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In vitro bacterial probiotic selection from *Nannostomus beckfordi*, an Amazon ornamental fish

[*Seleção* in vitro *de bactérias probióticas do peixe ornamental Amazônico* Nannostomus beckfordi]

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ABSTRACT

This study aimed to isolate and select *in vitro* bacteria with probiotic potential for the Amazon ornamental fish *Nannostomus beckfordi*. For isolate, twelve fish underwent surgery procedure to remove their intestinal tract, macerate and then inoculate in the plate petri containing de Man Rugosa Sharped Agar (MRS). After bacterial growth (48 hours at 35ºC), selected strains were inoculated in MRS broth and submitted to resistance test with NaCl (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0%), pH (4, 5, 6, 8 and 9) and bile salts (5% w/v). Inhibition test against pathogenic bacteria *Aeromonas hydrophila*, *Pseudomonas aeroginosa*, *Streptococcus agalactiae* and *Aeromonas Jandaei* was also performed. Within the isolated strains group (23 strains), only six (S1, S2, S3, S4, S5 and S6) showed probiotic potential. Strains S1 and S6 showed the greater resistance for NaCl (0.5% and 1%) and pH (5 and 6), but only S1 obtained better results to resist the bile salts. Even against pathogenic bacteria, the S1 showed the best results with inhibition halos greater than 9 mm. In the end, this bacterial strain (S1) was identified as *Enterococcus faecium* 11037CHB. Thus, this is the first report regarding isolated autochthonous bacterium *E. faecium* with probiotic potential of *N. beckfordi*.

Keywords: Amazon ornamental fish, autochthonous probiotic, *in vitro* assay

RESUMO

O objetivo deste estudo foi isolar e selecionar in vitro bactérias com potencial probiótico do peixe ornamental Amazônico Nannostomus beckfordi. *Para o isolamento, retirou-se o intestino de 12 espécimes, que foram macerados, homogeneizados e semeados em placa de petri contento Ágar Man Rogosa e Sharpe (MRS). Posteriormente ao crescimento bacteriano (48 horas a 35ºC), as cepas selecionadas foram mantidas em caldo MRS e submetidas a testes de resistência a NaCl (0,5, 1,0, 1,5, 2,0 e 2,5 e 3,0%), pH (4, 5, 6, 8 e 9) e sais biliares (5% p/v). O antagonismo foi realizado frente as bactérias patogênicas* Aeromonas hydrophila, Pseudomonas aeroginosa, Streptococcus agalactiae *e* Aeromonas jandaei. *Das cepas isoladas (23 cepas), apenas seis (C1, C2, C3, C4, C5 e C6) apresentaram potencial probiótico. As cepas C1 e C6 tiveram maior resistência (p<0,05) para o NaCl (0,5 e 1%) e pH (5 a 6), na presença de sais biliares somente a C1 teve a melhor resistência de crescimento. Para o antagonismo frente as bactérias patogênicas, a C1 apresentou halo de inibição maior que 9 mm. Sendo esta cepa bactéria (C1) identificada como* Enterococcus faecium *11037 CHB. Portanto, este é o primeiro relato do isolamento da bactéria autóctone* E. faecium *em* N. beckfordi *com potencial probiótico.*

Palavras-chave: peixe ornamental amazônico, probiótico autóctone, ensaio in vitro

INTRODUCTION

Ornamental fish farming is economically profitable (FAO, 2017; Evers *et al.*, 2019). However, the intensification of this activity has increased the incidence of fish diseases causing mortality outbreaks (Shameena *et al.*, 2020). Animal welfare can determine the success of ornamental fish rearing. Thus, the use of probiotics has been widely reported to show positive results, such as immune stimulation or the

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inhibition of pathogens, making it a useful strategy to prevent fish diseases (Sousa *et al.,* 2019; Yamashita *et al.,* 2020). The beneficial effects of probiotic supplementation in ornamental fish were reported in *Pterophyllum scalare* (Dias *et al.,* 2019; Sousa *et al.,* 2020), *Carassius auratus* (Jinendiran *et al.,* 2019), *Danio rerio* (Mohammadi *et al.,* 2019) and *Poecilia latipinna* (Ahmadifard *et al.,* 2019).

Nonetheless, most of the probiotics applied in ornamental fish farming originate from allochthonous sources (Azevedo *et al.,* 2016; Sousa *et al.,* 2020; Paixão *et al.,* 2020a) and sometimes have no beneficial effects on the host (Marengoni *et al.,* 2010). Thus, the selection of bacteria specific to the fish (autochthonous bacteria) can provide greater colonization of the intestine and resistance against pathogens (Dias *et al.,* 2019; Sousa *et al.,* 2019; Yamashita *et al.,* 2020). Thus, *in vitro* assay is extremely important in determining the bacterial resistance under physiological conditions (NaCl, pH and bile salts) and its capacity to inhibit pathogens (Dias *et al.,* 2019; Paixão *et al.,* 2020b). Based on *in vitro* tests, selecting bacteria strains with probiotic potential becomes easier and ensures benefits to the host (Dias *et al.,* 2019; Sousa *et al.,* 2019). Among the autochthonous probiotic bacteria, lactic acid bacteria, such as *Lactobacillus plantarum*, *Enterococcus faecium*, *Lactococcus lactis* and *Weissella cibaria*, were shown to benefit the fish (Mouriño *et al.,* 2016; Jatobá *et al.,* 2018; Dias *et al.,* 2019; Yamashita *et al.,* 2020; Paixão *et al.,* 2020a).

Amazon ornamental fish have a high popularity in the national and international markets because of their attractive patterns and colors. Among the reared species, *Nannostomus beckfordi* (*Lesbiasinidae* family) is important and has a fusiform form, calm behavior, and red color on the fins (Weitzman and Weitzman, 2003; Abe *et al.,* 2019).

There remains a gap in the scientific knowledge about the selection or even applicability of an autochthonous bacterium with probiotic potential for *N. beckfordi*. Therefore, this study aimed to isolate and select *in vitro* bacteria with probiotic potential for the Amazon ornamental fish *N. beckfordi*.

MATERIAL AND METHODS

To isolate lactic acid bacteria, twelve healthy fish (0.332±0.05g and 3.59±0.20cm) were caught (SISBIO 19515) in the Chumucui stream (01º12’38.3’’S, 046º47’31.7” W). The fish were starved for 24 h and received anesthetic (bath with Benzocaine 60 mg/L) before euthanasia by medullar section according to protocols by the Ethical Committee on the use of Animal at the UFPA (CEUA - 9202300420). Afterwards, they underwent a surgical procedure to remove the intestinal tract, homogenized in saline solution (NaCl 0.65%), conducted to serial dilution (1:10 factor) and then inoculated on a petri plate containing de Man Rugosa and Sharpe Agar (MRS Agar) with 1% aniline blue (1%). All plates were kept in a microbiological incubator for 48 hours at 35ºC (Mouriño *et al.,* 2016; Paixão *et al.,* 2020b).

The lactic acid bacteria selected (bacillus and coccus) with blue color, negative catalase and positive gram were inoculated in MRS broth and kept for 24 hours at 35ºC (Mouriño *et al.,* 2016). To determine growth kinetics, the strains were 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. 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). Based on these results, growth rate and final concentration were determined (FCU/mL) (Dias *et al.,* 2019; Paixão *et al.,* 2020b).

To determine the resistance under *in vitro* physiological conditions, an experiment in completely randomized design was conducted with different concentrations of NaCl (0, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0%), pH (4, 5, 6, 8 and 9) and bile salt (5% w/v), all of them with three replicates and carried out simultaneously. The Falcon tubes were then incubated for 24 hours at 35ºC. Afterwards, using a spectrophotometer, the percentage reduction of absorbance at 630 nm was determined (Dias *et al.,* 2019; Paixão *et al.,* 2020b).

For the inhibition of pathogenic bacteria, the isolated strains previously inoculated on the petri plates containing MRS Agar were used to remove

discs with a diameter of 0.8 cm. Each disc was placed on another petri plate containing Triptone Soya Agar with the pathogenic bacteria *Aeromonas hydrophila* (CPQBA22808 DRM), *Pseudomonas aeroginosa* (ATCC27853), *Streptococcus agalactiae* (LAQUA) and *Aeromonas jandaei* (LAQUA). This experiment was performed in a completely randomized design with tree replicates per treatment, after the incubation period (48 hours at 35ºC), inhibition halos (mm) were determined according to Dias *et al.* (2019). The bacterial strain with potential probiotic selected was identified used the method MALDI-TOF, using the molecular weight of ribosomal proteins with laser shots at a wavelength of 260-337 nm (Sousa *et al*., 2019; Paixão *et al*., 2020b).

All data were submitted to normality (Shapiro Wilk) and homoscedasticity (Levene's) tests. Afterwards, all data were submitted to analysis of variance (ANOVA) with post hoc Tukey test (p<0.05). The percentage data were then submitted to transformation with arc sin square root and microbiological counting with log (x+1).

RESULTS

Out of 23 strains selected, only six (S1, S2, S3, S4, S5 and S6) showed probiotic potential with the characteristics blue color, negative catalase, and positive gram. Four strains (S1, S3, S5 and S6) showed greater growth rate reaching 10^9 FCU/mL at 24 hours (Table 1). In the in vitro assay, the highest bacterial growth (p<0.05) was observed for strains S1 and S6 at concentrations of 0.5 and 1% NaCl (%) and in the pH range between 5 and 6 (Table 1). In the presence of bile salts (5%), the greatest resistance was observed for strain S1, followed by S5 and S6 (Table 1).

Table 1. Values (Mean±S.D) of Maximum growth rate per hour (MGR/h), final concentration after growth at 24 hours (FCU/mL) and resistance tests (%) to different concentrations NaCl, pH and Bile Salt for isolated strains of the intestinal tract of *N. beckfordi*

Strains	MGR/h	Final concentration	NaCl (0.5)	NaCl (1.0)	NaCl (1.5)	NaCl (2.0)	NaCl (2.5)	NaCl (3.0)
S1	0.77±0.02a	2.7±0.12x10^{9}a	78.7±1.83a	75.26±0.72a	64.38±1.33a	54.15±0.79a	51.49±1.52a	43.94±0.98a
S2	0.67±0.03b	1.8±0.11x10^{8}b	63.02±4.97b	64.51±2.16c	58.92±1.77b	52.28±1.02a	47.36±0.98b	28.75±1.64d
S3	0.78±0.03a	2.6±0.18x10^{9}a	70.64±3.56ab	68.46±1.40bc	46.32±1.67c	40.58±1.61b	48.42±0.87ab	29.72±0.86d
S4	0.65±0.04a	2.2±0.19x10^{8}b	72.75±3.59a	70.26±1.23b	43.13±2.08c	39.77±1.75b	50.37±1.59ab	33.81±1.14c
S5	0.77±0.02a	2.7±0.11x10^{9}a	71.80±2.55ab	69.06±1.53b	65.10±1.73a	54.07±1.75a	50.98±0.99a	39.20±1.13b
S6	0.76±0.04a	2.6±0.12x10^{9}a	73.03±2.60a	74.01±1.32a	65.76±2.49a	54.60±1.159a	50.78±1.11a	43.24±0.92a
p-value	0.00162	0.00140	0.00686	0.00097	0.00001	0.00003	0.00691	0.00018
Strains	pH 4	pH 5	pH 6	pH 8	pH 9	Bile Salt		
S1	23.29±0.54a	79.37±0.84a	84.94±1.12a	40.94±0.89a	38.33±0.66a	59.30±3.01a		
S2	22.43±0.72a	66.03±1.04c	71.75±0.92b	39.02±0.37b	34.74±0.97b	28.85±3.14cd		
S3	8.77±0.85b	68,49±0.71b	70.61±1.02b	31.88±0.59c	18.85±0.76c	34.86±3.26c		
S4	9.32±0.45b	67,62±0.85bc	69.34±1.30b	40.19±41.02ab	18.23±0.75c	21.78±2.09d		
S5	21.69±0.84a	62,19±0.81d	56.49±0.98c	38.72±0.47b	37.93±0.88a	48.26±2.29b		
S6	23.30±0.87a	77,15±0.95a	85.60±1.02a	41.26±0.45a	36.21±0.44	55.88±3.37ab		
p-value	0.00043	0.00217	0.00001	0.00011	0.00317	0.00402		

*Different letters in the same column indicate significant differences identified by Tukey test (5%).

The strain S1 also showed a larger inhibition halo (greater than 9 mm) against pathogenic bacteria (Table 2). The smaller inhibition halo was observed for strain S5 against *A. jandaei* and *S. agalactiae* (Table 2). Lastly, the bacterial strain (S1) was identified as *Enterococcus faecium* 11037CHB (MALDI-TOF score de 2.13).

Table 2. Values (Mean±S.D) of Inhibition halo (mm) of the probiotic strain isolated from the intestinal tract of *N. beckfordi* against pathogenic bacteria *A. hydrophila* (AH), *A. jandaei* (AJ), *P. aeroginosa* (PA) and *S. agalactiae* (SA)

Strains	AH	AJ	PA	SA
S1	9.66±0.57a	12.41±074a	11.74±0.56a	11.61±0.48a
S2	9.73±0.51a	9.68±0.58b	9.01±0.23c	9.08±0.17b
S3	8.27±0.67b	12.36±0.56a	10.38±0.57b	11.36±0.58a
S4	9.05±0.39ab	9.74±0.61b	9.03±0.15c	9.42±0.57b
S5	9.73±0.57a	1.33±0.61c	9.39±0.54bc	1.66±0.57c
S6	9.88±0.24a	10.87±0.32a	12.21±0.42a	9.44±0.48b
p-value	0.01524	0.00321	0.00029	0.00012

*Different letters in the same column indicate significant differences identified by Tukey test (5%).

DISCUSSION

The beneficial effects of probiotic supplementation have been reported for ornamental fish farming (Azevedo *et al.,* 2016; Jinendiran *et al.*, 2019; Sousa *et al.*, 2020; Paixão *et al*., 2020a). However, there are few scientific reports about *in vitro* tests of autochthonous probiotics for Amazon ornamental fish. Currently, only *P. scalare* is reported in the scientific literature as an autochthonous probiotic (Dias *et al.,* 2019). In the present study, *E. faecium* isolated from *N. beckfordi* showed greater values in resistance tests and a larger inhibition halo making it more efficient to colonize the intestine and promote modulating the gut microbiota, growth, and immune responses during phases in cultivating *N. beckfordi*.

For probiotic bacteria, colonization of the intestinal tract is the most important factor to promote the beneficial effects in the host (Sousa *et al*., 2019; Yamashita *et al*., 2020). Therefore, *in vitro* tests are necessary to select probiotic bacteria that present greater resistance to the host's physiological actions. (Paixão *et al.*, 2020a; Khan *et al*., 2021).

In the fish, bile salt has an important function in emulsifying lipid during digestion and acting as a bactericide breaking the bacterial cell wall (Jahangiri *et al*., 2018; Brandvold *et al*., 2019), as observed for *Lactobacillus plantarum* isolated from Clownfish *Amphiprion ocellaris* (Paixão *et al.,* 2020b). However, in the present study, *E. faecium* (strain 1) showed greater resistance to bile salt (growth above 60%) and a similar result was observed for *E. faecium* isolated from *P. scalare* (Dias *et al.,* 2019). Thus, resistance to high bile levels observed for *E. faecium* isolated from *N. beckfordi* bare related to the gene expression of *gltK* that encodes the glutamate/aspartate permease, increasing bile resistance of probiotic bacteria (Zhang *et al.*, 2013).

Sodium chloride concentration (NaCl) is another factor widely reported for selecting probiotic bacteria *in vitro* (Dias *et al*., 2019; Paixão *et al.*, 2020b). Fish have an ionic concentration to maintain their osmotic profile at the same level as the external environment, and ionic changes in the digestive system of the host can affect the probiotic bacterium, causing rupture of the membrane (Moniruzzaman *et al.,* 2018; Mortezaei *et al.,* 2020). This hypothesis corroborates with the findings by Vieira *et al.* (2013), which found reduced growth for *L. plantarum* in the presence of 1.5% NaCl. For this study, *E. faecium* (strain 1) showed greater growth performance in 0.5 to 3.0% NaCl, an important characteristic of this probiotic bacterium for *N. beckfordi*, even if there were an osmotic imbalance in the specie, a similar result to *E. faecium, Lactococcus lactis* and *Weissella oryzae* (Dias *et al.,* 2019; Mortezaei *et al.,* 2020).

Bacterial resistance to different levels of pH has extreme importance for selecting a probiotic bacterium. In fish, the pH value can modify the intestinal microbiota, reducing the beneficial bacteria (Sylvain *et al.,* 2016). According to Girijakumari *et al.* (2018), stomach acid can even reduce the bacterial load by 100%, affecting colonization (Sousa *et al.*, 2019). In this study, all strains showed growth in acidic (pH 4) or alkaline medium (pH 9), showing better results with pH 5 and 6, mainly for S1 (*E. faecium*). Different results were observed in Dias *et al.* (2019) and Girijakumari *et al.* (2018) with *P. scalare* and *Maylandia lombardoi*, respectively, obtaining reduced growth at 100% for pH 5 and 2. In this study, the resistance of *E. faecium* can be a factor to determine its probiotic potential for *N. beckfordi* because the resistance of this probiotic bacteria to pH values allows the necessary amount in colony-forming units (CFU) for colonization in the intestine and promotes beneficial effects in the host.

The inhibition of pathogenic bacteria has been the most important benefit reported for probiotic diets in fish farming. Probiotic strains can release inhibitory compounds affecting the bacterial growth of pathogens, reducing fish diseases (Jinendiran *et al.,* 2019; Yamashita *et al.,* 2020). Among the strains isolated from *N. beckfordi*, the *E. faecium* (strain 1) showed an inhibition halo greater than 9 mm against four pathogenic bacteria, mainly for *A. jandaei*. This antagonistic characteristic observed for *E. faecium* in the present study can favor the resistance of the species to bacterial diseases during cultivation, thus avoiding possible outbreaks of mortality.

The antagonistic potential of *E. faecium* has been reported against *A. hydrophila, P. aeruginosa, A. veronii, Staphylococcus haemolyticus, Vibrio*

parahaemolyticus, and *V. vulnifcus* (Dias *et al.*, 2019; Mao *et al.*, 2020; Paixão *et al.*, 2020b). Its ability is related to the capacity to release lactic acid, hydrogen peroxide, and bactericides, antimicrobial compounds that control pathogens, in addition to competing for specific space and binding sites in the intestinal tract (Ng *et al.*, 2020; Paixão *et al.*, 2020b). Therefore, the use of *E. faecium* (strain 1) as a probiotic may be a strategy for the aquaculture of the Amazon ornamental fish species *N. beckfordi*.

CONCLUSION

This is the first report on the autochthonous bacteria *E. faecium* isolated from *N. beckfordi*. With adequate resistance under physiological conditions and greater inhibitory capacity against pathogens, it can be recommended as a potential probiotic for the aquaculture of this ornamental fish species.

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