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Presence of Antimicrobial Activity in the Mucus of Chame Fish (*Dormitator latifrons*)

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The present work describes the presence of antimicrobial activity of chame fish mucus (*Dormitator latifrons*) over different Gram (+) and Gram (-) bacteria types. On this way, it was shown the inhibitory effect of chame mucus over 2 of 3 analyzed strains bacillus type. Furthermore, it was determined the existence of a strong inhibitory effect in chame mucus over *Vibrio vulnificus* and *Vibrio harveyi* strains. Also it was observed, though in lower magnitude, an inhibitory effect over a *Vibrio anguillarum* strain. The preliminary information obtained on this study suggests the presence of antibacterial agents in chame fish mucus, which could be used in the future with an application in animal and human health.

Key words: Chame, Mucus, *Vibrio, Bacillus,* Antibacterial activity.

Chame (*Dormitator latifrons*) belongs to the family Eleotridae, it is widely distributed along the Pacific coast of America, that comprises from the southern part of California to the northern part of Peru (Department of Lambayeque). In Ecuador, it is found in The San Lorenzo Estuary, the Esmeraldas Delta River, Chone Delta River, Portoviejo River, Guayas River and Santa Rosa Estuary. Because of the benefits this fish presents for aquatic farming, it is produced in an artisanal way especially in the Province of Manabi on traditional "chameras" (chame farms) (Bonifaz *et al.,* 1985). The present work deals with a microbiology framework related particularly to mucus glandular cells, they present a narrow bottleneck shape that extends to the inside of the animal dermis and it is broadly distributed among plane epidermis cells in chame. These cells secrete mucus; this glandular product meets multiple functions, they are: allowing the animal a better displacement on its medium, the mucus seems to accomplish the mission of expelling microorganisms, irritating substances. Chame's characteristic odor is due to the mucus. Thus, the mucus would accomplish an interspecific way of communication within shoals (Lagler *et al.,* 1984).

On natural conditions, marshes, favorite habitat for this fish, they represent open spaces where a good water quality is not provided, adding the abundant surrounding flora, this environment is suitable for pathogenic agents propagation, pejorative to these ecological niches, then through a hyper secretion of mucus on chame, its mucus

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glandular cells would block virus action and harmful bacteria.

It has been determined in other fish species, the presence of antimicrobial activity from protein extract in mucus (Subramaian *et al.,* 2008; Kuppulakshmi *et al.,* 2009), moreover the activity of certain enzymes such as L-amino-acid oxidase with antibacterial properties, it is put in evidence in black rock fish (*Sebastes schelegeli*) (Kitami *et al.,* 2007). These experiments show that the fish mucus can work as part of the fish innate immune system. This system would be the first defense barrier of the fish, protecting them in such way, against some microorganisms presented in their habitat. In other experiments, it is shown the presence of antimicrobial peptides found in mucus and epithelial cells of different species of fish such as pleurocidins in sole fish (Cole *et al.,* 1997; Douglas *et al.,* 2003), oncorhycin in trout (Fernandes *et al.,* 2004; Fernandes *et al.,* 2003), moronecidin in striped bass (Lauth *et al.,* 2002), myxinidin in hagfish (Subramanian *et al.,* 2009) and parasin in catfish (Park *et al.,* 1998).

The present work intends to show the presence of antibacterial activity in mucus secreted by epidermal cells in chame fish, using for this aim Gram (+) and Gram (-) bacteria. The use of both bacteria has the objective to demonstrate that antibacterial activity in mucus of chame has a broad action spectrum.

MATERIALS AND METHODS

Biological material

Fish used on this study were donated by a chame grow-out research facility located in the Rocafuerte Canton, Province of Manabi. Mucus samples were collected after obtaining them through fish epidermis massage, using surgical gloves; they were deposited in 1.5 mL tubes. Tested microorganisms for antimicrobial activity bioassays were strains identified as *Bacillus sp.* (1), *Bacillus sp.* (2), *Bacillus sp.* (3), *Vibrio harveyi*, *Vibrio alginolyticus*, *Vibrio anguillarum* and *Vibrio vulnificus*. Furthermore, it was also used the strain *Escherichia coli D31* donated by The National Center of Aquaculture and Marine Research "Edgar Arellano" (CENAIM as its acronym in Spanish) and The Services Center for Aquaculture (CSA, as its acronym in Spanish).

Bioassays of Antimicrobial activity through inhibition halos

Tubes containing a volume of 15-20 ml of Lennox broth were maintained in a 45°C (113 °F) water bath, to avoid its solidification. On the tubes were inoculated 2*106 bacteria and homogenized with the assistant of a vortex, to being immediately deposited in Petri dishes, assuring on this way bacteria growth in the whole volume of the media. Once the agar was solidified, five perforations were accomplished, 0.6 cm in diameter each, with a sterile Pasteur pipette. On the holes of every Petri dish were deposited 30 µl of sample (plasma) and 30 µl of a 1/10 dilution of the sample and a 1/100 dilution of the same sample. The 2 remaining wells stand for the bacteria growth control (adding sterile saline solution in the hole), and the bacteria growth inhibition compounded with Ethylenediaminetetraacetic acid (EDTA) at 5%. Petri dishes were incubated for more than 16 hours, necessary time for detection of bacteria growth inhibition halos around the wells.

Turbidimetric bioassay

The protocol for turbidimetric bioassays was the one performed by Tapia, 1997 but using Hepes buffer solution 0.1M at pH 7 for microorganisms' dissolution, as described in Lijima *et al.,* 2003. The bioassay was conducted with the addition of sample, bacteria and broth culture. In parallel, it was performed a positive control, a negative control and a control for inhibition effect using EDTA at 1% and 5%. The positive control was performed placing the bacteria and the culture broth. The negative control included a sample of lysate supernatant of circulating cells and the culture broth. Inhibition controls were accomplished using EDTA solution (1 % or 5 %) adding bacteria and broth culture. Each testing samples and controls were performed by triplicate. Results for each one of the strains are described below as absorbance raw values (each treatment) minus the value of absorbance in the negative control. As a means to estimate the capacity of inhibition in the different treatments, results are also presented as an inhibition percentage; the value of the inhibition percentage was obtained using the formula:

$$\text{Inhibition \%} = \frac{\text{Absorbance in treatment-Negative control}}{\text{Positive control}} \times 100$$

Statistical analysis

The statistical analysis was accomplished through one way ANOVA and then a comparison of mean values with Sheffe test, using the software Statistica.

RESULTS

In order to accomplish the evaluation of antimicrobial activity by inhibition halos and turbidimetric bioassays, it was necessary to establish the equivalence between absorbance and corresponding bacteria numbers through spectrophotometry. Starting from these analyses, it was established that the correspondence of an absorbance value of 1 was approximately equivalent to a density of 1.7 x 108 cells/ml of *Bacillus sp.* (1), 1.1 x 108 cells/ml of *Bacilus sp.* (2), 6.9 x 108 cells/ml of *Bacillus sp.* (3), 1.9 x 108 cells/ml of *V. harveyi*, 3.6 x 109 cells/ml of *V. alginolyticus* and 2.8 x 109 cells/ml of *V. anguillarum*, 7.2 x 108 cells/ml of *V. vulnificus* and 1.16 x 109 cells/ml of *E. coli (D31)*.

Once established the spectrophotometric relationship and the number of bacteria per mL, then the antibacterial activity analysis was performed. Results through inhibition halo of mucus in chame using as testing strain the bacterium *E. coli D31* showed the presence of antibacterial activity in the testing substance. Once the presence of antibacterial activity was demonstrated, it was necessary to perform bioassays by turbidimetric method, in order to quantify such activity in other studied strains.

Turbidimetric analysis of antibacterial activity accomplished over the strains identified as *Bacillus sp*. (1) in the raw mucus sample or in the mucus dilution 1/10 of chame, they presented absorbance values of 0.312±0.42 and 0.345±0.007. These values did not present significant differences ($p<0.05$) with respect to the obtained value of the growth control of this strain 0.379±0.50. Turbidimetric bioassays with strains identified as *Bacillus sp*. (2) treated with chame mucus presented absorbance values of 0.503±0.028. This value was significantly different ($p<0.05$) to the absorbance values obtained from strains of *Bacillus sp.* (2) treated with diluted chame mucus 1/10 (0.869±0.245) and the growth control of such strain (0.958±0.193). On the contrary, growth of *Bacillus sp.* (3) did not show significant differences ($p<0.05$) between the treatment with chame mucus (0.277±0.038), diluted mucus (0.272±0.012) and the growth control without treatment (0.272±0.015). The review of analysis of results is presented on figure 1.

Additionally, it was determined inhibition percentages for each values obtained with the different bacillus strains used on this work, they are presented on figure 2. Related to the presence of antimicrobial activity over Gram (-) bacteria, it was determined this activity against 4 different types of bacteria strains of vibrio genus. Thus, the growth measured as absorbance of bacteria identified as *V. algynoliticus* did not present significant differences ($p<0.05$) between the growth control treatment (0.255±0.013), raw chame mucus (0.290±0.08) and the diluted chame mucus 1/10 (0.277±0.055). For antimicrobial analysis performed with *V. anguillarum*, it was determined the level of absorbance in the growth control treatment (0.048 ±0.04), it presented significant differences with respect to the value obtained in the chame sample treatment (0.007±0.007). However, growth optical density values of *V. anguillarum* after the treatment with diluted chame mucus 1/10 (0.055±0.12) did not present significant differences ($p<0.05$) with respect to the growth control treatment. Absorbance result values in *V. vulnificus* growth subjected to the presence of raw mucus (0.11±0.020) and diluted mucus (0.065±0.013) presented significant differences ($p<0.05$) with respect to the control treatment. Also, it was possible to demonstrate significant differences in the absorbance obtained between *V. vulnificus* bacteria treated with chame mucus and chame diluted mucus samples 1/10. At the same time, optical density values in bacteria growth using the strain identified as *V. harveyi* treated with chame mucus (0.021±0.031) and diluted chame mucus (0.022±0.019) presented significant differences ($p<0.005$) in relation to the absorbance determined in the control treatment (0.182±0.012). Furthermore, it was not determined any significant difference ($p<0.05$) in the growth absorbance in bacteria identified as *V. harveyi* treated with chame mucus and diluted chame mucus 1/10 among them. The review of results determined over antibacterial activity of chame mucus in some strains of vibrio genus is presented on figure 3.

Inhibition percentage values found for

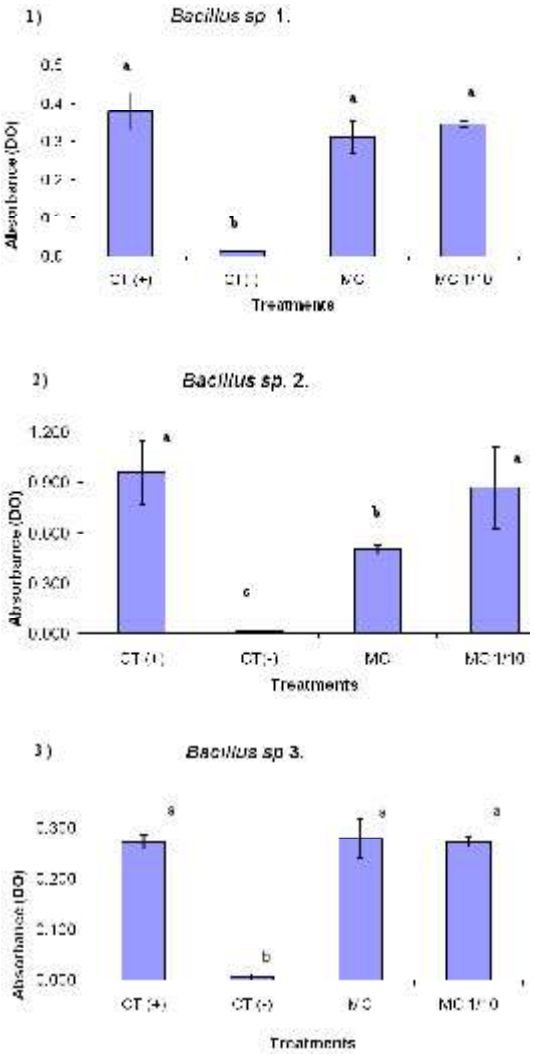

Fig. 1. Determination of bacteria growth through spectophotometry obtained at 650 çm in the growth of three bacillus strains. 1) Absorbance in the growth of *Bacillus sp.* 1 with different treatments. 2) Absorbance in the bacteria growth of the strain identified as *Bacillus sp.* 2 using different testing substances. 3) Absorbance in bacteria growth of *Bacillus sp.* 3 after its exposition to different substances. CT(+), Absorbance in growth of different bacillus without any treatment. CT(-), it represents the absorbance of bacteria growth of each one of the bacillus strains exposed to EDTA 1%. MC, it represents the absorbance in growth of different bacillus after being in contact with chame mucus. MC 1/10, it represents the absorbance in growth of different bacteria strains confronted respectively with diluted chame mucus 1/10.

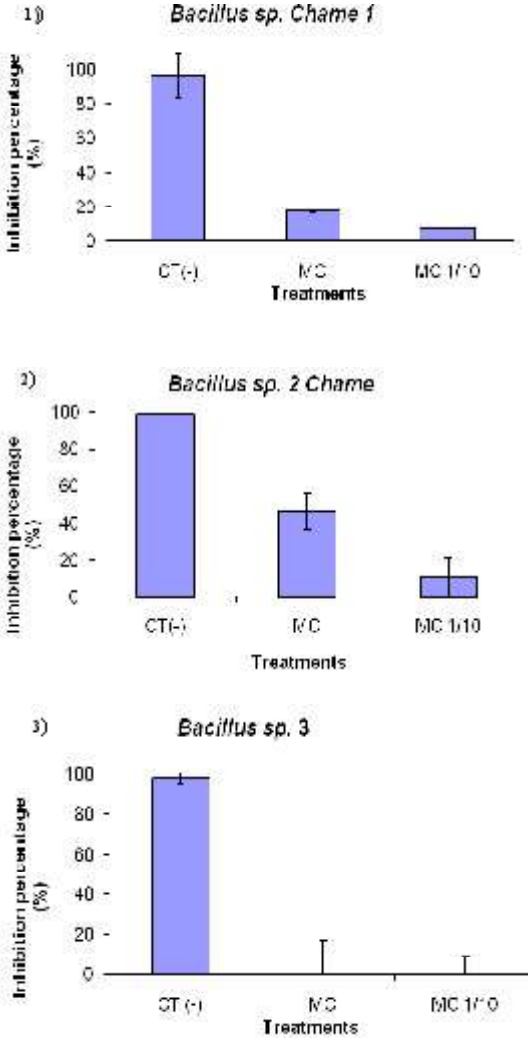

Fig. 2. Inhibition percentage of chame mucus against three different bacillus types. 1) Inhibition percentage determined against the strain identified as *Bacillus sp.* 1. 2) Inhibition percentage determined against the strain identified as *Bacillus sp.* 2. 3) Inhibition percentage determined against the strain identified as *Bacillus sp*. 3. CT (-), it represents the inhibition percentage of each one of the bacteria strains exposed to EDTA 1%. MC, it describes the growth inhibition percentage of bacteria strains after being in contact with chame mucus. MC 1/10, it represents the growth inhibition percentage of different bacteria strains confronted respectively with diluted chame mucus 1/10

each one of the strains of vibrio genus used on this study are presented on figure 4.

DISCUSSION

In order to establish suitable conditions for the accomplishment of antibacterial bioassays, it was determined through spectrophotometry the number of cells, according to the optical density for the different strains used on this study. Results in the control strain of this bioassay using *E. coli D31* presented a value 1.16 x 109 which it is closed to the theoretical expected value of 1.2 x 109 (Tapia, 1997), this validates the results obtained with different strains used on this bioassay.

The results obtained in the turbidimetric bioassays of antibacterial activity against Gram (+) strains present a slightly inhibitory effect of chame mucus over these strains. However, it could not be determined if a truly inhibitory effect exists over *Bacillus sp*. (1) and *Bacillus sp*. (3) strains, with low percentages of inhibition (17% and 10% respectively), they do not present values significantly different to the values in the control growth. This could be found in relationship with the sensibility of the technique, which probably could not permit to detect a low antibacterial activity or the absence of inhibitory effect of chame mucus over these strains. On the contrary, the inhibitory effect shown over Gram (+) strain *Bacillus sp*. (2) with the chame mucus was of 46%, these values are significantly different to those in the control growth, this demonstrates the existence of a truly inhibition. Thus, it is possible to infer that the chame mucus has an inhibitory effect over some Gram (+) bacteria strains Bacillus type.

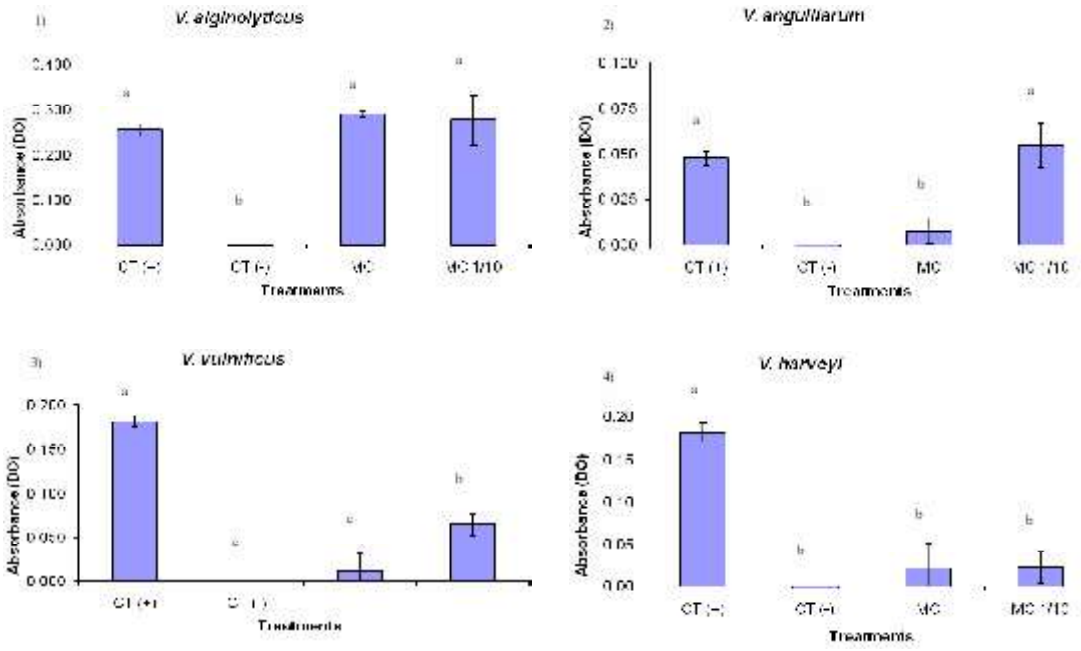

Fig. 3. Determination of bacteria growth through spectrophotometry obtained at 650 çm on t hree bacillus strains. 1) Absorbance in *V. alginolyticus* growth with different treatments. 2) Absorbance in bacteria growth with the strain identified as *V. anguillarum*, using different testing substances. 3) Absorbance in bacteria growth of *V. vulnificus*. 4) Absorbance in growth of *V. harveyi* after exposition to different treatments. CT (+), Absorbance in growth of different bacillus without any treatment. CT (-), it represents the absorbance in bacteria growth on each one of the bacillus strains exposed to EDTA 1%. MC, it represents the absorbance in growth of different bacillus after being in contact with chame mucus. MC 1/10, it represents the absorbance in growth of different bacteria strains confronted respectively with diluted chame mucus 1/10

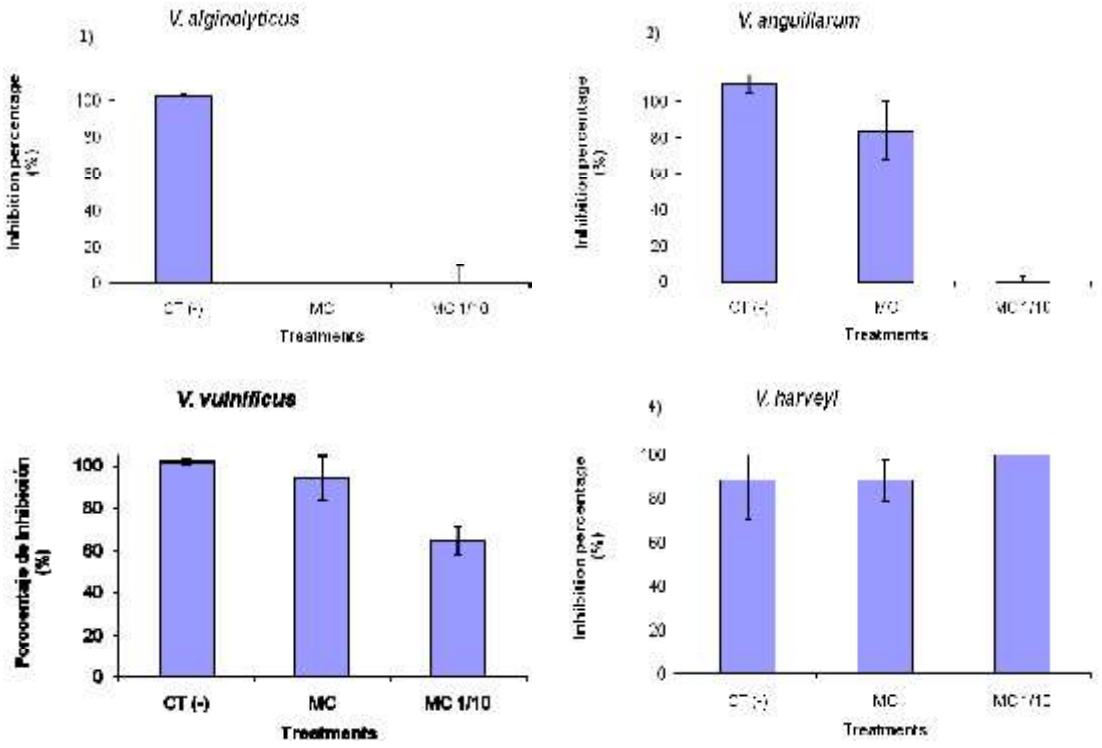

Fig. 4. Inhibition percentage in chame mucus against four different vibrio types. 1) Inhibition percentage determined against the strain identified as *V. alginolyticus*. 2) Inhibition percentage determined against the strain identified as *V. anguillarum*. 3) Inhibition percentage determined against the strain identified as *V. vulnificus*. 3) Inhibition percentage determined against the strain identified as *V. harveyi*. CT (-), it represents the inhibition percentage of every one of the bacteria strains exposed to EDTA 1%. MC, it describes the inhibition percentage in growth of bacteria strains after being in contact with chame mucus. MC 1/10, it represents the inhibition percentage in growth of different bacteria strains confronted respectively with diluted chame mucus 1/10

Regarding to Gram (-) vibrio strains used in the turbidimetric bioassays, it is clearly possible to determine a strong inhibitory effect of chame mucus over *V. harveyi* and *V. vulnificus* growth closed to 100% of inhibition. This activity was important over these strains even when diluted chame mucus 1/10 was used for the bioassays. Moreover, it was also detected an important antibacterial activity over *V. anguillarum* 83%, though this effect it is lost when the mucus is diluted 1/10. Finally, it was never detected antibacterial activity of chame mucus over *V. alguinolyticus* strain.

The presence of antibacterial activities of chame mucus over different bacteria Gram (+) and Gram (-) types, it implies that the inhibitory effect is of broad spectrum. On these instances, it is very difficult to determine the origin of this antibacterial activity found in the dermal secretions of chame fish. This could have different origins; one of those could be related to the presence of antibacterial peptides that could be presented in the dermal secretions as it has been shown in other fish either freshwater or marine fish. However, it cannot be discarded that such activity is possible due to the presence of some kind of enzyme or other component of different chemical nature that could be responsible of such activity. From other point of view, it is necessary to analyze that the antibacterial action put in evidence in the chame mucus does not only have its origin in only one component and it could be

the product of a synergism between different components.

From the physiological point of view, the present research gives indications about the resilience of this fish to develop itself in inhospitable places, and then the secretion of certain components in the chame mucus could confer some type of innate immunity. This immunity, as it was demonstrated on this research, could work in a selective way, based on the fact that some strains were not inhibited under exposition of chame mucus. This particular can indicate some type of physiological adaptation to certain environments in order to confer certain resistance to these animals, fact that allows defending themselves in a selective way against certain type of bacteria strains. Thus, it is possible to maintain the hypothesis, that no matter the origin of antibacterial activity put in evidence, this could be the evolutionary result of this fish to environments in which they inhabit.

The present research is according to our knowledge, the first attempt to establish the presence of antibacterial activity in a fish of the Ecuadorian littoral. Moreover, it presents the originality of the species. Because of their living conditions in an extreme habitat, it represents a good candidate for molecules search with antibacterial properties and probably original to the structure level.

CONCLUSIONS

The present research determined the presence of antimicrobial activity of chame mucus against Gram (+) and Gram (-) bacteria. Thus, it was possible to determine the inhibitory effect of chame mucus over strains codified as *Bacillus sp*. (2), *V. anguillarum*, *V. vulnificus* and *Vibrio harveyi*. This study opens new perspectives and basic sets, both to define the origin of this antibacterial activity and to determine its effect in the innate immune response of chame fish.

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