http://dx.doi.org/10.1590/1678-4162-12404

Arq. Bras. Med. Vet. Zootec., v.74, n.2, p.327-337, 2022

In vitro selection of autochthonous bacterium with probiotic potential for the neotropical fish piauçu *Megaleporinus microcephalus*

[*Seleção* in vitro *de bactéria autóctone com potencial probiótico para o peixe neotropical piauçu* Megaleporinus macrocephalus]

F.A.L. *Barros*[1], L.A. *Silva*[2], J.A.R. *Dias*[1], H.A. *Abe*[1], P.E.G. *Paixão*[3], N.C. *Sousa*[1], C.A.M. *Cordeiro*[1], R.Y. *Fujimoto*[4]

[1]Universidade Federal do Pará, UFPA, *Campus* Bragança, Bragança, PA, Brasil
[2]Graduate, Instituto Federal do Pará, IFPA, *Campus* Tucuruí, Tucuruí, PA, Brasil
[3]Universidade Tiradentes, Unit, Farolândia, SE, Brasil
[4]Empresa Brasileira de Pesquisa Agropecuária, Embrapa, Aracaju, SE, Brasil

ABSTRACT

The study aimed to isolate, identify, and apply *in vitro* tests on bacteria with autochthonous probiotic potential isolated from fifteen healthy specimens of *Megaleporinus macrocephalus*. The strains were selected from the intestinal tract of fish and inoculated in the Petri plate containing Sharp Man Rogosa Agar (MRS) for (48 hours at 35ºC). They were isolated based on a test of catalase, Gram stain, tolerance to different gradients NaCl (1, 2 and 3%), pH (4, 5, 6, 8 and 9) values and bile salts (2.5 and 5%), in addition to the inhibition zone against pathogens. Of the 42 strains isolated, ST1 and ST9 had higher values ($p<0.05$) for total viable cells (31.80±0.07 and 32.51±0.05 CFU/mL × 10^8) respectively. In the resistance tests, strains ST1 and ST9 presented the best results, with emphasis on ST9 in the gradients of pH, high values of bile salts and larger inhibition zones against *Aeromonas hydrophila* and *Aeromonas jandaei*. The strains with the best results in the tests, ST1 and ST9, were identified by the MALDI-TOF-MS method as *Enterococcus faecium*. Thus, the isolated *E. faecium* bacteria, may be recommended as for probiotic use in farming the *M. macrocephalus.*

Keywords: bacteria selection, lactic acid, inhibition of pathogens, specific species

RESUMO

O presente estudo visou isolar, identificar e aplicar testes in vitro *em bactérias com potencial probiótico, autóctones, isoladas de 15 espécimes saudáveis de* Megaleporinus macrocephalus. *As cepas foram selecionadas do trato intestinal dos peixes e inoculadas em placas de Petri contendo Man Rogosa Sharped Agar (MRS), por 48 horas, a 35ºC. Foram isoladas com base em teste de catalase, coloração de Gram, tolerância a diferentes gradientes de NaCl (1, 2 e 3%), valores de pH (4, 5, 6, 8 e 9) e sais biliares (2,5 e 5%), além do halo de inibição contra patógenos. Das 42 cepas isoladas, ST1 e ST9 apresentaram maiores valores (P<0,05) para células viáveis totais (31,80±0,07 e 32,51±0,05 UFC/mL × 10^8), respectivamente. Nos testes de resistência, as cepas ST1 e ST9 apresentaram os melhores resultados, com destaque para ST9 nos gradientes de pH, altos valores de sais biliares e maiores halos de inibição contra* Aeromonas hydrophila *e* Aeromonas jandaei. *As cepas com melhores resultados nos testes, ST1 e ST9, foram identificadas pelo método de MALDI-TOF-MS como* Enterococcus faecium. *Assim, a bactéria isolada* Enterococcus faecium, *pode ser recomendada para uso probiótico na criação do* M. macrocephalus.

Palavras-chave: seleção de bactérias, ácido lático, inibição de patógenos, espécie- específico

Corresponding author: alxlbarros@gmail.com
Submitted: May 24, 2021. Accepted: February 18, 2022.

INTRODUCTION

Globally, aquaculture production is continuously expanding, generating approximately USD 250 billion in 2018 (The state…, 2020). However, intensive production has provoked the outbreak of diseases, mainly because of bacterial infection, causing productive and economic losses (Madani *et al*., 2018). For pathogen control, several antibiotics are used by fish farm, sometimes inappropriately (Doan *et al*., 2018), with deleterious effects on water quality parameters. In addition, chemicals can bioaccumulate in reared aquatic organisms and promote the selection of resistant bacteria (Qi *et al*., 2020).

As alternatives to chemical substances, probiotics are commonly used as a prophylactic management strategy, improving growth and immunology (Doan *et al*., 2018; Sousa *et al*., 2019). However, to obtain its benefits, the microorganisms with probiotic potential must completely colonize the intestinal tract of the host. Thus, autochthonous microorganisms commonly show greater colonization efficiency than allochthonous ones because of their specific relationship with the host (Dias *et al*., 2019; Sousa *et al*., 2019; Yamashita *et al*., 2020).

In vitro assays can aid in the selection of autochthonous bacteria with probiotic potential, determining their survival in different physiological conditions as well as their inhibitory capacity against pathogens (Dias *et al*., 2019; Pereira *et al*., 2019; Paixão *et al*., 2020; Qi *et al*., 2020; Sousa *et al*., 2019). Some studies have reported positive results for both in vitro and vivo assays using autochthonous probiotic bacteria in aquaculture, such as *Lactobacillus* spp. from lambari *Astyanax bimaculatus* (Jatobá *et al*., 2017), *Bacillus cereus* of tambaqui *Colossoma macropomum* (Dias *et al*., 2018), *Enterococcus faecium* of the species pirarucu *Arapaima gigas* (Sousa *et al*., 2019) and *Lactococcus lactisa* selected from jandiá *Rhamdia quelen* (Yamashita *et al*., 2020). However, despite several reports for aquaculture, some native fish species with economic importance remain without any scientific information about the use of autochthonous probiotics.

The Anostomidae family is the second most diverse among the Characiformes, with approximately 150 species (Fricke *et al*., 2019; Garavello and Britski, 2003). While the genus *Leporinus* comprises a little more than half of all the diversity of the family, with about 80 valid species (Burns *et al*., 2014 Ramirez *et al*., 2016). Recent attempts have distinguished phylogenetic differences in the monophyletic groups of species as distinct genera and have included 9 species of *Leporinus* in a new genus *Megaleporinus*, including *macrocephalus* (Birindelli *et al*., 2020; Ramirez *et al*., 2017). The genus *Megaleporinus* was diagnosed by the reduction in the number of teeth (only three teeth in each mandible), the presence of a ZW sex chromosome and most of them have a large body, reaching up to 500 mm LS (Ramirez *et al*., 2017).

Among the neotropical fish species with national importance, the native fish piauçu *Megaleporinus macrocephalus* of Paraná River Bay plays an important role in aquaculture (Garavello and Britski, 1988; Ramirez *et al*., 2017). It has been introduced into the Northern region of Brazil in state of Acre (Martins *et al*., 2017). The species shows well-developed reproduction in captivity (Martins and Yoshitoshi, 2003), a large growth potential (Takahashi *et al*., 2004), and readily accepts industrial feed, either extruded or pellet rations (Soares-Júnior *et al*., 2013).

Even with the large potential for captivity production, there are no reports about the use of autochthonous bacteria as probiotics for piauçu. Thus, the current study aimed to isolate and apply in vitro tests in autochthonous bacteria with probiotic potential for the neotropical fish species *Megaleporinus macrocephalus*.

MATERIALS AND METHODS

For the isolation of bacteria with probiotic potential, 15 *M. macrocephalus* specimens from extensive rearing (0.785±0.12kg and 26.59±0.23cm) were anesthetized (benzocaine 20mg/L), sterilized with 70% alcohol, and euthanized by medullar section according to protocols of the Ethical Committee for Animal Use (CEUA number 3991300420). Afterward, the intestinal tracts were removed, selecting the anterior and medium parts

(approximately 1g), which were macerated in saline solution NaCl 0.65%, submitted to serial dilution (1:10 factor), and inoculated on petri plates containing de Man Rugosa Sharped Agar (MRS Agar) with 1% aniline blue. After inoculation, the plates were kept in an oven at 35ºC for 48 hours (Jatobá *et al*., 2008).

Only lactic acid bacteria with coccus and bacillus morphology, gram positive, catalase negative, and with a blue color were selected (Dias *et al*., 2019; Jatobá *et al*., 2008; Vieira *et al*., 2013). Developed blue colonies were isolated in petri plates containing MRS Agar (48 hours at 35ºC) through the streak plate technique to ensure the purity of the strain. To determine the bacterial growth kinetics, each strain was inoculated in MRS broth and incubated for 24 hours at 35ºC. During incubation, an aliquot (3mL) was collected every 2 hours to determine absorbance 630 nm via a spectrophotometer (Jatobá *et al*., 2008). At the same time, another aliquot (100 µL) was inoculated on a petri plate containing MRS Agar and incubated for 48 hours at 35ºC to determine the colony- forming unit (CFU/mL^{-1}). Based on these results, maximum growth rate and duplicating time of strains were calculated (Jatobá *et al*., 2008; Vieira *et al*., 2013).

In vitro assays were carried out with grown bacteria in MRS broth (24 hours at 35ºC) containing different levels of NaCl (0.0, 1.5, 3.0 and 4.5%), pH (4, 5, 6, 8, 9, and the control 7), and bile salt (2.5 and 5.0% w/v), all with four replicates (Jatobá *et al*., 2008; Vieira *et al*., 2013). Growth percentage was determined using absorbance at 63nm in a spectrophotometer (Jatobá *et al*., 2008; Vieira *et al*., 2013). The inhibitory ability against pathogens was evaluated measuring the inhibitory halo (Jatobá *et al*., 2008). Discs with a diameter of 0.8cm were removed from petri plates containing acid lactic bacteria and placed on petri plates containing Tryptone Soya Agar (TSA) previously inoculated with *Aeromonas hydrophila*, *Aeromonas caviae*, *Aeromonas jandaei*, *Pseudomonas aeruginosa*, and *Streptococcus agalactiae*. A positive control without probiotic bacteria, containing only antibiotic (oxytetracycline at 3 mg/L), was used to compare the results according to Vieira *et al*. (2013) and Paixão *et al*. (2020). After incubation (48 hours at 35ºC), the inhibition halo (mm) against the pathogen was determined. This experiment was performed in a completely randomized design with four replicates per treatment.

The bacterium with better performance regarding probiotic use was identified as the species level for method MALDI-TOF-MS (matrix-assisted laser desorption ionization time-of-flight mass spectrometry) using the molecular weight of ribosomal proteins with laser shots at a wavelength of 260-337 nm. Scores ≥1.7 (Paixão *et al*., 2020; Sousa *et al*., 2019).

In vitro tests data and bacterial counts were square root-transformed and subjected to normality and homoscedasticity tests (Shapiro Wilk and Levene, respectively). Analysis of variance (ANOVA one-way) with post hoc Tukey´s test ($p<0.05$) was used to compare means, using the statistical software Past 3.0.

RESULTS

Of the 42 isolated strains, only 12 showed probiotic potential after the analysis of affinity biochemical characterization with aniline blue dye, Gram stain and catalase test. (Table 1).

The antagonistic capacity against pathogens was determined by the diameter of the inhibition discs; strain ST9 showed the best results ($p < 0.05$). This strain showed a greater inhibition halo against fish pathogens such as *Aeromonas hydrophila* and *Aeromonas jandaei*, followed by ST8 and ST10 with high values for *Aeromonas caviae*. Strain ST10 showed inhibition values like those for ST9 regarding *Pseudomonas aeruginosa* and *Staphylococcus agalactiae*. Both ST1 and ST2 demonstrated the lowest values for *Staphylococcus agalactiae*, followed by ST11 and ST12 against *Aeromonas hydrophila*. Strain ST9 also showed greater values when compared to the positive control regarding *Aeromonas hydrophila* and *Aeromonas jandaei*, but similar values for *A. caviae*, *P. aeruginosa*, and *S. agalactiae* (Table 3).

Table 1. Determination of the biochemical characteristics of the isolated piauçu (*Megaleporinus macrocephalus*) strains

Strain	Aniline blue dye	Gram stain	Catalase test
ST1	+	+	-
ST2	+	+	-
ST3	+	-	+
ST4	+	-	+
ST5	+	-	+
ST6	+	+	-
ST7	+	+	-
ST8	+	+	-
ST9	+	+	-
ST10	+	-	+
ST11	+	-	+
ST12	+	-	+

* Affinity with aniline blue dye (+) or non-affinity (-); Gram stain: positive (+) or negative (-); catalase test: positive (+) or negative (-).

Two strains (ST1 and ST9) showed greater values ($p<0.05$) for total viable cells (31.80±0.07 and 32.51±0.05 CFU/mL × 10^8) and lower duplication periods (4.39±0.04 and 4.36±0.04 h), respectively. The maximum growth rate was observed for ST1 and ST11 (0.15±0.01 and 0.15±0.02 cells/hour) respectively. In the resistance tests, strains ST1 and ST9 showed the best results over NaCl, pH, and bile salt variation, highlighting ST9 ($p<0.05$) for pH at alkaline levels (8) and high values of bile salt (2.5% w/v) (Table 2).

Table 2. Bacterial growth kinetics: total viable bacteria count after 24 hours (TVB – CFU/mL^{-1} x 10^8), maximum growth rate (MGR), duplicating time (DT); and tests of resistance to NaCl values, pH scales of bile salts (BS) in reducing the absorbance of strains (%), of autochthonous bacteria isolated from piauçu (*Megaleporinus macrocephalus*)

Strain	TVB (CFU/mL-^{1} x 10^8)	MGR (h^{-1})	DT (h)	NaCl 1%	NaCl 2%	NaCl 3%	pH 4
ST1	31.80±0.07 a	0.15±0.01 a	4.39±0.04 a	77.86±0.40 a	65.30±1.12 a	61.22±1.51 a	4.27±4.27c
ST2	5.09±0.01 c	0.07±0.02 d	5.69±0.02 e	74.55±0.70 a	55.95±1.49 b	37.48±2.04 b	3.20±1.9c
ST3	5.04±0.01 c	0.09±0.00 c	5.45±0.01 d	36.43±2.20 d	31.70±1.73 d	23.47±1.19 d	2.26±0.08c
ST4	4.87±0.00 d	0.04±0.00 e	6.01±0.02 f	47.97±0.91 b	39.88±1.25 c	22.91±0.51 de	2.94±0.89c
ST5	1.54±0.00 e	0.07±0.00 d	5.41±0.04 d	30.02±1.65 e	38.13±1.43 c	20.48±0.57 e	0.46±0.03d
ST6	4.93±0.06 d	0.06±0.01 d	5.28±0.15 d	26.92±0.77 f	22.32±0.44 g	13.78±0.47 f	0.37±0.05d
ST7	4.93±0.04 cd	0.07±0.00 d	5.00±0.06 c	40.30±0.81 c	25.26±1.35 f	15.74±0.57 f	6.30±0.45bc
ST8	1.53±0.03 e	0.06±0.01 d	5.44±0.14 d	35.80±2.33 d	22.91±0.51 fg	15.19±0.99 f	7.69±0.89b
ST9	32.51±0.05 a	0.10±0.00 b	4.36±0.04 a	74.76±1.20 a	67.74±0.76 a	62.76±0.93 a	26.22±0.93a
ST10	1.55±0.12 e	0.11±0.01 b	4.81±0.13 b	31.27±0.16 e	27.90±1.81 e	16.61±1.06 f	6.41±0.21bc
ST11	5.73±0.07 b	0.15±0.02 a	4.82±0.03 bc	44.73±0.38 b	37.26±0.20 c	26.37±1.66 c	0.19±0.08d
ST12	1.53±0.01 e	0.07±0.00 d	5.10±0.04 cd	31.07±1.69 e	28.90±0.79 e	15.49±0.46 f	8.62±0.54d
P-value	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001

	pH 5	pH6	pH8	pH9	SB 2.5%	SB 5%
ST1	21.41±1.68c	59.30±1.68c	65.89±0.74b	10.83±1.33a	48.69±2.78b	32.52±2.87bc
ST2	23.91±0.74bc	60.75±1.16c	75.28±1.55ab	7.99±0.33b	49.00±0.36b	23.66±1.22e
ST3	24.72±1.90bc	87.07±1.22a	71.07±1.63ab	7.82±1.33b	43.30±2.30bc	35.25±0.99b
ST4	23.66±1.40c	64.35±1.57c	37.65±1.67b	0.47±0.08de	43.09±0.95bc	29.45±1.20cd
ST5	10.93±1.34d	69.03±1.82bc	70.98±1.46ab	4.31±0.96c	41.15±1.44c	28.81±2.40d
ST6	9.33±1.27d	61.51±1.91c	35.48±1.40d	0.08±0.03e	29.74±3.16d	21.85±0.81ef
ST7	27.10±0.68b	61.69±0.93c	62.53±1.75d	1.11±0.46d	47.75±1.97b	32.39±1.26bc
ST8	37.49±2.24a	69.29±1.87bc	38.34±0.89d	0.57±1.15d	32.51±1.97d	20.09±0.46f
ST9	36.92±1.72a	76.37±1.56b	80.40±1.26[a]	12.04±0.82a	80.78±0.44[a]	51.25±0.56[a]
ST10	29.19±1.14b	62.43±0.49bc	46.61±0.48cd	0.45±0.17de	44.92±3.72bc	23.94±0.20e
ST11	3.65±0.08e	62.26±1.13bc	52.95±1.83c	9.50±2.19ab	49.31±2.81b	29.02±0.50d
ST12	38.50±2.01a	66.83±0.72bc	41.12±1.16d	3.05±0.38c	25.03±2.07e	13.99±0.58g
P-value	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001

Mean values (mm) ± standard deviation with different letter in the column means statistical difference by Tukey test ($p<0.05$).

Table 3. Inhibition halos of autochthonous lactic acid bacteria strains isolated from piauçu (*Megaleporinus macrocephalus*) against pathogenic bacteria *Aeromonas hydrophila*, *Aeromonas jandaei*, *Aeromonas caviae*, *Pseudomonas aeroginosa*, *Staphylococcus agalactiae*

Strain	*A. hydrophila*	*A. jandaei*	*A. caviae*	*P. aeruginosa*	*S. agalactiae*
ST1	10.90±0.34 d	11.85±0.70 e	11.53±0.46 bc	10.70±0.29 cd	8.35±0.31 d
ST2	10.98±1.23 d	10.23±0.44 f	10.05±0.70 c	9.45±0.41 d	8.20±0.14 d
ST3	10.83±0.36 d	11.58±0.46 ef	12.08±0.36 b	11.18±0.35 bc	8.43±0.30 d
ST4	10.80±1.19 d	14.00±0.54 d	12.45±1.22 b	11.00±0.36 c	9.20±0.32 d
ST5	11.45±0.33 d	12.05±0.44 e	10.12±0.42 c	10.85±0.35 cd	11.18±0.56 c
ST6	10.28±0.30 de	15.55±0.83 c	11.83±0.66 bc	11.88±0.94 bc	11.75±0.99 c
ST7	13.48±0.73 c	15.18±0.37 cd	12.58±0.43 b	11.95±0.90 bc	13.93±0.39 b
ST8	12.63±0.30 cd	13.60±1.13 d	14.98±0.90 a	12.53±0.86 b	13.98±0.87 b
ST9	19.40±0.99 a	19.08±0.79 a	15.18±0.56 a	15.10±0.84 a	16.45±0.89 a
ST10	11.58±0.46 d	15.78±0.46 c	15.65±0.30 a	13.93±0.52 a	15.03±0.95 ab
ST11	8.28±0.21 e	14.55±0.44 cd	12.83±0.68 b	12.03±0.59 bc	14.58±1.22 b
ST12	8.53±0.41 e	10.83±0.36 ef	12.68±1.04 b	11.48±0.41 bc	8.85±0.37 d
*Antib.	16.05±1.11 b	17.38±0.53 b	15.50±1.20 a	14.38±0.70 a	16.73±0.98 a
p-value	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001

Mean values (mm) ± standard deviation with different letter in the column means statistical difference by Tukey test ($p<0.05$). *Antibiotic - positive control.

The strains were identified by the MALDI-TOF-MS method, with prevalence of the species *Enterococcus faecium*, *Klebsiella pneumoniae*, *Edwardsiella tarda* and at the genus level for *Salmonella* sp. (Table 4).

Table 4. Identification of autochthonous bacteria with probiotic potential isolated from the intestinal tract of piauçu (*Megaleporinus macrocephalus*) performed by the MALDI-TOF-MS method

Strain	Organism (Matched Pattern)	Score value	NCBI Identifier	Rank (quality)
ST1	*Enterococcus faecium* DSM 13589 DSM	1.61	1352	(-)
ST2	No Organism Identification Possible	1.43	_	_
ST3	*Klebsiella pneumoniae* RV_BA_03_B LBK	2.04	573	(+++)
ST4	*Edwardsiella tarda* CIP 68_6 CIP	2.07	636	(+++)
ST5	*Edwardsiella tarda* CIP 106473 CIP	2.03	636	(+++)
ST6	No Organism Identification Possible	1.39	_	_
ST7	No Organism Identification Possible	1.50	_	_
ST8	No Organism Identification Possible	1.44	_	_
ST9	*Enterococcus faecium* 20218_1 CHB	2.01	1352	(+++)
ST10	*Salmonella* sp (enterica st Dublin) Sa05_188 VAB	1.84	98360	(+)
ST11	*Edwardsiella tarda* CIP 103852 CIP	2.00	636	(+++)
ST12	*Edwardsiella tarda* CIP 68_6 CIP	1.96	636	(+)

* Score 2.00 to 3.00 and Rank (quality) (+++): High-confidence identification; Score 1.70 to 1.99 and Rank (quality) (+): Low-confidence identification and Score 0.00 to 1.69 and Rank (quality) (-): No Organism Identification Possible. Second similarity method of MALDI-TOF-MS. GenBank: National Center for Biotechnology Information (NCBI).

DISCUSSION

Several studies have reported the benefits of the use of autochthonous lactic acid bacteria for aquaculture (Dias *et al*., 2018; Pereira *et al*., 2019; Jatobá *et al*., 2017; Sousa *et al*., 2019; Yamashita *et al*., 2020). Among the bacteria with probiotic potential, *Enterococcus faecium* stands out because of its ability to completely colonize the intestinal tract of the host when compared with heterotrophic bacteria and which is most likely related to its rapid growth rate in the intestine (Dias *et al*., 2019; Souza *et al*., 2019). In addition, it is a non-hemolytic species, does not harm the host (Dias *et al*., 2019), therefore, hemolytic activity is necessary to evaluate the probiotic and determine its infectivity in the host (El-Jeni *et al*., 2016).

Strains ST1 and ST9 showed higher growth rates, including higher viable cell numbers, when compared to the isolated strains of *Pterophyllum*

scalare with 5 x 10^8 CFU/mL (Dias *et al*., 2019); the adequate value for probiotic supplementation is between 10^8 and 10^9 CFU/g (ANVISA, 2017). Thus, probiotic bacteria can provide additional protection to intestinal mucus, probably forming barriers against pathogenic bacteria and improving the immunological system of the host (He *et al*., 2017; Souza *et al*., 2019).

However, the viability of colonization for probiotic bacteria depends on environmental conditions throughout the dietary supplementation and ingestion by the host (Dias *et al*., 2019). The bacterial growth rate undergoes alterations because of changes in chemical and osmotic aspects in the intestine (Erkkilä and Petäjä, 2000). In the scientific literature, *E. faecium* demonstrates large resistance, above 60% at an NaCl concentration of 3% (Dias *et al*., 2019; Paixão *et al*., 2020) corroborating the present values for piauçu.

Different levels of salinity can affect bacterial growth (Vieira *et al*., 2013). Fish have an ionic concentration to maintain their osmotic profile at the same level as the external environment, driving the energy usage for moments of stress (Weirich *et al*., 1992). Thus, chemical and osmotic aspects of the intestinal tract can influence the survival and colonization of probiotic bacteria, provoking cell membrane rupture (Erkkilä and Petäjä, 2000). The resistance of isolated bacteria to saline stress in vitro may be an indication of great intestinal viability (Vieira *et al*., 2013). Similar results have been observed for *Lactobacillus plantarum* isolated from *Litopenaus vannamei* (Vieira *et al*., 2013) and *E. faecium* isolated from *P. scalare*, with resistance at up to 3% salinity.

In this study, ST9 showed resistance to pH values from 4 to 8 and high values of salt bile at 5%, with survival rates above 50%. For this reason, acid and alkaline values would be used for the control of pathogenic bacteria (El-Jeni *et al*., 2016). The resistance reported for *E. faecium* in this study could be a factor to determine its probiotic potential for the host. In the scientific literature, some genera, such as *Enterococcus*, *Bacillus*, and *Pseudomonas* are described as resistant strains at pH 6, 9, and 7, respectively (Dias *et al*., 2019; Paixão *et al*., 2020; Qi *et al*., 2020).

In the intestine, bile salt acts in the emulsification of fat and some vitamins, but it can also break bacterial cell membranes (Lambert *et al*., 2008), affecting the levels of phospholipids and fatty acids (Vieira *et al*., 2013). Some bacteria are resistant to bile salt by using specific enzymes, thereby reducing the bactericidal effect (Erkkilä and Petäjä, 2000). A study with gene variants showed that the resistance of *E. Faecium* to the action of bile emulsion is related to variations in ion gradients by ATPase type V, which are in the membranes and function as proton pumps or sodium ions through an ion gradient, losing ATP (Senior, 1990). Resistance to bile salts was observed by studying the GltK gene and confirmed its deletion that sensitized *E. faecium* E1162 to the action of bile. The GltK can encode glutamate/aspartate protein permease in the transport system and therefore plays a role in resistance to bile (Zhang *et al*., 2013). Thus, resistance to high bile levels, observed for *E. faecium* isolated from *M. macrocephalus*, affirms the probiotic potential of this strain for dietary supplementation. For these reasons, resistance to factors such as stomach acidity, saline levels, and bile gradients promotes efficient intestinal colonization (Paixão *et al*., 2020; Vieira *et al*., 2013).

Inhibition ability against pathogens stands out as the most desired characteristic among the lactic acid bacteria used as probiotics in aquaculture (Dias *et al*., 2018; Sousa *et al*., 2019). Among the strains isolated from piauçu, ST9 *E. faecium* showed inhibition ability against *Aeromonas hydrophila* and *Aeromonas jandaei*, similar to the positive control with antibiotics. Inhibition ability against *A. hydrophila*, *Pseudomonas aeruginosa*, *Enterococcus durans*, *Staphylococcus haemolyticus*, *Vibrio parahaemolyticus*, and *V. vulnificus* has been reported for *E. faecium* (Dias *et al*., 2019; El-Jeni *et al*., 2016; Mao *et al*., 2020; Vieira *et al*., 2013). The genus *Enterococcus* contains some species with resistance to antimicrobial compounds and antibiotics through mutagenic processes (Pietro *et al*., 2016). Bacterial synergisms have been observed with co-cultivation of the probiotic strain of *E. faecium* CMGB16 added to the fractions of the culture of *Bacillus cereus*, whose effect on the strain *Escherichia coli* O28 was a greater susceptibility to the effects of the antibiotic, besides

influencing its adherence patterns (Ditu *et al*., 2011).

Probiotic bacteria produce various compounds such as lactic acid, hydrogen peroxide, and bacteriocins to control pathogens, in addition to competing for specific space and binding sites in the intestinal lumen (Jatobá *et al*., 2017). Isolates of *E. faecium* strains showed enterokinase A and B compounds with anti-Listeria activity and high thermostability (Ghomrassi *et al*., 2016). Such characteristics make it attractive, in view of its probiotic potential in supplementing the species' diet, by its direct promotion of immunity and prevention of diseases in breeding. These results serve as a basis for future tests and applications in vivo.

The MALDI-TOF-MS analysis of the isolated *M. macrocephalus* strains identified three species, *Enterococcus faecium*, *Klebsiella pneumoniae*, *Edwardsiella tarda* and one of the genus *Salmonella* sp, with emphasis for ST1 and ST9 identified as *E. faecium*. However, the level of reliability and similarity of the analyses, highlighted the ST9 strain with a score of 2.01 as the most suitable candidate with probiotic potential for the piauçu. Thus, the diversity of bacteria isolated from the intestinal tract of piauçu reflects the variation of bacterial communities in the intestine of fish influenced by biotic factors such as host age, stage of development, intestinal structure, food, nutritional status and abiotic such as habitat, characteristics of water quality, competition and cultivation conditions (Ramirez and Romero, 2017; Roeselers *et al*., 2011; Salas-Leiva *et al*., 2017).

Dietary supplementation with *E. faecium* in fish promoted modulation of the immune system, associated with colonization of bacteria in the intestine, increasing mucus secretion and total proteins such as (immunoglobulin) and enzymatic activities (lysozymes) (Das *et al*., 2013; Lazado and Caipang, 2014; Van Doan *et al*., 2019). Furthermore, supplementation with *E. faecium* stimulates the increase in the number of defense cells such as intraepithelial T lymphocytes, production of antibodies (IgA), in addition to stimulating macrophages and dendritic cells in the production of compounds such as nitric oxide (Khalkhali and Mojgani, 2017).

Similar to the present study, enterobacteria were isolated from *A. gigas*, among them are *Klebsiella pneumoniae* and *Edwardsiella tarda*, they are gram-negative bacteria with pathogenic potential in aquaculture, highlighting the high resistance registered for k. pneumoniae against the tested antibiotics (Proietti-Junior *et al*., 2021. The *K. pneumoniae* bacterium isolated from a group of fish expressed three residence genes ESBL (bla SHV + bla CTX + bla TEM) against several tested antibiotics (Singh *et al.*, 2017). Recently, studies carried out with tilapia have registered an accentuated 100% mortality of infected fish compared to the control group, without *K. pneumoniae* injection (Vaneci-Silva *et al*., 2022). Thus, the severity of the spread of this etiological agent and its degree of infection in fish are pointed out, causing a great economic impact.

The *E. tarda* is a versatile pathogen that can infect a wide range of hosts, from fish to humans (Li *et al*., 2012). It is a facultative and mobile Gram-negative bacterium, causing Edwardsiellosis disease, which can generate great economic losses in aquaculture (Lima *et al*., 2008; Woo and Bruno, 2010). A study carried out with isolates of *Bacillus subtilis*, *Bacillus velezensis* and *Bacillus pumilus*, significantly reduced the pathogenicity of *E. tarda* in zebrafish larvae increasing survival by 50%, and those infections may have occurred via the skin, gills and intestine, thus he observed promoting health through the use of probiotics (Santos *et al*., 2021).

K. pneumoniae and *E. tarda*, however the pathogenesis of bacteria of the genus *Salmonella* sp. are unknown in fish (Fernandes *et al*., 2018). They are facultative anaerobic bacteria, gram-negative and can to survive in different environments, including the aquatic one (Popoff; Le Minor, 2005; Oliveira and Vaz, 2018). The occurrence of this pathogen in fish can be transient and it is related to the management of creation, form of industrialization, inefficient hygiene practices, equipment and inadequate handling of food (Fernandes *et al*., 2018). Thus, in vitro isolation and selection protocols for autochthonous probiotic bacteria can help prophylactically in the prevention of diseases in aquaculture, and as an alternative to the use of antibiotics that favor and select increasingly resistant bacterial strains.

CONCLUSION

This is the first report on the autochthonous probiotic *Enteroccus faecium* isolated from *Megaleporinus macrocephalus*. The ST9 strain was considered the most resistant to the challenges of chemical gradients, in the simulation of physiological conditions and great inhibiting capacity against pathogens. These findings point to positive probiotic properties that should potentially be considered as a probiotic use in breeding the species in aquaculture.

ACKNOWLEDGMENTS

The authors thank the Coordination for the Improvement of Higher Education Personnel (CAPES) the National Council of Scientific and Technological Development (CNPq) for financial support to Fujimoto, R. Y. (304533/2019-0).

REFERENCES

ANVISA, Agência Nacional de Vigilância Sanitária. Alimentos com alegações de propriedades funcionais e ou de saúde, novos alimentos/ingredientes, substâncias bioativas e probióticos. 9. lista de alegações de propriedade funcional, 2017. Available in http://www.anvisa.gov.br/alimentos/comissões/tecno lista alega.htm. Accessed in: 20 dec. 2020.

BIRINDELLI, J.L.; BRITSKI, H.A.; RAMIREZ, J.L. A new endangered species of *Megaleporinus* (Characiformes: Anostomidae) from the Rio de Contas basin, eastern Brazil. *J. Fish Biol*., v.96, p.1349-1359, 2020.

BURNS, M.D.; FRABLE, B.W.; SIDLAUSKAS, B.L. A new species of *Leporinus* (Characiformes: Anostomidae), from the Orinoco Basin, Venezuela. *Copeia*, v.2014, p.206-214, 2014.

DAS, A.; NAKHRO, K.; CHOWDHURY, S. *et al*. Effects of potential probiotic *Bacillus amyloliquifaciens* FPTB16 on systemic and cutaneous mucosal immune responses and disease resistance of catla (*Catla catla*). *Fish Shellfish Immunol.*, v.35, p.1547-1553, 2013.

DIAS, J.A.; ABE, H.A.; SOUSA, N.C. *et al*. Dietary supplementation with autochthonous Bacillus cereus improves growth performance and survival in tambaqui *Colossoma macropomum*. *Aquac. Res.*, v.49, p.3063-3070, 2018.

DIAS, J.A.; ABE, H.A.; SOUSA, N.C. *et al. Enterococcus faecium* as potential probiotic for ornamental neotropical cichlid fish, *Pterophyllum scalare* (Schultze, 1823). *Aquac. Int*., v.27, p.463-474, 2019.

DITU, L.M.; CHIFIRIUC, M.C.; BEZIRTZOGLOU, E. *et al*. Modulation of virulence and antibiotic susceptibility of enteropathogenic *Escherichia coli* strains by *Enterococcus faecium* probiotic strain culture fractions. *Anaerobe*, v.17, p.448-451, 2011.

DOAN, H.V.; HOSEINIFAR, S.H.; KHANONGNUCH, C. *et al*. Host-associated probiotics boosted mucosal and serum immunity, disease resistance and growth performance of Nile tilapia (*Oreochromis niloticus*). *Aquaculture*, v.491, p.94-100, 2011.

EL-JENI, R.; EL BOUR, M.; CALO-MATA, P. *et al*. In vitro probiotic profiling of novel *Enterococcus faecium* and *Leuconostoc mesenteroides* from Tunisian freshwater fishes. *Can. J. Microbiol*., v.62, p.60-71, 2016.

ERKKILÄ, S.; PETÄJÄ, E. Screening of commercial meat starter cultures at low pH and in the presence of bile salts for potential probiotic use. *Meat Sci*., v.55, p.297-300, 2000.

FERNANDES, D.V.G.S.; CASTRO, V.S.; CUNHA NETO, A. *et al. Salmonellas* pp. in the fish production chain: a review. *Ciênc. Rural*, v.48, p.e2018141, 2018.

FRICKE, R.; ESCHMEYER, W.N.; VAN DER LAAN, R. Eschmeyer's catalog of fishes. 2019. Available in: http://researcharchive.calacademy.org/ research/ichthyology/catalog/fishcatmain.asp. Accessed in: 5 Jan. 2022

GARAVELLO, J.C.; BRITSKI, H.A. Family anostomidae. In: REIS, R.E.; KULLANDER, S.O.; FERRARIS, C.J.JR. (Eds.). *Check list of the freshwater fishes of South and Central America*. Porto Alegre: EDIPUCRS, 2003. p.71-84.

GARAVELLO, J.C.; BRITSKI, H. A. *Leporinus macrocephalus* sp. da Bacia do Rio Paraguai (Ostariophysi, Anastomidae). *Naturalia*, v.13, 67-74, 1988.

GHOMRASSI, H.; BEN BRAIEK, O.; CHOISET, Y. *et al*. Evaluation of marine bacteriocinogenic enterococci strains with inhibitory activity against fish-pathogenic Gram-negative bacteria. *Dis. Aquat. Organ*., v.118, p.31-43, 2016.

HE, S.; RAN, C.; QIN, C. *et al*. Anti-infective effect of adhesive probiotic *Lactobacillus* in fish is cor-related with their spatial distribution in the intestinal tissue. *Sci. Rep*., v.7, p.13195, 2017.

JATOBÁ, A.; MORAES, A.V.; STECKERT, L.D. *et al*. Selection of autochtone probiotic for *Astyanax bimaculatus*. *Arq. Bras. Med. Vet. Zootec*., v.69, p.1645-1652, 2017.

JATOBÁ, A.; VIEIRA, F.D.; NETO, C.B. *et al.* Utilização de bactéria ácido-lácticas isoladas do trato intestinal de tilápia-do-nilo como probiótico. *Pesqui. Agropecu. Bras*., v.43, p.1201-1207, 2008.

KHALKHALI, S.; MOJGANI, N. *Enterococcus faecium*; um candidato probiótico adequado para modulação de respostas imunes contra patógenos. *Int. J. Sci. Basic Med*., v.2, p.77-82, 2017.

LAMBERT, J.M.; BONGERS, R.S.; DE VOS, W.M.; KLEEREBEZEM, M. Functional analysis of four bile salt hydrolase and penicillin acylase family members in *Lactobacillus plantarum* WCFS1. *Appl. Environ. Microbiol*., v.74, p.4719-4726, 2008.

LAZADO, C.C.; CAIPANG, C.M.A. Mucosal immunity and probiotics in fish. *Fish Shellfish Immunol*., v.39, p.78-89, 2014.

LI, M.F.; HU, Y.H.; ZHENG, W.J. *et al*. Inv1: an *Edwardsiella tarda* invasin and a protective immunogen that is required for host infection. *Fish Shellfish Immunol*., v.32, p.586-592, 2012.

LIMA, L.C.; FERNANDES, A.A.; COSTA, A.A.P. *et al.* Isolation and characterizaton of *Edwardsiella tarda* from pacu *Myleus micans*. *Arq. Bras. Med. Vet. Zootec*., v.60, p.275-277, 2008.

MADANI, N.S.H.; ADORIAN, T.J.; FARSANI, H.G.; HOSEINIFAR, S.H. The effects of dietary probiotic Bacilli (*Bacillus subtilis* and *Bacillus licheniformis*) on growth performance, feed efficiency, body composition and imune parameters of whiteleg shrimp (*Litopenaeus vannamei*) postlarvae. *Aquac. Res*., v.49, p.1926-1933, 2018.

MAO, Q.; SUN, X.; SUN, J. *et al*. A candidate probiotic strain of *Enterococcus faecium*

from the intestine of the crucian carp *Carassius auratus*. *AMB Express*, v.10, p.40, 2020.

MARTINS, M.L.; YOSHITOSHI, E.R. A new nematode species *Goezia leporini* n. sp. (Ascaridoidea) from cultivated freshwater fish *Leporinus macrocephalus* (Anostomidae) in Brazil. *Braz. J. Biol*., v.63, p.497-506, 2003.

MARTINS, W.M.O.; JUSTO, M.C.N.; CÁRDENAS, M.Q.; COHEN, S.C. Metazoan parasite communities of *Leporinus macrocephalus* (Characiformes: Anostomidae) in cultivation systems in the western Amazon. Brazil. *Acta Amazonica*, v.47, p.301-310, 2017.

OLIVEIRA, S.J.; VAZ, A.K. *Guia bacteriológico prático:* identificação, patogenicidade e imunidade. Canoas: Ulbra, 2018. 272p.

PAIXÃO, P.E.G.; COUTO, M.V.S.; SOUSA, N.C. *et al.* In vitro selection of autochthonous lactic acid bacterium from clownfish *Amphiprion ocellaris. Aquac. Res*.,

v.51, p.848-851, 2020.

PEREIRA, G.V.; PEREIRA, P.S.A.; SOARES, A. *et al*. Autochthonous probiotic bacteria modulate intestinal microbiota of Pirarucu, *Arapaima gigas. J. World Aquac. Soc*., v.50, p.1152-1167, 2019.

POPOFF, M.Y.; LE MINOR, L.E. Genus *Salmonella*. In: BRENNER, D.J.; KRIEG, N.R.; STALEY, J.T. (Eds.). *Bergey's manual of systematic bacteriology*. The Proteobacteria. Part B. The Gammaproteobacteria. 2.ed. New York: Springer, 2005. v.2, p.764-799.

PRIETO, A.M.G.; SCHAIK, W.V.; ROGERS, M.R. *et al.* Global emergence and dissemination of enterococci as nosocomial pathogens: attack of the clones? *Front. Microbiol*., v.7, p.1-15, 2016.

PROIETTI-JUNIOR, A.A.; LIMA, L.S.; GONÇALVES. *et al*. Cultivable autochthonous bacteria of the intestinal mucosa of *Arapaima gigas* (Pisces: Arapaimidae) with probiotic potential. *Aquac. Res.*, v.52, p.1788-1796, 2021.

QI, X.; XUE, M.; CUI, H. *et al.* Antimicrobial activity of *Pseudomonas monteilii* JK-1 isolated from fish gut and its major metabolite, 1-hydroxyphenazine, against *Aeromonas hydrophila*. *Aquaculture*, v.526, p.735-366, 2020.

RAMIREZ, C.; ROMERO, J. The microbiome of seriola lalandi of wild and aquaculture origin reveals differences in composition and potential function. *Front. Microbiol*., v.8, p.1844, 2017

RAMIREZ, J.L.; BIRINDELLI, J.L.O.; GALETTI, P. A new genus of Anostomidae (Ostariophysi: Characiformes): diversity, phylogeny and biogeography based on cytogenetic, molecular and morphological data. *Mol. Phylogenet. Evol*., v.107, p.308-323, 2017.

RAMIREZ, J.L.; CARVALHO-COSTA, L.F.; VENERE, P.C. *et al.* Testing monophyly of the freshwater fish *Leporinus* (Characiformes, Anostomidae) through molecular analysis. *J. Fish Biol*. v.88, p.1204-1214, 2016.

ROESELERS, G.; MITTGE, E.K.; STEPHENS, W.Z. *et al*. Evidence for a core gut microbiota in the zebrafish. *ISME J*., v.5, p.1595-1608, 2011.

SALAS-LEIVA, J.; OPAZO, R.; REMOND, C. *et al*. Characterization of the intestinal microbiota of wild-caught and farmed fine flounder (*Paralichthys adspersus*). *Lat. Am. J. Aquat. Res*., v.45, p.370-378, 2017.

SANTOS, R.A.; MONTEIRO, M.; RANGEL, F. *et al*. *Bacillus* spp. inhibit *Edwardsiella tarda* quorum-sensing and fish infection. *Mar. Drugs*, v.19, p.602, 2021.

SENIOR, A.E. The proton-translocating ATPase of escherichia coli. *Annu. Rev. Biophys. Biophys. Chem.*, v.19, p.7-41, 1990.

SINGH, A.S.; LEKSHMI, M.; PRAKASAN, S. *et al*. Multiple antibiotic resistant, extended spectrum-β-Lactamase (ESBL)-producing Enterobacteria in fresh seafood. *Microorganisms*, v.5, p.53, 2017.

SOARES JUNIOR, M.S.; CALIARI, M.; PEREIRA, D.E.P. Effect of soybean inclusion in extruded rations on performance of juvenile Piavuçu (*Leporinus macrocephalus* L.). *Ciênc. Anim. Bras.*, v.14, p.399-405, 2013.

SOUSA, N.C.; COUTO, M.V.S.; ABE, H. A. *et al*. Effects of an *Enterococcus faecium* based probiotic on growth performance and health of Pirarucu, *Arapaima gigas*. *Aquac. Res.*, v.50, p.3720-3728, 2019.

TAKAHASHI, L.S.; GONÇALVES, F.D.; ABREU, J.S.D. *et al.* Economic viability of the piauçu *Leporinus macrocephalus* (Garavello & Britski, 1988) production. *Sci. Agric.*,

v.61, p.228-233, 2004.

THE STATE of world fisheries and aquaculture. Sustainability in action. Rome: FAO, 2020. Available in: https://doi.org/10.4060/ca9229en. Accessed in: 5 Nov. 2020.

VAN DOAN, H.; HOSEINIFAR, S.H.; TAPINGKAE, W. *et al*. Boosted growth performance, mucosal and serum immunity, and disease resistance Nile Tilapia (*Oreochromis niloticus*) fingerlings using corncob-derived Xylooligosaccharide and *Lactobacillus plantarum* CR1T5. *Probiotics Antimicrob. Proteins*, v.22, p.1-12, 2019.

VANECI-SILVA, D.; ASSANE, I.M.; ALVES, L.O. *et al*. *Klebsiella pneumoniae* causing mass mortality in juvenile Nile tilapia in Brazil: Isolation, characterization, pathogenicity and phylogenetic relationship with other environmental and pathogenic strains from livestock and human sources. *Aquaculture*, v.546, p.737376, 2022.

VIEIRA, F.N.; JATOBÁ, A.; MOURIÑO, J.L.P. *et al*. *In vitro* selection of bacteria with potential for use as probiotics in marine shrimp culture. *Pesqui. Agropecu. Bras.*, v48, p.998-1004, 2013.

YAMASHITA, M.F.; FERRAREZI, J.V.; PEREIRA, J.G. *et al*. Autochthonous vs allochthonous probiotic strains to *Rhamdia quelen*. *Microb. Pathog.*, v.139, p.103897, 2020.

WEIRICH, C.R.; TOMASSO, J.R.; SMITH, T.I.J. Confinement and transport-induced stress in white bass *Morone chrysops* x striped bass *M. saxatilis* hybrids: Effect of calcium and salinity. *J. World. Aquac. Soc.*, v.23, p.49-57, 1992.

WOO, P.T.K.; BRUNO, D.W. Edwardsiella septicaemias. In: EVANS, J.J.; KLESIUS, P.H.; PLUMB, J.A., SHOEMAKER, C.A. (Eds.). *Fish diseases and disorders*. Wallingford: CABI International, 2010. p.512-534.

ZHANG, X.; BIERSCHENK, D.; TOP, J. *et al*. Functional genomic analysis of bile salt resistance in *Enterococcus faecium*. *BMC Genet.*, v.14, p.299, 2013.