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Assessment of the *in-vitro* aflatoxin B1 adsorption and probiotic capacity of yeasts isolated from Pacific white shrimp (*Litopenaeus vannamei*)

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ABSTRACT

This study aimed to isolate and identify yeasts present in the intestinal microbiota of Pacific white shrimp (*Litopenaeus vannamei*) cultivated in a tropical estuary and carry out *in-vitro* assessments regarding their probiotic and aflatoxin B_1 (AFB_1) adsorption capacity of isolated *Saccharomyces cerevisiae* strains. The isolation and identification of intestinal yeasts from 40 *L. vannamei* individuals were performed by molecular sequencing. Three *S. cerevisiae* strains (C2B, C2D and C9) were chosen for probiotic potential assessments through homologous inhibition, self-aggregation, co-aggregation, antibacterial activity, gastrointestinal condition viability, and AFB_1 adsorption analyses. The following species were identified: *Candida* spp., *Candida tropicalis*, *Lodderomyces elongisporus*, *Rhodotorula* spp., and *S. cerevisiae*. All isolated *S. cerevisiae* strains presented antibacterial activity, with the C9 strain displaying better performance in the antimicrobial activity, pH viability, and AFB1 adsorption assays. It was, thus, possible to isolate *Candida* spp., *C. tropicalis*, *Rhodotorula* spp. and *S. cerevisiae* from *L. vannamei* shrimp, and our study demonstrated for the first time that *L. elongisporus* may be present in the gut of this shrimp species in captive conditions. Furthermore, the isolated *S. cerevisiae* strains exhibited *in-vitro* probiotic and AFB_1 adsorption potential.

Keywords: *Candida* spp.; *Lodderomyces elongisporus*; *Rhodotorula* spp., *Saccharomyces cerevisiae*.

Avaliação *in vitro* da capacidade de adsorção de aflatoxina B_1 e probiótica de leveduras isoladas do camarão-branco-do-pacífico (*Litopenaeus vannamei*)

RESUMO

Com este estudo, objetivaram-se isolar e identificar leveduras presentes na microbiota intestinal de *Litopenaeus vannamei* cultivados em estuário tropical e testar *in vitro* a capacidade probiótica e adsorvente de aflatoxina B_1 (AFB_1) por cepas de *Saccharomyces cerevisiae* isoladas. Foram adquiridos 40 camarões da espécie *L. vannamei*, fizeram-se o isolamento e a identificação de leveduras intestinais por sequenciamento molecular, e foram escolhidas três cepas de *S. cerevisiae* (C2B, C2D e C9) para avaliar o potencial probiótico. Realizaram-se os seguintes testes: inibição homóloga, autoagregação, coagregação, atividade antibacteriana, viabilidade às condições gastrointestinais e também a análise de adsorção de AFB_1. Foram identificadas as seguintes espécies: *Candida* spp., *Candida tropicalis*, *Lodderomyces elongisporus*, *Rhodotorula* spp. e *S. cerevisiae*. As cepas de *S. cerevisiae* isoladas apresentaram atividade antibacteriana, e a cepa C9 obteve melhor desempenho nos testes de atividade antimicrobiana, na viabilidade em pH e na adsorção da AFB_1 do que as demais. Concluiu-se que é possível isolar *Candida* spp., *C. tropicalis*, *Rhodotorula* sp. e *S. cerevisiae*, e o estudo demonstrou pela primeira vez que *L. elongisporus* pode estar presente no intestino em viveiros. As cepas de *S. cerevisiae* isoladas do intestinos de *L. vannamei* possuem potencial probiótico e adsorvente de AFB_1 em testes *in vitro*.

Palavras-chave: *Candida* spp.; *Lodderomyces elongisporus*; *Rhodotorula* sp.; *Saccharomyces cerevisiae*.

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INTRODUCTION

The constant search for products with higher nutritional quality favored the increase in seafood consumption (Sidonio et al., 2012; Nascimento et al., 2015), and shrimp farming is one of the most developed agribusinesses worldwide (FAO, 2018).

One of the main concerns of the shrimp farming sector is the contamination of the feed by mycotoxigenic fungi, that can produce mycotoxins in inadequate conditions of storage of food products, which can affect animal health and, in turn, cause inestimable public health problems (Pereyra et al., 2010). Aflatoxins are mycotoxins mainly produced by *Aspergillus flavus* and *Aspergillus parasiticus*, with aflatoxin types B_1, B_2, G_1, and G_2 as the most noteworthy. Aflatoxin B_1 (AFB_1) is the most prevalent and biologically active among these metabolites, considered the most toxic for animals, triggering teratogenic, hepatotoxic, mutagenic, carcinogenic, and immunosuppressive effects (Cardoso Filho and Muratori, 2011; Rocha et al., 2014; Anater et al., 2016; Matejova et al., 2016).

The use of biological techniques, such as metabolite detoxification by yeasts with probiotic capacity, is noteworthy among mycotoxin control measures. When these microorganisms are added to contaminated foods, they can adsorb mycotoxins, reducing their concentrations and fermentative action without affecting nutritional and palatability characteristics (Pizzolitto et al., 2012; Rahaie et al., 2012; Ribeiro et al., 2015).

Saccharomyces cerevisiae is a probiotic microorganism with antibacterial properties and immunostimulating compounds (Armando et al., 2011; Caruffo et al., 2015) and may act as a mycotoxin decontaminating agent (Pinheiro et al., 2020). Therefore, the aim of the present study was to isolate and identify yeasts from the intestinal microbiota of Pacific white shrimp (*Litopenaeus vannamei*) cultivated in tropical estuary environments and evaluate the probiotic capacity and *in-vitro* aflatoxin B_1 adsorbent potential of isolated *S. cerevisiae* strains.

MATERIALS AND METHODS

Sampling

Probiotic and aflatoxin B_1-adsorbing yeast isolation and identification were carried out utilizing 40 *L. vannamei* with an average weight of 12 g from a shrimp farm located in Luís Correia, Piauí, Brazil (2°56'52.5"S; 41°26'42.1"W).

After harvesting, the animals were stunned and sacrificed by thermal shock, randomly sampled, placed in individual plastic bags and taken in an isothermal box containing ice, and transported to the Center for Studies, Research and Food Processing at the Universidade Federal do Piauí, in Teresina, Piauí, Brazil (5°02'32.3"S 42°47'04.3"W). An ethics statement was not required for this study.

Yeast strain isolation and characterization

The cephalothoraxes and intestines of each shrimp were aseptically removed in a laminar flow chamber, cut longitudinally to expose the mucosa, transferred to flasks containing 150 mL of peptone dextrose yeast extract (YPD) broth and incubated in a biochemical oxygen demand (BOD) oven at 37 °C for 48 h for enrichment (Yang et al., 2011).

Molecular yeast strain identification and selection

DNA extraction

The isolated yeast strains were resuspended in 100 µL of lysis buffer and incubated in a water bath at 65 °C for 35 minutes. The following reagents were used in the extraction protocol: 200 µL of chloroform:isoamyl alcohol (24:1), 70 µL of isopropanol and 200 µL of 70% ethanol. The tubes containing the pellets were left at room temperature for overnight drying. On the next day, the pellets were eluted in 50-µL Buffer TE (10 mM TRIS-HCL, 1 mM EDTA, pH 8) and incubated in an oven at 37 °C. The obtained DNA was measured using a Thermo Scientific NanoDrop® spectrophotometer (ND-1000; 220 to 750 nm) and diluted until reaching an approximate concentration of 200 $ng \cdot \mu L^{-1}$.

Polymerase chain reaction product purification and sequencing reactions

All obtained molecular profiles were selected and subjected to a reaction system using a pair of primers complementary to the ITS sequence, namely ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') 5.8S rDNA, in which the ITS segment was amplified.

The reaction was carried out employing a PCR Express thermocycler (Valpo protect, Eppendorf), with an initial denaturation step at 95 °C for 2 minutes. The amplicons generated by the polymerase chain reaction (PCR) were purified using EDTA. A total of 11.25 µL of 125 mM EDTA and 135 µL of absolute ethanol were added to 45 µL of the PCR product. The mixture was then subjected to centrifugation at 13,000 rpm for 25 minutes, in which the supernatants were discarded, and the

remaining ethanol was dried for 20 minutes at 37 °C. The DNA was then resuspended in 10 µL of sterile deionized q.s.p. water, and the obtained product was dosed using a NanoDrop ND 1,000 spectrophotometer.

For sequencing, the samples were collected using the Data Collection 3 program (Applied Biosystems), and the 5.8S ITS rDNA sequences and sequence comparisons were analyzed using the BLAST software (Basic Local 562 Alignment Serch Tool, BLAST 2.0 version 2.215).

In-vitro aflatoxin B_1 and probiotic adsorption capacity evaluations

Three *S. cerevisiae* strains (C2B, C2D and C9) isolated from the intestines of Pacific white shrimp (*L. vannamei*) were evaluated. The *in-vitro* tests to assess probiotic strain potential comprised homologous inhibition, self-aggregation, coaggregation, and antimicrobial activity assays. Subsequently, the yeasts were submitted to an AFB_1 adsorption test. Viability tests under gastrointestinal conditions were performed by artificially simulating the pH conditions and the feed passage time reported for shrimp.

Homologous inhibition

In this assay, the three isolated yeast strains were challenged against each other using the cross-striation method described by Muzzolón (2010). Suspensions (10^7 cells·mL^{-1}) were prepared from MEA tubes previously seeded with each of the yeast isolates. Each strain was centrally seeded in a straight line from the upper edge of the plate to the lower edge in Petri dishes containing YPD agar and incubated at 37 °C for 48 hours. After centerline growth, the strains were exposed to chloroform vapors for 10 minutes. The different strains were then sown by crossing streaks up to the edge of the central streak followed by incubation for 24 hours at 37 °C. The presence of growth inhibition halos (≥ 5 mm) around the central stria indicated positive results, and no growth or inhibition halos indicated negative results (< 5 mm).

Self-aggregation capacity

This test was performed according to Kos et al. (2003). Yeast cell counts were performed using a double mirrored Neubauer Chamber (Herka®) employing 10^7 cells·mL^{-1}. The yeast strains were incubated for 24 hours at 37 °C in 4 mL of YPD broth, followed by centrifugation at 5,000 rpm for 10 minutes. The supernatants were then discarded, and the cells washed with phosphate buffer saline (PBS) at pH 7.2. The resulting pellets were resuspended in 4 mL of the same PBS and subjected to homogenization employing a vortex mixer. The contents were incubated under non-agitation conditions for 2 hours at 37 °C using a 002CB temperature-controlled culture oven (Fanem LTDA®). Subsequently, 2 mL was collected from the upper portion of the solutions for optical density (OD) determinations using a Biospectro SP-220 spectrophotometer at 600 nm. Initial optical density was standardized to about 0.5. Auto-aggregation was expressed by Eq. 1:

$$\text{Auto-aggregation capacity \%} = [1 - (DO_{final}/DO_{initial})] \times 100 \quad (1)$$

In which: DO_{final}: final optical density; $DO_{initial}$: initial optical density.

Coaggregation capacity

This test was performed as described by Handley et al. (1987). Four microorganisms were employed: *Aeromonas hydrophila* INQS 00318 (IOC/FDA 110-36), *Escherichia coli* INQS 00033 (ATCC 25922), *Staphylococcus aureus* INQS 00015 (ATCC 25923), and *Streptococcus agalactiae* INQS 00128 (ATCC 27853). A culture of each pathogenic bacterium was prepared in brain-heart broth (BHI) incubated for 24 hours at 37 °C, followed by centrifugation at 5,000 rpm for 10 minutes. The supernatants were discarded, and the cells were then resuspended in PBS (pH 7.2).

The coaggregation test was performed in two stages, comprising a visual test followed by a spectrophotometric coaggregation test. In the visual coaggregation assay, the tested strains were cultured in BHI and the bacterial suspensions were assigned a coaggregation score from 0 to 4+ as proposed by Cisar et al. (1979). The formula proposed by Handley et al. (1987) was then applied to determine coaggregation percentages in the spectrophotomeric coaggregation assay, as Eq. 2:

$$\text{Coaggregation \%} = [1 - DO_{Mix} / (DO_{Pathogen} + DO_{Yeast} / 2)] \times 100 \quad (2)$$

In which: DO_{Mix}: optical density of the yeast + pathogen mixture; $DO_{Pathogen}$: optical density of the pathogen; DO_{Yeast}: optical density of the yeast.

Antimicrobial activity

Yeast strains were tested concerning the production of antimicrobial substances against pathogenic bacteria performed

by the Slab test method on YPD agar according to Strus (1998). The pathogenic bacterial strains used in this assay were *A. hydrophila* INQS 00318 (IOC/FDA 110-36), *E. coli* INQS 00033 (ATCC 25922), *S. aureus* INQS 00015 (ATCC 25923), and *S. agalactiae* INQS 00128 (ATCC 27853). The bacterial suspensions were standardized at 600 nm to an optical density of 0.7. The bacteria were then seeded on nutrient agar plates incubated at 37 °C for 48 hours and swabbed onto the surface of 150 × 15 mm Petri dishes containing Mueller-Hinton agar.

After 48 h, the 14 mm diameter discs were removed from the YPD agar containing the cultured yeasts under aseptic conditions and distributed over the surface of 150 × 15 mm Petri dishes containing Mueller-Hinton agar previously seeded with the test bacteria and incubated at 37 °C for 24 hours. Each treatment was performed in duplicate. The diameters of the growth inhibition halos around the agar plates were measured, and the Pz value (a/b) was calculated, expressed as the ratio between the diameter of the well (a) and the diameter of the lowest well plus the diameter of the precipitation halo around the proteolysis area (b).

Antimicrobial activity was then classified into four categories:

- Pz 1,000 = no antimicrobial activity;
- Pz between 0.999 to 0.7000 = low antimicrobial activity;
- Pz between 0.699 to 0.400 = moderate activity;
- Pz between 0.399 to 0.100 = high antimicrobial activity (Ramos et al., 2015).

Tolerance to acidic pH and bile salts

Two assays were performed to determine the viability and tolerance of the isolated yeast strains, consisting of a simulation of intestinal *L. vannamei* conditions at pH 7 (Alexandre et al., 2014) and a test employing variable pH values (2, 7, and 7 with the addition of bile salts).

Suspensions of each of the isolated strains (C2B, C2D and C9) were prepared in peptone water to obtain 10^7 cells·mL^{-1}, according to Van der Aa Kuhle et al. (2005), with modifications. A total of 100 µL of these solutions were mixed with 900 µL of YPD broth and adjusted to pH 2 with hydrochloric acid, PA. The solutions containing the inoculums were submitted to constant agitation at 150 rpm on an SL-180/DT (Solab®) table shaker at room temperature of ± 30 °C. Culture aliquots (100 µL) were collected at times 0 (control), 4, 8 and 12 h, and viable cell counts were performed by decimal dilution and seeding by spreading on YPD agar surfaces. The plates were then incubated for 24 hours at 37 °C, and the assays were performed in duplicate.

YPD broth supplemented with 0.5% bovine bile (Sigma – Aldrich®) was adjusted to pH 7 by adding 1 M sodium hydroxide solution. At the end of the different incubation times cited before, 100 µL aliquots were taken for viable cell counting by decimal dilution and seeding by spreading on YPD agar surfaces. The plates were then incubated for 24 hours at 37 °C, and the assays were performed in duplicate. Controls of the pH tolerance test were performed by submitting each strain to the same procedure already described, but with inoculation in YPD broth at pH 7 and without the addition of bovine bile.

AFB_1 adsorption assay

The AFB_1 adsorption assay was performed according to Bueno et al. (2007) and Poloni et al. (2015), with some modifications. The initial AFB_1 solution used in the assay was resuspended in acetonitrile from a dry extract of AFB_1 core. Stock solutions of AFB_1 (25 and 50 ng·mL^{-1}) were prepared in PBS (pH 2 and 7). The yeasts used in the assay were previously prepared in YPD broth, and their growth at 10^7 cells·mL^{-1} was standardized after 48 hours with the aid of a Neubauer chamber.

Subsequently, 1-mL aliquots of this solution were transferred to microtubes and subjected to centrifugation for 15 minutes at 5,000 rpm at room temperature. The samples were then washed with distilled water, centrifuged again, and mixed with 1 mL of a PBS pH 2 solution containing AFB_1, incubated at 30 °C for 30 minutes and shaken manually. Following another centrifugation step, the pellets were mixed with 1 mL of PBS at pH 7 containing AFB_1 at 25 and 50 ng·mL^{-1}, incubated at 30 °C for 60 minutes and subjected to manual agitation every 5 minutes. Cells were then pelleted by centrifugation for 15 minutes at 5,000 rpm at room temperature, and the supernatants containing unbound mycotoxins were collected and stored for high-performance liquid chromatography (HPLC) adsorption analyses. The positive (AFB_1 only) and negative (PBS only) controls were not included for testing. All experiments were performed in duplicate.

A high efficiency liquid chromatograph (RF-10AXL-SHIMADZU) was used for AFB_1 detection and quantification, with excitation and emission wavelengths of λ 360 and 440 nm, respectively, equipped with a 20-µL injection volume loop (Trucksess et al., 1994) and C_{18} silica gel reverse phase column (SHIM-PACK VP-ODS 150 × 4.6 mm column with 5 µm particle size). Aliquots (100 µL) of each sample extract were then mixed with 350 µL of a derivatizing solution composed of trifluoroacetic acid:glacial acetic acid:water (20:10:70, v/v). The mobile phase comprised an isocratic acetonitrile:methanol:water (17:17:66, v/v) system at a 1.5 mL·min^{-1} flow rate.

An aflatoxin analytical curve was employed measuring the areas, and its interpolation to a curve constructed with different concentrations of an AFB_1 standard dissolved in acetonitrile, with the limit of detection established as 0.4 $ng \cdot g^{-1}$ and the limit of quantification as 1.2 $ng \cdot g^{-1}$. The adsorbed AFB_1 quantifications were established by the correlation between the two peak areas of the samples and the standard curve. Adsorption percentages were calculated as Eq. 3:

$$\text{Adsorption \%} = (\text{supernatant area} / \text{toxin area of the positive control}) \times 100 \quad (3)$$

Statistical analyses

The treatments were distributed in an entirely randomized design as a 3×4 factorial scheme (three yeast strains, four viability times/four pathogenic bacteria strains) comprising two repetitions per treatment for the viability, coaggregation and antimicrobial activity assays. Regarding the adsorption test, a 3×2 factorial scheme was employed (three yeast strains, two AFB_1 concentrations), comprising two repetitions per treatment. Colony counts were transformed into $\log_{10}^{(x+1)}$. The obtained data were submitted to an analysis of variance and a comparison of means by Tukey's test at 5% significance ($p < 0.05$) employing the SAS® University Edition software.

RESULTS

Figure 1 displays the gel corresponding to the PCR reaction with primers ITS1 and ITS4 used to identify yeast species through sequencing.

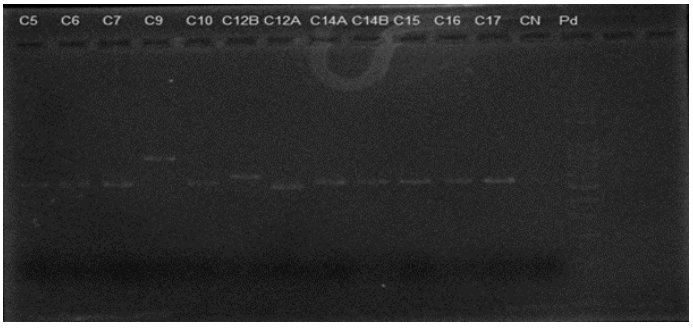

Figure 1. Band profiles of polymerase chain reaction using the ITS1 and ITS4 primers. Right to left: (Pd) molecular weight standard, (CN) negative control and yeast strains C5 to C17 isolated from Pacific white shrimp intestines (*Litopenaeus vannamei*).

Among the total of 40 intestines of *L. vannamei* analyzed, 15 yeast strains were obtained, being identified *Candida* spp., *Candida tropicalis*, *Lodderomyces elongisporus*, *Rhodotorula* spp. and *S. cerevisiae* (Table 1).

Table 1. Yeast species isolated from Pacific white shrimp (*Litopenaeus vannamei*) intestines.

Yeast species	Strain	Isolated	Frequency (%)
Candida spp.	C12B	1	6.7
C. tropicalis	C2E	1	6.7
L. elongisporus	C5, C6, C7, C10, C14A, C14B, C15, C16 e C17	9	60.0
Rhodotorula sp.	C12 A	1	6.7
S. cerevisiae	C2B, C2D e C9	3	20.0
Total		15	100

A statistically significant difference ($P<0.05$) was observed between *S. cerevisiae* strains regarding self-aggregation capacity, with emphasis on strains C2B and C2D (Table 2).

Table 2. Self-aggregating capacity (%) of *Saccharomyces cerevisiae* strains isolated from the intestinal microbiota of Pacific white shrimp (*Litopenaeus vannamei*) intestines*.

S. cerevisiae strains	Self-aggregating capacity (%)	Score	CV (%)
C2B	70.4 ± 5.8 [a]	+	8.19
C2D	70.3 ± 8.4 [a]	+	11.93
C9	50.4 ± 0.2 [b]	-	0.36

*Means followed by different lowercase letters in the same column differ significantly from each other by Tukey's test at a 5% probability ($p < 0.05$); -: aggregation ≤ 60; +: aggregation > 60 and < 80. Data are expressed as means ± standard deviations; CV: coefficient of variation.

All *Saccharomyces* strains isolated from the intestinal microbiota of *L. vannamei* shrimp presented a 3+ qualitative self-aggregation score with the tested pathogenic bacteria and self-aggregation percentages higher than 23%. The self-aggregation results are displayed in Table 3.

The tested *Saccharomyces* strains demonstrated similar antimicrobial activity capacity ($P = 0.667$) in inhibiting the pathogens investigated in this study. The Pz index indicated a different antagonistic activity according to the *Saccharomyces* strain and the tested pathogen, with C9 as the only strain

presenting high antimicrobial activity for all the tested bacteria (Table 4).

Table 5 summarizes the viability test results of the *S. cerevisiae* strains isolated from the intestinal microbiota of *L. vannamei* shrimp under different pH and bile salt conditions. The presence of pH 7 bile inhibited the multiplication of the C2B strain, while the other ones exhibited similar performance to the strains incubated with the controls. All strains remained viable at pH 2 during the cultivation period.

Table 3. Bacterial self-aggregation result and self-aggregation capacity percentages of the *Saccharomyces cerevisiae* isolated from the intestinal microbiota of *Litopenaeus vannamei* and the pathogenic bacteria strains tested herein*.

Bacteria strains tested	*S cerevisae* strains		
	C2B	C2D	C9
Aeromonas hydrophila	24.1 ± 4.5 ^{bA}	34.2 ± 2.2 ^{aA}	34.0 ± 0.1 ^{Aa}
Streptococcus agalactiae	35.8 ± 4.5 ^{abA}	40.2 ± 2.3 ^{aA}	32.1 ± 0.3 ^{Aa}
Staphylococcus aureus	50.7 ± 4.5 ^{aA}	40.8 ± 4.7 ^{aA}	37.2 ± 0.1 ^{Aa}
Escherichia coli	40.0 ± 3.6 ^{abA}	23.0 ± 2.8 ^{aA}	38.9 ± 0.3 ^{Aa}
Score	+++	++	++

*Means followed by different lowercase letters in the same line and uppercase letters in the same column differ significantly from each other by Tukey's test at a 1% probability (P = 0.019). Data are expressed as means ± standard deviations.

Table 4. Antimicrobial activity (Pz Index) of the *Saccharomyces cerevisiae* strains isolated from the intestinal microbiota of *Litopenaeus vannamei* shrimp against pathogenic bacteria strains*.

Bacteria strains tested	*S. cerevisiae* strains		
	C2B	C2D	C9
Aeromonas hydrophila	0.413^{a}	0.412^{a}	0.384^{a}
Streptococcus agalactiae	0.368^{a}	0.379^{a}	0.373^{a}
Staphylococcus aureus	0.417^{a}	0.413^{a}	0.373^{a}
Escherichia coli	0.384^{a}	0.384^{a}	0.395^{a}

*Means followed by different lowercase letters on the same line differ significantly from each other by Tukey's test at a 1% probability (P = 0.667); interpretation of the Pz index = 1,000: no antimicrobial activity; from 0.999 to 0.700: low antimicrobial activity; from 0.699 to 0.400: moderate activity; and from 0.399 to 0.100: high antimicrobial activity.

Table 5. Viability test results for *Saccharomyces cerevisiae* strains isolated from the intestinal microbiota of *Litopenaeus vannamei* shrimp under different pH and bile salt conditions*.

S. cerevisiae strains	pH	Time (hours) CFU/mL, in log			
		0	4	8	12
C2B	7 (Control)	7.39 ± 0.13^{b}	8.33 ± 0.04^{ab}	8.63 ± 0.12^{ab}	8.84 ± 0.02^{a}
C2D		7.22 ± 0.32^{b}	9.54 ± 0.51^{a}	8.77 ± 0.04^{a}	8.92 ± 0.09^{a}
C9		7.35 ± 0.76^{b}	8.11 ± 0.02^{ab}	8.72 ± 0.08^{ab}	8.98 ± 0.13^{a}
C2B	7 (Bile)	7.67 ± 0.10^{a}	8.52 ± 0.08^{a}	8.81 ± 0.13^{a}	8.80 ± 0.01^{a}
C2D		7.11 ± 0.59^{b}	7.94 ± 0.05^{ab}	8.99 ± 0.03^{a}	8.87 ± 0.10^{a}
C9		7.73 ± 0.27^{b}	8.00 ± 0.13^{ab}	8.61 ± 0.22^{ab}	8.94 ± 0.11^{a}
C2B	2	7.99 ± 0.58^{a}	7.39 ± 0.01^{a}	7.64 ± 0.90^{a}	7.46 ± 0.08^{a}
C2D		7.57 ± 0.03^{a}	7.61 ± 0.13^{a}	7.33 ± 0.09^{a}	7.86 ± 0.13^{a}
C9		7.74 ± 0.05^{a}	7.43 ± 0.04^{a}	7.75 ± 0.01^{a}	7.80 ± 0.01^{a}

*Means followed by different lowercase letters on the same line differ significantly from each other by Tukey's test ($p < 0.05$); CFU/mL, in log: viable cell counts; data are expressed as means ± standard deviation.

The tested *S. cerevisiae* strains were able to adsorb both 25 and 50 ng·mL^{-1} of AFB_1 in similar ways, except for the C9 strain, which displayed no adsorption at 25 ng·mL^{-1} (Fig. 2 and Table 6).

Table 7 summarizes the results of other assessments also employing *S. cerevisiae* strains from various sources in AFB_1 adsorption assays at 25 and 50 ng·mL^{-1}.

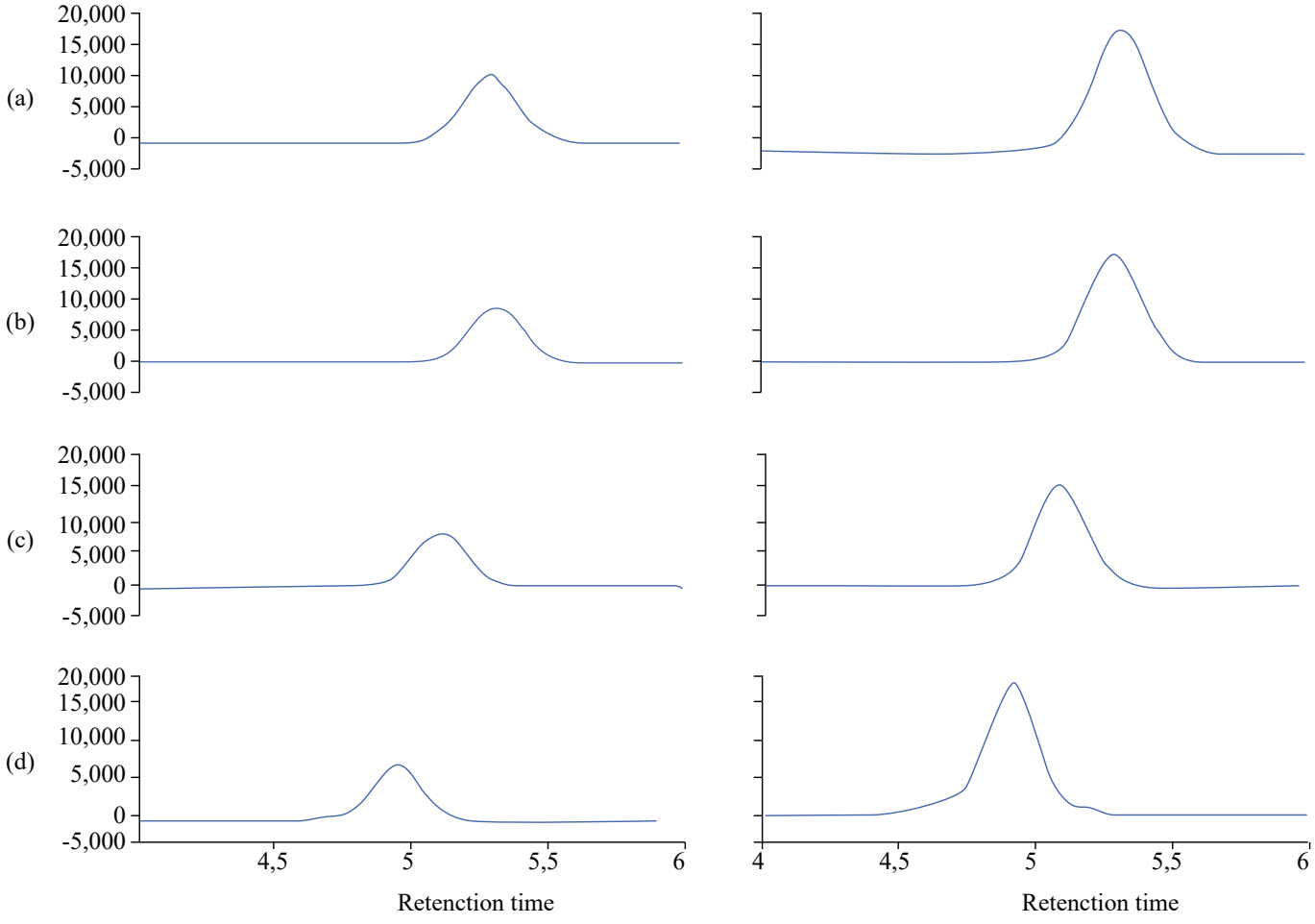

Figure 2. Chromatogram presenting AFB_1 adsorptions by *Saccharomyces cerevisiae* strains isolated from the intestinal microbiota of *Litopenaeus vannamei* shrimp. (a) Aflatoxin B1 control; (b) C2B strain; (c) C2D strain; (d) C9 strain.

Table 6. AFB_1 adsorption by three *Saccharomyces cerevisiae* strains isolated from the intestinal microbiota of *Litopenaeus vannamei* shrimp*.

***S. cerevisiae* strains**	**AFB_1 adsorption[1] (ng·mL^{-1})**			
	25 ng·mL^{-1}		**50 ng·mL^{-1}**	
	(Ẋ ± SD)	**%**	**(Ẋ ± SD)**	**%**
C2B	2.08 ± 0.94 [a]	28.59	3.11 ± 3.58[a]	24.08
C2D	4.85 ± 2.17 [a]	39.76	9.54 ± 1.31[a]	35.79
C9	12.50 ± 0.30 [a]	70.61	11.87 ± 0.51[a]	40.04

Ẋ: means; SD: standard deviations; *means followed by different letters in the same column differ significantly by Tukey's test at a 1% probability ($p < 0.01$).

Table 7. Aflatoxin B_1 adsorption assays results employing *Saccharomyces cerevisiae* strains isolated from different environments reported in other published studies.

Author	Origin of *S. cerevisiae*	Strain	AFB_1 ($ng \cdot mL^{-1}$)	AFB_1 adsorption (%)
Pinheiro et. al. (2020)	Brewery yeast	RC1	25	4.70
		RC3	25	15.20
	Fish farming environment	A8L2	25	19.80
Pizzolitto et al. (2012)	Poultry farm feces	01	50	38.60
		03	50	46.60
		05	50	33.40
		08	50	46.40
Armando et al. (2011)	Ration	RC008	50	67.60
		RC009	50	16.40
		RC0012	50	29.60
	Swine intestine	RC0016	50	82.00

DISCUSSION

The identification of yeasts from the intestinal microbiota of *L. vannamei* may favor the understanding of digestive physiology and prophylactic aspects shrimp concerning diseases caused by environmental stress. This study demonstrated that *L. elongisporus* can be present in the intestine of *L. vannamei* reared in cultivation ponds. Potentially pathogenic *Candida* sp. and *C. tropicalis* strains were also isolated. Due to the pathogenicity of these yeasts in other species, their probable effects on the health of cultivated *L. vannamei* requires investigation. *Rhodotorula* spp. and *S. cerevisiae*, which were also isolated, may exhibit protective characteristics against *L. vannamei* diseases.

The assays with the *S. cerevisiae* strains isolated from the intestinal microbiota of *L. vannamei* shrimp demonstrated natural development between Petri dish streaks with no inhibition. Other *S. cerevisiae* strains isolated from a fish culture environment cultured *in vitro* (Pinheiro et al., 2020) also exhibited similar homologous inhibition behavior.

Self-aggregation capacity may vary according to strain and isolation environment. For example, strains isolated from tilapia ponds have been reported as exhibiting self-aggregation capacity between 76 and 87.1% (Pinheiro et al., 2020), while strains isolated from corn silage and swine intestines ranged from 85.3 to 97.9% (Armando et al., 2011), and from chicken feed between 68.4 and 84.7% (Pizzolitto et al., 2012). The percentages obtained herein indicate that not all *S. cerevisiae* strains can self-aggregate, so strains must be tested prior to their application in probiotic assays concerning shrimp farming commercial products.

In general, the tested strains analyzed herein presented similar self-aggregation results ($p < 0.05$) with pathogenic bacteria, although the C2B strain did not exhibit as good aggregation capacity for *A. hydrophila* as for *Staphylococcus aureus*. Pinheiro et al. (2020) also observed that *S. cerevisiae* strains exhibited different coaggregation capacities: 45.7% for *E. coli*, 42% for *S. aureus*, and 59.5% for *Pseudomonas* spp. Pizzolitto et al. (2012) when evaluating *S. cerevisiae* strains reported values from 1.2 to 14.9% for *E. coli* and from 25.3 to 36% for *S. aureus*. These results suggest that yeast self-aggregation ability is linked to their competence to establish microorganism associations. Therefore, the self-aggregation capacity of *S. cerevisiae* may vary according to individual characteristics and bacterial species.

The *S. cerevisiae* var. *boulardii* strain tested by Rajkowska et al. (2012) only displayed antibacterial activity against *S. aureus*. However, the same species presented an inhibitory effect when tested against *Salmonella* spp., *Staphylococcus aureus*, *Pseudomonas* spp. (Pinheiro et al., 2020), *E. coli* (Armando et al., 2011; Pinheiro et al., 2020), *S. enterica*, and *E. cloacae* (Armando et al., 2011). The antagonism observed for the *Saccharomyces cerevisiae* strains isolated from the intestinal microbiota of *L. vannamei* shrimp demonstrates their ability to inhibit the investigated pathogens.

Concerning the *in-vitro* test, *S. cerevisiae* strains remained viable at pH 7, similar to the gastrointestinal tract of *L. vannamei* as indicated by Alexandre et al. (2014). After 12 hours of incubation at pH 7 (control), all strain counts increased significantly (Table 5) after 4 hours of incubation.

The tested *S. cerevisiae* strains exhibited desirable probiotic characteristics in terms of AFB_1 adsorption capacity. However, the C9 strain displayed better performance in the antimicrobial activity, pH viability, and AFB_1 adsorption assays compared to the other strains, although it did not exhibit the best efficiency in terms of self-aggregation capacity. Among the three tested strains, the C9 strain would, therefore, display the best performance as a probiotic in shrimp diets. The inclusion of this strain in shrimp diets may reduce the amount of AFB_1 in feed, in addition to probably improving the health of these animals. However, *in-vivo* studies should be performed to better evaluate the use of the isolated yeasts in shrimp farming.

Adsorption percentages may vary according to strain origin and aflatoxin concentration. Although the tested strains were isolated from shrimp inhabiting the same environment, varied AFB_1 adsorption performances were noticed (Table 7) in the present study, probably due to the *S. cerevisiae* cell wall composition, which consists mainly of proteins, lipids and polysaccharides, glucans and mannans (Vila-Donat et al., 2018), resulting in different binding mechanisms (hydrogen, ionic or hydrophobic interactions) (Ringot et al., 2006). Thus, despite the *S. cerevisiae* strains having been isolated from the same culture environment, their cell walls seem to behave differently in relation to AFB_1 adsorption at 25 and 50 $ng{\cdot}mL^{-1}$.

CONCLUSION

Candida spp., *C. tropicalis*, *L. elongisporus*, *Rhodotorula* spp., and *S. cerevisiae* yeasts are a part of the microbiota of *L. vannamei* shrimp cultivated in tropical estuary environments.

Lodderomyces elongisporus was reported herein for the first time in the intestinal microbiota of *L. vannamei*.

The *S. cerevisiae* strains isolated from the intestinal microbiota of *L. vannamei* exhibited probiotic and AFB_1 adsorbent potential in *in-vitro* tests.

CONFLICT OF INTERESTS

Nothing to declare.

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AUTHOR'S CONTRIBUTIONS

Conceptualization: Santos JTO, Costa JA; **Methodology:** Santos JTO, Costa JA, Sousa Junior JF, Oliveira FAA, Rocha MS; **Investigation:** Santos JTO, Costa JA, Sousa Junior JF, Oliveira FAA, Rocha MS; **Data curation:** Santos JTO, Sousa Junior JF, Rocha MS, Bacelar RGA, Muratori MCS; **Formal Analysis:** Sousa Junior JF, Rocha MS, Muratori MCS; **Resources:** Pinheiro REE, Muratori MCS; **Software:** Santos JTO, Rocha MS, Muratori MCS; **Project administration:** Santos JTO, Muratori MCS; **Funding acquisition:** Muratori MCS; **Supervision:** Pinheiro REE, **Visualization:** Pinheiro REE, Bacelar RGA; **Writing – original draf:** Santos JTO; **Writing – review & edition:** Sousa Junior JF.

DATA AVAILABILITY STATEMENT

All dataset were generatet/analysed in the current study.

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