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Effect of probiotic *Pediococcus acidilactici* on antioxidant defences and oxidative stress of *Litopenaeus stylirostris* under *Vibrio nigripulchritudo* challenge

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ABSTRACT

Antioxidant defences and induced oxidative stress tissue damage of the blue shrimp *Litopenaeus stylirostris*, under challenge with *Vibrio nigripulchritudo*, were investigated for a 72-h period. For this purpose, *L. stylirostris* were first infected by immersion with pathogenic *V. nigripulchritudo* strain SFn1 and then antioxidant defences: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (Gpx), Total antioxidant status (TAS), glutathiones and induced tissue damage (MDA and carbonyl proteins) were determined in the digestive gland at 0, 12, 24, 48 and 72 h post-infection (h.p.i.). In the meantime, TAS was also measured in the blood. Infection level of the shrimps during the challenge was followed by determining *V. nigripulchritudo* prevalence and load in the haemolymph of the shrimps. Changes in all these parameters during the 72-h.p.i. period were recorded for control shrimps and shrimps previously fed for one month with probiotic *Pediococcus acidilactici* MA18/5M at 10^7 CFU g^{-1} of feed.

Our results showed that immersion with *V. nigripulchritudo* led to maximal infection level in the haemolymph at 24 h.p.i. preceding the mortality peak recorded at 48 h.p.i. Significant decreases in the antioxidant defences were detected from 24 h.p.i. and beyond that time infection leaded to increases in oxidative stress level and tissue damage.. Compared to control group, shrimps fed the probiotic diet showed lower infection (20% instead of 45% at 24 h.p.i. in the control group) and mortality (25% instead of 41.7% in the control group) levels. Moreover, infected shrimp fed the probiotic compared to uninfected control shrimps exhibited very similar antioxidant status and oxidative stress level. Compared to the infected control group, shrimps fed the probiotic sustained higher antioxidant defences and lower oxidative stress level.

This study shows that bacterial infection leads to oxidative stress in *L. stylirostris* and highlighted a beneficial effect of *P. acidilactici*, suggesting both a competitive exclusion effect leading to a reduction of the infection level and/or an enhancement of the antioxidant status of the shrimps.

1. Introduction

In recent years, infectious and non-infectious disease and environmental pollution have seriously affected cultured shrimps [1]. Viral infection remains the main problem and is responsible for major economic loss in the aquaculture industry worldwide. Vibriosis has been also implicated as the cause of high mortality in juvenile penaeid shrimp [2]. For example, shrimp farming in New Caledonia today faces two diseases involving bacterial pathogens: “Syndrome 93” [3] and “Summer syndrome” [4]. Therefore the development of solutions for improved resistance and survival of shrimps in fluctuating environments and with pathogen infection is crucial to sustain the growth of the shrimp culture industry. Among the solutions proposed, the use of probiotic has shown promising results and is now widely accepted as a complementary tool for the alternative management of disease and for improving nutrition of aquatic animals [5].

All living organisms are under constant attack from free radicals, which can lead to serious cellular damage if produced in excess. Reactive oxygen species (ROS) are naturally produced in animals during normal aerobic metabolism [6]. Many stress conditions like temperatures at the edge of thermal windows of the specie or hypoxia lead to an increased production of ROS resulting in “oxidative stress” (OS) inside the cell (reviewed by Kassahn et al. [7]). For instance, superoxide is considered as the major free radical stress-produced by living cells [8]. However, ROS production also comes as part of the immune defence system and plays an important role in microbicidal activity [9]. In decapods crustaceans, haemocytes are involved in the immune response to pathogen infection via phagocytosis and melanin production through the prophenoloxidase system [10]. Phagocytosis is a common cellular defence response generally recognised as a central and important way to

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eliminate micro-organisms and foreign particles. Once a pathogen enters the haemolymph, NADPH-oxidase is then activated in the haemocyte of the host, which in turn reduces oxygen to the superoxide anion, subsequently leading to the production of ROS as hydrogen peroxide, singlet oxygen, hydroxyl radicals and numerous other reactive compounds [9]. This process, called respiratory burst, has been reported in several shrimp species, including *Litopenaeus stylirostris* [11]. However, although ROS play an important role in host defence, their over-expression and residual ROS can result in OS.

The antioxidant defence system of the organism, including enzymes such superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidise (Gpx), is set to maintain the lowest possible levels of ROS in the cell, and is recognised as an essential component of an organism's self-maintenance. Moreover, the antioxidant defence system and the immune system are closely linked in the response to pathogens [12]. Therefore the implication of the antioxidant defence system in the development of disease through its ability to limit OS induced by respiratory burst activity and/or deleterious effects of poor environmental conditions, even if poorly investigated in shrimp, may be crucial.

However, most of the studies on OS in shrimps are restricted to its presence and role during exposure to xenobiotics or environmental perturbations [6,13]. Experimental investigations on alteration of animal antioxidant defence system in pathogenic infectious disease remain scarce. Recent studies have shown changes in some antioxidant enzymes activity such as superoxide dismutase, catalase and glutathione peroxydase, and oxidative damages (lipid peroxydation) in various tissues in *Penaeus monodon* infected with WSSV [14,15]. In addition, several studies have also shown changes in SOD activity [16–18] and in SOD, Gpx and CAT expression following bacterial infection or virus challenges [19–21]. It has also been suggested that the antioxidant defences operate at a very much lower rate in infected shrimps despite the higher requirement for dismutation of harmful free radical formation during infection by pathogens [15].

Based on this claim, it has been proposed that dietary supplementation of products with antimicrobial and antioxidant properties may be a promising disease prevention option for increasing resistance of shrimps to pathogens [15]. For instance, Chiu et al. [22] reported that administering *Lactobacillus plantarum* can enhance the antioxidant status of *Litopenaeus vannamei* and could lead to increased resistance to *Vibrio alginolyticus* infection. However, except for SOD activity [23], up to day no study has been found reporting significant effect of such dietary products, including probiotics, on the antioxidant response in shrimps following pathogen challenges.

In a previous study we showed that one month feeding with a diet enriched with 1 g kg^{-1} of *Pediococcus acidilactici* MA18/5M resulted in an increased survival rate, changes in antioxidant enzymes activity and higher total antioxidant status (TAS) of *L. stylirostris* [24]. Moreover, this previous study also highlighted that after natural infection by *Vibrio nigripulchritudo*, probiotic-fed shrimps showed an increased survival rate and lower OS damages in their digestive gland. Therefore the present study was carried out in order to investigate, under controlled conditions, how probiotic *P. acidilactici* treatment influences antioxidant defences and OS development in shrimps experimentally challenged with *V. nigripulchritudo*.

2. Materials and methods

2.1. Experimental shrimps, impregnation period and feeding

The shrimps *L. stylirostris* used in our experiments were reared semi-intensively (without aeration) in earthen ponds (1000 m^2) at the Saint-Vincent station (Ifremer). Six hundred *L. stylirostris* juveniles (12.79 ± 2.72) were fished in earthen ponds, transferred to the laboratory and acclimated into 6 circular polyester tanks (capacity 1600 L) for one week prior to the beginning of the experiment. Each tank was continuously oxygenated by injection of high pressure air and supplied with seawater pumped into the lagoon with a water renewal rate of 200% per day. Temperature was measured continuously (every hour) using an automatic recording probe (Optic StowAway® Temp; Onset).

Tanks were then assigned to two different dietary treatments: four tanks were fed with a control diet, while the remaining two tanks received the probiotic diet. Shrimps were fed for one month with the two diets, distributed four times a day and provided *ad libitum*. The amount of feed was adjusted daily to minimise left-over feed. The water temperature was 26.0 ± 1.9 °C and salinity was $35^0/_{00}$ throughout.

2.2. Feeds and probiotic

Shrimps were fed an experimental feed processed in the laboratory as previously described [24]. The commercial probiotic preparation tested was Bactocell® PA 10 (Lallemand Animal Nutrition S.A., Blagnac, France) formulated with live *P. acidilactici* MA18/5M (Institut Pasteur, Paris, France). For the treated group, 1 g kg^{-1} of the probiotic (powder form) was top-coated on the pellets using 2% of fish oil as a carrier, giving a final concentration of 0.9×10^7 CFU of *P. acidilactici* per gram of diet. The control diet was also top-coated with 2% fish oil and, prior to use, checked for possible contamination by the probiotic strain. The feed was then stored in 5-litre boxes at 20 °C until use.

2.3. Challenge with V. nigripulchritudo by immersion

After one month of rearing, intermoult shrimps were transferred in the experimental room for immersion challenge with *V. nigripulchritudo*. The experimental challenge was made in a controlled area allowing the disinfection of the waste water before its release into the lagoon. Shrimps were assigned to twenty-four 300 L fibre glass tanks filled with 5 μm-filtered seawater, aerated and held at 27 °C. Shrimp were acclimatized for one week prior to the challenge test using a previously described protocol to reduce transfer stress and to lower physiological disturbances [25]. Shrimp were continuously fed at 2% of the tank biomass four times daily with one of the two diets according the treatment, and water was renewed continuously.

Shrimps were infected by immersion for 2 h with *V. nigripulchritudo*. A short contact period is preferred in order to prevent possible bacterial reinfection during the experimental survey [26]. The *V. nigripulchritudo* pathogenic strain SFn1 [4] was cultured beforehand in Marine Broth for 18 h at 30 °C under constant shaking, allowing the late exponential growth phase to be reached. The bacterial culture concentrations were evaluated by reading their optical density at 600 nm, as compared to a previously determined reference curve (data not shown). Three treatments were then carried out: control shrimps, infected control shrimps and infected probiotic shrimps. Shrimps were infected by inoculating tanks with 10^5 CFU ml^{-1} of the pathogenic *V. nigripulchritudo* strain. This density was shown in preliminary experiments to kill approximately half the population within 3 days (Goarant et al., unpub. results). At the end of this challenge, the tanks were emptied in order to remove the water-borne pathogen, and were immediately refilled with clean seawater.

For the susceptibility test 3 tanks each containing 20 shrimps were used for each treatment. Survival was tracked every 6 h over a 4-day period, as preliminary trials demonstrated that no significant mortality occurred subsequently (Goarant et al., personal communication).

For the determination of antioxidant defences and oxidative stress bio indicators following infection, tests were carried out on