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Oleic Acid and Diketopiperazines Produced by Marine Bacteria Reduce the Load of the Pathogen *Vibrio parahaemolyticus* in *Argopecten purpuratus*

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Abstract

The aquaculture industry must often deal in its cultures with bacterial contamination by *Vibrio parahaemolyticus*, which causes gastroenteritis in humans when they eat contaminated organisms. Until recently these pathogens were treated with antibiotics which are now forbidden because of their negative effects on humans and on the ecosystem. In the last few years there have been attempts to solve this problem by searching for active metabolites from antagonist bacteria. The objetive of this work was to evaluate the decrease of the load of the pathogen *V. parahaemolyticus* in *Argopecten purpuratus* scallops by the addition of oleic acid and diketopiperazines isolated from marine bacteria known for having an antibacterial effect against the pathogen, and with commercially available products similar to the molecular structures isolated from the bacteria. The decrease of the pathogen load was determined by the most probable number method from the absence in the samples of the gene tdh that codes for thermostable direct hemolysin (TDH), the main virulence factor in this species. The *A. purpuratus* bivalves treated with the oleic acid and diketopiperazines isolated from the bacteria showed preliminarily that they cause a reduction of the bacterial load of the *V. parahaemolyticus* pathogen. The same trend was seen when the scallops were treated with commercial oleic acid and diketopiperazines. Based on the inhibiting activity seen with the commercial products we suggest the possibility of experimenting with these products against *V. parahaemolyticus* or other pathogens in different commercially important organisms, mainly in depuration systems that require a short time (12 to 24 hours) to reduce the concentration of human pathogens like V. parahaemolyticus.

Keywords: Antibacterial; Oleic acid; Diketopiperazines; *Vibrio parahaemolyticus*; *Argopecten purpuratus*

Introduction

The marine ecosystem is a potential source of microorganisms that produce antibacterial compounds [1]. The search for marine microorganisms as producers of therapeutic agents began in the 20th century [2]. At present almost 10% of the 1.000.000 known biologically active metabolites are of microbial origin [3]. The first research wrongly attributed the active metabolites to marine eukaryotes, but later the activity of the microorganisms that live associated with them was recognized [4].

In the aquaculture industry pathogenic bacteria have become multiresistant to traditional antibiotics used as antifouling agents, antibacterials, antiparasitics, anesthetics, and disinfectants. The amounts of antibiotics used by these industries are not always available to the public [5], but it is known that their use has caused significant damage to marine ecosystems, resulting in an increase of clinical complications in cases that could previously have been treated successfully, longer hospitalization, and greater costs to society [6]. The possibility of implementing the use of bacterial active metabolites in the culture systems of commercial organisms through the direct injection of their extracts or live strains is an alterantive that is gaining importance due to the interest in optimizing the production of commercial organisms with environmentally fiendly alternatives. Growth, reduction of malformations, resistance to disease, and microbial equilibrium can be achieved through the modulation of the intestinal microbiota, increase of favorable bacteria, production of inhibiting compounds to compete with the pathogen, inhibiting the expression of virulence genes and improving immune response [7].

Bacteria of the genera *Vibrio* and *Bacillus* are known for their probiotic properties [8] detected 300 pathogen inhibiting bacteria of the family Vibrionaceae, and those with the highest activity were identified as *V. coralliilyticus, Vibrio neptunios* and *Photobacterium halotolerans*, from which two antibiotics identified as andrimid (from V. coralliilyticus) and holomycin (from *P. halotolerans*) were isolated . Mujeeb et al. [9] found inhibiting activity in bacteria of the genus Bacillus isolated from cultures of the shrimp *Macrobrachium rosenbergii* against pathogenic *Aeromonas hydrophila*, *Vibrio parahaemolyticus*, *Vibrio vulnificus*, and *Escherichia coli* bacteria. Similarly, Avella et al. [10] suggested beneficial effects on the growth of Sparus aurata fish by adding a mixture of bacteria of the genus *Bacillus* (*B. lincheniformis, B. pumilus* and *B. subtilis*). Somnath et al. [11] showed resistance to disease and survival in cultures of larvae of *Penaeus monodon* by the addition of probiotics (*Bacillus* MCCB 101, *Pseudomonas* MCCB 102/103 and *Arthrobacter* MCCB 104) that act against the pathogen *Vibrio harvey*, showing that *Bacillus* and *Arthrobacter* operate as probiotics through immunostimulating digestive enzymes. More recent studies by Qinghui et al. [12] show that diets supplemented with *Bacillus subtilis* in a dose of 1x107 CFU/g improve growth, feeding efficiency ratio, immune response and resistance to disease of juvenile *Larimichthys crocea*. However, the evaluation of the effect of the bioactive compounds from marine bacteria on the reduction of the pathogenic bacterial load in mollusks is scarce.

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Preliminary studies in our laboratory have identified metabolites active against the pathogenic bacteria *V. parahaemolyticus* from two marine bacteria: oleic acid from *Vibrio* sp. [1] and diketopiperazines from *Bacillus pumilus* [13]. Based on these data the present study has the purpose of verifying the anti-*V. parahaemolyticus* activity of oleic acid and diketopiperazines in the bivalve *Argopecten purpuratus* infected with the pathogen.

Materials and Methods

Source of the strains and product extraction

The inhibiting bacteria *Bacillus pumilus* (C32) and *Vibrio* sp. (C33), and the pathogenic *Vibrio parahaemolyticus* (strain PM48.5 who possesses the *tdh* gene) were obtained from the strain collection of the Laboratorio de Ecología Microbiana of the Universidad de Antofagasta. The three bacteria were kept in collections in Tryptone Soya Agar (TSA Oxoid Ltd., Basingstoke, Hampshire, England) culture medium supplemented with 2% NaCl (TSA Oxoid Co.) under axenic conditions at 20 ± 1°C and frozen in crioperls. *Vibrio* sp. was cultivated in minimum medium M9 [14] and *B. pumilus* in M9 medium modified (casamino acid 1 g l-1; Na_2HPO_4 1 g l^{-1}; KH_2PO_4 2 g l^{-1}; NH_4Cl 1 g l^{-1}; NaCl 4 g l^{-1}; DW 960 ml, 10 ml $MgSO_4$ (24 g l^{-1}); 10 ml $CaCl_2$ (1.5 g l^{-1}); 1 ml vitamin B1(10 g l^{-1}); 100 ml glucose (200 g l^{-1}); pH 4) at an initial concentration of 1×10^7 cells mL^{-1} at 20°C with constant stirring. Extraction of the antibacterial products was made at 96 hours of cultivation by adding 150 mL of ethyl acetate (Winkler ET-0790, Mexico) per liter of culture (bacteria+supernatant). The organic phase was recovered and allowed to stand for 20 min over sodium sulfate, (Merck, Germany), filtered through filter paper, concentrated to dryness in a rotavapor at 45°C, and placed for 24 hours in a lyophilizer to remove the moisture. The extract was stored at -20°C until its later use.

Evaluation of the best dose inhibiting bacterial product

The experiment was performed to determine the approximate dose of active product that provides the best inhibition of pathogenic bacteria. *V. parahaemolyticus* (*Vp*) was inoculated at an initial concentration of 1×10^5 cells mL^{-1} in test tubes with 10 mL of M9 culture medium. Growth was measured at 4, 8, 12, 24 and 48 hours by taking a 1 mL aliquot of each treatment and reading its spectrophotometric absorbance (600 nm). The concentrations used were the following: Vp+PC32 (*Vp*+1 mg mL-*1* of product from C32); Vp+C33 (*Vp*+1 mg mL^{-1} of product from C33), using Vp+M (*Vp*+methanol (Merck 1.07018.2511, Darmstadt, Germany) solvent with which the product was diluted) as control; Vp+C (*Vp*+1 mg mL^{-1} of commercial chloramphenicol antibiotic (Sigma-Aldrich CO378-25G, St. Louis, USA), an inhibitor of *Vp*). During the experiment the samples were kept at 20°C and sampling was made in triplicate.

Effect of the bacterial product on infected scallops

Previously acclimated *A. purpuratus* scallops were infected with *V. parahaemolyticus* (Vp) at a concentration of 1×10^5 cells mL^{-1} during 18 hours. Then, in covered plastic containers with 1 liter of seawater and 3 scallops (treated with UV radiation and filtered to 1 µm) to discard stress factors, the antibacterial product was inoculated at a concentration of 1 mg mL^{-1}. The treatments used were: CT (*Vp* without antibacterial product); P-C32 (*Vp*+1 mg mL^{-1} product from C32), and P-C33 (*Vp*+1 mg mL^{-1} product from C33). 24 hours after treating the infected scallops with the antibacterial products each organism was placed in a separate container with 100 mL of marine saline solution and they were blended with a hand blender. The presence of *V. parahaemolyticus* in the tissues of the mollusk was expressed by the most probable number (MPN) as described in the Bacteriological Analytical Manual of the Food and Drug Administration [15]. For that purpose each mixtrure was diluted into 3 series in triplicate in alkaline peptone water (APW) enriched culture medium (peptone 10 g, NaCl 10 g, distilled water 1000 mL, pH 8.4). The samples were incubated in a thermostatic bath at 37°C overnight, then 1 mL of each sample was boiled at 100°C for 15 min and centrifuged at 14.000 rpm for 10 min at 4°C. Finally, the presence of *tdh* (fwd 5'-GTA AAG GTC TCT GAC TTT TGG AC-3', rev 5'-TGGAAT AGA ACC TTC ATC TTC ACC-3') y *tlh* (fwd 5'-AAA GCG GAT TAT GCA GAA GCA CTG-3', rev 5'-GCT ACT TTC TAG CAT TTT CTC TGC-3') genes [16] of *V. parahaemolyticus* that indicate pathogenicity was determined qualitatively in agarose gels by the polymerase chain reaction (PCR).

Effect of oleic acid and diketopiperazine in infected scallops

In plastic containers with 50 L of seawater (previously treated with UV radiation and filtered to 1 µm) 22 scallops infected with *V. parahaemolyticus* (*Vp*) under the same conditions mentioned in the previous step were added. The experiment was conducted in a closed loop system of water. When the commercial antibacterial product 1 mg mL^{-1} of oleic acid (OA) (Sigma-Aldrich 364525-1L, St Louis, USA) and diketopiperazines (DKP) (Sigma-Aldrich 362557-5G, St Louis, USA) was added, the water level was reduced to 30 L, that will not affect the scallops, allowing the product to act for 40 min with manual stirring every 10 min, then the water level was restored to 50 L. The treatments were: CT (scallop uninfected and without antibacterial product); *Vp* (scallop with *Vp* without antibacterial product); C (scallop with *Vp*+chloramphenicol 1 mg mL^{-1}); DKP (scallop with *Vp*+diketopiperazine); OA (scallop with *Vp*+oleic acid). After 24 hours of the treatment of the infected scallops with the commercial antibacterial products, samples were taken in triplicate from each treatment and they were processed for the MPN method in the same way as in the previous step.

Statistical Analysis

The data were analyzed by one-way ANOVA [17] after determining fulfillment of the variance homogeneity assumptions (Bartlett's test), the normal distribution of the data (Kolmogorov-Smirnov test), the normality of the residuals (Anderson-Darling test), the independence and linearity of the model using the MINITAB 14 statistical software.

Results

Inhibition of the pathogen at different product concentrations

In our work the antibacterial product from strains C32 and C33 showed inhibition of the pathogen *V. parahaemolyticus* during the first 12 hours of culture, with significant differences with respect to the controls (cultivation of *V. parahaemolyticus* without the addition of products). At 24 and 48 hours an increase was seen in the cultures with the active products but lower than the controls (Figure 1).

Effect of the bacterial product on infected scallops

The MPN analysis of the infected *A. purpuratus* scallops treated for 24 hours with the antibacterial products showed a decrease of the pathogen load of bacteria C32 with 5.587 (MPN/100 g of scallop) and C33 with 2.634 (MPN/100 g of scallop) with respect to the control, 10.943 (MPN/100 g of scallop) (Figure 2).

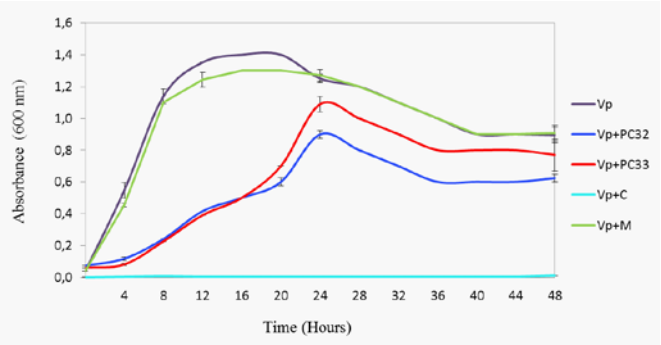

Figure 1: Effects of antibacterial products on the growth of the pathogen *V. parahaemolyticus*. **Vp** (*Vp* without antibacterial product). **Vp+PC32** (*Vp*+1 mg mL^{-1} of product from *Bacillus pumilus* C32); **Vp+C33** (*Vp*+1 mg mL^{-1} of product from *Vibrio* sp. C33); and **Vp+M** (*Vp*+methanol, solvent in which the product was diluted) as control; **Vp+C** (*Vp*+1 mg mL^{-1} of chloramphenicol) commercial antibiotic inhibitor of *Vp*.

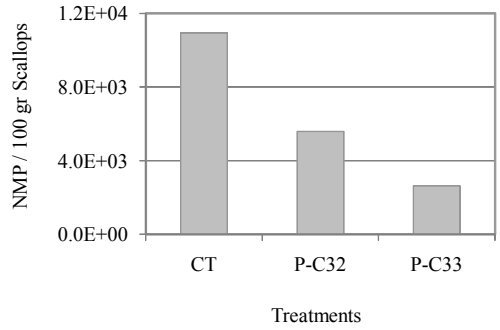

Figure 2: Evaluation of the inhibiting activity of the products isolated from the antagonist bacteria against the pathogen *V. parahaemolyticus* (*Vp*) shown by the decrease of the *tdh* gene indicator of the presence of the pathogen in the tissues of scallops. MPN (most probable number of pathogenic bacteria per 100 g of scallops); CT (*Vp* without antibacterial product); P-C32 (*Vp*+product from *Bacillus pumilus* C32), and P-C33 (*Vp*+product from *Vibrio* sp. C33).

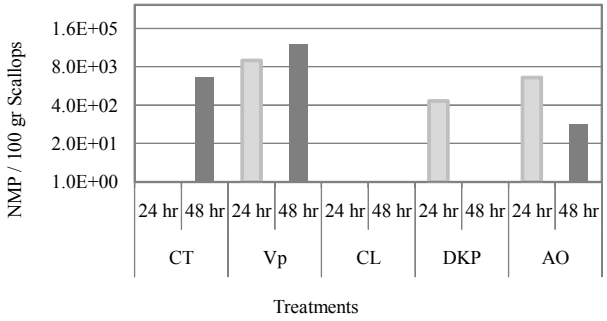

Figure 3: Evaluation of the inhibiting activity of the commercial products against the pathogen *V. parahaemolyticus* (*Vp*) shown by the decrease of the *tdh* gene indicator of the presence of the pathogen in the tissues of the scallop. MPN (most probable number of pathogenic bacteria per 100 g of scallops); CT (scallop uninfected and without antibacterial product); Vp (scallop with *Vp* without antibacterial product); CL (scallop with *Vp*+chloramphenicol); DKP (scallop with *Vp*+diketopiperazine); OA (scallop with *Vp*+oleic acid); hr (hours).

Effect of the commercial product on infected scallops

In the CT treatment (scallops uninfected and untreated with OA and DKP) the presence of the tdh gene was seen at 48 hours of culture, indicating that the scallops were bearers of the pathogen *V. parahaemolyticus*. The Vp treatment (scallops infected for 18 hours with the pathogen) shows that the scallops were infected and that the concentration of the pathogen had increased by 48 hours of culture. The CL treatment (scallops traeated with chloramphenicol) showed its good antibiotic effect as the presence of the *tdh* gene was not detected during the experiment, but it should be noted that 100% survival was seen in all the treatments and controls, except for the control treatment with cloramphenicol (CL), where 27% mortality was found at 24 hours and 100% mortality at 48 hours, showing the toxicity of chloramphenicol, in contrast with what was found in the treatments with the commercial OA and DKP, with which no mortality was seen in the treated scallops. The DKP treatment (scallops treated with commercial DKP) showed the best antibiotic effect against the pathogen, with a decrease from 46.736 (MPN/100 g of scallops) with the Vp treatment to 0 (MPN/100 g of scallops) with the DKP treatment at 48 hours of culture. The OA treatment also showed a decrease from 46.736 (MPN/100 g of scallops) with the Vp treatment to 91 (MPN/100 g of scallops) at 48 hours of culture (Figure 3).

Discussion

There is evidence of the beneficial effect of probiotic bacteria in culture systems. For example, García de la banda et al. [18] evaluated strains of the genus *Shewanella* in juveniles of *Solea senegalensis* (sole), finding that these bacteria affect the intestinal microbiota providing protection against the pathogen *Photobacterium damselae* subsp. *piscicida*. García de la banda et al. [19] tested the nutritional effects of *Shewanella putrefaciens* bacteria on the cultivation of *S. senegalensis* juveniles against the pathogen *P. damselae* subsp. *piscicida*, finding increased growth rates of the fish when the probiotic was inoculated fresh at a concentration of 1x109 cells/g. Al-Dohail et al. [20] mention that the probiotic *Lactobacillus acidophilus* is a good candidate for use as biocontrol against pathogenic bacteria such as *Staphylococcus xylosus, Aeromonas hydrophila* and *Streptococcus agalactiae* in cultures of *Claries gariepinus* fish juveniles. But once we have information on the probiotic effect of various bacteria it is necessary to identify the active metabolites to evaluate the feasibility of using them in culture systems.

The active products of bacteria *Bacillus pumilus* and *Vibrio* sp. used in this study were identified previously by Leyton et al. [1], but one of the problems found by the authors was due to the small amount of extracted product per liter of culture. Since the identified products are known and the objective of our work was to show the effectiveness of oleic acid and diketopiperazines in larger volumes, the work was done with commercially available compounds. Oleic acid is easy to obtain at a convenient price, but it was not possible to find the same diketopiperazine structures isolated by the authors, so the work was done with the most structurally similar DKPs available, whose cost was far higher than that of OA. The MPN results showed that the commercial DKP (Sigma-Aldrich 362557-5G) and OA (Sigma-Aldrich 364525-1L) reduced the load of the pathogen *V. parahaemolyticus* in *A. purpuratus* 48 h after treating the scallops with these products, with a reduction in the scallop's tissues of the tdh gene which codes for thermostable direct hemolysin (TDH), the main virulence factor in this species, and based on the preliminary results of the reduction of the pathogen in scallops treated with the products obtained from the bacteria, the reduction of the pathogen in the tissues of the scallop can be attributed to the OA and DKP molecules.

We report for the first time the benefits of using diketopiperazines and oleic acid to reduce the load of the pathogen *V. parahaemolyticus*

in adult individuals of *Argopecten purpuratus*, without affecting the viability of the bivalves. These results represent a contribution to the discussion by Bhatnagar and Kim [21], who state that marine microorganisms have not received the attention they deserve, and there is a very limited vision of the bioactive capacities and potential in the literature available to date. DKPs and their derivatives have previously been described as an interesting source of secondary metabolites with both antifungal and antibiotic bioactive properties [22]. For example, Fdhila et al. [23] found DKP activity against *Vibrio anguillarum* isolated from bacteria associated with cultures of *Pecten maximus*. The various acknowledged biological actions of DKPs make them more interesting for research on their clinical [24] and aquacultural potential. On the other hand, the antibacterial activity of OA is commonly attributed to the fact that its action mechanism is the inhibition of the synthesis of fatty acids [25]. For example, its inhibiting activity against the pathogenic oral bacteria *Streptococcus mutans* has been identified [26]. However, the possibility is open to do new research that will allow us to optimize the production of the metabolites generated by the bacteria for their use in culture systems. In this way the preparation of products on a larger scale for their later marketing would be facilitated. Merrifield et al. [7] state that current studies on the application of probiotics and prebiotics in aquaculture are quite varied, making it difficult to plan a strategy for their efficient application at a commercial level and suggest that future studies should be centered on providing practical applications on an industrial scale. Finally, in the CT treatment (scallops uninfected and untreated with OA and DKP) the presence of the *tdh* gene, indicating that the scallops were bearers of the pathogen *V. parahaemolyticus*. In this respect it should be mentioned that the presence of *V. parahaemolitycus* was reported in the Bay of Antofagasta during outbreaks in 1997 and 1998 [27].

Conclusions

Based on the inhibiting activity seen with the commercial products, we propose the possibility of testing these products against *V. parahaemolyticus* or other pathogens in different commercially important organisms, mainly in depuration systems that require short times (12 to 24 hours) to reduce the concentration of human pathogens like *V. parahaemolyticus*.

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