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Methanogenic communities and methane emissions from enrichments of Brazilian Amazonia soils under land-use change

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ABSTRACT

Amazonian forest conversion into agricultural and livestock areas is considered one of the activities that contribute most to the emission of greenhouse gases, including methane. Biogenic methane production is mainly performed by methanogenic Archaea, which underscores the importance of understanding the drivers shaping microbial communities involved in the methane cycling and changes in methane metabolism. Here, we aimed to investigate the composition and structure of bacterial and archaeal communities in tropical soils in response to land-use changes, emphasizing the methanogenic communities. We collected soil samples from primary forest, pasture, and secondary forest of the Amazonian region and used a strategy based on the enrichment of the methanogenic community with three different methanogenic substrates followed by measurements of methane emission, quantification of *mcrA* gene copies by qPCR, and total 16 S rRNA gene sequencing (metataxonomics). We observed variations in the structure of bacterial and archaeal communities of soils under different uses. The richness of methanogenic communities was higher in pasture than forest soils and this richness remained during the incubation period, and as a consequence, the enrichment induced earlier methane emission in pastures-derived samples. Furthermore, pastures enrichments exhibited methanogenic archaea networks more complex than primary and secondary forests. In conclusion, pastures harbor a richer and more responsive methanogenic community than forest samples, suggesting that conversion of forest areas to pasture may boost methane emission.

1. Introduction

The Amazon rainforest is of great importance due to its high biodiversity and influence on local and global climates by regulating precipitation and atmospheric gas exchanges (Malhi et al., 2008). Despite its ecological and economic values, the forest is under threat of deforestation and the loss of primary forest areas has several negative impacts, including biodiversity reduction (Lima et al., 2013), increase ecosystem vulnerability to environmental changes (Davidson et al., 2012), and disturbances in the carbon cycle. Alterations in the carbon cycle may increase greenhouse gases emissions (Grace et al., 2014), including the methane (CH_4). Methane is considered the second most important greenhouse gas in the atmosphere, after carbon dioxide (CO_2), but with potential to retain infrared radiation between 25 and 34 times more than CO_2 (Ciais et al., 2013).

Methane emissions are originated from thermogenic, pyrogenic, or biogenic sources (Ciais et al., 2013). The biogenic methane is mainly produced by Archaea, as a product of organic matter anaerobic

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decomposition (Hofmann et al., 2016). Microbial communities involved in the methane cycle are known to be affected by the conversion of primary forests to pasture. Paula et al. (2014) reported a reduction in the abundance of methane oxidation genes in pasture soils but did not observe changes in the content of methanogenesis-related genes. The authors suggested that pasture areas, especially those unmanaged, emitted more methane due to soil compaction. Compacted soils have a decreased oxygen diffusion, which leads to the formation of anaerobic microsites, increasing methanogenic activity (Lammel et al., 2015; Paula et al., 2014; Steudler et al., 1996). Meyer et al. (2017) observed through metagenomics data that the abundance of methanogenic genes did not differ among soils of primary forest, pasture, and secondary forest, however these authors observed differences in the composition of these communities.

Despite its importance, the ecological role of microorganisms involved in methane flux in Amazonian soils is poorly understood, especially in soils under forests impacted by land use changes. Here, we took the advantage of a culture enrichment approach to perform a study on the ecology of methanogens, which allowed us to monitor changes in microbial communities, to estimate methane production in different soil usages, as well as to test the effect of different carbon sources on methane emissions. We hypothesize that pastures soils may host a more abundant and responsive methanogenic community, and as a consequence, the methane emission in these soils may increase faster than in forest soils, along the enrichment.

2. Methods

2.1. Soil sampling and characterization

The soil samples were collected from the surface layer (0–10 cm depth) in the municipality of Belterra, Pará, Brazil, in May 2016. The soil samples of primary forest were collected in the Tapajós National Forest (TNF) (2◦51′23.9‘S, 54◦57′28.4′W), while the pasture (3◦07′52.9‘S, 54◦57′28.1′W) and secondary forest (3◦15′47.9‘S, 54◦53′36.0′W) soil samples were collected in an unmanaged area with low grazing intensity, and an area naturally recovering after deforestation, respectively (adjacent areas to TNF). Soil samples were vertically collected using cylinders of polyvinyl chloride (PVC) at equidistant points (100 m) along a line. These cylinders were then closed with lids at both ends to maintain the sample structure during transportation (Pazinato et al., 2010). Additionally, soil samples were collected using a volumetric Kopeck ring of 50 cm^3 and sent to the Department of Soil of the “Luiz de Queiroz” College of Agriculture - University of São Paulo (ESALQ-USP) for further soil density analysis.

2.2. Methanogen-enrichment culture

The enrichment medium was composed by mineral salts (in g L^{-1}: NH_4Cl 0.5 g; KH_2PO_2 0.4 g; $MgCl_20.6\ H_2O$ 0.1 g; $CaCl_20.2\ H_2O$ 0.05 g) (Zinder and Koch, 1984), trace metal solution [$FeSO_40.7\ H_2O$ 0.556 g; $MgSO_4$ 0.5 g; $MnSO{\cdot}7\ H_2O$ 0.5 g; Na_2MoO_4 0.24 g; $Na_2WO{\cdot}2\ H_2O$ 0.24 g; Na_2SeO_3 0.15 g; $NiCl_20.6\ H_2O$ 0.1 g; $CoCl_20.6\ H_2O$ 0.1 g; $ZnSO_40.7\ H_2O$ 0.1 g; $CuSO_40.5\ H_2O$ 0.01 g; $AlK(SO_4)_2$ 0.01 g; H_3BO_3 0.01 g], bicarbonate solution (0.1% w/v), vitamin solution 10 ml (folic acid 0.005 g, lipoic acid 0.005 g, nicotinic acid 0.005 g, biotin 0.005 g, calcium pantothenate 0.005 g, pyridoxine HCl 0.010 g, riboflavin 0.005 g, thiamine HCl 0.005 g, vitamin B12 0.0001 g and Milli-Q water 1000 ml) and cysteine solution (0.1% w/v). The enrichment medium was adjusted to pH 7, then stored in a different bottle (50 ml) and received 5 g of soil from each area. In addition, the enrichment samples has been separately supplemented with methanol, acetate or atmosphere of hydrogen H_2:CO_2 (80:20, v/v), comprising nine separate treatments (each bottle containing one methanogenic substrate and one soil sample) in three replicates, totaling 27 enrichments. The bottles were closed with butyl caps and aluminum seals, and their headspaces of enrichments were formed by using a simultaneous gas distribution system. The hydrogenotrophic enrichments received an atmosphere of H_2:CO_2 (80:20), and the enrichments supplemented with acetate (1% w/v) or methanol (1% w/v) received an atmosphere of N_2:CO_2 (70:20, v/v). The enrichments were incubated at 30 °C along 63 days, the period where the methane production declined in the enrichments supplemented with methanol.

2.3. Methane emission measurements

The methane emissions from the enrichments were monitored by gas chromatography (GC) every seven days, starting after 28 days of incubation. For this purpose, 100 μL of the headspace of each bottle were collected with a disposable 1 ml syringe and immediately injected into the Agilent® HP6890N gas chromatograph equipped with a flame ionization detector (FID), and a megabore column [Agilent HP-Plot “S” (Al_2O_3), 50 m * 0.53 mm* 0.15 μm]. The temperature for column chamber, inlet chamber and detector were 40 °C (isothermal), 250 °C and 250 °C, respectively. High purity hydrogen was used as the carrier gas at a flow rate of 32 ml ml^{-1}. The split ratio of gas sample in inlet chamber was 25:1. The flow rate for air and nitrogen were 250 and 25 ml min^{-1}, respectively. Methane concentrations were calculated through the equation given by:

$$x = \frac{4.44196y}{z}$$

where x is the area of the sample peak; z is the standard area determined by the average of five consecutive methane injections, with a standard deviation less than 1%; and y is methane concentration expressed in $mmol.L^{-1}$. Calculations were based on Clapeyron equation, considering temperature of 298.15 K (25 °C) and atmospheric pressure of 0.93 atm (conditions during chromatographic determinations).

2.4. Extraction of DNA for quantitative PCR and 16 S rRNA gene sequencing

Aliquots of 1.5 ml of the enrichment cultures were collected at 0, 15, 28 and 63 days for nucleic acids extraction. The deoxyribose nucleic acids were extracted using the PowerSoil® DNA extraction kit (MO BIO, Laboratories, Carlsbad, USA) according to the manufacturer’s instructions, but adding a first step of centrifugation for 4 min at 13,000 rpm and glass beads. The purity of the extracted DNA was checked with the Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) (260/280 nm ratio), and was quantified by Qubit® 2.0 fluorometer using the dsDNA BR Assay kit (Invitrogen™). The integrity of the DNA was also confirmed by electrophoresis in a 0.8% agarose gel with 1 X TAE buffer.

The quantitative PCR (qPCR) was performed to determine the *mcrA* gene copy numbers. The qPCR reactions contained 6 μL of SYBR® Green PCR MasterMix kit (Life Technologies™), 0.5 mM of $MgCl_2$, 400 μg ml^{-1} of Bovine Serum Albumin (Roche), 20 ng of the DNA, and 2.5 pmols of the primers mlas-F (GGT GGT GTM GGD TTC ACM CAR TA) and mcrA-R (CGT TCA TBG CGT AGT TVG GRT AGT)(Steinberg and Regan, 2009), in a total a volume of 10 μL per reaction. The qPCR reactions were carried out in the Real-Time PCR System StepOne (Applied Biosystems). The amplification conditions were as follows: initial denaturation at 95 °C for 10 min, followed by 35 cycles at 95 °C for 30 s, at 60 °C for 45 s, and at 72 °C for 30 s. For *mcrA* quantification, standards were produced from PCR products of 9 positive clones for *mcrA* gene, obtained from an enriched sample. The quantification efficiency was tested by logarithmic regression of dilutions values (R^2). The values obtained for each sample were used for absolute gene quantification and the final values were expressed in log copies ml^{-1} of enrichment.

Bacterial and archaeal 16 S rRNA genes were amplified using primers 515 F (5′- GTGCCAGCMGCCGCGGTAA-3′) and 926 R (5′-

CCGYCAATTYMTTTRAGTTT-3′) (Walters et al., 2016) for paired-end sequencing on the Illumina MiSeq platform (Caporaso, et al., 2012), at the René Rachou Institute (IRR/FIOCRUZ) NGS Sequencing Platform, according to the Earth Microbiome Project 16 S rRNA amplification protocol. Amplicon data analysis was performed following the Brazilian Microbiome Project recommendations (Pylro et al., 2014), using the BMPOS (Pylro et al., 2016). Microbial community analyses were carried out with the MicrobiomeAnalyst, a web-based platform in which differences among the soil communities was estimated with PERMANOVA using a dissimilarity matrix generated by Bray-Curtis distance (Chong et al., 2020; Dhariwal et al., 2017). Also, the Random Forests analysis was performed in the same web-based platform.

Non-metric multidimensional scaling (NMDS) using Bray-Curtis distance was plotted to visualize the structure of the microbial community enriched among the different treatments and over the incubation time. Co-occurrence and co-exclusion networks based in Pearson Correlation Coefficient were constructed using the Molecular Ecological Network Analysis Pipeline (MENAP) (accessible: http://ieg4.rccc.ou.edu/mena) (Zhou et al., 2011). The networks analysis was performed using the filtering relative abundance data for each soil profile enriched with each carbon source. Networks topological features were calculated in the MENAP by employing the Global network properties tool. The networks were visualized using the Cytoscape software 3.7.0 (Shannon et al., 2003). Finally, the sub-networks were generated by selecting operational taxonomy units (OTU's) previously assigned to methanogenic and the neighboring nodes directly connected using the Cytoscape tool "New Network from Selection".

2.5. Accession numbers

The 16 S rRNA sequences generated in this study have been deposited in the GenBank database under the BioProject PRJNA642296 with the accession numbers SAMN15391623 to SAMN15391731 (https://www.ncbi.nlm.nih.gov/sra/PRJNA642296).

3. Results

3.1. Soil characterization

The soil under pasture presented the highest density (1.40) and the lowest total soil porosity (47.33) values (%) indicate by Tukey test ($p < 0.05$), demonstrating that pasture soil is more compacted than forest samples. Secondary forest exhibited intermediate values of density (1.04) and porosity (58.01), and the primary forest soils presented the lowest density (0.83) and the highest total soil porosity (65.83) values (Table S1).

3.2. Microbial community composition of the soil samples

Prokaryotic communities structure differed among the three areas ($P < 0.001$), and the variable "area" accounted for ~ 55% of the observed variation (Fig. S1). The PCoA analysis depicting the beta-diversity showed an overlap of the primary and secondary forest communities, indicating similarity between these areas. (Fig. S1).

Random Forests analysis showed fluctuations of error rates in response to the trees-built number. For the different areas, it was observed, using only ~ 50 trees, that the algorithm was able to reach perfect prediction of each group (Fig.S2b). In addition, the taxa with highest contributions to classification accuracy were Firmicutes, Thaumarchaeota and Gal15 for primary forest samples, Verrucomicrobia and Candidate phylum TM6 for secondary forests, Bathyarchaeota, Saccharibacteria, Parcubacteria and Elusimicrobia for pastures samples. These groups can be considered potential biomarkers for each area and are presented in Fig. S2a.

When focusing on the *Archaea* domain, we observed the dominance of the phylum Thaumarchaeota in primary (72.47%) and secondary forest (67.76%) and pasture samples (68.14%). Pasture samples had a higher diversity of methanogenic groups, such as Methanosarcinaceae (5.59%), Methanobacteriaceae (1.15%), Methanocellaceae (2.64%) and Barthyarchaeota phylum (13.21%) in comparison to forest samples (Fig. S3).

3.3. Methane emission and quantification of mcrA gene

Pasture soil enrichments presented higher methane emissions in comparison with enrichments from primary and secondary forest soils, in the presence of any of the three methanogenic substrates (methanol, acetate or H_2:CO_2). However, distinct patterns for CH_4 emission were observed among soil and substrates (Fig. 1). In methanol enrichments, the difference was pronounced in the first four measurement points (Fig. 1b). This enrichment stimulated an earlier methane production, followed by acetate-supplemented enrichments (Fig. 1a) and then H_2:CO_2 atmosphere (Fig. 1c). After 63 days of incubation, methane emissions were similar among the soil profiles and among the methanogenic substrates.

At the initial stage of enrichment incubation, the *mcrA* gene was only detected in pasture soils and presented a mean value of 2.58×10^4 copies g.soil ($n = 6$). In the final stage of incubation, the *mcrA* gene was detected in all samples from forest and pasture and the three types of soil had similar values of *mcrA* copies 2.08×10^7 of copies.ml^{-1}. The same pattern was observed for enrichments supplemented with acetate ($f=0.28$), methanol ($f=0.89$) and H_2:CO_2 ($f=0.48$).

3.4. Microbial community structure along enrichments

The Non-metric multidimensional scaling (NMDS) showed that enriched communities were grouped according to land use in a consistent way for the three carbon sources applied, which was reinforced by the Adonis analysis: methanol [Pr(>F): 0.001), acetate (Pr(>F): 0.001) and H_2:CO_2 (Pr(>F): 0.001] (Fig. 2). Within each land use treatment, the communities were also grouped according to the incubation time, for the three carbon sources: methanol [Pr(>F): 0.001), acetate (Pr(>F): 0.002) and H_2:CO_2 (Pr(>F): 0.001] (Fig. 2).

3.5. Microbial methanogenic composition of enriched samples

The recovered reads derived from the enrichments assigned as methanogenic archaea were affiliated to the phyla Bathyarchaeota (Evans et al., 2015) and Euryarchaeota. The Euryarchaeota reads were affiliated to following genera: *Methanocella* and the Rice Cluster I group belonging to the Methanocellaceae family (Conrad et al., 2006), *Methanosarcina* (Metanosarcinaceae), *Methanobacterium* (Methanomicrobiaceae) (Narihiro and Sekiguchi, 2011) and *Methanomassillicocus* (Methanomassiliicoccaceae) (Iino et al., 2013).

In the acetate-supplemented enrichments, primary forest samples showed an increase in the relative abundance of the genus *Methanosarcina* at 63 days of incubation (2.82%) (Fig. 3a). Pasture enrichments showed an increase in methanogenic groups at 28 days, mainly those belonging to the *Methanosarcina* genus (2.99%) (Fig. 3b). At 63 days of incubation, the *Methanobacterium* genus was prevalent (1.29%). In the samples from the secondary forest at the final stage of incubation, *Methanosarcina* (4.95%) and Methanocellaceae-Rice Cluster I (1.10%) represented the most abundant taxa (Fig. 3c).

Methanol-supplemented enrichments showed a faster increase of methanogens; for example, primary forest samples showed an increase in the relative abundance of the *Methanosarcina* genus (4.14%) at 28 days of incubation (Fig. 3d). In the same incubation stage, pasture samples presented an increase of 7.08% for sequences classified under this genus after 15 days of incubation (Fig. 3e), and secondary forest enrichments showed a relative abundance of 7.55% to *Methanosarcina* (Fig. 3f).

Finally, samples of primary forests enriched with a H_2:CO_2

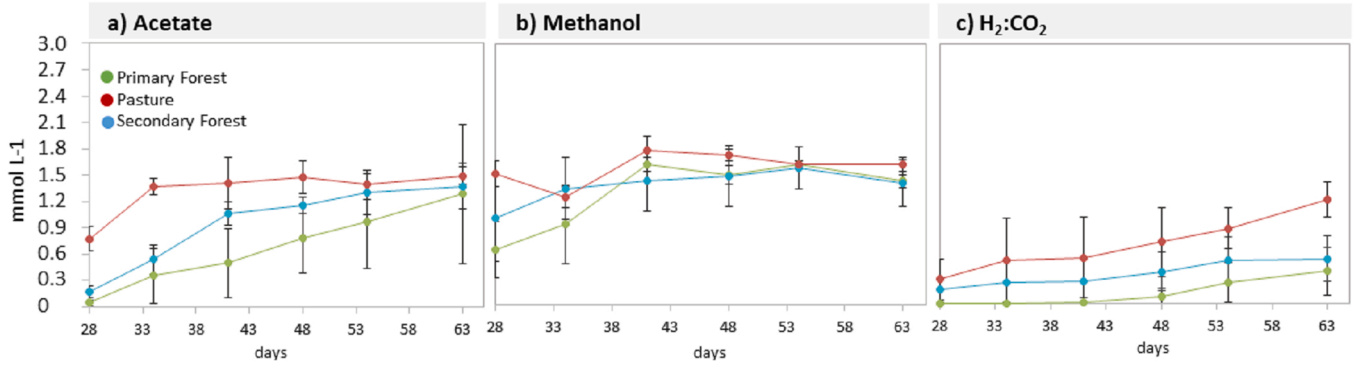

Fig. 1. Methane emissions by enrichments of Primary Forest, Pasture and Secondary Forest soils in media supplemented with: A) Acetate B) Methanol C) H_2CO_2.

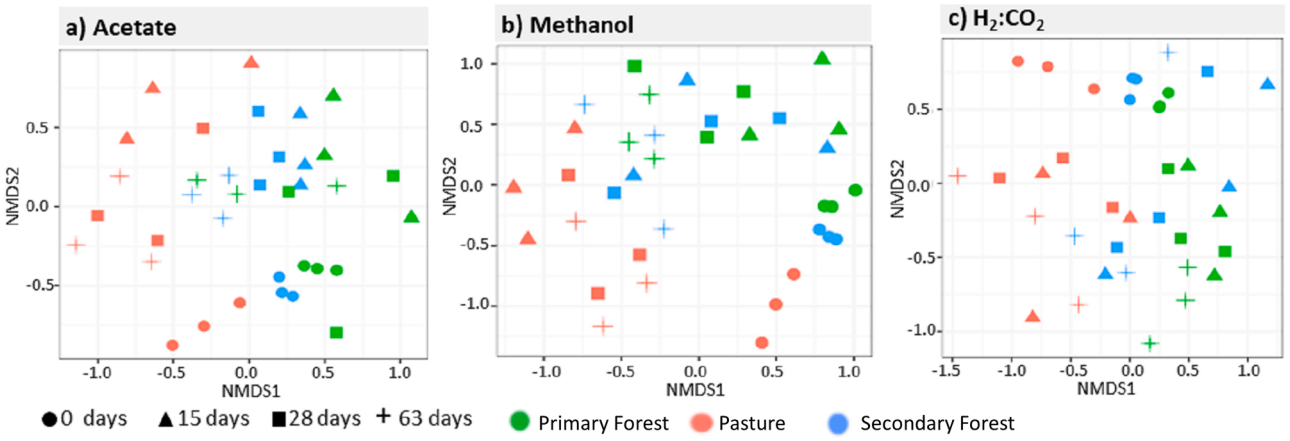

Fig. 2. Similarity among the microbial communities of the enriched samples from the three different areas along the incubation period indicated by a Non-Metric Multidimensional scaling (NMDS). A) Acetate-supplemented enrichments, B) Methanol-supplemented enrichments, C) Enrichments with H_2CO_2 atmosphere.

atmosphere showed an increase in the relative abundance of *Methanosarcina* (1.39%) at final stage of incubation (Fig. 3g). Pasture enrichments reached the maximum value of relative abundance of this genus (3.23%) at 15 days of incubation (Fig. 3h). Other groups which were also enriched in the final stage of pasture enrichments were Barthyarchaeota (0.50%), and *Methanomassiliicoccus* (0.90%). Finally, secondary forest presented a relative abundance of 1.72% of *Methanosarcina* at 63 days of incubation (Fig. 3i).

3.6. Co-occurrence patterns of the enriched microbial community

Networks generated from pasture enrichments were less complex, considering the total number of nodes and edges (Table 1). The enriched community from pastures also presented lower values of modularity and average path distance (GD) in comparison with primary and secondary forest enrichments. In addition, pastures networks demonstrated higher values of average clustering coefficient (Avg) than those obtained from primary and secondary forest networks. Results of network size, average path distance and average clustering coefficient followed a similar pattern in communities observed when the three carbon sources were used (Table 1).

Regarding the modules established by methanogenic archaea sub-networks, only the community from methanol-supplemented enrichment showed significant interactions for primary forest (Fig. 4a). This sub-network presented the genus *Methanosarcina* as a methanogenic node with two negative correlations: an uncultured bacterium of the Selenomonadales family (phylum: Firmicutes) and the *Sphingopyxis* genus (phylum: Proteobacteria) (Fig. 4a).

Sub-networks from enrichments supplemented with the three carbon sources were observed for secondary forest and pasture samples (Figs. 4b and 5). Addressing the secondary forest, the sub-network generated from acetate-supplemented enrichments presented the *Methanosarcina* as methanogenic node negatively correlated with a non-cultivable microorganism belonging to the Oligoflexales order (phylum: Proteobacteria) (Fig. 4b). Sub-network from methanol-supplemented enrichments presented *Methanosarcina* as methanogenic node with three negative correlations: *Bdellovibrio* genus (phylum: Proteobacteria), *Acidisphaera* genus (phylum: Proteobacteria) and uncultured bacterium belonging to the Thermosporotrichacea family (phylum: Chloroflexi). The community from the enrichment supplemented with a H_2:CO_2 atmosphere presented a sub-network with *Methanosarcina* as a methanogenic node with four negative correlations: *Thermosporothrix* genus (phylum: Chloroflexi), non-cultivable bacterium belonging to Ktedonobacteria class (phylum: Chloroflexi), *Rhodomicrobium* genus (phylum: Proteobacteria) and with a non-cultivable bacterium of the Rhodospirillales order (phylum: Proteobacteria).

With regard to the pasture samples, the sub-network generated from acetate-supplemented enrichments presented two methanogenic nodes: *Methanosarcina* and *Methanobacterium* genera (Fig. 5). *Methanosarcina* showed a positive correlation with uncultured bacterium belonging to the BRC1 phylum, while *Methanobacterium* presented three positive correlations with uncultured bacterium of Clostridia class (phylum: Firmicutes). The sub-network from methanol-supplemented enrichment presented two methanogenic nodes: *Methanocella* and *Methanosarcina* genera. *Methanocella* genus established 3 positive correlations with uncultured bacterium of Clostridia class while *Methanosarcina* genus established two positive correlations: with an uncultured bacterium from the Ruminococcaceae family (phylum: Firmicutes) and with the *Enterobacter* genus (phylum: Proteobacteria) (Fig. 5). Finally, the sub-network constructed from enrichments supplemented with a H_2:CO_2

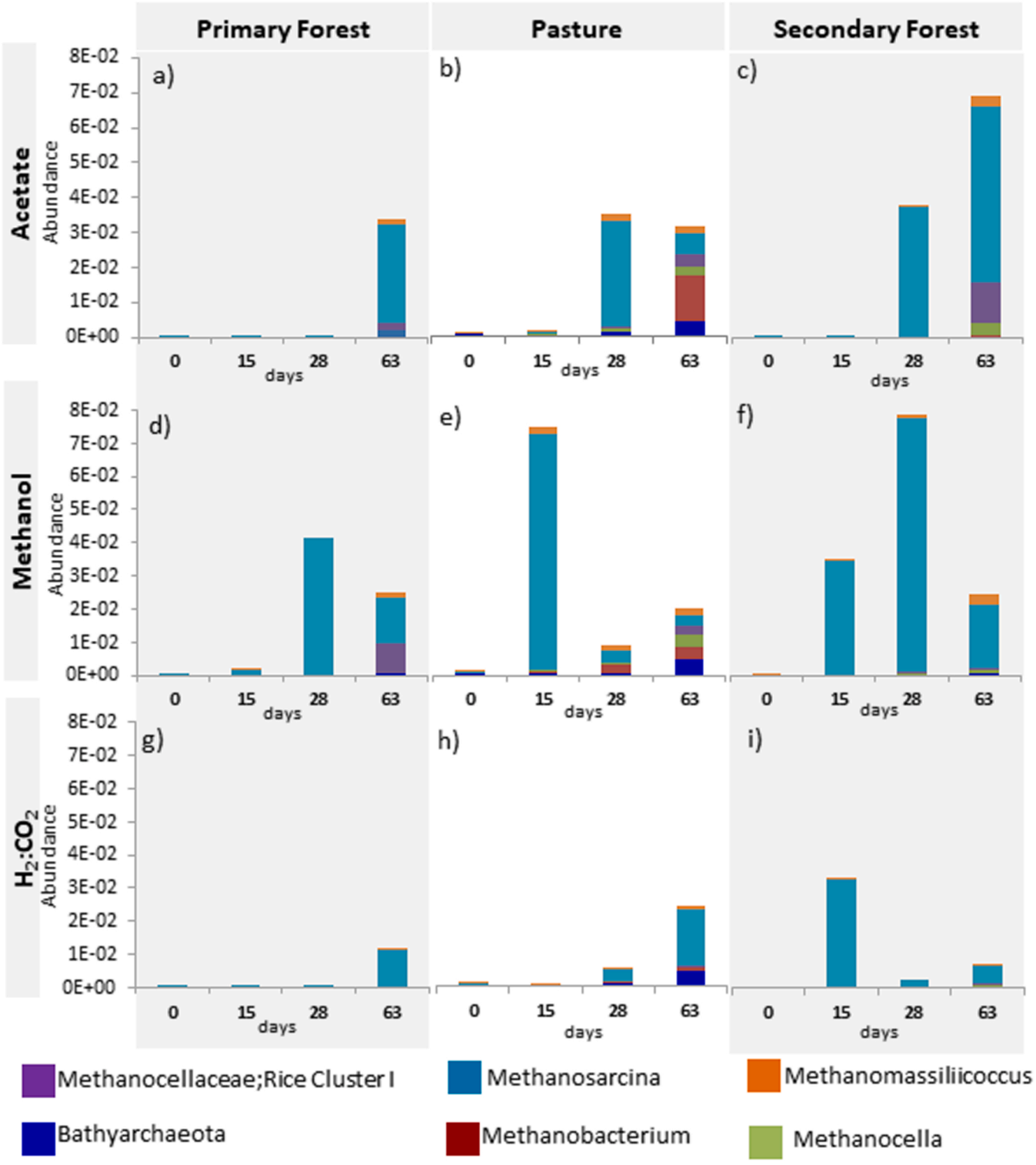

Fig. 3. Composition of methanogenic archaea from the enrichments of Primary Forest, Pasture and Secondary Forest supplemented with acetate, methanol and H_2CO_2.

atmosphere presented *Methanorsarcina* and Bathyarchaeota as methanogenic nodes. *Methanosarcina* established positive interaction with several genera, namely *candidatus* Koribacter (phylum: Acidobacteria), *Ruminiclostridium* (phylum: Firmicutes), *Bacillus* (phylum: Firmicutes), as well as with the Lentimicrobiaceae family (phylum: Bacteroidetes) (Fig. 4). Bathyarchaeota node, on the other hand, presented positive interaction with the genus *Geobacter* (phylum: Proteobacteria).

4. Discussion

Although important in the emission of greenhouse gases, the microbial communities acting in the methane generation and consumption are not properly addressed in most of environments. Part of this lack of information is based in the low abundance of such communities, such as the methanogenic, what lacks its proper detection with most of methods applied. The present work aimed to overcome this limitation, using a

Table 1
Topological features of microbial communities from enrichments of Primary Forest, Secondary Forest and Pasture enrichments supplemented with acetate, methanol and H_2:CO_2.

Enrichment substrate	Area	nº of nodes[a]	nº of edges[b]	Positive edges[c]	Negative edges[d]	Modularity[e]	Avg. Clustering[f]	Avg. path distance (GD)[g]
Acetate	PF	177	1493	3.75	96.25	0.23	0.18	3.01
	P	79	965	13.06	86.94	0.10	0.51	1.74
	SF	255	4664	2.70	97.30	0.13	0.21	2.14
Methanol	PF	78	331	8.16	91.84	0.18	0.23	2.33
	P	76	1219	9.76	90.24	0.07	0.54	1.59
	SF	246	3992	3.53	96.47	0.15	0.21	2.25
H_2:CO_2	PF	98	557	3.59	96.41	0.17	0.14	2.48
	P	80	1608	10.70	89.30	0.07	0.61	1.49
	SF	228	1816	2.53	97.47	0.23	0.13	2.68

PF: Primary forest; P: Pasture; SF: Secondary forest
[a] Microbial taxa with at least one significant correlation ($P > 0.01$) at Pearson correlation obtained by MENAP analyses
[b] Number of connections obtained by MENAP analyzes
[c] Positive correlations
[d] Negative correlations
[e] Measures the degree that network are organized into evidently delimited modules
[f] The degree of the nodes tend to cluster
[g] Measures the shortest path between two nodes

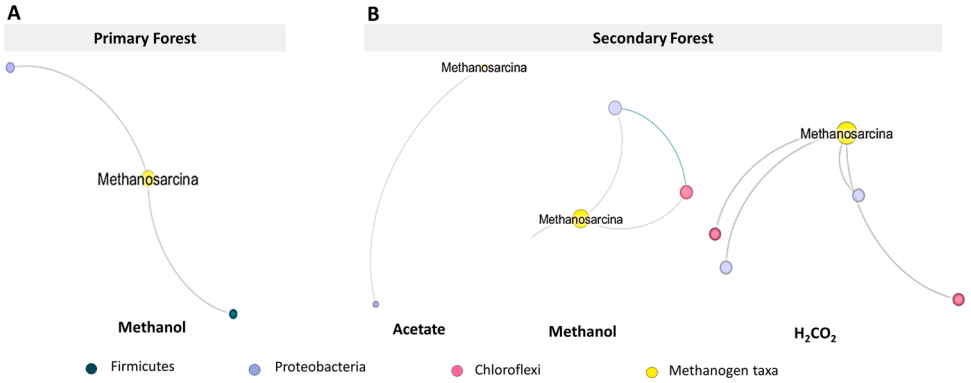

Fig. 4. Sub-network from forest enrichments created by selection of the OTUs linked to methanogenic OTUs. The gray edges represent the negative interactions. Each node represents taxa at genus level based on 16sRNA data. Node size is proportional to the number of direct edges linked to the node. A) Sub-network from primary forest. B) Sub-network from secondary forest.

combinatory approach of community enrichment followed by molecular assessments of methanogenic communities found in Amazonian soils under distinct usage.

Differences in the methanogenic communities were highlighted, where more groups of methanogens were found in the initial pasture samples than in those from primary and secondary forests. The differences in the structure of the prokaryotic community among the areas were maintained along the enrichment process, thus supporting the initial inferences. In addition, the pasture samples showed higher complexities in the microbial communities organization associated to methanogenesis, which was consistent with the earlier and pronounced methane emission observed in the pasture enrichments samples.

Accordingly, the results presented corroborate that land use change can alter the microbial community structure (Mendes et al., 2015) and gene composition in Amazon soils (Paula et al., 2014). In a previous work, secondary forests differed from primary forests, not only regarding taxonomical composition, or gene pool, but also concerning microbial functional profiles (Paula et al., 2014). Here this study reported that the methanogens are among the taxa that showed an increased abundance in pasture soils. The greater richness of methanogenic community reported in pasture soil samples can be partially attributed to soil compaction in unmanaged pastures. Soil compaction increases anaerobic microsites in the soil structure, limits oxygen diffusion and consequently favors the methanogenic metabolism (Lammel et al., 2015; Steudler et al., 1996). The detection of *mcrA* genes in these soil samples through qPCR reinforce that methanogens are in higher abundance and possibly more active in pasture soils than those in the primary and secondary forest samples.

4.1. *Methane emission of enrichments*

The incubation of samples in enrichment medium, coupled to methane measurements, allowed us to compare the soil microbiota response to three methanogenic substrates supplemented to the medium. The methanol-supplemented enrichments displayed faster methane emissions. This substrate is considered non-competitive (Conrad, 2009) and favors organisms with low energetic metabolism, as methanogenic archaea, what can explain the earlier methane emission. Although the methylotrophic pathway is considered uncommon in anaerobic soils, genes related to methylotrophy were found in primary and secondary forest Amazon soils, corroborating previous finds of a greater abundance of such genes in soils under pasture within the same ecosystem (Meyer et al., 2017). The present study suggests that, despite limited, methylotrophy is an active pathway in methane production of Amazon aerated soils and should be further investigated. In addition, we observe that although primary forest enrichment community was less favorable to methanogens, the exposure to anaerobic conditions stimulated methane production, evidencing the capacity of forests soil to become a methane source to the atmosphere when conditions are proper. It directly links the importance of the soil usage to the

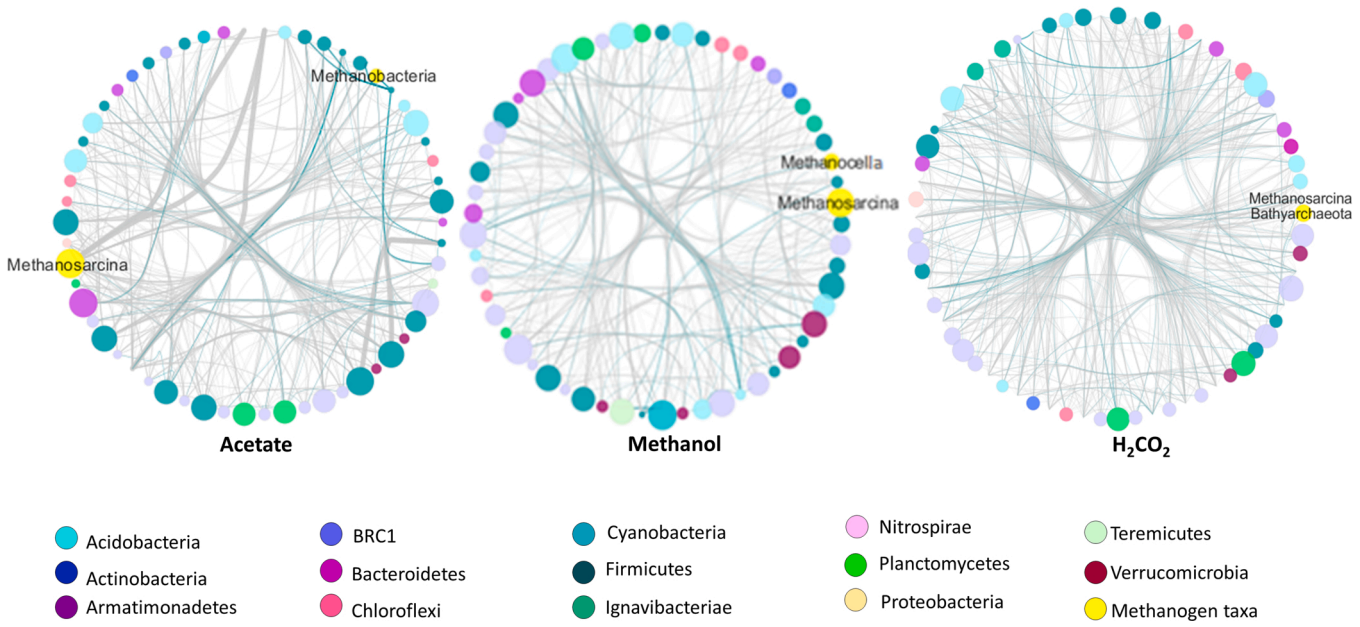

Fig. 5. Sub-networks from pasture enrichments. The sub-networks were created by OTUs linked to methanogenic OTUs. The blue edges represent the positive interaction between the nodes and gray edges represent the negative interactions. Each node represents taxa at genus level based on 16sRNA data. Node size is proportional to the number of direct edges linked to the node and the edge thickness is proportional to edge betweenness values.

production and emission of methane in Amazonian soils.

4.2. Microbial methanogenic composition of enriched samples

Enrichments showed different compositions of methanogenic groups among soil sources, but the *Methanosarcina* genus was generally prevalent. This genus can perform a wide range of methanogenic pathways (Youngblut et al., 2015), and has been reported as being widely distributed in the Amazonian soils (Meyer et al., 2017). In pasture samples, *Methanobacterium* was also found in high abundance, and it was previously reported in anaerobic cultivation of soil from flooded areas of the Amazon region, indicating the importance of this genus to methane production in this ecosystem (Pazinato et al., 2010). The *Methanomassiliicoccus* genus is a methanogenic archaea commonly found in aerated soils in China (Xie et al., 2017) and its presence in Amazon soils suggests a wide distribution of this genus. The Methanocellaceae family encompass important methane-producing genera and was also found in greater abundance in pasture soils when compared to those from primary forest (Meyer et al., 2017). Lastly, genomes belonging to the Barthyarchaeota phylum were described as capable to perform the three biosynthetic pathways of methane production (Evans et al., 2015; Lazar et al., 2016), which explains the enrichment of this group in the three substrates here evaluated. We observed that the methanogenic communities in secondary forest samples, especially the *Methanosarcina* genus, responded faster in abundance increase than primary forest samples, along the enrichment. These results suggest that the secondary forest studied here is still in recovery process and maintain a responsive methanogenic community.

4.3. Co-occurrence patterns of the enriched microbial community

Network analysis showed that forests and pasture-derived enrichments are different in topological features, especially in modularity, where forest samples showed a higher value than pasture. Higher modularity values suggest a more complex microbial community (Zheng et al., 2017), and our results indicate a modification in the complexity of microbial communities in response of change in soil use. However, pastures sub-networks presented a higher number of interactions between methanogenic and other taxa, suggesting the occurrence of a more intricate microbial organization directed to methanogenesis, possibly providing intermediate compounds used as methanogenic substrates. In addition, pasture communities showed a more connected network due to lower values of average path distance (GD) (Zheng et al., 2017), together with higher values of average clustering (Avg) (Zhou et al., 2011), which means that the nodes are strongly linked with its neighbors and confirms the complex interactions between methanogens and possible syntrophic microorganisms. These results support that microbial interactions differ in methanogenesis process during enrichments derived from different soils. In the sub-networks, the *Methanorsacina* genus was the most common methanogenic node. In the sub-networks from pasture, the microbial groups showing a positive interaction with methanogenic nodes are involved in degradation of polysaccharides and monosaccharides. The hydrolysis of these substrates may release low molecular weight compounds, which methanogenic archaea are ultimately able to use. The members of Clostridia, for example, are involved in hydrolysis and fermentation of organic compounds during the acidogenic phase of organic matter degradation (Hattori, 2008; Traversi et al., 2012). The *Enterobacter* genus (phylum Proteobacteria) is described as a facultative anaerobe, acting as a decomposer of initial monosaccharides present in anaerobic microsites of aerated forest soils (Degelmann et al., 2009). The *Candidatus* Koribacter (phylum Acidobacteria) is related to hydrolysis of several storage and structural polysaccharides (Rawat et al., 2012). The family Lentimicrobiaceae (phylum Bacteroidetes) has one species, *Lentimicrobium saccharophilum*, that is a strict anaerobe and degrades carbohydrates generating compounds as acetate, malate, formate and hydrogen (Sun et al., 2016). The *Bacillus* genus (phylum Firmicutes) are related to hydrolysis of organic matter and recalcitrant compounds (Winded et al., 2008). Also, the *Geobacter* genus is associated to methanogenesis in anaerobic consortium sharing electrons with *Methanosaeta* genus via direct interspecies electron transfer (DIET) in terrestrial ecosystem (Holmes et al., 2017). The interactions observed in the sub-networks confirmed that the ecological interactions of pasture related to methanogenic communities are more complex than in forest soils. Also, it sheds light on interactions related to methane production that was just possible to observe due to the enrichment of the samples.

To investigate the shifts in the methanogenic communities after land use-change we used a combinatory approach of direct soil analysis for

methanogenic communities and the enrichment strategy. This allowed us to zoom-in on these communities, being able to observe that pastures without a proper management host an active and more intricate methanogenic community. Also, the enrichment approach enables us to measure the potential for methane emission of environmental samples and to unveil the interactions among the methanogenic archaea and other microorganisms that together allow for methane production. In summary, our results suggest that the conversion of primary forest to unmanaged pastures in the Amazonia region alters the microbial composition of these soils, favoring methanogenesis in pastures.

5. Conclusion

Our results showed that the conversion of primary forests into pastures causes shifts in the prokaryotic community, increasing the abundance and richness of methanogenic archaea. As a consequence, pasture exhibits a more responsive methanogenic community, which was evidenced by the earlier and pronounced methane emission. Furthermore, the methanogenic community structure of pasture soils remained more diverse throughout the enrichment experiment than forest-derived soils. These findings corroborated with our hypothesis that pastures soils host a more abundant, responsive methanogenic community, and have the potential to exhibit an earlier and more pronounced methane emission in comparison with forest soils. Our results suggest that future studies should consider the methylotrophic metabolism an important pathway in methane production of Amazon aerated soils. Also, the combinatory approach adopted here can be applied to study other microorganisms involved in methane cycle occurring in low abundance in soils. Finally, we highlight the importance of conserving forest areas of Amazon region, as a climate change mitigation strategy.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.micres.2022.127178.

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