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Original Article

Pioneer access of the foam nest bacterial community of Leptodactylidae frogs and its biotechnological potential

Acesso pioneiro à comunidade bacteriana de ninhos de espuma de sapos Leptodactylidae e seu potencial biotecnológico

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Abstract

Many anuran amphibians deposit their eggs in foam nests, biostructures that help protect the eggs and tadpoles from predators. Currently, there are no other identification and description studies of the cultivable microbiota role in the nests of the Leptodactylid frogs such as *Physalaemus cuvieri, Leptodactylus vastus* and *Adenomera hylaedactyla*. This study aimed to isolate and identify the culturable bacteria from these three anuran species' nests, as well as to prospect enzymes produced by this microbiota. Foam nests samples and environmental samples were diluted and viable cell count was determined. Bacterial morphotypes from foam nest samples were isolated through spread plate technique. Isolates' DNAs were extracted followed by rRNA 16S gene amplification and Sanger sequencing. To evaluate their enzymatic potential, the isolates were cultured in ATGE medium supplemented with starch (0.1% w/v), gelatin (3% w/v) and skimmed milk (1% w/v), to verify amylase and protease activity. A total of 183 bacterial morphotypes were isolated, comprising 33 bacterial genera. *Proteobacteria* phylum was the most abundant in all the three nests (79%). The genera *Pseudomonas* and *Aeromonas* were the most abundant taxon in *P. cuvieri* and *L. vastus*. In *A. Hylaedactyla*, were *Enterobacter* and *Bacillus*. Regarding enzymatic activities, 130 isolates displayed protease activity and 45 isolates were positive for amylase activity. Our results provide unprecedented information concerning culturable bacterial microbiota of the foam nests of the Leptodactylid frogs, as well as their potential for biomolecules of biotechnological interest.

Keywords: culturable, enzymes, prospection, *Leptodactylus vastus*, *Physalaemus cuvieri*, *Adenomera hylaedactyla*, amphibian.

Resumo

Muitos anfíbios anuros depositam seus ovos em ninhos de espuma, bioestruturas que ajudam a proteger os ovos e girinos de predadores. Atualmente, não existem relatos de identificação e descrição do papel da microbiota cultivável nos ninhos de sapos da família Leptodactylidae, como *Physalaemus cuvieri*, *Leptodactylus vastus* e *Adenomera hylaedactyla*. Este estudo teve como objetivo isolar e identificar as bactérias cultiváveis presentes nos ninhos dessas três espécies de anuros, além de prospectar enzimas produzidas por essa microbiota. Amostras de ninhos de espuma e amostras ambientais foram diluídas, e a contagem de células viáveis foi determinada. Os morfotipos bacterianos das amostras de ninhos de espuma foram isolados por meio da técnica de spread plate. O DNA dos isolados foram extraídos, seguidos pela amplificação do gene rRNA 16S e sequenciamento Sanger. Para avaliar o potencial enzimático, os isolados foram cultivados em meio ATGE suplementado com amido (0,1% p/v), gelatina (3% p/v) e leite desnatado (1% p/v), para verificar a atividade de amilase e proteases. Um total de 183 morfotipos bacterianos foi isolado, abrangendo 33 gêneros bacterianos. O filo Proteobacteria foi o mais abundante em todos os três ninhos (79%). Os gêneros *Pseudomonas* e *Aeromonas* foram os taxons mais abundantes em *P. cuvieri* e *L. vastus*. Em *A. hylaedactyla*, foram *Enterobacter* e *Bacillus*. Em relação às atividades enzimáticas, 130 isolados exibiram atividade de protease e 45 isolados foram positivos para atividade de amilase. Nossos resultados fornecem informações inéditas sobre a microbiota bacteriana cultivável dos ninhos de espuma dos sapos da família Leptodactylidae, bem como seu potencial para biomoléculas de interesse biotecnológico.

Palavras-chave: cultiváveis, enzimas, prospecção, *Leptodactylus vastus*, *Physalaemus cuvieri*, *Adenomera hylaedactyla*, anfíbios.

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1. Introduction

Amphibian foam nests are established during the amplex, when the female produces a clocal liquid rich in surfactant proteins and the male and/or female vigorously rotate its legs to incorporate air and create an emulsion. This reproduction pattern helps species to overcome environmental barriers, by protecting the eggs against dehydration, UV light, degradation, predators, pathogens, deoxygenation and acting as incubators. Furthermore, these structures can act as food reserves during offspring development (Cooper et al., 2005; Hissa et al., 2008; Méndez-Narváez et al., 2015; Pereira et al., 2017; Shahrudin et al., 2017). The nest site can vary by species to species, being classified as aquatic (puddles or currents), terrestrial (construction of pits), or arboreal (Dalgetty and Kennedy, 2010).

Anuran foam nests are composed mainly of surfactant proteins, carbohydrates, and lectins. The proteins and lectins assist the formation and maintenance of the nest's structure during several days (Alcaide et al., 2009; Fleming et al., 2009; Hissa et al., 2016). Foam nests also have an associated microbiota that are barely known in terms of composition, function, and influence on the health of these animals (Hissa et al., 2008). Knowing the microbial composition and its role in foam nests are a first step in the conservation of amphibian's species.

Anurans are important organisms to the overall biodiversity maintenance, and serve as model organisms in several studies. They are also bioindicators of climate change due to their skin breathing mode, making them vulnerable to temperature fluctuations and pollutants (Relyea et al., 2005; Hopkins, 2007). However, the amphibian population has been constantly suffering with epidermal infections caused by the fungus *Batrachochytrium dendrobatidis* (Bd) (Vieira et al., 2013). This deadly fungus has extinguished 90 species and led to a decline in 501 frog populations (Scheele et al., 2019).

Due to the impact caused by this fungus, several strategies aimed at the conservation of amphibian species have been developed. One of them is the study of skin-associated microbiota, to evaluate microorganisms' potential to inhibit this pathogen. Among this microbiota, the main genera of bacteria reported are *Pseudomonas*, *Aeromonas*, *Chromobacterium* and *Stenotrophomonas*, presented in bacterial community of several different species of anurans (Muletz-Wolz et al., 2017; Rebollar et al., 2019; Kruger, 2020). However, the knowledge related to the foam nests-associated bacteria and its potential applicability against Bd are not yet available.

The skin-associated microbiome is inherited from both vertical and environmental transmission. Remarkably, studies like McGrath-Blaser et al. (2021) suggest that some key microbes acquired from the initial foam nest can persist on the adult skin**.** This information highlights the importance of the foam nest as a carrier of the amphibian's initial microbiome and its ecological role.

For prospection of biotechnological products, amphibian skin is the subject of many studies, such as Silva et al. (2019), on the skin secretion of *Phyllomedusa.* This genus' skin secretion has been reported to inhibit the growth of *Pseudomonas aeruginosa* and the protozoan *Trypanosoma cruzi*.

Comparable to amphibian skin, foam nests are a rich environment for prospecting biomolecules, mainly from their bacterial community. Among these molecules, the enzymes are of great interest. These biomolecules have increased participation in the pathogen interactions, in various modern industrial processes, providing a promising and robust alternative for chemical catalysts (Kamble et al., 2019). Enzymes of microbial origin are highly effective and more stable than plant and animal enzymes (Liu and Kokare, 2017). Due to the large enzymatic framework and ease of cultivation, the microbial platform has stirred great interest from researchers to prospect enzymes with greater stability in mild conditions of temperature and pressure, to meet industry needs more easily than enzymes from plants or animals.

Considering the unknown knowledge of the culturable microbiota and its enzyme repertoire from foam nest microbiome, this study focused on the identification of the culturable bacteria of three foam-nesting frog species (*Physalaemus cuvieri, Leptodactylus vastus* and *Adenomera hylaedactyla*), to assess the relevance of these microorganisms in maintaining the health and biodiversity of frogs, as well as the biotechnological/enzymatic potential of this microbiota. Such studies are crucial to better understand all resources from anuran's biodiversity taking into account that anurans are the group of vertebrates with the highest extinction rates.

2. Materials and Methods

2.1 - Collection of foam nests

Foam nests from *Adenomera hylaedactyla, Leptodactylus vastus,* and *Physalaemus cuvieri,* were collected during the beginning of the rainy season in January/2019 at the Private Reserve of Monte Alegre Natural Heritage (RPPN Monte Alegre - Latitude: 03º 95'S & Longitude: 38 54'W), Pacatuba, state of Ceará. Collection procedure followed Brazilian regulations through authorization SISBIO 8036-1 released by the Ministry of the Environment and registration of the National Management System for Genetic Heritage and Associated Traditional Knowledge (SisGen) A35A5E2. The nests were collected from puddles of water or from pits with moist soil. The environmental samples from the site of the foam nests were also collected for future comparison with the bacterial community of the foam nests. All samples were kept in sterile tubes and at room temperature and later stored in a -20 °C freezer at the Genetic Resources Laboratory (LaRGen), in the Department of Biology of the Federal University of Ceará (UFC).

2.2. Isolation of culturable microbiota

The foam nest samples were treated beforehand, using sterile tweezers for removal of solid residues (leaves and branches), and had their weight standardized to approximately 4 g. Afterwards, aliquots were diluted in sterile saline solution (0.9%) to the concentration of 10^{-5}. For the environmental samples, 1 mL of puddle water and

4.04 g of soil were serial diluted and homogenized using sterile saline solution (0.9%) until the 10^{-4} dilution. Finally, the last three dilutions of each sample were plated using the spread plate method in ATGE medium (15 g/L agar, 5 g/L tryptone, 1 g/L glucose and 2.5 g/L yeast extract) and incubated at room temperature for 32 hours.

Total viable count was then performed, calculating the colony-forming units per mL in each of the collected samples. Subsequently, each distinct morphotype was described according to the morphological characteristics (color, shape, margin, elevation, and aspect of the colony), then isolated into a new plate, incubated at room temperature for 16 hours. After bacterial growth, each isolate was transferred to TGE broth (5 g/L tryptone, 1 g/L glucose and 2.5 g/L yeast extract) and incubated at 30ºC and 150 rpm for 16 hours. Finally, 900 µl of grown culture were added in 600 µl of 50% glycerol (v/v), for conservation in final concentration of 20% glycerol (v/v) and stored in sterile cryogenic tubes at -20 °C and -80 °C, for the establishment of the microbiome collections of foam nests.

2.3. DNA extraction, amplification and sequencing of 16S gene

For total DNA extraction, two different protocols were used. First, a thermolysis protocol based on Sá et al. (2013) with temperature modifications. From a fresh culture plate, 10 isolated colonies were diluted in 500 µL of sterile ultrapure water in 1.5 mL microtubes and homogenized. Then, they were placed in the dry bath at 99 ºC for 11 min and immediately put in the freezer at - 80 ºC for 4 min. After cooling, the tubes were centrifuged at 12,000 rpm for 5 min and 300 µL of the supernatant was transferred to new microtubes. If the thermolysis protocol failed to extract DNA, the total DNA extraction protocol based on the CTAB 2X cetyltrimethyl ammonium bromide method Warner (1996) was used. The concentration of the genomic DNA obtained was verified by measuring the absorbance at 260 nm (A260) in spectrophotometer Nanodrop ND100 (Nanodrop, Wilmington, DE, USA). The rations 260/280 nm and 260/230 nm provided the DNA quality. Afterwards, the samples were diluted in ultrapure water for concentration of 10 ng/µL and stored at -20 ºC.

For 16S rRNA gene amplification, the primers 27F (5'-AGAGTTTGATCMTGGCTCAG–3') and 1525R (5'-AGAAAGGAGGTGATCCAGCC–3'), or 63F (5′-CAGGCCTAACACATGCAAGTC-3′) and 1389R (5′-ACGGGCGGTGTGTGTACAAG-3') were used. The PCR reactions were carried in an Eppendorf Mastercyler thermal cycler (Epperndorf, Hamburg, Germany) using the following protocol: 95 ºC for 2 min, followed by 30 cycles of 95 ºC for 1 min, 55 ºC for 1 min, 72 ºC for 1 min and 72 ºC for 10 min. In some cases, to improve the amplification of reactions, lower concentration of DNA and primers were used, as well as a reduction of annealing time from 1 min to 30 sec. The specificity of amplification and the size of the sequences of interest were verified by electrophoresis on 1% agarose gel (w/v), stained by SYBR ™ safe.

The PCR products with confirmed amplification were purified and precipitated with potassium acetate and alcohol protocol. For that, 72 µL of PCR product were added to 7.2 µL of 3 M potassium acetate solution (pH 5.5) with twice the solution's total volume of 100% ethanol. After homogenization, the solution was refrigerated at -80 ºC for 30 min, then centrifuged at 14,000 rpm, at 4 ºC, for 15 min, and the supernatant was discarded. The pellet was resuspended with 158.4 µL of cold 70% ethanol and centrifuged at 14,000 rpm, at 4ºC, for 5 min. After centrifugation, the supernatant was discarded, and the pellet was dried in a dry bath at 36 ºC for approximately 20 min. When complete evaporation of the alcohol was observed, the purified DNA was resuspended in 30 µL of RNase-free and DNase-free ultrapure water. Confirmation of the removal of impurities from the samples were observed by the final concentration (ng/µL) and by the ratios 260/230 nm and 260/280 nm above of 1.8 when quantified by Nanodrop ND100 (Nanodrop, Wilmington, DE, USA).

DNA was sequenced by Sanger sequencing, using the primers 518F (5'-CCAGCAGCCGCGGTAATACG-3') and 800R (5'- TACCAGGGTATCTAATCC -3') for amplification of the 16S rRNA gene region, by Macrogen. Sequence treatment was done using Geneious Prime 2019 software and the extremities bases with Phred quality below 30 were removed. The sequences were *de novo* assembled, forming contig sequences. Subsequently, the contig sequence was subjected to molecular identification in the local alignment tool BLAST (Altschul, 1990), using the nucleotide collection database. The isolates that matched with only one genus of bacteria, with identity greater than 97%, were considered to belong to the genera.

2.4. Enzymatic assays

Each isolate was plated on ATGE medium (15 g/L agar, 5 g/L tryptone, 1 g/L glucose and 2.5 g/L yeast extract) and incubated at room temperature for 32 hours. Then, the microorganisms were cultured in plates with ATGE + starch (0.1% w/v) to test for amylase activities, ATGE + gelatin (3% w/v) and ATGE + skim milk (1% w/v) to detect activities of different proteases. After 16 hours, enzymatic activity was measured qualitatively, through the presence of degradation halos around the colony. The addition of lugol (I_2 1%/KI 2%) to reveal amylase activity and of 4.1 M ammonium sulfate ($(NH_4)_2SO_4$) for proteinase activity in the medium containing gelatin were necessary.

3. Results

3.1. Culturable microbiota in foam nests

The samples from the foam nests of *P. cuvieri* and *L. vastus* revealed a culturable bacterial community with similar abundance, being estimated, respectively, at 2.82 ± 0.93 x 10^7 CFU/g and 2.74 ± 0.57 x 10^7 CFU/g. The viable count of *A. hylaedactyla* foam nests presented an abundance of 8.1 ± 0.76 x 10^6 CFU/g, approximately 100 times less colony forming units than the samples from *P. cuvieri* and *L. vastus* foam nests.

When compared to the environmental samples, the foam nests from *P. cuvieri* and *L. vastus* exhibited higher

mean viable count than the bacterial communities found in the water (1000 times higher) or the soil (10 times higher) where the nests were deposited (Table 1). In contrast, samples from *A. hylaedactyla* foam nests exhibited a similar mean viable count to the ones from the soil where the nest was. From these samples, a total of 183 bacteria from the three foam nests were isolated, 98 from *P. cuvieri*, 55 from *L. vastus* and 30 from *A. hylaedactyla.*

3.2. Molecular identification of the bacterial isolates

The identification of the foam nests isolates revealed the presence of four main phyla: Proteobacteria, Bacteroidetes, Firmicutes, and Actinobacteria. The phylum Proteobacteria being the most abundant in the bacterial communities of all three nests (79%). The remaining isolates belong to the phylum Bacteroidetes (10%), Firmicutes (9%), and Actinobacteria (2%). However, the abundance of each phylum varies according to the nest species from which these microorganisms were isolated. The isolates from *A. hylaedactyla* have Firmicutes as the second most abundant phylum (31%) and have no Actinobacteria representatives (Figure 1).

The microorganisms were classified in a total of 33 different genera (Figure 2), the most abundant genera were represented by the phyla Proteobacteria and Firmicutes. Using the 16S ribosomal gene, 85% of the isolates were identified at the genus level. Therefore, the Operational Taxonomic Unit (OTU) used in this work refers to genus.

The genera *Pseudomonas* and *Aeromonas* were the most abundant in the isolates of *P. cuvieri* (43.2%; 6.8%) and *L. vastus* (22.9%; 12.5%). In *A. hylaedactyla*, most representative isolates were *Enterobacter* (34.6%) and *Bacillus* (26.9%). The nest microbiota from the three species had 5 OTUs in common (*Bacillus*, *Enterobacter*, *Comamonas*, *Chryseobacterium*, and *Serratia*). The genera *Aeromonas*, *Kurthia*, *Pseudomonas*, *Rhizobium*, *Acidovorax*, and *Citrobacter* were represented only in *P. cuvieri* and *L. vastus*. The genus *Stenotrophomonas* was found only in

Table 1. Colony-forming units (CFU/g or CFU/mL) in the different samples.

Sample	Viable cell count
Foam nest of *P. cuvieri*	2.82 ± 0.93 x 10^7 CFU/g
Foam nest of *L. Vastus*	2.74 ± 0.57 x 10^7 CFU/g
Foam nest of *A. hylaedactyla*	8.10 ± 0.76 x 10^6 CFU/g
Puddle water from *P. cuvieri*'s nest site	3.33 ± 0.57 x 10^4 CFU/mL
Soil from *L. vastus*'s nest site	1.41 ± 0.73 x 10^6 CFU/g
Soil from *A. hylaedactyla*'s nest site	2.53 ± 0.61 x 10^6 CFU/g

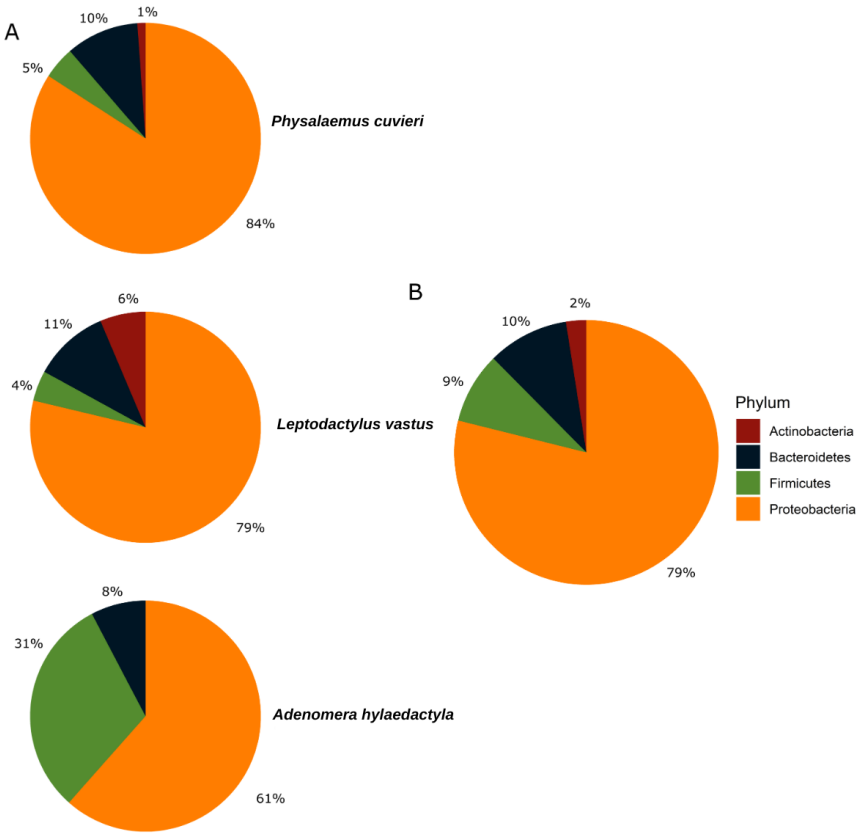

Figure 1. Taxonomic distribution of foam nests isolates. (A) Abundance of the phyla represented by the isolates of each foam nest; (B) Total abundance of the phyla belonging to the cultivable bacterial community of the foam nests.

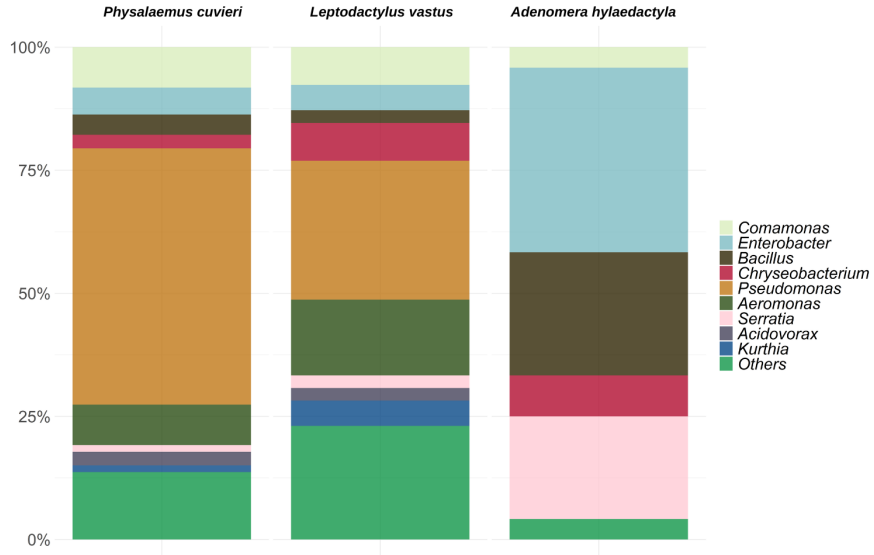

Figure 2. Relative abundance of identified genera in foam nests (%). Others: OTUs identified in only one of the nests with a single representative.

Table 2. Exclusive OTUs in each foam nest.

	Exclusive OTUs
P. cuvieri	*Acinetobacter, Curtobacterium, Empedobacter, Flavobacterium, Klebsiella, Ochrobactrum, Rheinheimera, Shewanella, Sphingobacterium, and Vogesella.*
L. vastus	*Brevundimonas, Burkholderia, Dyella, Herbaspirillum, Microbacterium, Niabella, Pandoraea, Pedobacter, and Streptomyces.*
A. hylaedactyla	*Lysinibacillus*

P. cuvieri and *A. hylaedactyla*. No OTU was found exclusively in *L. vastus* and *A. hylaedactyla*.

In addition to sharing OTUs, the nests also have genera that were only observed in one species of the foam nests, referred as exclusive culturable microbiota. Both *P. cuvieri* and *L. vastus*' nests presented ten exclusive OTUs, whereas *A. hylaedactyla*'s nest presented only the genus *Lysinibacillus* in its exclusive OTU (Table 2).

3.3. Enzymatic activities

In all analyzed species, the number of foam nest isolates that showed protease activity was greater than those with amylase activity. Of the 99 isolates of *P. cuvieri*, 66 displayed protease activity and 23 amylase activity. Of the 55 isolates of *L. vastus*, 45 were positive for protease activity and 14 for amylase. Of the 30 isolates from the nest of *A. hylaedactyla*, 19 displayed positive activity for protease and 8 for amylase. Altogether, only 34 foam nest isolates showed positive activity for all tested enzymes (4 from the nest of *A. hylaedactyla*, 20 from the nest of *P. cuvieri* and 10 from the nest of *L. vastus*). The most abundant genera able to use all tested substrates were *Aeromonas* (29%), *Chryseobacterium* (18%) and *Bacillus* (15%) (Figure 3).

4. Discussion

This research unlocks unprecedented knowledge about culturable microorganisms residing within the foam nests of three amphibian species: *A. hylaedactyla*, *L. vastus*, and *P. cuvieri*. The identification of the bacterial community in the foam nests will help in understanding important correlations between the microbiota and the protection of frogs in the juvenile phases. Biomolecules obtained from the metabolism of these microorganisms may also contribute to the identification of ecological information and biotechnological applications.

Nests are fundamental structures for the microbial colonization of tadpoles. They constitute the environment inhabited by newborns before they are exposed to the direct influence of external conditions (Campos-Cerda and Bohannan, 2020). Nests differ in form, function, parental involvement in their making, and are structured to incubate and hatch eggs or to enable birth (Barber, 2013; Mainwaring et al., 2014; Campos-Cerda and Bohannan, 2020). The characteristics of the nest have been reported as potentially responsible for shaping the first interactions between neonates and microorganisms, functioning as a source of microorganisms and filtering those that can successfully establish themselves in the nest (Ruiz-

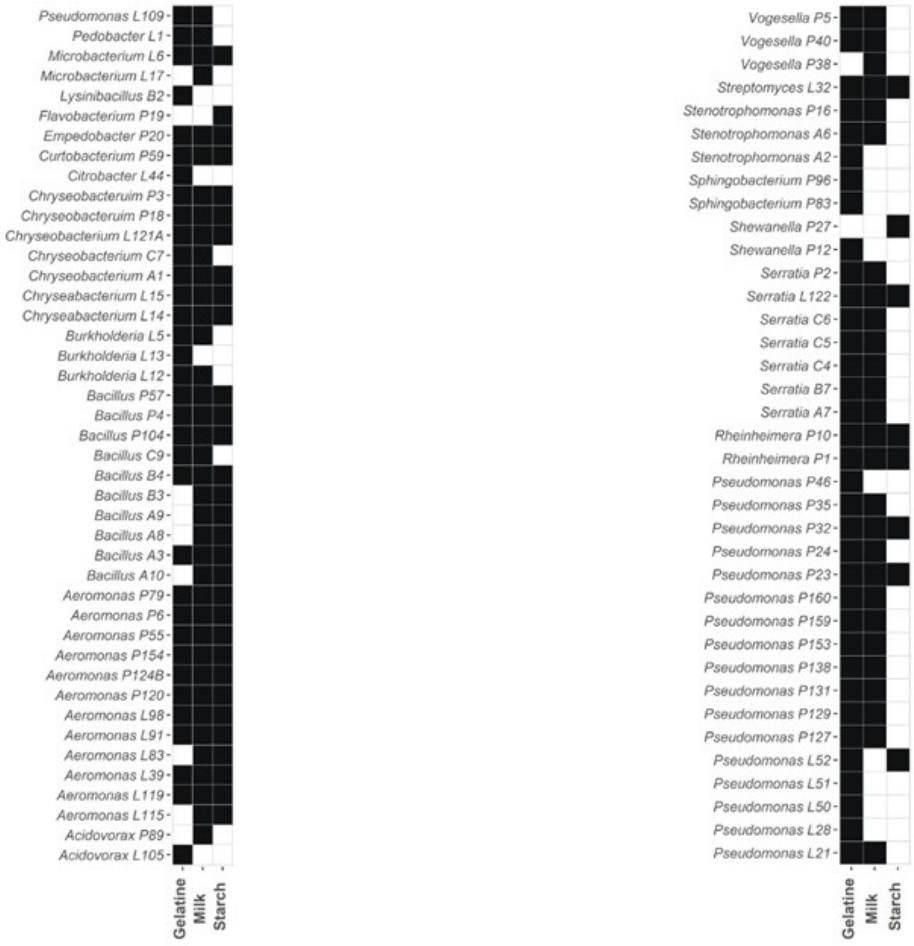

Figure 3. Positive enzymatic activities from at least one of the subtract tested on milk, gelatin, and starch. Bacterial with no positive activity are not included. Black: with activity. White: no activity.

Castellano et al., 2016; Shukla et al., 2018; Teyssier et al., 2018; Campos-Cerda and Bohannan, 2020).

Associations between animals and their resident microbial community contribute to physiology, development, and fitness, often performing essential functions for the animal organism. As this microbiota has been present since birth, selected by parental transference and by the environment, its establishment becomes a crucial ecological and evolutionary occurrence (Lynch and Pedersen, 2016; Campos-Cerda and Bohannan, 2020; Rebollar et al., 2020)

The data on the culturable microbiota present in the foam nests strengthen the hypothesis that the nest provides an environment for the development of microorganisms, results that were also found in other studies from this group (Hissa et al., 2008). Supporting this hypothesis, the microbial diversity found in foam nests was greater when compared to their respective environmental samples, by at least 10 times higher for *L. vastus*. These results are supported by the work of McGrath-Blaser et al. (2021), in which the diversity of the microbial communities of three nest-forming frogs (*Polypedates leucomystax*, *Polypedates macrotis*, and *Polypedates otilophus*) was found to be greater inside the nest than outside. The work also points out differences between inherent nest communities and the microbiota of tadpoles' skin, showing a specific community for the foam nests.

The microbial OTU associated with anuran foam nests reveals important functions for the host, regarding the presence of exclusive OTUs and phyla abundance. For example, a metagenomic approach of the microbiome from the nests of Bornean foam-nesting frogs evidenced the abundance of similar top phyla of Proteobacteria (67%), Bacteroidetes (18%), Firmicutes (10%), Actinobacteria (4%), and Tenericutes (0.4%), when comparing it to adults and tadpoles' microbiome samples (McGrath-Blaser et al., 2021). Similarly, in this study the phylum Proteobacteria (79%) was shown to be the most abundant, followed by Bacteroidetes (10%), Firmicutes (9%), and Actinobacteria (2%). Several skin microbiome studies designate the same percentage placement in amphibians, even with variations of habitats, species, and stages of development (Vences et al., 2016). Moreover, our group's metagenomic investigations have revealed the prevalence of specific

bacterial genera, including *Pseudomonas*, *Vogesella*, and *Chryseobacterium*, as the most abundant within the nests of three distinct frog species (Monteiro et al., 2023). In this study, we discovered that, using cultivable isolation methods, we are also able to successfully identified representatives of these genera.

The *Chryseobacterium* group, belonging to the order Flavobacteriales, is present in all three foam nests (8% in *A. hylaedactyla*, 6.25% in *L. vastus,* and 2.27% in *P. cuvieri*) and has several notable characteristics. Some species of *Chryseobacterium* are known for their protective role in the skin of amphibians, being able to inhibit up to three different genotypes of Bd (Muletz-Wolz et al., 2017). The genus is also reported as a promoter of growth in plants (Sang et al., 2018), on top of having antifungal (Wang et al., 2012) and antimicrobial activities (Dahal et al., 2021). Therefore, the presence of this OTU is very promising for applications in the biotechnology market.

Another frequent group was *Serratia* (20% *A. hylaedactyla*, 2% in *L. Vastus,* and 1.3% in *P. cuvieri)*. Individuals in this group are often found on the skin of anurans and stand out because of the production of prodigiosin, a red pigment with antifungal (Berg, 2000; Woodhams et al., 2018) and antibacterial activity (Lapenda et al., 2015), being able to inhibit the growth of Bd in vitro in minimal concentrations (Woodhams et al., 2018). The presence of this genus in the nests may provide evidence of its role in defense against pathogens.

Bacteria belonging to the genus *Pseudomonas* are quite common in nature, acting as bioindicators of metal contamination in soils (Brandt et al., 2006), producers of biosurfactants (Kuiper et al., 2004) and of enzymes with industrial applications (Hasan et al., 2006; Furini et al., 2018). Although not frequent in animals, this genus proved to be part of the resident microbiota of the amphibian skin (Jiménez and Sommer, 2017), and in the cultivable foam nest microbiome of *P. cuvieri* (43%) and *L. vastus* (22.9%). In addition, several amphibian skin isolates belonging to this genus were able to inhibit the Bd fungus (Lam et al., 2010), concluding that the presence of this OTU may carry a similar protective role in the nest.

Aeromonas are also present in the associated foam nest microbiota of *P. cuvieri* (6%) and *L. vastus* (12.5%). This genus is known by production of several secreted enzymes (Peixoto et al., 2012). In the present study, ten of the 12 stains were able to use all substrate tested. In addition, some strains were able to grow in crude oil and produce biosurfactants (Ilori et al., 2005). This genus, however, is known for having species that cause disease in frogs (e.g., *Aeromonas hydrophila*). Furthermore, this specie is not inhibited by antimicrobial peptides present in the skin of anuran (Hird et al., 1983; Tennessen et al., 2009). The abundance of this OTU on the foam nest associated microbiota needs to be investigated.

There was a high abundance of the genus *Enterobacter* (36%) in the foam nest of *A. hylaedactyla* (less frequent in *L. vastus* and *P. cuvieri*). This genus is mainly present in the intestinal microbiota of female anurans and has an important role in the hydrolysis of chitin, the most abundant component of the insect carapace. An increase of this OTU in the intestine may also be related to nematode infection (Shu et al., 2019). Thus, the abundance of the genus in the foam nest of *A. hylaedactyla* may be related with cloacal transference during the amplex.

Besides the microbial ecological role, the presence of hydrolases in foam nest microbiome enzymatic repertoire could potentially be justified due to the nest's nutritional environment, rich in proteins and carbohydrates (Fleming et al., 2009; Hissa et al., 2016). In this study, ten hydrolase producers were identified as members of the *Bacillus* group, and half were able to use all tested substrates (milk, gelatin, and starch). The *Bacillus* group is one of the most representative bacterial genera in the production of industrial enzymes and earned relevant biotechnological interest. *Bacillus* stands out as it produces a diversity of extracellular proteases, mainly alkaline, comprising mostly serine proteases, cysteine proteases, and metalloproteases (Contesini et al., 2018). *Bacillus* produces alpha-amylases, capable of hydrolyzing the starch present in dextrin, maltose, and glucose. These amylases are also critical in the detergent, food, pharmaceutical, and textile industries (Simair et al., 2017) , and are necessary for the enzymatic hydrolysis of biomass used in the production of bioethanol (Wood et al., 2016).

As for *Chryseobacterium*, its representatives are also known as producers of proteases. Here, 88.8% of *Chryseobacterium* strains were able to use all tested substrates. The ability of these organisms to produce extracellular amylases using organic kitchen waste has also been reported and proved to be of great interest to the industry (Hasan et al., 2017). However, the high protease activity may be involved as virulence factors in pathogenic strains of humans (Pan et al., 2000) and even in hydrolysis of melanin and keratin in bird feathers (Gurav et al., 2016).

The two *Rheinheimera* strains had positive activity for all tested enzymes. This OTU is exclusive of *P. cuvieri* and has shown potential for biotechnological applications. This genus has been reported as belonging to the resident microbiota of amphibian skin (Hernández-Gómez et al., 2020) and provides antimicrobial proteins capable of inhibiting the growth of gram-negative and gram-positive bacteria, yeasts, and algae (Chen et al., 2010). In addition, it produces extracellular pigments in media supplemented with arginine (Grossart et al., 2009).

Some of the most abundant OTUs have already been reported for their production of biosurfactants, which could contribute to the stability of these biofoams. Bacteria of the genus *Pseudomonas* are known to produce rhamnolipid biosurfactant, used in liquid detergent formulations (Jadhav et al., 2019) to improve the degradation of hydrocarbons co-contaminated with toxic metals (Maier and Soberón-Chávez, 2000), and in skin care products(Sekhon Randhawa and Rahman, 2014) . Bacteria of the genus *Bacillus* are kwon producers of lipopeptide biosurfactants, whose applications range from biological control in plants (Geissler et al., 2019), reduction of biofilm formation of pathogens in equipment in the food industry, to antimicrobial additives in toothpaste (Bouassida et al., 2017). Biosurfactants are molecules of biotechnological interest due to their several applications, including cleaning, cosmetics, food, agricultural, pharmaceutical, coal processing, and bioremediation industries (Fakruddin, 2012).

The description of an exclusive community in these foam nests with a diversity of characteristic genera provides evidence of the potential of these organisms to defend against pathogens and predators, and to maintain the micro-habitat through the production of secondary metabolites and the availability of nutrients. In addition, it represents a great potential for the biotechnology industries. However, the role of microbial enzymes in foam nests is still unknown, and further studies that focus on investigating these pathways and their relationship with newborn development are crucial. A possible function of these enzymes is to assist the tadpoles in the digestion of the foam nest, as it has been shown that the supplementation of foam nest solution significantly promotes the growth of the tadpoles (Tanaka and Nishihira, 1987).

It is noteworthy that Brazil is the country with the greatest richness of anuran species and it is still estimated that 25% of anuran species in Brazil are yet to be discovered; containing, therefore, a great amount of diversity not yet described. Considering that 41% of amphibian's species are categorized as threatened, part of amphibian's biotechnological repertoire may be lost even before studied, showing the importance to value studies that access this biodiversity(Guerra et al., 2020; Rocha et al., 2008).

5. Conclusion

This work contributed to the understanding of the culturable microbiota associated with the foam nests built by the three anuran species *Adenomera hylaedactyla, Leptodactylus vastus*, and *Physalaemus cuvieri*. The diversity of characteristic genera in the foam nests provides evidence about their importance to the development of the tadpole. The enzymatic potential of this community was also highlighted, with emphasis on the production of amylases and proteases by strains of the genera *Aeromonas* (29%), *Chryseobacterium* (18%) and *Bacillus* (15%). However, further analyses are needed to identify their biochemical potential, as well as their importance for nest maintenance, nutrient availability and larvae development.

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