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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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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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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