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Microbial community structure and chemical composition from dark earth in a native archaeological site of the lower Amazon

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The microbial community structure from dark earth in a native archaeological site of the Lower Amazon was analyzed by PCR-DGGE, using 16S rRNA gene for prokaryote population and 18S rDNA and ITS regions (using clamp GC) for the eukaryote population. The bands were excised from gel and re-amplified for sequencing. The diversity found according to the region of amplification showed same profiles for the two primers pairs. The bacteria genus were: *Bacillus, Klebsiella, Pantoea, Enterobacter, Lactobacillus, Escherichia, Leuconostoc* and actinobacterias as *Streptomyces* and *Microbacterium*. Among the fungal community was *Zygosaccharomyces, Lachancea, Saccharomyces, Cladosporium, Candida, Penicillium* and Uncultured ascomycota and zygomycete were found. Molecular approaches revealed microbial groups that have never been reported in Lower Amazon soil as the *Leuconostoc mesenteroides, Lactobacillus casei* and *Lactobacillus paracasei* bacteria's and *Lachancea meyersii* yeast. The soil pH was ~6.5; the soil had high levels of minerals with exception of Na (not detected) and Al (~0.2 mg/dm^3). The organic matter was 3.5 dag/kg. This study also shows that the Amazon soil is rich in minerals. This can be an important factor in the species richness in the Amazon region. The present data show that the Lower Amazon represents a vast resource for the biotechnology area.

Key words: PCR-DGGE, fungi and yeast, soil microdiversity, soil chemical.

INTRODUCTION

The soils are biodiverse ecosystems. Microorganisms are a component of these ecosystems (Pereira et al., 2006). Microorganisms are involved in biodegradation, decomposition and mineralization, and inorganic nutrient cycling in soils.

The Amazon soil contains great unexplored ground. The Terra Preta are anthropogenic soil (Lehmann et al., 2003) which are fertilized through increasing the cation exchange capacity and the nutrient content (Lehmann et al., 2003; Kim et al., 2007).

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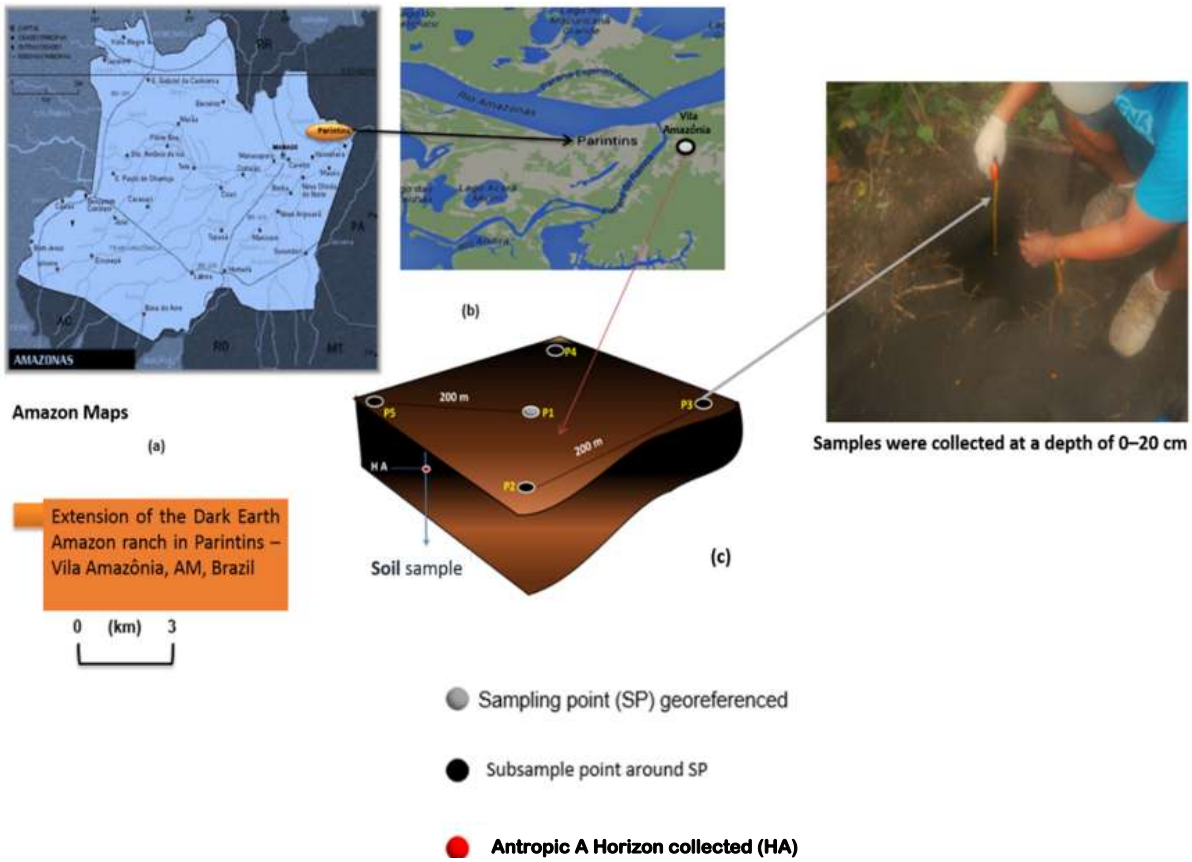


Figure 1. (a) Location of native archaeological site in Amazon, Brazil. **(b)** Site collected the samples. **(c)** Distribution of sampling point. Sampling point scheme: one composed soil sample (30 sub-samples) was collected around each sampling point.

The microbial diversity present in Amazon soils is little known. Therefore, the Brazilian government provides financial support for research. The application of molecular techniques in the microbial diversity and structure analysis of these communities has been used previously (Bossio et al., 2005; Pereira et al., 2006; Castro et al., 2008; Taketani et al., 2013; Brossi et al., 2014). The molecular approaches have been proven to be powerful tools in providing an inventory of the microbial diversity in environmental samples (Ascher et al., 2010; Silva et al., 2012).

The assessment of diversity in Amazon soil is an important aspect in the quest for maintenance of soil biodiversity (Brossi et al., 2014). A characterization of the bacterial and fungal microbial community associated with Amazon soil from dark earth in a native archaeological site of the Lower Amazon is lacking. Furthermore, it is likely that richness of these bacterial and fungal communities is affected by anthropogenic modification of environment. The objectives of this study were: Firstly, molecular survey of the bacterial and fungal communities associated with Amazon soil, based on the sequencing of different rDNA regions, and secondly to analyze the physicochemical characteristics of this soil.

MATERIALS AND METHODS

Soil sampling

Anthropogenic Dark Earth soil samples were collected from 5 points (duplicate) in a native archaeological site of the Lower Amazon, Brazil (Figure 1).

Composited samples were collected at a depth of 0 to 20 cm. The soils were stored in sterile Nasco® plastic in 4°C bags for further use.

DNA extraction and PCR-DGGE

Sample (approximately 0.25 g soil wet weight) of soil was used for DNA extraction by using a NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany). Total DNA was used for PCR amplification of prokaryote and eukaryote ribosomal target regions, for PCR-DGGE analyses. Two primers sets were used for the microbial population. Primers and analysis conditions are given Table 1.

Bands from the PCR-DGGE gels were excised and were reamplified using the same primers for prokaryote and eukaryote (Table 1). The amplicons were sequenced by Macrogen Inc. (Seoul, South Korea). GenBank searches

Table 1. DGGE-PCR primers used to detect eukaryote and prokaryote community from dark earth in a native archaeological site of the Lower Amazon.

Primer	Sequence (5' – 3')	Population	Target	PCR	DGGE	References
968Fgc	AAC GCG AAG AAC CTT AC GC clamp connected to the 5' end of 968f	Bacterial	V6-V8 region of the 16S rRNA gene	Condition 1	16h at 70 V at 60°C.	[b]Magalhães et al. (2010)
1401r	CGG TGT GTA CAA GAC CC					
ITS1fGC	TCC GTA GGT GAA CCT GCG G GC clamp connected to the 5' end of ITS1gc	Fungal	ITS region of the rDNA	Condition 1	16h at 70 V at 60°C.	Wallis et al. (2010)
ITS4r	TCCTCCGCTTATTGATATGC					
338fGC	GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG GC clamp connected to the 5' end of 338fgc	Bacterial	V3 region of the 16S rRNA gene	Condition 1	6h at 70 V at 60°C.	Magalhães et al. (2010)
518r	ATT ACC GCG GCT GCT GG					
NS3fGC	GCA AGT CTG GTG CCA GCA GCC GC clamp connected to the 5' end of NS3gc	Fungal	18S region of the rDNA	Condition 2	16h at 70 V at 60°C.	Magalhães et al. (2010)
YM951r	TTG GCA AAT GCT TTC GC					

GC clamp - CGC CCG CCG CGC GCG GCG GGC GGG GCG GG , f - forward primer; r - reverse primer; Condition 1 - Denatured for 5 min at 95°C. 30 cycles: denaturing at 92°C for 60s, annealing at 55°C for 60s and extension at 72°C for 60s. Final extension for 10 min at 72°C. condition 2 - 35 cycles instead of 30.

(http://www.ncbi.nlm.nih.gov/BLAST/) were performed to determine the species of the obtained sequences. The PCR-DGGE gels were analyzed for Bio-Numerics software (version 1,5, Applied Maths, Kortrijk, Belgium) for determining the amplicons diversity.

Physico-chemical analysis of the soil

The physical and chemical characteristics of Amazon soil from dark earth in a native archaeological site of the Lower Amazon were analyzed. The soils were analyzed in duplicate according to Embrapa (1997). The followings were determined: The value of pH, concentration of hydrogen + aluminium (H + Al), calculation of exchangeable bases (SB), zinc (Zn), iron (Fe), manganese (Mn), copper (Cu), nickel (Ni), arsenic (As), potassium (K), phosphorus (P), aluminum (Al), magnesium (Mg), organic matter (OM), cadmium (Cd), lead (Pb), sodium (Na) and mercury (Hg). For statistical analysis, the SAS System 9.1 (SAS Institute Inc., Cary, NC, USA) was used.

RESULTS

Use of different primers to assess eukaryote and prokaryote communities from dark earth in a native archaeological site of the Lower Amazon

The bacterial and fungal DGGE profiles of Amazon dark earth in a native archaeological site of the Lower Amazon are shown in Figure 2. These profiles exhibited the species abundance. For eukaryote, primer pair ITS1fGC/ITS4r and NS3fGC/YM951r were able to provide a diversity of bands and to differentiate filamentous fungi (*Penicillium* and *Cladosporium)* and yeasts (*Saccharomyces, Zygosaccharomyces, Candida* and *Lachancea).*

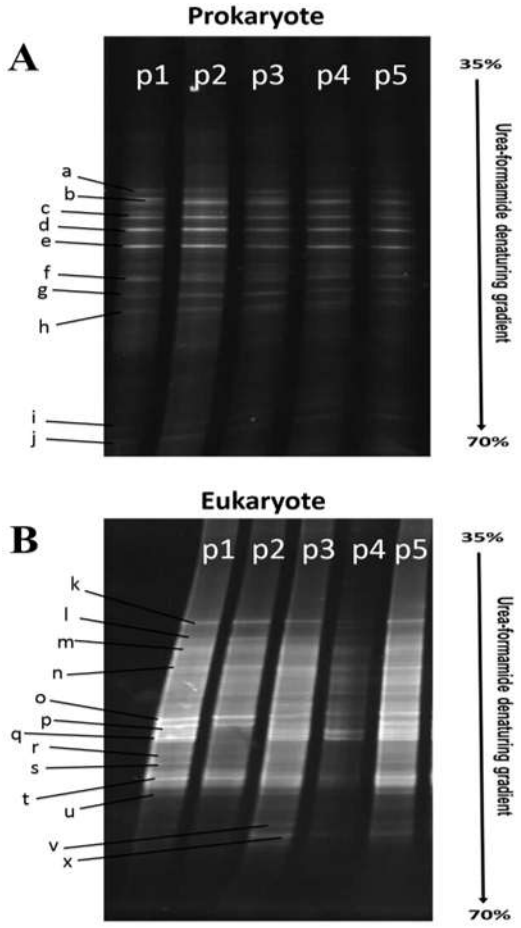
For eukaryote, the primers 338fGC/518r and 968fGC/1401r were able to demonstrate variety of bands with the appearance of *Bacillus, Klebsiella, Enterobacter, Pantoea, Escherichia, Lactobacillus, Leuconostoc* and actinobacterias.

The obtained results using different pairs of primers, show a diverse PCR-DGGE profile, suggesting the presence microbial consortium. This has high relevance in terms of the efficiency of the characterization of microbial diversity of Amazon dark earth in a native archaeological site of the Lower Amazon.

Identification of microbial communities from dark earth in a native archaeological site of the Lower Amazon

Table 2 and Figure 2 showed the molecular diversity of bacterial and fungal from dark earth in a native archaeological site of the Lower Amazon. Identical profiles were obtained for the following primers pairs: Prokaryote (968fGC/1401r and 338fGC/518r) and eukaryote (ITS1fGC/ITS4 and NS3fGC/YM951r). The sequencing bands exhibited equal and higher than 98% identity with sequences available in the Gen-Bank.

In relation to the prokaryotic community analysis, the bands a to j (Figure 2a) were identified as different bacteria species. The band a was identified as *Bacillus macerans* (AB281478), band b - *Klebsiella pneumonia*



Identical profiles were obtained for the following primers pairs:
Prokaryote (968fGC/1401r and 338fGC/518r)
Eukaryote (ITS1fGC/ITS4 and NS3fGC/YM951r)

Figure 2. PCR-DGGE profiles of the Prokaryote (A) and Eukaryote communities (B) in rDNA fragments amplified from dark earth in a native archaeological site of the Lower Amazon.(A) The closest relatives of the fragments sequenced, based in search of GenBank (≥99% similarity), werebands: A - *Bacillus macerans* (AB281478), b - *Klebsiella pneumonia* (CP000964), c –*Pantoea agglomerans,* d - *Enterobacter cowanii* (FJ357832) , e - *Lactobacillus casei* (EU626005.1), f -*Escherichia coli* (EU026432), g – *Leuconostoc citreum* (FJ378896.1), h - *Streptomyces gelaticus* (EU741111.1),i - *Microbacteriumazadirachtae*(EU912487.1), j -*Lactobacillus paracasei* (AB368902.1). (B) The closest relatives of the fragments sequenced, based on a search of GenBank (≥98% similarity), were bands: k -*Zygosaccharomyces* sp. (AF017728.1), l –*Lachancea meyersii* (AY645661.1), m -Uncultured Ascomycota (GQ404775), n -Uncultured zygomycete (AY969178), o - *Saccharomyces cerevisiae* (EU019225.1), p - *Cladosporium oxysporum* (AJ300332.1), q - *Cladosporium* sp. (FJ950740), r – *Candida glabatra* (AY939793.1),s – *Candida tropicalis* (EF194842.1),t – *Candida orthopsilosis* (FN812686.1), u –*Candida* sp. (G1190714325), v - *Penicillium oxalicum* (JF309107), x - *Candida labiduridaru* (FJ623629.1). Abbreviations: p = collected points.

(CP000964), band c - *Pantoea agglomerans* (FJ388592.4),, band d -*Enterobacter cowanii* (FJ357832), band e - *Lactobacillus casei* (EU626005.1), band f - *Escherichia coli* (EU026432), band g - *Leuconostoc citreum* (FJ378896.1), band h - *Streptomyces gelaticus* (EU741111.1) ,band i - *Microbacterium azadirachtae* (EU912487.1) and band j as *Lactobacillus paracasei* (AB368902.1).

In relation to the eukaryotic community analysis (Figure 2b), the band k was identified as *Zygosaccharomyces* sp. (AF017728.1), band l - *Lachancea meyersii* (AY645661.1), band m -Uncultured Ascomycota (GQ404775), band n - Uncultured zygomycete (AY969178), band o - *Saccharomyces cerevisiae* (EU019225.1), band p - *Cladosporium oxysporum* (AJ300332.1), band q - *Cladosporium* sp. (FJ950740), band r - *Candida glabatra* (AY939793.1), band s - *Candida tropicalis* (EF194842.1), band t - *Candida orthopsilosis* (FN812686.1),band u – *Candida* sp. (G1190714325), band v - *Penicillium oxalicum* (JF309107), and band x as *Candida labiduridaru* (FJ623629.1).

C. labiduridaru (FJ623629.1) yeast and *P. oxalicum* (JF309107) filamentous fungi were found in only samples of the points 3, 4 and 5 numbers. Figure 3 describes the microbial genus abundance in dark earth in a native archaeological site of the Lower Amazon. The bacterial genus of greater abundance was *Lactobacillus* and the fungal genus of greater abundance was *Candida*.

Physico-chemical properties from dark earth in a native archaeological site of the Lower Amazon

Chemical and biochemical properties of dark earth in a native archaeological site of the Lower Amazon are given in Table 3. The soil pH value was close to neutral (~6.5). The soil had high levels of minerals with exception of Na and Al (Table 3). The organic matter was found as 3.5 dag/kg.

DISCUSSION

Studies of microbiota and soil chemistry show that the fertility of anthropogenic soils results from combination of mineral and organic components (Navarrete et al., 2010; Taketani et al., 2013). The microbial population profiles can be dependent on the PCR primers used (Anderson and Cairney, 2004; Schwarzenbach et al., 2007). When Amazon soil samples were subjected to analysis by PCR-DGGE, important information became available. Species present in low concentrations can be detected by PCR-DGGE (Pereira et al., 2011).

The results show that the eukaryote population in the Amazon soils reveals a microbial diversity, *Aspergillus* sp., *Penicillium* sp., *Trichoderma* sp., *Zygosaccharomyces* sp. *Lachancea* sp., *Saccharomyces* sp., *Cladosporium* sp., and *Candida* sp. The large

Table 2. Molecular diversity of bacteria and fungal from dark earth in a native archaeological site of the Lower Amazon.

Point	Bacteria	Yeasty	Filamentousfungi
1	*B. macerans* (AB281478), *K. pneumonia* (CP000964), *P. agglomerans* (FJ388592.4), *E. cowanii* (FJ357832) , *Lactobacillus casei* (EU626005.1), *E. coli* (EU026432), *L. Citreum* (FJ378896.1), *S. gelaticus* (EU741111.1), *M. Azadirachtae* (EU912487.1), *L. paracasei* (AB368902.1)	*Zygosaccharomyces* sp. (AF017728.1), *L. meyersii* (AY645661.1),Uncultured Ascomycota (GQ404775), Uncultured zygomycete (AY969178), *S. cerevisiae* (EU019225.1), *C. glabatra* (AY939793.1), *C. tropicalis* (EF194842.1), *C. orthopsilosis* (FN812686.1), *Candida* sp. (G1190714325)	*C. oxysporum* (AJ300332.1), *Cladosporium* sp.(FJ950740)
2	*	*	*
3	*	**Candida labiduridaru* (FJ623629.1)	**Penicillium oxalicum* (JF309107)
4	*	**C. labiduridaru* (FJ623629.1)	**P. oxalicum* (JF309107)
5	*	**C.labiduridaru* (FJ623629.1)	**P. oxalicum* (JF309107)

*Species same of the Point 1.

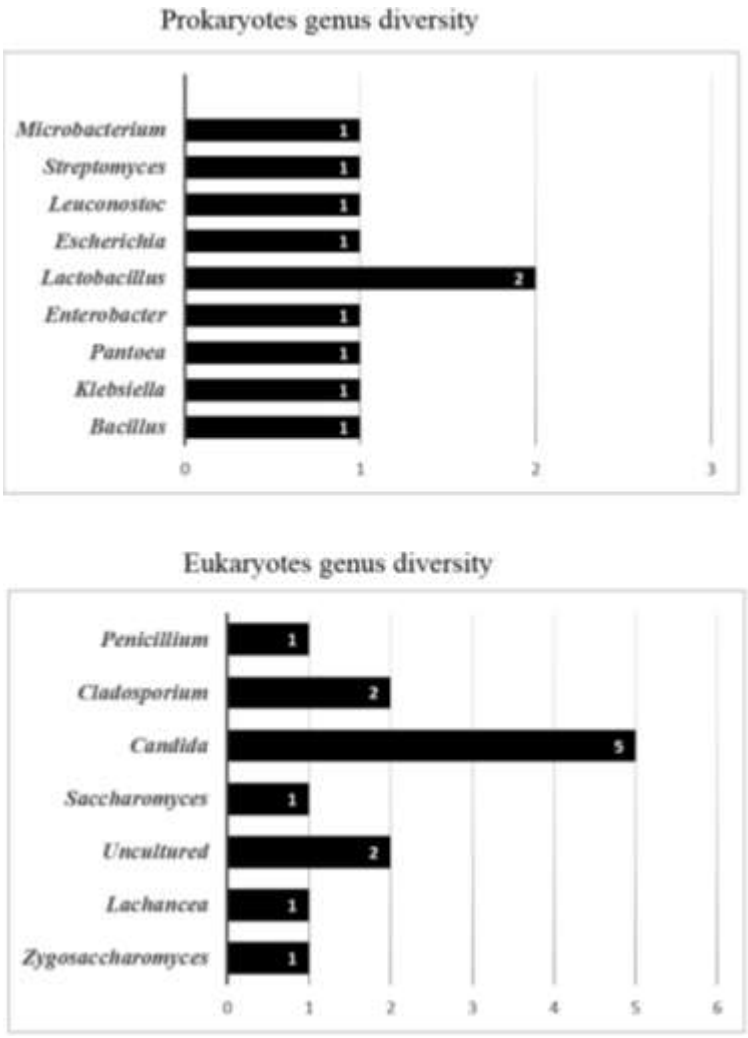


Figure 3. Microbial genus abundance from dark earth in a native archaeological site of the Lower Amazon (Numbers mean species abundance).

Table 3. Chemical and physical characteristics from dark earth in a native archaeological site of the Lower Amazon.

Point	pH	P mg/dm^3	K mg/dm^3	Mg mg/dm^3	Al mg/dm^3	Zn mg/dm^3	Fe mg/dm^3	Mn mg/dm^3	Cu mg/Kg	Cr mg/Kg
1	6.3±0.1^a	51.2±0.1^a	25±1^a	2.1±0.0^a	0.1±0.1^a	6.4±0.1^a	23.4±0.1^a	24.3±0.1^a	16.3±0.1^a	12.9±0.1^a
2	6.4±0.1^a	51.3±0.1^a	26±2^a	2.1±0.0^a	0.2±0.1^a	6.5±0.1^a	23.0±0.1^a	24.4±0.1^a	16.4±0.1^a	12.7±0.1^a
3	6.5±0.1^a	51.2±0.1^a	25±1^a	2.2±0.0^a	0.2±0.1^a	6.6±0.1^a	23.1±0.1^a	24.3±0.1^a	16.3±0.1^a	12.6±0.1^a
4	6.5±0.1^a	51.1±0.1^a	24±1^a	2.1±0.0^a	0.1±0.1^a	6.6±0.1^a	23.5±0.1^a	24.5±0.1^a	16.5±0.1^a	12.8±0.1^a
5	6.4±0.1^a	51.2±0.1^a	25±1^a	2.1±0.0^a	0.1±0.1^a	6.7±0.1^a	23.4±0.1^a	24.2±0.1^a	16.2±0.1^a	12.8±0.1^a
	Ni mg/kg	**As mg/Kg**	**Cd mg/Kg**	**Pb mg/Kg**	**Hg mg/Kg**	**Ca Cmol/dm^3**	**H+Al Cmol/dm^3**	**OM dag/Kg**	**SB mg/dm^3**	**Na mg/dm^3**
1	3.2±0.1^a	1.9±0.1^a	0.14±0.1^a	8.8±0.1^a	1.02±0.1^a	7.1±0.1^a	4.04±0.1^a	3.2±0.1^a	9.16±0.1^a	n.d
2	3.4±0.1^a	1.9±0.1^a	0.14±0.2^a	8.7±0.1^a	1.04±0.1^a	7.2±0.1^a	4.04±0.1^a	3.4±0.1^a	9.14±0.2^a	n.d
3	3.5±0.1^a	1.6±0.1^a	0.14±0.1^a	8.5±0.1^a	1.02±0.1^a	7.1±0.1^a	4.05±0.1^a	3.5±0.1^a	9.16±0.1^a	n.d
4	3.5±0.1^a	1.6±0.1^a	0.14±0.1^a	8.5±0.1^a	1.02±0.1^a	7.3±0.1^a	4.05±0.1^a	3.5±0.1^a	9.16±0.1^a	n.d
5	3.4±0.1^a	1.7±0.1^a	0.14±0.1^a	8.6±0.1^a	1.04±0.1^a	7.1±0.1^a	4.04±0.1^a	3.4±0.1^a	9.14±0.1^a	n.d

Data are average values of duplicate ± standard deviation. Different letters indicate significant differences ($P < 0.05$). K, Potassium; P, phosphorus; Al, aluminum; Mg, magnesium; OM, organic matter; pH; H + Al, hydrogen + aluminium; Zn, zinc; Fe, iron; Mn, manganese; Cu, copper; Ni, nickel; As, arsenic; Cd, cadmium; Pb, lead; Na, sodium; Hg, mercury; (SB, exchangeable bases; n.d. not detected.

variability is due the incorporating of different types and quantities of organic matter (Bissett et al., 2013). Recently, Bresolin et al. (2010) observed microbiota present in Brazilian Cerrado soil using DNA analysis by PCR-DGGE. The microbial species isolated were related to Uncultured soil fungus and Uncultured soil bacteria. Schwarzenbach et al. (2007) observed the presence of Ascomycota yeasts in sandy loam soil sample situated in Central Switzerland.

Among the genus and species of yeast and fungi found in this study; *Lachancae meyersii* is the first species in the collected genus from Amazon soil. According to Fell et al. (2004), all of the isolations of the other species of *Lachancea* have been from plants, plant products or plant-associated insects, fruits or food. The specific ecological niche of *L. meyersii* has not been determined. *Zygosaccharomyces* is a yeast genus as synonymous with spoilage. *Zygosaccharomyces* includes osmotolerant (Thomas and Davenport, 1985). These characteristics of resistant microorganisms to different environments are related to presence in soils. The *Cladosporium* genus constitutes one of the largest genus of Hyphomycetes (Mukherjee and Mittal, 2005). *Cladosporium* grows when there is not enough ventilation; sometimes on walls and wallpaper in rooms (Mukherjee and Mittal, 2005). However, recent data suggest that they are present in soil samples (Paul et al., 2008). The *Candida* genus is commonly found in soils in forest soil in Taiwan including the new species *Candida jianshihensis, Candida yuanshanicus, Candida dajiaensis* and *Candida sanyiensis* (Meyer et al., 1998). Shin et al. (2001) also found Candida genus in Korean soils.

The obtained results showed the presence of several bacterial species in Amazon soil (Figures 2 and 3 and Table 2). The bacterial species *Bacillus* sp. commonly are found in soil samples (Quirino et al., 2009).

Klebsiella, Enterobacter, Pantoea, and *Escherichia* genera were found in this study. These genera are found naturally in soil, water, and plants (Quirino et al., 2009). *Leuconostoc* and *Lactobacillus* genera are not commonly found in soil, however it was found in this study. The presence of this specie in soil is associated with plants and fruits present in vegetation Amazon.

This study also showed the presence of actinobacterias as *Streptomyces gelaticus* (EU741111.1) and *Microbacterium azadirachtae* (EU912487.1). Actinobacteria are genus colonizers in soils. Many species produce enzymes for degradation of cellulose, chitin and, in part, starch. Actinobacteria often occur in degraded organic materials (Schäfer et al., 2010). In the present work, the Streptomyces genus was also found. The Streptomyces genus is focus of research because of the produced substances and has been modified with advances in molecular biology (Souza et al., 2008). *Microbacterium* genus can be isolated from air, soil and water. Many *Microbacterium* spp. play a significant role in oil, lactone and xylan degradation, production of bio-surfactants, and as a growth promoter in plants (Lin et al., 2012).

The soil physico-chemical properties are important and how these properties could be related to microbial profiles in different soils must be evaluated (Peixoto et al., 2010). The properties affect the native microbial populations (Bresolin et al., 2010). This study showed small amounts of aluminum in the Amazon soil (Table 3). High quantities of aluminum in the soil promote the soil impoverishment (Ruggiero et al., 2002). This study also showed the Lower Amazon soil is rich in minerals. This can be an important factor in the species richness in the Amazon region.

Conclusion

In summary, this research has furthered our knowledge about microbial community structure and chemical composition of archaeological site of the Lower Amazon. The application of PCR-DGGE technique based approaches for prokaryote and eukaryote population analysis has confirmed that microbial ecosystems of Amazon soil support a wide diversity of microorganisms that may be responsible for some characteristics these soils. On the other hand, molecular approaches revealed microbial groups that had never been reported in native archaeological site of the Lower Amazon as the *Leuconostoc mesenteroides, Lactobacillus casei* and *Lactobacillus paracasei* bacteria's and *L. meyersii* yeast. The data presented adds important information that will help future studies in these environments.

Conflict of interests

The authors have not declared any conflict of interests.

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Abbreviations: ITS, Internal transcribed spacer, **PCR-DGGE,** polymerase chain reaction -denaturing gradient gel electrophoresis.

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