

Isolation and Identification of a Potential Amylolytic Probiotic Bacterium from the Gut of Jundiá Catfish, *Rhamdia quelen*.

Tatiana Vieira Poletto¹, Cleide Rosana Werneck Vieira², Carlos Peres Silva³, Debora Machado Fracalossi^{1*};

¹Federal University of Santa Catarina - Aquaculture Department; ² Universidade Federal de Santa Catarina ✓ - Department of Food Science and Technology; ³ Universidade Federal de Santa Catarina ✓ - Biochemistry

ABSTRACT

This study aimed to isolate a potential probiotic amylolytic strain from the gut of jundiá catfish to improve carbohydrate digestibility in fish. Two of 31 strains isolated from the foregut of *Rhamdia quelen* were able to grow on starch-agar medium and were considered amylolytic. The strain that presented higher amylolytic potential, based on a qualitative amylase assay, was chosen. The strain was phenotypically characterized and analysed to determine bile and pH tolerance and extracellular quantitative amylase activity. The probiotic candidate, identified as *Aeromonas veronii*, showed the ability to survive stresses from a range of pH and bile salt conditions and secreted an interesting enzymatic profile, which may exhibit a synergistic effect when combined with the enzymes secreted by the jundiá catfish, improving carbohydrate digestion in the host. The results demonstrated the potential of *A. veronii* to improve the digestion process in jundiá by providing exogenous enzymes for the breakdown and absorption of nutrients.

Keywords: enzymes, probiotics, aquaculture, intestinal microbiology, *Aeromonas veronii*



* Author for correspondence: debora.fracalossi@ufsc.br

INTRODUCTION

Feed production for aquaculture remains dependent on expensive protein-rich ingredients to meet the nutritional requirements of fish ^{1,2}. Nutritional requirements and capacity to utilize diverse ingredients vary according to fish gut physiology and morphology ^{1,3}, making the search for alternative ingredients difficult for some fish species.

Carbohydrate is an interesting type of macronutrient that can be included in formulated diets as a source of energy, representing an efficient way to decrease costs and contribute to the protein-sparing effect ^{4,5}. However, there is no evidence of specific dietary requirement for carbohydrate in fish ⁶, and fish do not use dietary carbohydrate as efficiently as terrestrial animals.

The jundiá catfish *Rhamdia quelen* is widely distributed and is a species of interest in the aquaculture sector in Brazil, Uruguay and Argentina. This fish species is a good model for carbohydrate utilization, as despite its omnivore feeding habits, it exhibits a short intestine, which hinders dietary carbohydrate utilization ⁷⁻⁹ compared with other typical omnivores such as Nile tilapia (*Oreochromis niloticus*) ^{3,9}.

Probiotic bacteria play roles in the digestive and nutritional processes of aquatic animals ¹⁰ through the production of enzymes ¹¹. There is vast potential for research linked to the use of these enzyme-producing bacteria to the increase feed efficiency and, consequently, improve the growth of the host. Therefore, the aim of this study was to isolate a probiotic strain with amylolytic potential from a native freshwater fish (*R. quelen*) to improve carbohydrate digestibility.

MATERIAL AND METHODS

Preparation of the Foregut from Jundiá Catfish

Ten female jundiá catfish (95.45 ± 6.66 g) were starved for 48 h to clean the gastrointestinal tract before dissection ¹⁴. All of the fish were sacrificed through a sharp blow to the head, and their proximal intestine (foregut) was removed aseptically, then cut into pieces and flushed three times using a syringe with a chilled sterile 0.9% sodium chloride solution. The proximal intestines were homogenized in the chilled sterile 0.9% NaCl solution ¹⁵ with a sterile mortar. All of the procedures used in the present study complied with the guidelines for animal care of the local Ethics Committee on the Use of Animals (CEUA-UFSC, protocol number PP00815).

Detection of Amylase-Producing Bacteria

The foregut homogenate was used after 10-fold dilutions were obtained, which were spread in triplicate aliquots (100 μ l) on tryptic soy agar (TSA) to characterize the total viable counts of autochthonous aerobic bacteria. The plates were incubated at 32 °C for 24 h, and colonies were purified by streaking ¹⁴ and then transferred to starch-agar medium (SA) ¹⁶, followed by incubation at 32 °C for 24 h. The strains that grew under these conditions were considered amylolytic ¹⁷.

Determination of Possible Harmful Effects of the Bacterial Isolates on the Host

To verify that the selected isolates were not pathogenic to the host, 36 juveniles of jundiá (28.4 ± 5.0 g) were stocked in three aquaria (50 L glass; one for each treatment,

consisting of the two probiotic candidates and the control), with 70% daily water exchange, continuous aeration and a constant temperature (28 °C) for 120 h.

Both probiotic candidates were grown in tryptic soy broth medium (TSB) in a shaking incubator at 32 °C overnight. After incubation, the cells were harvested via centrifugation (5 000 g, 5 min), washed twice in sodium chloride solution and re-suspended in the same sterile saline solution. Dilution plating was used to verify the relationship between the OD₆₀₀ and the number of colony-forming units (CFU) per millilitre. The absorbance at 600 nm was adjusted to 0.60 ± 0.05 to standardize the number of bacteria (10^9 CFU mL⁻¹).

All fish were inoculated intraperitoneally with 0.1 mL of saline containing 10^9 CFU mL⁻¹ of respective selected isolate, except for the control group, which was injected with sterile phosphate-buffered saline (PBS) alone. During the experiment, the behaviour of the fish was monitored, and after 120 h, all fish were sacrificed as described above, and their organs were examined for evidence of disease. To check for the presence of the respective probiotic candidate strain, swabbed materials from the peritoneal cavity and kidneys of all fish were inoculated onto TSA and incubated at 32 °C for 24 h.

Phenotypic Characterization

The probiotic candidates were grown on TSA at 32 °C for 24 h, and the following characteristics of the isolates were tested: Gram reaction (KOH method), shape and motility (phase contrast microscopy), cytochrome oxidase activity, catalase formation (H₂O₂, 3% v/v) and glucose metabolism (O/F test).

Qualitative Amylase Assay

The isolated bacteria were inoculated on starch-agar plates, which were incubated at 32 °C for 24 h after Lugol's iodine solution [(w/v) 1% iodine in 2% potassium iodide] was poured over the plates. Extracellular amylase activities were detected as clear zones (halos) around the colonies, and the halo diameter was estimated using a calliper rule. The probiotic candidate presenting the largest halo around its colonies was chosen for further experiments.

Genotypic Identification

For DNA isolation, the selected strain was grown in TSB at 32 °C for 24 h. Bacterial genomic DNA was isolated using the DNeasy® Tissue Kit (QIAGEN, CA, USA). Sequences of the 16S rDNA gene were amplified using the universal bacterial primers 8F (5'-AGAGTTTGATCCTTGGCTCAG-3'), 519R (5'-GTATTACCGCGGCTGCTG-3'), 519F (5'-CAGCAGCCGCGGTAATAC-3') and 1492R (5'-GCYTACCTTGTTACGACTT-3'). PCR amplification was performed using a thermal cycler (2720, Applied Biosystems), with an initial denaturation step of 4 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 45 s at 50 °C, and 1 min at 72 °C, and a final extension step of 10 min at 72 °C. Five microliters of the DNA extract was used for amplification in a total volume of 50 µl containing 1.25 U of AmpliTaq DNA polymerase LD (Applied Biosystems, CA, USA). After purification, the PCR products were directly sequenced via the Sanger sequencing method using an ABI PRISM 350 Genetic Analyzer with the ABI BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems). The obtained DNA sequence was compared with the GenBank database of the National Center for Biotechnology Information (NCBI) using the

BLAST program. A phylogenetic analysis was carried out using Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0.

Quantitative Amylase Assay

The selected probiotic candidate was grown previously in 25 mL of TSB medium overnight at 32 °C. A 0.5 mL aliquot of the liquid culture that was produced was subsequently inoculated into a flask with starch “liquid” medium, consisting of the starch-agar medium without the agar^{14,16,17}. The culture flask was incubated at 32 °C for 24 h. The cells were then harvested via centrifugation (10 000 g, 10 min, 4 °C), and the cell-free supernatant solution, which was used as the enzyme extract, was divided in aliquots in 15 mL Eppendorf tubes and stored at -80 °C until analysis. Amylase activity was measured via the 3,5-dinitrosalicylic acid (DNS) method (Noelting and Bernfeld 1948). The reaction mixture consisted of 25 µl of a 1% (w/v) starch solution as a substrate, 25 µl of Tris-HCl buffer [50 mmol, pH 8.5] and 50 µl of the sampled enzyme extract. The reaction mixture was diluted with distilled water, and the absorbance at 550 nm was recorded using a microplate reader (Tecan Infinite 200). This method is based on the estimation of reduced sugar using maltose as the standard. Quantitative enzyme activity was expressed as units (U). One amylase unit was defined as the amount of enzyme per millilitre of culture filtrate releasing 1 microgram of reducing sugar per minute. Total protein determination was performed according to Bradford (1976) using bovine serum albumin as a standard. Specific activity was defined in terms of the enzyme activity units per mg of total protein.

Enzymatic Characterization

Enzymatic characterization of the potential probiotic strain was performed using the API ZYM system (bioMérieux, Marcy l’Etoile, France) according to the manufacturer’s instructions.

pH and Bile Tolerance

The selected probiotic candidate was grown previously in TSB medium overnight at 32 °C. The absorbance (A_{600 nm}) was adjusted to 0.25 ± 0.05 to standardize the bacterial concentration (10⁶ to 10⁷ CFU mL⁻¹). Aliquots of the bacterial suspension were centrifuged (5 000 g, 10 min) and resuspended in sterile PBS, which was adjusted to different pH levels (2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0) using 1% HCl or NaOH. The samples were then incubated for 2 h at 32 °C. After each incubation period, the OD₆₀₀ was measured, and the samples were serially diluted in sterile PBS to check the viable counts through plate counting using TSA. Tolerance to different bile concentrations was determined using an initial suspension (10⁶ to 10⁷ CFU mL⁻¹) of the selected strain grown in TSB medium. Aliquots of the bacterial suspension were centrifuged (5 000 g, 10 min) and then resuspended in sterile PBS with 0, 0.15 or 0.3% (w/v) ox-bile. The samples were subsequently incubated for 2, 4 and 8 h at 32 °C. After each incubation period, the optical density was measured with a Genesis 10S spectrophotometer (Thermo Scientific, MA, USA) at 600 nm, and the samples were serially diluted in sterile PBS to check the viable counts through plate counting using TSA. All *in vitro* experiments were executed in triplicate.

RESULTS AND DISCUSSION

Two of the 31 isolates obtained on (TSA)(2.2% of the total), which came from the foregut of jundiá, were able to grow on Starch-agar medium and were therefore considered amylolytic. There was no observed mortality and no evidence of any harmful effects of either selected strain (designated M2 and E7 until proper identification) on jundiá juveniles, insofar as there was a total absence of any signs of disease following challenge via the intraperitoneal route. The M2 isolate was a Gram-negative, motile, rod-shaped bacterium, presenting non-fermentative glucose metabolism and catalase and oxidase positivity. The other isolate, E7, was a Gram-negative, motile, rod-shaped, glucose-fermenting, catalase- and oxidase-positive bacterium.

To assess the intensity of extracellular amylolytic enzyme production by the two isolated strains, a qualitative assay was conducted, and the halo-forming zone expressed in one culture (E7) reached 4 mm, compared with the formation of a 0.5 mm halo by M2 strain. Considering this result, only the E7 strain was retained, as it showed better results regarding qualitative amylolytic enzyme production. Based on 16S rDNA sequence analysis, the selected E7 strain was identified as *Aeromonas veronii*, with homology of 99%.

Perhaps the most important criterion for selecting a potential probiotic is the ability of the bacterium to colonize the intestine of the host^{19,20}. Based on adaptation allowing attachment to intestinal tissues, an adequate method for selecting a probiont is to extract it directly from the GI tract of the host²¹. This procedure does not limit the potential of the probiont merely to that of a transit strain²², even though transient bacteria can produce a positive influence in the host²³.

The probiotic candidate isolated in this study probably belongs to the autochthonous microbiota, as neither of the two previous procedures performed to guarantee the removal of allochthonous bacteria impaired the ability of the strain to remain adhered to the jundiá intestine. Another study showed the high adhesive ability of *A. veronii* in contact with the carp intestine under feeding or no-feeding conditions, which resulted in bacterial representativeness of 50% in both groups, in contrast to the other isolates²⁴. This evidence corroborates the positive colonization status of *A. veronii* observed in the intestine of jundiá in the present study.

The genus *Aeromonas* is widely described as an emerging pathogen in aquatic organisms and humans²⁵, but there is still a missing link between this virulent genus and a specific species or originating source. This situation becomes more obvious when one species is able to present multiple virulence factors without showing definite virulence potential²⁶. The utilization of a bacterium from this exceptionally diverse genus as a probiotic can have positive effects as long as probiotic strains compete effectively. It is important to note that it would be highly desirable to obtain such strains belonging to the same pathogenic species that have been searching for nutrients and space to attach to a fish's intestine²⁷. In the present study, *A. veronii* did not present any harmful effects in jundiá catfish, providing an initial basis for a mutualistic relationship²⁸. Moreover, the high observed nucleotide variability^{29,30} and lack of an established pathogenic consensus about Aeromonads in humans²⁵ leave many gaps to be filled to enable the use of such strains as possible probionts.

In this study, the amylase enzyme activity measured in *A. veronii* was 28.57 U mL⁻¹, and the specific activity was 3.57 U mg⁻¹. In addition, an enzymatic profile determined using the API ZYM system was established for the probiotic candidate and is summarized in Table 1.

API ZYM is a semi-quantitative assay for screening 19 hydrolytic enzymes, among which eight carbohydrases are considered. However, only N-acetyl- β -glucosaminidase presented positive activity in *A. veronii* in the present study. All of the other carbohydrases presented negative activity, including α -glucosidase, which decreases the range of susceptible carbohydrates to allow complete degradation by this exogenous

enzyme for aquaculture feed usage, as α -glucosidase is adapted to hydrolyse many carbohydrates, such as α -D-glucosides (ρ -nitrophenyl- α -D-glucoside and methyl- α -D-glucoside), oligosaccharides (maltodextrins), and polysaccharides (amylose, amylopectin, and glycogen)³¹. On the other hand, the host itself may secrete this α -glucosidase enzyme, thus avoiding the limitation imposed by *A. veronii*. This hypothesis is supported by the fact that the digestion of jundiá is improved by the inclusion of ingredients from plant sources with higher levels of maltose, instead of starch, within tested diets³². Moreover, the promising synergistic effect of the combination of α -glucosidase and α -amylase resulted in starch granule hydrolysis that was 8 to 11 times greater than the sum of the hydrolysis achieved by two enzymes alone³¹.

Table 1 Enzymatic profile (API ZYM test) of *A. veronii* probiotic candidate

Biochemical test	<i>A. veronii</i>
Alkaline phosphatase	-
Esterase (C4)	+
Esterase lipase (C8)	+
Lipase (C4)	-
Leucine arylamidase	+
Valine arylamidase	-
Cystine arylamidase	-
Trypsin	-
α -Chymotrypsin	+
Acid phosphatase	+
Naphthol-AS-BI-phosphohydrolase	+
α -Galactosidase	-
β -Galactosidase	-
β -Glucuronidase	-
α -Glucosidase	-
β -Glucosidase	-
N-acetyl-b-glucosaminidase	+
α -Mannosidase	-
α -Fucosidase	-

The selected strain presented high tolerance to acidic conditions (Fig. 1). Resistance of the isolate was observed after exposure to acidified media, except at a pH of 2. However, the greatest tolerance of *A. veronii* occurred between pH 5 and 9. The tolerance of *A. veronii* in the presence of bile salts was also analysed, and the results showed that it was able to survive at both of the concentrations tested (0.15 and 0.3%) (Fig. 2). Our findings showed that *A. veronii* presents pH and bile tolerance, starting at exposure to a pH of 3 and in all of the tested concentrations of bile salts (0.15% - 0.3%). Despite the great importance attributed to probiont gastric survival thus far, studies have shown that it is possible to achieve sufficient viability of acid-sensitive strains in the stomach by inducing pH elevation through the addition of feed³³ or via offering physical protection mechanisms to the microorganisms³⁴. In this context, an essential criterion for the selection of probiotics, in addition to bile salt tolerance^{22,35}, should be the ability to tolerate an alkaline pH³⁴ close to 8.5, which occurs in the region posterior to the pylorus³⁶, thus enabling probiont survival and colonization of the small intestine. According to this scenario, the present study showed promising results, as *A. veronii* was able to tolerate

pH levels ranging from neutral up to pH 9, which was the maximum level tested in this study.

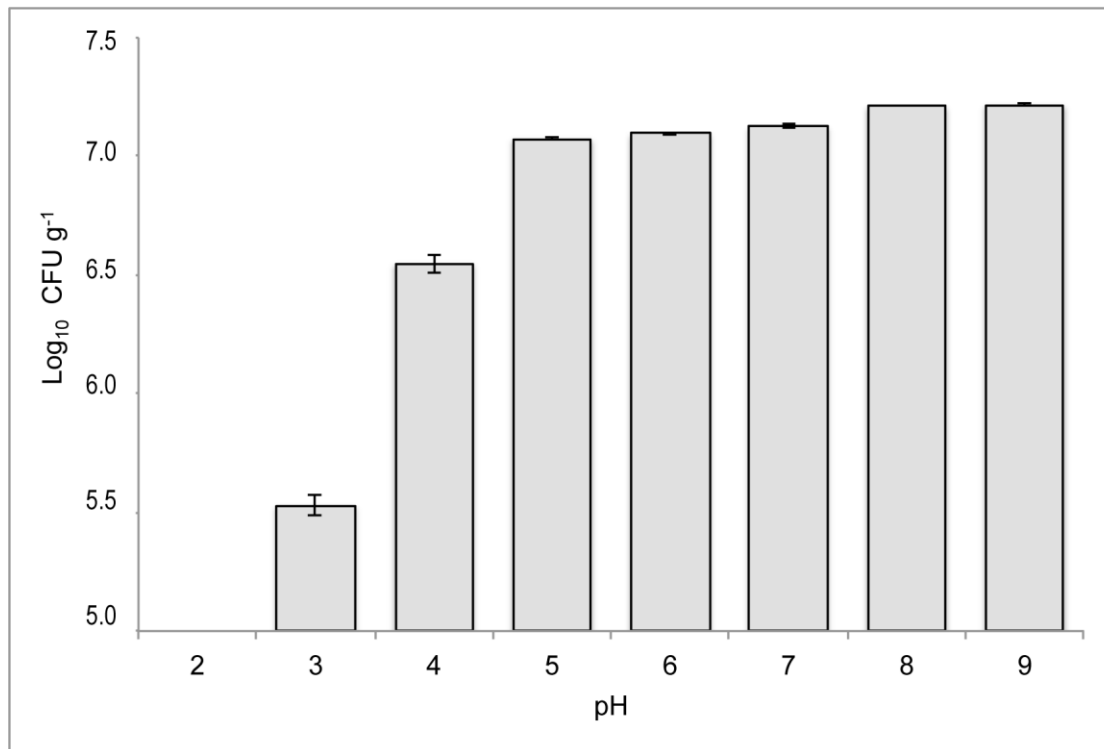


Figure 1 – pH tolerance of *A. veronii* in TSB after 2 h of incubation. Results are expressed as Log₁₀ CFU g⁻¹ of bacteria survival in different pH. Each value is the mean ± SEM.

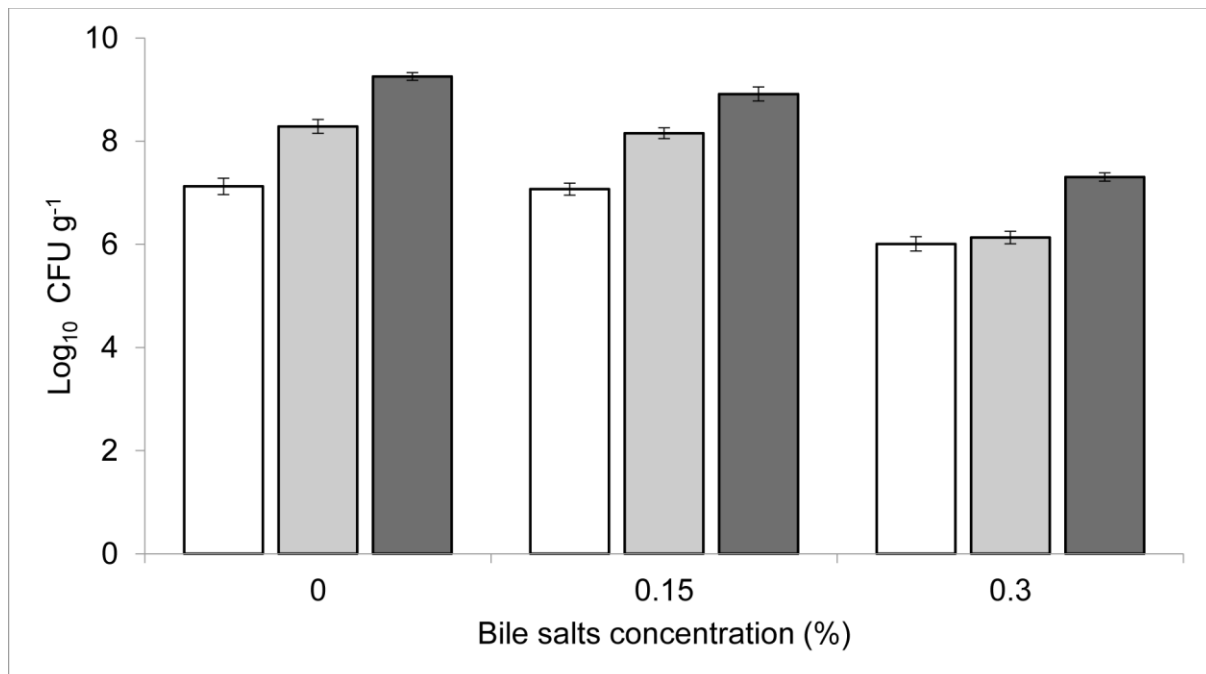


Figure 2 - Bile salt tolerance of *A. veronii* in TSB after 2 h (□), 4 h (◻) and 8 h (◼) of incubation. Results are expressed by Log₁₀ CFU g⁻¹ of bacteria survival in different concentrations of bile salts (%). Each value is the mean ± SEM.

In conclusion, the results of this study demonstrated the potential of the isolated *A. veronii* strain to improve the digestion process of jundiá, by providing exogenous enzymes for the breakdown and absorption of nutrients. The bacterium isolated from the intestinal tract of jundiá was able to produce enzymes that are commonly used in the digestion process, including phosphatase, lipase, esterase, peptidase and carbohydrase. Another favourable feature of *A. veronii* is its ability to survive stress exerted by a range of pH and bile salt conditions. Efforts to improve the utilization of enzyme-producing bacterial isolates as probiotics in feed formulated with higher contents of carbohydrates are essential to reduce costs and decrease dependence on ingredients of animal origin, thereby enhancing the sustainability of aquaculture.

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