

SCREENING OF PROBIOTIC BACTERIA FOR ARAPAIMA GIGAS AND FIRST REPORT OF DISEASE CAUSED BY ENTEROBACTER CLOACAE

**TRIAGEM DE BACTÉRIAS PROBIÓTICAS PARA ARAPAIMA GIGAS E
PRIMEIRO RELATO DE DOENÇA CAUSADA POR ENTEROBACTER
CLOACAE**

Ciências Biológicas, Ciências Agrárias • 10/10/2026

REGISTRO DOI: [10.70773/revistatopicos/791611131](https://doi.org/10.70773/revistatopicos/791611131)

Cynthia Rafaela Monteiro da Silva Maia¹

Kamila Oliveira dos Santos²

Suzana Kotzent³

Roselene Nogueira Rodrigues⁴

Cintia Mara Costa de Oliveira⁵

Sílvia Umeda Gallani⁶

Guilherme Campos Tavares⁷

Gustavo Moraes Ramos Valladão⁸

ABSTRACT

Arapaima gigas is an important and strategic species for aquaculture in South America, carrying significant economic and social impacts, especially in the Amazon region. However, sanitary challenges can limit its productivity. This study carried out the isolation, characterization, and identification of autochthonous bacteria from the intestine of *A. gigas* from Amazonian aquaculture, aiming to identify bacterial isolates with probiotic potential. A total of 120 initial isolates were obtained, of which 25 representative strains were subjected to *in vitro* screening, including tolerance to acidic pH and bile salts, hemolytic activity, autoaggregation, biofilm formation, and antagonism, followed by *in vivo* safety tests. The results revealed promising and harmless strains, including *Bacillus cereus*, *Enterococcus faecalis*, *Enterococcus gallinarum*, and *Lactococcus lactis subsp. lactis*. Notably, *Enterococcus faecium* stood out for its physiological robustness and antagonistic activity against the pathogen *Aeromonas jandaei* (MT02). In contrast, *Enterobacter cloacae*, although isolated from healthy hosts, exhibited acute virulence in *in vivo* assays, resulting in 100% mortality. This is the first record of this bacterium as an opportunistic pathogen in *A. gigas*, with the reproduction of the disease confirmed through controlled experimental infection. This study also provides perspectives on biotechnological innovations through the identification of autochthonous strains with probiotic potential that may contribute to sustainable health management in *A. gigas* farming. However, *in vivo* validation remains essential to ensure microbial safety before their application in aquaculture.

Keywords: *Arapaima gigas*; aquatic probiotics; fish health; microbial safety; pathogenicity.

RESUMO

O "Arapaima gigas" é uma espécie importante e estratégica para a aquicultura na América do Sul, gerando impactos econômicos e sociais significativos, especialmente na região amazônica. No entanto, desafios sanitários podem limitar sua produtividade. Este estudo realizou o isolamento, a caracterização e a identificação de bactérias autóctones do intestino de "A. gigas" provenientes da aquicultura amazônica, visando identificar isolados bacterianos com potencial probiótico. Um total de 120 isolados iniciais foi obtido; destes, 25 cepas representativas foram submetidas a uma triagem "in vitro", incluindo testes de tolerância a pH ácido e sais biliares, atividade hemolítica, autoagregação, formação de biofilme e antagonismo, seguidos por testes de segurança "in vivo". Os resultados revelaram cepas promissoras e inócuas, incluindo "Bacillus cereus", "Enterococcus faecalis", "Enterococcus gallinarum" e "Lactococcus lactis" subsp. "lactis". Destaca-se "Enterococcus faecium", que sobressaiu pela sua robustez fisiológica e atividade antagonista contra o patógeno "Aeromonas jandaei" (MT02). Em contrapartida, "Enterobacter cloacae", embora isolada de hospedeiros saudáveis, exibiu virulência aguda em ensaios "in vivo", resultando em 100% de mortalidade. Este é o primeiro registro dessa bactéria como patógeno oportunista em "A. gigas", com a reprodução da doença confirmada por meio de infecção experimental controlada. O estudo também apresenta perspectivas de inovações biotecnológicas através da identificação de cepas autóctones com potencial probiótico, as quais podem contribuir para o manejo sanitário sustentável na criação de "A. gigas". Contudo, a validação "in vivo" permanece essencial para garantir a segurança microbiana antes de sua aplicação na aquicultura.

Palavras-chave: Arapaima gigas; probióticos aquáticos; saúde de peixes; segurança microbiana; patogenicidade.

1. INTRODUCTION

Arapaima gigas is one of the most important species for aquaculture production in South America (Valladão *et al.*, 2018; Ferreira *et al.*, 2020). The adaptability of this species to intensive production systems has been the subject of several studies, with promising results obtained even outside the Amazon biome. A notable example is its cultivation in California, USA (Ohs *et al.*, 2021), which highlights its potential for diversifying aquaculture on a global scale. Currently, pirarucu production has gained prominence in Brazil, reaching approximately 1,947,305 kg in 2023 and showing steady growth over recent decades (IBGE, 2024). However, several challenges such as reproduction, nutritional aspects, diseases, and a scarcity of species specific inputs still limit the development of the production chain (Escudero *et al.*, 2024; Gonçalves *et al.*, 2024).

In this context, using biotechnology such as developing probiotics to enhance the fish production chain can provide significant sanitary and productive benefits. These additives aim to improve the nutritional quality of diets, optimize feed efficiency, and promote reproductive performance (Eissa *et al.*, 2024; Enzeline *et al.*, 2024). Furthermore, probiotics are defined as live microorganisms that can minimize stress, stimulate the immune system, and increase fish resistance to various diseases (Wuertz *et al.*, 2021; Hoseinifar *et al.*, 2023; Torres-Maravilla *et al.*, 2024).

Despite these general benefits, the effectiveness of probiotic supplementation depends directly on the ability of the microorganisms to colonize and persist in the gastrointestinal tract. Recent studies indicate that using autochthonous probiotics (isolated from the host itself) offers significant advantages over

allochthonous or commercial strains, as these microorganisms are already adapted to the physiological and immunological conditions of the target species (Rwezawula *et al.*, 2025). This biological compatibility promotes greater adhesion to the intestinal mucosa and more efficient competitive exclusion of pathogens, making the bioprospecting of native strains a promising strategy to maximize health and zootechnical performance in intensive farming systems (Doan *et al.*, 2019; Fachri *et al.*, 2024). For this reason, the study of autochthonous strains derived from this Amazonian fish deserves particular attention.

Both *in vitro* and *in vivo* tests are routinely employed to select bacteria with probiotic potential. These assays aim to confirm essential characteristics such as resistance to adverse gastrointestinal conditions including low pH and the presence of bile salts (Hrabar *et al.*, 2025) and to evaluate autoaggregation capacity, the inhibition of intestinal pathogenic bacteria, antimicrobial resistance, and benefits to host health (Kotzent, 2017). Additionally, efforts are made to confirm the absence of pathogenicity in the isolates and guarantee bacterial stability during storage (Sanson *et al.*, 2020). Considering the importance of *A. gigas* for national and international trade, this study aimed to isolate, characterize, and identify autochthonous bacteria from the intestines of *A. gigas* reared in aquaculture systems in Amazonas, Brazil. For this purpose, the strains were subjected to *in vitro* tests and *in vivo* safety assays to prospect for potential probiotics for the species.

2. MATERIALS AND METHODS

2.1. Ethics Statement

The experimental procedures were approved by the Animal Ethics Committee (CEUA) of Nilton Lins University (Protocol No. 010/2021) and conducted in accordance with the guidelines of the Federal Council of Veterinary Medicine (CFMV).

2.2. Isolation And Initial Characterization Of Autochthonous Bacteria

For the isolation process, 25 healthy juvenile *A. gigas* of varying sizes and weights (mean $\pm$ SD: 562.26 $\pm$ 982.88 g) were used. These fish were obtained from five distinct fish farms located across the following municipalities in the state of Amazonas: Iranduba, Itacoatiara, Manacapuru, Manaus, and Presidente Figueiredo.

For sample collection and bacterial isolation, the fish were euthanized in a benzocaine solution (0.3 g/L) following the protocol described by Wang et al. (2024). Prior to necropsy, the external body surface was sterilized using water, neutral soap, 70% alcohol, and 1% iodized alcohol to ensure aseptic access to the coelomic cavity. The intestines were then removed and dissected, and the fecal content was washed out with sterile PBS. Subsequently, the washed intestinal fragments were homogenized in a sterile saline solution (0.65%) at a 1:1 ratio (w/v) to preferentially isolate resident bacteria (adhered to the mucosa) rather than transient ones. Serial dilutions (10^{-1} to 10^{-6}) were prepared in PBS (pH 7.0) using 100 μ L of the homogenate. Aliquots of 100 μ L from each dilution were plated using the spread plate technique onto Man, Rogosa, and Sharpe agar (MRS, Sigma-Aldrich) and tryptic soy agar (TSA, Neogen Culture Media). The simultaneous use of these media was intended to select potential probiotic lactic acid bacteria (LAB) on the MRS medium and to quantify total cultivable heterotrophic bacteria on the TSA

medium. The plates were then incubated at 35 °C for 24 h for colony isolation and the enumeration of colony-forming units (CFU).

2.3. Microbiological And Biochemical Characterization

The bacterial isolates were screened based on morphology using the Gram staining technique (Beveridge, 2001) and examined under a light microscope (Bel Photonics) with a 1000× objective lens using immersion oil. Initial biochemical characterization included catalase (Abdou *et al.*, 2018) and oxidase (Kovács, 1956) tests.

2.4. Identification Of Bacteria

Following preliminary screening using biochemical methods (catalase and oxidase), pure bacterial cultures were provisionally identified via Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF). Mass spectrometer calibration was performed with the Bruker bacterial test standard (Bruker Daltonics), following the method detailed by Assis *et al.* (2017).

Molecular identification was carried out by extracting total genomic DNA using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol for Gram-positive and Gram-negative bacteria. The universal primer sets described by Corinaldesi *et al.* (2005) were used to amplify and sequence the 16S rRNA genes: 16S-F (5' prime - AGA GTT TGA TCC TGG CTC AG- 3' prime) and 16S-R (5' prime - GGT TAC CTT GTT ACG ACT T – 3' prime). Sequencing of the amplified and purified products was conducted at the Center for Biological Resources and Genomic Biology (CREBIO) at São Paulo State University (UNESP, São Paulo, Brazil).

3. IN VITRO TESTS

3.1. Hemolytic Activity

The biological safety of the isolates was assessed by evaluating hemolytic activity on blood agar plates supplemented with 5% sheep blood, as described by Kuebutornye *et al.* (2022). The plates were incubated at 35 °C for 24 h, and hemolysis was evaluated based on erythrocyte lysis around the colonies. Isolates were classified as β -hemolysis (clear halo, total lysis), α -hemolysis (greenish halo, partial lysis), or γ -hemolytic (absence of a halo). This screening step is crucial for distinguishing pathogenic isolates from safe probiotic candidates; hemolytic activity is a primary virulence factor associated with tissue destruction and anemia in fish, making it a standard exclusion criterion for probiotic selection (Fachri *et al.*, 2024). However, strains exhibiting hemolytic activity in this study were not excluded from subsequent evaluations. This approach was adopted to investigate whether *in vitro* conditions influence the expression of traits that could be suppressed or induced *in vivo*, thereby avoiding the premature exclusion of potential probiotic candidates.

3.2. Tolerance To Bile Salts

Tolerance to bile salts is a fundamental prerequisite for probiotic selection, as bile constitutes a stressful physicochemical barrier within the gastrointestinal tract (Mohammed *et al.*, 2025). Consequently, the ability to survive physiological bile concentrations (approximately 0.3%) is essential for viable microorganisms to reach the intestine, enabling their colonization and metabolic proliferation (Kuebutornye *et al.*, 2022). The previously characterized isolates

(based on Gram staining, catalase, oxidase, and hemolysis) were subjected to a bile salt tolerance test according to Kusada *et al.* (2021) and Sreepathi *et al.* (2023). The strains were incubated at 35 °C for 24 h in MRS broth supplemented with 0.3% and 1.0% bile salts (Sigma-Aldrich), alongside a bile-free control. Growth was monitored by measuring optical density at 600 nm (OD), and the survival rate was calculated using the following equation:

$$\text{Survival (\%)} = \frac{\text{OD}_{\text{Treatment}}}{\text{OD}_{\text{Control}}} \times 100$$

3.3. Low PH Tolerance

Tolerance to acidic pH was evaluated according to the protocol described by Mazlumi *et al.* (2022). Washed bacterial suspensions in PBS were inoculated at 2% (v/v) into TSB broth adjusted to pH 3.0 and 5.0, alongside an unadjusted control, and incubated at 35 °C. Growth was monitored by measuring the optical density at 600 nm (OD) after 3 and 24 h, and the tolerance rate was determined using the following equation:

$$\text{Tolerance (\%)} = \frac{\text{OD}_{\text{Treatment}}}{\text{OD}_{\text{Control}}} \times 100$$

3.4. In Vitro Antagonistic Activity

Isolates that demonstrated tolerance to low pH and bile salts were subjected to an antagonism assay. The pathogenic strain *Aeromonas jandaei* (MT-02), provided by the Laboratory of Microbiology Applied to Aquatic Organisms (Nilton Lins University), was used in this assay. This pathogen was selected due to its epidemiological relevance in *A. gigas* farming, as it is the etiologic agent responsible for hemorrhagic septicemia outbreaks associated

with high mortality rates (Proietti-Junior *et al.*, 2021). The assays were based on protocols described by Hütt *et al.* (2006) and Balouiri *et al.* (2016), employing three complementary methodologies: agar block diffusion, co-culture competition, and well diffusion with supernatant (bacterial metabolites).

3.5. Evaluation Of Autoaggregation Capacity

The autoaggregation capacity of the preselected isolates (following the pH, bile salts, and antagonism assays) was evaluated as described by Zuo *et al.* (2016). Bacterial cultures grown in TSB (24 h, 35 °C, 170 rpm) were harvested by centrifugation (3,000 × g, 10 min, 4 °C), washed with PBS, and adjusted to an optical density at 600 nm (OD) corresponding to 10⁸ cells/mL. Aliquots of 5 mL were incubated at 35 °C under static conditions, and the absorbance of the upper suspension was measured after 1, 2, and 4 h. The percentage of autoaggregation was calculated using the following equation:

$$\text{Autoaggregation (\%)} = (1 - A_t/A_0) \times 100$$

where A_t is the absorbance after 1, 2, or 4 h, and A_0 is the absorbance at time 0.

3.6. Biofilm Production

Biofilm production was evaluated in all 25 isolates using the Congo red agar method (Freeman *et al.*, 1989). The bacterial strains were plated onto Mueller-Hinton agar (pH 7.0) supplemented with 0.8 g/L of the dye (INLAB) and incubated at 35 °C for 24 h. The development of black colonies was used as the criterion for positive biofilm production.

3.7. In Vivo Safety Evaluation

In vivo safety was evaluated for nine selected autochthonous isolates (*Bacillus cereus*, *Bacillus vietnamensis*, *Enterobacter cloacae*, *Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus gallinarum*, *Lactococcus petauri*, *Lactococcus lactis subsp. lactis*, and *Lactococcus lactis*). The bacterial inocula, cultivated in TSB (35 °C, 24 h) and washed with PBS, were adjusted to a concentration of 10^8 cells/mL. Juvenile *A. gigas* (10.48 g $\pm$ 2.26) were acclimated for 7 days, anesthetized with benzocaine (0.1 g/L), and inoculated via the intraperitoneal route at a dose of 0.1 mL/10 g of body weight (n = 7 fish per aquarium), alongside a control group injected solely with PBS. The experimental design considered the aquarium as the experimental unit and the individual fish as the sampling unit, thereby mitigating pseudoreplication (Ruohonen, 1998; Zar, 1999; Knud-Hansen, 2017). Commercial feed was provided twice daily, and clinical signs and mortality were monitored for 14 days.

4. RESULTS

From the five sampled farms, 120 intestinal bacterial isolates from *A. gigas* were obtained, distributed among approximately 10 genera. Primary screening prioritized the morphological diversity of the colonies. Following purification and preliminary biochemical characterization (catalase and oxidase), 25 representative strains were selected for subsequent assays, as shown in Tables 1 and 2.

As detailed in Table 1, of the 25 selected strains, 10 were characterized as Gram-positive and 15 as Gram-negative. Regarding their biochemical profile, 18 isolates were catalase-positive and 24 were oxidase-negative. Concerning hemolytic activity, the majority of the

isolates (n = 18) were classified as γ -hemolytic, while α -hemolysis and β -hemolysis were observed in only four and three isolates, respectively.

Regarding tolerance to bile salts, most strains exhibited high viability at 0.3%, with survival above 80%. The increase to 1.0% caused a dose-dependent and species-specific reduction in viability for some strains; however, survival generally remained $\geq 50\%$. *E. gallinarum* stood out, with rates of 167% (0.3%) and 146% (1.0%), suggesting proliferation under bile stress. *Enterococcus faecalis*, *Lactococcus lactis subsp. lactis*, and *Laribacter hongkongensis* also demonstrated robustness, maintaining 100% viability at 0.3%. In contrast, the lowest survival rates at 1.0% were recorded for *Lactococcus petauri* (33%) and *Lactococcus garvieae* (34%).

As presented in Table 2, the exposure of the strains to pH 3.0 for 24 h proved to be a stringent selective barrier, resulting in total lethality for all isolates. In contrast, most species showed high viability at pH 5.0, with notable resilience observed in *Enterococcus gallinarum* (100%), *Enterobacter cloacae* (96%), and *Aeromonas jandaei* (93%).

The autoaggregation indices indicate that the bacteria possess proteins or polysaccharides that facilitate the association of identical cells. This adhesion capacity is crucial, as it enables cellular attachment and prevents the microorganisms from being cleared by intestinal flow, ensuring their persistence within the host (Zhao *et al.*, 2024; Pepoyan *et al.*, 2025).

All evaluated strains demonstrated some degree of autoaggregation, which increased progressively over the 4-h incubation period. Notably, *Aeromonas jandaei* exhibited the

highest aggregation capacity among all isolates, with values of 33.61%, 43.2%, and 53.7% at 1, 2, and 4 h, respectively. A slightly lower degree of autoaggregation (< 50%) was observed for the remaining strains.

Although the autoaggregation indices were relatively low, the literature emphasizes that intestinal colonization is not solely determined by this parameter. According to Zuo *et al.* (2016), adhesion to the host can occur independently of cellular aggregation, mediated by specific adhesins that facilitate direct interaction between the bacterium and intestinal mucosal receptors. Furthermore, autoaggregation is also associated with biofilm formation in microorganisms. Among the tested strains, *Enterobacter asburiae*, *Enterobacter cloacae*, *Escherichia coli*, *Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus gallinarum*, and *Lactococcus lactis subsp. lactis* exhibited a positive phenotype for this trait.

The 25 strains were evaluated for antagonistic activity using three *in vitro* methodologies: agar block diffusion, co-culture competition, and well diffusion (using supernatant containing bacterial metabolites). Among the tested strains, *Acinetobacter pittii*, *Enterococcus faecium*, *Klebsiella pneumoniae*, and *Proteus mirabilis* demonstrated antagonism against *Aeromonas jandaei* (MT-02). This activity was observed exclusively in the agar block diffusion assay; no inhibition of the pathogen was detected in the co-culture competition or well diffusion assays.

Table 1. Results of the initial *in vitro* tests of the selected *A. gigas* strains.

Identified strains	Gram	Cat.	Oxi.	Hemolysis	Bile t (0.3%)
<i>Aeromonas jandaei</i>	-	+	-	β	60%
<i>Acinetobacter pittii</i>	-	+	-	γ	75%
<i>Bacillus cereus</i>	+	+	-	β	99%
<i>Citrobacter</i>	-	+	-	α	9.6%

⚠ Esta tabela possui muitas colunas e foi cortada para impressão. Para visualizá-la completa, acesse o artigo original em: <https://revistatopicos.com.br/artigos/screening-of-probiotic-bacteria-for-arapaima-gigas-and-first-report-of-disease-caused-by-enterobacter-cloacae?noblockage>

(Cat = catalase. Oxi = oxidase). (*ND = Not determined) (NT = Not tested *in vivo* in this study). (Aj = *Aeromonas jandaei*).

Table 2. Results of the *in vitro* tests of tolerance to low pH, autoaggregation, biofilm production, antagonism and *in vivo* assays.

Identified strains	Tol. pH 3 (3 h)	Tol. pH 5 (3 h)	Tol. pH 3 (24 h)	Tol. pH 5 (24 h)	Autoaggregation (h)
<i>Aeromonas jandaei</i>	2%	6%	0%	93%	53%
<i>Acinetobacter pittii</i>	1%	24%	0%	66%	16%
<i>Bacillus cereus</i>	2%	7%	0%	43%	39%

⚠ Esta tabela possui muitas colunas e foi cortada para impressão. Para visualizá-la completa, acesse o artigo original em: <https://revistatopicos.com.br/artigos/screening-of-probiotic-bacteria-for-arapaima-gigas-and-first-report-of-disease-caused-by-enterobacter-cloacae?noblockage>

(Cat = catalase. Oxi = oxidase). (*ND = Not determined) (NT = Not tested in vivo in this study). (Aj = *Aeromonas jandaei*).

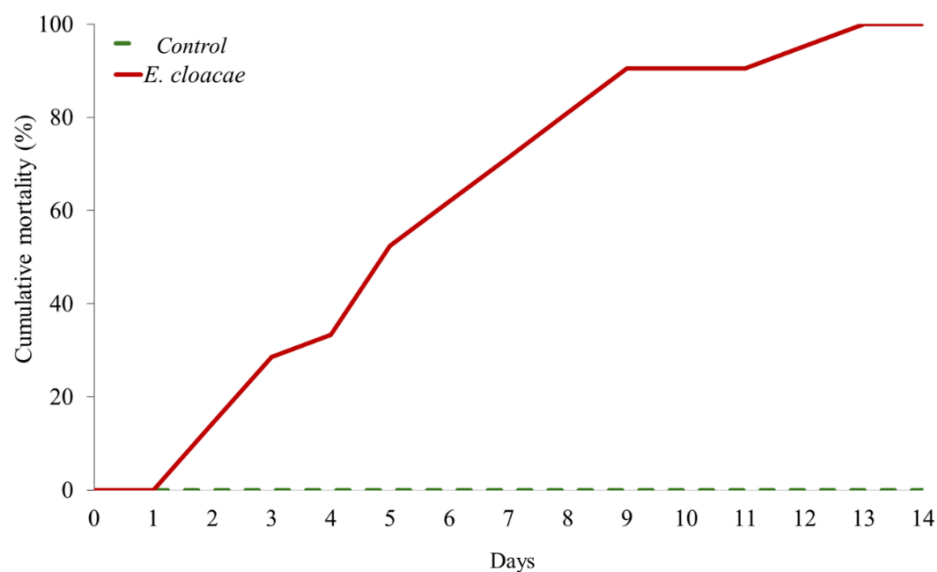
4.1. *In Vivo Safety Test*

The strains *Bacillus cereus*, *Bacillus vietnamensis*, *Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus gallinarum*, *Lactococcus petauri*, and *Lactococcus lactis* were harmless to juvenile *A. gigas*. By contrast, *Enterobacter cloacae* (CRBP111) caused 100% mortality, accompanied by clinical signs that included gill lesions, fin erosion, diffuse hemorrhages, and cutaneous petechiae (Fig. 1). Pathogenicity was confirmed by experimental reinfection, with the identity of the agent verified by MALDI-TOF following reisolation from the kidney, brain, and liver. Cumulative mortality (Fig. 2) showed progressive deaths up to 14 days post-infection.

Figure 1. Juvenile *A. gigas* showing gill lesions (white arrows) fin erosion, diffuse hemorrhages and petechiae along the body (black arrows) caused by *E. cloacae*. Source. Author's personal archive.



Figure 2. Cumulative mortality (%) of *A. gigas* affected by *E. cloacae*.



5. DISCUSSION

This study enabled the isolation, characterization, and identification of autochthonous bacteria from the intestinal tract of *A. gigas* to select strains with probiotic potential. For this purpose, 25 strains were subjected to an initial screening that included *in vitro* assays: Gram staining, enzymatic profiling (catalase and oxidase), biological safety testing via hemolysis assays, antagonism against *Aeromonas jandaei* (MT-02), and autoaggregation capacity. The physiological robustness of the strains was also evaluated under low pH and bile salt conditions, alongside *in vivo* safety assays for the selected strains.

One of the fundamental criteria for probiotic selection is the absence of hemolytic activity, a characteristic that reinforces the biological safety profile of the isolates. This requirement aligns with FAO/WHO guidelines, which aim to ensure that the introduction of these strains does not pose a pathogenic risk to the host (Elsegeny *et al.*, 2025). Despite this, in the present study, strains exhibiting α -, β -, and γ -hemolytic patterns were retained for subsequent analyses. This decision was made to avoid the premature exclusion of isolates that, although showing hemolytic activity *in vitro*, may still possess

relevant probiotic potential without necessarily compromising fish health.

Our results indicated that the evaluated strains exhibited bile tolerance, with survival rates exceeding 60% when exposed to 0.3% bile salts for 24 h, corroborating the findings of Al-Noor *et al.* (2023). Exposure to a higher concentration (1.0%) resulted in reduced viability, with an average decrease in survival of approximately 40%. Notably, *Enterococcus gallinarum* stood out by maintaining viability above 100% at both concentrations, suggesting not only resistance but also an ability to proliferate under prolonged bile stress conditions. The evaluation of tolerance to low pH (pH 5.0 for 3 and 24 h) suggests that these isolates possess traits that may allow them to survive the physiological stresses associated with gastrointestinal transit, indicating a potential for colonization, albeit possibly at reduced population levels (Shija *et al.*, 2025).

The autoaggregation capacity of the strains ranged from 10% to 39%. Regarding antagonistic potential, the isolates *Acinetobacter pittii*, *Enterococcus faecium*, *Klebsiella pneumoniae*, and *Proteus mirabilis* demonstrated inhibitory activity against the pathogen *Aeromonas jandaei* (MT-02), reinforcing their potential as biological control agents in aquaculture. These properties are associated with the ability of the strains to adhere to the intestinal epithelium, thereby promoting the competitive exclusion of pathogenic microorganisms (Hussain *et al.*, 2025; Palusiak, 2022; Bello-López *et al.*, 2024). Nevertheless, these bacteria require careful further investigation, as they include species with known pathogenic potential in humans.

Regarding biofilm formation capacity, 8 of the 25 studied isolates exhibited a positive phenotype. This trait may enhance the probiotic

potential of these candidates in aquaculture, as biofilms act as protective matrices that facilitate adhesion to the intestinal epithelium, thereby increasing strain persistence. According to Liaqat *et al.* (2025), biofilm production by beneficial bacteria in fish can contribute to competitive exclusion by creating a physical barrier that limits the attachment of opportunistic pathogens.

The *in vivo* safety assays evaluated 9 of the 25 preselected strains, chosen based on the *in vitro* tests and an extensive literature review. Among the tested isolates, only *Enterobacter cloacae* (CRBP 111), although originally isolated from the intestinal tract of healthy *A. gigas*, proved harmful to juveniles of the same species. This finding highlights the risk of opportunistic pathogenicity, reinforcing that an autochthonous origin alone does not guarantee the safety of a microorganism. As noted by Elsegeny *et al.* (2024), *in vivo* validation is a decisive step, as strains demonstrating probiotic potential *in vitro* may exhibit virulence when interacting with the host immune system under farming conditions

Although *in vitro* and *in vivo* assays provide important evidence, the bioprospecting of autochthonous bacteria and the understanding of their functional benefits for the productivity and health of *A. gigas* remain insufficiently explored. This knowledge gap underscores the need for further studies to validate the use of native microorganisms as a sustainable strategy for Amazonian aquaculture.

In vitro probiotic tests pose significant challenges, particularly in selecting and applying methodologies that accurately reflect the interactions between microorganisms and their hosts. Byakika *et al.* (2019) argue that many existing tests may require updated validation methods to reliably produce conclusive results regarding bacteria–

fish interactions. Furthermore, Vinderola *et al.* (2017) provide a critical, in-depth discussion of the correlation between *in vitro* and *in vivo* assays, highlighting approaches that minimize methodological biases and offer a more comprehensive assessment for probiotic validation.

In the present study, bacterial genera widely documented in the literature as potential probiotics were isolated, including *Bacillus*, *Enterococcus*, *Lactobacillus*, and *Lactococcus*. A notable finding, however, was the pathogenicity of *Enterobacter cloacae* (CRBP 111) in *A. gigas*. The detection of *E. cloacae* in the intestinal tract of healthy specimens, followed by the manifestation of acute virulence under experimental conditions (during *in vivo* safety assays), reveals the opportunistic nature of this pathogen in *A. gigas*. The observed clinical signs of hemorrhagic septicemia and fin erosion are consistent with reports of mortality in other fish species, such as *Pangasianodon hypophthalmus* in the rivers of Thailand and Vietnam (Kumar *et al.*, 2013) and *Mugil cephalus* in coastal waters of tropical and subtropical regions (Sekar *et al.*, 2008). These findings establish the *Enterobacter cloacae* (CRBP 111) strain as a reemerging pathogen (Mabrok *et al.*, 2024), representing the first official record of its pathogenicity in *A. gigas* in the state of Amazonas, Brazil.

This finding highlights a critical limitation of conventional screening protocols, as the pathogenic strain did not exhibit hemolytic activity *in vitro*, which would theoretically classify it as safe (Proietti-Junior *et al.*, 2021). This discrepancy reinforces the premise that an autochthonous origin or the absence of hemolysis does not guarantee safety, making *in vivo* validation a mandatory step to distinguish true probiotic candidates from hidden epidemiological risks.

In light of the identification of opportunistic pathogens in *A. gigas*, the strain *Enterococcus faecium* stands out as the most promising probiotic candidate in this study. It was the only isolate to simultaneously demonstrate *in vivo* safety and antagonistic activity against *Aeromonas jandaei* (MT-02). Notably, its biological safety in the host contrasts with the partial hemolysis phenotype observed *in vitro*, suggesting that traditional exclusion criteria may be overly conservative.

The potential of *Enterococcus faecium* in aquaculture is well supported in the literature (Van Doan *et al.*, 2019; Suphoronski *et al.*, 2021), particularly regarding its inhibitory action against *Aeromonas* spp. (Gopalakannan e Arul, 2011; Mao *et al.*, 2020). In Amazonian fish, *E. faecium* has already been associated with improvements in zootechnical performance and parasitic resistance (Sousa *et al.*, 2019), fulfilling the biotechnological prerequisites proposed by Vinderola *et al.* (2017). This strain also exhibited important traits in *in vitro* assays, including tolerance to bile salts and acidic pH, as well as moderate autoaggregation and biofilm formation capabilities that are essential for persistence in the gastrointestinal tract.

Although they did not exhibit significant results in the *in vitro* assays, particularly regarding bacterial antagonism, the strains *Bacillus cereus*, *Enterococcus gallinarum*, *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis*, and *Enterococcus faecalis* proved safe for *A. gigas* under the evaluated conditions. These species already have documented probiotic potential in other fish (Sorroza *et al.*, 2013; Yang *et al.*, 2021; Torres-Maravilla *et al.*, 2024; Enzeline *et al.*, 2024). This scenario reinforces the premise that more detailed *in vivo* studies are essential, as complex interactions with the host can trigger beneficial responses not reproduced in laboratory settings.

Caution is warranted, however, since some strains within these genera are described as opportunistic pathogens (Krawczyk *et al.*, 2021). Furthermore, most research has focused on allochthonous microorganisms, highlighting a considerable knowledge gap regarding the biotechnological potential of the autochthonous microbiota of *A. gigas* (Pereira *et al.*, 2019; Proietti-Junior *et al.*, 2021).

CONCLUSION

The study revealed that the strains *Bacillus cereus*, *Enterococcus faecalis*, *Enterococcus gallinarum*, and *Lactococcus lactis subsp. lactis* showed promising results in *in vitro* assays, indicating potential as probiotic candidates for *A. gigas*. Among these, the strain *Enterococcus faecium* emerged as the most promising, demonstrating physiological robustness, biofilm-forming ability, and specific antagonism against *Aeromonas jandaei* (MT-02). This research also provides the first report of *Enterobacter cloacae* as an emerging pathogen for this species in Brazil, underscoring the need for rigorous *in vivo* validation to complement laboratory assays. Overall, the results provide a solid foundation for developing native probiotic additives aimed at promoting sustainability and health in the intensive production of this important Amazonian fish.

Funding: Foundation – FAPEAM for granting the scholarship through the Stricto Sensu Postgraduate Support Program - POSGRAD INPA – 2021 Edition, which enabled the realization of this work. I also acknowledge the support from the programs CT&I Priority Areas, Productivity-CT&I, PDCA/AM, and PDCTR/AM. To the Coordination for the Improvement of Higher Education Personnel (CAPES) through the call PROCAD/AMAZÔNIA 2018 (process no.: 88881.200614/2018-01).

This study was funded by Foundation de Amparo à Pesquisa do Estado do Amazonas - FAPEAM (ST&I Programs in Priority Areas N° 01.02.016301.03427/2021-36; PDCTR/AM N° 01.02.016301.00759/2022-40; PDCA/AM N° 01.02.016301.04070/2022-94).

Data Availability Statement: The original contributions presented in this study are included in the Article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Acknowledgments: The authors thank the members of the Laboratory of Parasitology and Pathology of Aquatic Organisms – LAPPA and Laboratory of Microbiology Applied to Aquatic Organisms – LAMIC of UNL for their valuable participation, data collection and support throughout the experiments.

Special thanks to Prof. Dr. Spartaco Astolfi Filho for his contribution to the publication of this paper.

The authors used Gemini 3 Pro (Google) to improve the language, clarity, and readability of this manuscript. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the final version of the publication.

Conflicts of Interest: The authors have no relevant financial or non-financial interests to disclose.

Ethics approval: This study was approved by the Ethics Committee on the Use of Animals (CEUA) at Nilton Lins University under protocols 010/2021, which is consistent with the ethical principles of animal experimentation adopted by the Brazilian College of Animal Experimentation (COBEA).

REFERENCES

- Abdou, A. M., Hedia, R. H., Omara, S. T., Mahmoud, M. A. E., Kandil, M. M., Bakry, M. A. (2018). Interspecies comparison of probiotics isolated from different animals. ***Veterinary World***, 11(2), 227–230. <https://doi.org/10.14202/vetworld.2018.227-230>
- Al-Noor, J. M., Najim, S. M., Al-Waely, W. A. (2023). Isolation and characterization of lactic acid bacteria from the fish intestine for application as probiotics in young common carp *Cyprinus carpio* L. diet. ***International Journal of Aquatic Biology***, 11(3), 200–212. <https://doi.org/10.22034/ijab.v11i3.1942>
- Assis, G. B., Pereira, F. L., Zegarra, A. U., Tavares, G. C., Leal, C. A., Figueiredo, H. C. (2017). Use of MALDI-TOF mass spectrometry for the fast identification of gram-positive fish pathogens. ***Frontiers in Microbiology***, 8, 1492. <https://doi.org/10.3389/fmicb.2017.01492>
- Balouiri, M., Sadiki, M., Ibnsouda, S. K. (2016). Methods for in vitro evaluating antimicrobial activity: A review. ***Journal of Pharmaceutical Analysis***, 6(2), 71–79. <https://doi.org/10.1016/j.jpha.2015.11.005>
- Bello-López, E., Escobedo-Muñoz, A. S., Guerrero, G., Cruz-Córdova, A., Garza-González, E., Hernández-Castro, R., Zarain, P. L., Morfín-Otero, R., Volkow, P., Xicohtencatl-Cortes, J., Cevallos, M. A. (2024). *Acinetobacter pittii*: The emergence of a hospital-acquired pathogen analyzed from the genomic perspective. ***Frontiers in Microbiology***, 15, 1412775. <https://doi.org/10.3389/fmicb.2024.1412775>
- Beveridge, T. J. (2001). **Use of the Gram stain in microbiology.** *Biotechnic & Histochemistry*, 76(3), 111–118.

<https://doi.org/10.1080/713682857>

Brazilian Institute of Geography and Statistics (IBGE). (2021). **Pirarucu**. <https://www.ibge.gov.br/explica/producao-agropecuaria/pirarucu/br>

Byakika, S., Mukisa, I. M., Byaruhanga, Y. B., Muyanja, C. (2019). A review of criteria and methods for evaluating the probiotic potential of microorganisms. **Food Reviews International**, 35(5), 427–466. <https://doi.org/10.1080/87559129.2019.1584815>

Corinaldesi, C., Danovaro, R., Dell'Anno, A. (2005). Simultaneous recovery of intracellular and extracellular DNA from marine sediments. **Applied and Environmental Microbiology**, 71(12), 7973–7981. <https://doi.org/10.1128/AEM.71.12.7973-7981.2005>

Doan, H. V., Hoseinifar, S. H., Ringø, E., Esteban, M. Á. (2020). Host-associated probiotics: A key factor in sustainable aquaculture. **Reviews in Fisheries Science & Aquaculture**, 28(1), 16–42. <https://doi.org/10.1080/23308249.2019.1643288>

Eissa, E. S., El-Sayed, A. F. M., Hendam, B. M., Ghanem, S. F., Elnabi, H. E. A., El-Aziz, Y. M. A., Abdelnour, S. A., Eissa, M. E. H., Dighiesh, H. S. (2024). The regulatory effects of water probiotic supplementation on the blood physiology, reproductive performance, and its related genes in Red Tilapia (*Oreochromis niloticus* X *O. mossambicus*). **BMC Veterinary Research**, 20, 351. <https://doi.org/10.1186/s12917-024-04190-w>

Elsegeny, S. R., Radwan, F. S., Elshamy, Y. M., Amer, S. M., Mohamed, R. A., Shokrak, N. M., Abdella, B. (2025). A comprehensive overview of probiotics in aquaculture: From efficacy evaluation to diverse

applications. **Annals of Microbiology**, 75(1), 35.
<https://doi.org/10.1186/s13213-025-01825-7>

Enzeline, V., Agus, O. S., Alimuddin, A., Hasan, N., Widanarni, W. (2024). Improving reproductive performance and larvae survival by dietary administration of probiotic *Bacillus cereus* NP5 in female African catfish *Clarias gariepinus*. **Aquaculture International**, 32, 7629–7646. <https://doi.org/10.1007/s10499-024-01532-1>

Escudero, M. A. G., De La Vega, A. J. M. (2024). Reproductive aspects of the Amazon giant paiche (*Arapaima gigas*): A review. **Fisheries and Aquatic Sciences**, 27(2), 57–65.
<https://doi.org/10.47853/FAS.2024.e7>

Fachri, M., Amoah, K., Huang, Y., Cai, J., Alfatat, A., Ndandala, C. B., Chen, H. (2024). Probiotics and paraprobiotics in aquaculture: A sustainable strategy for enhancing fish growth, health and disease prevention-a review. **Frontiers in Marine Science**, 11, 1499228.
<https://doi.org/10.3389/fmars.2024.1499228>

Ferreira, G., Marcovitch, J., Val, A. L. (2020). A systematic review of the production chain of the *Arapaima gigas*, the giant fish of the Amazon. **Management of Environmental Quality**, 31(2), 349–363.
<https://doi.org/10.1108/MEQ-11-2019-0238>

Freeman, D. J., Falkiner, F. R., Keane, C. T. (1989). New method for detecting slime production by coagulase negative staphylococci. **Journal of Clinical Pathology**, 42(8), 872–874.
<https://doi.org/10.1136/jcp.42.8.872>

Gonçalves, A. C. T., Santos, L. C. R. D. (2024). Bioeconomia na Amazônia Central: Manejo participativo de pirarucu (*Arapaima*

gigas). **Revista de Administração Contemporânea**, 28, e240195.
<https://doi.org/10.1590/1982-7849rac2024240195.en>

Gopalakannan, A., Arul, V. (2011). Inhibitory activity of probiotic *Enterococcus faecium* MC13 against *Aeromonas hydrophila* confers protection against hemorrhagic septicemia in common carp *Cyprinus carpio*. **Aquaculture International**, 19, 973–985.
<https://doi.org/10.1007/s10499-011-9415-2>

Hoseinifar, S. H., Maradonna, F., Faheem, M., Harikrishnan, R., Devi, G., Ringø, E., Van Doan, H., Ashouri, G., Gioacchini, G., Carnevali, O. (2023). Sustainable ornamental fish aquaculture: The implication of microbial feed additives. **Animals**, 13(10), 1583.
<https://doi.org/10.3390/ani13101583>

Hrabar, J., Babić, I., Jozić, S., Trumbić, Ž., Pioppi, A., Nielsen, L. J. D., Mladineo, I. (2025). Prospecting microbiota of Adriatic fish: *Bacillus velezensis* as a potential probiotic candidate. **Animal Microbiome**, 7(1), 64. <https://doi.org/10.1186/s42523-025-00429-5>

Hussain, N., Mirza, A. F., Muccee, F., Haddad, A. H. A., Afzal, M. I. (2025). In vitro analysis of probiotic characteristics of gut-associated bacteria from *Solea solea*. **Frontiers in Veterinary Science**, 12, 1581675.
<https://doi.org/10.3389/fvets.2025.1581675>

Hütt, P., Shchepetova, J., Lõivukene, K., Kullisaar, T., Mikelsaar, M. (2006). Antagonistic activity of probiotic lactobacilli and bifidobacteria against entero- and uropathogens. **Journal of Applied Microbiology**, 100(6), 1324–1332. <https://doi.org/10.1111/j.1365-2672.2006.02857.x>

Knud-Hansen, C. F. (2017). Experimental design and analysis in aquaculture. In **Dynamics of Pond Aquaculture** (pp. 325–375). CRC Press.

<https://www.taylorfrancis.com/books/mono/10.1201/9780203759028/dynamics-pond-aquaculture?refId=5234314d-3f5a-4fe6-95b8-2c8c32a8bbf1&context=ubx>

Kotzent, S. (2017). **Bactérias com potencial probiótico do intestino de tambaqui (*Colossoma macropomum*)** (Dissertação de mestrado, Universidade Estadual Paulista). Repositório Institucional UNESP.

Kovács, N. (1956). Identification of *Pseudomonas pyocyanea* by the oxidase reaction. **Nature**, 178(4535), 703. <https://doi.org/10.1038/178703a0>

Krawczyk, B., Wityk, P., Gałęcka, M., Michalik, M. (2021). The many faces of *Enterococcus* spp. commensal, probiotic and opportunistic pathogen. **Microorganisms**, 9(9), 1900. <https://doi.org/10.3390/microorganisms9091900>

Kuebutornye, F. K. A., Abarike, E. D., Lu, Y. (2019). A review on the application of *Bacillus* as probiotics in aquaculture. **Fish & Shellfish Immunology**, 87, 820–828. <https://doi.org/10.1016/j.fsi.2019.02.010>

Kuebutornye, F. K. A., Lu, Y., Wang, Z., Mraz, J. (2022). Functional annotation and complete genome analysis confirm the probiotic characteristics of *Bacillus* species isolated from the gut of Nile tilapia. **LWT**, 163, 113541. <https://doi.org/10.1016/j.lwt.2022.113541>

Kumar, K., Prasad, K. P., Raman, R. P., Kumar, S., & Purushothaman, C. S. (2013). Association of *Enterobacter cloacae* in the mortality of *Pangasianodon hypophthalmus* (Sauvage, 1878) reared in culture

pond in Bhimavaram, Andhra Pradesh, India. **Indian Journal of Fisheries**, 60(3), 147–149. <https://doi.org/10.21077/>

Kusada, H., Morinaga, K., Tamaki, H. (2021). Identification of bile salt hydrolase and bile salt resistance in a probiotic bacterium *Lactobacillus gasseri* JCM1131T. **Microorganisms**, 9(5), 1011. <https://doi.org/10.3390/microorganisms9051011>

Liaqat, I., Qaiser, I., Aftab, M. N., Ali, S., Latif, A. A., Naseem, S., Khalid, A. (2025). Anti-biofilm potential of some fish probiotics, alone and in combination with antibiotics against isolated aquaculture pathogens; A preliminary data. **Microbial Pathogenesis**, 203, 107437. <https://doi.org/10.1016/j.micpath.2025.107437>

Lopes, E. M., Silva, A. V., Barros, F. A. L., Santos, A. F. L., Cordeiro, C. A. M., Couto, M. V. S., Sousa, N. C. (2022). Seleção in vitro de bactérias probióticas do peixe ornamental Amazônico *Nannostomus beckfordi*. **Arquivo Brasileiro de Medicina Veterinária e Zootecnia**, 74(1), 111–116. <https://doi.org/10.1590/1678-4162-12499>

Mabrok, M., Algammal, A. M., El-Tarabili, R. M., Dessouki, A. A., ElBanna, N. I., Abd-Elnaby, M., El-Lamie, M. M. M., Rodkhum, C. (2024). *Enterobacter cloacae* as a re-emerging pathogen affecting mullets (*Mugil* spp.): Pathogenicity testing, LD50, antibiogram, and encoded antimicrobial resistance genes. **Aquaculture**, 583, 740619. <https://doi.org/10.1016/j.aquaculture.2024.740619>

Mao, Q., Sun, X., Sun, J., Zhang, F., Lv, A., Hu, X., Guo, Y. (2020). A candidate probiotic strain of *Enterococcus faecium* from the intestine of the crucian carp *Carassius auratus*. **AMB Express**, 10, 40. <https://doi.org/10.1186/s13568-020-00973-0>

Mazlumi, A., Panahi, B., Hejazi, M. A., Nami, Y. (2022). Probiotic potential characterization and clustering using unsupervised algorithms of lactic acid bacteria from saltwater fish samples. **Scientific Reports**, 12(1), 11952. <https://doi.org/10.1038/s41598-022-16322-z>

Mohammed, E. A. H., Ahmed, A. E. M., Kovács, B., Pál, K. (2025). The significance of probiotics in aquaculture: A review of research trend and latest scientific findings. **Antibiotics**, 14(3), 242. <https://doi.org/10.3390/antibiotics14030242>

Nakharuthai, C., Boonanuntanasarn, S., Kaewda, J., Manassila, P. (2023). Isolation of potential probiotic *Bacillus* spp. from the intestine of Nile tilapia to construct recombinant probiotic expressing CC chemokine and its effectiveness on innate immune responses in Nile tilapia. **Animals**, 13(6), 986. <https://doi.org/10.3390/ani13060986>

Ohs, C., Hill, J., Wright, S., Giddings, H. M., Donahou, A. D. (2021). Candidate species for Florida aquaculture: *Arapaima* *Arapaima* *gigas*. **EDIS**, 2021(4). <https://doi.org/10.32473/edis-fa231-2021>

Palusiak, A. (2022). *Proteus mirabilis* and *Klebsiella pneumoniae* as pathogens capable of causing co-infections and exhibiting similarities in their virulence factors. **Frontiers in Cellular and Infection Microbiology**, 12, 991657. <https://doi.org/10.3389/fcimb.2022.991657>

Pepoyan, A., Grigoryan, L., Harutyunyan, N., Mirzabekyan, S., Balayan, M., Galstyan, A., & Manvelyan, A. (2025). Auto-aggregation properties of probiotic *Lactobacillus delbrueckii* str. IAHAH1: From genes to function. *Bulletin of University of Agricultural Sciences and*

Veterinary Medicine Cluj-Napoca. Food Science and Technology, 82(1), 197–208. <https://doi:10.15835/buasvmcn-fst:2024.0030>

Pereira, G. V., Pereira, S. A., Soares, A., Mouriño, J. L. P., Merrifield, D. (2019). Autochthonous probiotic bacteria modulate intestinal microbiota of Pirarucu, *Arapaima gigas*. **Journal of the World Aquaculture Society**, 50(6), 1152–1167. <https://doi.org/10.1111/jwas.12638>

Proietti-Junior, A. A., Lima, L. S., Roges, E. M., Rodrigues, Y. C., Lima, K. V. B., Rodrigues, D. P., Tavares-Dias, M. (2021). Experimental co-infection by *Aeromonas hydrophila* and *Aeromonas jandaei* in Pirarucu *Arapaima gigas* (Pisces: Arapaimidae). **Aquaculture Research**, 52(4), 1688–1696. <https://doi.org/10.1111/are.15021>

Ruohonen, K. (1998). Individual measurements and nested designs in aquaculture experiments: A simulation study. **Aquaculture**, 165(1-2), 149–157. [https://doi.org/10.1016/S0044-8486\(98\)00252-4](https://doi.org/10.1016/S0044-8486(98)00252-4)

Rwezawula, P., Waiswa Mwanja, W., Vereecke, N., Bossier, P., Vanrompay, D. (2025). Advancing aquaculture probiotic discovery via an innovative protocol for isolation of indigenous, heat and salt tolerant, quorum quenching probiotic candidates. **Frontiers in Microbiology**, 16, 1558238. <https://doi.org/10.3389/fmicb.2025.1558238>

Samson, J. S., Choresca, C. H., Quiazon, K. M. A. (2020). Selection and screening of bacteria from African nightcrawler, *Eudrilus eugeniae* (Kinberg, 1867) as potential probiotics in aquaculture. **World Journal of Microbiology and Biotechnology**, 36(1), 16. <https://doi.org/10.1007/s11274-019-2795-2>

Sekar, V. T., Santiago, T. C., Vijayan, K. K., Alavandi, S. V., Raj, V. S., Rajan, J. J. S., Sanjuktha, M., Kalaimani, N. (2008). Involvement of

Enterobacter cloacae in the mortality of fish, *Mugil cephalus*. **Letters in Applied Microbiology**, 46(6), 667–672. <https://doi.org/10.1111/j.1472-765X.2008.02365.x>

Shija, V. M., Amoah, K., Li, Y., Zhong, Y., Zhong, X., & Cai, J. (2025). In vitro selection, assessment, and identification of potential probiotic features of the *Bacillus* strains isolated from the intestine of Nile tilapia (*Oreochromis niloticus*). **Acta Oceanologica Sinica**, 44(7), 116–131. <https://doi.org/10.1007/s13131-024-2411-y>

Sorroza, L., Real, F., Acosta, F., Acosta, B., Déniz, S., Román, L., El Aamri, F., Padilla, D. (2013). A probiotic potential of *Enterococcus gallinarum* against *Vibrio anguillarum* infection. **Fish Pathology**, 48(1), 9–12. <https://doi.org/10.3147/jsfp.48.9>

Sousa, N. C., Couto, M. V. S., Abe, H. A., Paixão, P. E. G., Cordeiro, C. A. M., Monteiro Lopes, E., Ready, J., Alves Jesus, G. F., Martins, M., Pereira Mouriño, J. L., Carneiro, P. C. F., Maria, A. N., Fujimoto, R. Y. (2019). Effects of an *Enterococcus faecium*-based probiotic on growth performance and health of Pirarucu, *Arapaima gigas*. **Aquaculture Research**, 50(12), 3720–3728. <https://doi.org/10.1111/are.14332>

Sreepathi, N., Kumari, V. B. C., Huligere, S. S., Al-Odayni, A. B., Lasehinde, V., Jayanthi, M. K., Ramu, R. (2023). Screening for potential novel probiotic *Levilactobacillus brevis* RAMULAB52 with antihyperglycemic property from fermented *Carica papaya* L. **Frontiers in Microbiology**, 14, 1168102. <https://doi.org/10.3389/fmicb.2023.1168102>

Suphoronski, S. A., de Souza, F. P., Chideroli, R. T., Mantovani Favero, L., Ferrari, N. A., Ziemniczak, H. M., Gonçalves, D. D., Lopera Barrero, N. M., Pereira, U. P. (2021). Effect of *Enterococcus faecium* as a water

and/or feed additive on the gut microbiota, hematologic and immunological parameters, and resistance against francisellosis and streptococcosis in Nile Tilapia (*Oreochromis niloticus*). **Frontiers in Microbiology**, 12, 743957. <https://doi.org/10.3389/fmicb.2021.743957>

Torres-Maravilla, E., Parra, M., Maisey, K., Vargas, R. A., Cabezas-Cruz, A., Gonzalez, A., Tello, M., & Bermúdez-Humarán, L. G. (2024). Importance of probiotics in fish aquaculture: Towards the identification and design of novel probiotics. **Microorganisms**, 12(3), 626. <https://doi.org/10.3390/microorganisms12030626>

Valladão, G. M. R., Gallani, S. U., Pilarski, F. (2018). South American fish for continental aquaculture. **Reviews in Aquaculture**, 10(2), 351–369. <https://doi.org/10.1111/raq.12164>

Van Doan, H., Soltani, M., Ringø, E. (2021). In vitro antagonistic effect and in vivo protective efficacy of Gram-positive probiotics versus Gram-negative bacterial pathogens in finfish and shellfish. **Aquaculture**, 540, 736581. <https://doi.org/10.1016/j.aquaculture.2021.736581>

Vinderola, G., Gueimonde, M., Gomez-Gallego, C., Delfederico, L., Salminen, S. (2017). Correlation between in vitro and in vivo assays in selection of probiotics from traditional species of bacteria. **Trends in Food Science & Technology**, 68, 83–90. <https://doi.org/10.1016/j.tifs.2017.08.005>

Wang, X., Yao, Y., Ge, H., Zhang, J., Zhang, J., & Yan, Q. (2024). Isolation and identification of probiotic *Bacillus subtilis* AJQ03 from the intestinal tract of *Anguilla japonica* (Japanese eel). **Frontiers in Microbiology**, 15, 1446299. <https://doi.org/10.3389/fmicb.2024.1446299>

Wuertz, S., Schroeder, A., & Wanka, K. M. (2021). Probiotics in fish nutrition: Long-standing household remedy or native nutraceuticals? **Water**, 13(10), 1348. <https://doi.org/10.3390/w13101348>

Yang, H. L., Liu, Z. Y., Jian, J. T., Ye, J. D., & Sun, Y. Z. (2021). Host-associated *Bacillus siamensis* and *Lactococcus petauri* improved growth performance, innate immunity, antioxidant activity and ammonia tolerance in juvenile Japanese seabass (*Lateolabrax japonicus*). **Aquaculture Nutrition**, 27(6), 2739–2748. <https://doi.org/10.1111/anu.13399>

Zar, J. H. (1999). **Biostatistical analysis** (4th ed.). Prentice Hall.

Zhao, J., Zhao, J., Zang, J., Peng, C., Li, Z., & Zhang, P. (2024). Isolation, identification, and evaluation of lactic acid bacteria with probiotic potential from traditional fermented sour meat. **Frontiers in Microbiology**, 15, 1421285. <https://doi.org/10.3389/fmicb.2024.1421285>

Zuo, F., Yu, R., Feng, X., Chen, L., Zeng, Z., Khaskheli, G. B., Ma, H., & Chen, S. (2016). Characterization and in vitro properties of potential probiotic *Bifidobacterium* strains isolated from breast-fed infant feces. **Annals of Microbiology**, 66, 1027–1037. <https://doi.org/10.1007/s13213-015-1187-x>

¹ Graduate Program in Aquaculture, Nilton Lins University/National Institute of Amazonian Research (INPA), Manaus, Amazonas, Brazil, 69058-030. Division de Biotechnology, Centro de Apoiol Multidisciplinário (CAM), Federal University of Amazonas, Manaus, Amazonas, 69067-005, Brazil. E-mail: [acesse o artigo original para visualizar o e-mail](#); [acesse o artigo original para visualizar o e-mail](#)

² Graduate Program in Aquaculture, Nilton Lins University/National Institute of Amazonian Research (INPA), Manaus, Amazonas, Brazil, 69058-030.

³ Graduate Program in Aquaculture, Nilton Lins University/National Institute of Amazonian Research (INPA), Manaus, Amazonas, Brazil, 69058-030.

⁴ Graduate Program in Aquaculture, Nilton Lins University/National Institute of Amazonian Research (INPA), Manaus, Amazonas, Brazil, 69058-030.

⁵ Graduate Program in Aquaculture, Nilton Lins University/National Institute of Amazonian Research (INPA), Manaus, Amazonas, Brazil, 69058-030.

⁶ Graduate Program in Aquaculture, Nilton Lins University/National Institute of Amazonian Research (INPA), Manaus, Amazonas, Brazil, 69058-030.

⁷ Department of Preventive Veterinary Medicine, School of Veterinary Medicine, Federal University of Minas Gerais—UFMG, Belo Horizonte 31270-901, MG, Brazil.

⁸ Graduate Program in Aquaculture, Nilton Lins University/National Institute of Amazonian Research (INPA), Manaus, Amazonas, Brazil, 69058-030.