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Original Article - Zootechnics

Prospection of *Lactobacillus plantarum* and *Enterococcus faecium* with potential species-specific probiotic use in ornamental aquaculture of *Betta splendens* Regan, 1910

[*Prospecção de* Lactobacillus plantarum *e* Enterococcus faecium *com potencial uso probiótico espécie-específico na aquicultura ornamental de* Betta splendens *Regan, 1910*]

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ABSTRACT

The research evaluated the isolation, identification, and prospection of species-specific beneficial bacteria in fish farming of ornamental fish *Betta splendens*. For this, the microbiological material was obtained from the intestinal tract of healthy specimens, with bacterial growth in selective culture medium Man Rogosa Sharped (MRS). Sixteen strains were isolated based on the response of *in vitro* tests of catalase, Gram, nilin blue, hemolytic activity and antibiogram to pathogens *Aeromonas hydrophila, Pseudomonas aeroginosa, Enterococcus durans* and *Escherichia coli*. Of the isolated strains C1BS and C5BS, they showed the best responses, which were later identified by the Maldi-TOF method as *Lactobacillus plantarum* and *Enterococcus faecium*. Due to the performance of lactic acid strains in *in vitro* tests and the bibliographic record of their performance as probiotics, the species have great potential for species-specific use in the ornamental production of *Betta splendens.*

Keywords: Maldi-TOF, microbiology, ornamental fish, pathogenic inhibition

RESUMO

A pesquisa avaliou o isolamento, a identificação e a prospecção de bactérias benéficas espécie-específico na piscicultura do peixe ornamental Betta splendens. *Para isso, o material microbiológico foi obtido do trato intestinal de espécimes sadios, com crescimento bacteriano em meio de cultura seletivo Man Rogosa Sharped (MRS). Dezesseis cepas foram isoladas com base na resposta dos testes* in vitro *de catalase, Gram, azul de anilina, atividade hemolítica e antibiograma aos patógenos* Aeromonas hydrophila, Pseudomonas aeroginosa, Enterococcus durans *e* Escherichia coli. *Das cepas isoladas, C1BS e C5BS foram as que apresentaram melhores respostas, as quais posteriormente foram identificadas, pelo método Maldi-TOF, como* Lactobacillus plantarum *e* Enterococcus faecium. *Devido ao registro bibliográfico e ao desempenho das cepas ácido lácticas nos testes* in vitro, *essas apresentam um grande potencial para uso probiótico espécie-específico na produção ornamental de* Betta splendens.

Palavras-chave: Maldi-TOF, microbiologia, peixe ornamental, inibição patogênica

INTRODUCTION

Ornamental aquaculture is a traditional and profitable activity that reaches a worldwide turnover of more than 15 million dollars (Evers *et al.*, 2019), in which more than 2 billion species are traded (Vasanthakumaran *et al.*, 2020). In Brazil, this segment presents a high growth, an aspect that is related to the greater demand by the final consumer and the exponent profit in the commercialization of native and exotic species (Brasil, 2012; Gomes *et al.*, 2019).

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However, in this market there is no consolidated official statistics, but in general the most commercialized species are exotic organisms such as beta (*Betta splendens*); kinguio ou japonês (*Carassius auratus*); carpa or koy (*Cyprinus carpio*) and the guppy or lebiste (*Poecilia reticulata*) (Millington *et al.*, 2022).

With the commercialization of *Betta splendens* mainly concentrated in Thailand, Indonesia, Singapore, China, Malaysia, Japan, USA, and Mexico, which are its main producers (Thongprajukaew *et al.* 2011), the animal is raised intensively with high stocking densities, sanitary and feeding management that are often inappropriate, which compromise its productive performance, health, and animal welfare indices (Willis, 2015).

In this way, it is essential to improve breeding techniques in ornamental aquaculture, with food additives standing out, such as exogenous enzymes, prebiotics, probiotics, and symbiotics that have shown positive results on fish performance and immunity (Abasali and Mohamad, 2011; Azevedo *et al.*, 2016; Gomes *et al.*, 2019).

In this scenario, probiotics are live microorganisms that, when inserted into the animal production system, colonize the digestive tract of the host, and stimulate its immune system from the lymphatic tissue associated with the intestine, in addition to acting antagonistically to pathogenic agents, through competition for space, nutrients and production of inhibitory metabolites (Balcázar *et al.*, 2008; Gatesoupe, 1999, 2008).

Benefits have been proven in the aquaculture of the *Carassius auratus* (Jinendiran *et al.*, 2019), *Poecilia latipinna* (Ahmadifard *et al.*, 2019), *Danio rerio* (Arani *et al.*, 2019) and *Pterophyllum escalare* (Dias *et al.*, 2019). However, for the successful use of probiotics, it is necessary to select species-specific microorganisms that can provide greater effects (Dias *et al.*, 2018, 2019, 2022; Yamashita *et al.*, 2020).

However, probiotic species-specific selection requires several *in vitro* tests to determine the effectiveness of microorganisms for therapeutic purposes, such as resistance to physiological and environmental conditions (Ramirez *et al.*, 2006 Dias *et al.*, 2019), as well as its viability in inhibiting the occurrence of endogenous pathogenic strains and in the breeding system (Dias *et al.*, 2019; Barros *et al.*, 2022).

Therefore, the objective of the research was to isolate and select in vitro strains with species-specific probiotic potential in aquaculture of *Betta splendens*.

MATERIAL AND METHODS

The assays were approved by the Animal Experimentation Ethics Committee of the Federal University of Para (CEUA nº 9202300420).

The adult specimens of *Betta splendens* were purchased from commercial aquarium shops in the greater metropolitan region of Belem-Pa, Brazil. For this, a brief on-site assessment of the health status of the animals was carried out, based on the macroscopic analysis of the parasite and the specific physiological behavior of the species.

After acquiring the specimens, they were transported to the Probiotics Laboratory of the Federal University of Para, University Campus of Bragança-Pa, Brazil.

For species-specific bacterial isolation, five healthy specimens of the *Betta splendens*, after a 24-hour fast, for the emptying of the digestive system, and then the individuals were anesthetized with eugenol ($60mg.L^{-1}$) and from the deepening of the anesthetic plane, the animals were euthanized by sectioning the spinal cord according to the guidelines of the Brazilian Guide of Good Practices for Euthanasia in Animals (Guia, 2012), disinfected externally with a 70% alcohol solution and then transferred to a laminar flow oven under sterile conditions, to perform excision of the intestine.

After removal of the intestine, it was macerated in sterile saline solution at 0,65% in proportion 1:1 (w/v), using mortar and porcelain pestle. Subsequently, the macerated material was transferred to collection tubes containing 10 mL of culture medium broth Man Rogosa and Sharpe (MRS), which were homogenized with a vortex tube shaker and incubated for 24 hours at a temperature of 35°C in a stove with forced air circulation (Jatobá *et al.*, 2008; Dias *et al.*, 2018; Paixão *et al.*, 2020).

After microbiological growth in broth, the culture medium was diluted in factor 1:10, until 10^5, and then sown 1mL in petri dishes containing the culture medium MRS Agar enriched with 10% of aniline blue as a possible initial indicator of probiotic strains, homogenized with micropellet spheres and bacterial growth carried out in an oven at 35 °C during 48h (Vieira *et al.*, 2013; Fujimoto *et al.*, 2014). The colonies that absorbed the indicator dye continued to identify the morphotype using the Gram staining method (Dias *et al.*, 2019).

Thus, Gram-positive cocci and bacillus colonies were isolated and seeded in a new MRS Agar culture medium for plate exhaustion (Oplustil *et al.*, 2010; Dias *et al.*, 2018, 2019). And subsequent evaluation of catalase and hemolytic activity, the negative catalase colonies and those that did not present homolytic activity, continued the analysis of isolation of potentially probiotic bacteria, as described in the protocols of Vieira *et al.* (2013) and Dias *et al.* (2019).

The *in vitro* inhibition against pathogenic agents was performed by the method of Tagg and McGiven (1971), adapted by Ramirez *et al.* (2006) and Dias *et al.* (2019). For the experiment, the strains of isolated bacteria were grown in test tubes with MRS Broth culture medium, kept for 24 hours and incubated in an oven at 35°C, later the material was seeded in petri plates containing Agar MRS culture medium and incubated at 35°C for 48 hours. Where four discs with a diameter of 0.8 cm were removed from an agar plate with the potentially probiotic bacteria strains and were superimposed on a Muller Hinton Agar culture medium, recently seeded with pathogens *Aeromonas hydrophyla*, *Pseudomonas aeroginosa*, *Enterococcus durans* and *Escherichia coli*, which were incubated at 30 °C for 24 hours.

For further analysis of the antibacterial capacity of potentially probiotic strains against pathogenic agents, which were analyzed by the development or not of the diffusion halo, named by the diameter of the strains inhibitory zone (Hjelm *et al.*, 2004).

For the identification of strains with prospect of probiotic use in species-specific fish farming of Betta splendens, the time-of-flight matrix-assisted laser desorption ionization method was used-MALDI-TOF (Cunha *et al.*, 2006; Assis *et al.*, 2011; Dias *et al.*, 2022).

For this, newly grown colonies were used in the MRS Agar culture medium, and subsequently transferred to the plate of MALDI (Bruker Daltonics), added of 1µL matrix solution HCCA [α-cyano-4-hydroxycinnamic (Sigma-Aldrich), in the concentration of $5mg.mL^{-1}$ in a solution of 50% acetonitrile and 2.5% trifluoroacetic acid (v/v)], until the complete drying of the reaction in a stainless steel plate for later analysis in the MALDI-TOF. which considered the degree of confidence in the physical-chemical identification of the strains up to the species level, from scores greater than 2.0 (Bier *et al.*, 2017).

The inhibitory diameters obtained by the *in vitro* pathogenic test were submitted to tests of homoscedasticity and normality assumptions, by Levene and Shapiro-Wilk, respectively, for further analysis of variance (ANOVA), that when significant, Tukey's test was used to separate the means at 5% probability, analyzed with the aid of the statistical program BioEstat.

RESULTS

A total of 16 autochthonous strains of *Betta splendens* were isolated, 10 reacted with the aniline blue dye (Figure 1), six were classified as Gram positive, with cell morphology of cocci (6) and bacilli (4), and three showed no catalase and hemolytic activity (Figure 1).

Thus, three cultures proceeded to the inhibition test against pathogens, which were named C1BS, C5BS and C14BS. All strains showed inhibition halos with diameters greater than 11 mm for all evaluated pathogens (Table 1), with a significant difference ($p<0.05$) for two strains (C1BS and C5BS), in which the highest inhibitory measures were observed, compared to the C14BS strain.

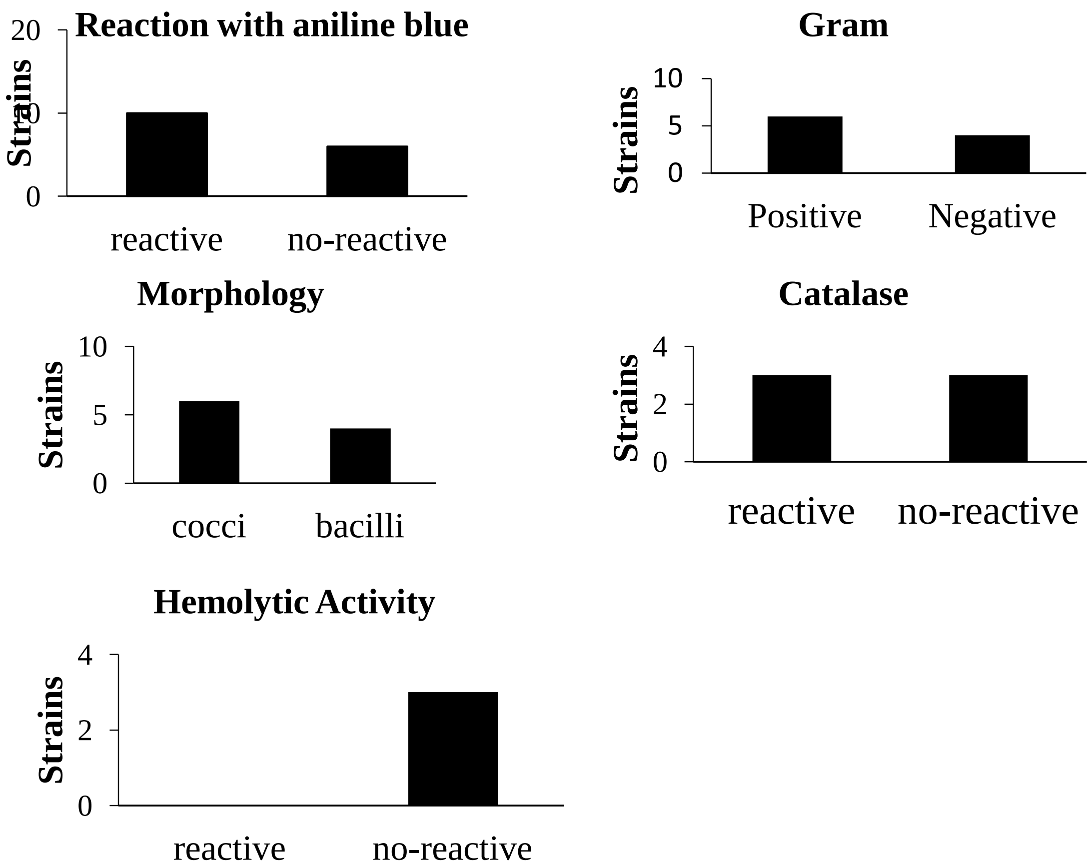

Figure 1. Selection of autochthonous bacteria in *Betta splendens* fish farming, based on *in vitro* reaction tests with aniline blue dye, Gram stain, catalase, and hemolytic activity.

Table 1. *In vitro* evaluation of bacteria with potential probiotic use isolated from the digestive tract of the ornamental *Betta splendens* submitted to the antagonistic challenge, halo of inhibition (mm), with the pathogens *Aeromonas hydrophila* (AH), *Pseudomonas aeroginosa* (PA), *Enterococcus durans* (ED) and *Escherichia coli* (EC)

Strains	AH(-)	PA(-)	EC(-)	ED(+)
C1BS	20.14 ± 0.27A	18.22 ± 0.380A	18.31 ± 1.08A	18.89 ± 1.00A
C5BS	19.03 ± 0.60A	17.87 ± 0.43A	19.32 ± 1.07A	19.11 ± 0.90A
C14BS	11.10 ± 0.51B	13.65 ± 0.52B	14.58 ± 0.93B	12.66 ± 0.95B

(-) Gram negative bacteria, (+) Gram positive bacteria. Different letters in the same column indicate significant difference Tukey test ($p<0.05$).

With this, the bacteria that stood out in the processes of selection and *in vitro* pathogenic challenge for potential species-specific probiotic use in ornamental aquaculture of *Betta splendens*, were identified from two genera, *Lactobacillus* and *Enterococcus*, for a species of *Lactobacillus plantarum* and two varieties for *Enterococcus faecium*, both belonging to the lactic-acid bacteria group.

DISCUSSION

The autochthonous selection process for the prospection of bacteria for potential probiotic use is of great relevance to the probability of an effective final product and guarantee of food health in the host (Dias *et al.*, 2018, 2019). For this, it is necessary to meet some *in vitro* prerequisites that enable the feasibility of use after protocols that simulate microbiological not-pathogenesis and probiotic benefits for a possible healthy microbiome in the target animal in production (Dias *et al.*, 2018, 2019; Paixão *et al.*, 2020).

Thus, the requirements for the safety of supplementation of a beneficial microorganism to act as a promising probiotic must meet the affinity to the aniline blue reagent, as indicative of Gram positive lactic acid bacteria, having as a principle that most aquatic pathogenic agents belongs to the group of Gram negative bacteria, in addition to being catalase negative, it does not present hemolytic activity and inhibit the growth of potentially pathogenic bacteria in animal intestinal environment (Vieira *et al*., 2013; Dias *et al.*, 2018).

Aniline blue is a reagent that, when added in small concentrations (10%) in culture media for microbiological growth, adheres to newly grown colonies from the occurrence of lactic acid, the main carbohydrate that is expelled during the process of fermentation by lactic acid bacteria (Ramirez *et al.*, 2006; Vieira *et al.*, 2013; Dias *et al.*, 2019).

This group of microorganisms has a diversity of representatives with therapeutic purposes with the genera *Carnobacterium, Enterococcus, Lactococcus, Lactobacillus, Lastosphaera, Leuconostoc, Oenococcus, Pedicoccus, Streptococcus, Tetragenococcus, Vagococcus* and *Wessella*, and as the main species applied in aquaculture *Lactobacillus plantarum, Lactobacillus acidophilus*, *Pediococcus acidilactici* and *Enterococcus faecium* which are Gram-positive bacteria, with bacilli or cocci morphology, non-sporulating, obligate anaerobic or aerotolerant, which produce antimicrobial peptides (bacteriocins) during the process of enteric colonization in the host (Mello *et al.*, 2013).

Another method that contributes to the not-pathogenicity of microorganisms isolated for probiotic purposes is the absence of hemolytic activity of the strains, for which the bacteria must not carry out the production of hemolysin, which acts as an enzymatic co-factor in the retention of iron ions and consequently causes the anemic clinical picture in the host (Vesterlund *et al*., 2007; Husain, 2008).

Thus, the group of probiotic acid-lactic bacteria does not propose this ion for its production, an advantageous and safe characteristic in natural and anthropic habitats, which in many cases compete for nutrients and space with pathogenic microorganisms, as occurs in the intestinal microbiome (Elli *et al.*, 2000).

The antagonistic activity of a potential probiotic is of great importance due to its inhibitory zone, which will compete with undesirable cultures from the production of organic acids, hydrogen peroxide and bacteriocins, to inhibit the pathogenic microbiological growth and thus contribute to the sanitation, health and infectious prevention factors in ornamental aquaculture (Vaseeharan *et al.*, 2005; Pridmore *et al.*, 2008).

With this, it can be inferred that the inhibition of autochthonous strains of *Betta splendens*, in the face of the challenged pathogens, presented an effective prospect for probiotic use, based on the inhibition of the growth of both Gram-positive and Gram-negative pathogenic agents. This may be related to the action of organic acids (lactic and propionic) produced by lactic acid strains, which have both an action in regulating the intestinal pH of the host and in inhibiting Gram-negative pathogens. (Ghadban, 2002; Bairagi *et al.*, 2002), as verified in the performance of the isolated strains against the pathogens of *Aeromona hydrophyla*, *Pseudomonas aeroginosa* and *Escherichia coli*.

As for the inhibition of Gram-positive pathogens, bacteriocins may have presented the greatest spectrum of action (Gillor *et al.*, 2008; Sugita *et al.*, 2007), as observed by the exclusion of the microorganism *Enterococcus durans*, when exposed to the challenge of antagonism with potentially probiotic autochthonous bacteria from the *Betta splendens.*

CONCLUSION

The results obtained in the present research indicate that the acid-lactic bacteria of *Lactobacillus plantarum* and *Enterococcus faecium*, isolated from the digestive tract of *Betta splendens*, meet the *in vitro* requirements for animal probiotic use. However, despite the effectiveness of the strains showing probiotic potential *in vitro* tests, other factors may influence their success in ornamental aquaculture, such as the route of administration, dosage, and time of supplementation. Therefore, future studies are necessary to prove its effectiveness in improving zootechnical, sanitary, physiological and immunological parameters, to better elucidate this technology and route of administration in the target species.

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