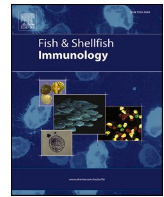
Fish and Shellfish Immunology 119 (2021) 623–634

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi



Acute inflammatory response in the skin of gilthead seabream (Sparus aurata) caused by carrageenin

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A R T I C L E I N F O

Keywords:
Carrageenin
Skin inflammation
Gene expression
(Sparus aurata)
Teleosts
Aquaculture

A B S T R A C T

Although inflammation is a well-characterized process in mammals, few studies have dealt with the mechanisms involved in this process in fish. The present study evaluated the expression of inflammation-related genes in the skin of fish injected with carrageenin, which has previously been used in inflammatory models in mammals. In our case, fish were injected subcutaneously with PBS (as control) or carrageenin (1%), and skin samples from the injection site were collected 1.5, 3 and 6 h post-injection. The gene expression of inflammatory markers (*csfr1*, *mhc-ii* and *phox40*), several pro-inflammatory cytokines (*il1b, tnfa, il6, il8* and *il18*) and other molecules related (such as *myd88* and *c-rel*) were up-regulated at 1.5 and 3 h in fish injected with carrageenin compared with control levels. By contrast, the gene expression of anti-inflammatory molecules (*nlrx1, nlrc5* isoform 1, *ctsd* and *ctss*) was down-regulated in fish injected with carrageenin and sampled 3 h post injection, again compared to the gene expression in control fish. According to our results, carrageenin can be considered not only a good stimulator to study skin inflammation in gilthead seabream but also this method might be use to study the modulation of fish inflammatory process caused by internal or external factors.

1. Introduction

Inflammation is a complex reaction of the innate immune system that is frequently involved in different diseases and which affects all tissues [1]. It also plays a critical role in alerting cells to prepare effective immune responses, and initiate wound repair and healing processes to help recover physiological homeostasis [1]. Generally, this reaction is initiated as a response to a stimulus, such as the presence of pathogens or irritants, injury or trauma. Such stimuli trigger a series of cascading reactions in order to resolve the situation [2]. Among the most characteristic symptoms of inflammation are heat, redness, swelling, pain and loss of function [3]. Although these acute symptoms are usually temporary and local, in some circumstances, they may persist to become a chronic response [4]. In humans, chronic inflammation is related to numerous diseases, [5–7]. At molecular level, NF-κB, a pivotal transcription factor consisting of five subunits (p65/RELA, RELB, c-REL, p50/NF-κB1 and p52/NF-κB2), controls the gene expression of numerous inflammation-associated molecules, including proinflammatory cytokines, such as IL-1β, IL-6, IL-8, and TNF-α, as well as genes involved in ROS production, playing a key role in the modulation of the inflammatory response [8]. Inflammation needs to be studied in greater depth in fish, particularly in those species that are of economic interest in aquaculture, one of the fastest-growing food-related sectors in the world in the last decades [9]. It is well known that intensive fish production conditions, whereby high numbers of fish are confined in a small volume of water, increase the occurrence of injuries and diseases in farmed animals. These, in turn, are very frequently associated with the appearance of wounds or ulcers in the fish skin, triggering an inflammatory response and causing serious economic losses in the aquaculture industry [10,11].

In order to improve our knowledge of the inflammatory process, carrageenin was used, as a possible inflammation trigger in fish. Carrageenin is a high-molecular-weight sulphated mucopolysaccharide obtained from the cell walls of a red seaweed(*Chondrus crispus*) [12]. In ionic solutions, κ- and ι-carrageenins self-associate into helical structures that form rigid or flexible gels, respectively, and these gels seem to be related to the immunostimulant properties in teleost fish against bacterial infections [13–15]. In contrast, λ-carrageenin does not form

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https://doi.org/10.1016/j.fsi.2021.10.009
Received 24 July 2021; Received in revised form 4 October 2021; Accepted 11 October 2021
Available online 14 October 2021
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helicoidal structures and is non-gelling. The local injection of λ-carrageenin into soft tissues in rodents induces acute inflammation [15]. Due to its properties, carrageenin has been used traditionally as inflammatory model in rats, mice and guinea pigs, since it produces a local inflammation in their paws, associated with hyperalgesia, oedema, erythema and an exacerbated response to thermal and mechanical stimuli common [16–22]. Furthermore, this polysaccharide is also able to induce inflammation in human experiments carried out *in vitro* [23,24], and it is even used commercially in the food industry as a thickener and gelling agent.

In fish, carrageenin has also been used to provoke inflammation in several parts of the fish body. In a histopathological study, carrageenin was inoculated in the myotomal muscle on the dorsal side of plaice (*Pleuronectes platessa*), and chronic inflammation was observed [25]. In another study, carrageenin was injected into the swim bladder of tilapia (*Oreochromis niloticus*) and a local inflammatory reaction was evident after 3 h, reaching a peak at 24 h. In this case, the symptoms observed in the inflamed area consisted of vascular congestion, thrombocytes, rare granulocytes and oedema [26]. Similarly, an acute inflammation was induced by carrageenin in the swim bladder of pacu (*Piaractus mesopotamicus*) but after acute stress caused by air exposure. In this situation, carrageenin injection provoked congestion, interstitial haemorrhage, dissociation of the collagen sheaf and inflammatory infiltrate, accompanied by the presence of macrophages and thrombocytes in the injection area [27]. More recently, the intraperitoneal administration of carrageenin in adult zebrafish (*Danio rerio*) was seen to induce significant abdominal oedema and an increase in the levels of TNF-α and iNOS proteins and in the leucocyte marker, myeloperoxidase (MPO), were also observed [28]. In another study, the intraperitoneal injection of carrageenin as a proinflammatory agent was used in trahira (*Hoplias malabaricus*) in order to test the effect of two anti-inflammatory drugs: diclofenac and dexamethasone. Carrageenin stimulated leucocyte migration and an increase in the number of polymorphonuclear cells, but also led to a reduction in the number of mononuclear cells in the peritoneal cavity [29].

Taking into account all these considerations, the aim of the present study was to evaluate the possible use of a subcutaneous injection of carrageenin to stimulate inflammation in the skin of the gilthead seabream (*Sparus aurata*). The gene expression of several cytokines, cell markers, Toll-like receptors (TLR), Nod-like receptors (NLR), cathepsins, as well as of different molecules involved in the cholinergic reflex and in the NF-κB – transduction pathway were assessed. To the best of our knowledge, this is the first study to use carrageenin as an activator of this process in order to trigger inflammation-related gene expression in fish, and thereby contribute to unravelling the mechanism of this inflammatory process in gilthead seabream.

2. Material and methods

2.1. Animals

Twenty-four specimens (20.875 ± 5.6 g mean weight) of the seawater teleost gilthead seabream (*Sparus aurata* L.), obtained from a local farm (Mazarrón, Spain), were kept in re-circulating seawater aquaria (450 L) in the Marine Fish Facilities at the University of Murcia (Spain) for a quarantine period of one month. The water temperature was maintained at 20 ± 2 °C with a flow rate of 900 L h^{-1}, 28‰ salinity, a photoperiod of 12 h light to 12 h dark and with continuous aeration. Fish were fed with a commercial diet (Skretting, Spain) at a rate of 2% body weight day^{-1} and were kept 24 h without feeding before the trial. All experimental protocols were approved by the Ethical Committee of the University of Murcia.

2.2. Experimental design and sample collection

Fish were randomly selected, anesthetized with clove oil (20 mg L^{-1}, Guinama®), subcutaneously injected in the left flank, beneath the lateral line at the level of the second dorsal fin, before being divided into two duplicate experimental groups (6 fish in each of four tanks, 50 L, 800 L h^{-1} and continuously aerated). The following groups were established: *i)* fish in two tanks (12 fish) were subcutaneously injected with 50 μl of Phosphate-Saline Buffer (PBS; 11.9 mM Phosphates, 137 mM NaCl, and 2.7 mM KCl, pH 7.4) (Fisher Bioreagents) (control group); and *ii)* fish in the other two tanks were injected with 50 μl of carrageenin (1%) (Sigma-Aldrich) diluted in PBS. After 1.5, 3 and 6 h, two fish from each tank were weighed, skin from the injected area was collected with a biopsy metal punch of 4 mm diameter (Stiefel) (Supplementary Figs. 1 and 2) and the fish were returned to the aquaria. Immediately, the skin samples were stored in TRIzol® (Invitrogen) at −80 °C for gene expression analysis.

2.3. Gene expression analysis by real-time qPCR

The sequences of the selected genes were obtained from a gilthead seabream database [30]. The Open Reading Frames (ORF) were located using the ExPASy translation software (SIB Bioinformatics Resource Portal) and an additional check was performed using NCBI BLAST sequence alignment analysis (NIH). Primers used (Table 1) were designed with the Thermo Fisher OligoPerfect™ tool.

Total RNA was extracted from samples of 0.5 g of gilthead seabream skin using TRIzol Reagent [31], following the manufacturer's instructions and quantification and purification were assessed using a Nanodrop® spectrophotometer; the 260:280 ratios were 1.8–2.0. Then, the RNA was treated with DNase I (Promega) to remove genomic DNA contamination, and complementary DNA (cDNA) was synthesized from 1 μg of RNA using the reverse transcriptase enzyme SuperScriptIV (Life Technologies) with an oligo-dT_{18} primer. The expression of the selected genes (see Table 1) was analysed by real-time qPCR with QuantStudio™ Real-Time PCR System Fast (Life Technologies). The reaction mixtures [containing 5 μl of SYBR Green supermix, 2.5 μl of primers (0.6 μM each) and 2.5 μl of cDNA template] were incubated for 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 1 min at 60 °C, and finally 15 s at 95 °C, 1 min at 60 °C and 15 s at 95 °C. The gene expression was analysed using the $2^{-\Delta Ct}$ method [32], which was performed as described elsewhere [33]. The specificity of the reactions was analysed using samples without cDNA as negative controls. For each mRNA sample, gene expression was normalized with the geometric mean of ribosomal protein (*s18*), elongation factor 1-alfa (*ef1α*) and beta-actin (*actb*) RNA content. Gene names follow the accepted nomenclature for zebrafish (http://zfin.org/). In all cases, each PCR was performed on triplicate samples.

2.4. Statistical analysis

The results were expressed as mean ± standard error of the mean (SEM). Data were analysed by student t-test, and one-way ANOVA (followed by Tukey tests) to determine differences between experimental groups and each group respect to time, respectively. Normality of the data was previously assessed using a Shapiro-Wilk test and the homogeneity of variance was also verified using the Levene test. Non-normally distributed data were log-transformed to perform parametric tests, and, when the data did not meet parametric assumptions, a non-parametric Kruskal-Wallis test, followed by Dunn's multiple comparison test, were used. All statistical analyses were conducted using the computer package SPSS (25.0 version; SPSS Inc., Chicago, IL, USA) for Windows. Correlation analysis was performed to identify pairwise associations (direct or inverse) between the different gene markers using the "corrplot package" from Wei and Simko (2021) [34] in the R software (R Development Core Team, 2020). The level of significance used was $p < 0.05$ for all statistical tests.

Table 1
Primers used for real-time qPCR.

Gene name	Gene abbreviation	GenBank number	Primer sequences (5′→3′)	Melting temperature	Primer efficiencies
Colony-stimulating factor receptor 1 receptor	*csfr1*	AM050293	F: ACGTCTGGTCCTATGGCATC R: AGTCTGGTTGGGACATCTGG	27,43 °C	87.79%
Major histocompatibility complex class IIa	*mhcIIa*	DQ019401	F: CTGGACCAAGAACGGAAAGA R: CATCCCAGATCCTGGTCAGT	24,85 °C	100.00%
NADPH oxidase, subunit Phox22	*Phox22*	FM148169	F: CATCAAGAATCCCCCTCAGA R: TGACAGAGATGGGGTTGTCA	24,19 °C	96.97%
NADPH oxidase, subunit Phox40	*Phox40*	AM749961	F: GCGGAGTTGAACCTGAAGAG R: TCACCTTCTGTGTCGCTGTC	24,84 °C	99.65%
Toll-like receptor 2	*tlr2*	B008611	F: TCCATGCTTTCGTCCAGGAC R: ACTGTGTTGAGCAAGGCCTC	31,80 °C	95.71%
Toll-like receptor 5	*tlr5*	B001824	F: CAACTTGAGCTCCAACGCAC R: GGCTGGAGATAGGTCAAGGC	28,79 °C	89.83%
Toll-like receptor 7	*tlr7*	B004477	F: CCAACAATGGGAGCATGGTG R: ATGGTGAGAGTCAGGTTGGTG	30,81 °C	89.20%
Toll-like receptor 8	*tlr8*	B024796	F: CCAGAGCAATTCCAGGGCTA R: TGTCCAGCCCTTTGAACTCTG	30,69 °C	99.97%
Toll-like receptor 9	*tlr9*	B003345	F: GATCACACCGTTCACTGTCTC R: GGAGGAGAGGGACTGGATTC	29,86 °C	87.29%
Toll-like receptor 13	*tlr13*	B008611	F: CCTCCCTGCCTTGACGTATC R: TGTCTGGTTGTTGCTCTGCA	30,46 °C	101.40%
Tumor necrosis factor receptor superfamily, member 1a	*tnfrsf1a*	B006439	F: TCTTGCGTCTGCTCTCAGTG R: CCTCAGCATCTGGTACTGCC	28,65 °C	95.1%
Tumor necrosis factor receptor superfamily, member 1b	*tnfrsf1b*	B026296	F: TACCGCAGCTCTTCACGATC R: ACTGTGTGGGGATGCTGATC	26,67 °C	104.30%
MYD88 innate immune signal transduction adaptor	*Myd88*	B013233	F: GCCTTCATCTGCTACTGCCA R: TCTGTCGAACACGCACAGTT	25,07 °C	98.45%
TNF receptor-associated factor 6	*traf6*	B010645	F: ACCTGTGTCGTGCCAAGATT R: TCACAGTACTGGCACGTCAC	27,78 °C	97.41%
Interleukin-1 receptor-associated kinase 1	*irak1*	B011814	F: TGGTGCTGCTGGAGATTCTG R: AACCGTTCGGACTTTCCTCC	26,28 °C	100.82%
Signal transducer and activator of transcription 3	*stat3*	B015325	F: ACATCCTTGGCACCAACACA R: ACCATTGCCACACCTCTGTT	22,87 °C	97.10%
inhibitor of nuclear factor kappa B kinase regulatory subunit gamma	*Ikbkg*	B006470	F: GAAGGAGGAGGTGGAGCAAC R: CTCTCTCGCTTCTCGCTCTG	26,77 °C	98.44%
v-rel avian reticuloendotheliosis viral oncogene homolog A	*Rela*	B030837	F: GAACCCCACCCTCATGAGTG R: GTTCTGGGCAGCAGTAGAGG	24,97 °C	95.15%
v-rel avian reticuloendotheliosis viral oncogene homolog B	*Relb*	B012502	F: ACAGAGGAGGTGGAGGTCAG R: TATGGATCTGGGTTGTGCGG	26,67 °C	100.08%
v-rel avian reticuloendotheliosis viral oncogene homolog	*Rel*	B018958	F: AAGCAAGAGCCCCAGATCAC R: TAGGGCGAGGAAGCAAGTTG	25,88 °C	98.70%
Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	*nfkb1*	B005908	F: CCGACAGACGTTCACAGACA R: TCTTCAGCTGGACGAACACC	27,41 °C	100.39%
Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2	*nfkb2*	B012900	F: ATCACAGCGCAGAGATCGAG R: TGCGGGATGTAGGTGAACTG	27,56 °C	104.06%
Interleukin 1	*il1b*	XM030416076.1	F: GCGAGCAGAGGCACTTAGTC R: GGTAGGTCGCCATGTTCAGT	18,95 °C	82.34%
Tumor necrosis factor alpha	*Tnfa*	AJ413189	F: CTGTGGAGGGAAGAATCGAG R: TCCACTCCACCTGGTCTTTC	33,88 °C	84.78%
Interleukin-6	*il6*	AM749958	F: AGGCAGGAGTTTGAAGCTGA R: ATGCTGAAGTTGGTGGAAGG	28,91 °C	91.88%
Interleukin-7	*il7*	JX976618	F: GATCTGGAAAACACCGGAGA R: TGGACGTGCGTTCTGGTAGC	28,94 °C	99.65%
Interleukin-8	*il8*	AM765841	F: GCCACTCTGAAGAGGACAGG R: TTTGGTTGTCTTTGGTCGAA	23,99 °C	88.74%
Interleukin-18	*il18*	JX976626	F: TTGAGGGGTTGTCCTGTTTC R: AGTTTTTACCCCAGCCCTGT	27,62 °C	100.13%
Interleukin-10	*il10*	XM030420872.1	F: CTCACATGCAGTCCATCCAG R: TGTGATGTCAAACGGTTGCT	27,81 °C	92.78%
Transforming growth factor 1 beta	*tgf1b*	AF424703	F: GCATGTGGCAGAGATGAAGA R: TTCAGCATGATACGGCAGAG	26,47 °C	99.97%
NLR family, CARD domain containing 3	*nlrc3*	B000011	F: CTGCCAGTGGTCAAAGCCTC R: AGGACTGGGAGCTGAGAACT	22,82 °C	95.68%
NLR family, CARD domain containing 5 (Isoform 1)	*nlrc5 (isof.1)*	B003870	F: AGCAGCTAGTTTGGCCTCTG R: GGCGATGTGTTTGATCCCTG	27,76 °C	90.57%
NLR family, CARD domain containing 5 (Isoform 2)	*nlrc5 (isof.2)*	B003870	F: CAAGAGTGATGCCCCTGTGT R: GACTGTGAGGCTCTGAGCAG	32,85 °C	100.83%
NLR family member X1	*nlrx1*	B002577	F: AGGTGTACCAAAGAGCCACG R: CTGAGGATGGGATGCCAGTC	27,26 °C	91.58%
Cathepsin D	*Ctsd*	B000122	F: TCGCTGCCTGTTGTCTCTTT R: GCCCGACAGACAGATTGACA	25,46 °C	95.70%
Cathepsin L	*Ctsl*	B019572	F: ATGATGAGCCAGACTGCAGC R: AGACCCCAGCTGTTCTTGAC	28,54 °C	89.31%
Cathepsin S	*Ctss*	B007924		24,61 °C	85.37%

(*continued on next page*)

Table 1 (*continued*)

Gene name	Gene abbreviation	GenBank number	Primer sequences (5′→3′)	Melting temperature	Primer efficiencies
			F: AACCTGGTGGACTGTTCGTC R: GCGTCAGAGTCGATACCCTG		
Acetylcholinesterase	*Ache*	B017377	F: CGGAGTGGATGGGTGTGATC R: GTCGGCTCAGTTTCTCCTCC	30,93 °C	94.94%
Butyrylcholinesterase	*Bche*	B013682	F: CAGGTACTCCCAACACGGTG R: ATCTCGTAGCCGTGCATGAC	24,64 °C	104.22%
Cholinergic receptor, nicotinic, alpha 7	*chrna7*	B000251	F: AATGCCAGCCACAGAGATCC R: TGATTTGGGTCCAGCTCTGC	32,03 °C	102.24%
Ribosomal protein S18	*rps18*	AM490061	F: CGAAAGCATTTGCCAAGAAT R: AGTTGGCACCGTTTATGGTC	11,67 °C	105.07%
Elongation factor-1 alfa	*ef1a*	AF184170	F: TGTCATCAAGGCTGTTGAGC R: GCACACTTCTTGTTGCTGGA	20,59 °C	88.97%
Actin beta	*Actb*	X89920	F: GGCACCACACCTTCTACAAATG R: GTGGTGGTGAAGCTGTAGCC	23,92 °C	88.54%

3. Results

The expression profile of four cell markers (Fig. 1), twenty-four pro-inflammatory genes (Fig. 2), and twelve anti-inflammatory genes (Fig. 3) was analysed by real-time PCR in fish skin samples collected 1.5, 3 and 6 h after carrageenin or PBS injection. A heatmap was elaborated in order to help to better visualize the pro-inflammatory effect of carrageenin on gilthead seabream (Fig. 4). Regarding Pearson's correlation, the coefficients among the three previously established groups (cell markers, pro-inflammatory and anti-inflammatory genes) are shown in Table 2 and Fig. 5 for both treatments and sampling times.

3.1. Cell markers

The gene expression of *csfr1* (macrophage marker; 0.00074 ± 0.00009) (Fig. 1A), *mhcIIa* (antigen-presenting cell marker; 0.00676 ± 0.00136) (Fig. 1B), and *phox40* (acidophilic granulocyte marker; 0.00520 ± 0,00097) (Fig. 1C) was up-regulated in fish sampled 1.5 h post-carrageenin injection (p.i.), compared with the expression measured in the skin of specimens injected with PBS (control group). Similarly, *phox22* (acidophilic granulocyte marker; 0.02151 ± 0.00524) (Fig. 1D) and *phox40* (0.02447 ± 0,00779) gene expression was up-regulated in the fish skin of the p.i. group sampled 3 and 6 h, respectively, compared to the values found in the control group.

Considering the time factor, only the expression of *phox22* gene (0.07678 ± 0,02543) (Fig. 1D) had increased in the fish sampled 6 h p.i., compared with the levels measures in the fish sampled at 1.5 h. By contrast, no significant variations were observed in the gene expression of fish from control group.

3.2. Proinflammatory markers

Regarding proinflammatory markers, the gene expression of *myd88* (0,00252 ± 0,00012) was seen to be up-regulated 1.5 h p.i. with respect to the levels measured in the control group (Fig. 2A). However, 3 h p.i. the expression of the following proinflammatory cytokines were seen to

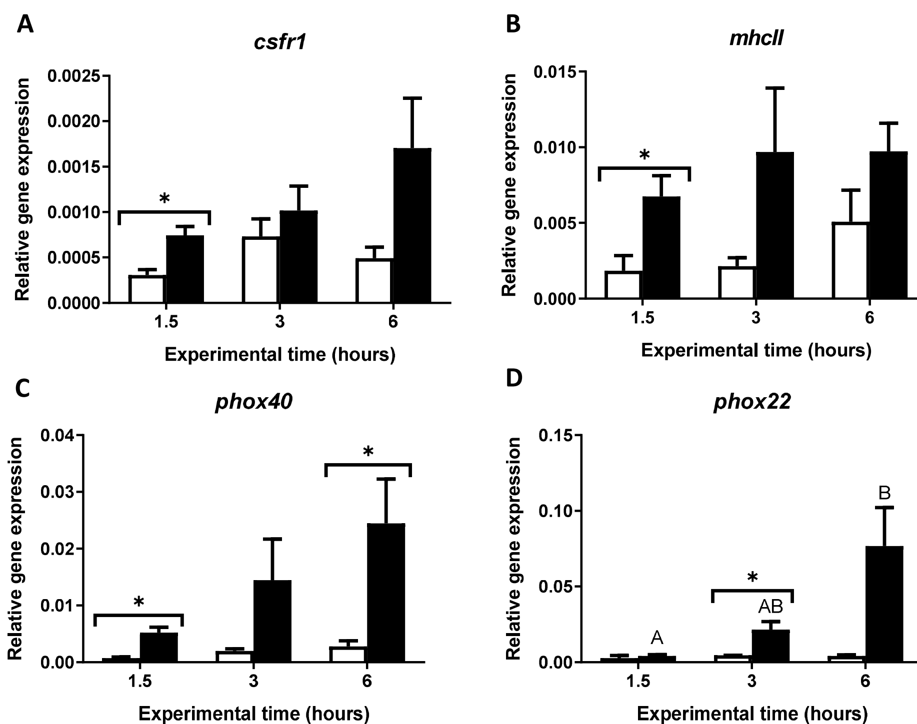

Fig. 1. Relative expression of the cell markers *csfr1* (A), *mhc-ii* (B), *phox40* (C) and *phox22* (D) in skin samples of gilthead seabream injected with PBS (control, white bars) or carrageenin (1%, black bars) and sampled at 1.5, 3 and 6 h. Error bars of columns denote standard error of means (n = 4). Asterisks denote significant differences between control and treatment groups (T-test; $p < 0.05$) and different letters indicate differences between time points (ANOVA; $p < 0.05$).

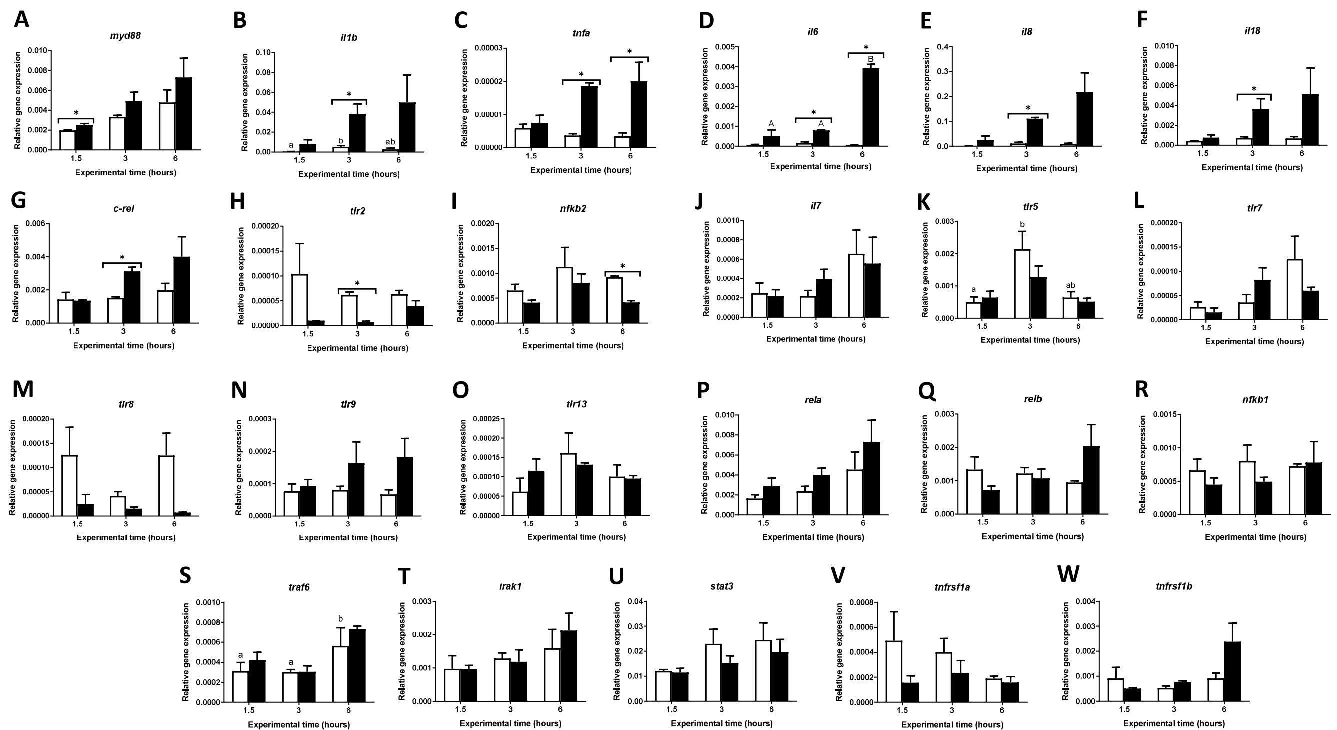

Fig. 2. Relative expression of the proinflammatory markers *myd88* (A), *il1b* (B), *tnfa* (C), *il6* (D), *il8* (E), *il18* (F), *c-rel* (G), *tlr2* (H), *nf-kb2* (I), *il-7* (J), *tlr5* (K), *tlr7* (L), *tlr8* (M), *tlr9* (N) and *tlr13* (O), *rela* (P), *relb* (Q), *nfkb1* (R), *traf6* (S), *irak1* (T), *stat3* (U), *tnfrs1a* (V) and *tnfrs1b* (W) in skin samples of gilthead seabream injected with PBS (control, white bars) or carrageenin (1%, black bars) at 1.5, 3 and 6 h. Error bars of columns denote standard error of means (n = 4). Asterisks denote significant differences between control and treatment groups (T-test; $p < 0.05$) and different letters indicate differences between time points (ANOVA; $p < 0.05$).

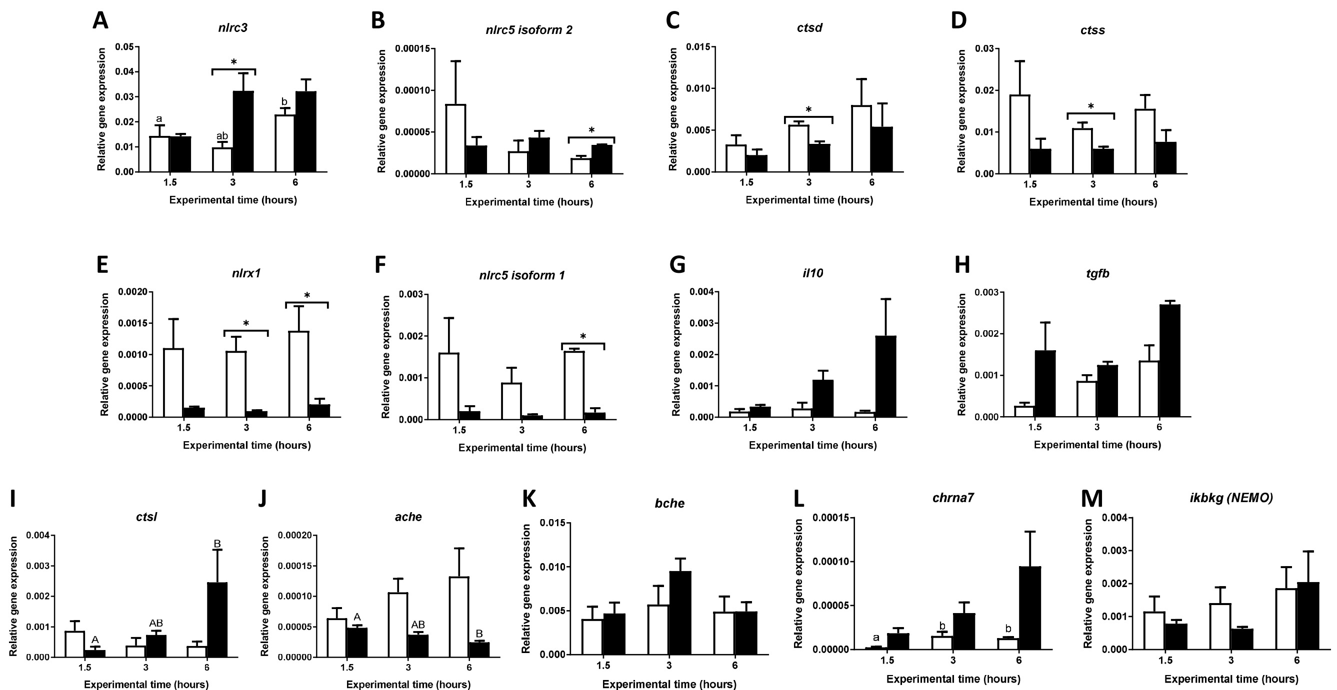

Fig. 3. Relative expression of the anti-inflammatory markers *nlrc3* (A), *nlrc5 Isoform 2* (B), *ctsd* (C), *ctss* (D), *nlrx1* (E), *nlrc5 isoform 1* (F), *il10* (G), *tgfb* (H), *ctsl* (I) *ache* (J), *bche* (K), *chrna7* (L) and *ikbkg* (NEMO) (M) in skin samples of gilthead seabream injected with PBS (control, white bars) or carrageenin (1%, black bars) at 1.5, 3 and 6 h. Error bars of columns denote standard error of means (n = 4). Asterisks denote significant differences between control and treatment groups (T-test; $p < 0.05$) and different letters indicate differences between time points (ANOVA; $p < 0.05$).

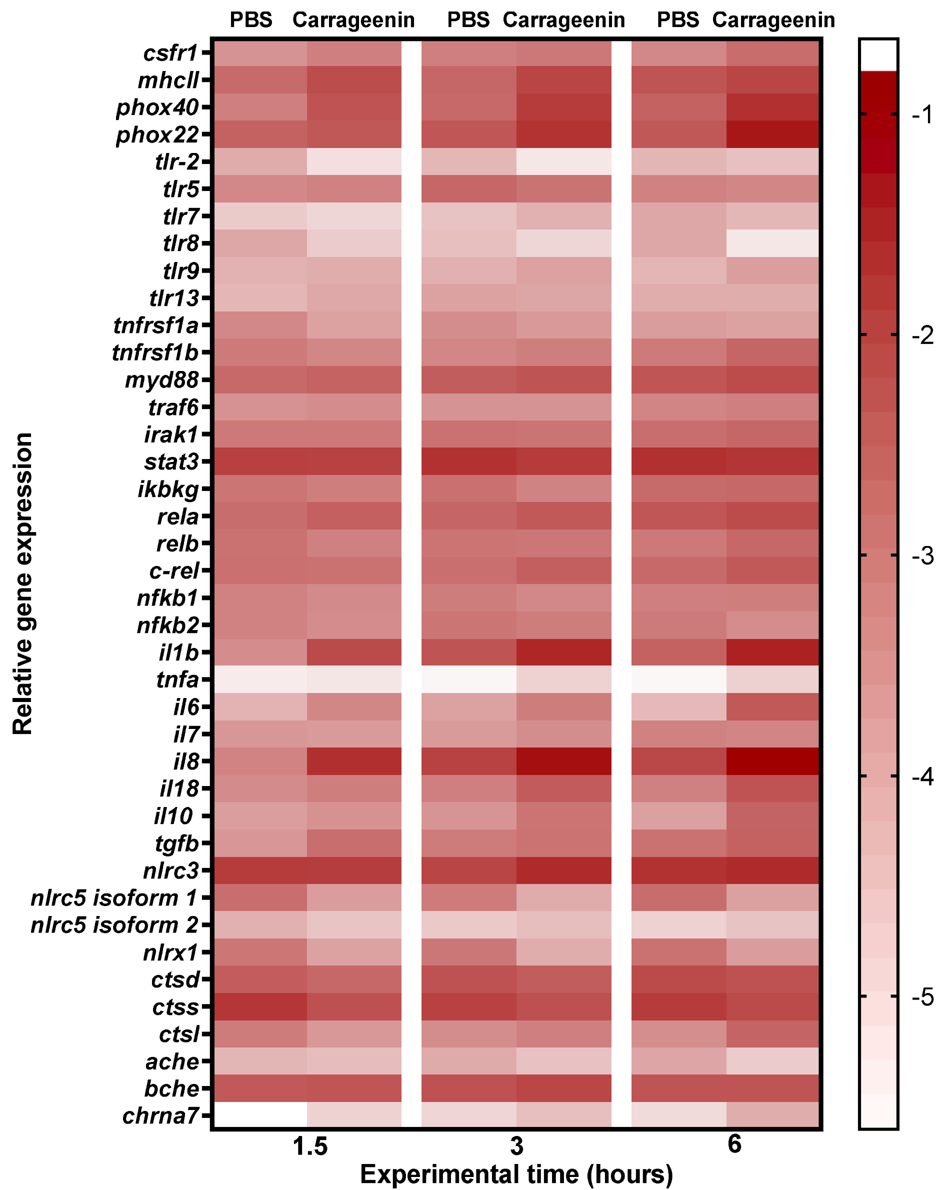

Fig. 4. Heatmap of the relative gene expression in skin samples of gilthead seabream injected with PBS (control) or carrageenin (1%) at 1.5, 3 and 6 h. The colour scale on the right of the heatmap represents the gene expression level, whereas the colours white and red indicate low, and high expression level on the skin of fish, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Fig. 5. Graphical display of correlation matrix among the different target genes in skin samples of gilthead seabream injected with PBS (control) or carrageenin (1%) at 1.5, 3 and 6 h. The colour scale on the right of the correlation matrix represents the correlation level, whereas the colours blue and red indicate direct, and inverse correlation level on the skin of fish, respectively. Asterisks denote significant differences between control and treatment groups (Correlation test; $p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Fig. 6. Possible inflammatory model of carrageenin-transduction pathway in acidophilic granulocytes in the skin of gilthead seabream. Symbols + and – represent induction and repression interactions, respectively.

be upregulated: *il1b* (0.0383 ± 0.00994), *tnfa* (0.000019 ± 0.000001), *il6* (0.0007 ± 0.00002), *il8* (0.11078 ± 0.0054), *il18* (0.00363 ± 0.00104) and c-*rel,* (0.00311 ± 0.00025) compared with the levels expressed in the skin of control fish (Fig. 2B–G). However, at the end of the trial (6 h p.i.), only the gene expression of the cytokines *tnfa* (0.00002 ± 0.000006) and *il6* (0.00392 ± 0.00019), remained up-regulated with respect to the expression found in control fish (Fig. 2C and D). By contrast, the expression of *tlr2* (0.000007 ± 0.000002) and *nfκb2* (0.00041 ± 0.000038) genes was down-regulated at 3 and 6 h, respectively, in p.i. fish respect to values found in control fish (Fig. 2H and I). Interestingly, the gene expression of the proinflammatory markers *il7, tlr5, tlr7, tlr8, tlr9, tlr13, rela, relb, nfkb1, traf6, irak1, stat3, tnfrsf1a,* and *tnfrsf1b* (Fig. 2J–W) did not show any significant variation in fish injected with carrageenin compared with control levels at any experimental time.

Taking into account the time factor, the expression of genes analysed in the skin of fish from the control group did not show any change, with the exception of the proinflammatory markers *il1b* (Fig. 2B), *tlr5* (Fig. 2K) and *traf6* (Fig. 2S). The expression of *il1b* (0.0383 ± 0.00994) and *tlr5* (0.00127 ± 0.00034) genes was up-regulated at 3 h p.i. compared with values found at 1.5 h p.i. fish. In the case of *traf6* gene (0.0003 ± 0.00005), its expression was still upregulated at 6 h into the trial compared to the levels found in sampling taken at 1.5 and 3 h. In fish from the carrageenin group, only *il-6* gene expression (0.00392 ± 0.00019) was up-regulated at 6 h compared with the levels found in samples taken at 1.5 and 3 h (Fig. 2D).

3.3. Anti-inflammatory markers

The administration of carrageenin triggered up-regulation of the expression of n*lrc3* (0.03235 ± 0.00703) and isoform 2 of *nlrc5* (0.000035 ± 0.0000003) genes, as measured 3 h and 6 h, respectively, compared with the control group (Fig. 3A and B). In contrast, the gene expression of *ctsd* (0.00335 ± 0.00031), *ctss* (0.00599 ± 0.00051) and *nlrx1* (0.000096 ± 0.000014) was down-regulated when measured 3 h p.i. of carrageenin compared with control fish (Fig. 3C–E). Furthermore, the expression of *nlrx1* (0.0002 ± 0.00008), as well as, isoform 1 of *nlrc5* gene (0.00017 ± 0.0001) was still down-regulated at 6 h after carrageenin administration compared with the values observed in the skin from control fish (Fig. 3E and F). The gene expression of the anti-inflammatory cytokines *il10* and *tgfb* was not affected by injection with carrageenin (Fig. 3G and H). Similarly, the expression of *ctsl, ache, bche, chrna7* and *ikbkg* (NEMO) genes did not show any significant variations in fish injected with carrageenin compared with fish from the control group (Fig. 3I–M).

As regards the time factor, in the control group the gene expression of *nlrc3* (0.03223 ± 0.00472) (Fig. 3A) was seen to be up-regulated at 6 h compared with the level measured at 1.5 h, while the expression of *chrna7* (0.00004 ± 0.00001; 0.00009 ± 0.00004) (Fig. 3L) gene was up-regulated at 3 and 6 h compared with the values observed at 1.5. Finally, in fish injected with carrageenin, the expression of *ctsl* gene (0.00246 ± 0.00106) was up-regulated and that of *ache* gene (0.000025 ± 0.000003) down-regulated at 6 h compared with the measurements made after 1.5 h (Fig. 3I and J andFig. 4).

3.4. Correlation analysis

The results of correlation confirmed that the gene expression of cell markers (*csfr1, phox22* and *phox40)* were positively correlated with the expression of *tlr9*, adapter molecules (*myd88, traf6, irak1* and *ikbkg*), NF-κB subunits (*rela, relb, c-rel* and *nfkb2*), the cytokines (*il1b, il6, il7, il8, il18, tgfb* and *il10*), *nlrc3* and *ctsl* (Fig. 5). In addition, the expression of TLRs genes (*tlr2*, *tlr7*, *tlr9* and *tlr13*), adapter proteins, NF-κB subunits, cytokines and cathepsins (*ctsd* and *ctss*) was also positively correlated among themselves. The strongest positive correlation was observed when analysing the expression of *phox22* and the cytokines *il8, il18* and *tgfb*. Interestingly, only the expression of *il1b* was negatively correlated with the expression of *nlrx1* gene.

4. Discussion

Carrageenin has been used for decades as a model for the study of the inflammatory response mainly in rats [35]. Assuming the possible conservation of some pathways during the evolution of vertebrates [36], we studied the local and acute inflammation process produced by a subcutaneous carrageenin injection in gilthead seabream, a fish species selected due to its high importance in the marine aquaculture of the Mediterranean area. The present study was inspired by an experiment in which carrageenin was injected into the paw of rats [37]. Based on this experiment, and regarding both the anatomic differences between mammals and fish, and the different sensitivity to wounds between the skin placed above and below the lateral line of gilthead seabream [25,27,38,39] we selected conscientiously the area for the subcutaneous injection of carrageenin for assessing the inflammation process. Furthermore, the concentration of carrageenin injected per fish (50 µl of carrageenin 1%) and the sampling times (1.5, 3 and 6 h p.i.) were previously tested, offering a local and systemic vision of the process triggered by carrageenin [40,41]. Additional time points (12, 24 h) were discarded since homeostasis mechanism seemed to be just initiated after the experimental times selected in the current study [36]. In addition, it would be important to mention that both criteria (carrageenin concentration and sampling times) were used to produce a peak of inflammation in higher vertebrates and other fish species, while lower doses failed to elicit substantial changes in the inflammatory response [17,18,20,35,38]. Since the signal transduction pathways here studied has been preserved in fish [36] and although the number of available genes was restricted due to the fact that the genome of gilthead seabream is not completely sequenced or annotated, it was thought that an analysis of the genes studied in the present work might identify the main mechanisms involved in the acute inflammatory response triggered in gilthead seabream after carrageenin injection. For this, and according to the results obtained in the correlation assay, we followed the schema shown in Fig. 6 to facilitate understanding of the obtained results. These results should be of interest not only for basic research but also for applied reasons related with the aquaculture sector.

As it is known from mammalian studies, in response to a harmful stimulus, such as carrageenin, inflammation is triggered locally and the recruitment of immune cells to the damaged area is initiated [42]. In our study, the up-regulation of cells' marker genes, which encode leucocyte receptors of *csfr1* (macrophage marker) [43,44], antigen-presenting cells (*mhciia*), and acidophilic granulocytes (functionally equivalent to mammalian neutrophilic granulocytes) (*phox22* and *phox40*) [45–47], respectively, suggested the activation or migration of these cells in the inflammation focus at 1.5 h of the carrageenin injection [20]. Leucocytes from gilthead seabream are mainly produced in head-kidney, from which granulocytes are recruited to the damage site and they can be recruited rapidly from the head-kidney and persist there for a certain time (at least 6 h according to our study), while newly recruited monocytes could become activated and differentiate into macrophages, showing their antigen presentation capacities and the expression of proinflammatory cytokines [46,48,49]. One of the main function of macrophages and acidophilic granulocytes is the phagocytosis, although granulocytes are also responsible for releasing reactive oxygen intermediates from their granules, so it could be assumed that the high molecular weight and peculiar structure of carrageenin could trigger its internalization and storage in phagosomes, in other words, both cells would be actively involved in the elimination of the carrageenin from the extracellular medium [20,47]. This fact could also be supported due to the correlation analysis here developed which associated the expression of cell markers of acidophilic granulocytes and macrophages with the expression of TLRs, adapter molecules, NF-κB subunits, cytokines, NLRs and cathepsins, all of them molecules responsible for the

triggering and regulation of the immune response. In agreement with these results, our previous immunohistochemistry assay demonstrated not only the presence and implication of acidophilic granulocytes and monocytes/macrophages in the skin inflammation of gilthead seabream, but also the one of non-specific cytotoxic cells and mucus-secreting cells. These results revealed the constitution of an inflammatory environment produced by the molecules released by both resident cells and immune cells recruited to the focus of inflammation [40]. Nevertheless, more studies are needed at the protein level in order to characterize the cells that are being modulated by carrageenan, since the activation of cellular regulatory mechanisms could affect their gene expression independently of their protein function [50].

Otherwise, the possible recognition of carrageenin by a specific or a nonspecific receptor that triggers the activation of one or more inflammatory pathways cannot be rejected from the present data. In fact, carrageenin was able to induce activation of NF-κB and inflammation through the surface member of the family of innate immune receptors TLR4 in human colonic epithelial cells, and via TLR2/6 and TLR4/6 heterodimers in HEK-293 cells [23,51]. Nonetheless, the presence of TLR4 orthologs in the gilthead seabream is unknown and it explains its resistance to the toxic effect of bacterial lipopolysaccharide [52]. In this sense, our results demonstrated that the gene expression of other conserved receptors such as *tlr5, tlr7, tlr8, tlr9,* or *tlr13* did not show significant variations in fish injected with carrageenin at any sampling time (1.5, 3, and 6 h), possibly meaning minor participation of these TLRs in the signal transduction pathway.

In contrast, in the present study the expression of *tlr2* gene, whose protein is able to recognize the teichoic acid of Gram-positive bacteria, was down-regulated 3 h after p.i. of carrageenin, which could be considered as a preventive mechanism of activation of other proinflammatory pathways triggered by different pathogen-associated molecular patterns (PAMPs) [53]. These results agree with previous studies that demonstrated that teichoic acids alone were able to activate the TLR2 pathway and induce the expression of anti-inflammatory cytokines such as IL-10 in order to regulate inflammation, while the presence of teichoic acids with *Lactobacillus casei* induced the expression of proinflammatory cytokines through this receptor [54]. These facts point to the need for future studies in order to clarify the possible involvement of TLRs in the fish inflammatory response. In any case (either by phagocytosis or by recognition by receptors), carrageenin was able to activate inflammatory mechanisms of gilthead seabream and transducing the signal to the NF-κB transcription factor, which is the crucial piece of inflammation. Perhaps, as it has been previously suggested, the carrageenin effects were mediated through adaptor proteins such as MyD88, IRAK1 and TRAF6, which bind to and tightly regulate TLRs [8,55,56]. In our study, only myd88 gene expression was up-regulated to 1.5 after p.i. of carrageenan, which could have been linked to TLRs that participate in the signal transduction pathway to NF-κB. Alternatively, since MyD88 was activated in mouse lung epithelial cells for the purpose of inducing neutrophil recruitment through chemokine production, it could play an alternative role outside the TLR pathway [57,58].

It is important to emphasize that in mammals, in absence of an inflammatory stimulus, IκB (κB inhibitor) is responsible for hiding the nuclear localization domain (NLD) of NF-κB, preventing its translocation to the nucleus, being forced to remain in the cytosol [59]. However, in presence of a trigger of inflammation, IκB kinase (IKK), whose structure is formed by a 3-subunits complex, assembles and then, it is able of phosphorylating 2 serine residues of the IκB regulatory domain, which is modified through the ubiquitination process and degraded in the proteasome complex [60]. Once IκB has been degraded, the NF-κB complex would be able of translocating from the cytosol to the nucleus and binds to DNA response elements present in promoters or enhancers of target genes involved in the triggering of inflammation and its regulation [61,62]. In fact, NF-κB shares DNA-binding domains and dimerization domains that can combine with homo or heterodimers and, after activation, mainly form a heterodimer consisting of p50/NF-κB1: p65/RELA, which constitutes its canonical activation pathway [62,63]. Interestingly, among all the genes analysed in our study, the positive regulation of *c-rel* gene expression that was found in fish from the carrageenan group sampled at 3 h p.i. suggests a different activation of the inflammation pathway in fish. Furthermore, with respect to the components of the NF-κB family, while c-REL, RELA and RELB have a C-terminal domain involved in the transcription of their target gene, NF-κB2 and NF-κB1 do not have this domain, so they could act as transcriptional repressors [59,64]. Our results showed that the gene expression of *nfκb2* was down-regulated in the skin of fish from the carrageenin group sampled 6 h p.i. compared the values recorded in skin from control fish. This interesting observation suggests that the NF-κB transcription factor is expressed as a result of the union of homodimers of c-REL or heterodimers of c-REL with other components of NF-κB, but not homodimers of NF-κB2. The main function of the NF-κB transcription factor is to activate proinflammatory cytokines [63], which agrees with the present results on the up-regulation of the gene expression of different proinflammatory cytokines (*il1β, tnfα, il6, il8* and *il18*) observed 3 p.i. of carrageenin. In addition, *il-6* and *tnf-α* gene expression remained up-regulated at 6 h in the skin of fish from the carrageenin group compared with the control fish. It is known that under inflammatory conditions, TNF-α is able to activate endothelial cells, which increased the expression of adhesion molecules (such as selectins and integrin ligands), and chemokines, participating in the adhesion-recruitment cascade of neutrophils [65]. Likewise, IL-6 not only participates in cell migration, but also in cell proliferation, invasion, differentiation, and angiogenesis [66–72]. Thus, our results strongly suggest that TNF-α and IL-6 could be the main cytokines involved in the recruitment of the acidophilic granulocytes observed 6 h after carrageenin administration. These results will also imply that carrageenin provokes an acute phase response in gilthead seabream.

As the masterpiece of this intricate puzzle, the activation or inhibition of NF-κB transcription factor is tightly regulated endogenously by multiple factors, such as proteins located upstream of its transduction pathway, other related proteins involved in the process like Nod-like receptor (NLR) proteins, and even external mediators and molecules released from the extracellular space like neuropeptides or cathepsins [42,62,73]. Proteins of NLR family are a group of intracellular pattern recognition receptors (PRRs), characterized by stimulating cellular signalling of inflammation, which were analysed in our study [73]. For instance, mitochondrial NLRX1, which is able to interfere with the interaction of the TRAF6 and IKK protein complex and limit the NF-κB activation, was down-regulated at 3 and 6 h p.i. of carrageenin in the present study, allowing the inflammation cascade [74]. Similarly, NLRC3, although is a protein that might negatively affect the expression of NF-κB transcription factor by ubiquitinating the TRAF6 signal adapter protein, also may participate in the establishment of the inflammasome, which, in agreement with our results (it was up-regulated in our study at 3 h in the skin of gilthead seabream injected with carrageenin), could suggest its notable action in the development of an inflammatory state in this fish species [75,76]. Otherwise, it has been documented that, while in humans and mice the protein NLRC5 could interact with IKKα protein, thus inhibiting the catalytic activity and consequently interfering in the NF-κB pathway [77], in Atlantic salmon (*Salmo salar*) it was able to activate the inflammasome [78]. Interestingly, since two variants of NLRC5 (NLRC5 isoform 1 and NLRC5 isoform 2) were described in the species under study with probably several functions and specificity of cellular type, and regarding the possible pathway conservation, the present results could insinuate possible participation of NLRC5 in the functions previously described. The IKKα protein could be inhibited by isoform 1, whereas isoform 2 could participate in the activation of the inflammasome [79]. Future studies should attempt to ascertain whether similar results are obtained in other fish species of interest.

The inflammatory response may also be regulated through the release of acetylcholine (ACh) from the parasympathetic SNA terminals [80,81]. ACh can inhibit both TNF-α secretion and the proinflammatory

cytokine synthesis of IL-1β, IL-6 and IL-8 in visceral macrophages [3,80]. This inhibitory intracellular signal is transduced by immune system cells which express acetylcholine receptors (AChR) such as α7nAChR, which is a key player in the inflammatory reflex, regulating cytokine expression in macrophages by binding to ACh. Through the α7nAChR receptor, AChE (pseudocholinesterase) and BChE (butyrylcholinesterase) recognize ACh and hydrolyse it into choline and acetate [81,82]. In our experiment, although no significant variations were found in the gene expression of *ache*, *bche* and *chrna7* between the experimental groups, *ach*e gene expression was down-regulated in the skin of fish sampled at 6 h compared with the results obtained in those fish from the carrageenin group sampled at 1.5 h p.i. Considering that AChE is the main hydrolytic enzyme of the anti-inflammatory molecule ACh, the gradual down-regulation of this gene expression with time suggests that an "anti-inflammatory environment" is generated to prevent an excessive tissue damage [83]. In this sense, the fact that the gene expression of proinflammatory cytokines (*il1b, tnfa, il6, il18*) was seen to be modulated in our study, especially at 3 h, suggests that the cholinergic system was negatively modulated by carrageenin.

As regards other regulators of the inflammation process, cathepsins are lysosomal cysteine endonucleases that also negatively modulate inflammation, in this case, because they activate final mechanism of cellular apoptosis [84]. In our study, the expression of *ctsd* and *ctss* genes was down-regulated at 3 h in the skin from fish injected with carrageenin coinciding with the up-regulation of *phox22* at the same time and compared with the expression levels obtained in the skin from control fish, supporting the action of the recruited acidophilic granulocytes in the damaged area and avoiding apoptosis. In addition, and regarding the time factor, *ctsl* gene expression was up-regulated in fish from the carrageenin group at 6 h compared with the levels recorded at 1.5 h. Cathepsin L is related to cell adhesion through cadherin E and neutrophil recruitment in mammals [85], which, once again, is consistent with our hypothesis.

To conclude, our results indicate that the subcutaneous injection of carrageenin triggers the inflammatory process in the skin of gilthead seabream. Carrageenin produced an up-regulation of the expression of several proinflammatory genes and other genes involved in the preparation of the cellular environment for later recovery The measurement of gene expression of pro- and anti-inflammatory markers can provide sensitive and rapid information regarding the acute local inflammatory response, which can be considered for developing new studies aimed at modulating the inflammation process in fish of commercial interest to the aquaculture sector.

CRediT authorship contribution statement

Jose Carlos Campos-Sánchez: Writing – original draft, formal analysis, performed the experiment and data generation, analysed the experimental data. **Javier Mayor-Lafuente:** Formal analysis, and performed the experiment and data generation, analysed the experimental data. **Daniel González-Silvera:** Formal analysis, performed the experiment and data generation. **Francisco A. Guardiola:** Writing – original draft and work on data curation . **María Ángeles Esteban:** Designed the experiment and manage the funding. All authors have reviewed the manuscript and approved the final version.

Declaration of competing interest

All authors declare that they have no competing interests.

Acknowledgements

This work was supported by the Spanish Ministry of Economy and Competitiveness (MINECO) co-funded with the European Regional Development Funds (ERDF/FEDER) (Grant no. AGL-2017-83370-C3-1-R) and *Fundación Séneca de la Región de Murcia* (*Grupo de Excelencia* 19883/GERM/15).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2021.10.009.

Data availability statement

All the data supporting the findings of this study are available on request from the corresponding author.

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