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Marine macroalgae as a dietary source of genoprotection in gilthead seabream (*Sparus aurata*) against endogenous and exogenous challenges

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Abstract

DNA integrity and stability are essential to organisms' health and survival. However, it has been neglected in what concerns to fish farming, disregarding the potential impact of endogenous/ exogenous factors. As marine macroalgae constitute a source of natural compounds with a large spectrum of biological activities, this study, situated in the interface of nutritional-genetic research and development of algae practical applications, aimed to evaluate the genoprotective properties of a macroalgae-enriched diet (total percentage of 5 %, incorporating equal percentages of *Ulva rigida*, *Gracilaria gracilis* and *Fucus vesiculosus*) in gilthead seabream (*Sparus aurata*). Protection was assessed in relation to a basal genome integrity and against an exogenous genotoxic challenge (cyclophosphamide; CP). Fish were reared for 30 days with the supplemented diet, being then injected with CP and sampled at days 3 and 10 post-injection (p.i.). To evaluate whether the favorable effects remain after the end of supplementation, a fish subgroup previously fed with algae-enriched diet was submitted to a diet reversion at day 3 p.i., being thereafter fed with the standard diet. Genetic damage was evaluated through the erythrocytic nuclear abnormalities (ENA) and comet assays and complemented by the assessment of the antioxidant system. Results pointed out that algae-enriched feed exhibits anti-genotoxic properties, mostly expressed in relation to the exogenous pressure, manifest in relation to DNA strand breaks and chromosomal lesions, also reducing oxidative DNA damage. Nonetheless, blood antioxidants were only punctually altered by the supplemented diet (*e.g*. catalase and glutathione-S-transferase). Analyzing the effect persistence, it was perceived that 7 days without algae uptake was enough to partially reduce the protection efficacy. Overall, these findings are promising towards the benefits of macroalgae inclusion in fish diet, and thus, to invigorate mariculture activity and the commercial use of algae, also providing new insights on the DNA protection mechanisms.

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Keywords: Seaweeds, fish, genetic damage, anti-genotoxic potential.

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1. Introduction

The DNA integrity and stability are essential to organisms' health, fitness and, ultimately, to survival (Clancy, 2008), being a challenging achievement due to the recurrent manifestation of endogenous and exogenous sources of genotoxic stress. In fact, DNA is constantly damaged in aerobic organisms due to its susceptibility to reactive oxygen species (ROS), endogenously formed as part of physiological processes (Oliveira et al., 2010), concomitantly to a plethora of agents present in the environment with potential to alter the structural integrity of the DNA molecule (Geacintov and Broyde, 2010). A single alteration in the DNA molecule of an organism could originate serious biological consequences, disrupting normal cell processes and leading to cell death. The loss of DNA integrity is considered one of the first events that occur in organisms exposed to a genotoxic pressure, highlighting thus the importance of its early evaluation (Frenzilli et al., 2009; Guilherme, 2012).

Within the context of intensive aquaculture, the problematic of genome instability has been largely neglected. This is surprising considering the exposure (intentional or accidental) of aquacultured species to agents with the capacity (potential or demonstrated) to create stressful conditions passible to affect DNA integrity, such as disinfectants (Ibrahim, 2015; Silva et al., 2011), anesthetics (Barreto et al., 2007; Siekel, 1990), anti-parasitic (Speit and Merk, 2002) and antibiotics (Rodrigues et al., 2017), as well as contaminants carried by water (FDA, 2011; Justino et al., 2016) and aquafeeds (CAST, 2003; Johnston and Savage, 1991). On the other hand, the manipulation of rearing conditions to get a fast-growing performance (Alonso-Alvarez et al., 2007), as well as the exposure of farmed organisms to hypoxia/anoxia followed by reoxygenation (Almeida et al., 2007; Hermes-Lima and Zenteno-Savín, 2002), are responsible for the formation of ROS that can oxidatively damage DNA. In the long-term, this situation may compromise growth performance and animal welfare, causing a reduction in revenues (Fazio et al., 2015; Leal et al., 2011). Hence, the adoption of aquafeeds able to promote genome stability in farmed fish may contribute to mitigate those constrains.

In recent years, the number of studies on marine macroalgae addressing their chemical composition and physiological properties has grown exponentially (Fleurence, 2016; Mohamed et al., 2012), mainly triggered by scientific and commercial interests on their potential as functional or health-promoting foods in humans (Patarra et al., 2011). Macroalgae are rich in soluble dietary fibers, proteins, minerals, vitamins, antioxidants, and polyunsaturated fatty acids, in parallel with a low caloric value. A wide range

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of bioactives has been identified with potential applications in various areas, including pharmaceutical, cosmeceutical, nutraceutical and functional food industries (Mendis and Kim, 2011; Pangestuti and Kim, 2011). In addition, as algae are at the base of the aquatic food chains, representing a food resource that wild fish are adapted to consume (Norambuena et al., 2015), they are receiving increasing attention as a novel feed ingredient in pisciculture (Batista, 2008). It was demonstrated that dietary macroalgae supplementation may be used to improve growth performance, feed utilization, nutrient retention, carcass quality, stress response, survival, resistance and unfavorable conditions and disease (Emre et al., 2013; Magnoni et al., 2017; Norambuena et al., 2015; Peixoto et al., 2016b; Wassef et al., 2005), in association with a strengthening of antioxidant systems (Andrade et al., 2013; Augusto et al., 2014; Surget et al., 2017). Notwithstanding the publications previously mentioned, it is manifest a scarcity of information on the macroalgae action protecting genome integrity of fish. As a *Proof of Concept* towards a genoprotective potential of marine macroalgae in fish, it was demonstrated that a *Kappaphycus alvarezii* (red algae) extract exhibits potent anti-genotoxicity effects in Jarbua fish (*Terapon jarbua*) against DNA damage induced by mercury (Hg) (Nagarani et al., 2012). Moreover, Zinadah et al. (2013) showed that, when flathead grey mullet (*Mugil cephalus*) diet was supplemented with 10-20 % of *Ulva lactuca* or *Caulerpa prolifera* (green algae), a considerable DNA protection was achieved. This macroalgae potential is reinforced by research on non-fish models. Hence, *in vitro* data suggested protective effects of red [*Palmaria palmate* (Yuan and Walsh 2006) and *Porphyra* sp. (Kwon and Nam 2006)] and brown [*Laminaria setchellii, Macrocystis integrifolia* and *Nereocystis luetkeana* (Yuan and Walsh 2006)] algae against different cancer types. Furthermore, the benefits of *Ulva rigida*, *Fucus vesiculosus* and *Codium tomentosum* extracts were evaluated in human lymphocytes (*in vitro*), showing no cytotoxic effects, in parallel with strong anti-genotoxic and anti-clastogenic effects against the chemotherapeutic agent mitomycin-C (Celikler et al., 2009, 2008).

In order to fulfill a knowledge gap, the central goal of the present research, situated in the interface of nutritional-genetic research and development of algae practical applications, concerns the evaluation of genoprotective properties of a macroalgae-enriched diet in gilthead seabream (*Sparus aurata*). The choice of gilthead seabream is justified mostly by the fact that, though mainly carnivorous, it can be accessorily herbivorous, thereby accepting well macroalgae in the diet. The tested diet was supplemented with three different species of - *Ulva rigida* (green algae), *Gracilaria gracilis* (red algae) and *Fucus vesiculosus* (brown algae) – in a total percentage of 5 % (incorporating equal percentages of

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each algae). Macroalgae species were selected for their taxonomic representativeness (one species from each phyla - Chlorophyta, Rhodophyta and Ochrophyta), due to the expected co-occurrence with gilthead seabream in the wild, which makes them passible to be included in its natural diet, and because they are common in the Atlantic coast and relatively easy to grow in aquaculture systems. It was intended to discriminate between a beneficial action in relation to a genome integrity baseline (endogenous challenge) or against an exogenous challenge, using a model genotoxicant – cyclophosphamide (CP; i.p. injection of 40 mg. kg^{-1}), as well as to clarify if the potentially favorable effects persist beyond the end of supplementation. Two types of genetic damage were evaluated in blood cells, *viz*. DNA strand breaks (primary and reparable damage), measured with the comet assay, and chromosomal damage (potentially more permanent damage), measured through the erythrocytic nuclear abnormalities (ENA) assay. Complementary, it was assessed the involvement of antioxidant system modulation on the potential defense mechanisms under investigation, evaluating enzymatic [superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST), glutathione reductase (GR) and glutathione peroxidase (GPx)] and non-enzymatic [total glutathione (GSHt)] antioxidants.

The information to obtain would invigorate mariculture activity, enabling the improvement of fish farming practices and the commercial use of algae, being of paramount importance within the framework of a “blue growth” development model.

2. Materials and methods

2.1. Chemicals

DNA lesion-specific repair enzymes, namely formamidopyrimidine DNA glycosylase (FPG) and endonuclease III (EndoIII), were purchased from Professor Andrew Collins (University of Oslo, Norway).

Cyclophosphamide and all the other chemicals were obtained from the Sigma-Aldrich Chemical Company (Madrid, Spain).

2.2. Experimental diets

Two diets were prepared (2.0-mm pellet size), composed by the same basic ingredients: a standard diet (S; formulation adjusted according to recommendations for *S. aurata*), a diet enriched with algae

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(A). Algae supplementation concerned a total percentage of 5 %, incorporating three different species (Mix macroalgae) - *Ulva rigida* (green algae; Chlorophyta), *Gracilaria gracilis* (red algae; Rhodophyta) and *Fucus vesiculosus* (brown algae; Ochrophyta) - in an equal percentage (approx. 1.67 % each). The percentage of algae incorporation was based on a previous study carried out in the same fish species (Magnoni et al., 2017). Algae were reared at ALGAplus, Lda. (Ílhavo, Portugal), an integrated multi-trophic aquaculture (IMTA). Diets were produced by SPAROS, Lda. (Faro, Portugal), and the respective formulations are presented in table 1. Fish were hand-fed once a day (10 a.m.), at a daily rate of 3% (as percentage of fish biomass).

2.3. Fish and holding conditions

Gilthead seabream (*Sparus aurata* L.) specimens, with an initial average body weight of 72.95 ± 27.90 g and a total length of 17.69 ± 2.17 cm, were supplied by the semi-intensive fish farm Materaqua, Lda. (Ílhavo, Portugal). Fish condition was assessed through the Fulton's condition factor (K), according to the expression $K = (W \times 100)/L^3$, where W = weight (g) and L = total length (cm), being the initial K of 1.26 ± 0.06.

Prior to the experiment described below, fish were acclimatized to the experimental tanks/conditions, including to the standard diet (S), for one week. Acclimation and the first experimental period of 30 days took place in 8000 L tanks, while 1000 L tanks were adopted in the post-injection periods (Fig. 1). All the tanks were kept under a natural photoperiod, as open systems (each thank was independently supplied by a flow-through seawater system pumping from Aveiro lagoon, corresponding to a water renewal rate of 6 times per day), with the following physico-chemical conditions: salinity 35, temperature 17±1 ˚C, ammonia 0.5±0.4 mg L^{-1}, nitrite 0.05±0.02 mg L^{-1} and dissolved oxygen 12.5±1.4 mg L^{-1}.

Fish well-being and all the procedures were supervised by a certified operator, in accordance with national and international guidelines (Directive 2010/63/EU) to ensure minimal animal use and discomfort.

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2.4. Experimental design and sampling

A lot of 130 fish was divided into two tanks as follows: 55 fish corresponding to the group reared with a standard diet (S); 75 fish corresponding to the group reared with the algae-enriched diet (A) (Fig. 1). Following acclimation, and just before the experiment beginning, 10 fish (randomly collected from both tanks) were sampled and used as the initial reference group (time zero; t_0). As a first step of the experiment, fish were reared for 30 days either with the S or A diet. Then, fish from both experimental groups were intraperitoneally injected with 40 mg kg^{-1} cyclophosphamide (CP; dissolved in saline solution), corresponding to S_{CP} and A_{CP} groups, or with a saline solution (S and A groups), and sampled at days 3 and 10 post-injection (p.i.), keeping the diet unaltered. To calculate the volume to inject (both for CP solution and saline solution), respecting the intended CP dose as a function of body weight (b.w.), fish were weighted, and the volume of an 8 mg/mL CP solution was proportionally defined according the ratio 500 µL/100 g b.w.

Additionally, to evaluate whether the potentially favorable effects of algae remain after the end of supplementation, a subgroup of fish previously fed with algae-enriched diet was submitted to a diet alteration at day 3 p.i., being then fed with the standard diet for 7 days (sampled at day 10 p.i.). This diet reversion was applied to both CP treated (A_{CP}/S) and untreated (A/S) groups.

Fish were not fed on the day before sampling. At each sampling time point, 10 fish were sampled per experimental group (n=10). Immediately after collection, fish were anesthetized with 0.5 mg L^{-1} tricaine methanesulfonate (MS-222) for approximately 15 min (Gilderhus and Marking, 1987). After being weighed (to nearest 0.1 g) and measured (total length; to the nearest 0.1 cm), fish were sacrificed by cervical transection.

Fish blood was drawn from the posterior cardinal vein, using heparinized (27 mg mL^{-1} heparin) Pasteur pipettes, and placed into 2 mL microtubes (two microtubes per fish). Thus, one microtube containing 0.002 mL of blood diluted in 1 mL of chilled PBS (pH=7.4; 0.01M), constituted the cell suspension for comet assay, and the other, with the remaining blood volume, was assigned for antioxidants analysis. Aliquots for comet assay were kept cold up to further procedures, while the aliquots for antioxidants determination were immediately frozen in liquid nitrogen. Additionally, blood smears were immediately prepared for ENA assay.

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2.5. Evaluation of genetic damage

2.5.1. Exogenous genotoxic insult

Cyclophosphamide was the model genotoxicant selected to induce an acute challenge to genome integrity of *S. aurata*. The target for CP action in the cells is predominantly DNA, where cross-linkages occur, leading to DNA strand breaks and, ultimately, to inability to synthesize DNA and inhibition of mitotic division (Mazur and Czyzewska, 1994). This drug is an alkylating agent that causes alkylation of the purine ring and, as a result, there is miscoding and blockade of DNA replication, being thus the mutagenic usually adopted as a positive control in *in vivo* tests of short duration (Ali et al., 2008). It appeared to be a pure clastogen (agent giving rise to or inducing disruption or breakages of chromosomes, leading to sections of the chromosome being deleted, added, or rearranged), also showing a weak aneugenic activity (effect on mitotic spindle apparatus, resulting in the loss or gain of total chromosomes) (Vanparys et al., 1990). The CP dose and exposure duration currently tested were based on previous studies with fish (Cavalcante et al., 2008; Grisolia and Starling, 2001; Pacheco and Santos, 1998).

2.5.2. Comet assay

The assay was conducted according to the technique described by Collins (2004) as adapted by Guilherme et al. (2010), with the proper adjustments to the procedure with an extra step of digesting the nucleoids with endonucleases. All slides were freshly prepared. A system of eight mini-gels per slide was adopted, based on a model created by Shaposhnikov et al. (2010), to increase the assay output. Each individual is represented by 2 replicate mini-gels, being 4 different individuals represented in each slide. Briefly, 0.02 mL of cell suspension were mixed (with 0.07 mL of 1% low melting point agarose, in PBS, and eight drops with 0.007 mL of cell suspension were placed onto the pre-coated slide (with 1% normal melting point agarose) as two rows of 4, without coverslips. The mini-gels were left for ±5 min at 4 ˚C in order to solidify agarose, and then immersed in a lysis solution (2.5 M NaCl, 0.1 M EDTA, 0.01 M Tris, 1% Triton X-100, pH 10) at 4 ˚C, and kept overnight. After lysis of agarose-embedded cells, slides were washed 3 times with buffer (0.1 M KCl, 0.0005 M EDTA, 0.04 M HEPES, 200 mg L^{-1} bovine serum albumin, pH 8) at 4 ˚C and three sets of slides were prepared: two sets were incubated with endonucleases FPG or EndoIII, which convert oxidized purines and pyrimidines into extra DNA single

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strand breaks, respectively (Azqueta et al., 2009), and a third set was incubated only with buffer. Hence, 0.03 mL of each enzyme (diluted in buffer) were applied in each mini-gel, together with a coverslip, prior to incubation at 37 ˚C for 30 min, in a humidified atmosphere. The slides were then placed in the electrophoresis tank, immersed in electrophoresis solution (20 min) for alkaline treatment. DNA was allowed to migrate at a fixed voltage of 25 V, with 1.04 V cm^{-1} and a current of 300 mA (achieved by adjusting the solution volume in the electrophoresis tank), during 15 min. Later, the slides were stained with ethidium bromide (0.035 mL for each 4 mini-gels) and fifty nucleoids were observed per mini-gel, using a Leica DMLS fluorescence microscope (400× magnification). Slides were coded and scored blind. The DNA damage was quantified by visual classification of nucleoids into five comet classes, according to the tail intensity and length, from 0 (no tail) to 4 (almost all DNA in tail) (Collins, 2004).

The total score expressed as a genetic damage indicator (GDI) was calculated multiplying the percentage of nucleoids in each class by the corresponding factor, according to the following formula:

GDI = [(% nucleoids class 0) × 0] + [(% nucleoids class 1) × 1] + [(% nucleoids class 2) × 2] + [(% nucleoids class 3) × 3] + [(% nucleoids class 4) × 4]

GDI values were expressed as arbitrary units in a scale of 0 to 400 per 100 scored nucleoids (as average value for the 2 gels observed per fish). When the comet assay was performed with additional FPG and EndoIII steps, GDI values were calculated in the same way but the parameter designated GDI_{FPG} and $GDI_{EndoIII}$, respectively. Additional DNA breaks corresponding to net enzyme-sensitive sites alone (NSS_{FPG} or $NSS_{EndoIII}$) were also expressed.

2.5.3. ENA assay

The assay was carried out in mature peripheral erythrocytes, according to the procedure of Pacheco and Santos (1996). Briefly, one blood smear per animal was fixed with methanol during 10 min and stained with Giemsa (5%) during 30 min. Slides were coded and scored blind. From each smear, 1000 erythrocytes were scored, under 1000x magnification (microscope Olympus BX50), to evaluate the relative frequency of the following nuclear lesions: kidney shaped nuclei (K), lobed nuclei (L), segmented nuclei (S), vacuolated nuclei (V) and micronuclei (MN). Results were expressed as the sum of frequencies for all the categories observed (K + L + S + V + MN).

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2.6. Evaluation of antioxidant system status

2.6.1. Tissue preparation and fractionation

Whole blood samples (stored at -80 ˚C) were lysed through homogenization in a 1:6 ratio (blood volume:buffer volume), using a Potter-Elvehjem homogenizer, in chilled phosphate buffer (0.1 M; pH 7.4). This lysate was then centrifuged (Eppendorf 5415R centrifuge) at 12 000 rpm for 20 min, at 4 °C, to obtain PMS (post-mitochondrial supernatant) fraction. All the aliquots were stored in microtubes at -80 ˚C until analyses.

2.6.2. Measurement of enzymatic and non-enzymatic antioxidants

All measurements were carried out in a SpectraMax 190 microplate reader, at 25 °C.

CAT activity was assayed in PMS by the method of Claiborne (1985), with slight modifications. Briefly, the assay mixture consisted of 0.190 mL phosphate buffer (0.05 M, pH 7.0) with hydrogen peroxide (H_2O_2; 0.010 M) and 0.010 mL of PMS, in a final volume of 0.2 mL. Change in absorbance was measured in appropriated UV-transparent microplates (UV-Star® flat-bottom microplates, Greiner Bio-One GmbH, Germany), recorded at 240 nm and CAT activity was calculated in terms of μmol H_2O_2 consumed min^{-1} mg^{-1} protein using a molar extinction coefficient (ε) of 43.5 M^{-1} cm^{-1}.

SOD activity was assayed in PMS with a Ransod kit (Randox Laboratories Ltd., UK). The method employs xanthine and xanthine oxidase to generate superoxide radicals, which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5- phenyltetrazolium chloride (INT) to form a red formazan dye determined at 505 nm. SOD activity is then measured by the degree of inhibition of this reaction, considering that one unit of SOD causes a 50% inhibition of the rate of reduction of INT, under the conditions of the assay. Results were expressed as SOD units mg $protein^{-1}$.

GPx activity was determined in PMS according to the method of Mohandas et al. (1984) and modified by Athar and Iqbal (1998). The assay mixture consisted of 0.09 mL phosphate buffer (0.05 M, pH 7.0), 0.03 mL ethylenediaminetetraacetic acid (EDTA; 0.010 M), 0.03 mL sodium azide (0.010 M), 0.03 mL glutathione reductase (GR; 2.4 U mL^{-1}), 0.03 mL reduced glutathione (GSH; 0.010 M), 0.03 mL nicotinamide adenine dinucleotide phosphate-oxidase (NADPH; 0.0015 M), 0.03 mL H_2O_2 (0.0025 M) and 0.03 mL of PMS in a total volume of 0.3 mL. Oxidation of NADPH to $NADP^+$ was recorded at 340 nm

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and GPx activity was calculated in terms of nmol NADPH oxidized min^{-1} mg $protein^{-1}$ ($\varepsilon = 6.22 \times 10^3$ M^{-1} cm^{-1}).

GR activity was assayed in PMS by the method of Cribb et al. (1989), with some modifications. The assay determines indirectly the GR activity by measuring the NADPH disappearance associated with a reduction of oxidized glutathione (GSSG) catalyzed by GR. Briefly, the assay mixture contained 0.050 mL of PMS fraction and 0.250 mL of reaction medium consisted of phosphate buffer (0.05 M, pH 7.0), NADPH (0.0002 M), glutathione disulfide (GSSG; 0.001 M) and diethylenetriaminepentaacetic acid (DTPA; 0.0005 M). The enzyme activity was determined by measuring the oxidation of NADPH at 340 nm and calculated as nmol NADPH oxidized min^{-1} mg $protein^{-1}$ ($\varepsilon = 6.22 \times 10^3$ M^{-1} cm^{-1}).

GST activity was determined in PMS with CDNB (1-chloro-2,4- dinitrobenzene) as a substrate, according to the method of Habig et al. (1974). The assay mixture consisted in 0.1 mL of PMS and 0.17 mL of phosphate buffer (0.2 M, pH 7.9) and GSH (0.0018 M). The reaction was initiated by addition of 0.03 mL of CDNB (0.01 M), and the increase in absorbance was recorded at 340 nm. The enzyme activity was calculated as nmol CDNB conjugate formed min^{-1} mg^{-1} protein ($\varepsilon = 9.6$ mM^{-1} cm^{-1}).

For GSHt content determination, PMS was precipitated with trichloroacetic acid (TCA 12%) for 1 h and then centrifuged at 12 000 g for 5 min at 4 °C. GSHt was determined (in deproteinated PMS) adopting the enzymatic recycling method using GR excess, whereby the sulfhydryl group of GSH reacts with 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB; Ellman's reagent) and produces a yellow colored 5-thio-2-nitrobenzoic acid (TNB) (Baker et al., 1990; Tietze, 1969). The rate of TNB production is directly proportional to this recycling reaction, which is in turn directly proportional to the GSH concentration in the sample. The assay mixture consisted in 0.2 mL sodium phosphate buffer (0.143 M, pH 8), EDTA (0.0063 M), DTNB (0.001 M) and NADPH (0.00034 M), added to 0.04 mL of deproteinated PMS. The reaction was initiated with 0.04 mL of GR (8.5 U mL^{-1}). Formation of TNB was measured at 415 nm. It should be noted that GSSG is converted to GSH by GR in this system, which consequently measures total GSH. The results were expressed as nmol TNB formed min^{-1} mg $protein^{-1}$ ($\varepsilon = 14.1 \times 10^3$ M^{-1} cm^{-1}).

Total protein content was determined according to the Biuret method (Gornall et al., 1949), using bovine serum albumin (E. Merck-Darmstadt, Germany) as a standard.

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2.7. Statistical analysis

Statistica 8.0 software was used for statistical analysis. All data were first tested for normality and homogeneity of variance to meet statistical demands.

For the pre-injection sampling moment, one-way Analyses of Variance (ANOVA) was applied. The remaining data were analyzed through a two-way ANOVA, followed by a post hoc Tukey test, in order to compare the different treatment groups between each other. Temporal variations were tested using the independent samples t-test by groups. In all the analyses, differences between means were considered significant when $p < 0.05$ (Zar, 1996).

3. Results

No fish mortality was observed during the experiment. Though feeding was not strictly monitored, no relevant alterations on fish feeding response were perceptible in dependence to the diet profile, since fish were very active and come to feed immediately in both diets; however, a visual evaluation of feeding response, mainly during the first 30-day period, suggested that fish fed with algae-enriched diet displayed a slightly higher appetite. At the end of this period, gilthead seabream specimens reached, respectively for S and A groups, an average body weight of 116.92 ± 27.59 g and 131.58 ± 26.47 g, a total length of 19.12 ± 1.93 cm and 20.53 ± 1.25 cm, with values of 1.67 ± 0.20 and 1.50 ± 0.08, showing no significant differences.

3.1. DNA damage as comet assay

Concerning the first thirty days, when fish were fed with the different diets (S *vs*. A), without any genotoxic challenge, no statistically significant differences were found on GDI values (Figure 2). Three days after injection, both groups treated with cyclophosphamide (S_{CP} and A_{CP}) revealed a significantly higher DNA damage, measured as GDI, in comparison with the corresponding untreated diet-group. No significant differences were observed between both CP treated groups at this moment. In relation to the last sampling moment (ten days after injection), a similar pattern was revealed when CP treated and untreated groups were compared, depicting significant GDI increases. However, CP treated groups

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previously fed with algae-enriched diet (A_{CP} and A_{CP}/S) displayed GDI values significantly lower than the CP treated group feed with standard diet (S_{CP}). Paralleling the samplings after injection, all groups, with the exception of S_{CP}, showed a significant decrease in GDI values over time.

Taking into account the comet assay improved with the extra-step, involving the DNA lesion-specific endonucleases EndoIII and FPG, the same pattern as previously described for GDI was observed for both $GDI_{EndoIII}$ and GDI_{FPG} parameters (Figs. 3A and 4A), with the exception of S group at 10 days p.i. that was no longer significantly lower than the corresponding group in the previous sampling moment.

Bearing in mind specifically the DNA breaks corresponding to net endonuclease-sensitive sites (Fig. 3B), differences were only observed ten days after injection, when groups S_{CP} and A_{CP}/S were significantly lower than the corresponding untreated diet-groups. In addition, at that time, all groups (except S_{CP}) were significantly different from the previous sampling moment, being notorious a time-related increase in untreated groups (S, A and A/S) while the opposite variation was observed in CP treated groups (A_{CP} and A_{CP}/S).

Taking into account the NSS_{FPG} values (Fig. 4B), at three days after injection it was observed a significant increase in S_{CP} when compared to S group. In what concerns ten days after injection, it was observed the same pattern as described for $NSS_{EndoIII}$ parameter, with the exception that A group was no longer different over time.

3.2. Chromosomal damage as ENA frequency

The first thirty days of dietary trial showed no significant alterations when total ENA frequency was considered (Fig. 5). However, similarly to comet assay results, three days after injection, both groups submitted to cyclophosphamide injection presented a significant ENA increase in comparison with the corresponding untreated groups. No significant differences were observed between both CP treated groups at this moment.

In what concerns to ten days p.i., groups previously injected with CP had a significantly higher total ENA frequency (when compared to the respective untreated groups), with the exception of A_{CP} group. Furthermore, groups previously injected with CP and fed with algae-enriched diet, with (A_{CP}/S) or without (A_{CP}) feed reversion, showed a significantly lower ENA frequency than the CP treated group fed

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with standard diet (S_{CP}). Paralleling the samplings after injection, S_{CP} and A_{CP} showed significant differences, translated in time-related increase and decrease, respectively.

3.3. Antioxidant responses

Concerning the evaluation of *S. aurata* antioxidant system (Figs. 6 and 7), significant alterations were observed in CAT and GST activities as well as GSHt content.

Taking into account CAT activity, ten days after injection, A group was significantly higher than S and A/S groups, and no temporal variations were observed. Bearing in mind GST activity, ten days after injection, S_{CP} and A_{CP}/S groups were significantly higher than non-injected groups; however, the most interesting result is the comparison between CP treated groups, i.e. A_{CP} group was significantly lower than groups fed with standard feed (S_{CP}) or submitted to feed reversion (A_{CP}/S).

Temporal variations of GST activity were observed at three days after injection in A group, showing a reduction in relation to the precedent sampling moment, as well as in all groups at ten days p.i., which were also significantly lower than the corresponding groups at 3 days p.i., with the exception of A_{CP}/S group.

Considering GSHt content, at three days p.i., group A was significantly lower than S, having these two groups also decreased comparing to the previous sampling. In relation to ten days p.i., A_{CP}/S was significantly higher than the corresponding untreated group, while S and A/S groups displayed a time-related decrease.

4. Discussion

The genome integrity of fish raised in aquaculture systems can be affected through several pathways, associated either to endogenous (*e.g*. ROS produced under conditions of stress and accelerated growth) or exogenous (*e.g*. waterborne contaminants, therapeutants, disinfectants and anaesthetics) insults (Alonso-Alvarez et al., 2007; Rodrigues et al., 2017; Silva et al., 2011). In both cases, the structural integrity of the DNA molecule may be protected by some mechanisms among which stands out the antioxidant systems. Notwithstanding the previous statements, the assessment of the

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balance between pro-genotoxic and anti-genotoxic processes in farmed fish has been disregarded in aquaculture research, overlooking the medium to long-term consequences for the organism health and the potential (direct or indirect) impact on productivity.

Despite the recent developments in fish nutrition towards a functional and environmentally oriented aquafeeds (*e.g*. Li et al. 2009) and the assumption that a nutritional-genetic combined approach has the potential to provide critical knowledge, this area of research has been mainly focused on the impact on the physiology of muscle growth (*e.g*. Kwasek et al. 2012). To the best of the authors' knowledge, no studies have been done on the diet manipulation towards an improved capacity of farmed fish to protect their DNA.

Since the comet and the ENA assays allow the detection of different types of genetic damage, both can provide independent and significant data and should be adopted as complementary genotoxic endpoints (Wirzinger et al., 2007). Therefore, the following discussion will focus first on the macroalgae protection in relation to each type of genetic damage separately, *viz*. primary DNA damage (measured as comet assay) or potentially more persistent alterations (chromosomal damage measured as ENA assay), and then on the interplay mechanisms with antioxidants system.

The current determination of biometric parameters intended, primarily, to discard the possibility that macroalgae may contain anti-nutritional factors, rather than to evaluate growth (experiment was not designed to fulfill the requirements of a growth trial, namely because fish were not fed *ad libitum*). Indeed, the condition factor (K) did not show significant differences between diets (the only significant changes in K are attributable to CP). This result is in accordance with previous findings (*e.g*. Wassef et al. 2005) indicating that feeding *S. aurata* with 10% of *Pterocladia* or 5% of *Ulva* meal didn't affect negatively the growth and even promoted feed utilization, nutrient retention, and survival. In the same direction, Batista (2008) and Magnoni et al. (2017) demonstrated that the incorporation of *Ulva* or *Gracilaria* species in the diet of *S. aurata* did not significantly affect the growth.

4.1. Protective effects of macroalgae against primary DNA damage

Analyzing the present results after thirty days of fish rearing with the two tested diets, it was perceptible that algae supplementation didn't improve the basal DNA integrity, as depicted by the parameters signaling both non-specific and oxidative damage. This was not a surprising result as the

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main hypothesis of the present study concerned the possibility of algae play a protective action in the presence of an exogenous genotoxic insult. Accordingly, the confirmation that the challenging agent – cyclophosphamide – increased DNA strand breaks appears as a precondition to analyze the potential protective effects of the macroalgae-enriched diet. Hence, at 3 days' p.i., CP induced DNA damage in blood cells of *S. aurata*, in both diet-groups, measured as GDI as well as both $GDI_{EndoIII}$ and GDI_{FPG}. This is in agreement with previous studies describing CP genotoxic potential to fish (*Prochilodus lineatus*), measured through comet assay (Cavalcante et al., 2008; Monteiro et al., 2011). In addition, when DNA strand breaks resulting specifically from FPG activity (NSS_{FPG}) are under analysis, a CP capacity to affect purine rings was perceptible (in S_{CP} group at 3 days' p.i.), as previously stated by Ali and Seehy (2008).

Considering 3 days' post-injection data, and in what concerns the overall DNA damage (either non-specific - GDI, or updated with oxidative lesions - $GDI_{EndoIII}$ and GDI_{FPG}), the algae-enriched diet didn´t display a protective action against CP genotoxic insult. Differently, when considering specifically the indicator of purine oxidation (NSS_{FPG}), it was clear that this DNA damaging process was prevented by the algae supplementation. This result is a clear evidence of an improved antioxidant defense promoted by algae supplementation. This is in agreement with previous studies reporting high antioxidant activity in *Fucus* sp., *Ulva* sp. and *Gracilaria* sp. (Queiroz et al. 2014; Magnoni et al. 2017).

With the passage of time after injection, it was notorious that genotoxicity still occurs (at 10 days p.i.) in all groups injected with CP. Nevertheless, a positive interference of algae supplementation was perceptible since groups with an algae-enriched feed history (A_{CP} and A_{CP}/S) showed an improvement on their condition, translated in a lower extent of DNA damage, when compared to S_{CP} group. This beneficial effect is strengthened by the analysis of the temporal evolution of DNA damage from 3 to 10 days' p.i., namely as $GDI_{EndoIII}$ and GDI_{FPG}, as a time related decline was observed in fish groups fed with the algae-enriched diet, though extensible to both CP treated and untreated groups. This particular result suggests an additional protection of algae in relation to the basal levels of DNA damage (*i.e.*, in the absence of an identified exogenous insult).

It was evident that the anti-genotoxic action of algae supplementation gained preponderance in the course of p.i. time, which can be explained by fish physiological adjustments and/or by a decline of CP internal levels of exposure. This can be regarded as an indication of a higher efficacy of algae components to cope with a moderate genotoxic challenge (rather than an extreme insult like that

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posed by 40 mg kg^{-1} at 3 days' p.i.), which is more in line with the extent of the genotoxic pressures likely to occur in rearing environment.

Analyzing the DNA breaks at EndoIII- and FPG-sensitive sites ($NSS_{EndoIII}$ and NSS_{FPG}, respectively), at 10 days' p.i., a surprising response profile was noticeable, since in both parameters lower levels of DNA oxidatively damaged were detected in S_{CP} and A_{CP}/S groups (comparing to the respective untreated group). Though this is not a classical pattern of response in genotoxicology, it has been often reported in the literature (*e.g*. Marques et al. 2014b). According to Marques et al. (2014b), it is plausible that under a low/moderate attack by genotoxicants, fish can trigger compensatory mechanisms able to prevent the DNA damaging effect of an external threat as well as of that caused by endogenous factors. Hence, two mechanisms can be hypothesized (alternatively or in combination): enhancement of oxidative DNA damage repair and mobilization of the antioxidant system as a response to ROS over-generation, whose efficacy can be able to bring down the DNA oxidative damage below the control levels. Interestingly, the phenomenon above described was not so distinguishable in the fish group continuously fed with the algae-enriched diet (A_{CP}), suggesting that an additional protection provided by algae made unnecessary the fish adjustment hypothesized for the other CP treated groups. The increase of DNA breaks corresponding to net EndoIII- and FPG-sensitive sites observed from 3 to 10 days' p.i. in unchallenged fish seems to be more pronounced in the group fed with the standard diet, which, in parallel with the opposite time-related variation displayed by CP challenged groups, seems to corroborate the previous hypothesis.

In an attempt to evaluate whether the potentially favorable effects of algae persist beyond the end of supplementation, fish fed with algae-enriched diet (for 30 + 3 days) were also submitted to a diet reversion to the standard feed. Thus, the analysis of these particular results as overall DNA damage (non-specific and updated with oxidative lesions) revealed no repercussions of the feeding alteration, pointing out a persistence of algae components in fish body, or subsequent favorable biochemical milieu, for at least one week. Nevertheless, data concerning DNA breaks at EndoIII- and FPG-sensitive sites denunciated a dissimilar response profile when comparing groups always fed with algae-enriched diet and groups in which the standard diet was reset in the last 7 days. This can be an indication that algal components with oxidative DNA damage-protecting capacity substantially declined in a 7-day period without uptake. However, this temporal kinetics profile was not extensible to all the algal components involved in the anti-genotoxic action.

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4.2. Protective effects of macroalgae against chromosomal damage

The present results as ENA frequency, after the first step of 30 days rearing and 3 days' p.i., followed a pattern similar to that described for comet assay. Thus, after 30 days, the fish baseline condition was not affected by the diet, which was expectable, once the ENA baseline frequencies were low, comparing for instance with Teles et al. (2005) study, limiting the possibility to operate a reduction. Concerning 3 days' p.i., it was evident the genotoxic capacity of CP, since it was observed a significant increase of ENA~~s~~ frequency in both injected groups (S_{CP} and A_{CP}), regardless the diet. This finding is in agreement with Monteiro et al. (2011), who also detected a CP genotoxic effect, measured through ENA assay, in *Prochilodus lineatus*.

Taking into account 10 days' p.i., some noteworthy differences were perceptible in relation to the response profile described for comet assay. It was particularly relevant the observation that ENA induction by CP increased from 3 to 10 days' p.i. in the group fed with standard diet (S_{CP}), pointing out a progression of the genotoxic effect, which didn't occur in the groups with algae-enriched feed history (A_{CP} and A_{CP}/S). Moreover, in A_{CP} group the ENA frequency returned to the levels corresponding to the untreated group, highlighting an effectiveness of algae supplementation on providing protection against the genotoxic pressure experimentally imposed. According to Marques et al. (2014a), and assuming morphologic nuclear anomalies as hardly reparable lesions, the recovery detected in A_{CP} group relies on the removal of erythrocytes containing abnormal nuclei and/or a dilution effect resulting from erythropoiesis (releasing new normal cells into circulation). These mechanisms were probably coupled with a strong reduction of new erythrocytes carrying abnormal nucleus.

An intermediate condition in terms of anti-genotoxic defense was perceived from the comparative analysis of the A_{CP}/S group with the other CP treated groups, since the chromosomal damage was still detectable, but a significant improvement was achieved (compared to S_{CP} group). Besides reinforcing the protective action of algae supplementation, this analysis also indicates a clear decline of that protection as a consequence of the supplementation suppression.

Unlike the comet assay data, it may be noted that, by the end of the experiment, the fish basal condition (in the absence of an exogenous insult), measured as ENA frequency, was maintained overtime.

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4.3. DNA and chromosomal damage *vs*. antioxidants system status

Considering that CP-induced DNA oxidative damage was signalized by the enzyme-modified comet assay, namely as purine oxidation at 3 days' p.i., it would be expectable a correspondent reaction of the antioxidant system. However, the blood antioxidants assessed were (almost) completely irresponsive to CP exposure at that time. These results seem to support the idea that under the tested conditions (species/concentration), the threshold limit to trigger the antioxidant machinery was not reached in blood. Nevertheless, this seems to be in accordance with Ahmad et al. (2006), who stated that the oxidative damage cannot be predicted only based on antioxidant variations.

The predominantly unaltered levels of enzymatic antioxidant activities following algae supplementation is in line with previous studies involving the same algae species as dietary supplement in *S. aurata* (Augusto et al., 2014) and *D. labrax* (Peixoto et al., 2016a).

Regarding CAT activity, the increased values found in fish fed all the time with algae-enriched diet is corroborated by Raji et al. (2018), that explored fishmeal replacement with two freshwater microalgae in African catfish (*Clarias gariepinus*) diet, and observed that diet with 75% replacement with *Chlorella vulgaris* showed significantly higher CAT activities. In the same way, and using one of the species from our study, Hoseinifar et al. (2018) tested the effects of 8 weeks *Danio rerio* fed on different concentration of *Gracilaria gracilis* powder (0.25 - 1%) on CAT gene expression, reporting significantly higher values in dietary supplemented groups. These authors suggested that CAT elevated gene expression reflects a positive effect of *Gracilaria* powder on zebrafish antioxidant defense, being the presence of antioxidant compounds also established in *G. gracilis* by Francavilla et al. (2013).

GSH is an important antioxidant involved in numerous cellular activities, including detoxification, antioxidant defense and maintenance of cellular redox status (Basu et al., 2015). Identically to enzymatic antioxidants, the studies above cited (Augusto et al., 2014; Peixoto et al., 2016a) reported unaltered GSHt content in association with algae supplementation. Moreover, at 3 days' p.i., fish reared with algae-enriched diet displayed lower levels of GSHt, comparing with the standard diet and irrespectively of CP treatment. A possible explanation for this alteration concerns a reduction of GSH synthesis by fish, as a counterbalance reaction to the exogenous source of other non-enzymatic antioxidants provided by algae supplementation, namely vitamins. Nevertheless, this hypothesis was

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not confirmed at 10 days' p.i., since a significant increase of GSHt content was observed in A_{CP}/S group comparing to A/S.

Concerning the enzymatic activities modulation at 10 days' p.i., it is noteworthy that only GST revealed significant alterations. GSTs are evolutionarily conserved enzymes, important in the detoxification of numerous xenobiotic compounds. These enzymes catalyze the conjugation of GSH to electrophilic substrates, thus producing compounds that are generally less reactive and more soluble. This facilitates the removal of these compounds from the cell via membrane-based GSH conjugate pumps. The broad substrate specificity of GSTs allows them to protect cells against a wide range of toxic chemicals (Gumulec et al., 2013). Apart from their essential functions in intracellular transport and the biosynthesis, GSTs have a critical role in defense against oxidative damage and peroxidative products of DNA and lipids (George, 1994). Hence, a marked GST induction was currently observed in response to CP treatment (at 10 days' p.i.), but only perceptible in S_{CP} and A_{CP}/S groups. This is a clear evidence of lower oxidative pressure occurring in the fish group continuously fed with algae-enriched diet. The supplementation suspension for 7 days showed again to have a negative impact on the fish condition, here expressed as a need to keep GST activity upregulated. This GST response pattern fits well on the genotoxicity endpoints profile, being particularly in line with the DNA oxidative damage ($NSS_{EndoIII}$ and NSS_{FPG}). Therefore, lower levels of DNA bases oxidation coincided with higher levels of GST activity, as an expression of defense processes mobilization (augmented biotransformation/detoxification activity and/or increased antioxidant protection), also corroborating the hypothesis raised in point 4.1.

Considering that the present study was undertaken as a first approach regarding this subject, a comprehensive screening of the bioactive compounds that might be the source of the antigenotoxic action should be carried out in the future. Nevertheless, it is considered as well that, since the ingestion of the whole macroalgae is in equation, the identification of the biologically active ingredient(s) causally linked to the genome integrity maintenance, besides being a herculean task (owing to the complex net of interactions likely to occur), loses some relevance. Anyhow, based on previous phytochemical characterizations, a battery of compounds can be hypothesized, probably involving a synergistic action. For instance, it should be considered vitamins A and E (Paiva et al. 2014), polyphenols, sulfated polysaccharides and chlorophyll-related compounds as components of *Fucus* sp. (Marques et al., 2018), carotenoids (Pinteus et al., 2017), vitamins A, C and E, chlorophylls a and b and phenolic compounds as

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constituents of *Ulva* sp. (Yildiz et al., 2012), and sulfated polysaccharides (Imjongjairak et al., 2016), high phenolic content (Yangthong et al., 2009) and vitamin C (Norziah and Ching 2000) as components of *Gracilaria* sp.. Given the nature of these compounds, a strengthening of the antioxidant shielding emerges as the most probable genoprotective mechanism associated to macroalgae effects (although not finding a strong support in the present antioxidant data).

Summarizing and providing an answer to the central goal of the present research, it was demonstrated that a macroalgae-enriched diet supplemented with *Ulva rigida* (Chlorophyta), *Gracilaria gracilis* (Rhodophyta) and *Fucus vesiculosus* (Ochrophyta), in a total percentage of 5 %, exhibits genoprotective properties in gilthead seabream (*Sparus aurata*) blood cells. This beneficial action of macroalgae was apparent in relation to a primary and reparable damage (DNA strand breaks) and to a potentially more permanent damage (chromosomal lesions), though it appeared more pronounced in the latter type of genotoxicity expression. Unsurprisingly, benefits were mostly expressed in relation to an exogenous genotoxic challenge (cyclophosphamide - CP), rather than in relation to a basal genome integrity. A clear oxidative DNA damage-protecting activity was displayed, particularly in the presence of a strong genotoxic insult occurring 3 days after CP injection, when purine oxidation was prevented by algae supplementation. Nonetheless, blood antioxidants were not altered by the supplemented diet, with the exception of GST activity that was induced as response to CP treatment, but only in S_{CP} and A_{CP}/S groups. This was a clear evidence of a lower oxidative pressure occurring in the fish group continuously fed with algae-enriched diet. Clarifying if the favorable effects of algae persist beyond the end of supplementation, it was demonstrated that 7 days without uptake was enough to partially reduce the protection efficacy, namely in what concerns the algal components with oxidative DNA damage–protecting capacity.

Overall, these results seem to be promising towards the benefits of macroalgae inclusion in fish diet, offering a potential strategy to strengthen fish fitness, and thus, to invigorate aquaculture activity (both algae and fish cultivation), also providing new insights on the mechanisms of DNA protection in fish.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Figure 1: Schematic overview of the experimental design. Each horizontal line represents an experimental group: grey lines correspond to fish groups fed with the standard diet (S), while green ones represent fish groups fed with the algae-enriched diet (A). Fish were reared for 30 days with the two different diets. At that time, fish were intraperitoneally injected with cyclophosphamide (CP; as subscript in groups' abbreviation) or with a saline solution (no subscript text in groups' abbreviation). Then, fish were sampled at 3 and 10 days' post-injection (p.i.), keeping the diet unaltered. In addition, fish fed with algae-enriched diet, were submitted to a diet alteration at 3 days p.i., being then fed with the standard diet for 7 days (sampled at 10 days p.i.). This diet reversion was applied to both CP injected and not injected fish, giving rise to the groups A_{CP}/S and A/S, respectively. Vertical lines correspond to sampling moments. t_0 corresponds to sampling just before the experiment beginning.

Figure 2: Mean values of genetic damage indicator (GDI), expressed as arbitrary units, measured by comet assay in blood cells of *S. aurata* at the different sampling moments (t_0 corresponds to sampling just before the experiment beginning). Bars represent the standard error. Statistically significant differences ($p < 0.05$) are: (*) between groups within the same sampling moment; (a) between the corresponding groups at 3 and 10 days' post-injection (p.i.). S = standard feed (grey columns); A = algae-enriched feed (green columns); A/S corresponds to a diet reversion from algae-enriched to standard feed (half green-grey columns); CP = cyclophosphamide.

Figure 3: Mean values of DNA damage, measured by the comet assay in blood cells of *S. aurata* at the different sampling moments (t_0 corresponds to sampling just before the experiment beginning). Values resulted from the assay with an extra step of digestion with endonuclease III (EndoIII) to detect oxidized pyrimidine bases: **(A)** overall damage ($GDI_{EndoIII}$) and partial scores, i.e. genetic damage indicator (GDI) after the standard comet assay and additional DNA breaks corresponding to net EndoIII-sensitive sites ($NSS_{EndoIII}$; dark grey/green); **(B)** $NSS_{EndoIII}$ alone. Bars represent the standard error. Statistically significant differences ($p < 0.05$) are: (*) between groups within the same sampling moment; (a) between the corresponding groups at 3 and 10 days' post-injection (p.i.). S = standard feed (grey columns); A = algae-enriched feed (green columns); A/S corresponds to a diet reversion from algae-enriched to standard feed (half green-grey columns); CP = cyclophosphamide.

Figure 4: Mean values of DNA damage, measured by the comet assay in blood cells of *S. aurata* at the different sampling moments (t_0 corresponds to sampling just before the experiment beginning). Values resulted from the assay with an extra step of digestion with formamidopyrimidine DNA glycosylase (FPG) to detect oxidized purine bases: **(A)** overall damage (GDI_{FPG}) and partial scores, i.e. genetic damage indicator (GDI) after the standard comet assay and additional DNA breaks corresponding to net FPG-sensitive sites (NSS_{FPG}; dark grey/green); **(B)** NSS_{FPG} alone. Bars represent the standard error. Statistically significant differences ($p < 0.05$) are: (*) between groups within the same sampling moment; between the corresponding groups at 3 and 10 days' post-injection (p.i.). S = standard feed (grey columns); A = algae-enriched feed (green columns); A/S corresponds to a diet reversion from algae-enriched to standard feed (half green-grey columns); CP = cyclophosphamide.

Figure 5: Mean values of erythrocytic nuclear abnormalities (ENA) frequency (‰) in peripheral erythrocytes of *S. aurata* at the different sampling moments (t_0 corresponds to sampling just before the experiment beginning).

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Bars represent the standard error. Statistically significant differences ($p < 0.05$) are: (*) between groups within the same sampling moment; between the corresponding groups at 3 and 10 days' post-injection (p.i.). S = standard feed (grey columns); A = algae-enriched feed (green columns); A/S corresponds to a diet reversion from algae-enriched to standard feed (half green-grey columns); CP = cyclophosphamide.

Figure 6: Mean **(A)** superoxide dismutase (SOD) and **(B)** catalase (CAT) activity in peripheral blood of *S. aurata* at the different sampling moments (t_0 corresponds to sampling just before the experiment beginning). Bars represent the standard error. S = standard feed (grey columns); A = algae-enriched feed (green columns); A/S corresponds to a diet reversion from algae-enriched to standard feed (half green-grey columns); CP = cyclophosphamide; p.i. = post-injection.

Figure 7: Glutathione related parameters in peripheral blood of *S. aurata* at the different sampling moments (t_0 corresponds to sampling just before the experiment beginning): mean values of **(A)** glutathione-S-transferase (GST), **(B)** glutathione reductase (GR) and **(C)** glutathione peroxidase (GPx) activities, as well as **(D)** total glutathione (GSHt) content. Bars represent the standard error. Statistically significant differences ($p < 0.05$) are: (*) between groups within the same sampling moment; (a) concerning the preceding sampling moment and the corresponding group. S = standard feed (grey columns); A = algae-enriched feed (green columns); A/S corresponds to a diet reversion from algae-enriched to standard feed (half green-grey columns); CP = cyclophosphamide; p.i. = post-injection.

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Table 1: Formulation and chemical composition of the experimental diets.

	Standard diet	Algae-enriched diet
Ingredients (%DM)		
Fishmeal LT 70	27	26.5
Fishmeal 60	20	20
CPSP 90	3	3
Blood meal	5	5
Soy protein concentrate	8	8
Wheat gluten	10	10
Wheat meal	7.5	3
Pea starch	5.1	5.1
Fish oil	13	13
Vit & Min Premix	1	1
Binder	0.4	0.4
Mix macroalgae	0	5
As fed basis		
Crude protein (%DM)	50.3	50.3
Crude fat (%DM)	17.7	17.6
Fiber (%DM)	0.6	0.4
Starch (%DM)	7.8	5.1
Ash (%DM)	12.1	12
Total P (%DM)	1.4	1.4
Gross energy (kJ g^{-1} DM)	20.8	20.8

DM = dry matter

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Highlights

- Macroalgae-enriched diet exhibits genoprotective properties in gilthead seabream
- Reduction of oxidative DNA damage owing to an exogenous insult was achieved
- Seven days without algae uptake was enough to partially weaken the protection
- Promising results to invigorate aquaculture activity (both algae and fish cultivation)

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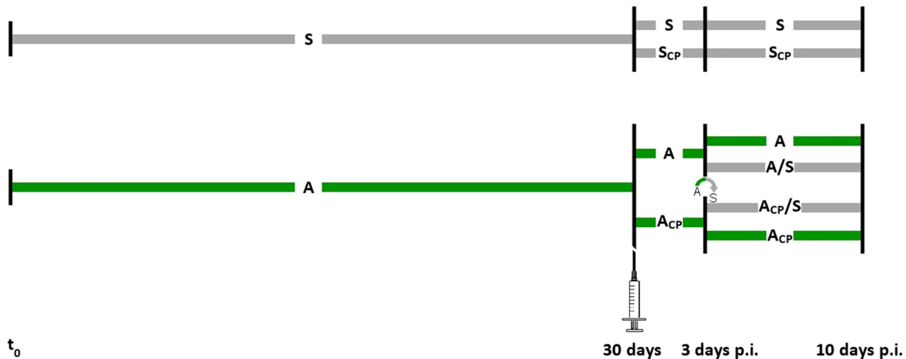

Figure 1

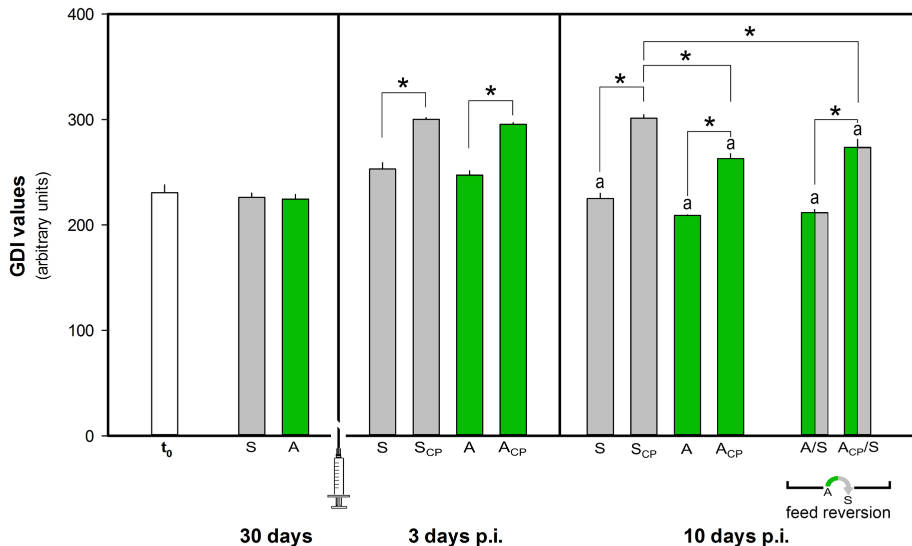

Figure 2

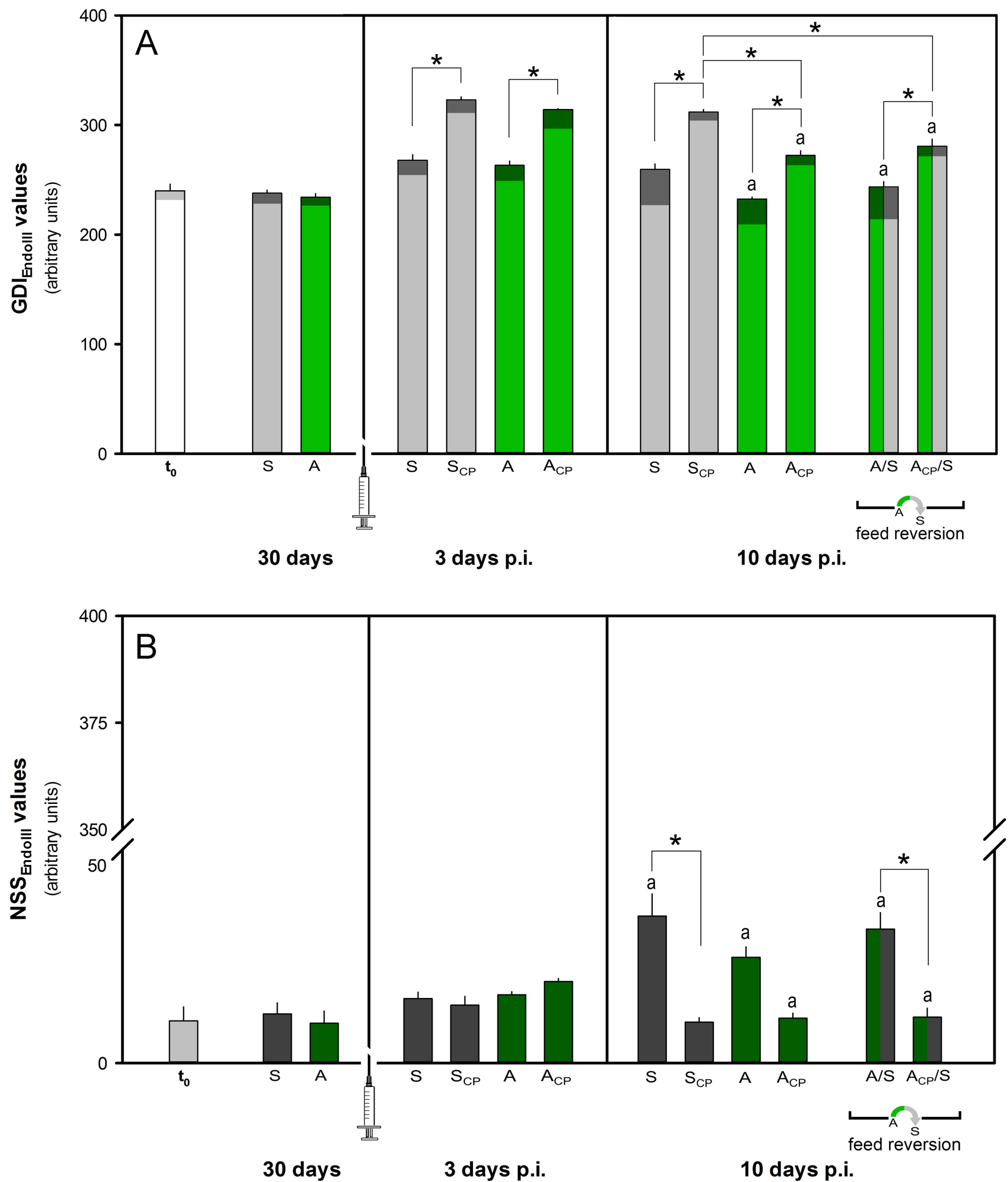

Figure 3

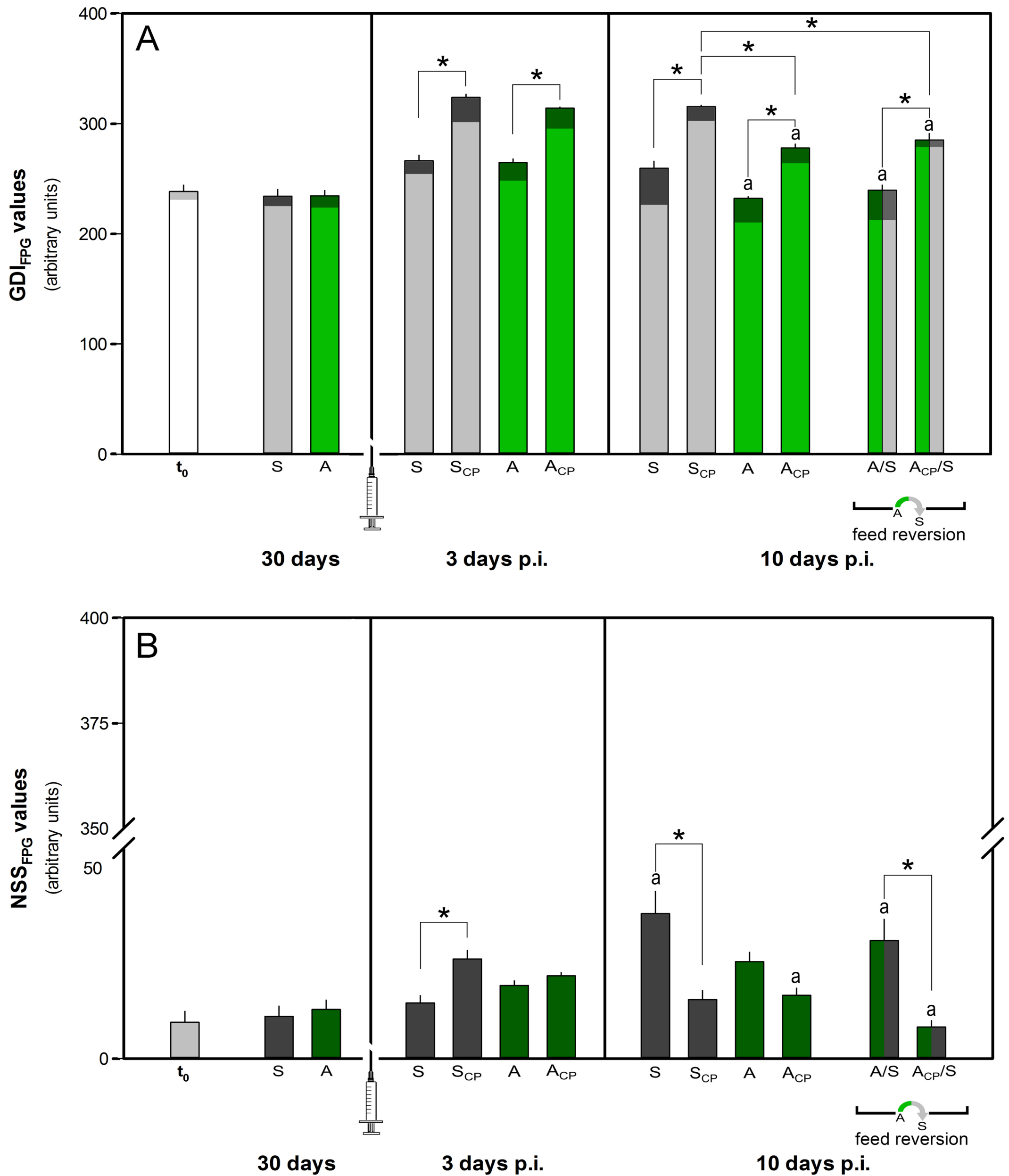

Figure 4

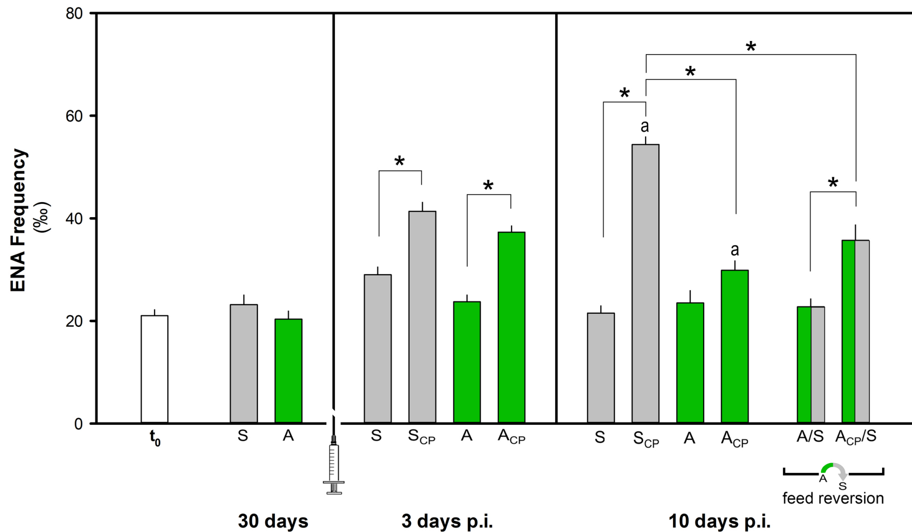

Figure 5

Figure 6

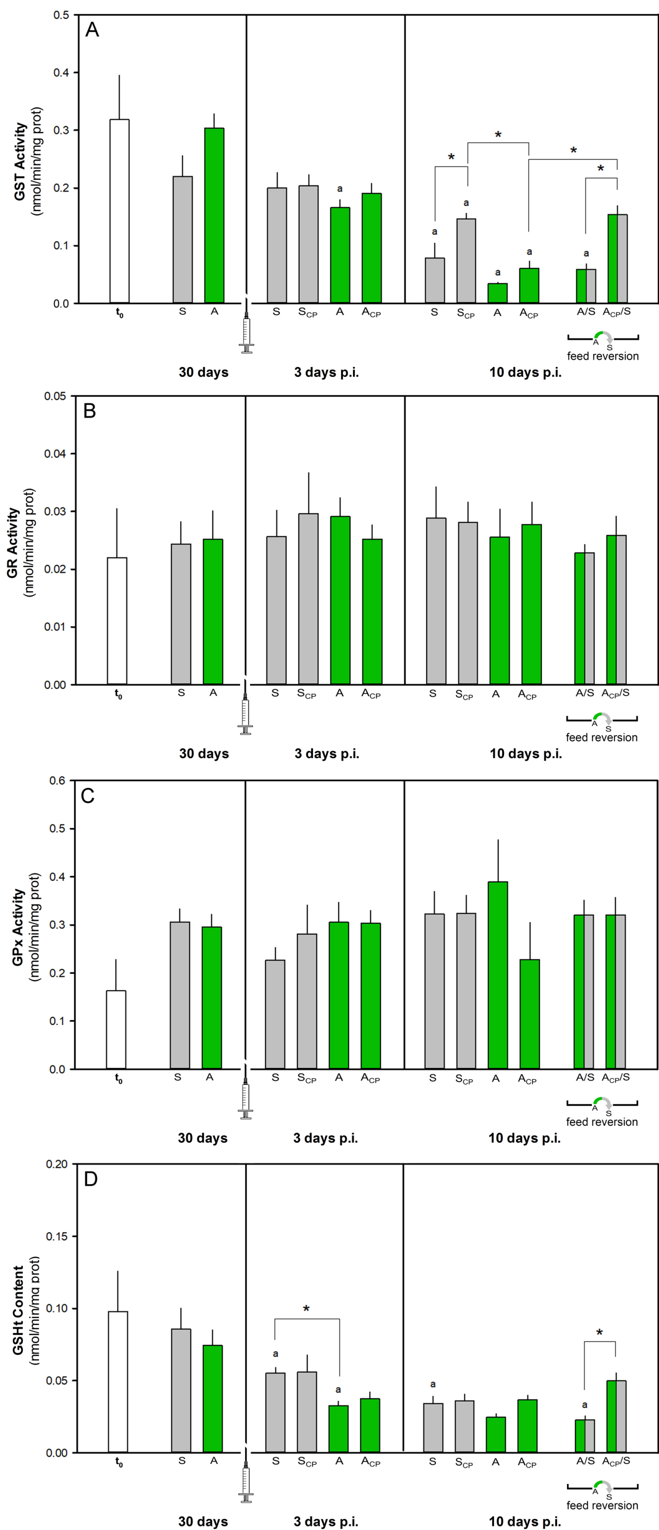

Figure 7