Fish & Shellfish Immunology 42 (2015) 367–378

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Full length article

Long-term feeding with *Euglena gracilis* cells modulates immune responses, oxidative balance and metabolic condition in *Diplodon chilensis* (Mollusca, Bivalvia, Hyriidae) exposed to living *Escherichia coli*

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ARTICLE INFO

Article history:
Received 8 July 2014
Received in revised form
13 November 2014
Accepted 14 November 2014
Available online 24 November 2014

Keywords:
Paramylon
Immunomodulation
Antioxidant responses
Growth
Starvation

ABSTRACT

We evaluated the modulating effect of long-term feeding with lyophilized *Euglena gracilis* cells on immune response, oxidative balance and metabolic condition of the freshwater mussel *Diplodon chilensis*. Mussels, previously fed with *Scenedesmus vacuolatus* (SV) or *E. gracilis* (EG) for 90 days, were challenged with an environmentally relevant concentration of *Escherichia coli* in water for 5 days, under feeding or starvation conditions. EG diet increased overall phagocytic activity and tissue hemocyte accumulation (gill and mantle), and favored hemocyte viability upon *E. coli* challenge. Tissular hemocyte accumulation, and humoral bacteriolytic activity and protein content were similarly stimulated by EG and *E. coli*, with no further effect when both stimuli were combined. Both, *E. coli* challenge and EG diet reduced gill bacteriolytic activity with respect to nonchallenged SV mussels, while no effect was observed in challenged EG mussels. Gill and digestive gland protein contents, along with digestive gland bacteriolytic activity were higher in EG than in SV mussels. Both SV and EG mussels showed increased gill mass upon *E. coli* challenge, while digestive gland mass was increased by bacterial challenge only in SV mussels. Bacterial challenge produced no effect on humoral reactive oxygen species levels of both groups. Total oxyradical scavenging capacity levels was reduced in challenged SV mussels but remained unaffected in EG ones. In general, EG diet decreased glutathione S-transferase and catalase activities in gill and digestive gland, compared with SV diet; but increased enzyme activity was evident in challenged mussels of both groups. Gill and digestive gland lipid peroxidation levels were higher in EG than in SV mussels but *E. coli* challenge had stronger effect on SV mussels. Adductor muscle RNA:DNA ratio was higher in EG mussels than in SV ones, and increased upon *E. coli* challenge in mussels of both groups. *E. gracilis* can be suggested as a nutritional and protective diet complement suitable for filtering bivalves. However, our results obtained from starved mussels show that starvation periods after supplying this diet should be avoided, since these could revert part of the acquired benefits and/or exacerbate detrimental effects.

Abbreviations: SV, mussels fed with *Scenedesmus vacuolatus*; EG, mussels fed with *Euglena gracilis*.

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http://dx.doi.org/10.1016/j.fsi.2014.11.022
1050-4648/

1. Introduction

Euglena species cells constitute a rich source of the β-1,3-glucan paramylon [1], proteins [2], polyunsaturated fatty acids and antioxidants, such as polyphenols, flavonoids, tannins, β-carotene, vitamin C and vitamin E [3–6]. These cells are commonly used in fish aquaculture to improve the diet nutritional value [7,8], while β-glucans have been tested for improving somatic growth in crustaceans and fish, e.g. Refs. [9–12]. Paramylon extracted from *Euglena gracilis* increases immune responses against infection in fish [12], exerts antioxidant protective action on acute hepatic injury in rats [13] and potentiates the resistance of shrimps against stress conditions during growing and handling [8]. It has also been suggested that highly nutritious proteins contained in *Euglena* species cells would strengthen the effect of paramylon; whereas antioxidant compounds would exert a direct protective action against oxidative stress [1]. Basanta et al. [14] have found that fingerlings of the fish *Labeo rohita* fed with *Euglena viridis* are more resistant to *Aeromonas hydrophila* infection.

Particularly for bivalves, *in vitro* experiments and studies based on injection of β-glucans show increases in nitric oxide production, peroxidase and antibacterial activity and phagocytosis [15–18]. Although individuals of a Euglenaceae species have been identified in the diet of oysters [19], the possible effects of applying Euglenaceae cells in the diet of bivalves have not been studied yet.

In the aquatic environment, bivalves are exposed to a wide range of harmful microorganisms, among which pathogenic bacteria have received most attention [20–23]. However, non-pathogenic bacteria released into the water bodies by sewage discharges may threaten bivalves' immune competency leading to health deterioration [24]. *Escherichia coli*, a frequent fecal gram-negative bacterium, may be filtered [25], digested [26] and accumulated in tissues [27,28] by bivalves. This bacterium is recognized by hemocytes, triggering mainly stress-activated signaling pathways [29,30]. The mussel *Hyriopsis cumingii*, challenged with *E. coli* DNA, increases *in vitro* bactericidal activity and *in vivo* antibacterial, lysozyme and prophenoloxidase activities [31]. Furthermore, daily feeding with *E. coli* augmented antioxidant defenses and lipid peroxidation in the digestive gland of *Diplodon chilensis*, after 5–6 weeks [25].

Laboratory studies for evaluating responses to β-glucans and bacterial challenges are frequently performed by *in vitro* exposure or by injection of bacteria or isolated pathogen associated molecular patterns (PAMPs) [17,18,32,33]. In bivalves, this methodology allows avoiding first line defenses, such as shell, mantle epithelium, gills' mucus and resident microbiota [34,35]. However, the effects elicited *in vitro* or by injection may differ from those obtained by oral administration as it has been shown for fish. Selvaraj et al. [36] have reported that intraperitoneal injection of β-glucan increases relative survival in the carp *Cyprinus carpio* upon exposure to *A. hydrophila*, and also increases immune defense variables like total leukocyte counts and bacteria killing capacity; while oral administration of β-glucan has no effect at the same doses.

The freshwater mussel *D. chilensis* has been proposed as an efficient tool for bioremediating polluted and eutrophicated waters due to its ability for clearing suspended bacteria by filter feeding [25,26], algae and nutrients concentrations [37,38]. However, mussels inhabiting sewage-polluted waters in Northwest Patagonia may suffer alterations in their oxidative balance, growth rate and population structure [25,39]. The protective action of feeding with *Euglena* sp. cells may enhance *D. chilensis* resistance against bacteria present in polluted sites. This may also be applicable to other bivalves used in bioremediation strategies for both sewage and aquaculture effluents.

In general terms, very little is known about how continuous and long-term stimulation can be applied on non-specific immune systems without risking harmful effects, such as the loss of response [10], which could lead to increased susceptibility. In this work, we evaluate the modulating effect of long-term supplied *E. gracilis* cells on the immune response, oxidative balance and metabolic condition of the freshwater mussel *D. chilensis* submitted to *E. coli* challenge (environmentally relevant *E. coli* concentrations in water).

Using bivalves for bioremediation purposes implies relocation and depuration strategies [37,40], which may include short starvation periods. Therefore, *E. coli* challenge is applied to both, fed and starved mussels previously fed with either *E. gracilis* or the green algae, *Scenedesmus vacuolatus*. This experiment aims to assess whether dietary supply of *E. gracilis* can enhance the response to and/or reduce the damage produced by this bacteria, and whether this protective effect is affected by starvation.

2. Material and methods

2.1. Experimental diets

S. vacuolatus (BAFC CA4 strain, provided by the Laboratory of Phycology, Department of Biodiversity and Experimental Biology, Faculty of Exact and Natural Sciences, University of Buenos Aires) was grown at 20 °C in Bold's basal medium (BBM; Bischoff and Bold [41]), under continuous cool-white fluorescent light (11 W). *E. gracilis* (UTEX 753 strain, from the Culture Collection of Algae of Texas University, USA) was grown at 20 °C in *E. gracilis* medium (EGM; CCAP [42]). Dark conditions were set to enhance paramylon production [1]. Cells from both cultures were recovered by centrifugation for 15 min at 4000× *g*, for *S. vacuolatus* and at 1000× *g*, for *E. gracilis*, lyophilized and kept at −20 °C.

According to Sabatini et al. [43], 3×10^6 cells of *S. vacuolatus* were provided to each mussel per feeding (0.133 mg of lyophilized cells). Since *S. vacuolatus* and *E. gracilis* differ in cell size, diets were set at equal biomass instead of equal number of cells. Paramylon was extracted and purified from *E. gracilis* cells according to Kiss and Triemer [44]. Lyophilized cells (11 mg) were re-suspended in buffer Tris–HCl (0.125 M) with 2% sodium dodecyl sulfate and incubated at 37 °C for 30 min. Paramylon granules were precipitated by centrifugation at 3500× *g* for 20 min. This procedure was repeated until obtaining a translucent supernatant. Paramylon was then washed three times with distilled water at 70 °C and dried at 60 °C until constant weight. The quality of the extract was evaluated for presence of proteins [45] and then the calculated paramylon content value was corrected.

2.2. E. coli

E. coli JM109 strain (provided by the Department of Biological Chemistry, Faculty of Exact and Natural Sciences, University of Buenos Aires) was grown at 37 °C in nutritive agar medium (CM0003, OXOID) for 24 h. Bacteria were then inoculated in sterile saline solution (NaCl 0.9%, Merck) to obtain a suspension of 1.5×10^8 cell/mL, estimated as 0.5 in McFarland's scale (0.080–0.100 abs at 625 nm). Mussels' exposure was set at a final concentration of 2.4×10^4 cells/100 mL. This was the maximum fecal bacteria concentration measured in an area of the Lacar lake (Northwest Patagonia) inhabited by *D. chilensis* and affected by sewage pollution [25,46].

2.3. Mussel collection and handling

The experiments were performed during the non-reproductive season of *D. chilensis* [47], in order to reduce possible additional

stress. According to their shell length [39], relatively young adult mussels ($n = 48$; 66.67 ± 0.59 mm shell length; 24.72 ± 6.8 g) were collected by a diver at 3 m of depth at a reference site located in the north coast of Lacar lake (40° 10′ S, 71° 31′ 30″ W). Mussels were transported in the cold to the laboratory and sorted into four aerated tanks (150 individuals/m^2) filled with sterile dechlorinated tap water (5 L). Mussels were fed three times a week with *S. vacuolatus*, during 21 days of acclimation. Water was changed before feeding and work temperature was 12.5 ± 2.5 °C.

2.4. Experimental design

After acclimation, two groups of mussels were set: SV: fed with *S. vacuolatus* ($n = 24$) and EG: fed with *E. gracilis* ($n = 24$). Experimental diets were supplied three times a week for 90 days with water changes before feeding. After this period, mussels from both dietary treatments (SV, EG) were sorted into four groups ($n = 6$ each). Mussels were individually placed in aerated glass containers with 500 mL of sterile dechlorinated tap water and submitted to one of the following experimental treatments: 1) Feeding every two days (SV or EG); 2) Daily alternated feeding with the corresponding diet of either SV or EG, and *E. coli*; 3) Starvation; 4) Daily alternated starvation and *E. coli* (Fig. 1). Mussels were processed after 5 days of treatment.

2.5. Immune response

2.5.1. Hemocyte response

Mussels ($n = 6$ per treatment) were anesthetized on ice and 2 mL of hemolymph per individual were collected from the adductor muscle with a sterile syringe. Hemolymph was aliquoted in sterile microcentrifuge tubes, kept on ice and used for analysis within 1 h from collection.

Total and viable hemocytes were counted in Neubauer's chamber using Trypan Blue (0.2%) dissolved in sterile anticoagulant solution (3 g glucose and 0.36 g trisodium citrate per L, pH 7), according to Bianchi et al. [48]. Total hemocyte were expressed as cells/mL of hemolymph and viability was expressed as the proportion of viable cells (not stained with Trypan Blue)/total cells [24]. Hemocyte phagocytic activity was evaluated against Congo red stained yeast cells and expressed as the phagocytized yeast cells/hemocyte ratio [48].

Histological examinations were carried out in gills and mantle ($n = 4$ for each treatment) to evaluate hemocyte accumulation in these organs. In gills, strips of 3–5 mm width were cut transversally, from the middle section of the external demibranch, while in mantle, tissue portions of 0.25 cm^2 were extracted from the distal zone, including both muscle and connective tissue. Tissue portions were carefully extracted and fixed in buffered formalin solution 10%. Samples were then dehydrated through an alcohol series, cleared in xylene, and embedded in synthetic paraffin (Histoplast®, Biopack, Argentina). Gill and mantle samples were sectioned (7 μm thick) in a transverse plane and stained with haematoxylin and eosin ($n = 6$ per tissue sample). Slides were examined by light microscopy (200×) (Nikon Eclipse E600) and photographed using a digital microscope imager (Celestron #44421). Tissue area measurements and hemocyte counts were carried out using the Image J 1.48j software [49]. Results were expressed as cells/mm^2.

2.5.2. Humoral response

Hemolymph was centrifuged at 500× *g* for 20 min (4 °C) and bacteriolytic activity (BA) of hemolymph supernatant was measured against *E. coli* JM109 strain at 1.5 × 10^8 cell/mL [48] and expressed as BA/mL. This assay allows measuring the total lytic activity in the sample, since the decrease in absorbance values indicates the breakdown of bacteria cells. Humoral protein content was measured according to Bradford [45], using a bovine seroalbumin standard curve.

2.5.3. Tissue measurements

Bacteriolytic activity in gills and digestive gland ($n = 6$ per treatment) was evaluated in supernatants obtained as described in Section 2.6.2. Measurements of BA/mg of protein were made following the same procedure as for plasma samples. The morphometric relationships ($n = 6$ per treatment), gill factor (GF) and digestive gland factor (DGF) were calculated for each mussel according to Bianchi et al. [48]. The organ wet mass (g) was divided by the shell length^3 (cm) and multiplied by 100. Protein content was measured in gill and digestive gland ($n = 6$ per treatment) according to Bradford [45] (1976), using a bovine seroalbumin standard curve. Results were expressed as mg/g.

2.6. Oxidative balance

2.6.1. Humoral ROS and TOSC

Extracellular ROS [32] and the total oxyradical scavenging capacity (TOSC) ($n = 6$ per treatment) were measured in plasma by a

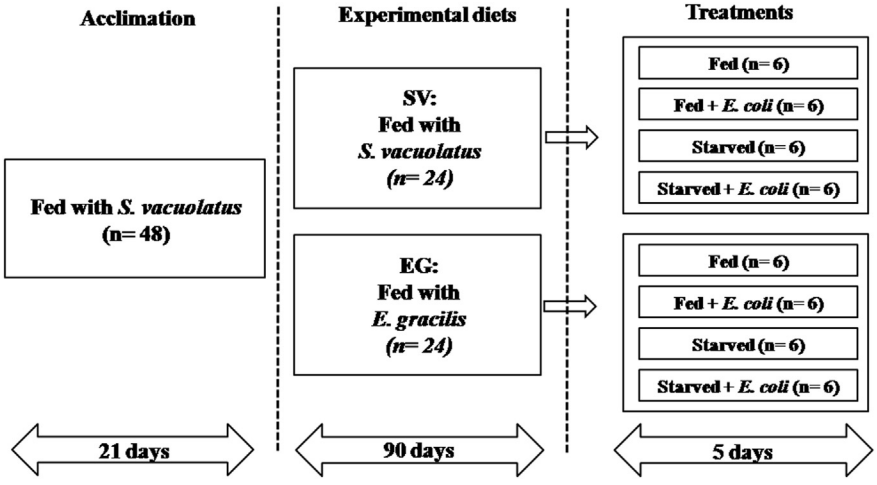

Fig. 1. Experimental design to test the effects of long-term supplied *Euglena gracilis* on the immune response, oxidative balance and metabolic condition of *Diplodon chilensis* challenged with *Escherichia coli*, under feeding or starvation conditions.

fluorometric method, employing 2′,7′-dichlorofluorescein diacetate (H_2DCF-DA, Sigma) as substrate, according to Amado et al. [50]. Briefly, each plasma sample was separated into two groups of three replicates, which were incubated at 35 °C for 5 min in fluorometer tubes (Qubit assay tubes Q32856, Invitrogen). In the first group, 50 μL of plasma (obtained as described in Section 2.5.2) were mixed with 130 μL of reaction buffer (30 mM Hepes, 200 mM KCl; 1 mM MgCl, pH 7) and 10 μL of ABAP solution (4 mM 2,2′-Azobis(2-methylpropionamidine) dihydrochloride, Sigma). In the second group, ABAP was replaced by 10 μL of buffer. After incubation, 10 μL of H_2DCF-DA were added to each tube and incubation was continued at 35 °C for 12 min. The change in fluorescence (485/530 nm) was recorded during 18 min in a Qubit™ fluorometer, Invitrogen. ROS content was referred to a H_2O_2 standard curve with the fluorescent probe and results were expressed as meq H_2O_2/mL of plasma. TOSC was calculated from the relative area between the curves obtained with and without ABAP and referred to mL of plasma.

2.6.2. Tissue GST and CAT activities, and lipid peroxidation

Gills and digestive gland were extracted and separately homogenized (Omni 1000 motorized homogenizer at 20,000 rpm) in cold phosphate buffer (100 mM, pH 7.0) 1:5 w/v, containing protease inhibitor (0.2 mM phenylmethylsulfonil fluoride, Sigma). Supernatants were obtained after centrifugation at 11,000 × g for 15 min at 4 °C and then used for biochemical analysis.

Since protein content in tissue samples varied significantly among treatments (Section 3.3.2), results for enzyme activities, and TBARS content were referred to wet tissue mass (g) and not to protein concentration.

GST activity was measured using chloro-2,4 dinitrobenzene as substrate, according to Habig et al. [51]. One GST Unit was defined as the amount of enzyme needed to catalyze the formation of 1 μmol of GS-DNB per min at 25 °C. Results were expressed as U GST/g. CAT activity was measured by the decomposition of H_2O_2 (10 mM in 50 mM potassium phosphate buffer, pH 7) [52]. One CAT Unit was defined as the amount of enzyme needed to catalyze the hydrolysis of 1 μmol of H_2O_2/min at 25 °C. Results were expressed as U CAT/g. Lipid peroxidation was estimated by the thiobarbituric acid reactive substances method (TBARS) [53] and expressed as μmol of TBARS/g.

2.7. Metabolic condition: RNA/DNA ratio

Adductor muscle tissue was preserved in RNA Later (Ambion) and kept at −20 °C for further analysis. Muscle tissue was then rinsed in cold saline phosphate buffer (100 mM, pH 7) and homogenized in extraction/lysis buffer (10 mM Tris–HCl, 1 mM EDTA, 1% sodium dodecyl sulfate, pH 8) modified from Serafim et al. [54]. RNA and DNA contents were measured according to manufacturer instructions (Invitrogen), using a Quant-it RNA Assay kit (Q32852) and Quant-it DNA BR Assay kit (Q32850), respectively.

2.8. Statistical analysis

Data were expressed as mean ± standard error. Normal distribution and homogeneity of variance were checked by Kolmogorov–Smirnov and Levene's tests, respectively. When statistical assumptions were not met, values were transformed by $\mathrm{Log}_{10}(x)$ or $\mathrm{Log}_{10}(x+1)$, when appropriate. Differences between diets, under feeding or starvation, in challenged and nonchallenged mussels were tested by three-way ANOVA (diet * feeding condition * challenge). Based on the results of this analysis, significant differences among treatments were assessed by Newman–Keuls post hoc comparisons for two or three way interaction terms (significance $P < 0.05$). Differences between corresponding treatments from both diets were assessed by pairwise comparisons from the Newman–Keuls' matrix (significance $P < 0.05$).

3. Results

3.1. Paramylon content

Paramylon accounted for 92.91% of the total mass of lyophilized *E. gracilis* cells, after subtraction of a 3.53% of protein detected in the

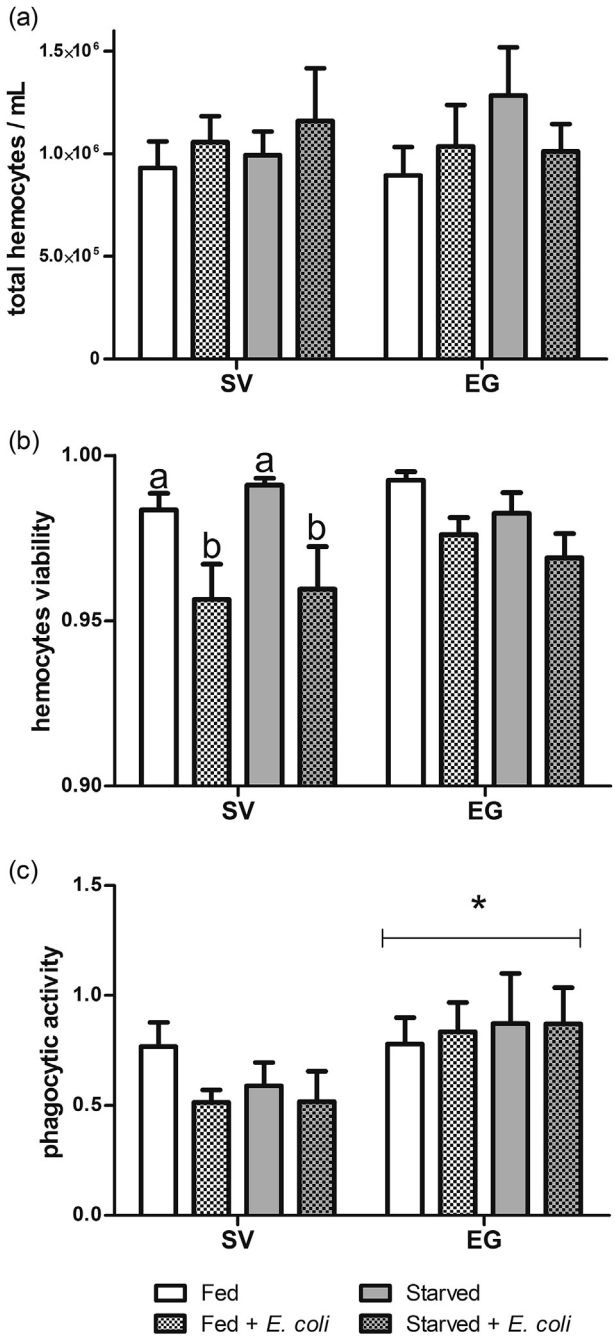

Fig. 2. Hemocyte response in *Diplodon chilensis* fed with *Scenedesmus vacuolatus* (SV) or *Euglena gracilis* (EG) and challenged with *Escherichia coli*, under feeding or starvation conditions ($n = 6$ for each treatment). Results for total hemocyte number (a), hemocyte viability (b) and phagocytic activity (c) are expressed as mean ± standard error. Different characters denote significant differences ($P < 0.001$) between challenged (fed + starved) and nonchallenged (fed + starved) SV mussels in (b), while * denotes $P < 0.05$ between diets (SV *vs.* EG) in (c).

extract. According to this, each mussel received 128 mg of paramylon per feeding.

3.2. Immune response

3.2.1. Hemocyte response

The total number of hemocytes in hemolymph showed no changes upon diet or *E. coli* challenge, neither under feeding nor under starvation conditions (Fig. 2(a)). Diet * challenge two-way interaction was significant ($P < 0.05$) for hemocyte viability (Fig. 2(b)). This variable decreased by 3% in both, fed and starved SV mussels, when these were challenged with *E. coli* ($P < 0.001$), while no significant effect was observed in EG mussels. Phagocytic activity was higher in all EG mussels compared to SV ones (diet effect, $P < 0.05$) and was affected neither by starvation nor by *E. coli* challenge (Fig. 2(c)).

Diet * challenge two-way interaction was significant for hemocyte number in gills ($P < 0.01$) (Fig. 3(a)). This variable increased significantly only in challenged SV mussels both, under feeding (38%) and starvation conditions (73%), compared to nonchallenged mussels ($P < 0.001$). In mantle, diet * feeding condition * challenge three-way interaction was significant at $P < 0.001$ (Fig. 3(b)). In this tissue, *E. coli* challenge augmented hemocyte number in fed SV mussels by 28% ($P < 0.001$) but diminished the same variable in starved mussels by 37% ($P < 0.05$). Hemocyte number in gills and mantle was affected by the diet ($P < 0.001$) (Fig. 3(a) and (b)). EG mussels showed overall significantly higher numbers of hemocytes accumulated in both tissues than SV ones ($P < 0.001$, for both tissues). EG hemocyte number did not differ among treatments and was similar to the maximum values recorded in challenged SV mussels. Fig. 4 shows histological preparations from gill and mantle, with infiltrating hemocytes, in fed challenged SV mussels.

3.2.2. Humoral response

Diet * challenge two-way interaction was significant for both bacteriolytic activity ($P < 0.01$) and protein content ($P < 0.05$) in the cell-free hemolymph (humoral fraction) (Fig. 5(a) and (b)). These variables were increased by *E. coli* challenge in SV mussels, with respect to nonchallenged SV mussels ($P < 0.05$ for both). Bacteriolytic activity and protein content in humoral fraction of EG mussels had similar levels to those in challenged SV mussels but did not respond to *E. coli* challenge. Additionally, starvation significantly incremented the values of both variables by about 25% ($P < 0.001$ for both) considering SV and EG mussels together. Since protein content in humoral fraction varied significantly among treatments, results for bacteriolytic activity were referred to mL of sample and not to protein concentration, in order to avoid biasing data.

3.2.3. Tissue response

Diet * challenge two-way interaction was significant for gill bacteriolytic activity ($P < 0.01$). This activity decreased by 45% in fed SV mussels and by 34% in starved ones ($P < 0.05$), upon *E. coli* challenge. Additionally, this activity was 42% lower in fed nonchallenged EG mussels than in the corresponding group receiving SV diet (pairwise comparisons, $P < 0.05$) (Fig. 6(a)).

Diet * feeding condition * challenge three-way interaction was significant for digestive gland bacteriolytic activity ($P < 0.01$) (Fig. 6(b)). A negative effect of *E. coli* challenge on this activity was evident in starved mussels of SV and EG groups ($P < 0.01$ for both), reducing this activity by about 63%. Digestive gland bacteriolytic activity was c.a. 28% higher in fed EG mussels than in fed SV ones (pairwise comparison, $P < 0.05$).

Diet * feed condition * challenge three-way interaction was significant for GF ($P < 0.05$), which was increased by *E. coli* challenge in fed SV mussels and in fed EG ones with respect to their corresponding unchallenged controls ($P < 0.05$ for both). In addition, this variable was higher in challenged starved EG mussels than in nonchallenged ones ($P < 0.05$) (Fig. 7(a)). Diet * feeding condition * challenge three-way interaction was also significant for DGF ($P < 0.01$) (Fig. 7(b)). This variable was enhanced by *E. coli* challenge only in fed SV mussels (30%, $P < 0.05$) and in starved EG ones (35%, $P < 0.01$). Both, GF and DGF were higher in challenged starved EG mussels than in challenged starved SV ones by 50% and 40%, respectively (pairwise comparisons, $P < 0.05$ for both) (Fig. 7(a) and (b)).

Diet * feeding condition two-way interaction was significant for gill protein content ($P < 0.001$) (Fig. 7(c)). In fed EG mussels, this variable was 66% higher than in the corresponding group of SV mussels ($P < 0.001$). Starvation suppressed the stimulant effect of the EG diet ($P < 0.001$). For protein content in digestive gland (Fig. 7(d)), feeding condition * challenge and diet * feeding condition two-way interactions were significant ($P < 0.001$ and $P < 0.01$, respectively). The protein content of this tissue was positively affected by *E. coli* challenge in fed mussels from both groups

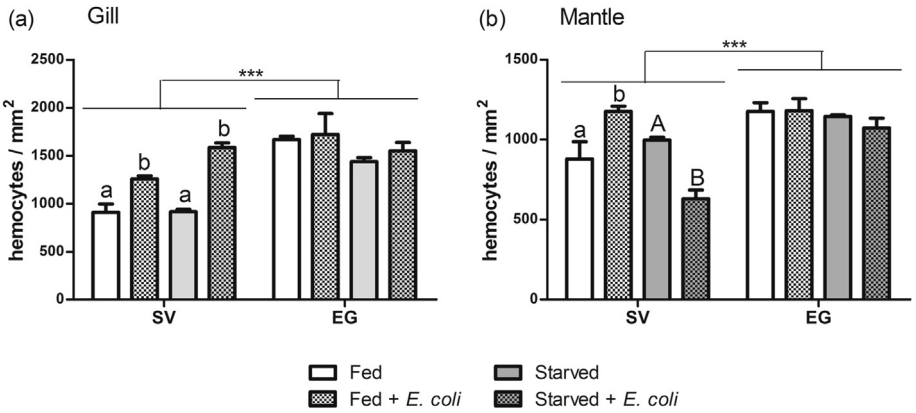

Fig. 3. Hemocyte number in gills (a) and mantle (b) of *Diplodon chilensis* fed with *Scenedesmus vacuolatus* (SV) or *Euglena gracilis* (EG), and challenged with *Escherichia coli*, under feeding or starvation conditions ($n = 6$ for each treatment). Results are expressed as mean ± standard error. Different characters denote significant differences ($P < 0.001$) between challenged (fed + starved) and nonchallenged (fed + starved) SV mussels in (a). In (b), different characters denote significant differences between challenged and nonchallenged SV mussels, under feeding ($P < 0.001$; lowercase letters) and starvation conditions ($P < 0.05$; uppercase letters). Lines with *** denote significant differences ($P < 0.001$) between diets (SV *vs.* EG).

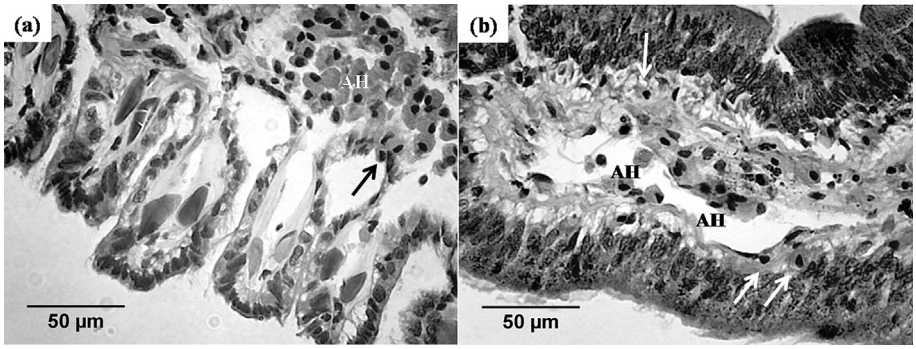

Fig. 4. Infiltrating hemocytes (arrows) in gill epithelium (a) and in mantle connective tissue (b) of fed challenged SV mussels. Aggregated hemocytes in hemolymph space are depicted by AH, in (a) and (b).

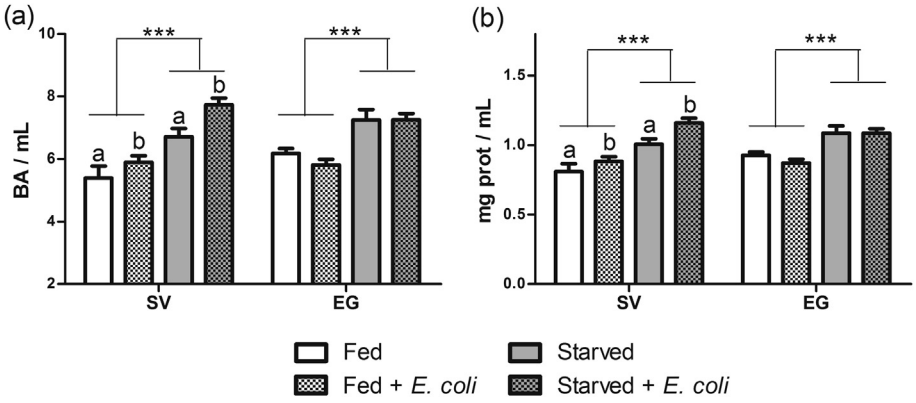

Fig. 5. Humoral response of *Diplodon chilensis* fed with *Scenedesmus vacuolatus* (SV) or *Euglena gracilis* (EG) and challenged with *Escherichia coli*, under feeding or starvation conditions ($n = 6$ for each treatment). Results for bacteriolytic activity (BA) (a) and protein content (b) per mL of humoral fraction are expressed as mean ± standard error. Different characters denote significant differences between challenged (fed + starved) and nonchallenged (fed + starved) SV mussels in (a) and (b) ($P < 0.05$ for both). Lines with *** denote significant differences ($P < 0.001$) between fed and starved mussels.

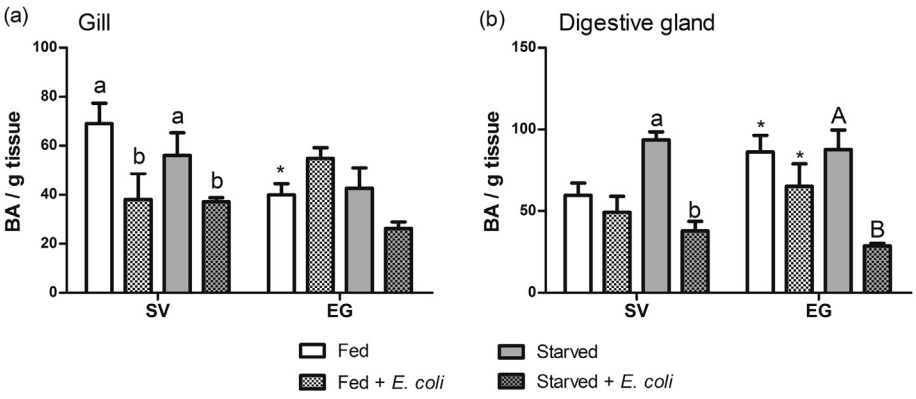

Fig. 6. Bacteriolytic activity (BA) in gills (a) and digestive gland (b) of *Diplodon chilensis* fed with *Scenedesmus vacuolatus* (SV) or *Euglena gracilis* (EG) and challenged with *Escherichia coli*, under feeding or starvation conditions ($n = 6$ for each treatment). Results are expressed as mean ± standard error. In (a), different characters denote significant differences between challenged (fed + starved) and nonchallenged (fed + starved) SV mussels ($P < 0.05$). In (b), different characters denote significant differences between challenged and nonchallenged starved mussels in SV ($P < 0.01$; lowercase letters) and in EG ($P < 0.01$; uppercase). * denotes $P < 0.05$ between fed nonchallenged SV and fed nonchallenged EG mussels in (a) and (b), and between fed challenged SV and fed challenged EG in (b).

($P < 0.05$), while a negative effect of bacterial challenge was observed in starved individuals ($P < 0.05$). Digestive gland protein content was higher in fed EG mussels than in fed SV ones ($P < 0.001$), with increments of 25% and 50%, for nonchallenged and challenged mussels, respectively. In addition, starvation increased protein content in starved SV mussels with respect to fed ones ($P < 0.001$).

3.3. Oxidative balance

3.3.1. Humoral ROS and TOSC

Diet * feeding condition * challenge three-way interaction was significant for extracellular ROS ($P < 0.01$) (Fig. 8(a)). This variable was significantly increased only in starved SV mussels challenged with *E. coli* ($P < 0.001$), reaching values 300% higher than those of

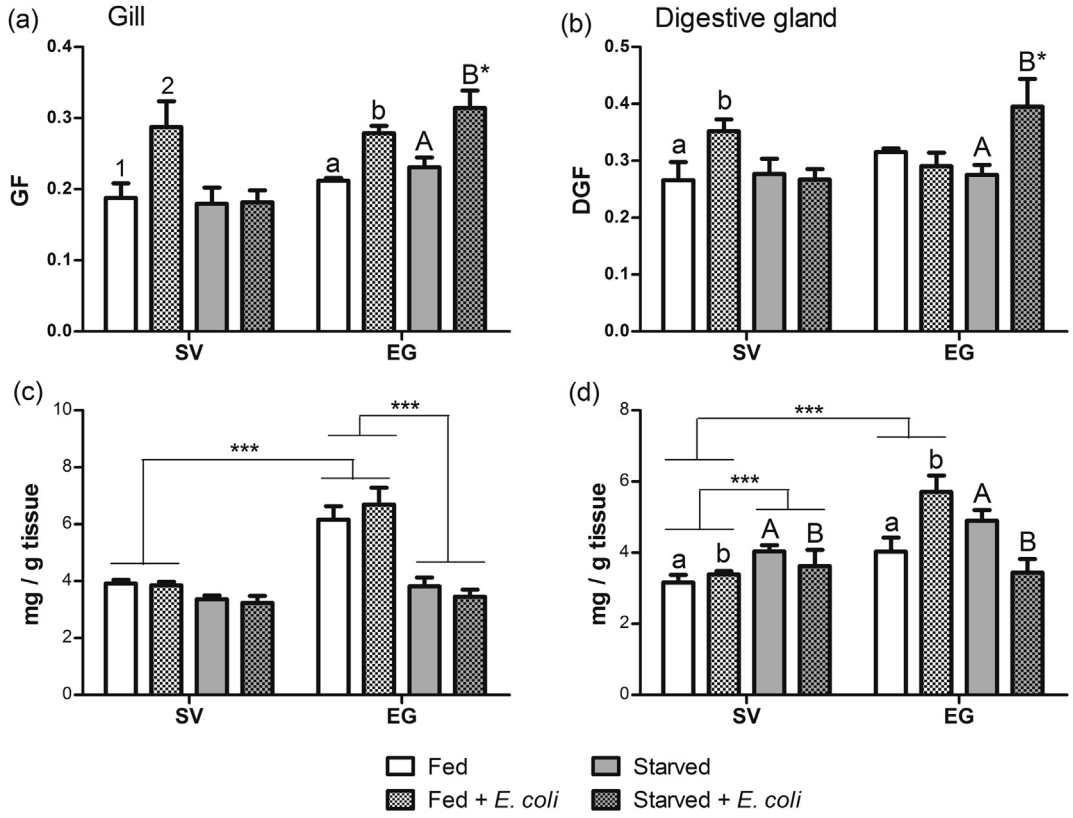

Fig. 7. Gill factor (GF) and protein content (a, c), digestive gland factor (DGF) and protein content (b, d) of *Diplodon chilensis* fed with *Scenedesmus vacuolatus* (SV) or *Euglena gracilis* (EG), and challenged with *Escherichia coli*, under feeding or starvation conditions ($n = 6$ for each treatment). Results are expressed as mean ± standard error. Different characters (numbers, lowercase letters, uppercase letters) denote significant differences between challenged and nonchallenged mussels: in (a) for fed SV mussels, for fed EG mussels and for starved EG mussels ($P < 0.05$ for all comparisons); in (b), for fed SV mussels ($P < 0.05$) and for starved EG ones ($P < 0.01$); in (d), for fed (SV + EG) mussels and for starved (SV + EG) mussels ($P < 0.05$ for both comparisons). * denotes significant differences ($P < 0.05$) between diets for a particular treatment in (a) and (b). Lines with *** indicate $P < 0.001$ for starvation effects and differences between fed SV and EG mussels (challenged + nonchallenged) in (c, d).

the rest of the treatments. Diet * challenge two-way interaction and starvation effect were significant for TOSC ($P < 0.01$; $P < 0.001$, respectively) (Fig. 8(b)). This variable was diminished by 50% with starvation both, in SV and EG mussels ($P < 0.001$) although *E. coli* challenge reduced TOSC only in SV mussels (11% for fed and 60% for starved mussels, $P < 0.05$).

3.3.2. Tissue GST, CAT and lipid peroxidation

Diet * feeding condition * challenge three-way interaction was significant for gill GST activity ($P < 0.01$) (Fig. 9(a)). *E. coli* challenge significantly increased this activity in fed SV (60%) and fed EG (800%) mussels ($P < 0.001$ for both comparisons). Pairwise comparisons showed that this enzyme activity was decreased in

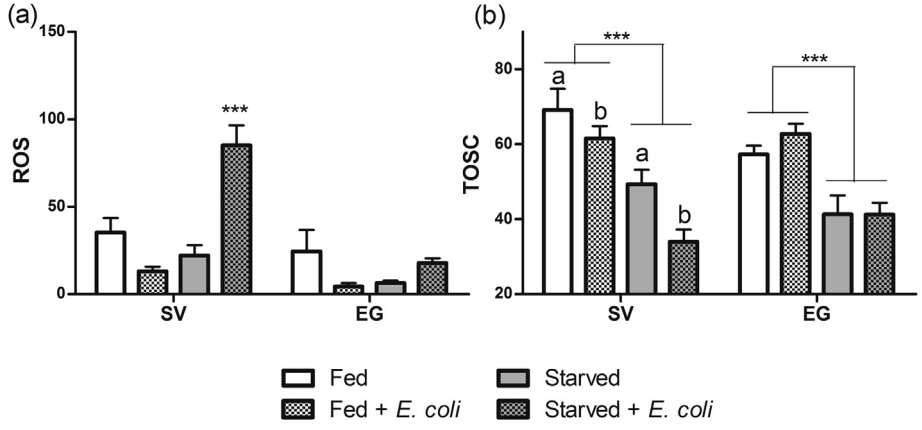

Fig. 8. Reactive oxygen species (ROS) (a) and total oxyradical scavenging capacity (TOSC) (b) in plasma from *Diplodon chilensis* fed with *Scenedesmus vacuolatus* (SV) or *Euglena gracilis* (EG), and challenged with *Escherichia coli*, under feeding or starvation conditions ($n = 6$ for each treatment). Results are expressed as mean ± standard error. In (a), *** denotes significant differences at $P < 0.001$ between starved challenged SV mussels and the rest of the treatments. In (b), different characters denote significant differences ($P < 0.05$) between challenged (fed + starved) and nonchallenged (fed + starved) SV mussels. Lines with *** denote $P < 0.001$ between fed (SV + EG) and starved (SV + EG) mussels.

nonchallenged (84%, $P < 0.001$) and challenged (19%, $P < 0.05$) EG mussels compared with the corresponding SV treatments.

Diet * feeding condition, diet * challenge and feeding condition * challenge two-way interactions were significant for gill CAT activity ($P < 0.001$, $P < 0.01$, $P < 0.05$, respectively) (Fig. 9(b)). This activity was significantly stimulated by *E. coli* challenge in SV mussels (fed + starved) (60%, $P < 0.001$). In fed mussels (SV + EG), challenged individuals had also higher CAT activity than nonchallenged ones ($P < 0.01$). Pairwise comparisons showed that gill CAT activity was significantly lower in challenged (70%, $P < 0.001$) and nonchallenged (63%, $P < 0.05$) EG mussels compared with challenged and nonchallenged SV mussels, respectively. This variable was negatively affected by starvation only in SV mussels ($P < 0.001$).

Diet * feeding condition two-way interaction was significant for gill TBARS levels ($P < 0.05$) (Fig. 9(c)). Starvation increased gill TBARS respect to fed individuals only in the EG group ($P < 0.05$). Pairwise comparisons showed that fed nonchallenged EG mussels had significantly higher gill TBARS levels (75%, $P < 0.05$) than the corresponding SV mussels. Starved EG nonchallenged and challenged mussels had 55% ($P < 0.05$) and 69% ($P < 0.01$) higher gill TBARS levels than the corresponding SV mussels. The levels of this variable were increased by *E. coli* challenge only in fed SV mussels ($P < 0.05$).

In the digestive gland, feeding condition * challenge two-way interaction was significant for GST activity ($P < 0.001$). This enzyme activity increased with *E. coli* challenge only in fed mussels (SV + EG) (208% and 65%, respectively, $P < 0.001$). Pairwise

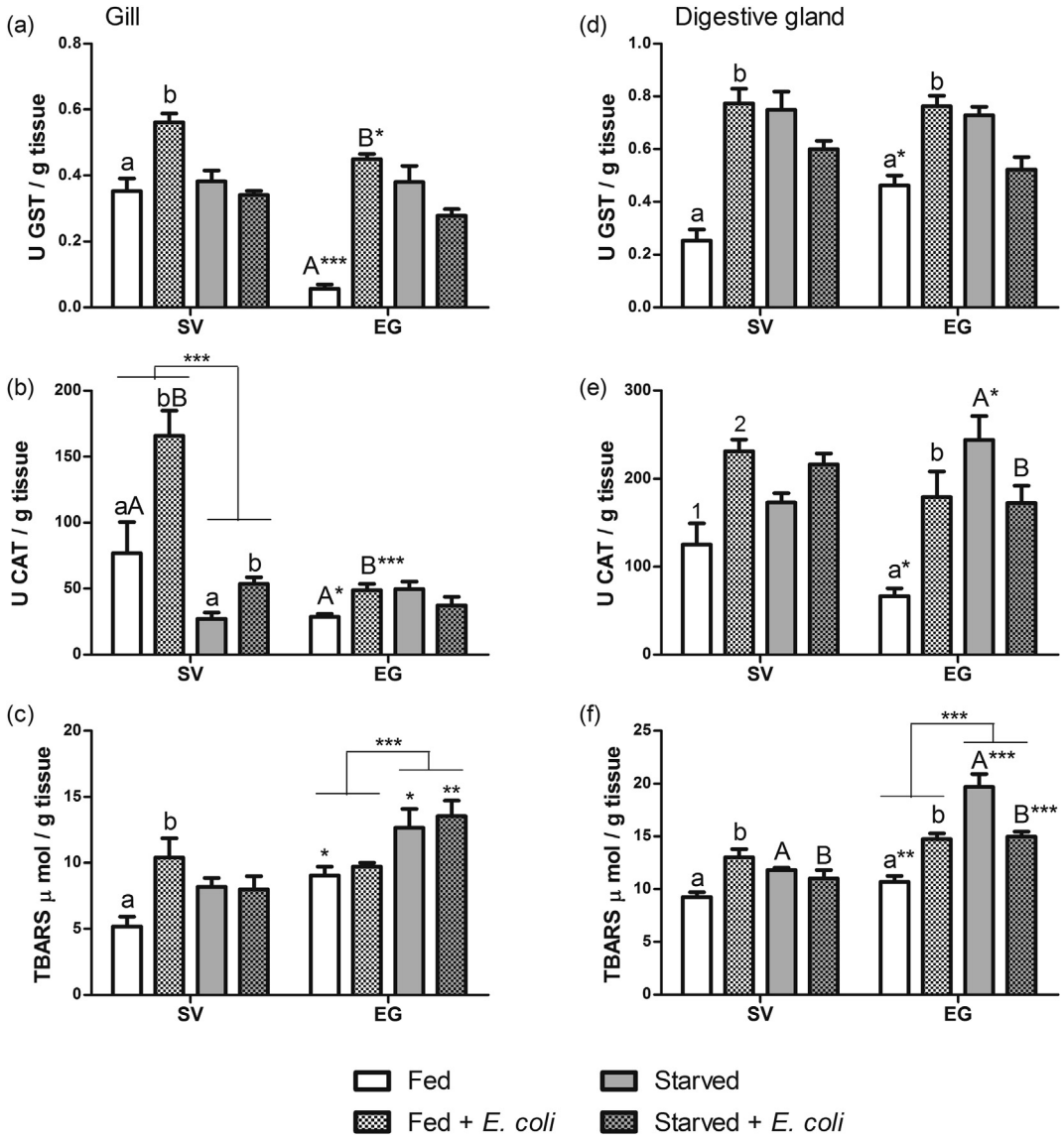

Fig. 9. Glutathione S-transferase activity (GST), catalase activity (CAT) and lipid peroxidation (TBARS) in gills (a–c) and digestive gland (d–f) of *Diplodon chilensis*, fed with *Scenedesmus vacuolatus* (SV) or *Euglena gracilis* (EG), and challenged with *Escherichia coli*, under feeding or starvation conditions ($n = 6$ for each treatment). Results are expressed as mean ± standard error. Different characters (numbers, lowercase letters, uppercase letters) denote significant differences between challenged and nonchallenged mussels: in (a) for fed SV and fed EG mussels ($P < 0.001$ for both comparisons); in (b) for SV (fed + starved, $P < 0.001$) and for fed (SV + EG, $P < 0.01$) mussels; in (c) for fed SV mussels ($P < 0.05$); in (d) for fed mussels (SV + EG, $P < 0.001$); in (e) for fed SV ($P < 0.01$), for fed EG ($P < 0.001$) and for starved EG ($P < 0.01$) mussels; in (f) for fed (SV + EG, $P < 0.001$) and for starved (SV + EG, $P < 0.05$) mussels. *, ** and *** above bars denote significant differences between diets for a particular treatment at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively. Lines with *** denote $P < 0.001$ between fed and starved mussels in (b), (c) and (f).

comparisons showed that digestive gland GST activity was higher in fed nonchallenged EG mussels than in SV ones (84%, $P < 0.05$).

Diet * feeding condition * challenge three-way interaction was significant for digestive gland CAT activity ($P < 0.01$). *E. coli* challenge increased this activity in fed SV (85%, $P < 0.01$) and EG mussels (168%, $P < 0.001$) with respect to their respective nonchallenged controls. In starved EG mussels CAT activity was decreased by *E. coli* challenge (30%, $P < 0.05$). In addition, pairwise comparisons showed that CAT activity was lower in EG than in SV fed nonchallenged mussels (56%, $P < 0.05$) and higher in EG than in SV starved nonchallenged mussels (41%, $P < 0.05$).

Diet * feeding condition and feeding condition * challenge interactions were significant for digestive gland TBARS levels ($P < 0.001$ for both). Starvation increased TBARS level only in the EG group ($P < 0.001$). In addition, fed challenged mussels of both groups (SV + EG) showed an increase of about 50% in TBARS levels compared to nonchallenged ones ($P < 0.001$). In starved mussels (SV + EG), *E. coli* challenge decreased TBARS ($P < 0.05$). Pairwise comparisons showed that digestive gland TBARS levels were higher in fed nonchallenged, starved nonchallenged and starved challenged EG mussels than in the corresponding SV treatments ($P < 0.01$).

3.4. Metabolic condition: RNA/DNA ratio

Feeding condition * challenge two-way interaction and diet effect were significant for adductor muscle RNA/DNA ratio ($P < 0.05$ for both) (Fig. 10). *E. coli* challenge increased significantly ($P < 0.01$) this ratio in fed mussels by 150% for SV and 80% for EG, while no change was detected in starved mussels. EG mussels showed higher RNA/DNA ratio than SV mussels ($P < 0.001$).

4. Discussion

We studied, for the first time, the effects of continuous and long-term feeding with *Euglena* cells, which are rich in the β-glucan paramylon, on immunological and oxidative stress parameters of a freshwater mussel. Lyophilized *E. gracilis* supplied for 90 days (EG) positively modulated the immunological response of *D. chilensis*, especially at the cellular level. This treatment also improved the metabolic condition through the increase of protein content in tissues and RNA/DNA ratio.

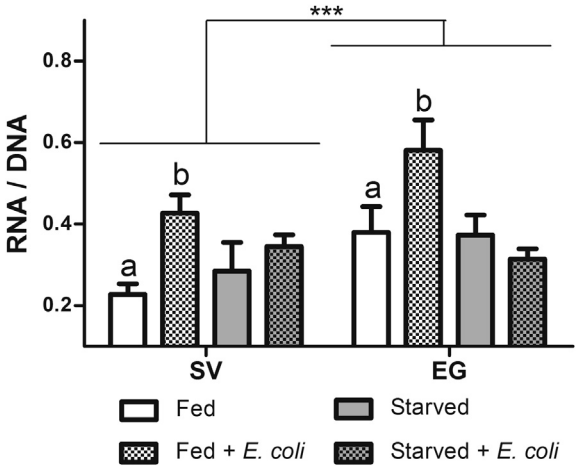

Fig. 10. RNA/DNA ratio in adductor muscle of *Diplodon chilensis* fed with *Scenedesmus vacuolatus* (SV) or *Euglena gracilis* (EG), and challenged with *Escherichia coli*, under feeding or starvation conditions ($n = 6$ for each treatment). Results are expressed as mean ± standard error. Different characters denote $P < 0.01$ between challenged fed mussels (SV + EG) and nonchallenged fed mussels (SV + EG). *** denote $P < 0.001$ between diets (SV *vs.* EG).

4.1. Immune response

4.1.1. Hemocyte and humoral response

Out of five cellular immune variables studied, three: phagocytic activity, number of hemocytes accumulated in gills, and number of hemocytes accumulated in mantle, showed increased values in EG mussels, independently of *E. coli* challenge. In addition, *E. coli* challenge significantly reduced hemocyte viability in control (SV) but not in EG mussels, suggesting protective effects of the EG diet. Accordingly, Anderson et al. [55] reported that β-glucans, injected for three days, stimulated cellular immune responses, like number of circulating hemocytes and hemocyte aggregation in the oyster *Crassostrea virginica*.

Studies in which immune variables were recorded during and after continuous dietary administration of β-glucans, showed increases in total hemocyte counts in shrimps [10] and in white blood cells counts in fish [56]. These changes were followed by a loss of significant effects, referred by the former authors as “immunity fatigue”. In contrast, *D. chilensis* hemolymph total hemocyte count did not change with diet neither with *E. coli* challenge. Since in the present study immune variables were measured only at the end of the experiments, this lack of effect could not be assigned to either immune fatigue or lack of stimulation. However, the fact that hemocyte resistance against *E. coli*, phagocytic activity, and number of hemocytes accumulated in gill and mantle were higher in EG than in SV mussels after 90 days suggests that there is no immunity fatigue. Accordingly, Misra et al. [56] reported that phagocytic activity and resistance against pathogenic bacteria remained stimulated in fingerlings of the fish *L. rohita* supplied with β-glucan for 56 days. Furthermore, increased accumulation of hemocytes in gill and mantle tissues of EG mussels could indicate that the total hemocyte number was actually augmented in our study, even if it was not evident in hemolymph samples, as has been suggested by Oubella et al. [57].

Bacterial challenge stimulated the hemocyte accumulation in gill of all SV mussels and in mantle of fed SV mussels, along with bacteriolytic activity and protein concentration in cell-free hemolymph. The fact that these hematologic variables were similarly stimulated by EG and *E. coli*, with no further effect when both stimuli were combined, suggests that paramylon and LPSs activate the same kind of receptors. In this sense, LGBP (lipopolysaccharide and β-glucan binding protein) and PRP (pattern recognition protein) genes were reported to respond to both bacterial LPSs and β-glucans, in decapod crustaceans and in the mollusk *Haliotis discus*, respectively [11].

On the other hand, in *D. chilensis*, phagocytic activity was enhanced by EG but not by *E. coli*. This suggests that phagocytic activity responded to paramylon or to other compounds contained in *E. gracilis* cells through a regulatory pathway, which is not sensitive to LPSs, although the time of exposure to *E. coli* (five days) could have been too short to stimulate phagocytic activity. Nevertheless, multiple binding sites for glucans, LPSs or other foreign components were reported for fish and crustaceans [11] but little is known about such binding sites in mollusks, e.g. Refs. [23,58]. Thus, it becomes interesting to explore the existence of different glucan and LPS binding sites and the related immune response mechanisms in *D. chilensis* and in other mollusks.

On the other hand, starvation affects humoral responses of *D. chilensis* under both experimental diets. The increase of the humoral protein content observed in starved individuals could be associated to energetic reserve mobilization [59,60]. Additionally, the increase of bacteriolytic activity observed in starved *D. chilensis*

could respond to starvation-related stress, which activated some immunological responses [61]. In general terms, it could be suggested that bacteriolytic activity is exerted by elements of protein nature, since variations in this activity and protein content in cell-free hemolymph showed the same trend.

4.1.2. Tissue response

Gill bacteriolytic activity responded in the opposite direction but with similar patterns with respect to hemocyte recruitment in gill tissue and humoral bacteriolytic activity. *E. coli* challenge and EG diet similarly reduced the values of this variable with respect to those of nonchallenged SV mussels. There was no further effect when *E. coli* was applied to EG mussels. Although gill bacteriolytic activity has mainly a host defense function, e.g. Ref. [8], the overall immune response in bivalves has been generally attributed to the presence of phagocytic hemocytes [62,63]. In agreement, as it was discussed in Section 4.1.1, the gill immune defense of *D. chilensis* seems to be based on hemocyte recruitment and phagocytic capacity, and on humoral bacteriolytic activity.

It was reported that bacteria constitute part of bivalve's diet, e.g. Ref. [64], and that digestive gland lysozyme was adaptively differentiated for digestive function [65–67]. Particularly, the ability of *D. chilensis* to digest *E. coli* and to incorporate bacterial fatty acids was previously reported by Lara et al. [26] and Rocchetta et al. [39], respectively. In this work, digestive gland bacteriolytic activity was higher in fed EG than in SV mussels, and decreased upon bacterial challenge only in starved mussels of both groups. These results suggest a differential bacteriolytic response to EG in gill and digestive gland related to differences in the enzymes involved, e.g. organ specific lysozyme paralogs [65] and/or activation of different receptors or regulatory pathways by foreign molecules.

E. coli challenge increased GF in fed SV mussels, and in fed and starved EG mussels. This increase in gill mass with respect to shell length upon bacterial challenge could be related to inflammatory processes involving hemocyte infiltration and dilatation of hemolymphatic sinuses [24,68]. In this regard, the increased GF in challenged SV mussels is in accordance with the enhanced hemocyte accumulation observed in this organ upon bacterial challenge. However, hemocyte accumulation was also augmented in starved SV mussels, which did not show increased GF. Besides, EG stimulated hemocyte accumulation in gill of both fed and starved individuals and increased protein content only in fed ones, regardless of bacterial challenge.

β-glucans have been reported to have anti-inflammatory effects in gut of *C. carpio* challenged with *Aeromonas salmonicida* [69] and in spleen of *Oncorhynchus mykiss* challenged with bacterial LPS [70]. In addition, Falco et al. [69] found that the inflammatory process modulated by β-glucan could be organ-specific. In *D. chilensis*, the response of DGF to *E. coli* challenge varied with diet and with feeding condition. Fed SV mussels showed increased DGF upon challenge, while fed EG ones did not. In contrast, among starved mussels, only those of the EG group had increased DGF.

Inflammation in gill would be related to active immune responses as those observed in fed SV and EG mussels. Contrarily, the lack of digestive gland inflammation observed in fed EG mussels would favor nutrient transport and metabolic processes, as suggested for *C. carpio* gut [69]. This may explain the increased gill and digestive gland tissue protein content, and the higher muscle RNA/DNA ratio in fed EG mussels compared to SV ones (discussed in Section 4.3).

4.2. Oxidative balance

Oxidative burst has been reported to increase upon β-glucan and LPS challenge; however, duration and dose of the stimulus may modulate this response [11,18]. In this study, bacterial challenge significantly increases humoral ROS level in starved SV mussels and reduces humoral TOSC both in fed and starved mussels of this group. In contrast, both TOSC and ROS levels remain unaffected by bacterial challenge in EG mussels. The maintenance of humoral antioxidant capacity upon *E. coli* challenge in EG could explain, at least in part, the lower sensitivity of hemocytes to such stressful conditions. On the other hand, the decrease of TOSC levels upon starvation in both SV and EG mussels may be the result of the depletion of energetic reserves. A similar trend was reported for hemolymphatic superoxide dismutase and peroxidase activities in starved Sydney rock oysters (*Saccostrea glomerata*) [71]. Thus, it could be speculated that *E. gracilis* diet favors the maintenance of the hemolymphatic oxidative balance in *D. chilensis*, allowing the display of adequate responses even after five days of starvation.

The activation of detoxifying and antioxidant defenses protects tissues from oxidative damage during bacterial challenge and immune response [23]. In the scallop *Chlamys farreri*, injected β-glucans may enhance hemocyte antioxidant response, such as superoxide dismutase, peroxidase and myeloperoxidase upon temperature changes [72]. In the abalone, *Haliotis* sp (Gastropoda), bacterial challenge stimulated GST activity in several tissues, including gill and digestive gland [73]. Nevertheless, to the best of our knowledge there is no information about the modulating effect of β-glucans on the antioxidant responses of bivalves subjected to bacterial challenge.

In our study, fed SV and fed EG mussels showed significant activation of gill and digestive gland GST and CAT activities upon bacterial challenge. However, this response was not enough to avoid oxidative damage. In fed nonchallenged mussels, gill and digestive gland had significantly lower CAT activities and higher TBARS levels in EG than in SV, while differences in GST activity were organ-dependent. Thus, it seems that EG diet did not reinforce gill and digestive gland antioxidant capacity under the experimental conditions of this study.

On the other hand, exogenous antioxidants, such as flavonoids, provided by *E. gracilis* cells [6] may complement the action of antioxidant enzymes. This could explain the reduced activities of gill GST, and gill and digestive gland CAT, in fed nonchallenged EG mussels. The pronounced increase in gill and digestive gland lipid peroxidation as well as the decreased humoral TOSC caused by starvation in EG mussels may be explained by the loss of such exogenous antioxidants upon starvation. Furthermore, an increase in lipid susceptibly to oxidation due to the incorporation of high amounts of unsaturated fatty acids from *E. gracilis* cells [74], may not be discarded.

4.3. Metabolic condition

The RNA:DNA ratio has been used to assess nutritional condition and growth in fish [75] and bivalves [76,77]. It could be interpreted that the elevated RNA:DNA ratio of EG mussels reflected the energetic advantage of incorporating β-glucans and/or other highly nutritious compounds contained in *E. gracilis* cells [1–6]. The significant increase in gill and digestive gland protein content in fed EG mussels further suggests a higher nutritional status. Accordingly, Bai et al. [10] and Misra et al. [56] reported augmented specific growth rate after prolonged dietary administration of β-glucan, for fish and shrimps, respectively. Lopez et al. [78] suggested that degradation of β-glucans in the digestive gland by glucanases to produce energy favors the utilization of proteins for growth. However, up to date the mechanisms involved in β-glucans' growth promoting properties have not been elucidated. The increase of RNA:DNA ratio in fed challenged *D. chilensis* compared to the respective not challenged controls, might indicate that the

combination of SV or EG diet with *E. coli* would favor mussels growth through the input of a higher variety and/or quantity of nutrients. Alternatively, the synthesis of proteins related to the response against invading microorganisms [79] could also explain the RNA:DNA ratio increase in fed challenged mussels of both groups. The increased digestive gland protein content in challenged EG mussels could also respond to either higher nutritional status or activated synthesis of defensive proteins.

Although starvation was expected to reduce the RNA:DNA ratio [76,80], this ratio was similar in starved mussels of both groups compared with the corresponding fed individuals. This lack of effect could be explained by glycogen or lipid reserves mobilization [60]. Nevertheless neither muscle RNA:DNA ratio nor gill or digestive gland protein content were increased in response to bacterial challenge in starved mussels.

5. Conclusions

An *E. gracilis*-rich diet positively modulates *D. chilensis* immune response, avoiding the damage caused by exposure to *E. coli*. This modulation is mainly based on cellular responses, e.g. higher phagocytic capacity, enhanced hemocyte viability and increased number of hemocytes in target organs such as gill and mantle. Furthermore, *E. gracilis* supply favors mussels' proteins synthesis and humoral antioxidant capacity.

Our results suggest that *E. gracilis* cells may be applicable as a nutritional and protective diet complement suitable for filtering bivalves. However, starvation periods after supplying this diet should be avoided, since these could revert part of the acquired benefits, e.g. the increment of gill protein content induced by EG diet is lost upon starvation. Additionally, starvation exacerbates detrimental effects of EG, such as increased tissular TBARS levels.

Some immunological parameters of *D. chilensis* respond differentially to *E. gracilis*, while others respond similarly to *E. gracilis* and *E. coli*, with no further effect of combined stimuli. This suggests the existence of different types of immune receptors in freshwater mussels, which requires further study.

Acknowledgments

This study was supported by grants CONICET PIP 0282 and ANPCYT PICT 00214 to CML; ANPCYT PICT 1293 to IR and CONICET PIP 0283 and UBACYT 01/W290 to VC; with permission N° 815 from the National Parks Administration. We acknowledge material support from Centro de Ecología Aplicada del Neuquén and Cooperativa de Agua Potable y Saneamiento de San Martín de los Andes. The authors wish to thank Flavia Bieczynski and Santiago Franzoni for scuba diving. Special thanks to the anonymous reviewers who helped to improve this manuscript.

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