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Short Communication

Occurrence and diversity of yeast species isolated from fish feed and tambatinga gut

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ABSTRACT. The present study aimed to identify yeasts species isolated from fish feed and the intestinal tracts of tambatinga fish (*Colossoma macropomum* × *Piaractus brachypomum*) cultivated in a Brazilian fish farm. Twenty tambatingas and 30 fish feed samples from different brands were acquired from two commercial establishments in the state of Piauí, Brazil. The sampled guts were divided into three equal parts, namely the anterior, medium and posterior portions, totaling 60 samples. Molecular identification was performed by PCR amplification and sequencing of the D1/D2 regions of the large rRNA subunit gene. Sixteen *Candida nivariensis* strains in the gut were identified. The fish feed samples showed a higher variability of yeast species, with the following species isolated: *Hyphopichia burtonii* (23.08%), *Lodderomyces elongisporus* (15.39%) and *Trichosporon asahii* (15.39%), *Candida nivariensis* (7.69%), *C. orthopsilosis* (7.69%), *C. parapsilosis* (7.69%), *Rhodotorula minuta* (7.69%), *Sterigmatomyces elviae* (7.69%), *Cryptococcus liquefaciens* (7.69%). Yeast found in feed, however, was not always isolated from tambatinga gut. Molecular identification allowed for the isolation of yeast species not previously reported in fish feed and gut samples.

Keywords: *Candida nivariensis*, *Colossoma macropomum*, *Piaractus brachypomum*, aquaculture.

The tambatinga, a hybrid fish, obtained from a cross between *Colossoma macropomum* (tambaqui) and *Piaractus brachypomus* (pirapitinga), is one of the most cultivated species in Brazil, especially in the northern, northeastern and central-western regions of the country (IBAMA, 2008). National fish production in 2014, which includes species from the *Colossoma* and *Piaractus* genera, including tambaqui, pacu, pirapitinga, and hybrids, have shown a surprising expansion in cultivation activities, with the production of 186 thousand tons of fish (Kubitza, 2015). The tambatinga, in particular, is of utmost importance in both Brazilian aquaculture activities and cuisine (Pessoa, 2009).

The type of feed ingested by a fish can directly affect its inherent microbiota (Ringo *et al*., 2010). Fish gastrointestinal tracts contain many different microorganisms which are part of a complex ecosystem reflecting their environment, microbial composition, and diet (Fjellheim *et al*., 2007; Nayak, 2010). In this sense, the preservation of microbial balance is essential to maintain adequate nutrition (Ringo *et al*., 2010; Omar *et al*., 2012). Yeasts play key roles in several ecosystem processes, establishing ecological relations with other organisms. These have been identified as part of wild-caught as well as farm-raised fish microbiota and display an ability to interfere with the fish's nutrition and sanitary conditions (Gatesoupe, 2007; Navarrete & Tovar-Ramírez, 2014). Yeasts have been isolated from many aquatic ecosystems, with higher prevalence among the genera: *Candida*, *Cryptococcus*, *Hansenula*, *Rhodotorula*, *Torulopsis*, *Saccharomyces* and *Trichosporon* (Rawls *et al*., 2004; Gadanho & Sampaio, 2005; Kutty *et al*., 2013).

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Fish bacterial microbiota displays beneficial effects and contributes to critical biological processes, such as nutrient processing, absorption, and development of the mucosa immunological system (Rawls *et al*., 2004). To fully understand their participation in the health and nutritional aspects of fish from different environments, yeasts as part of the intestinal microbiota of farm-raised fish need to be investigated, with the aim of improving sanitary conditions and productive performance. In this context, the present study aimed to identify yeast species present in the intestinal tracts of farm-raised tambatinga, as well as in their feed.

Twenty adult tambatinga specimens were obtained from two aquaculture environments, the Ademar Braga Fish Culture Station, at the National Department of Drought Prevention (DNOCS) in Piripiri, PI, and the aquaculture vivarium at the Department of Zootechny (DZO), Center of Agrarian Sciences (CCA), UFPI, Teresina, PI, Brazil. This study was approved by the Ethics and Animal Research Experimentation Commission of the Federal University of Piauí (UFPI), Teresina, PI, under protocol no. 013/14. The mean weight of the obtained tambatingas was of 839.2 ± 272.5 g from the "Ademar Braga" Fish Culture Station and 591.0 ± 151.4 g from the aquaculture vivarium. For the microbiological analyses, ten fish were collected from each location and placed in polypropylene containers filled with 20 L of water and 10 mL of eugenol alcohol solution at 5.0%. Following desensitization, the fish were euthanized by medullar section, stored in plastic bags, identified and placed in refrigerated boxes containing recyclable ice for transportation to the Microbiology Nucleus of Feed Study, Research, and Processing Laboratory (NUEPPA) at the Department of Animal Morphophysiology, CCA, UFPI, in Teresina, PI. In total, 30 fish feed samples from three different commercial brands (A, B and C), and different feeding phase diameters were collected from fish farms and tanks from nearby properties.

The fish were weighed, and the intestines were classified as belonging to three different regions: anterior, medium, and posterior, based on the anatomical topographic location of the segment and morphological aspect of the mucosa. Ten-centimeter portions were sectioned from each region, and a longitudinal cut was made in each fragment, thus exposing the mucosa. Each intestine sample was inoculated in 150 mL of yeast peptone dextrose (YPD) broth for 48 h at 37°C in a microbiological chamber (Keller, 2012). Isolations were performed using the serial decimal dilution methodology. Serial decimal dilutions up to 10^{-5} in yeast peptone dextrose (YPD) broth were carried out. 0.1 mL aliquots of the respective dilutions were spread onto plates containing Dichloran Rose-Bengal Chloramphenicol (DRBC) and YPD agar media for yeast isolation. The isolation of yeasts from the commercial feed samples was also carried out according to the serial decimal dilution methodology, where 10 g of each feed sample was homogenized with 90 mL of sterile 0.1% peptone water obtaining an initial dilution of 10^{-1}. Serial decimal dilutions up to 10^{-3}, were performed. From each dilution, 0.1 mL aliquots were plated in yeast peptone dextrose (YPD) agar. Subsequently, all plates were incubated at 25°C for up to seven days in a microbiological chamber. Yeast DNA extraction was performed in the Yeast Ecology and Biotechnology Laboratory (ICB/UFMG). The pure colonies grown on MEA were transferred to a microtubule and re-suspended in 100 µL CTAB 2% (2% CTAB-Sigma; 1.4 M NaCl; 20 mM EDTA; 100 mM Tris-HCl 1M-pH8) buffer, preheated to 65ºC and 10 µL of 2-mercaptoetanol (Merck), shaking the mixture in a vortex for 5 min. The microtubules were then incubated for 1 h at 65°C. Following this period, 500 µL of chloroform isoamyl alcohol (24:1 v/v) mixture was added, and this was shaken for 1 min and centrifuged for 15 min 12000 rpm. Three hundred µL of the aqueous phase was transferred to a sterile microtubule to which the same volume of isopropanol was added. The mixture was homogenized through inversion and incubated for 1-2 h at -20ºC. After the incubation time, the microtubules were centrifuged at 12000 rpm for 10 min, and the supernatant was discarded. The residue was washed with 700 µL of cold 70% ethanol (Biopack) and dried at room temperature for 2 to 3 min, inverting the tubes over absorbent paper. Finally, the residues were re-suspended in 100 µL TE buffer (Tris-HCl 10 mM, EDTA 1.0 mM, pH 8). The residues were stored at -20°C until their quantification, which was performed using NanoDrop® ND-1000 (220-750 nm) spectrophotometer (Thermo Scientific) apparatus.

Yeast into similar profiles, a PCR Finger-printing using the EI1 primer (5'- CTGGCTTGGTGTATG-3') was employed to group the yeast into similar profiles, according to the protocol described by Barros-Lopes *et al*. (1996, 1998). The PCR reaction was performed using 2.5 µL 10X buffer (MBI Fermentas), 1 µL of 2.5 mM dNTP (Invitrogen, USA), 1.5 µL of 25 mM $MgCl_2$ (MBI Fermentas), 2 µL of the 10 pmol EI1 primer (Invitrogen, USA), 0.2 µL of 1.25 U taq DNA polymerase (MBI Fermentas) and 1.0 µL of DNA. The final volume of the reaction was completed with deionized water up to 25 µL. The PCR was performed using a Mastercycler thermocycler (Eppendorf) under the following conditions: initial denaturation at 94°C for 3 min, 33 denaturation cycles at 94°C for one minute, annealing at 45°C for 2 min and extension at

74°C for a minute and a half, followed by final extension at 74°C lasting 5 min. For identification of the isolates, the nucleotide sequences of the D1/D2 domain of the large subunit (26S) ribosomal DNA gene of all the isolates was analyzed as described in Lachance *et al*. (1999). The primers used for the amplification were NL1 (5'-GCATATCAATAAGCG GAGGAAAAG-3', forward) and NL4 (5'-GGTCCGT TGTTTCAAG ACGG-3', reverse). The reaction was conducted in a final volume of 50 µL containing 5.0 µL of 10X PCR buffer (MBI Fermentas), 3.0 µL of 25 mM $MgCl_2$ (MBI Fermentas), 1.0 µL of 2.5 mM dNTP (Invitrogen, USA), 1.0 µL of 10 pmol NL1 primer (Invitrogen, USA), 1.0 µL of 10 pmol NL4 primer (Invitrogen, USA), 0.2 µL of 1.25 U taq DNA polymerase (MBI Fermentas) and 1.0 µL of DNA. A PCR Express (Valpo.protect - Eppendorf) thermocycler was used to carry out the reaction, under the following conditions: initial denaturation at 95°C during 2 min, 35 denaturation cycles at 95°C during 15 sec, annealing at 54°C during 25 sec and extension at 72°C during 20 sec, followed by a final extension at 72°C lasting 10 min. The amplicons generated by the PCR reaction were purified using EDTA and absolute ethanol. The obtained products to be used in the sequencing reactions were quantified using a NanoDrop ND 1000 spectrophotometer (NanoDrop Technologies). The DNA sequencing reactions were performed using the Big Dye Kit, version 3.1 (Applied Biosystems EUA) combined with an automated ABI 3730 sequencer. The sequences were analyzed by BLASTn software (Basic Local 562 Alignment Search Tool version 2.215 of BLAST 2.0), available at the NCBI portal (http:// www.ncbi.nlm.nih.gov/blast/), and were compared to sequences stored within GenBank.

Figure 1 shows the gel corresponding to the PCR reaction fingerprint using the EI1 primer. Grouping similar profiles compared the band patterns of the isolates. The following bands presented similar profiles: Profile A (P1, P2, P3, P4, P5, P6, P7, P8, P9, P10, P11, P12, P13, P14, P15, P16); profile B (R20 and R21); and profile C (R28 and R29). The use of the EI1 primer for lineage differentiation simplified the identification of yeast strains isolated after the PCR reaction, favoring the grouping of similar species. The samples showing differential bands were then selected for the PCR reaction with the NL1/NL4 primers.

The total number of isolates obtained was: sixteen *Candida nivariensis* strains isolated from the gut. *C. nivariensis* were identified by conducting BLAST searches within the GenBank database with ITS sequences as the query. The anterior portion of the intestine showed a high frequency of this species (50%), followed by the medium (31.25%) and posterior portions (18.75%) (Table 1).

In the feed samples several yeast species were found (Table 2). Brand A recovered the highest number of yeast isolates (nine strains), followed by brand B with three strains (*Cryptococcus liquefaciens*, *Candida parapsilosis* and *Rhodotorula minuta*), while brand C recovered only a single strain (*Lodderomyces elongisporus*). Diets with larger diameter pellets given to fish during the growth phase (6.0 to 8.0 mm) showed a higher number of isolates compared to the smaller particle sizes used for the juvenile (2.0 to 6.0 mm), fingerling (1.0 to 2.0 mm) and post-larvae (<1.0 mm) stages. The species *Candida nivariensis* found in the fish gut were also isolated from feed brand A with particle sizes ranging from 4.0 to 6.0 mm, which are given to fish in the juvenile stage. The other yeast species identified in the feed were not found in tambatinga gut samples.

Demands for large-scale commercial fish production require high density in the rearing of stocked fish, leading to the emergence of diseases caused by stress during cultivation, high mortality and economic losses (García-Marengoni & Menezes-Albuquerque, 2015). This fact associated with knowledge regarding morphological and physiological characteristics, behavioral and feeding management of fish species is crucial for aquaculture success. Intestinal colonization in fish plays an important role in stimulating the development of the immune system, and imbalances are associated with susceptibility to infections and immune disorders, with the predominance of pathogenic strains (Walker, 2008). *Debaryomyces hansenii*, *Candida* sp., *Saccharomyces cerevisiae*, *Leucosporidium* sp., and *Rhodotorula* sp., have frequently been isolated as the dominant yeast found in rainbow trout intestine (Gatesoupe, 2007). The *Rhodotorula* genus is commonly observed in the fish microbiota, as are *Cryptococcus* and *Trichosporon* (Gatesoupe, 2007). These genera were also observed in the present study, although only in the powdered feed used for the fingerling.

The only isolated yeast species from the intestines was *C. nivariensis*. This species was prevalent in the tambatinga gut with a 50% frequency in the initial portion of the intestine. Invasive fungal infections caused by *Candida* spp. are the primary cause of morbidity and mortality in immunocompromised hosts (Ruhnke, 2006). An increasing number of infections have been attributed to *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. lusitaniae* and *C. krusei* which have emerged in recent years as significant opportunistic pathogens (Nucci & Marr, 2005). *Candida nivariensis* is associated with human infections,

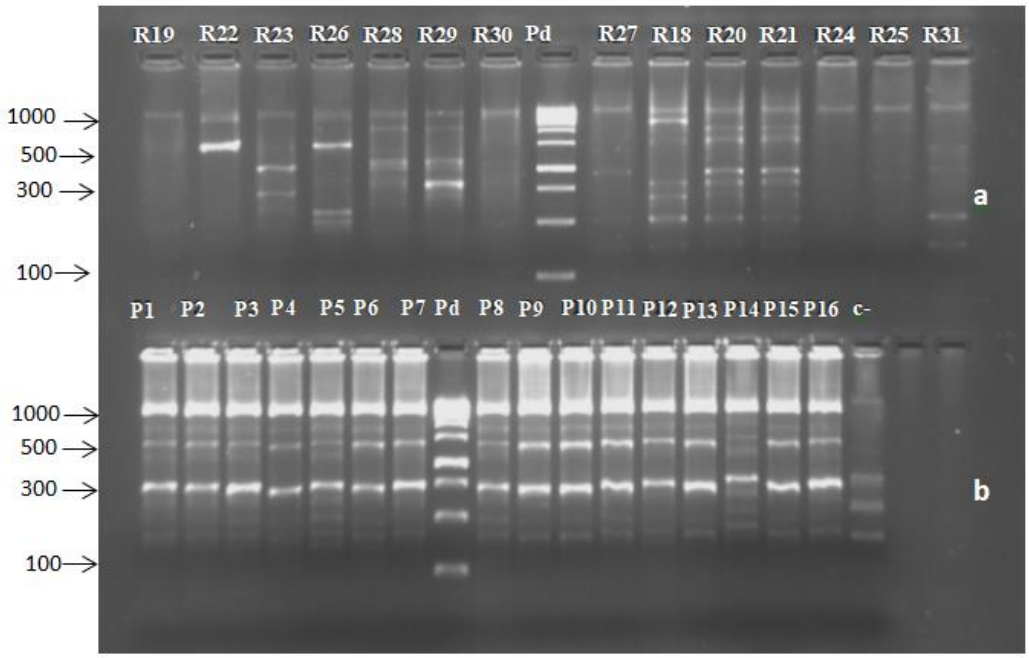

Figure 1. Profile bands obtained from the PCR reaction using EI1 as a primer. a) From left to right: yeasts strains (R19 to R31) fish feed, (Pd), b) yeasts strains (P1 to P16) isolated from tambatinga intestines, (Pd) molecular weight standard, and c) negative control. The sizes of base pair markers are indicated on the left.

Table 1. Yeast species isolated from tambatinga gut samples from two cultivation environments. Frequency (%) of yeasts: number of isolates of each intestinal section ×100/number total of isolated.

Yeast	Intestinal section	Number of isolates	Frequency (%)
Candida nivariensis	Anterior	8	50.00
Candida nivariensis	Medium	5	31.25
Candida nivariensis	Posterior	3	18.75
Total		16	100.00

and it is reported as an opportunistic agent, presenting resistance to many antifungal agents (Alcoba-Florez *et al*., 2005). Thus, *C. nivariensis* is regarded as a clinically important emerging pathogenic yeast (Borman *et al*., 2008). According to Gatesoupe (2007), who summarized the present knowledge concerning the importance of yeast in fish gut, most studies on freshwater species such as rainbow trout or other *Oncorhynchus* species indicate that *Debaryomyces hansenii* has frequently been isolated in these organisms, although *Candida* sp., *Saccharomyces cerevisiae*, and *Leucosporidium* sp. were also prevalent in some trout intestinal samples.

C. nivariensis may be a natural inhabitant of the tambatinga gut and may be responsible for physiological homeostatic processes alongside other bacteria since no pathological processes were observed in the investigated specimens. However, reports on their occurrence in the fish gut were not found, and knowledge regarding yeast species in fish is still scarce.

Some species belonging to the *Candida, Cryptococcus, Malassezia, Trichosporon* and *Geotrichum* genera can be pathogenic to animals (Cabañes, 2010). For example, *Candida* sp., *Cryptococcus* sp., and *Trichosporon* sp. can generate pathological disorders in fish, such as internal lesions or a swim bladder swollen with dense material and exophthalmos (Gatesoupe, 2007). Other species such as *Cryptococcus, Rhodotorula*, and *Sporobolomyces* are widespread across various oceanic regions (Kandasamy *et al*., 2012). Some yeast species and their components, such as β-glucans and mannoproteins, can stimulate the immune systems and are used as antioxidants by the host. Understanding the participation of yeast microbiota in fish health and nutrition may improve both their sanitary conditions and production performance (Navarrete & Tovar-Ramírez, 2014).

The intestinal microbiota of aquatic organisms, especially fish, is strongly related to aquatic microbiota,

Table 2. The frequency of isolated yeast from different commercial fish feed brands. Granulometric size interval according to the fish farming phase; extruded feed.

Brands	Yeast	Diameter of the feed/ cultivation phase	Number of isolates	Frequency (%)
A	*Hyphopichia burtonii*	6.0 to 8.0 mm/growth	3	23.08
A, C	*Lodderomyces elongisporus*	6.0 to 8.0 mm/growth	2	15.39
B	*Cryptococcus liquefaciens*	6.0 to 8.0 mm/growth	1	7.69
A	*Candida nivariensis*	4.0 to 6.0 mm/juvenile	1	7.69
A	*Sterigmatomyces elviae*	2.0 to 4.0 mm/juvenile	1	7.69
A	*Candida orthopsilosis*	1.0 to 2.0 mm/fingerling	1	7.69
A	*Trichosporon asahii*	1.0 to 2.0 mm/fingerling	2	15.39
B	*Candida parapsilosis*	< 1.0 mm/post-larvae	1	7.69
B	*Rhodotorula minuta*	< 1.0 mm/post-larvae	1	7.69
Total			13	100.00

which, in turn, is closely related, both quantitatively and qualitatively, to the physical, chemical and microbiological aspects of the culture environment (Dal Pupo, 2006). *C. parapsilosis*, *R. minuta*, and *Hyphopichia burtonii* have been isolated from marine environments such as fish and fishponds (Gadanho & Sampaio, 2005). Yeast distribution is dependent on the concentration and the kind of organic material available in fishponds and fish feed. In this study, *C. nivariensis* was found both in the feed and in the tambatinga gut, suggesting that its occurrence may be due to the presence of this yeast species in the feed. This study allowed for the identification of new yeast species isolates from fish feed and intestine, some of which have been identified for the first time in these environments.

This study enabled a better characterization of yeast populations in fish feed and their intestines. Molecular methods allowed for the identification of yeast species not previously reported in fish feed and tambatinga intestinal tracts. The yeast species *H. burtonii*, *L. elongisporus*, *T. asahii*, *C. nivariensis*, *C. orthopsilosis*, *C. parapsilosis*, *R. minuta*, *S. elviae* and *C. liquefaciens* were identified in feed samples used in fish-farming with *C. nivariensis* being detected in the intestinal biome of tambatingas in two culture environments. New investigations need to be carried out with the purpose of relating the yeast species found in aquatic environments with the sanitation of these animals.

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