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Peracetic acid can be used as a disinfectant for gilthead sea bream (Sparus aurata) juveniles without affecting fish welfare

F. Acosta [a,*], J. Bravo [a], L. Monzón-Atienza [a], J. Galindo-Villegas [b], S. Torrecillas [a], D. Montero [a]

[a] Grupo de Investigación en Acuicultura (GIA), IU-ECOAQUA, Universidad de Las Palmas de Gran Canaria, Crta, Taliarte s/n, Telde, Las Palmas, Canary Islands, Spain
[b] Faculty of Biosciences and Aquaculture, Nord University, Bodø, Norway

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ABSTRACT

Peracetic acid (PAA) is a European Union Commission authorized disinfectant for use in animal health care. It has shown a strong inactivation potential for bacteria, viruses, fungi and bacterial spores. A stress-related adaptive response after an exposure to PAA has been described in different species of fish such as carp (*Cyprinus carpio*), rainbow trout (*Oncorhynchus mykiss*). The present study aims to evaluate the response of gilthead seabream systemic (plasma) as mucosal (gill and skin) defences after PAA a unique exposure (5 min), by measuring cortisol, glucose and lactate in plasma, as well as the expression of several genes (*glutathione peroxidase, glucocorticoid receptor, superoxide dismutase 2* and *superoxide dismutase* involved in the response to oxidative stress in mucosal tissues). study. We observe how seabream (*Sparus aurata*) copes with oxidative stress induced by PAA. PAA exposure did not induce an important antioxidant response in fish, whereas induced a mild response to stress, with a fast and effective recovery of basal levels after 24 h. Although PAA triggers a mild stress response, the response described in our study reflect that it can be used for sea bream in the concentration tested with no severe physiological consequences.

1. Introduction

Peracetic acid (PAA) is an European Union Commission authorized disinfectant for use in animal health care (Assefa and Abunna, 2018; Scenihr, 2012). PAA is very reactive it has shown a strong inactivation potential for bacteria, viruses, fungi and bacterial spores (Zhang et al., 2020). The mechanism of action of PAA is based on a direct and powerful action on cell membranes through hydroxyl radicals (Acosta et al., 2021). Different studies have demonstrated that PAA acts as a disinfectant with a great future projection in its use for the development of biosafety in aquaculture, due to its rapid decomposition into neutral waste (Soleng et al., 2019) and its effectiveness against a wide variety of pathogens such as *Vibrio harveyi*, *Photobacterium damselae* subspecies *piscicida*, *Vibrio anguillarum* and *Vibrio alginolyticus* (Acosta et al., 2021), *Aeromonas salmonicida* and *Yersinia ruckeri* (Meinelt et al., 2015), *Piscirickettsia salmonis* (Muniesa et al., 2019), *Yersinia ruckeri* (Yamasaki et al., 2017) and infectious salmon anaemia virus (Straus et al., 2018). Basically, the disinfecting activity of PAA is categorized from highest to lowest as follows: bacteria> viruses> bacterial spores> protozoan cysts (Wessels and Ingmer, 2013). The exact mechanism how PAA oxidizes and destroys a microorganism remains arguable due to the complexity of the reaction route. In contrast with other similar disinfectants, such as H2O2, PAA results in simple oxidation by dihydroxylation of double bonds and formation of free radicals (Wessels and Ingmer, 2013), which contribute to its effectiveness as disinfectant with lipid solubility properties (Lazado and Voldvik, 2020) and positions it as potential powerful antimicrobial agent than H2O2, due to its solubility in fats (Lazado and Voldvik, 2020). However further studies must be conducted to determine other still undescribed PAA mode of actions as disinfectant for specific aquaculture related pathogens.

An stress-related adaptive response after an exposure to PAA has been described in different species of fish such as carp (*Cyprinus carpio*) (Elia et al., 2006), rainbow trout (*Oncorhynchus mykiss*) (Gesto et al., 2018; Liu et al., 2017b, 2017a) and Atlantic salmon (*Salmo salar*) (Soleng et al., 2019). This response to PAA has been described as an early increase in circulating cortisol and a rapid recovery of basal levels (Gesto et al., 2018; Soleng et al., 2019), corresponding to a typical pattern of acute response to an oxidant agent exposition, subsequent activation of the antioxidant response system (Soleng et al., 2019), and proper recovery of basal levels.

Thus, the aim of the present study to evaluate the response of gilthead seabream systemic (plasma) as mucosal (gill and skin) defences

* Corresponding author.
E-mail address: felix.acosta@ulpg.es (F. Acosta).

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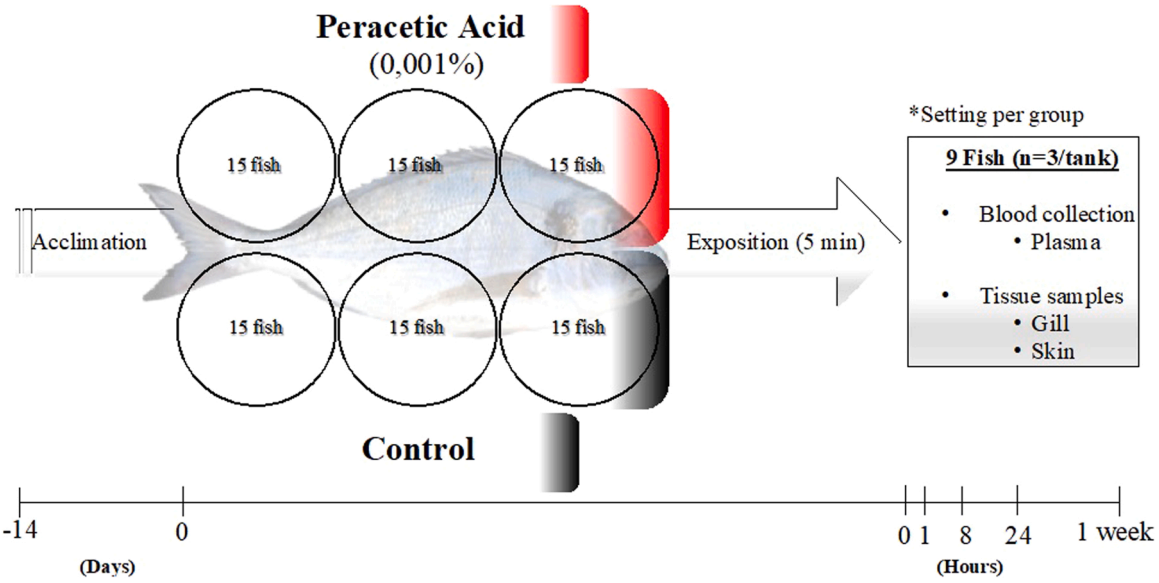

Fig. 1. Experimental setup. After the acclimation period, one group of gilthead seabream (*Sparus aurata)* was stimulated with peracetic acid (PAA) at 0.001% (v/v) for 5 min, the control group was treated equally with PBS. Samples of plasma were obtained at time 0, 1, 8, 24 and 1 week after PAA exposition (n = 3 fish/tank). Gill and skin were also sampled for gene expression studies.

after PAA exposure, by measuring cortisol, glucose and lactate in plasma, as well as the expression of several genes involved in the response to oxidative stress in mucosal tissues, to test if PAA is a fish/welfare-friendly disinfectant for sea bream after single acute exposure.

2. Material and methods

2.1. Ethics approval

This experiment was conducted according to the Spanish legislation (RD 53/2013) and European Union Directive (2010/63/EU). The Bioethics Committee of the University of Las Palmas de Gran Canaria approved the experimental procedure (REF. OEBA-ULPGC 03/2020 R2).

2.2. Experimental fish and husbandry conditions

Ninety sea bream (*Sparus aurata*) juveniles with 20.0 g of average body weight produced at the aquaculture facilities of the Universitary EcoaAqua Institute of the University of Las Palmas de Gran Canaria (ULPGC) were randomly distributed in three cylindrical–conical 500 L tanks (30 fish per tank) and acclimatized for three weeks. Along this period, RAS system tanks were supplied with a constant flow rate of 0.4 m3/h, photoperiod maintained at 12 L: 12D, temperature at 21 ± 1 °C and dissolved oxygen kept at 5–6 ppm. Fish were fed a commercial diet (Skretting, Alterna, 3 mm) to apparent satiation. The fish for this experiment never had been expose to PAA before.

2.3. Peracetic acid exposure test

After acclimation period, fish were fasted for 24 h and divided in six 200 L tanks (15 fish per tank) for peracetic acid solution (40%) (obtained from Sigma-Aldrich Chemical, USA (Cat. 94,329)) test exposure. The fish rested for 10 min before adding the PAA solution at 0.001% (v/v) concentration in 3 of the experimental tanks, being the other three tanks acting as a control treatment tanks. To correct dilution of the PAA was ensured by vigorous tank aeration. The concentration of PAA tested was chosen based on a previous study carried out addressing the determination of PAA toxicity levels for this fish species, and reflects a dosage enough to be effectively used as disinfectant for this species (Acosta et al., 2021). While exposure to PAA was being carried out, the water flow remained closed and during the 5 min of PAA exposure fish were under sedation with tricaine methanesulfonate (MS222) 20 mg/L and submerged all the time. After treatment, fishes were removed from the PAA tanks to new tanks (Fig. 1).

2.4. Sampling

Sample collection was done at 0, 1, 8, 24 h and 1week after PAA exposure (Fig. 1). From each tank, 3 fish were taken and euthanized with an overdose of MS222 at 250 mg/L. A sample of blood was obtained from the caudal sinus using a heparinized syringe, centrifuged at 1000 g for 10 min at 4 °C, and the plasma was kept at − 80 °C until analyses. For RNA isolation, samples of the skin from the dorsal area and of second branchial arch were obtained and kept in RNA-Later (Ambion, USA) at room temperature overnight and subsequently stored at − 80 °C until RNA extraction.

2.5. Plasma stress indicators (PSI)

Commercially kits were used to check the quantity of glucose, lactate, and cortisol. Plasma cortisol was analysed using an ELISA kit (Neogen, USA), following the manufacturer's instructions. Plasma glucose and lactate concentrations were quantified using a Glucose Assay Kit (Abcam, USA) and a Lactate Assay Kit (Sigma-Aldrich, USA), respectively. All samples were analysed in duplicates.

2.6. Total antioxidant capacity (TAC) assay

To quantify the TAC in fish plasma, a commercial kit (Sigma-Aldrich, Spain) based in a colorimetric technique was used. The TAC level was expressed in relation to 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), a water-soluble analogue of the vitamin E.

2.7. Expression of oxidative stress- related genes

The total RNA was isolated from skin and gill samples (Fig. 1) using E.Z.N.A.® Total RNA Kit (OMEGA), quantified by suing a NanoDrop 8000 spectrophotometer (Thermo Scientific, USA) and reverse transcribed into cDNA using the iScript ™ cDNA synthesis kit (BIO-RAD, USA). Relative gene expression of the genes *glutathione peroxidase* (*gpx*),

Table 1
Primers used in the present study.

Gene name	Acronym	GenBank	Primer sequence (5′−3′)
Elongation factor-1 α-tubulin	*ef1α*	AF184170	CCCGCCTCTGTTGCCTTCGCAGCAGTGTGGTTCCGTTAGC
Ribosomal protein S18	*rps18*	AM490061	CGAAAGCATTTGCCAAGAATAGTTGGCACCGTTTATGGTC
β-actin	*β-actin*	X89920	GGCACCACACCTTCTACAATGGTGGTGGTGAAGCTGTAGCC
Glutathione peroxidase 1	*gpx1*	DQ524992	GAAGGTGGATGTGAATGGAAAAGATGCTGACGGGACTCCAAATGATGG
Glutathione reductase	*gr*	AJ937873	TGTTCAGCCACCCACCCATCGGGCGTGATACATCGGAGTGAATGAAGTCTTG
Superoxide dismutase [Mn]	*sod2*	JQ308833	CCTGACCTGACCTACGACTATGGAGTGCCTCCTGATAT TTCTCCTCTG
Cu/Zn superoxide dismutase	*sod*	FJ860004	TGTTGGAGACCTGGGAGATGATTGGGCCTGTGAGAGTGAG

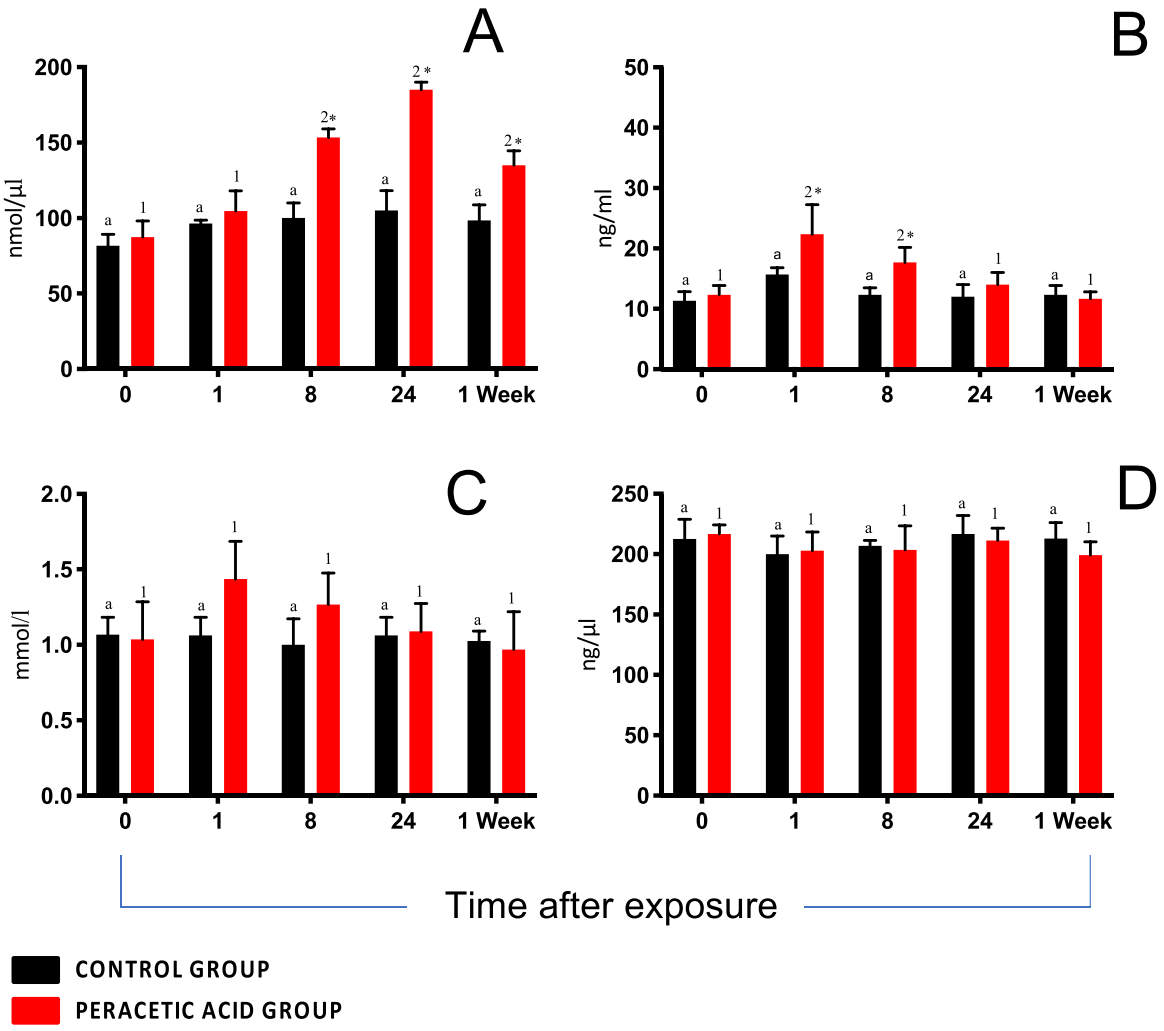

Fig. 2. Gilthead seabream (*Sparus aurata*) plasmatic levels of A) total antioxidant capacity (TAC), B) cortisol, C) glucose and D) lactate after peracetic acid exposition (0.001% v/v; PerA). The level of total antioxidant capacity (TAC) is expressed relative to Trolox standards. An asterisk (*) denotes significant differences between PAA group and the control group at each sampling time ($p < 0.05$). Different numbers denote significant differences between different times for PerA group ($p < 0.05$), and different letters indicate significant differences ($p < 0.05$) for control group along the challenge. Values expressed as mean SE of nine individual fish. Two-way ANOVA followed by Tukey post-hoc test.

glutathione reductase (*gr*), *manganese superoxide dismutase* (*sod2*) and *copper/zinc superoxide dismutase* (*sod*) were determined by real time qPCR in a CFX96 Touch Deep Well real-time PCR system (BIORAD, USA) using β-actin, 18 S ribosomal RNA and elongation factor 1 alpha as housekeeping genes. All PCR reactions were carried out in a final volume of 20 µl, with 8 µl of Brillant SYBR Green QPCR Master Mix (Bio-Rad Hercules, CA, USA), 1 µl of each primer (10 mM), 6 µl of cDNA (1:10 dilution) and 5 µl of MiliQ water. MiliQ water also replaced cDNA in blank control reactions. Relative gene expression was calculated following the Livak and Schmittgen (Livak and Schmittgen, 2001) after normalization in relation to housekeeping genes. Primers sequences are shown in Table 1.

2.8. Statistics

Statistical analyses were performed using GraphPad Prism software version 8.4.2 for macOS (GraphPad Software, San Diego, USA). Normality and homogeneity of variance were tested by Shapiro-Wilk and Brown-Forsyth tests, respectively. Two-way ANOVA was used to test the differences between the groups over time, and the Tukey post-hoc test was used to identify differences by pairs. $P < 0.05$ was defined as statistical significance for all tests.

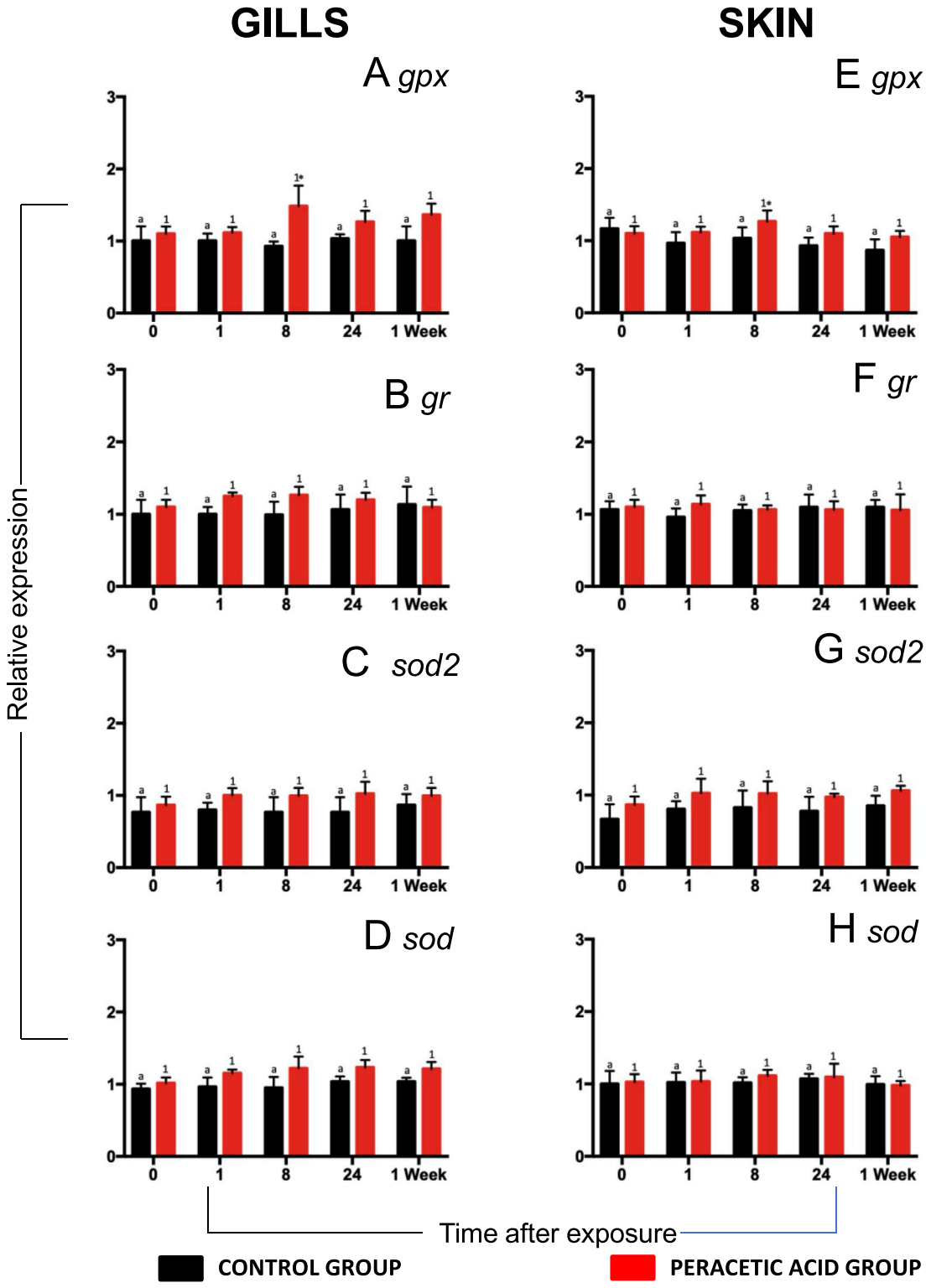

Fig. 3. Expression levels of antioxidant genes (*gpx=glutathione peroxidase, gr=glutathione reductase, sod2 =manganese superoxide dismutase and sod=copper/zinc superoxide dismutase*) in gills (A; B; C; D) and skin (E; F; G; H) of gilthead seabream (*Sparus aurata*) juveniles. An asterisk (*) denotes significant differences between PAA group and the control group at each sampling time ($p < 0.05$). Different numbers denote significant differences between different times for PAA group ($p < 0.05$), and different letters indicate significant differences ($p < 0.05$) for control group along the challenge. Values expressed as mean SE of nine individual fish. Two-way ANOVA followed by Tukey post-hoc test.

3. Results and discussion

PAA has been recognized as a disinfectant in seabream aquaculture for its relevant advantages over other biocides, but information on the effect of this strong oxidant in fish is still scarce. The use of any biocidal must be demonstrated to produce no harmful effects on fish that can compromise health and welfare of fish.

3.1. Total Antioxidant Capacity (TAC)

PAA has been described as a powerful oxidant, and hydroxyl radical forms and other reactive oxygen species are obtained from their components and decomposition (Kitis, 2004). The increase of TAC has been described by different authors and indicates that the redox balance undergoes a decrease as a result of oxidative stress, mobilizing antioxidants to counteract the alterations (Wu et al., 2017). In the present study, gilthead seabream exposed to PAA presented a significant ($p < 0.05$) increase in TAC from 8 h to a week after exposure compared to control treatment (Fig. 2). TAC increase represented a 53%, 80% and 36.7% at 8, 24 and 1 week respectively in the exposed group when compared to control fish, it was significant in these three sampling points (Fig. 2 A). The two-way ANOVA showed a significant interaction between the factors Time and Treatment at 8, 24 h and 1week with $P = 0,04$, $P = 0,01$ and $P = 0,03$ respectively (Fig. 2 A). TAC levels were not changed in the control group along the challenge (Fig. 2 A), which indicates a clear mobilization of the fish antioxidant defences against PAA-induced oxidative stress caused from 8 h after challenge onwards, as previously described in salmon (Soleng et al., 2019).

3.2. Plasma stress indicators

The handling procedure used in the present study induced the classic cortisol response expected for teleost fish (Cádiz et al., 2015; McCormick, 2001) (Fig. 2B). However, when comparing the response of both groups, the exposure to PAA induced a different pattern of response. Fish exposed to PAA presented a higher ($p < 0.05$) plasma cortisol level in the first hours after exposure compared to control group, despite basal levels were recovered in both treatments after 24 h of exposure, indicating non-severe fish response to the oxidant. In particular, fish cortisol levels after 1 and 8 h post PAA exposure were significant ($p < 0.05$) higher than those observed for control fish at the same sampling point. When we observed the changes in each group in the time, we observed that control group did not present changes, and in the treated group statistical differences were found at 1 and 8 h (Fig. 2B). The two-way ANOVA showed a significant interaction between the factors time and treatment at 1and 8 h with $P = 0042$ and $P = 0032$ respectively (Fig. 2B). Our results are according with previous studies of exposure to peroxides (Bowers et al., 2002; Chalmers et al., 2018; Gesto et al., 2018; Lazado et al., 2021; Osório et al., 2022) and to PAA (Liu et al., 2017b; Soleng et al., 2019). However, the level of plasmatic cortisol after stress or PAA exposition was lower when compared to other studies in gilthead seabream exposed to acute stress (Samaras et al., 2018; Vargas-Chacoff et al., 2020) indicating a moderated stress response, with an adequate recovery of cortisol baseline levels 24 h post- PAA exposure. Plasma glucose and lactate were not affected neither by the handling procedure nor the exposition to PAA, being those results similar to those obtained for the same fish species under different husbandry conditions (Chupani et al., 2014; Soleng et al., 2019), supporting the moderated stress response obtained in cortisol levels after exposition to 0001% PAA for 5 min

3.3. The antioxidant defences in mucosal tissues

Skin and gills are mucosal tissues that function as the first barrier of defence and are very sensitive to any environmental variation (Cabillon and Lazado, 2019), including to the reactive oxygen species (ROS) levels. The two-way ANOVA showed a significant interaction between the factors time and treatment at 8 h with $P = 0022$ (Fig. 3A). The expression of *gpx* (Fig. 3A) in the gills of the fish exposed to PAA was significantly ($P < 0.05$) higher after 8 h of PAA exposition compared to the control group, suggesting certain production of reactive species (Teles et al., 2019). Differences in *gpx* levels were observed in the treated group along the challenge at 8 h (Fig. 3A). Indeed, gilthead sea bream exposed to PAA at 0.001% did not show any change in the gene expression levels of the genes related with the endogenous antioxidant system functioning evaluated (Fig. 3B-D). Similar changes were observed in the skin where the expression of *gpx* was upregulated ($p < 0.05$) in fish exposed to the oxidant at 8 h after the exposure compared to the control group (Fig. 3E) and no effects were observed for the rest of genes studied (Fig. 2G-H), denoting again the important role of *gpx* in antioxidant defence against increases of ROS on mucosal surfaces (Khan et al., 2018; Lazado et al., 2015; Soleng et al., 2019). Differences in *gpx* levels were observed in the treated group along the challenge at 8 h (Fig. 3E). In general, that the expression without significant changes of antioxidant genes in skin and gill mucosal tissues between control and untreated fish, suggests that the PAA concentrations tested (0.001%) did not elicit a large mobilization of the antioxidant response, indicating that 0.001% PAA is an adequate and biosafe disinfectant dose in terms of potential ROS-induced damage to mucosal surfaces. In addition, the tested concentration always effectively eradicates the most complex and resistant community association formed by different bacterial species, such as biofilms (Acosta et al., 2021).

4. Conclusions

PAA is a very promising disinfectant to be used in aquaculture. Gilthead sea bream can adapt its mucosal and systemic response after exposure to 0.001% PAA. PAA exposure did not induce an important antioxidant response in fish, whereas induced a mild response to stress, with a fast and effective recovery of basal levels after 24 h. Although PAA triggers a mild stress response, the response described in our study reflect that it can be used for sea bream in the concentration tested with no severe physiological consequences. Even when the results of the present study could be reflecting the minimum effect of the use of PAA in the antioxidative response in seabream, studies on larger and/or repetitive exposures exposure to the PAA are necessary to conclude the general benefits at farm level. The results presented in this work must be considered specific to the formulation of the PAA product and that we cannot rule out or guarantee that other formulations may give rise to different reactions.

Authorship contributions

Please indicate the specific contributions made by each author (list the authors' initials followed by their surnames, e.g., Y.L. Cheung). The name of each author must appear at least once in each of the three categories below.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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