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Intestinal Explant Cultures from Gilthead Seabream (Sparus Aurata, L.) Allowed Determining the Mucosal Sensitivity to Bacterial Pathogens and the Impact of a Plant Protein Diet

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Abstract

Background

Although high levels of fish meal replacement by alternative protein sources have been achieved without relevant alterations in terms of growth performance, negative effects on immune status were detected. The diet and fish immunity interactions at gut level have been widely discussed, although *in vivo* approaches have reported several limitations. In this sense, intestine explant culture system can be a valuable complementary tool to study the interactions between pathogenic bacteria and fish gut response, and the possible influence of environmental, breeding, rearing conditions or dietary components on the responsiveness of the innate immune system in fish.

The object of this study was to test the impact of total substitution of fish meal by plant protein on the intestinal health of seabream (12g) in two growth stages: phase I (90 days), up to 68 g, and phase II (305 days), up to 250g. In phase II, the effects of the long term and short exposure (15 days) to plant protein diet were determined. In order to determine the effect of plant protein feeding on the innate immune response to bacterial pathogens, and an *ex vivo* procedure of intestine explants culture was implemented.

Results

Fish showed less tolerance to dietary plant protein in phase I than in phase II, while the *ex vivo* assays indicated that the intestine from fish fed at short-term plant diets showed a higher immune response than at long term feeding.

In relation to the immune response to bacterial challenge, a significant expression in pro-inflammatory cytokines IL-1β and IL-6 after 6 hours of exposure to *V. algynoliticus*, while COX-2 expression was significantly induced by *P. damselae* subsp. *pisicida*, showing positive high correlation between them.

Conclusions

A differential health status was observed depending of growth stage, being stricter to the plant protein inclusion the younger fish. The new experimental system based on fish intestinal explants culture has been successfully implemented, becoming an effective methodology for *ex vivo* studies. Under ex vivo conditions, the bacterial challenge induced inflammatory and immune intestinal response, responding stronger those intestine of fish fed during a short-term with a total substitution of fish meal.

Background

In addition to the digestion and absorption of nutrients, the fish intestine is a complex biological system that represents a major defence barrier against pathogens and plays a crucial role in osmoregulation, immune and inflammatory response [1]. Furthermore, the intestine participates in the modulation of gastrointestinal microbiota inducing inflammatory responses against pathogenic bacteria or developing immunotolerance to luminal bacteria.

The interactions between fish intestinal immunity, pathogen and commensal microbiota in the gut have been widely reviewed [2]. Bacterial challenges *in vivo* require specialized settings, expensive operating costs, and a high number of fish, and is difficult to perform and achieve the desired experimental working conditions [3]. In this regard, systems have been developed, based on the *ex vivo* maintenance of intestine fragments, to evaluate successfully the effect of different bacterial strains on the intestinal health and providing very reliable information on the interactions between the bacteria and the host. The *ex vivo* intestinal sack method [4] has been used to assess the histological and microbial changes in fish in response to bacteria exposure [3, 5–9]; however, this method is highly restricted by the tissue viability under experimental conditions [3, 4]. The development of new experimental models based on tissue explants culture has allowed maintaining the tissue lifespan, as well as immune and histological features [10–12]. These systems have been used to register responses to exposure to specific bacteria in human tissue explant cultures, also at gene expression level [12].

On the other hand, the necessity to replace fish meal by alternative protein sources in aquafeeds has led researchers to focus on the impact of the inclusion of alternative ingredients, such as plant protein sources. Although its use at high levels without impairing growth performance is feasible [13, 14], negative effects on immune capacity have been observed [15]. Previous findings revealed that during early growth stages the inclusion of plant protein has a great impact [16, 17]; at intestinal level, the inclusion of plant sources in diets has been related to morphological alterations, changes in the intestinal bacterial community, inflammatory events and lack of capacity to regulate the intestinal epithelial integrity [18]. Similar results have been reported *in vivo* at molecular level, with an altered gene expression pattern [19–21].

Research related to the impact of fish meal replacement becomes even more relevant for carnivorous species, such as gilthead seabream. In this species, previous studies with different levels of substitution and alternative ingredients, have been assayed to evaluate zootechnical parameters and survival [16, 22, 23], and the immune *in vivo* response to fish meal replacement [24–26] or bacterial infection [27–30].

The *ex vivo* response of intestinal tissue from fish feed with different protein sources to bacterial challenge has been previously addressed in other species [3]. However, to the best of our knowledge, this is the first study involving gene expression determination in fish intestinal explants after *ex vivo* bacterial exposure.

Therefore, in the current work, it was developed an intestine explant culture system to evaluate a possible differential inflammatory and immune response to *ex vivo* bacterial challenge in fish after a long-term of total fish meal replacement at different stages of growing. Additionally, the effect of a short-term of

total fish meal replacement by a plant mixture in on growing fish was also assayed.

Materials And Methods

Ethics statement

The experiment was reviewed and approved by the Committee of Ethics and Animal Welfare of the Universitat Politècnica de València (UPV), following the Spanish Royal Decree 53/2013 on the protection of animals used for scientific purposes [31].

Fish, rearing system conditions, diets and feeding conditions

A total number of 240 juveniles of gilthead seabream (averagare weigth 7,5 g and 60 days) were obtained from the fish farm BERSOLAZ (Bersolaz Spain, S.L.U, Culmarex Group) located in Port de Sagunt (Valencia, Spain) and transported to the facilities at the Universitat Politècnica de València, where the growth trial was conducted after 15 days of adaptation to experimental conditions. Features of the system and water parameters set were described in previous growth trials carried out in these facilities [32,33]. Lighting conditions were determined by the natural photoperiod. Temperature, pH, oxygen, ammonia, nitrite and nitrate concentrations were monitored along the experiment. The fish were daily fed by hand to apparent satiation two times per day (9:00h and 17:00h). The pellets were slowly distributed, allowing fish to eat, in a weekly regime of six day of feeding and one day of fasting.

Diets were prepared by cooking extrusion process using a semi-industrial twin-screw extruder (CLEXTRAL BC-45, St. Etienne, France). A fish meal based control diet (FM), in which most of the protein was provided by fish meal (59%), and a plant protein based diet (PP), in which all the fish meal was replaced by plant sources and synthetic amino acid were added to meet the minimum amino acid requirement for gilthead seabream juveniles [34]. Ingredients and proximate composition are shown in Table 1. Prior to diet formulation, dry matter, crude protein, crude lipid, ashes and crude fibre (CF) of different sources and ingredients used were analysed according to AOAC procedures [35]. All analyses were performed in triplicate. Amino acids of raw diets were also analysed by reverse phase – high performance liquid chromatography [36]. Macronutrients and essential amino acid content were determined in the experimental diets, and they are shown in Table 1.

Table 1
Ingredients, proximal composition and essential amino acids profile of experimental diets

	FM	PP
Ingredients (g kg^{-1})		
Fish meal	589	
Wheat meal	260	
Wheat gluten		295
Broad bean meal		41
Soybean meal		182
Pea meal		41
Sunflower meal		158
Krill meal		
Squid meal		
Fish oil	38.1	90
Soybean oil	92.9	90
Soy Lecithin	10	10
Vitamin-mineral mix*	10	10
Calcium phosphate		38
Arginine		5
Lysine		10
Methionine		7
Taurine		20
Threonine		3
Proximate composition (% dry weight)		
Dry matter	88.1	93.9
Ashes	10.1	7.4
Crude lipid	18.5	19.8
Crude fiber	0.8	4.3
Crude protein	44.2	45.0
Essential aminoacids (g 100 g^{-1})		
Arginine	3.39	3.30
Histidine	1.00	0.82
Isoleucine	1.47	1.17
Leucine	3.24	2.98
Lysine	3.68	2.26
Methionine	1.16	1.06
Phenylalanine	1.80	1.87
Threonine	1.98	1.44
Valine	2.01	1.47

Bacterial strains and growth conditions

Cultures of *Pseudomonas anguilliseptica* CECT 901, *Vibrio algynoliticus* CECT 521 and *Photobacterium damselae* subsp. *piscicida* CECT 7198 were obtained from the Colección Española de Cultivos Tipo (CECT, Valencia, Spain). The culture medium used for *P. anguilliseptica* was Tryptic Soy Broth (containing per litre of water, 15.0 g tryptone, 5.0 g soy peptone and 5.0 g NaCl, pH 7.3), and *V. algynoliticus* and *P. damselae subsp. piscicida* were grown on Marine Broth (containing per litre of water, 5.0 g Bacto peptone, 1.0 g yeast extract, 0.10 g Fe(III) citrate, 19.45 g NaCl, 0.16 g Na_2CO_3, 3.24 g $NaSO_4$, 1.80 g $CaCl_2$, 8.80 g $MgCl_2$, 0.55 g KCl, 0.08 g KBr, 34.00 mg $SrCl_2$, 22.00 mg H_3BO_3, 4.00 mg Na-silicate, 2.40 mg NaF, 1.60 mg $(NH_4)NO_3$ and 8.00 mg Na_2HPO_4, pH 7.6). Bacteria were grown under agitation at 26º C for 2 days (*P. anguilliseptica* and *P. damselae* subsp. *piscicida*) and at 30º C for 1 day *(V. algynoliticus).* Then, 1.5 g/L of bacteriological agar were added to these media to prepare solid medium in petri dishes. For the bacterial challenge, optical density (600 nm) of the bacterial cultures was determined and bacterial cell number was estimated using the standard curves established for each strain. Then, bacterial cultures were centrifuged at 4.000 g for 20 min, washed once with PBS, and re-suspended in CO_2-independent cell culture medium (Gibco, ThermoFisher) to a final concentration of $3{\cdot}10^7$ ufc/mL in the case of *V. algynoliticus*, and $1{\cdot}10^7$ ufc/mL of *P. damselae* subsp. *piscicida* and *P. anguilliseptica*.

Experimental design

The aim of this work was to evaluate the impact of dietary fish protein substitution by plant protein on the intestinal health status and its immune response capacity. For this purpose, fish were fed with fish meal (FM) or plant protein (PP) based diets, and animals were sacrificed and processed at two critical growth phases of sea bream [16,17]: Phase I (90 days; from 12g to~ 68g) and Phase II (305 days; up to 250g). Each diet was assayed in tanks per triplicate. Fig 1. illustrates the experimental design. At each sampling time intestine fragments were collected, part of them were used to determine basal expression of selected genetic markers and other fragments were used in the culture explant procedure (*ex vivo* assay) to test the immune competence of the intestinal mucosa when challenged with different bacterial pathogens.

Phase I: Up to 68 g

Fish were fed with FM or PP diets, in tanks per triplicated (40 fish per tank), up to 90 days, being scattering 2 fish per group to submit bacterial challenge (68±37.8g). For basal gene expression two fragments from foregut (FG) and hindgut (HG) from each fish were placed in an eppendorf tube containing 500 µl of RNA Later® (Qiagen, Valencia, CA) for subsequent total RNA (tRNA) extraction. Additionally, four fragments from of FG and HG fragments were used for the *ex vivo* assay and exposed to pathogens challenge (see below). Gene expression was determined in all samples to evaluate the inflammatory and immune status of the intestinal mucosa due to changes in the diet and the bacterial challenge.

Phase II: Up to 250 g

Fish, ~30 fish per tank, were fed with the same diets, FM and PP, up to 305 days when the mean weight was 252±70.1g (Fig 1). In this second phase, in addition to the impact of long-term feeding with 100% of PP diets, also a short-term exposure (15 days) of total fish meal substitution was evaluated. For this purpose, fish bred with FM diet (n=~15 fish per tank) were changed to a PP diet two weeks before the termination of the experiment (from day 290 to day 305) (PP* group). Three fish from the FM group and two from the PP and PP* were sacrificed to obtain FG and HG explants for *ex vivo* assays. As in the previous assay, for basal gene expression two fragments from each fish were placed in RNA Later® for subsequent total RNA (tRNA) extraction, and four pieces for *ex vivo* study.

Ex vivo assays and bacterial challenge

Before tissue preparation, fish were sacrificed by immersion in benzocaine (60 ppm) during 15 min. Then, they were dissected and the intestine was obtained and separated in two sections (foregut and hindgut). Each section was cut with a scalpel in small pieces (4 mm x mm), which were immediately placed in culture filter plates (15 mm diameter wells with 500 µm bottom-mesh, Netwell culture systems, Costar, Cambridge, MA) with the epithelial surface facing up. Filters were placed into wells containing 1 mL of the different bacterial solutions (one of them was preserved without bacteria as control; *Ex vivo* Unchallenged group) in CO_2-independent cell culture medium (Gibco, ThermoFisher). 100 µL of the corresponding bacterial solutions were finally added to epithelial surface to ensure that samples were completely submerged. At the end of the incubation time, samples were carefully collected from the culture filter plates and stored in 100 mM Tris-HCl at 4ºC or RNA later at -80º C for lactate dehydrogenase (LDH) activity evaluation or RNA isolation, respectively. Changes in pH of the explant culture medium due to different bacterial treatments were monitored. Explants of foregut (FG) and hindgut (HG) from two fish per group were incubated during 4 and 6 h at 22º C in independent CO_2 atmosphere, depending on the experiment. The bacterial species used in the pathogen challenge were: *Photobacterium damselae* subsp, *Pseudomonas anguilliseptica* and *Vibrio algynoliticus*. *Pseudomonas anguilliseptica* was discarded in phase II, because bacterial concentration could not be determined due to aggregate formation. After explant assay, the samples were placed into RNA Later (Qiagen, Valencia, CA) for subsequent tRNA extraction. All conditions (fish/section/stimuli) were assayed in duplicate and gene expression was determined in all samples to evaluate the intestinal inflammatory and immune status based on experimental diet and bacterial challenge.

LDH activity assay

In order to determine the tissue integrity, the LDH activity was determined [37] in the tissue (U/mg protein) and explant culture medium (U/L) at different times of the incubation (0, 4, 6 and 24 h). LDH activity was analysed measuring the nicotinamide adenine dinucleotide (NADH) absorbance at 340 nm using the commercial kit (BioSystems S. A., Barcelona, Spain). Tissue was weighed, homogenised in Tris-Hcl 100 mM while maintaining the tubes on ice, centrifuged at 12.000 rpm and 4º C for 15 min and supernatant was collected for LDH assessment. Total protein in tissue extracts was determined using Bradford [38].

Gene expression assay of intestinal inflammatory and immune markers

Based on the gene expression analysis used in previous studies in this species (*Sparus aurata*) to evaluate the intestinal inflammatory and immune status [32] tRNA was extracted from intestinal tissue samples using the phenol/chloroform method with Trizol Reagent (Invitrogen, Spain) and treated with DNAse I (Roche) to remove DNases. Total RNA concentration, quality and integrity were assessed using a NanoDrop 2000C Spectrophotometer (Fisher Scientific SL, Spain). The integrity of 28S/18S was also determined by gel electrophoresis. 1 µg of total RNA was used for cDNA synthesis reaction using the qScript cDNA synthesis kit (Quanta BioScience), according to the manufacturer's instructions. An Applied Biosystems 2720 Thermal Cycler was used with the following cycling conditions: 22 ºC for 5 min, 42 ºC for 30 min, and 85 ºC for 5 min. cDNA samples were stored at -20º C until gene expression was analysed.

Four housekeeping candidate genes (Table 2) were tested to be used as reference genes, and for assessing RNA integrity along the assay. The Cq of the four genes was determined in six pooled samples from Experiment 1 (two dietary groups: FM and PP; three times: 0, 4 and 6 h). Relative gene expression of six genes was determined in the foregut and hindgut samples. The genetic markers monitored in this assay were three pro-inflammatory markers, IL1-β, IL-6 and COX-2, the main immunoglobulin, IgM, the main intestinal mucin, I-Muc, and the occludin gene, Ocl, with primers listed in Table 2.

Table 2
Primer sequences of candidate genes (reference and target genes) in the RT-qPCR assay

Gene	Abbreviation	GeneBank ID	Primer Forward	Primer Reverse	Lenght	Reference
REFERENCE GENES						
Elongation Factor 1α	EF-1α	AF184170	CTGTCAAGGAAATCCGTCGT	TGACCTGAGCGTTGAAGTTG	87	[35, 36]
Glyceraldehide 3-phosphate dehydrogenase	GAPDH	DQ641630	CCAACGTGTCAGTGGTTGAC	AGCCTTGACGACCTTCTTGA	80	[37]
Ribosomal Protein S18	Rps18	AM490061	AGGGTGTTGGCAGACGTTAC	CGCTCAACCTCCTCATCAGT	97	[37]
β-Actin	β-Act	X89920	TCTGTCTGGATCGGAGGCTC	AAGCATTTGCGGTGGACG	113	[38]
TARGET GENES						
Interleukin 1β	IL-1β	AJ277166	GCGACCTACCTGCCACCTACACC	TCGTCCACCGCCTCCAGATGC	131	[37]
Interleukin 6	IL-6	AM749958	AGGCAGGAGTTTGAAGCTGA	ATGCTGAAGTTGGTGGAAGG	101	[35]
Cyclooxygenase 2	COX-2	AM296029	GAGTACTGGAAGCCGAGCAC	GATATCACTGCCGCCTGAGT	192	[35, 36]
Intestinal Mucin	I-Muc	JQ277712	GTGTGACCTCTTCCGTTA	GCAATGACAGCAATGACA	102	[38]
Immunoglobulin M	IgM	JQ811851	TCAGCGTCCTTCAGTGTTTATGATGCC	CAGCGTCGTCGTCAACAAGCCAAGC	131	[39]
Occludin	Ocl	JK692876	GTGCGCTCAGTACCAGCAG	TGAGGCTCCACCACACAGTA	81	[35, 36]

All qPCR assays and expression analyses were performed using the Applied Biosystems 7500 Real-Time PCR with SYBR® Green PCR Master Mix (ThermoFisher Scientific, Waltham, Massachusetts, USA). After an initial Taq activation of polymerase at 95 ºC for 10 min, 42 cycles of PCR were performed with the following cycling conditions: 95 ºC for 10 s and 60 ºC for 30 s in all genes. In order to evaluate assay specificity, a melting curve analysis was directly performed after PCR cycles by slowly increasing the temperature (1º C/min) from 60 to 95 ºC, with continuous registration of changes in fluorescent emission intensity. The total volume for every PCR reaction was 20 µl, performed from diluted (1:20) cDNA template (5 µl), forward and reverse primers (10 µM, 1 µL), SYBR® Green PCR Master Mix (10 µl), ROX (2 µL, 10 nM) and nuclease-free water up to 20 µl. The analysis of the results was carried out using the $2^{-\Delta\Delta Ct}$ method. The target gene expression quantification was expressed relative to the expression of the selected reference gene. A cDNA pool from all the samples was included in each run and acted as a calibrator, and a non-template control for each primer pair, in which cDNA was replaced by water, was run on all plates. Reference and target genes in all samples were run in duplicate PCR reactions.

Statistics

Statistical data analysis were performed with Statgraphics© Centurion XVI software (Statistical Graphics Corp., Rockville, MO, USA).

LDH enzymatic activity in tissues and the supernatant was statistically analysed by one-way analysis of variance (ANOVA) using Newman-Keuls test to determine possible differences across the assay (0, 4, 6 and 24 hours) in FG and HG.

The expression stability of reference genes was assessed using the BestKeeper program, basing on the arithmetic means of the Cq values [39]. Lower deviation in the expression is related to better stability.

The evaluation of intestinal inflammatory and immune status was performed through the gene expression of the target genes both *in vivo* and *ex vivo* conditions. The relative gene expression was statistically analysed by ANOVA. Gene expression of cultured pieces was normalised with the expression of *ex vivo* unchallenged samples at 4 and 6 hours. Multifactorial analysis was used to determine the significance ($p<0.05$) of different factors considered (dietary treatment: FM/PP, intestinal section: FG/HG and bacterial stimuli*: P. damselae* subsp. *piscicida*/*P. anguilliseptica*/*V. algynoliticus*) at different times and to determine differences in normalised gene expression between dietary groups, sections and bacterial stimuli, using Newman-Keuls test. Data was expressed with the mean and the standard error of the normalised expression values, and differences were considered statistically significant when $p<0.05$.

Additionally, with the aim to evaluate if the bacterial challenge is individually inducing the target genes or a combination of a set of genes, a correlation analysis was carried out and the Pearson product-moment coefficient was obtained for each pair of genes.

Finally, in order to confirm the assay reproducibility, the gene expression of the biological replicate samples was randomly assigned to different variables (x and y). Data consistency was evaluated for each gene by simple regression analysis using the model $y = ax$. 95% confidence intervals for a ($a \pm 1.96\sigma$) were obtained for each gene to validate the hypothesis $a=1$ ($y=x$).

Results

With the aim to evaluate the effect of PP diet on inflammatory and immune gene expression in the gut, 240 fish were fed on FM and PP for 305 days. The effect of diet was investigated in a first stage at day 90 (phase I) and the experiment continued until day 305 (phase II). Two weeks before the end of the experiment, a group of animals from the FM group was introduced to the PP diet, in order to assess a possible short term effect of the PP diet in adult fish. Intestine fragments of fish harvested in phase I and phase II were used for the *ex vivo* pathogen challenge assay, but also basal gene expression was determined on intestinal samples.

Optimal conditions for intestinal explant culture were set up through different approaches. Tissue and cellular integrity were monitored by the release of LDH activity at 0, 4, 6 and 24 h of incubation. No relevant differences were observed at tissue level, but a significant increase was registered at 24 h of incubation in the explant culture medium (Additional file Fig. 1). Candidate housekeeping genes for real time qPCR were tested at different times, indicating also that 6 h was an appropriate time of incubation (Additional file Fig. 2). On the other hand, if the effect of *ex vivo* procedure is evaluated, significant differences were observed in most of the genetic markers analysed (Additional file Fig. 3). Therefore, in the following assays, gene expression was normalised based on the *ex vivo* unchallenged samples.

Phase I: Up to 68 g

With the aim of assessing the effect of a total fish meal substitution on intestinal inflammatory and immune status at early growth stages (68 g fish weight), basal gene expression was determined and *ex vivo* trials were carried out with 6 h of challenge of pathogen bacterial cultures.

Basal gene expression

After 90 days fed with PP and FM diets, fish intestines showed a significantly different gene expression profile, as the PP group had a higher expression level for IL-1β and lower for COX-2 (Fig. 2).

Ex vivo assay

The explant culture system was used to determine the immune response of intestinal fragments from on-growing seabream specimens fed with different diets (Fig. 3A). Additionally, the intestine was divided into two segments: FG and HG to evaluate a possible differential immune response (Fig. 3B). At 6 h, gene expression was statistically different between the PP and FM diets in most of the markers, and the induction of IL-1β and Ocl in fish under PP diet was particularly remarkable (Fig. 3A). Gene expression responses of the different intestinal sections were quite similar, with higher expression of IL-6 in FG at 4 h (Additional file Fig. 4) and at 6 h HG presented higher expression of Ocl (Fig. 3B).

After the bacterial challenge, fish belonging to PP group showed, in general, a greater response to the bacterial stimuli (Fig. 4), with significant differences for IL-1β and IL-6 genes. This tendency to a higher response in PP group was not observed at 4 h of incubation, not registering significant differences between diets or bacterial stimuli (Additional file Fig. 5).

If only the bacteria variable is considered, significant differences were only observed with *V. alginolyticus* after 6 h of incubation, specifically with a higher expression of COX-2 and notably of IL-1β (Additional file Fig. 6B), indicating that this bacterium elicited a great response in all conditions. Finally, if a multifactorial analysis of variance is performed taking into account all the factors, IL-6 and Ocl were significantly altered by the section and the diet at 4 hours of incubation, respectively. Nevertheless, it was necessary 6 h of exposition to register differences caused by bacterial stimulus (Additional file Table 1).

Phase II: Up to 250 g

This second assay tried to evaluate the impact of a total substitution of fish meal at longer term feeding, up to 252 g (305 day; PP) on intestinal gene expression. Additionally, the differential response of total fish meal substitution with plant protein (PP) during a short-term period (15 days; PP*) was evaluated.

Basal gene expression

No gene expression differences were observed between fish fed FM diet and plant based diets at long-term (PP) as well as at short term (PP*), except to IgM in PP* group (Fig. 5).

Ex vivo assay

The expression of pro-inflammatory genes (IL-6 and COX-2 genes) increased after 6 h of incubation in the *ex vivo* unchallenged group respect to the basal values, confirming the results of previous assay (Additional file Figure S7). Hence, expression results in samples incubated with the different bacteria were normalised with the expression of the control samples, for each experimental factor.

The consistency of *ex vivo* assay for the biological replicates was checked by a correlation analysis for each biological replicate pair (Additional file Figure S8). The adjustment to the lineal model is particularly good for pro-inflammatory genes (IL1-β, IL-6, COX-2). I-Muc expression reported a high variability between duplicates and no significant relationship can be established between x and y data, consequently, it was not considered in further analyses.

A multifactorial ANOVA of gene expression taking into consideration the diet, section and bacterial challenge (stimuli), underlined a significant linkage of PP diet with COX-2 and Ocl. As expected, the expression of pro-inflammatory genes (IL-1β, IL-6 and COX-2) was bound to the pathogen stimuli (Table 3), while again no differences were reported between sections. Therefore, due to the lack of statistical differences ($p < 0.05$) between intestinal sections, the following analyses were performed joining both sections.

Table 3
Effect of different factors on normalised gene expression values in phase II

	IL-1β	**IL-6**	**COX-2**	**IgM**	**Ocl**
Diet	0.533	0.601	0.025*	0.120	0.044*
Section	0.157	0.138	0.168	0.864	0.486
Stimuli	0.003*	0.036*	0.021*	0.218	0.163

p-values obtained for each factor in the multifactorial analysis. Significant values are indicated by *

The exposition to *V. algynoliticus* and *P. damselae* subsp. *piscicida* induced a remarkable increase of IL-1β and IL-6 expression respect to unchallenged samples (Fig. 6). Regarding the sensitivity to the pathogen as function of diet, the PP* group showed a remarkable tendency to have higher expression values for all tested genes in response to bacteria, but only COX-2 and Ocl showed significant differences with respect to groups FM and PP (Fig. 6D and Fig. 6F). Of note, *V. algynoliticus* induced significant IL-1β response in all diet groups (Fig. 6B). Finally as expected, there was a high correlation (Pearson's coefficient) between the expression of IL-1β, a known master regulator of innate immune response and inflammation, and IL-6 and COX-2 (IL-1β / IL-6 = 0.74; IL-1β / COX-2 = 0.72) (Fig. 7).

Discussion

It has been extensively proven that the inclusion of alternative plant proteins can lead to nutritional imbalances [23, 40] and immune dysfunctions [24, 41], particularly in carnivorous fish, increasing their susceptibility to pathogenic invasion, disease and finally death. Findings of present study suggest that the fish gut response to the total dietary substitution of fish meal by plant protein meals can differ at short term and long-term feeding and the fish size. Previous studies in seabream demonstrated that the use of plant proteins induced significant alterations of the gut microbiota, gut gene expression and gut proteomic profile [32, 42, 43]. Nonetheless, these alterations can be affected to different extent by the feeding period or fish stage. In this study, the effect of a total fish meal (FM) substitution by plant protein (PP) was evaluated in two stages of fish growth: from 12 to 68 g (phase I) and up to 252 g (phase II). In phase II, the effect on intestinal health status of FM substitution by PP throughout the whole period (305 days) was compared with that of a shorter term feeding (15 days).

An explant culture assay has been implemented in this work to evaluate the intestinal health status of fish exposed to PP diet. *Ex vivo* approaches based on explants culture proved to be useful to analyse pro-inflammatory responses [12]. In fish, several works have been attempted to evaluate the pathogen-host [44], especially by the intestinal sack method [3, 7–9]. In the present work, significant differences were clearly observed with incubations of the intestine explants of 6 h, and these experimental conditions showed to preserve tissue integrity. Furthermore, explants obtained from the same section of each fish demonstrated a sound consistency in the response to pathogens. Although a different immunological performance has been attributed to the foregut and hindgut [45], using this experimental set up no significant differences could be found between gene expression in both segments. This allowed increasing the number of explant fragments from each single intestine, then improving the experimental efficiency and reducing the number of fish and, hence, reducing individual variability.

In the present experiment, three pro-inflammatory markers have been monitored to assess the inflammatory status (IL-1β, IL-6, COX-2) [46, 47]. IgM is considered as the most abundant immunoglobulin in plasma, high levels in fish fed with plant sources based diets have been reported [48] and its

expression was induced in mucosal tissues as response to pathogen infection [49]. I-Muc is the main intestinal mucin, and it is involved in epithelial protection, bacteria adhesion and growth, and has been suggested as a resilience biomarker to inflammation in fish [50]. Finally, Ocl is a key protein in the regulation of tight junctions between enterocytes, and therefore in the permeability of the epithelial barrier [51].

The stage of fish growth and the feeding period have a clear influence on the response of fish performance to dietary plant protein and previous studies reported that juvenile fish tolerates less dietary plant protein than commercial size fish [16, 52]. In phase I, intestine explants of seabream fed with plant protein for 90 days showed a high basal inflammatory response, particularly of pro-inflammatory genes IL-1β, IL-6 and also COX-2, as shown before. IL-1β is a known canonical master regulator of pro-inflammatory processes, it is secreted in response to gram negative bacteria and its release is followed by the production of IL-6 and other cytokines in the pro-inflammatory cascade [46, 53]. Modification in diet composition changed the expression of IL-1β, in agreement with previous studies, thus indicating that after plant protein diet rendered an already sensitized intestine to inflammatory/stress stimuli [32, 53–55].

During infection, IL-1β is induced, and increased expression of IL-1β has been reported in the intestine of different species, including gilthead seabream [29], after intraperitoneal challenge with gram negative bacteria. Increased expression of IL-1β and COX-2 has been reported before after *in vitro* challenge of gilthead seabream immune cells with bacteria or commercial pathogen associated molecular pattern (PAMP) solutions [56–59]. Although COX-2 is a typical oxidative stress marker, its expression is also induced by inflammatory mediators [58, 60], including IL-1β [61], as it takes part in the production of reactive oxygen species (ROS) and NO that have antibacterial activity and are also part of the innate immune system in higher vertebrates and carp macrophages [62, 63]. Also genes related to the maintenance of the epithelial tissue integrity, such as Ocl (expressing occludin), are influenced by inflammatory processes [64, 65], and its regulation depends on several cytoskeletal, scaffolding, signalling and polarity proteins [51] and it is definitely related to epithelial barrier functions *in vivo* and *in vitro* [65]. In mice, all these genes are possibly connected for the maintenance if intestinal barrier [66].

In phase II (up to 250 g), there were no significant differences between experimental groups (FM and PP), thus supporting that older fish are more tolerant to diet plant proteins than fish in earlier growth stages [16]. It is noteworthy the lack of inflammatory response in PP* group (short PP exposure), supporting that PP long term feeding has deeper alterations in fish gut and contributes to changes in the microbiota and an increase of fish mortality [32], in agreement with previous studies [33, 67, 68],

In so far as deficient diets could be considered a stress factor, long term feeding could determine suppressive or depressive effects on the immune mechanisms [24, 28, 32, 33]. The down-regulation of mRNA expression of some immune related genes with the increased plant proteins in the diet has also been reported in other species [69]. On the other hand, lower gene expression values reported in FM group might be related to a higher protection in host from bacterial adhesion and growth. Nevertheless, results should be analysed with caution since a wide individual variation of inflammatory and immune genes expression has been reported in other species [70] and level of expression before the *ex vivo* trial conditions the inflammatory and immune capacity registered after bacterial exposure [41].

Nevertheless, a longer stimulation with PP may lead to a degree of tolerance. This would explain why in the *ex vivo* challenges with bacterial pathogens, stimulation in seabream samples from PP* group was higher than in PP group.

Finally, bacterial pathogens have been selected for the challenge in the *ex vivo* assay due to their proven pathogenic activity in farmed gilthead seabream. *Photobacterium damselae* subsp. *piscicida* is the causal agent of pasteurellosis (Romalde, 2002), *Pseudomonas anguilliseptica* is related to 'winter disease' [71] and *Vibrio algynoliticus* has been described as the causal agent of vibriosis [72], also associated to other *Vibrio* species in high mortality outbreaks [73]. This work showed that *Vibrio algynoliticus* CECT 521 (ATCC 17749) had a very powerful inflammatory effect displaying a very high induction of IL-1β. This agrees with the fact that the published genome of this strain displayed a great toxigenic potential [74], with at least a gene encoding a pore forming RTX family toxin, among others (KEGG Genomes, toxin search on assembly GCA_000354175.2).

Conclusion

Plant protein diets showed to alter the mucosal immune homeostasis. In early stages of development, fish are very sensitive to plant diets, while adult fish seem to become tolerant to a constant PP diet, although maintaining a high threshold of inflammatory signals. The exposure to PP fed for short-term in adults led to a greater response to bacterial challenge. *V. algynoliticus* triggered the highest immune and inflammatory response. The successful evaluation of inflammatory and immune responses and pathogen challenge has been achieved by means of a new experimental system that implemented fish intestinal explants culture in gilthead seabream, a system that might be easily adapted to other teleost species. The use of such *ex vivo* methods constitutes an amenable technique that renders reliable results and it helps in reducing the number of animals per assay.

Abbreviations

ANOVA: analysis of variance; **AOAC**: Association of Analytical Communities; **β-Act**: β-Actin; **cDNA**: complementary desoxiribonucleic acid; **CECT**: Colección Española de Cultivos Tipo; **COX-2**: cyclooxygenase 2; **FM**: fish meal based FM diet; **DMEM**: Dulbecco's Modifed Eagle's Medium; **EF-1α**: Elongation Factor 1α; **FG**: foregut; **GAPDH**: glyceraldehyde-3-phosphate dehydrogenase; **HG**: hindgut; **IgM**: immunoglobulin M; **IL-1β**: interleuk¡ne 1β; **IL-6**: interleukine 6; **I-Muc**: intestinal mucin; **LDH**: lactate deshydrogenase; **NADH**: nicotinamide adenine dinucleotide; **Ocl**: ocludin; **PP**: plant protein based diet; PP*: plant protein based diet (short term); **qPCR**: quantitative polymerase chain reaction; **RNA**: ribonucleic acid; **Rps18**: ribosomal protein S18

Declarations

Ethics approval and consent to participate

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

Consent for publication

Not applicable.

Availability of data and materials

The datasets during the current study are available from the corresponding authors on reasonable request

Competing interests

The authors declare that they have no competing interests that could influence the content of the paper.

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Author’s contributions

CB, MJC, GPM, DSP and SML designed the assay. GE, CB and ATV carried out the experiments. GE analysed the data. GE, DSP, GPM and SML prepared the manuscript and discussed the results. All authors read and approved the final manuscript.

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Figures

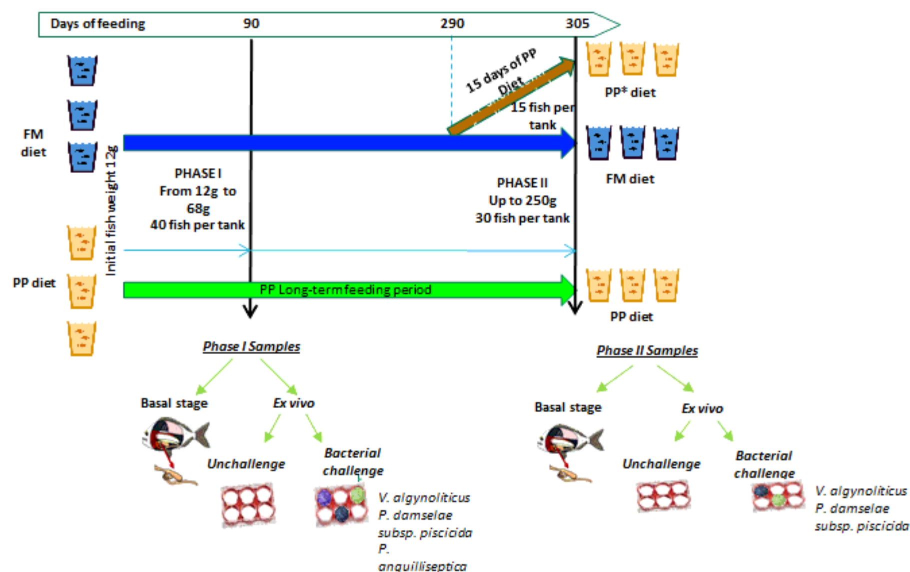

Figure 1

Summary of the experimental design. The impact of dietary fish protein substitution by plant protein in fish immune response to ex vivo bacterial challenge was evaluated at two on-growing phases: 12-68 g (90 days) and up to 250g (305 days). Additionally, a group was included at 305 days to estimate the effect of short-term fish meal substitution (15 days; PP*).

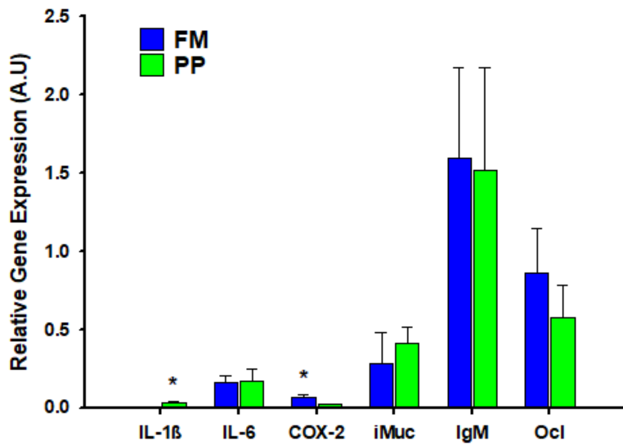

Figure 2

Intestinal basal expression of fish exposed to PP diet in Phase I (68 g). Relative gene expression (A. U.) of the different genes is expressed by the mean and standard error. Different superscripts on the bars indicate significant differences ($p<0.05$).

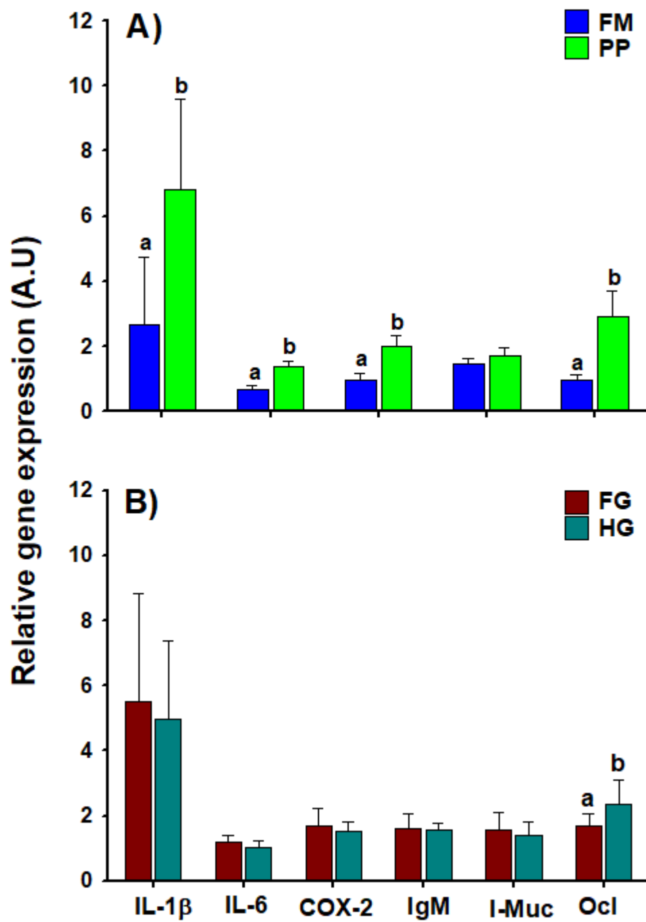

Figure 3

Effect of diet and intestinal section after 6h of ex vivo bacterial exposition in Phase I (68 g). Relative gene expression (A. U.) of the different genes is expressed by the mean and standard error. Different superscripts on the bars indicate significant differences between different conditions (diet/section) for each gene ($p<0.05$) at 6h of incubation. A) Effect of dietary treatment B) Effect of intestinal section.

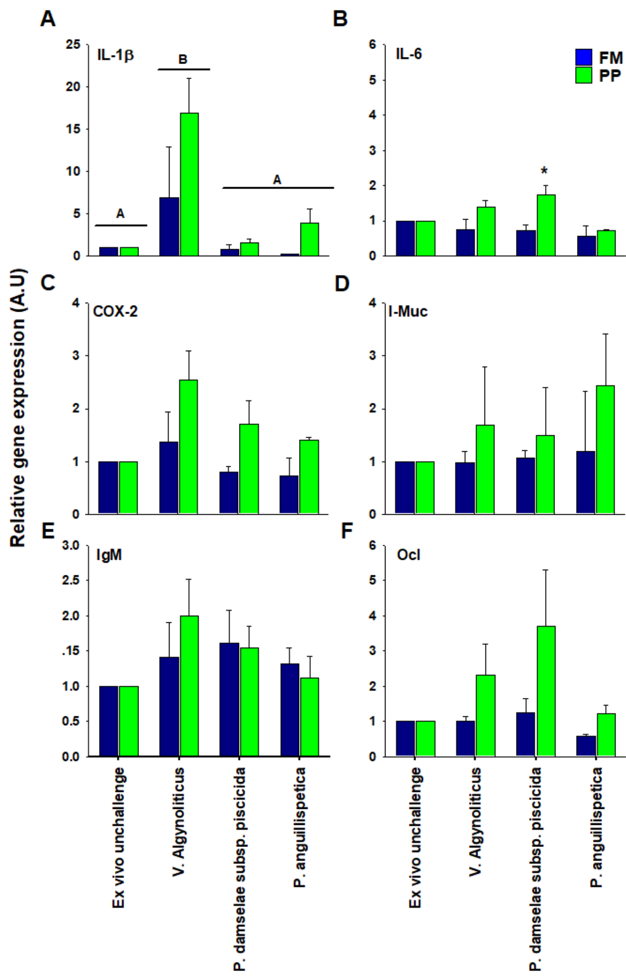

Figure 4

Effect of bacterial challenge based on the diet after 6h of ex vivo bacterial exposition in Phase I (68 g). Relative gene expression (A. U.) of the different genes is expressed by the mean and standard error. Asterisks on the bars indicate significant differences between diets, meanwhile different letters indicate differences between bacterial stimuli for each gene (p<0.05) at 6h of incubation. Gene expression of A) IL-1β B) IL-6 C) COX-2 D) I-Muc E) IgM F) Ocl

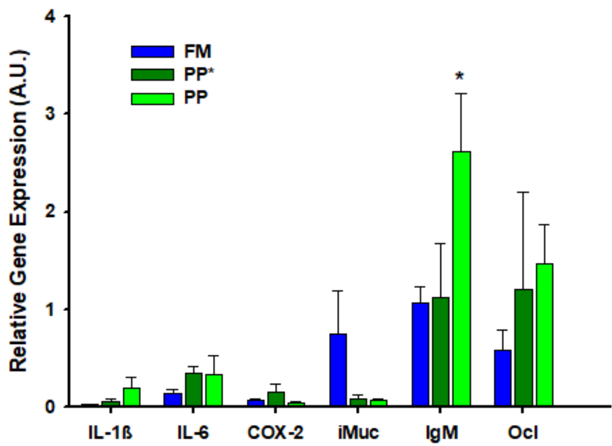

Figure 5

Intestinal basal expression of fish exposed to long (PP) or short term (PP*) diet in Phase II (250 g). Relative gene expression (A. U.) of the different genes is expressed by the mean and standard error. Asterisk on the bars indicates significant differences (p<0.05).

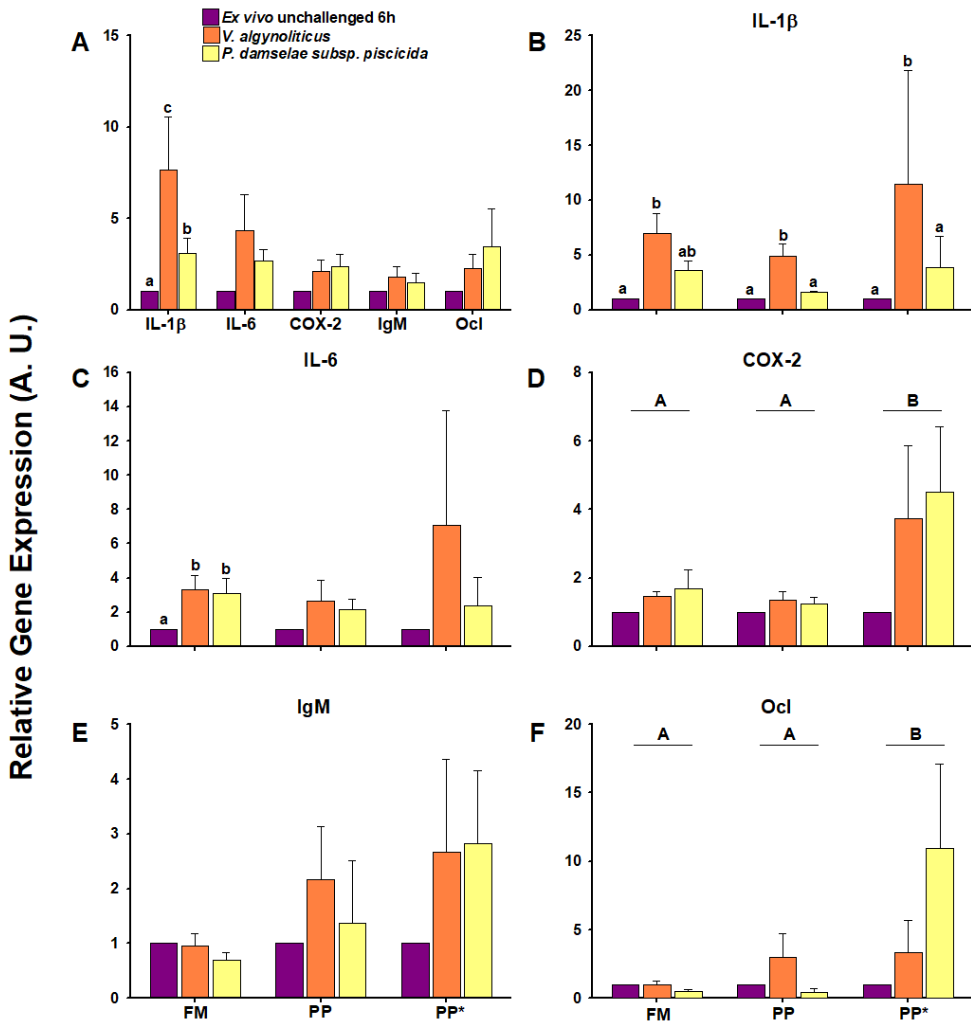

Figure 6

Effect of bacterial challenge based on the diet after ex vivo bacterial exposition in Phase II (250 g). Relative gene expression (A. U.) of the different genes is expressed by the mean and standard error. Capital letters indicate differences between diets, meanwhile lower case letters indicate differences between bacterial for each gene (p<0.05) after 6h of incubation. A) Effect of bacterial challenge independently of diet. Gene expression of B) IL-1β C) IL-6 D) COX-2 E) IgM F) Ocl.

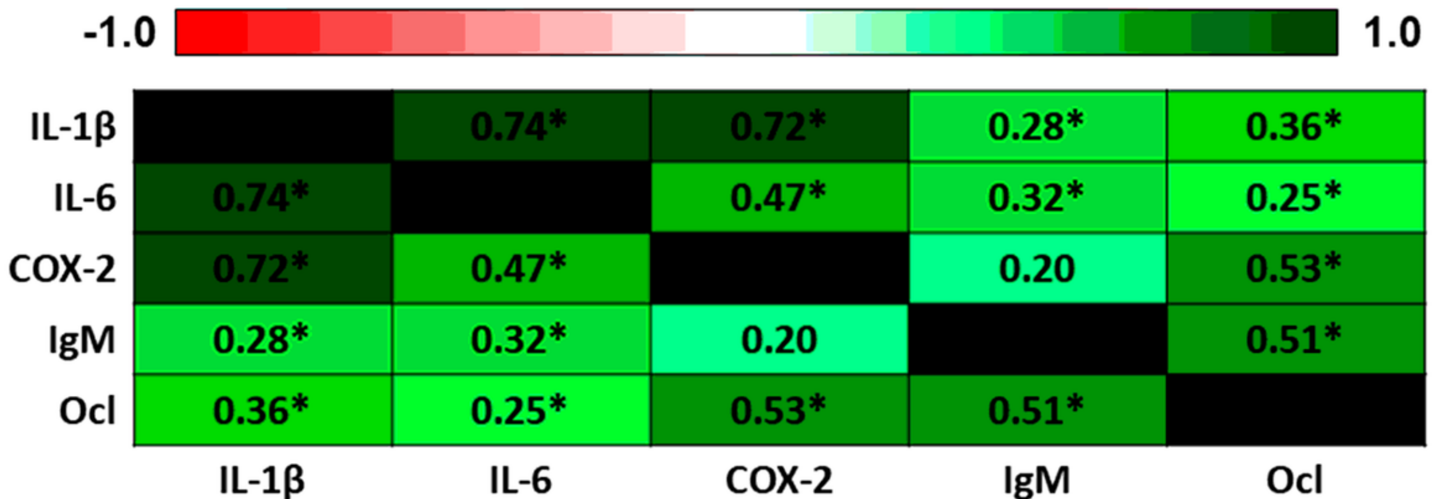

Figure 7

Correlation analysis of gene expression determined in samples after the ex vivo assay. Pearson product-moment coefficients between each pair of genes. Significant correlations are indicated with a *.

Supplementary Files

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- AdditionalFileTable1.docx
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- AdditionalfileFigure7.bmp
- AdditionalfileFigure6.bmp
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