biomass as the main feed ingredients of a FM-free diet. The achieved results highlighted that the new proposed formulation is able to support optimal growth and a healthy condition in fish for facing a parasite infective insult. Furthermore, growth performance before parasite challenge and afterwards in non-challenged fish was in the upper range for the class of fish size and rearing conditions (Martos-Sitcha et al., 2019; Simó-Mirabet et al., 2018; Solé-Jiménez et al., 2021).

In the present study, CTRL and NoPAP-SANA diets contained 1.61 and 1.77% EPA + DHA, respectively. Therefore, the levels of n-3 long chain polyunsaturated fatty acids (LC-PUFA) were above the recommended level for marine fish species (0.7–0.9% DM) (Skalli and Robin, 2004). In consequence, when revisiting the hepatic gene expression profile of highly responsive markers of deficiencies in n-3 LC-PUFA, the relative expression level of *fads2*, *elovl6* and *scd1a* enzymes was reduced in CTRL and NoPAP SANA diets in comparison to fish fed diets with a maximal replacement of vegetable oils or semisynthetic diets formulated to be deficient in n-3 LC-PUFA (Ballester-Lozano et al., 2015; Benedito-Palos et al., 2016; Perera et al., 2020). The up-regulated expression of these lipogenic enzymes contribute to mitigate the signs of deficiencies in n-3 LC-PUFA (Regost et al., 2003; Torno et al., 2018), though it is necessary to limit hepatic lipogenesis to avoid the lipotoxic effects of

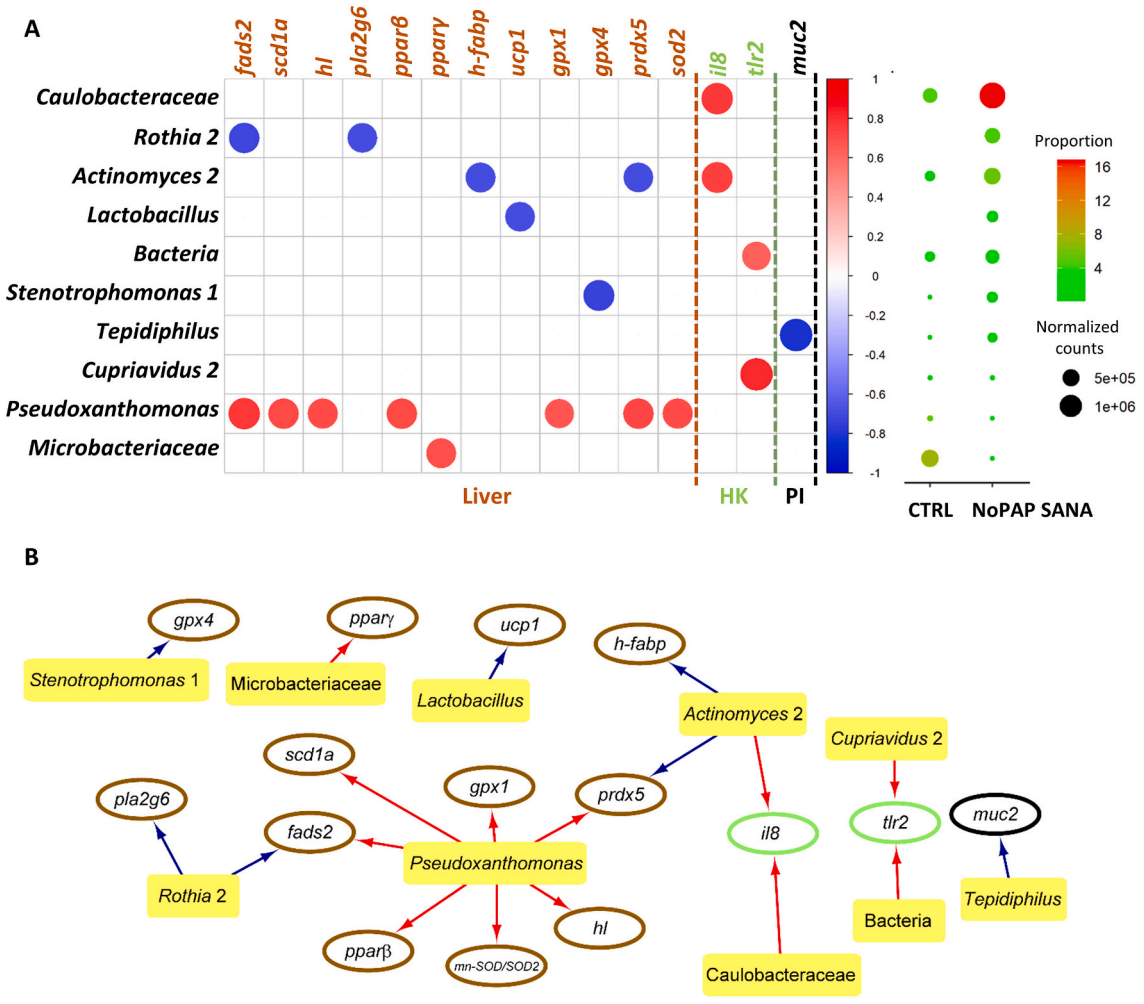

Fig. 5. Correlation between discriminant OTUs and differentially expressed genes in liver, head kidney (HK) and posterior intestine (PI). The dot plot map in A shows statistically significant ($P < 0.001$) positive (red) and negative (blue) correlations between OTUs and genes. On the right, dot plot depicting the mean abundance of each OTU in proportion (colour scale) and normalized counts (dot size) for each group (CTRL and NoPAP SANA). In B, a correlation network shows the different correlations depicted in A. Yellow squares represent OTUs and brown, green and black circles represent differentially expressed genes in liver, head kidney and posterior intestine, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

excessive fat accumulation (Perera et al., 2020). Indeed, *scd1a* is the rate limiting enzyme in the synthesis of MUFA, especially oleic acid (OA, 18:1n-9) and palmitoleic acid (16:1n-7) from stearoyl-CoA and palmitoyl-CoA, respectively. Likewise, *elovl6* is responsible for the elongation of SFA and MUFA of 12, 14 and 16 carbons to form 18-carbon FA (Weiss-Hersh et al., 2020). Therefore, the up-regulation of *scd1a* and *elovl6* would enhance the biosynthesis of MUFA, increasing the unsaturation index of FA membrane phospholipids. This is reinforced by our previous observation (Perera et al., 2020) that *scd1a* is epigenetically regulated in gilthead sea bream broodstocks fed alpha-linolenic acid (ALA, 18:3n-3) enriched diets. On the other hand, the expression of *cyp7a1,* a rate limiting enzyme of cholesterol biosynthesis, was also reduced in the present study, but overall the down-regulated expression of fatty acids and cholesterol biosynthesis was more evident in fish fed the NoPAP SANA diet rather than in CTRL diet, which might be due, at least in part, to changes in dietary EPA + DHA content and/or the dietary DHA/EPA ratio (0.55 and 0.99 in Control and test diet, respectively). This expression feature was related to the general down-regulation of hepatic markers of oxidative metabolism (*h-fabp, nd5, coxii*) and antioxidant defence (*ucp1, gpx4, prdx3, prdx5)*, which is not surprising given that lipogenesis is the most energy-demanding processes in liver (Rui, 2014), and reduced lipid biosynthetic processes during fasting or temperature drops are linked to a pronounced down-regulated expression of enzyme subunits of the mitochondrial respiratory chain (Bermejo-Nogales et al., 2014, 2015; Simó-Mirabet et al., 2020). Also in gilthead sea bream, several genes related to the antioxidant defence were down-regulated in fish fed macroalgae supplemented diets, being this finding attributed to the fact that the diets containing compounds with antioxidant properties have reduced requirements for host antioxidant enzymes (Magnoni et al., 2017). Besides, the NoPAP SANA diet contained higher amounts of black soldier meal and bacterial by-products, which have already been described to possess antioxidant activity (Medvedkova et al., 2009; Mouithys-Mickalad et al., 2020).

At the level of head kidney and posterior intestine, very few genes were regulated, demonstrating the capacity of NoPAP SANA diet to maintain the organism's homeostasis. In head kidney, only up-regulation of *il8* and *tlr2* was detected. Typically, Tlr2 is known to recognise gram-positive bacterial ligands (Ribeiro et al., 2010), but it is also able to interact with other components of gram-negative bacteria (Lien et al., 1999). Therefore, the positive correlation with the higher presence of the gram-negative Proteobacteria *Cupriavidus* is not senseless, but remains to be demonstrated. Activation of bacterial sensing receptors is not unexpected in a context of microbiota reshaping, but surprisingly, in the current study this was only detected in head kidney and not in posterior intestine. A similar effect was found for the chemotactic proinflammatory cytokine Il8, which is also able to respond

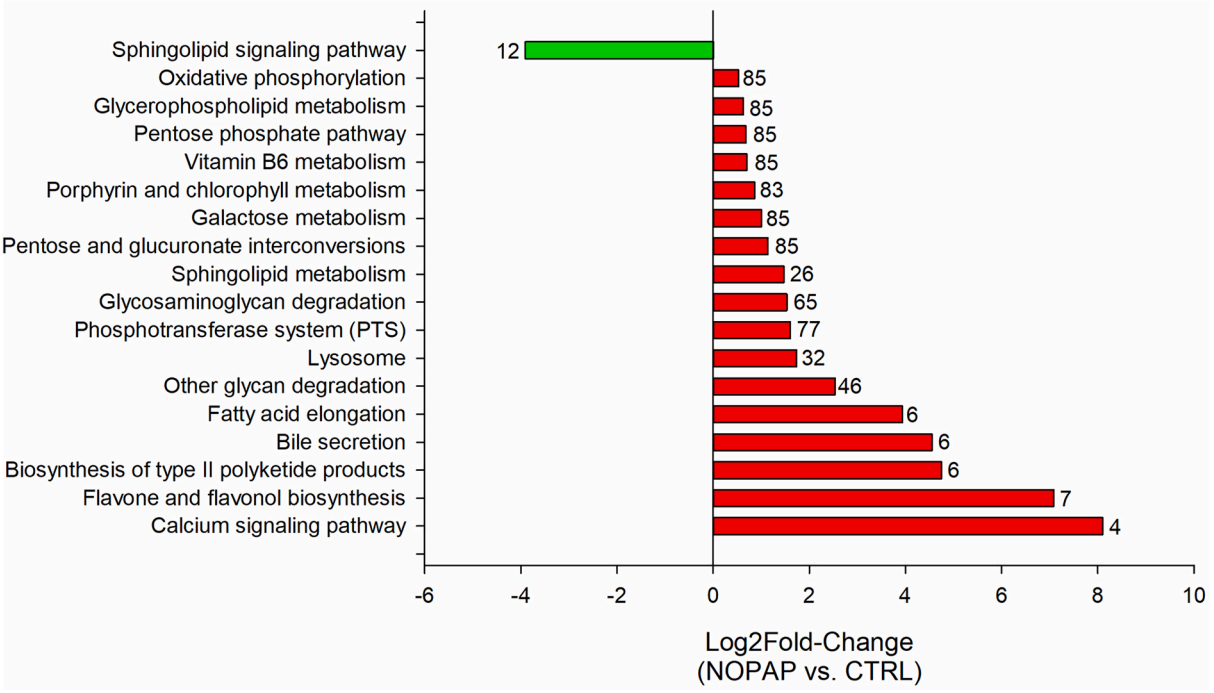

Fig. 6. Pathway analysis performed with the predicted metagenome obtained from the discriminant OTUs with VIP > 1. Bars show the Log_2 fold change of differentially over- (red) or under-represented (green) pathways when comparing NoPAP SANA group with the CTRL. Numbers in the bars indicate the number of discriminant OTUs that can potentially express genes related to each pathway. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

to a variety of symbiont bacteria (Peyyala et al., 2011), and in this case was correlated with the higher presence of *Actinomyces* and Caulobacteraceae. However, in the current study, its up-regulation was not linked with any other pro-inflammatory marker that could indicate a systemic inflammatory response towards the NoPAP SANA diet. In fact, in the intestine, down-regulation of the cell-mediated cytotoxicity markers *il12β* and *cd8β* (Bontkes et al., 2007) was found, supporting that the experimental diet is not inducing a pro-inflammatory profile, at least, at a local level. The expression of epithelial integrity markers was also maintained with the NoPAP SANA diet, with only a slight down-regulation of *cdh17*. Although this cadherin is important in cell-cell adhesion in the intestinal epithelium, the aberrant expression of this molecule has been correlated with major gastrointestinal malignancies in mammals (Su et al., 2008). Lastly, *muc2* was also down-regulated in intestine with the experimental diet. Muc2 is a large secreted gel-forming mucin normally constituting the dense inner mucus layer attached to the epithelium that is impenetrable for bacteria (Johansson et al., 2008) and is among the predominant mucins in gilthead sea bream's intestinal tract (Pérez-Sánchez et al., 2013). Down-regulation of this mucin has been found in mammalian and fish intestinal inflammation models (Johansson et al., 2008; Pérez-Sánchez et al., 2013), but the current expression profile does not indicate an intestinal inflammatory process, so this regulation might be more related to a slight reshape of the mucus layer of the posterior intestine upon a diet change. *Tepidiphilus* was found to be negatively correlated with the expression of *muc2;* regretfully, there is very scarce information about this genus as a symbiont so no hypothesis can be drawn from this association at the moment.

Intestinal microbiota is directly correlated with health status due its key roles in nutrient absorption and metabolism (Ganguly and Prasad, 2012; Semova et al., 2012), epithelial differentiation (Bates et al., 2006), immunity (Gómez et al., 2008) and defence against pathogens (de Bruijn et al., 2018). However, it is difficult to establish a specific ideal microbiota composition as these populations are extremely variable and the architecture at lower taxonomic levels can significantly change with many factors (Louca et al., 2016). Factors affecting fish intestinal microbiota composition have been widely studied in many model and aquaculture species (de Bruijn et al., 2018). For gilthead sea bream, in particular, diet, age, sex and genetic background showed to be important modulators of the intestinal microbiota (Magalhães et al., 2020; Moroni et al., 2021; Naya-Català et al., 2021b; Piazzon et al., 2017, 2019, 2020; Solé-Jiménez et al., 2021). Intestinal microbiota also changes in different parts of the intestine within the same individual due to their physiological differences (Jones et al., 2018; Ringø et al., 2006). Thus, it is important to remark that the current study focused on the posterior intestinal segment, unlike most of the studies performed in gilthead sea bream under similar conditions that targeted the anterior intestinal segment (Moroni et al., 2021; Naya-Català et al., 2021b; Piazzon et al., 2019, 2020; Solé-Jiménez et al., 2021), so differences, particularly at lower taxonomic levels, are expected. This typical plasticity reflects metabolic adaptations to different conditions and are not to be interpreted as dysbiosis, characterized by a heavily decreased microbial function and diversity, impaired epithelial barrier, bacterial translocation and inflammation (de Oliveira, 2019). In fact, in the current study, although significant changes in bacterial populations occurred when animals were fed the NoPAP SANA diet, the expected composition in terms of dominant phyla was found and no important changes in genes related to intestinal integrity or inflammation were detected, pointing towards a non-pathological reshape of the microbial communities. Thus, intestinal bacteria arise as dynamic and adaptable populations reciprocally interacting with the macroscopic host.

As the purpose of this study was to evaluate the whole formulation and not the effects of each ingredient separately, it is not possible to determine which changes are correlated with specific components of the diet. Nonetheless, some common trends in gut microbial composition were observed in similar studies evaluating specific ingredients. For instance, Fusobacteria increased in gilthead sea bream intestinal microbiota when fed black soldier fly meal diets (Panteli et al., 2021). In rainbow trout fed diets containing black soldier fly meal, the Bacteroidetes phylum and Vibrionales family decreased, whereas the genera

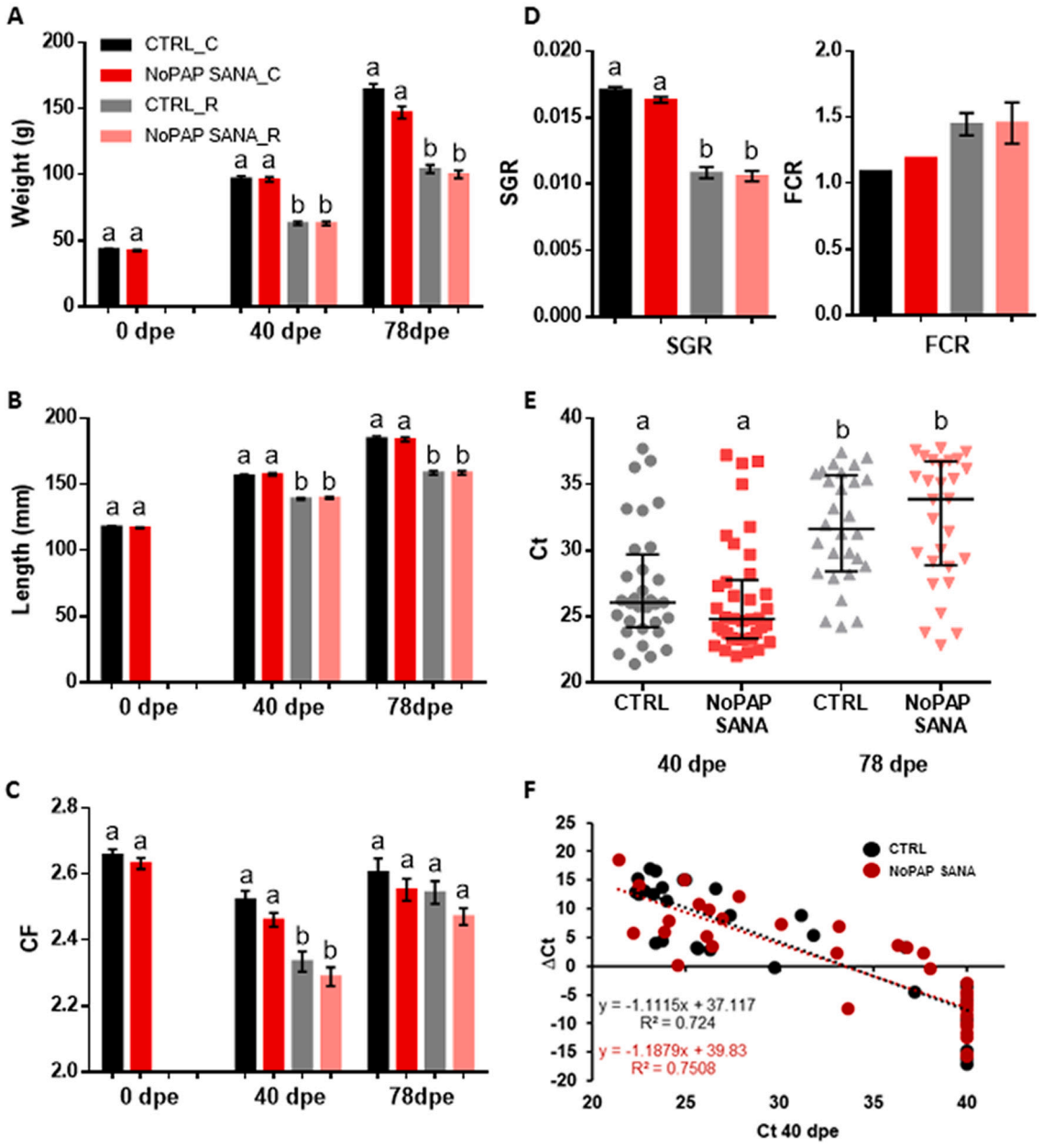

Fig. 7. Biometric parameters obtained during the infection challenge of gilthead sea bream fed CTRL and NoPAP diets. Weight (A), length (B) and condition factor (CF; C) of control uninfected [C] and *Enteromyxum leei* recipient [R] fish were measured immediately before infection (0 days post-exposure, dpe) and at 40 and 78 dpe. Individual specific growth rates (SGR) and tank feed conversion ratios (FCR; D) were calculated for the whole challenge period (0–78 dpe). Different letters indicate significant differences within each sampling point (one way ANOVA + Tukey's test, $P < 0.05$). No statistic was performed for FCR. The legend for A-D is located in A. Intensity of infection (E) was evaluated by the Ct values of the diagnostic PCR (lower Ct values = higher parasite load) and is represented as median ± interquartile range. Different letters indicate significant differences among groups (Kruskal-Wallis + Dunn's tests, $P < 0.05$). The recovery trend of each individual fish (F) was determined by plotting the ΔCt (Ct at 78 dpe – Ct at 40 dpe) against the Ct at 40 dpe. The negative slopes showed by the equations indicate a trend to increased Cts at the final sampling point with no differences between groups.

Lactobacillus and *Actinomyces* increased (Huyben et al., 2019; Terova et al., 2019), coinciding with what was found in the current study in gilthead sea bream. Most of these changes had been attributed to the chitin content of the diet. It has been hypothesized that chitin might act as a prebiotic, stimulating the colonization and growth of certain types of bacteria with the ability to digest chitin as a source of nutrients. Indeed, chitin was suggested to be a preferential substrate for lactic acid bacteria in the intestine of salmonids (Bruni et al., 2018). Also, *Actinomyces* members had been defined by their chitinolytic capacity (Beier and Bertilsson, 2013) as well as many other intestinal bacteria (Ringø et al., 2012). These similarities between distant fish species, with very different intestinal bacterial compositions, are interesting and highlight common trends to consider when looking for microbial biomarkers of health in aquaculture.

Few studies have addressed the effect of feeding bacterial biomasses on fish intestinal microbiota (Solé-Jiménez et al., 2021; Xie et al., 2021). In mammals, the non-commensal, non-pathogenic methanotropic soil bacterium *Methylococcus capsulatus* prevents experimentally induced colitis affecting dendritic cell maturation, shifting the cytokine profile and directing T cell polarization (Indrelid et al., 2017). Feeding *M. capsulatus* lysates to mice improves glucose and fat metabolism by inducing changes in the host microbiota (Jensen et al., 2021). The fish

intestinal symbiont, *Cetobacterium somerae* is known to promote fish health and enhance the gut epithelial barrier due to its production of vitamin B12 and butyrate (Li et al., 2015; Navarrete et al., 2012). Common carp (*Cyprinus carpio*) fed plant protein-based diets supplemented with fermentation product of *C. somerae* showed an alleviation of the pro-inflammatory profile and liver fat accumulation induced by the plant substitution of FM by regulating gene expression and gut microbial composition (Xie et al., 2021). The current results showed that fish fed NoPAP SANA diet shift the composition of their intestinal microbiota inducing a higher presence of large numbers of bacteria that can potentially be implicated in glucose and fat metabolism (Fig. 6). Also, several bacteria showed a significant correlation with liver fat metabolism gene expression (*Rothia*, *Pseudoxanthomonas*, Microbacteriaceae) and antioxidant defence (*Pseudoxanthomonas*, *Stenotrophomonas* and *Actinomyces*). Although the exact mechanisms and the cause-effect of these interactions remain to be determined, it is clear that intestinal microbiota and host-gene expression (not only local, but also systemic) are interacting and efficiently adapting to this novel formulation with alternative sustainable ingredients.

The results explored so far indicate that NoPAP SANA diet is an adequate sustainable alternative to traditional gilthead sea bream diets, at least in terms of growth, gene expression and intestinal microbiota. However, all these parameters were measured in healthy fish and the real proof of concept of a diet effect on health should be performed under challenging conditions. For that reason, fish were challenged with the enteric myxozoan parasite *Enteromyxum leei*, an economically important pathogen in Mediterranean sparid farms (Palenzuela, 2006). *Enteromyxum leei* invades the paracellular space of the intestinal epithelium producing severe desquamative enteritis that impairs nutrient absorption causing delayed growth, anorexia and cachexia. It first establishes in the posterior intestine and slowly progresses along the intestine producing changes at local and systemic levels (Estensoro et al., 2012, 2013; Piazzon et al., 2018). In *E. leei* infections, the severity of the disease signs has been previously correlated with the nutritional background of the host. Healthy gilthead sea bream fed plant-based diets did not display any negative side effects in terms of growth performance, histopathology, antioxidant status and muscle fatty acid profiles (Benedito-Palos et al., 2008; Saera-Vila et al., 2009). However, when these animals were challenged with *E. leei* a worse disease outcome was evidenced in terms of disease signs, prevalence and intensity of infection (Estensoro et al., 2011). The current results showed no major final differences in infection-associated parameters between fish fed CTRL and NoPAP SANA diets, indicating that this formulation is not inducing undesired effects upon a pathogenic challenge. Both R groups showed similar disease signs in terms of growth performance with a final weight loss of 37.64% and 40.9% in NoPAP SANA and CTRL diets, respectively. These values are in consonance with other laboratory challenge trials conducted also with warm temperatures and the same challenge route at 70 dpe (36.2% of weight loss and 72.4% prevalence of infection), using a commercial diet containing 15% FM (Palenzuela et al., 2020). In addition, both dietary groups showed similar signs of disease recovery during the last phase of the challenge trial. In general, high temperature is better for parasite proliferation, but *E. leei* proliferation showed to be suppressed at temperatures higher than 30 °C (China et al., 2014). In the current experiment, the high temperature registered between the intermediate and the final sampling (29 °C) together with the higher activation of the adaptive immune system of gilthead sea bream under these conditions (Picard-Sánchez et al., 2020), could explain the final decrease in the intensity of infection, which was similar in both dietary groups, in agreement with the recovery of the CF values and the lower decrease of the SGR.

In summary, the novel feed formulation tested in this study promoted adequate growth and nutrient utilization, arising as a good alternative for FM-free diets in gilthead sea bream aquaculture. The fish adapted to this new diet formulation through changes in gene expression, and gut bacterial composition to sustain proper growth without showing increased susceptibility against intestinal parasites, as often observed in low FM/FO diets. Moreover, the results show that intestinal microbiota can tightly interact with host-gene expression, not only locally, but also at a systemic level. Further studies are underway to unravel and better understand the root and mechanisms of these interactions.

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CRediT authorship contribution statement

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Declaration of Competing Interest

G-VP, JD and LEC were employed by the company SPAROS (Olhão, Portugal). WGN-O was employed by Adisseo (Dendermonde, Belgium). The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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