Bacteria associated with *Bostrychia calliptera* and *Rhizoclonium riparium* with antimicrobial and probiotic potential for use in aquaculture

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Abstract

The growth of the global population has driven the development of aquaculture as an alternative means of meeting the increasing demand for food. However, this sector is susceptible to the potential transmission of pathogens that can affect both the organisms in culture and consumers. The use of probiotics represents a promising solution, as it takes advantage of the capacity of certain microorganisms to exert antibacterial activity against pathogens. Given the paucity of research examining the diversity and probiotic potential of microorganisms associated with marine algae, despite their high diversity, this study aimed to assess the antimicrobial activity and probiotic potential of bacteria isolated from *Bostrychia calliptera* and *Rhizoclonium riparium*, epiphytic algae from mangroves on the Colombian Pacific coast. Initially, 52 bacterial strains were isolated on trypticase soy agar, nutrient agar, synthetic seawater, and soy flour mannitol agar. The antibacterial activity of these isolates was evaluated through biocontrol tests against six fish and shellfish pathogens, resulting in the selection of *Bacillus* sp. AB08, *Bacillus* sp. AB17, *Bacillus* sp. AN35, and *Pseudomonas mosselii* AR37 as probiotic candidates on the basis of their outstanding capacity to inhibit *Staphylococcus aureus*. None of the selected strains formed biofilms, which is a positive finding from the perspective of pathogenicity. In the antibiotic susceptibility and tolerance tests to temperature and pH variations, *Bacillus* sp. AB08 and AN35 exhibited notable susceptibility to all tested antibiotics and maintained viable counts exceeding 10^6 CFU/mL, characteristics that position them as promising candidates for use as probiotics. Nevertheless, further *in vitro* studies are advised to more accurately define their probiotic characteristics, as well as *in vivo* studies in aquaculture systems to substantiate their efficacy and safety in aquaculture.

Introduction

As the global population has continued to grow, aquaculture has played an important role in meeting food needs by providing products that constitute an essential part of the human diet (*Abdel-Latif et al., 2022; Fazle-Rohani et al., 2022; Wang, Li & Li, 2015*). The majority of seafood and fish consumed worldwide are produced ~~on~~ in aquaculture farms, the output of which has been steadily increasing over the past decades (*Khouadja et al., 2017; Wang, Li & Li, 2015*). For example, approximately 2.5 million tons of aquaculture products were produced in the Americas in 2010, increasing to 4.9 million tons by 2022 (*FAO, 2024*). However, these products serve as a conduit for the transmission of pathogenic microorganisms (*Wang, Li & Li, 2015*).

Aquaculture is an intensive production practice that increases the susceptibility of farmed aquatic species to a variety of diseases, resulting in considerable economic losses. These losses are attributable to damage to aquatic organisms during their various stages of development, high mortality rates, the occurrence of epidemics, and the difficulty in controlling disease outbreaks (*Fazle-Rohani et al., 2022; Mujeeb et al., 2022*). Furthermore, the ingestion of contaminated aquaculture products can lead to diseases in humans (*Mendes et al., 2023; Teplitski, Wright & Lorca, 2009*). These include listeriosis, botulism, cholera, and infections that may result in severe diarrhea, abdominal discomfort, dehydration, vomiting, inflammatory responses, and, in extreme instances, mortality (*Ali et al., 2020; Elbashir et al., 2018; Feldhusen, 2000*). The most commonly pathogens in fish and shellfish include *Escherichia coli*, *Klebsiella* spp., *Clostridium botulinum*, *Listeria monocytogenes*, *Salmonella* spp., *Staphylococcus aureus*, *Aeromonas hydrophila,* and *Vibrio* spp. (*Ghaderpour et al., 2014; Poharkar et al., 2016; Wang, Li & Li, 2015*).

In view of the above, there has been a growing concern to ensure the asepsis of aquaculture food products, particularly those traditionally consumed raw *(Mendes et al., 2023; Teplitski, Wright & Lorca, 2009)*. The management of pathogens in aquaculture has primarily involved the use of antibiotics, sterilization agents, prophylactic products and chemotherapeutics *(Fazle-Rohani et al., 2022; Mujeeb et al., 2022)*. However, the excessive use of these substances has resulted in adverse effects, including the emergence of antibiotic-resistant pathogens, the transmission of harmful chemical compounds, and their accumulation in the environment, which has led to significant environmental contamination *(Abdel-Latif et al., 2022; Butkhot et al., 2020; Fazle-Rohani et al., 2022; Mujeeb et al., 2022).*

Consequently, the search for new strategies or alternative therapeutic agents to address fish and shellfish pathogens in an economical and environmentally safe manner has been intensified (*Abdel-Latif et al., 2022*). In this context, probiotics, defined as a live microbial supplement from a single or mixed culture that, when administered in adequate amounts, has a beneficial effect on the host *(Bidhan et al., 2014; Verschuere et al., 2000)*, have been identified as a very promising solution in aquaculture due to their efficacy, cost-effectiveness, promotion of gut microbiota and non-invasive application *(Butkhot et al., 2020; Khouadja et al., 2017)*.

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In aquaculture, the beneficial effects include the improvement of the environmental or host-associated microbial community, as well as increased feed use efficiency through the production of digestive enzymes and improved feed conversion, which result in an increase in the nutritional value and growth rate of the organisms. Moreover, probiotics contribute to improved water quality by influencing the bacterial composition within the water column and sediments, thereby promoting a healthier aquatic ecosystem. Furthermore, probiotics bolster the host immune response to disease by stimulating immunity, competition for nutrients, energy or adhesion sites, and the production of pathogen-inhibitory compounds *(Bidhan et al., 2014; Mujeeb et al., 2022; Verschuere et al., 2000; Vieira et al., 2013)*.

Nevertheless, for probiotics to yield these benefits, it is imperative that they satisfy specific safety criteria and that they can readily and effectively access the target organs of aquatic species. It is therefore essential to evaluate pathogenicity factors, such as biofilm formation, since these structures have the potential to increase antibiotic resistance and facilitate tissue colonization during bacterial infections *(Cheong et al., 2021; Mujeeb et al., 2022)*. Additionality, the candidate bacteria should be evaluated for antibiotic resistance and their ability to tolerate adverse conditions common in aquaculture systems or during the production of the final product, such as low pH and high temperatures, while maintaining counts above 10^6 CFU/mL the minimum recommended for probiotics to be effective *(Ding & Shah, 2007; Graf et al., 2019; Jiang et al., 2018; Mujeeb et al., 2022; Pang, Ransangan & Hatai, 2020; Reichling, 2020; Rosini & Margarit, 2015; Sarkodie, Zhou & Chu, 2019; Tripathi & Giri, 2014).*

A promising strategy for the identification of new probiotics in aquaculture is the study and isolation of symbiotic microorganisms, such as bacteria associated with marine algae in mangroves. These ecosystems harbor a great microbial diversity with a wide range of adaptive strategies developed in response to variable physicochemical conditions such as salinity, flooding, light and temperature, among which the production of bioactive compounds stands out, with potential applications in the nutraceutical, pharmaceutical, agrochemical and food industries, among others *(Bouchez et al., 2013; Pereira et al., 2023; Chukwudulue et al., 2023; Mujeeb et al., 2022; Hwanhlem, Chobert & H-Kittikun, 2014; Rishad et al., 2016)*. In addition, the diversity of bacteria closely associated with the algal surface plays a pivotal role in safeguarding the algae against detrimental microorganisms by producing antimicrobial compounds *(Chukwudulue et al., 2023)*. This activity is crucial for the survival of algae, which lack an immune system and are continuously exposed to a variety of biotic factors, and thus rely on secondary chemical defenses to protect against fouling and potentially pathogenic microorganisms *(Busetti, Maggs & Gilmore, 2017; Chukwudulue et al., 2023; De Mesquita et al., 2019)*, suggesting that algal-associated bacteria represent a valuable source of microorganisms with probiotic potential whose antimicrobial capabilities can be exploited for aquaculture and other industries.

The bacterial biodiversity of macroalgae present in mangrove ecosystems is of great relevance; however, there has been a paucity of research into the knowledge and exploration of these

ecosystems for the isolation of microorganisms with antibacterial and probiotic potential *(Chukwudulue et al., 2023; Ravisankar, Gnanambal & Sundaram, 2013)*. In Colombia, research in this area is still in its early stages, despite the fact that mangrove forests constitute an integral part of the Pacific region's ecosystem and serve as habitat to a multitude of epiphytic macroalgae species *(Peña-Salamanca, 2008; Rengifo-Gallego, Peña-Salamanca & Benitez-Campo, 2012)*. The most commonly occurring species in this region are *Bostrychia calliptera* and *Rhizoclonium riparium*. *B. calliptera* is a member of the phylum Rhodophyta, family Rhodomelaceae, and typically grows in tufts on wet rocks or mangrove roots in coastal areas. In its own right, *R. riparium* represents a filamentous algae belonging to the Chlorophyta phylum, the Cladophoraceae family, which develops epiphytically within intertidal zones, on muddy substrates, and on mangrove roots *(Cantera & Londoño, 2017; Thatoi et al., 2013)*. Both species have been observed in association with the roots of *Rhizophora mangle* (red mangrove) and the pneumatophores of *Avicennia germinans* (black mangrove), the most prevalent mangrove species in the Colombian Pacific *(Peña-Salamanca, 2008)*.

Based on the above, this research evaluated the antimicrobial activity and potential probiotic properties of bacteria isolated from the surface of *B. calliptera* and *R. riparium*, species found in two mangrove habitats along the Colombian Pacific coastline.

Materials & Methods

Sampling

Sampling was conducted in a mangrove forest located at the mouth of the Dagua River, in front of Buenaventura Bay, in the department of Valle del Cauca, Colombia. At this location, two sampling stations were established (station 1: 3°51'25.9"N 77°04'16.9"W, station 2: 3°51'5.161"N 77°3'39.409"W). For bacterial isolation, three trees of R. mangle and three of *A. germinans* were selected, each of which had roots and pneumatophores with attached algae of *B. calliptera* and *R. riparium*. The samples were collected by swabbing the algal surface with sterile cotton swabs, which were immediately transferred to Falcon tubes containing synthetic seawater (AMS), prepared according to the methodology described by *Nguyen (2018)*. The samples were stored at 4°C and subsequently transported to the Microbiological Research Laboratory (LIM) of the Biology Department of the Universidad del Valle in Cali, Colombia, for further processing.

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Additionally, *B. calliptera* algae were gathered, placed in airtight bags, and stored at 4°C. The algal material was maintained in the laboratory in an aquarium with F/2 medium *(Lananan et al., 2013)*, at a salinity of 20 ppm.

The Universidad del Valle has been granted a Collection Framework Permit by Autoridad Nacional de Licencias Ambientales (ANLA) of the Ministry of Environment and Sustainable Development (Resolution 1070 of August 28, 2015). This permit covers the academic programs, research groups, and professors engaged in the collection of specimens of wild species of biological diversity for non-commercial scientific research purposes.

Isolation and morphological characterization of bacterial strains

Replicates of each algal sample smear were integrated into a single tube, from which 10 mL were extracted and inoculated into Erlenmeyers containing 90 mL of AMS *(Nguyen, 2018)*, soy flour mannitol medium (SFM) *(Hobbs et al., 1989)* and tryptic soy broth (TSB), were subsequently incubated at 28°C with constant shaking at 180 rpm. The Erlenmeyers containing SFM and TSB were incubated for 24 hours, while the AMS medium was incubated for 21 days. Subsequently, 100 µL replates were performed in the respective culture media using the standard plate count method. Furthermore, bacterial isolates associated with *B. calliptera*, maintained in the laboratory, were obtained through swabbing and subsequently plated on the surface of Petri dishes containing SFM, trypticase soy agar (TSA), AMS, and nutrient agar (AN). All Petri dishes were incubated at 28°C for 24 to 48 hours.

Once growth was observed in the different culture media, a characterization of the macroscopic morphology of the colonies was performed. Those that exhibited morphological differences were considered to be distinct strains and were replicated until individual colonies with uniform morphology were observed. Gram staining and microscopic characterization were then performed, describing cell staining, shape, and size.

Molecular identification of the bacterial isolates of *B. calliptera* and *R. riparium*

Bacterial isolates were inoculated into eppendorf tubes containing 1 mL of TSB, LB broth, or AMS, as appropriate, and incubated at 28°C for 24 to 48 hours. Subsequently, the samples were centrifuged at 13,300 rpm for 6 minutes, the supernatant was discarded, and two washes were performed, with 1 mL of phosphate buffered saline (PBS) added and the samples centrifuged under the aforementioned conditions. The supernatant was discarded, and the pellet was resuspended in 100 µL of PBS in order to proceed with DNA extraction using the Monarch extraction kit (New England Biolabs), in accordance with the manufacturer's instructions.

The 16S rRNA gene was amplified using the universal primers 63F (5′-CAG GCC TAA CAC ATG CAA GTC-3′) and 1387R (5′-GGG CGG WGT GTA CAA GGC-3′) proposed by *Marchesi et al. (1998)*. The master mix was prepared according to the following procedure: A total of 2.5 µL of 10X Buffer, 0.5 µL of dNTPs, 0.5 µL of each of the primers (F and R), 0.125 µL of Taq polymerase, 1 µL of DNA, and the requisite amount of ultrapure water to reach a total volume of 25 µL were combined. The thermal program for amplification consisted of one cycle of 30 seconds at 95°C, 30 three-phase cycles at 95°C for 30 seconds, 55°C for one minute, and 68°C for 1:20 minutes, followed by one cycle of 10 minutes at 68°C. The PCR products were sequenced by Macrogen, Korea, and the DNA sequences were analyzed using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) to identify the bacterial isolates.

Biocontrol testing against fish and shellfish pathogens

The biocontrol capacity of 56 bacterial strains isolated from *B. calliptera* and *R. riparium* was evaluated against a panel of bacteria reported as fish and shellfish pathogens. The following

bacterial strains were used in the evaluation: *Staphylococcus aureus* (ATCC 29737), *Escherichia coli* (ATCC 11229), *Listeria monocytogenes* (ATCC 13932), *Salmonella bongori* (ATCC 43975), *Vibrio brasiliensis* (RR81) and *Aeromonas hydrophila* (RB65) *(Ghaderpour et al., 2014; Poharkar et al., 2016; Wang, Li & Li, 2015).*

First, a preliminary test was performed to select strains with antibacterial activity against the aforementioned pathogens. To achieve this, the bacteria isolated from the algae and the pathogens were inoculated in test tubes with 2 mL of TSB, LB broth, or AMS, as appropriate, and incubated at 28 °C for 24 hours. Subsequently, the concentrations of the bacterial cultures were adjusted by measuring their optical density (OD), obtaining inocula with an OD_{625} between 0.08 and 0.1, which corresponds to the 0.5 standard of the McFarland scale *(EUCAST, 2024)*.

250 µL of the algae-associated bacteria inocula were added to a 96-well microtiter plate. Subsequently, the pathogenic bacteria were surface-plated in 150 x 25 mm Petri dishes with Müller-Hinton agar (MHA) using sterile swabs. The bacteria present within the microtiter plate were transferred to the center of the Petri dishes, which had been inoculated with the pathogens, using a 96-pin microplate replicator. For the positive control, a 30 µg chloramphenicol disk was utilized, whereas for the negative control, a puncture was made with a toothpick using sterile saline solution. The tests were performed in triplicate at 28°C and 37°C, which correspond to the typical breeding temperature of tropical fish and shellfish *(Boyd, 2018)* and the human body temperature *(Cramer et al., 2022)*. The incubation period was 18 hours.

Subsequently, the strains that demonstrated an inhibition zone were selected for a second round of biocontrol tests, employing the disk diffusion method, with the objective of verifying the measurement of the inhibition zone and eliminating the size variability associated with colony growth that was observed in the initial tests. The bacterial inocula were prepared once more and the inocula of the pathogenic bacteria were distributed across the surface of Petri dishes containing MHA. Conversely, 13 µL of the selected strains' inocula were added to sterile Whatman filter paper discs with a diameter of 6 mm and left to dry. The dry discs were then placed on MHA dishes that had been inoculated with the pathogens. The tests were performed in triplicate, using the same controls and incubation conditions that had been described for the preliminary biocontrol tests.

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Following the incubation period, the radius of the inhibition zones was measured ~~in mm,~~ from the edge of the disk to the limit of the generated inhibition zone. The results were interpreted in accordance with the criteria established by *Wanja et al. (2020)*, with certain modifications. Inhibition was classified as follows: No inhibition: 0 mm, incipient inhibition: 1 to 5 mm, moderate inhibition: 6 to 9 mm, and strong inhibition: > 10 mm. The strains that demonstrated the most effective biocontrol capabilities were subsequently subjected to further trials.

Phylogenetic analysis

To construct a phylogenetic tree, partial 16S rRNA gene sequences from the four isolates selected from the biocontrol tests were used, along with the sequences of the most closely related bacterial species obtained by BLAST and representative sequences of the corresponding genera. To root the tree, the sequences of *Streptomyces griseus* strain KACC 20084 (NR 042791.1) and *Streptomyces nigrescens* strain NRRL ISP-5276 (NR_116013.1) were incorporated, as both species are members of a phylogenetically distant group from the bacteria under investigation.

Sequences were aligned using the Muscle algorithm *(Edgar, 2004a; Edgar, 2004b)* and the phylogenetic tree was constructed using Bayesian inference (BEAST) in the BEAST2 software version 2.6.7. In order to analyze the data, the General Time Reversible (GTR) substitution model, as proposed by *Tavaré (1986)* was applied, and the robustness of the topologies was assessed using a Markov Chain Monte Carlo (MCMC) analysis with 10 million generations.

Biofilm formation test

The selected strains were inoculated into tubes containing 2 mL of TSB and incubated at 28 °C for 24 hours. Subsequently, the concentrations of the bacterial cultures were adjusted by measuring their OD at 600 nm, thus ensuring a uniform concentration for all strains, with inocula having an OD_{600} between 0.1 and 0.5. Subsequently, 100 µL of the prepared inocula were added in quadruplicate to microtiter plates containing 150 µL of TSB per well. As controls, 250 µL TSB was included as a blank, *E. coli* (ATCC 11229) was utilized as a negative control, and *Pseudomonas aeruginosa* (ATCC 27853) was employed as a positive control *(El-Abed et al., 2011)*. The plates were incubated at 28 °C for 48 hours.

The culture medium was removed from the wells using a multichannel micropipette, and three washes were performed with 200 µL of phosphate buffered saline (PBS) with a pH of 7.2 and at room temperature. Subsequently, the plate was left to dry upside down for approximately 10 minutes on sterile absorbent paper. For fixation of biofilm-forming bacteria, the plate was subjected to a 60°C oven temperature for one hour. 200 µL of Gram crystal violet (CV) was added to each well for staining, and the solution was allowed to act for 10 minutes at room temperature. The excess dye was removed, and the wells were rinsed gently with running water, allowing the plates to air dry. Then, the CV was resolubilized by adding 200 µL of 95% ethanol, and the solution was allowed to act for 30 minutes without shaking and with the plate covered to prevent evaporation. Finally, 125 µL of the resolubilized CV was transferred to a new microtiter plate. Optical density measurements of each well were performed using a microtiter plate reader at a wavelength of 570 nm. The results were interpreted in accordance with the classification system proposed by *Stepanović et al. (2007)*, which categorizes strains into four distinct groups: non-producers, weak producers, moderate producers, and strong producers of biofilms.

Antibiotic susceptibility test

The selected strains were inoculated in 2 mL of TSB and incubated at 28 °C for 24 hours. The concentration of the bacterial cultures was adjusted, resulting in inocula with an OD_{625} between 0.08 and 0.1. From these, a surface sowing was performed on Petri dishes containing MHA.

Subsequently, sterile tweezers were employed to place discs impregnated with the antibiotics on the agar surface. The antibiotics used were streptomycin (10 µg), ciprofloxacin (5 µg), tetracycline (30 µg), oxytetracycline (30 µg), ampicillin (20 µg), chloramphenicol (30 µg), kanamycin (30 µg), and penicillin G (10 IU). The Petri dishes were then incubated at 28 °C for 18 hours.

Following this period, the radius of the inhibition zones in mm was measured in accordance with the aforementioned methodology and the results were interpreted in alignment with the proposals put forth by *Patel et al. (2009)* and *Ramesh et al. (2015)*, with certain modifications. The antibiotic susceptibility was classified as follows: Resistant: ≤5 mm, sensitive: 6–9 mm and highly sensitive: ≥10 mm.

Tolerance testing for temperature and pH

The temperature tolerance test was performed by inoculating the selected strains into tubes containing 2 mL of TSB and incubating them at temperatures at 25, 28, 37, 45, 50, 55 and 60 °C for 16 h. After this time, the colony forming units (CFU/mL) were counted using the massive stamping plate drop method proposed by *Corral-Lugo et al. (2012)*, with certain modifications. In 150 x 25 mm Petri dishes containing TSA, 10 µL of the 10^{-3} to 10^{-7} dilutions were deposited on the agar surface with a multichannel micropipette. This procedure was conducted in triplicate, with the plates incubated for 16 hours at the same temperatures as the tubes.

On the other hand, for pH tolerance tests, the strains were re-inoculated in TSB with the pH adjusted to 2, 3, 4, 5, 6, 7, 8, and 9 with HCl and NaOH, and incubated at 28°C for 24 h. The count was then performed using the same methodology described in the temperature tolerance tests, with the Petri dishes incubated at 28°C for 16 hours.

Statistical analysis

The capacity of the temperature and pH tolerances was subjected to statistical analysis using the RStudio software, version 4.3.3. The data were previously transformed using a logarithm base 10, and subsequently an analysis of variance (ANOVA) was performed to determine the statistical significance of the results, with a p-value < 0.05.

Results

Molecularly identified algae-associated bacteria

A total of 56 bacterial strains were isolated from the algae *B. calliptera* and *R. riparium*, associated with the trees *R. mangle* and *A. germinans*, of which 52 were identified (Table 1). The strains were found to belong to 19 genera, with 68.42% of these falling within the Pseudomonadota phyla (*Acinetobacter*, *Aeromonas*, *Alteromonas*, *Brenneria*, *Enterobacter*, *Klebsiella*, *Pantoea*, *Pseudomonas*, *Raoultella*, *Serratia*, *Stutzerimonas*, *Photobacterium* and *Vibrio*), 21.05% in Bacillota (*Bacillus*, *Staphylococcus*, *Exiguobacterium* and *Lysinibacillus*) and 10.53% within Actinomycetota (*Kocuria* and *Paenarthrobacter*). Additionally, 69% of the

strains were determined to be Gram-negative, while the remaining 31% were classified as Gram-positive.

Fig. 1 illustrates the biodiversity and origin of the isolated genera, indicating that the algae-tree association exhibiting the highest diversity was that of *B. calliptera* with *R. mangle*, comprising five unique genera (26.31%). This was followed by the associations of *R. riparium* with *R. mangle* and *R. riparium* with *A. germinans*, which had four unique genera each (21.05%). The algal species *R. riparium* exhibited the greatest diversity, with a total of nine unique genera representing 47.36% of the total. Additionally, *Stutzerimonas* was isolated exclusively from laboratory-preserved *B. calliptera*. Notably, the genera *Pseudomonas*, *Bacillus*, and *Raoultella* were isolated from all algae-tree associations.

On the other hand, the genus *Bacillus* was the most prevalent among the isolated genera, comprising 21.15% of the total, followed by *Pseudomonas* (17.31%), *Acinetobacter* (13.46%), and *Raoultella* (9.62%). Furthermore, 11 genera were identified, collectively representing 21.12% of the strains and each has only one representative (1.92%). The proportion of identified genera is illustrated in Fig. 2.

Biocontrol of fish and shellfish pathogens

In the preliminary biocontrol test performed with the mass printing method, 12 strains (21.42% of the isolates) were identified as having the capacity to inhibit at least one of the seven fish and shellfish pathogens evaluated. Fig. 3 illustrates ~~an example of~~ a positive result, wherein several of the bacterial isolates demonstrated the capacity to inhibit the growth of *S. aureus*.

The results of the second round of biocontrol tests, conducted using the disk diffusion method with the 12 selected strains, are presented in Table 2. In this test, it was observed that strains AB08, AB17, AR37, and AN35 demonstrated moderate inhibition of *S. aureus* at 28°C, while at 37°C, inhibition of this pathogen was incipient. Furthermore, strains AB01, AB02, AB07, AB09, AR20, AR28, AR29, and AR31 demonstrated incipient inhibition of *S. aureus* at both temperatures. In the assays with *A. hydrophila*, the majority of the aforementioned strains (AB01, AB07, AB08, AB09, AB17, AR29, AR37, and AN35) demonstrated incipient inhibition at both temperatures, with the exception of AB02, which exhibited this inhibition only at 37°C. In addition, none of the strains evaluated demonstrated the capacity to inhibit the growth of *V. brasiliensis*, *S. bongori*, *L. monocytogenes* or *E. coli*. In light of these findings, strains AB08, AB17, AR37, and AN35, which demonstrated the most pronounced inhibitory activity against *S. aureus*, were selected as candidates for evaluation as potential probiotics for aquaculture, with further *in vitro* testing.

Morphological characterization of the selected bacterial strains

Of the four strains selected from the biocontrol tests, AB08, AB17, and AN35 were isolated from *B. calliptera* associated with *A. germinans* (in the case of the first two) and from *B. calliptera* preserved in the laboratory (AN35), were observed microscopically as Gram-positive rod-shaped

bacteria with sizes between 1.96 and 2.45 µm (Figs. 4a, 4b, and 4c). In AN, these strains formed colonies with similar characteristics, appearing cream-colored, circular, mucous, and slightly opaque. They exhibited an entire border and flat elevation (Figs. 4e, 4f, and 4g).

In contrast, AR37, a Gram-negative rod-shaped bacterium of approximately 0.98 µm (Fig. 4e), isolated from *R. riparium* associated with *A. germinans*, formed small, opaque, circular, cream-colored, whole-bordered, flat-elevated colonies (Fig. 4h) in AN and produced a slight yellowish coloration in the medium.

Phylogenetic analysis

The phylogenetic tree, constructed using Bayesian inference (Fig. 5), demonstrated that *Bacillus* sp. strains AB08, AN35, and AB17 constituted a well-supported clade with members of this genus, exhibiting a posterior probability value of 100%. However, strains AN35 and AB17 did not cluster with any specific species, while AB08 was related to *Bacillus stercoris* D7XPN1 (NR_181952.1) but with a very low support value (22.23%), thus precluding precise species determination of these three isolates. On the other hand, strain AR37 was found to be closely related to the sequence of *P. mosselii* CFML 90-83 (NR_024924.1) with a posterior probability of 100%. This indicates a high probability of belonging to this species and confirms the molecular identification that was performed using BLAST. Considering these results, the four selected strains were identified as *Bacillus* sp. AB08, *Bacillus* sp. AB17, *Bacillus* sp. AN35 and *P. mosselii* AR37.

Biofilm formation and susceptibility to antibiotics

Following an evaluation of the safety requirements of the strains selected as probiotic candidates, the results of the biofilm formation tests demonstrated that none of the strains exhibited the capacity to form such structures.

On the other hand, the antibiotic susceptibility tests revealed that the strains of the *Bacillus* genus exhibited the highest overall susceptibility. Notably, both *Bacillus* sp. AB08 and *Bacillus* sp. AN35 demonstrated sensitivity to all tested antibiotics, displaying an identical sensitivity pattern. However, *Bacillus* sp. AB17 exhibited resistance to streptomycin. Ultimately, the isolate *P. mosselii* AR37 exhibited the highest degree of antibiotic resistance, demonstrating sensitivity only to ciprofloxacin and kanamycin (Table 3).

Temperature tolerance

The *Bacillus* strains (AB08, AB17, and AN35) exhibited the most extensive temperature tolerance ranges, with the ability to survive between 25 and 55°C and maintain growth at a level equal to or greater than 10^7 CFU/mL within this range. These strains demonstrated growth inhibition only at 60°C. *P. mosselii* AR37 demonstrated the capacity to survive within a temperature range of 25 to 37°C, exhibiting counts above 10^8 CFU/mL. However, its growth was inhibited at 45°C. The results of the statistical analysis indicated that there were statistically

significant differences between the strains in their response to the temperatures that were evaluated ($p < 0.0001$) (Fig. 6).

pH tolerance

The *Bacillus* strains (AB08, AB17, and AN35) exhibited the most extensive pH tolerance ranges, demonstrating the capacity to survive at all pH levels evaluated (2 to 9). However, *Bacillus* sp. AB17, presented a growth of 10^5 CFU/mL at pH 2 ($p < 0.0001$), which is below the minimum recommended count for effective probiotics, while the growth of *Bacillus* sp. AB08 and *Bacillus* sp. AN35 was maintained at a level of 10^6 CFU/mL or greater throughout the pH range. In contrast, *P. mosselii* AR37 was unable to survive in the pH range of 2 to 4, but from pH 5 onwards, it demonstrated growth greater than 10^7 CFU/mL. The results of the statistical analysis indicated that there were statistically significant differences between the strains in their response to the different pH values evaluated ($p < 0.0001$) (Fig. 6).

Discussion

Marine ecosystems, including mangrove forests, are renowned for their exceptional microbial diversity, encompassing both free-living forms and those associated with natural and artificial surfaces *(Mieszkin, Callow & Callow, 2013; Kaur et al., 2023; Ravisankar, Gnanambal & Sundaram, 2013)*, so red algae (Rhodophyta) such as *B. calliptera* and green algae (Chlorophyta) such as *R. riparium* are no exception. In this study, a total of 19 bacterial genera were isolated from the algae using AN, TSA, AMS, and SFM media. Of these, nine were unique to *R. riparium*, seven were unique to *B. calliptera*, and both algae shared only three genera (Fig. 1). This pattern, whereby both algal species exhibited such exclusive diversity of genera despite being associated with the same tree species and collected from the same sampling sites, is consistent with that reported in other studies which have observed a high degree of specificity in the bacterial communities of each algal species. This indicates that the similarity between bacteria of the same algal species is greater, even in different environments, compared to bacteria associated with different algal species that inhabit the same habitat *(Florez et al., 2017; Goecke et al., 2013)*.

The most commonly occurring bacterial phyla associated with algae are Pseudomonadota, Bacillota, Bacteroidota, Actinomycetota, Cyanobacteriota, Planctomycetota, Verrucomicrobiota, and Deinococcota *(Florez et al., 2017; Kaur et al., 2023)*. Of these, Pseudomonadota is the most abundant in mangrove ecosystems *(Moreno-Chacón, 2013; Palit et al., 2022)*, a characteristic that was verified the present study, in which 68.42% of the isolated genera belonged to this phylum, 21.05% to Bacillota, and 10.53% to Actinomycetota. These findings are consistent with the results of *Thilakan, Chakraborty & Chakraborty (2016)*, who reported that a large proportion of bacterial strains isolated from brown and red algae belonged to the phylum Pseudomonadota (40%), followed by the phylum Bacillota (31%), with the genus *Bacillus* as the predominant genus, which was also reflected in the results obtained, where this genus had the highest percentage of isolated strains (Fig. 2). Furthermore, 36 of the 52 isolated strains were Gram-

negative, which is consistent with the findings of *Albakosh et al. (2016)*, who noted that the majority of marine bacteria associated with algae, particularly in intertidal zones such as mangroves, are Gram-negative.

On the other hand, although *B. calliptera* and *R. riparium* are among the most prevalent algal species in the mangroves of the Colombian Pacific *(Cantera & Londoño, 2017; Thatoi et al., 2013)*, the bacterial diversity associated with these algae has been relatively understudied. To the best of our knowledge, the only previous report on this topic is that of *Sedanza et al. (2016)*, who isolated *Micrococcus flavus* L11 from *R. riparium* var. *implexum*. Nevertheless, studies of other algal species have documented the presence of several bacterial genera obtained from their associated microbial community, such as *Pseudomonas*, *Vibrio*, *Alteromonas*, *Bacillus*, *Kocuria*, *Staphylococcus*, *Serratia*, *Acinetobacter*, *Klebsiella*, *Aeromonas*, *Enterobacter*, *Photobacterium*, *Exiguobacterium*, *Lysinibacillus*, *Stutzerimonas* and *Pantoea (De Mesquita et al., 2019; Goecke et al., 2010; Ismail et al., 2018; Karthick et al., 2015; Malik et al., 2020; Vega-Portalatino et al., 2024)*, while *Raoultella*, *Brenneria* and *Paenarthrobacter* have not been previously documented in association with algae or in mangroves, but have been identified in marine water and sediments *(Cherak et al., 2021; Dwinovantyo et al., 2015; Rosas-Díaz et al., 2021)*. In light of the above, the bacteria isolated in this study (Table 1) represent the first report of the biodiversity of the microbiota associated with the algae *B. calliptera* and *R. riparium*.

Bacteria associated with algae present a remarkable capacity to produce antimicrobial compounds. A number of studies have indicated that between 35% and 50% of bacteria isolated from algae exhibit antimicrobial activity *(Albakosh et al., 2016; De Mesquita et al., 2019; Goecke et al., 2010; Ismail et al., 2018; Thatoi et al., 2013)*. In accordance with the aforementioned findings, the present study identified that 21.42% of the isolates exhibited biocontrol activity against *S. aureus* and *A. hydrophila*. The most prominent isolates were identified as *Bacillus* sp. AN35, *Bacillus* sp. AB08, *Bacillus* sp. AB17, and *P. mosselii* AR37, which showed moderate inhibition (6 to 9 mm radius) at 28°C and incipient inhibition (1 to 5 mm radius) at 37°C on the aquatic pathogen *S. aureus* (Table 2). These findings reinforce the notion that algal-associated bacteria represent a valuable source for the discovery of novel antimicrobial compounds with biotechnological potential. Moreover, the observation that these strains exhibited heightened efficacy at 28°C, which coincides with the optimal temperature for fish and shellfish farming in tropical regions *(Boyd, 2018)*, suggests that they could be promising candidates for aquaculture applications in the tropical zone.

The results of the biocontrol experiment, performed with the three *Bacillus* strains and *P. mosselii* AR37, align with the expectations derived from prior research. These genera are well-documented for their antimicrobial properties, which confer upon them a competitive advantage against other potentially pathogenic microorganisms *(Albakosh et al., 2016; Chukwudulue et al., 2023; Goecke et al., 2010; Kim & Anderson, 2018; Kolndadacha et al., 2011; Shah et al., 2021; Singh, Kumari & Reddy, 2015; Verschuere et al., 2000)*. In fact, similar results have already been reported for bacteria of these genera. In the case of *Bacillus*, *Susilowati, Sabdono &*

Widowati (2015) observed that strain IB.6a.1 (*Bacillus subtilis*), isolated from brown algae *Sargassum* spp., exhibited inhibitory activity against methicillin-resistant *S. aureus* and *Staphylococcus epidermidis*, with halos of 3.75 and 5.3 mm, respectively. Similarly, *Prieto et al. (2012)* identified *Bacillus* strains isolated from red, brown, and green algae that inhibited pathogenic bacteria, including methicillin-resistant *S. aureus*, *Salmonella typhimurium*, *E. coli*, and *L. monocytogenes*, with inhibition halos greater a 3 mm radius. With regard to *Pseudomonas*, *Gram et al. (2001)* reported that the probiotic candidate strain AH2 (*Pseudomonas fluorescens*) generated significant inhibition zones (21 mm) against *Aeromonas salmonicida*. Likewise, *Albakosh et al. (2016)* underscored the biocontrol capabilities of strain NA_1 (*Pseudomonas* sp.), isolated from the surface of the brown alga *Splachnidium rugosum*. This strain demonstrated notable biocontrol activity, with inhibition zones reaching 10 mm against pathogens such as *Bacillus cereus*, *S. epidermidis*, *Mycobacterium smegmatis*, *Micrococcus luteus*, and *Pseudomonas putida*.

Additionally, various studies have demonstrated the efficacy of probiotic candidate species belonging to the *Bacillus* and *Pseudomonas* genera in the control of pathogens in fish and shellfish aquaculture such as *A. salmonicida*, *A. hydrophila*, *Saprolegnia* sp., *Edwardsiella tarda*, *Photobacterium damselae*, and different *Vibrio* species *(Amoah et al., 2019; Irianto & Austin, 2002; Gram et al., 2001; Kuebutornye et al., 2020; Vaseeharan & Ramasamy, 2003; Verschuere et al., 2000)*. It can thus be concluded that the strains tested have the potential to serve as highly effective biocontrol agents against pathogens in aquaculture, such as *S. aureus*.

With regard to the molecular identification and phylogenetic analysis of the four isolates selected as probiotic candidates, only strain AR37 was identified to the species level as *P. mosselii*, while the three *Bacillus* strains were identified to the genus level. The 16S rRNA gene has been extensively utilized as a molecular marker for identification and phylogenetic analysis in prokaryotes. Its advantages include ubiquity, constant function, high conservation, minimal horizontal transfer, and a relatively long length (~1500 nucleotides) with nine hypervariable regions that offer valuable insights for phylogeny and identification *(Schleifer, 2009; Valenzuela-Gónzalez et al., 2015; Vera-Loor et al., 2021)*. However, identification based on a single gene can have resolution limitations, and in many cases, amplification of the 16S region is insufficient. Therefore, it is necessary to complement with other genes or even the entire genome to obtain conclusive results. Moreover, this marker is often inadequate for distinguishing between species within certain genera, such as *Bacillus (Schleifer, 2009; Suárez-Contreras & Yañez-Meneces, 2020; Vera-Loor et al., 2021)*.

The genus *Bacillus*, which encompasses over 200 species, is a bacterial genus with a complex definition and a confusing phylogenetic history. It exhibits a wide range of phenotypic characteristics and polyphyly, and thus lacks a unifying phenotypic or molecular characteristic that distinguishes all species within the genus *(Patel & Gupta, 2020)*. This heterogeneity is a consequence of the imprecise historical criteria that grouped various endospore-forming species under the name *Bacillus*, including some species with phenotypic and biochemical properties

that do not align with those of the type species, *B. subtilis*. This has resulted in challenges in accurately identifying and understanding the phylogenetic relationships within the genus *(Patel & Gupta, 2020)*. Indeed, in recent decades, numerous studies have been conducted with the aim of reclassifying several *Bacillus* species into more than 10 new genera *(Ahmed et al., 2007; Ash et al., 1991; Grazia-Fortina et al., 2001; Heyndrickx et al., 1999; Nazina et al., 2001; Patel & Gupta, 2020; Waino et al., 1999; Wisotzkey et al., 1992; Yoon et al., 2001)*. This complexity was evident in the phylogenetic tree constructed (Fig. 5), where many of the relationships between *Bacillus* species demonstrated low support values (less than 70%), which precludes the drawing of accurate conclusions about the phylogeny and species-level identification of strains in this genus.

Nevertheless, for a candidate probiotic bacterium to be suitable for use in aquaculture, it must not only demonstrate biocontrol activity but also meet certain criteria to guarantee its quality, efficacy, and safety for the host. These include: 1) They must not be pathogenic or have unfavorable side effects, 2) they must not be resistant to drugs or antibiotics, 3) they must survive inside and outside the host digestive tract, and 4) they must ensure that the final product has an adequate amount of probiotics to confer benefits to the host *(FAO & WHO, 2006; Mujeeb et al., 2022)*.

The initial two conditions are fundamental to ensure the security of probiotics. First and foremost, it is of paramount importance that the bacteria do not present any pathogenic factors or generate any unwanted effects, such as the formation of biofilms, which is a common characteristic observed in pathogenic bacteria, including those of significant importance in aquaculture, such as Aeromonas and Vibrio *(Arunkumar et al., 2020; Graf et al., 2019; Lubis et al., 2024; Reichling, 2020; Rosini & Margarit, 2015)* The formation of biofilms allows for the colonization of tissues in pathogenic processes and resistance to antibacterial agents and the host's immune defenses, which makes it challenging to eliminate disease outbreaks *(Cai & Arias, 2017; Cheong et al., 2021; Graf et al., 2019; Reichling, 2020; Rosini & Margarit, 2015; Sarkodie, Zhou & Chu, 2019).* Even if probiotics are not directly pathogenic to aquatic species, biofilms on surfaces in aquaculture systems can serve as pathogen reservoirs for fish and shellfish *(Cai & Arias, 2017; Freitas de Oliveira, Moreira & Schneider, 2019).* Secondly, it is imperative to prevent the transfer of antibiotic resistance genes to potential pathogens or the gut microbiome of aquatic organisms, in order not to promote the dissemination of such resistance *(Mujeeb et al., 2022; Sanders et al., 2010)*, particularly in aquatic environments that act as a primary conduit for the spread of these genes to disparate ecosystems *(Cabrera-Alaix et al., 2023)*.

The third condition ensures that probiotics can persist in both aquaculture systems and the target organs of the hosts, which are typically the digestive system *(Diwan, Harke & Panche, 2023; Endo & Gueimonde, 2015; Pang et al., 2020)*. It is therefore imperative that the bacteria are capable of withstanding the fluctuations in pH that occur along the gastrointestinal tract, which ranges from acidic in the stomach to neutral and alkaline in the intestine *(Ding & Shah, 2007;*

Endo & Gueimonde, 2015; Pang et al., 2020; Solovyev et al., 2015; Wendel, 2022), and temperature fluctuations in aquatic ecosystems, which are exacerbated by climate change *(Mugwanya et al., 2022)*. Moreover, the survival of the probiotic product during processing is of paramount importance, as the industry grapples with the challenge of delivering viable microorganisms while avoiding heat death *(Bidhan et al., 2014; Pang et al., 2020; Kosin & Rakshit, 2010; Sanders et al., 2010; Wendel, 2022)*. Ultimately, the fourth requirement ensures the efficacy of the product for which it is recommended that the product contain and maintain a minimum of 10^6 CFU/mL *(Ding & Shah, 2007; Jiang et al., 2018; Tripathi & Giri, 2014)*.

Based on these criteria, the biofilm formation tests revealed that none of the selected strains possessed this capacity, which is a favorable indication for their consideration as probiotic candidates. This is because, although the capacity to form biofilms has been proposed as a beneficial trait for probiotics, facilitating colonization of the intestinal tract and prolonging their residence in the host mucosa *(Salas-Jara et al., 2016)*, and it plays a role in maintaining balanced nitrogen and carbon cycles in aquaculture systems *(Cai & Arias, 2017)*, these structures are strongly implicated in a wide range of bacterial infections in aquatic organisms and humans *(Arunkumar et al., 2020; Barzegari et al., 2020; Reichling, 2020)*. Biofilms also can detach, disperse, and adhere to other areas of the host, such as wounds, forming new colonies that result in the recurrence of previously controlled infections, potentially leading to significant economic losses *(Arunkumar et al., 2020; Reichling, 2020)*. Moreover, these structures facilitate the persistence of pathogens by acting as reservoirs that allow them to resist disinfectants and antibiotics, thereby exacerbating the situation *(Cai & Arias, 2017; Freitas de Oliveira, Moreira & Schneider, 2019)*. Therefore, the inability of the four selected strains to form biofilms indicates a reduced risk of pathogenicity. Nevertheless, it is imperative to supplement these findings with further investigations into pathogenicity factors, including motility, capsule formation, or hemolysin production *(Pasachova-Garzón, Ramirez-Martinez & Muñoz-Molina, 2019; Paz-Zarza et al., 2019; Sarkodie, Zhou & Chu, 2019)*. Additionally, it is crucial to assess their *in vivo* impact to ascertain their safety in diverse aquaculture species.

In antibiotic susceptibility testing, the *Bacillus* isolates demonstrated sensitivity to all antibiotics that were tested, with the exception of *Bacillus* sp. AB17, which exhibited resistance to streptomycin. In contrast, strain AR37 demonstrated resistance to streptomycin, tetracycline, oxytetracycline, ampicillin, chloramphenicol, and penicillin G (Table 3). These findings suggest that the *Bacillus* sp. AN35 and AB08 strains meet the safety criterion that a prospective probiotic strain should not harbor transmissible antibiotic resistance genes, which is of paramount importance to impede the dissemination of resistance and the emergence of novel resistant pathogens *(Chauhan & Singh, 2019)*. As previously noted by *Uzun Yaylacı (2022)*, the absence of resistance in these strains indicates that they do not possess resistance genes that can be transferred to other microorganisms. This suggests that they are promising candidates for future probiotic evaluations.

However, as *Endo & Gueimonde (2015)* have noted, before discarding isolates that are resistant to a few antibiotics as potential probiotic candidates, as in the case of the strain *Bacillus* sp. AB17, which is resistant only to streptomycin, it is essential to perform additional tests to determine whether the resistance genes are transferable or whether this resistance is an intrinsic trait. Intrinsic antibiotic resistance is encoded in the core genome of the microorganism, in contrast to acquired resistance, which is obtained through horizontal gene transfer *(Langendonk Neill & Fothergill, 2021)*. Consequently, intrinsic resistance does not represent a significant safety risk and carries a low risk of spread *(Compaoré et al., 2013; Endo & Gueimonde, 2015).* This type of resistance has been documented in other *Bacillus* species *(Compaoré et al., 2013).* Indeed, *Zhao et al. (2024)* observed that the strain *B. subtilis* SOM8 was intrinsically resistant to streptomycin, the same antibiotic to which *Bacillus* sp. AB17 was resistant, reinforcing the importance of confirming the transfer capacity of these genes before discarding any isolate. This underscores the necessity of conducting tests to ensure the preliminary selection of safe bacteria for use as probiotics in aquaculture, thereby reducing the risk of introducing strains with harmful characteristics into the aquatic environment.

In tolerance tests, both temperature and pH were found to exert significant effects on bacterial survival and growth (Figs 6 and 7). The *P. moselii* strain AR37 demonstrated a narrow survival range, tolerating temperatures between 25 and 37°C and ppH levels between 5 and 9, exhibiting a growth of 10^7 to 10^9 CFU/mL. These results indicate that this strain may not be capable of withstanding the processing of the final probiotic product or the acidic conditions of the animal stomach. As indicated by *Leelagud et al. (2024)* and *Dieppois et al. (2015)*, members of the *Pseudomonas* genus, including *P. entomophila*, a close relative of *P. mosselii*, are capable of tolerating temperatures between 4 and 42°C. This is consistent with the findings of the temperature tolerance test of AR37, which could not survive above 45°C. However, the pH tolerance results of this strain differ from those observed by *Devi et al. (2022)*, who reported that *P. mosselii* COFCAU_PMP5 survived in the pH range of 2 to 9.

In the case of *Bacillus* sp. AB17, although it demonstrated a remarkable survival capacity in a wide range of temperatures (25 to 55 °C) and pH (2 to 9), its count at pH 2 was significantly lower compared to the other pH values ($p < 0.0001$), reaching barely 10^5 CFU/mL. This suggests that in acidic environments, such as the animal stomach, it would not reach the minimum required for probiotics to be effective. In contrast, *Bacillus* sp. AB08 and AN35 exhibited a noteworthy capacity to survive within the same temperature and pH ranges, with counts reaching 10^6 to 10^9 CFU/ mL. The survival demonstrated by these strains was anticipated, as it has been observed that numerous probiotic strains of the *Bacillus* genus are capable of withstanding extreme pH and temperature ranges *(Amoah et al., 2019)*. Additionally, their capacity to form endospores enables them to exhibit enhanced viability and resilience in hostile environments *(Amoah et al., 2019; Butkhot et al., 2020; Kuebutornye et al., 2020; Shah et al., 2021; Verschuere et al., 2000; Zhang et al., 2020)*.

These results suggest that *Bacillus* sp. AB08 and AN35 strains are promising candidates for future probiotic applications, due to their ability to maintain counts above the minimum recommended 10^6 CFU/mL under a broader range of adverse environmental conditions, which is essential for their efficacy in aquaculture *(Ding & Shah, 2007; Endo & Gueimonde, 2015)*. *Pang et al. (2020)* reached similar conclusions when evaluating the tolerance of five potentially probiotic strains to disparate temperatures (18 to 60 °C) and pH (2 to 9). In their study, *Alcaligenes faecalis* and *Staphylococcus saprophyticus* were unable to grow at temperatures exceeding 37 °C or at pH levels of 2 and 3. Conversely, *Bacillus thuringiensis*, S*kermanella stibiiresistens*, and *Enterobacter cloacae* exhibited enhanced tolerance to these conditions, positioning themselves as more suitable candidates for probiotic applications in aquaculture.

Conclusions

This study represents the first report on the cultivable bacterial diversity associated with the surface of the mangrove algae *B. calliptera* and *R. riparium*, present on the Colombian Pacific coast, and represents a significant advancement in the exploration of the symbiotic microbiota of algae, with the objective of identifying bacteria with probiotic potential.

Mangrove seaweeds, a source of potentially probiotic bacteria that has been poorly explored to date, were found to be a valuable reservoir of microorganisms with pathogen biocontrol capacity, as evidenced by the finding of 12 strains (21.42% of isolates) that exhibited antibacterial activity against *S. aureus* and A. hydrophila. Among the isolates, *Bacillus* sp. AB08 and *Bacillus* sp. AN35 exhibited the most promising characteristics for use as probiotics in aquaculture. These strains demonstrated a notable biocontrol response against *S. aureus*, an inability to form biofilms, susceptibility to all tested antibiotics, and the capacity to maintain viable counts above 10^6 CFU/mL under adverse conditions, such as wide temperature and pH ranges.

As this study represents a preliminary analysis, further trials are recommended to evaluate additional probiotic properties, such as tolerance to bile and other gastrointestinal stress factors, as well as to investigate other pathogenicity factors, such as capsule or hemolysin production. Moreover, *in vivo* testing is essential to substantiate the efficacy of *Bacillus* sp. AB08 and AN35 in aquaculture systems, evaluate their influence on aquatic organisms and their interaction with native microbiota in these environments, and ensure their efficacy and safety for prospective commercial applications.

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