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Evaluation of the antibacterial activity of the skin mucus of the common octopus *Octopus bimaculatus* against pathogenic bacteria of farm animals

Evaluación de la actividad antibacteriana de la mucosidad de la piel del pulpo común *Octopus bimaculatus* contra bacterias patógenas de animales de granja

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ABSTRACT

Studies were conducted to evaluate the antibacterial potential of skin mucus collected from the common octopus *Octopus bimaculatus* against certain pathogenic strains for crustaceans, mollusks, fish, and bovine; *Escherichia coli*, *Staphylococcus aureus*, *Vibrio harveyi*, *Vibrio parahaemolyticus,* and *Staphylococcus pasteuri*. The antibacterial activities were measured in terms of inhibition halos in mm and compared with two antibiotics amikacin and chloramphenicol. The amount of protein in octopus mucus was also estimated in mg mL^{-1}. Inhibition halos produced by three different mucus concentrations were observed for the selected pathogens, except for the *V. parahaemolyticus* strain, which showed no inhibition at the lowest concentration. Furthermore, at the highest concentration, strain *V. parahaemolyticus* was the least inhibited, with a diameter of 3.9 ± 0.2 mm. The two antibiotics inhibited all strains, with the *E. coli* strain being the most inhibited. Therefore, these results have demonstrated that mucus extracted from octopus skin exhibits antibacterial activity and that they contain proteins. These components could be subjected to purification processes for potential use as an alternative to antibiotics in the control of pathogens in both aquaculture and terrestrial facilities.

KEY WORDS: Cephalopods, Protein, Inhibition, Antibacterials.

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RESUMEN

Se realizaron estudios para evaluar el potencial antibacteriano de la mucosidad de la piel recolectada del pulpo común *Octopus bimaculatus* contra ciertas cepas patógenas para crustáceos, moluscos, peces y bovinos; *Escherichia coli*, *Staphylococcus aureus*, *Vibrio harveyi*, *Vibrio parahaemolyticus* y *Staphylococcus pasteuri*. Las actividades antibacterianas se midieron en términos de halos de inhibición en mm y se compararon con dos antibióticos amikacina y cloranfenicol. También se estimó la cantidad de proteína en la mucosidad del pulpo en mg mL^{-1}. Se observaron halos de inhibición producidos por tres diferentes concentraciones de mucosidad en los patógenos seleccionados, con la excepción de la cepa *V. parahaemolyticus*, que no mostró inhibición en la concentración mínima. Además, en la concentración más alta, la cepa *V. parahaemolyticus* fue la menos inhibida, con un diámetro de 3.9 ± 0.2 mm. Los dos antibióticos inhibieron todas las cepas, siendo la cepa *E. coli* la más inhibida. Por lo tanto, estos resultados han demostrado que la mucosidad extraída de la piel del pulpo exhibe actividad antibacteriana y que contienen proteínas. Estos componentes podrían ser sometidos a procesos de purificación para su potencial utilización como una alternativa a los antibióticos en el control de patógenos tanto en instalaciones acuícolas como terrestres.

PALABRAS CLAVE: Cefalópodos, Proteína, Inhibición, Antibacteriano.

Introduction

Marine ecosystems are very complex, and organisms are known to possess bioactive compounds for defense and for the protection of eggs and embryos. Some have chemical mechanisms to synthesize compounds *de novo* to protect themselves from pathogens (Lauritano & Ianora, 2020).

Currently, about 16,000 natural products have been discovered from marine organisms, for example, in 1949 the first antibacterial product of marine origin, Cephalosporin C, was obtained from a strain of marine fungus Cephalosporium (Pandey, 2019). Among marine organisms, marine invertebrates offer a good source of potential antibacterial drugs (Di Costanzo *et al*., 2019; Lauritano *et al*., 2020).

Cephalopods are found in all marine habitats of the world, such as benthic-cryptic; epibenthic, and pelagic in bays and the open sea. However, cephalopods do not have a shell to protect themselves, so they use chemical means, coloration change, and toxins (Monolisha *et al*., 2013). Cephalopod skin consists of two layers, the epidermis, and the dermis. These layers cover the external surface of the body showing different regional specializations, such as suckers

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and arms. In addition, the skin is continued with the internal surfaces of the siphon and paleal cavity (Anadon, 2019).

To evaluate the condition of the animals, it is essential to carry out various tests on their organs, including the skin. The characteristics that should be noted on the skin are the presence of desquamation, hematomas, ulcers, and mucosal hypersecretion (FAO, 2011). The skin of most organisms contains mucosal epithelia with important antiparasitic, antifungal, antibacterial, and antiviral properties (Fast *et al*., 2002). Currently, marine and terrestrial farms present problems arising from diseases transmitted by pathogenic bacteria (OIE, 2019; Prachumwat *et al*., 2020; Deng *et al*., 2020; de Lorgeril *et al*., 2018; Palomares-Reséndiz *et al*., 2021; Rivera-Benitez *et al*., 2014; Burniston *et al*., 2015; Giraldo-Cardona *et al*., 2019, Pulido-Villamarín *et al*., 2021). For this reason, this research focuses on the skin of octopuses mainly in the study of mucus (González-Costa *et al*., 2020; Villanueva *et al*., 2021).

Recently, there have been many studies on the antibacterial properties of skin mucus from many species of marine organisms against various pathogenic microorganisms for humans and fish (Wei *et al*., 2010; Bragadeeswaran & Thangaraj, 2011; Vennila *et al*., 2011; Fuochi *et al*., 2017; Reverter *et al*., 2018), of freshwater fishes (Nwabueze, 2014; Pethkar & Lokhande, 2017), marine fishes and catadromous fishes (Fuochi *et al*., 2017; Pethkar & Lokhande, 2017), elasmobranchs (Coelho *et al*., 2019; Ritchie *et al*., 2017), anemones (Subramanian *et al*., 2011; Stabili *et al*., 2015), mollusks (Pales Espinosa *et al*., 2013; Kamiya *et al*., 2006; Suárez *et al*., 2021).

The main components of the mucus are glycoproteins and proteoglycans, and to a lesser extent, proteolytic enzymes, lysozymes, immunoglobulins, etc. Unlike other mollusks (Smith, 2002), the chemical nature of mucus in the genus Octopus is limited (Smith & Morin, 2002).

Each species has its own behavior and habitat, lives in different types of aquatic environments, and consumes different types of food, which may influence the amount of mucus secretion and its components within the same species or between different species and may be useful to provide a variety of immune responses. Therefore, the aim of the present study was: To analyze the antibacterial effect of the skin mucus of the common octopus *Octopus bimaculatus*, against a collection of pathogenic strains for terrestrial and aquatic farm animal collection.

Material and Methods

Specimen Collection

Five octopuses of the genus *O. bimaculatus* weighing approximately 500 to 700 grams were captured in the community of El Sauzoso, B.C.S. Mexico (24°18’44.5 “N 110°38’26.0 “W) with traps. They were placed in tanks on board of the boat with clean, sterile water. They were washed with a 4% solution of potassium permanganate ($KMnO_4$) before being placed in the tanks and before mucus collection. No chemicals or anesthesia were administered to the octopuses for skin mucus collection. Mucus was collected from five octopuses. Mucus was carefully scraped

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from the body surface by moving a sterile plastic spatula in an anteroposterior direction, from the head toward the tentacles, and mucus was collected at regular intervals (10 attempts in 3 hrs). Accumulation of mucus from the ventral area was avoided to eliminate intestinal and urogenital contamination (Chong *et al*., 2005). The mucus samples were brought on ice to the laboratory where they were centrifuged at 13,200 rpm for 10 min at 4°C. The supernatant was placed in a sterile tube and centrifuged again on ice at 11,000 rpm for 30 min. The precipitate was discarded, and the raw mucus extract was stored at -70°C before analysis.

Preparation of raw octopus mucus

Raw octopus mucus was treated with SIGMA® lysozyme enzyme at a final concentration of 10 mg mL^{-1} in phosphate buffer pH 7.0. The mixture was incubated at optimum pH and temperature for the enzyme to break the peptidoglycan layer of the bacterial cell wall for 2 hrs. The antibacterial activity was performed by a diffusion method in Petri dishes with Müller-Hinton agar using wells (Guerra & Pastrana, 2002).

Reactivation of pathogenic strains

The strains used for the *in vitro* antibacterial evaluation of the prepared octopus mucus were: *Escherichia coli* (Amador, 2018), *Staphylococcus aureus* (Unpublished data), *Vibrio harveyi* (Marino, 2012), *Staphylococcus pasteuri* (Amador, 2018), *Vibrio parahaemolyticus* (Amador, 2021) (Table 1). All bacterial strains were obtained from the collection of the Food Science and Technology Laboratory of at Autonomous University of Baja California Sur.

Bacterial strains were cultured according to the protocols and microbiological safety conditions of Eder *et al.* (2009). Each strain was incubated at 37 °C in nutrient broth (0.5% peptone, 0.5% NaCl, 0.3% meat extract, distilled water, pH 6.8 at 28 °C) for 18-24 h. Subsequently, a viable count of each culture was made and colony-forming units per mL (CFU/mL) were calculated (Barbosa *et al*., 1995).

Protein estimation

The amount of protein in octopus mucus was determined using the method of Bradford Kruger (2009).

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Table 1. Strains from the collection of the Food Science and Technology Laboratory of at Autonomous University of Baja California Sur.

Isolated	Genus	Species	Place
Oyster	*Escherichia*	*coli*	BCS, México.
Bovine	*Staphylococcus*	*aureus*	BCS, México.
Shrimp	*Vibrio*	*harveyi*	BCS, México.
Oyster	*Staphylococcus*	*pasteuri*	BCS, México.
Shrimp	*Vibrio*	*parahaemolyticus*	BCS, México.

Petri dish diffusion assay with wells in agar

The antibacterial effect of octopus mucus (OM) extracts on the selected bacterial strains was analyzed by the agar well diffusion method (Valgas *et al*., 2007). Petri dishes were prepared with 15 mL of Müller-Hinton agar and then the bacterial strains (Table 1) were seeded by surface spreading with $1x10^7$ CFU mL^{-1}. Subsequently, wells with a diameter of 6 mm were aseptically punched with a tip and extracts of the prepared octopus mucus were added (10, 50, and 100 µL mL^{-1} of mucus extract per well), as positive controls the antibiotics amikacin and chloramphenicol were used at a concentration of 40 µg mL^{-1} and 20 µg mL^{-1}, respectively, and physiological saline solution (0.85% NaCl) was used as a negative control. The Petri dishes already inoculated and with the extracts were incubated at 37 °C for 24 h. The evaluation of the bactericidal effect was performed by measuring the diameter of the IH formed around the well (Jorgensen & Turnidge, 2015). The diameter of the IH was measured in millimeters (mm) with a vernier, the measurement was made by taking the total diameter of the halo minus the 6 mm of the well. The results of antibacterial activity were compared with positive controls.

Statistical analysis

Results were subjected to Barlett's homoscedasticity test and D'Agostino-Pearson normality test with an $\alpha = 0.05$ and then a one-way analysis of variance (ANOVA) was performed to compare significant variation in octopus mucus and antibiotics. Determination of factors contributing to significant differences was performed using the LSD multiple comparison test (Sokal & Rohlf, 1980).

Results and Discussion

Octopus mucus may represent a good source of biologically active compounds to be used for different biotechnological purposes. The biological interface between the octopus and its marine environment consists of a mucus layer composed of biochemically diverse secretions of epidermal and epithelial cells (Accogli *et al*., 2017). It has been suggested that mucus secretion serves an antibacterial purpose, and also helps to collect bacteria from the surrounding waters, and

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promotes the accumulation of microbial cells, including *Vibrio* and other marine microorganisms (Troll *et al*., 2010).

The effects of a new antibacterial peptide (OctoPartenopin), extracted from the suckers of *O. vulgaris*, against *Pseudomonas aeruginosa*, *Staphylococcus aureus,* and *Candida albicans* were also demonstrated *(*Maselli *et al*., 2020). In this research, octopus mucus was used to inhibit the growth of some strains of pathogenic bacteria, where the higher the concentration, the greater the inhibition.

In another study, in *vitro* and *in vivo* assays were conducted using octopromycin, a peptide derived from *Octopus minor* against *Acinetobacter baumannii,* and revealed that infected fish exhibited a significantly higher relative survival percentage of 37.5 % than infected fish treated with (placebo)PBS 16.6 %, (Rajapaksha *et al*., 2021).

Soluble proteins

The protein content in the mucus of the selected octopuses varied very little. The general trend of protein content in the epidermal mucus of the species was 3.684 mg mL, while in another research a protein content of 1.0716 mg mL^{-1} for *O. dolfusii* and 1.3620 mg mL^{-1} for *O. aegina* was found (Monolisha *et al*.,2013). However, no further information on protein quantification in octopus mucus was found, but in fish, it was shown that raw and partially purified epidermal mucus from *Tachysurus dussumieri* presented a protein content of 0.48 ± 0.02 mg mL^{-1} and 0.82 ± 0.05 mg mL^{-1} (Arulvasu *et al*., 2012). Similarly, protein was found to be the major component in different skin mucus extracts of *Channa micropeltes*, *Cytusis striatus*, *Oreochromis niloticus,* and *Mystus nemurus*, which ranged from 4.32 ± 0.28 to 5.79 ± 0.32 mg mL^{-1} (Rao *et al*., 2015). Possibly due to the high protein content in the mucus extract of *O. bimaculatus* had a higher IH against the pathogens selected in this study, as mucus secretion has also been suggested to have an antibacterial purpose (Troll *et al*., 2010), and in addition to lectins, uncharacterized glycoproteins have also been associated with binding to bacteria in the hemolymph of *Octopus vulgaris (*Rögener *et al*., 1987).

Bacterial antagonism assay

The extract of raw octopus mucus and the two antibacterials, chloramphenicol and amikacin, exhibited a strong bactericidal effect against all selected pathogenic strains except for the pathogen *V. parahaemolyticus.*

In the bioassay, the inhibition of bacterial strains was evaluated using different concentrations of mucus and specific antibiotics. The results showed inhibition halos (IH) (Table 2), where it was observed that at the mucus concentration of 10 µL $mL^{-1,}$ there was a higher IH for the *E. coli* strain with 19.168 ± 3 mm, the lowest halo for *S. aureus* with 8.0 ± 0.4 mm and no IH for the *V. parahaemolyticus* strain. At the concentration of 50 µL $mL^{-1,}$ the highest IH was for *E. coli* with 20.21 ± 0.4 mm, and the lowest was for *V. parahaemolyticus* 1.56 ± 0.02. At the concentration

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of 100 µL mL^{-1} inhibition was shown in all strains, the highest halo was presented by the *S. aureus* strain with 14.2 mm ± 0.5 mm and the lowest halo was for *V. parahaemolyticus* with 3.9 ± 0.2 mm. The antibiotic chloramphenicol 20 lg mL^{-1} showed the highest inhibition halo for *E. coli at* 14.6 ± 0.5 mm and the lowest for *S. aureus* with 2.12 ± 0.1 mm and the antibiotic amikacin 40 lg mL^{-1} showed the highest IH for *S. aureus* strain with 8.4 ± 0.6 mm and the lowest for *V. parahaemolyticus* with 4.0 ± 0.2 mm.

Table 2. Halos of inhibition were shown by selected bacteria exposed to the three different concentrations of octopus mucus and two commercial antibiotics.

	Octopus mucus concentrations HI (mm)			HI antibiotics (mm)	
	10 µL mL-1	50 µL mL-1	100 µL mL-1	Chloramphenicol 20 lg mL-1	Amikacin 40 lg mL-1
Strains					
E. coli	19.168 ± 0.3	20.21± 0.4	5.52 ± 0.4	14.6 ± 0.5	5.52 ± 0.4
V. parahaemolyticus	0	3.9 ± 0.2	3.9 ± 0.2	2.4 ± 0.2	4.0 ± 0.2
S. pasteuri	10.32 ± 0.9	10.5 ± 0.45	12.7 ± 0.7	4.4 ± 0.43	5.52 ± 0.4
V. harveyi	8.82 ± 0.3	10.2 ± 0.5	12 ± 0.9	4.4 ± 0.3	6.04 ± 0.5
S. aureus	8.0 ± 0.4	10 ± 0.5	14.2 ± 0.5	2.12 ± 0.1	8.4 ± 0.6

HI= Inhibition halos. Halo Diameter of the inhibition halo minus the diameter of the well with an antibacterial.

The five pathogenic strains presented IH in the presence of the two antibiotics. With chloramphenicol 20 µg mL^{1}, the highest IH was of the following strains: *E. coli* with 14.6 ± 0.5 mm, *V. harveyi* with 4.4 ± 0.4 mm, *S. pasteuri* with 4.4 ± 0.43 mm and the lowest of *S. aureus* with 2.12 ± 0.1 mm. With amikacin 40 lg mL^{-1} the highest IH was presented by the *S. aureus* strain with 8.4 ± 0.6 mm, followed by *V. harveyi* with 6.04 ± 0.5 mm, *E. coli* and *S. pasteuri* with 5.52 ± 0.4 mm and the lowest was *V. parahaemolyticus* with 4 ± 0.2 mm.

The results of the inhibition halos of the selected bacterial strains due to mucus at a concentration of 10 µL mL^{-1} showed significant differences ($p > 0.05$) among all strains, however, *E. coli* was the most inhibited. At the concentration of 50 µL mL^{-1} there were no differences between the strains *S. pasteuri*, *V. harveyi*, *S. aureus*, but there were differences against *V. parahaemolyticus* and *E. coli* ($p > 0.05$) with the latter having the highest IH of 20.28 mm. With the 100 µL mL^{-1} mucus significant differences ($p > 0.05$) were shown between almost all strains: *S. aureus* and *V. harveyi* and the *E. coli* strain had the highest IH of 21.44 mm, except for *S. pasteuri.* The antibiotic chloramphenicol 20 lg mL^{-1} did not cause significant differences between the IH of the strains *S. pasteuri* and *V. harveyi*, between *V. parahaemolyticus* and *S. aureus*, but it did cause significant differences between these groups and against *E. coli, the* latter presenting the highest IH of 14.8 mm. The antibiotic amikacin 40 lg mL^{-1} did not cause significant differences between the IH of *E. coli* and *V. harveyi* between *S. pasteuri* and *S. aureus*, but these two groups did against *V. parahaemolyticus* ($p > 0.05$), here the strain with the highest IH was *S. aureus* with 8.4 mm.

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Importantly, the *V. parahaemolyticus* strain was the least inhibited at all three concentrations and antibiotics.

Other reports have shown the antimicrobial activity of aqueous and ethanolic extracts of mucus from different marine organisms. Antimicrobial activity assays carried out with ethanolic extracts of tissues from *Octopus dolfusii* and *Octopus aegina* organisms against *V. parahaemolyticus* showed IH of 34 and 28 mm on average respectively (Monolisha *et al*., 2013).

Likewise, in methanolic extracts of *O. dollfusi* tissue, the highest IH of 17 mm was observed against *E. coli*, another extract of *O. aegina* showed an IH of 15 mm against *V. parahaemolyticus* and another of *O. aerolatus* against *S. aureus* presented IH of 13 and 14 mm and the lowest IH of 8 mm was observed against *Streptococcus* sp. with extracts of *O. dollfusi*, *O. aegina* and *O. aerolatus* (Pasiyappazham *et al*., 2011). In this study, the surface mucus of *O. bimaculatus* against different pathogens showed the highest IH against *E. coli* of 21.44 mm, against *S. aureus* of 14.2 mm, against *S. pasteuri* of 12.75 mm and against *V. harveyi* of 12.36 mm. The lowest IH was 4.6 mm in *V. parahaemolyticus*, similarly, Monolisha *et al*. (2013) using ethanolic extracts of *O. dolfusii* tissue against *V. anguillarum*, *V. alginolyticus*, *V. sclintis,* and *V. harveyi* reported IH of about 27, 22, 21 and 15 mm respectively, also used extracts of *O. aegina extracts* against the same pathogens reported IH of 25, 21, 21 and 18 mm respectively, which suggests that the genus *Octopus* presents antibacterial activity in the superficial mucus and could also be in all the internal mucosa of these organisms. Similar studies with extracts of carp mucus showed that the maximum inhibitory effect of carp mucus *Hypophthalmichthys nobilis* against *E. coli* reached an IH of 31.00 ± 0.47 mm, that of *Ctenopharyngodon idella* against *P. aeruginosa* was 34.33 ± 0.13 m, significantly higher than amikacin 33.33 ± 0.13 mm and that of *O. bimaculatus* against *S. aureus* ranged from 10.32 to 12.75 mm (Kumari *et al*., 2019). It was also reported that *S. aureus* was more susceptible than other Gram-negative bacteria to growth inhibition by gastropod extracts (Benkendorff *et al*., 2001), however, no antibacterial activity against *E. coli* was detected in *Mytilus galloprovincialide* hemolymph (Hubert *et al*., 1999). It has been suggested that the resistance of *E. coli* to antibacterials was due to the complex structure of the Gram-negative cell wall, particularly the lipopolysaccharides of the outer membrane, which excludes most of the active compounds (Im & Khalid, 2020).

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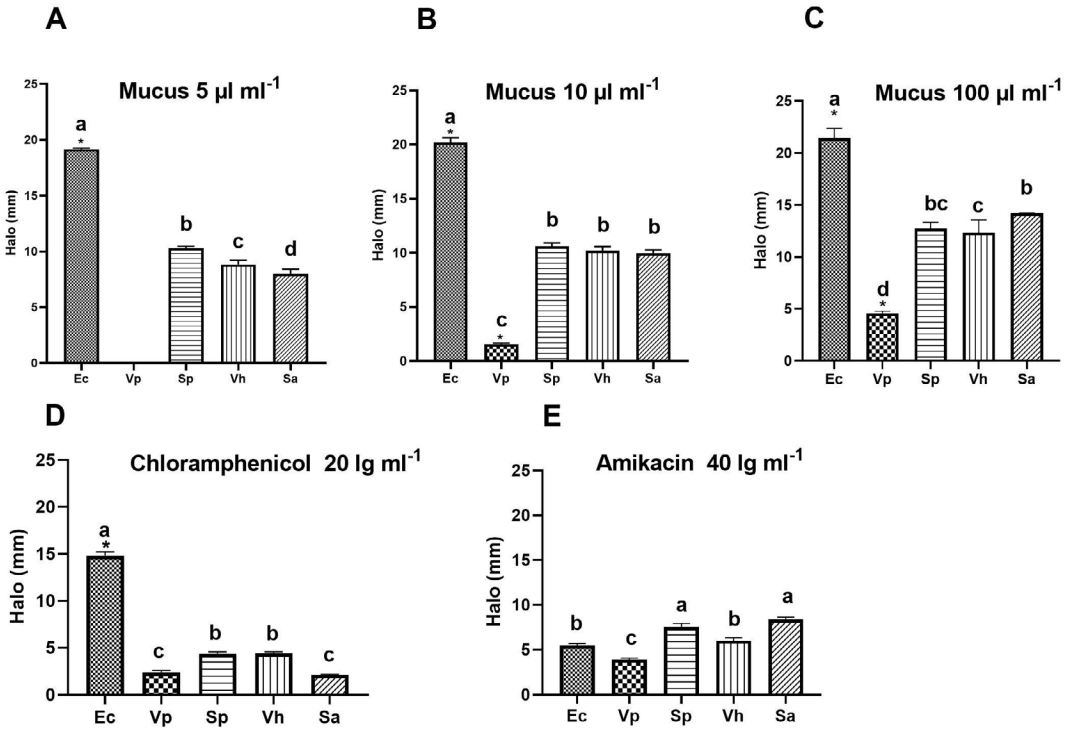

Figure 1. Halo of inhibition (HI) caused by the octopus mucus extract and two antibiotics on the growth of different selected pathogenic microbial strains. Ec (*E. coli*), Sa (*S. aureus*), Vh (*V. harveyi*), Sp (*S. pasteuri*), Vp (*V. parahaemolyticus*).

Identical letters denote no significant differences ($p > 0.05$). In Figure A, there are significant differences between all strains (a) Ec, (b) Sp, (c) Vh, and (d) Sa. Figure B, letter (b) Sp, Vh, Sa there are no differences between them; (a) Ec and (c) Vp means significant differences against a. In figure C, Sp (bc), Sa (b), and Vh (c) there are no differences between them, but there are significant differences (a) Ec and (d) Vp. Figure D no differences between (b) Sp and Vh, (c) Vp and Sa, and significant differences compared to (a) Ec. Figure E, no differences between (a) Sp and Sa, and significant differences in comparison with (c) Vp ($p > 0.05$) (Tukey HSD).

Conclusions

In the present study, it has been demonstrated that the skin mucus extract of the cephalopod *O. bimaculatus* has antimicrobial activity against bacteria pathogenic to fish, crustaceans, mollusks, and bovine, and it has been determined that the extract is rich in protein. This suggests that the mucus extract or subsequently purified antimicrobial compounds can be used as an alternative to antibiotics and could perhaps be used in aquaculture and terrestrial farms for chickens, bovine, etc. Since this extract is a natural product, it would help to reduce the problems of resistance to antibiotics that we currently have.

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Authors' contribution

Conceptualization of work (MSA, JSHR, MACC). Methodology development (MSA, NFPG, MACC). Software management (MSA, JSHR). Experimental validation (MSA, MRC). Analysis of results (MSA, JSHR, MACC, MRC, NFPG). Data management (MSA, JSHR). Writing and preparation of the manuscript (MSA, MRC). Writing, review, and editing (MSA, JSHR, MRC). Project administrator (MSA). Funding Acquisition (MSA).

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Conflicts of Interest

The authors declare no conflict of interest.

Declaration of principles for the ethical use of animals.

The authors declare that they have complied with current national and international regulations, which require ethical handling of animals.

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