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RESEARCH ARTICLE

Amazon forest-to-agriculture conversion alters rhizosphere microbiome composition while functions are kept

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One sentence summary: Although the forest-to-agriculture conversion leads to a loss of diversity, the soybean rhizosphere selects a specific microbial community in order to keep important functions in a long-term cropping in Amazon soils.

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ABSTRACT

The conversion of native forest to agriculture is the main cause of microbial biodiversity loss in Amazon soils. In order to better understand this effect, we used metagenomics to investigate microbial patterns and functions in bulk soil and rhizosphere of soybean, in a long-term forest-to-agriculture conversion. Long-term forest-to-agriculture led to microbial homogenization and loss of diversity in both bulk soil and rhizosphere, mainly driven by decreasing aluminum concentration and increased cations saturation in soil, due to liming and fertilization in long-term no-till cropping. Data revealed that long-term no-till cropping culminated in a decrease in Acidobacteria, Actinobacteria and Proteobacteria abundances. However, α- and β-Proteobacteria abundances were higher in the rhizosphere than in bulk soil, regardless of the time after forest-to-agriculture conversion. Changes in functional potential occurred predominantly in bulk soil, with decreases in functions related to potassium metabolism and virulence, disease and defense, while functions related to nucleic acids metabolism increased. Functions in the soybean rhizosphere remained stable, except for those related to potassium metabolism, which decreased after 20-year no-till cropping. Together, our results show that the soybean root system selects microbial taxa via trade-offs, to maintain functional resilience in the rhizosphere microbiome over time.

Keywords: Amazon biodiversity; homogenization; metagenomics; plant microbiome; soybean rhizosphere

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INTRODUCTION

Microorganisms inhabiting the rhizosphere play a key role in plant health and defense (Bakker *et al.* 2013; Mendes, Garbeva and Raaijmakers 2013; Mendes *et al.* 2017b), stress response (Castrillo *et al.* 2017), nutrition (Mendes *et al.* 2014; Fitzpatrick *et al.* 2018) and promoting plant growth (Sugiyama *et al.* 2014). The rhizosphere is considered a dynamic 'hotspot' of microbial diversity and ecological interactions across the plant–soil system. It comprises a thin layer (3–5 mm width) of soil surrounding and sometimes adhered to the roots of superior vascular plants (Hartmann, Rothballer and Schmid 2008). The roots release a variety of exudates, mucilage and other compounds to the rhizosphere, via rhizodeposition (Zhu *et al.* 2014), serving as source of carbon and energy to microorganisms, as well as chemotaxis signals that lead members of microbial communities in the surrounding soil, called bulk soil, to recognize and occupy niches in this region (Philippot *et al.* 2013). Thus, microbial diversity and abundance in the rhizosphere zone can be largely higher compared with its main source in free-roots surrounding soil (Huang *et al.* 2014). Moreover, the microbial community structure in the rhizosphere soil can be largely different from that in the bulk soil, resulting in a potential functional differentiation (Fan *et al.* 2017).

Except for some endophytes that come to soil adhered to or even colonizing the seeds of cultivated plants, the main source of microbial diversity in the rhizosphere is the bulk soil (Sugiyama *et al.* 2014). Niche occupancy in the rhizosphere is believed to be dependent on the source and quantity of those substrates released by the roots (Huang *et al.* 2014), root architecture (Saleem *et al.* 2018), plant species or genotype (Garbeva, Elsas and Veen 2007; Schlemper *et al.* 2017; Fitzpatrick *et al.* 2018) and development stage (Qiao *et al.* 2017). In some cases, authors have found that the influence of the plant root system can surpass the effect of soil type or management for both assembly (Smalla *et al.* 2001; Vega-Avila *et al.* 2015) and functional potential (Colin *et al.* 2017).

Forest-to-agriculture conversion is often found to be detrimental to microbial diversity (Rodrigues *et al.* 2013). However, literature is controversial when linking taxa trade-offs with the consequences to functional potential and ecosystem services (Colin *et al.* 2017; Mendes *et al.* 2017a). Furthermore, the low number of environmental variables generally measured leads to the assumption that the same set of soil factors, markedly pH (Rousk *et al.* 2010; Bartram *et al.* 2014), rule microbial shifts when converting forest to agriculture systems. A multidimensional approach, linking taxonomy, functions and a broader set of environmental variables, could enable researchers to depict correlations among diversity and niche occupancy, as well as to define the real factors modulating ecological patterns in agriculture soils (Mendes *et al.* 2015b; Goss-Souza *et al.* 2017). Thus, it is possible for microbial ecologists to depict those combinations, resulting in a better understanding of changes in microbial community assembly related to disturbances such as deforestation (Mueller *et al.* 2014), changes in soils management (Pérez-Jaramillo, Mendes and Raaijmakers 2016) or even natural ecosystems transitions over time (Dini-Andreote *et al.* 2014).

In this study, we hypothesize that soybean roots act as filters, selecting microbial communities via taxa trade-offs according to niche, to maintain functional resilience. Thus, we aimed to identify the microbial community patterns, in bulk soil and soybean rhizosphere, in a long-term forest-to-agriculture conversion chronosequence, in Eastern Amazon.

MATERIALS AND METHODS

Site description and soil sampling

Sampling fields are located into the Amazon Rainforest Biome, within the 'Alto Xingú' water basin, which is currently recognized as 'the last agricultural border' in the Southeastern Brazilian Amazon. Bulk soil samples were collected in January 2013, in agricultural fields, located in the municipality of Querência (12°22'S; 52°15'W), Mato Grosso State, Brazil (supplementary Fig. S1a available online). The climate of the region is Am (Köppen-Geiger classification), with annual average temperature of 27°C and annual precipitation of 1400 mm in 2013, composed of well-defined periods of wet and drought (supplementary Fig. S2 available online). In order to evaluate long-term microbial dynamics we established a chronosequence varying from 1-year cultivation after deforestation, to 10- and 20-year cultivation in a no-till cropping system, with successive rotation of cultures. All areas were deforested via slash-and-burn, followed by cultivation with common rice (*Oryza sativa*) for one season, in order to prepare the soil for further cropping. Since that, the selected areas have been cultivated in a no-till cropping system, with successive rotation, including: millet (*Pennisetum glaucum*), ryegrass (*Lolium multiflorum*) and black oat (*Avena strigosa*) in the winter season, as cover plants, and maize (*Zea mays*) and soybean (*Glycine max*) in the summer season, as main crops. After deforestation, both areas received liming in the first year and each fifth year to increase and keep pH around 6. Fertilizers and pesticides had been regularly applied over time, according to cultivation demand and technical recommendation. We collected soil samples from the 0–20 cm profile, between lines of soybean plants at V6 stage, in a cartesian-geogrid scheme (supplementary Fig. S1b available online). Eight samples were mixed to form one composite sample × six replicates × three areas, totalling 18 composite soil samples. The straw layer was removed from topsoil and used as cover in the further greenhouse experiment, in order to keep soil cover conditions. All samples were transported to the laboratory within 48 h after sampling for implementation of the mesocosms experiment.

Greenhouse mesocosm experiment

Soil samples collected in the field were used to grow soybean plants in mesocosms at CENA-University of São Paulo, Brazil. The experiment was carried out in greenhouse in order to normalize the influence of environmental parameters, such as temperature and moisture. In order to simulate field conditions we used seeds of soybean (*G. max* L. Merril, cultivar BRS 232) inoculated with a commercial solution containing *Bradyrhizobium elkanii* and *Bradyrhizobium japonicum* (1:1, V/V) in a final concentration of 10^{10} viable cells mL^{-1}, followed by treatment with the fungicide Vitavax-Thiram 200 SC®, according to the manufacturer's instructions. Mesocosms consisted of pre-sterilized plastic vases (25 cm height × 25 cm diameter) with a 4 cm pebble layer at the bottom (for drainage). The vases were filled with 5 kg of soil from each composite sample. Then, six soybean seeds were sown in each vase. The straw collected in each chronosequence area was distributed in the vases according to the quantity found in each sampling point in the field. The

experiment was carried out with 36 vases, consisting of 18 vases with plants, to evaluate the rhizosphere effect, and 18 vases with no plant, to evaluate the bulk soil effect, totalling 36 vases (three areas × two soil fractions × six replicates). The soil moisture in all vases was corrected for 60% of water holding capacity in the beginning of the experiment, and maintained via irrigation with deionized water (every 2 days). Soybean seeds were germinated at 28/20°C (day/night) and 12-h photoperiod. Ten days after germination, seeds with lower vigor were removed from the vases, keeping three plants per vase. The experiment was conducted until stage R1 (50% flowering plants), 65 days after sowing, from January to March 2013. Plants were carefully harvested and transported in dry ice to the laboratory. Immediately, roots were briefly shaken to separate bulk from rhizosphere soil. The soil that remained attached to the roots was defined as rhizosphere soil and extracted from the roots with the aid of a sterile brush. Soil samples from the control vases, with no plant, were collected and considered as bulk soil.

Environmental analyses

After harvest, we collected 500 g of soil and 200 g of straw for environmental analyses. We measured 54 environmental variables: 27 soil physicochemical attributes, 19 straw characteristics, 5 soil microbial enzymatic activities and 3 geographical coordinates (used as constraining variables for statistical analyses). Soil and straw physicochemical analyses were performed at the Soil and Vegetal Tissue Analysis Laboratory, University of São Paulo, Piracicaba, Brazil, following routine methodology (Keeney and Nelson 1982; Gee and Bauder 1986; Tedesco *et al.* 1995; Claessen *et al.* 1997; Dhaliwal *et al.* 2011). Soils enzyme activities were measured at the Biogeochemistry Laboratory, São Paulo State University, Jaboticabal, Brazil, following routine methodology compiled by Melo *et al.* (2010). Details regarding laboratory procedures and methodology for physicochemical and enzymatic analyses can be found in supporting information available as supplementary data online..

Soil DNA extraction and metagenomics sequencing

Total DNA extraction (250 mg) was performed for 36 soil samples (three areas × two soil fractions × six replicates) using PowerLyzer PowerSoil DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, USA). DNA quality was verified in 1% Tris-buffered saline with sodium boric acid (Brody and Kern 2004) agarose gel electrophoresis. DNA concentration was measured with the Qubit fluorometer (Thermo Fischer Scientific, Waltham, MA, USA). Nextera DNA Sample Preparation Kit (Illumina Inc., San Diego, CA, USA) was used to construct soil metagenomic libraries. A total of 36 barcoded samples were obtained with the Nextera Index Kit (Illumina Inc.). The product was purified with the Agencourt AMPure XP reagents (Beckman Coulter, Brea, CA, USA). |In order to quantify the DNA concentration of the library pool, a quantitative polymerase chain reaction (qPCR) of the purified barcoded product was performed with the KAPA SYBR FAST Universal qPCR Kit (Kapa Biosystems, Woburn, MA, USA) in a StepOne Plus thermocycler (Thermo Fischer Scientific). We performed high-throughput sequencing with the MiSeq Reagent Kit V2 in a MiSeq Desktop Sequencer (Illumina Inc.). High-throughput sequencing of metagenomic libraries consisted of a paired (2 × 250 bp) 500-cycle/39-h run. Above steps were performed at the Cellular and Molecular Biology Laboratory, CENA-USP, Piracicaba, Brazil. For details regarding metagenomic library preparation and sequencing procedure, see Goss-Souza *et al.* (2017) or access supporting information available as supplementary data online from this manuscript.

Annotation and analysis of metagenome libraries

DNA sequences, assigned through barcoding, were filtered to discard low-quality bases (quality score <20), using MiSeq software default parameters (Illumina Inc.). Remaining paired-end sequences were merged using the software FLASH version 1.2.11 (Magoč and Salzberg 2011). Additional quality trimming was performed using Phred algorithm with SeqyClean script (Zhbannikov *et al.* 2017). The sequences were annotated with Metagenomics Rapid Annotation (MG-RAST) pipeline version 3.6 (Wilke *et al.* 2016). Taxonomic and functional potential profiles were generated by matches to the M5nr (Wilke *et al.* 2012) and SEED (Overbeek *et al.* 2013) databases, respectively. To identify hits, BlastX searches with a minimum alignment length of 50 bp and an *E*-value cut-off of 1×10^{-5} was used (Smith *et al.* 2012; Mendes *et al.* 2014; Goss-Souza *et al.* 2017). We used the 'metagenomeseq' R package (Paulson *et al.* 2013) to normalize the abundances of taxa and functional potential, through a mixture model that implements a zero-inflated Gaussian distribution to account for varying depths of coverage. Tables of frequency of hits to each individual taxa level (taxonomy) or subsystem (functional potential) for each metagenome were generated. We used the resulting data matrices for downstream statistical analyses. Shotgun metagenome data are available at MG-RAST under the project ID 7830.

Statistical analyses

All statistical analyses were performed using the following design: three areas representing the no-till cropping chronosequence (1-, 10- and 20-year), two soil fractions (bulk soil and rhizosphere) and six replicates. Soil, straw and enzymatic values were compared by analysis of variance, followed by Tukey's test. Shannon's alpha-diversity and Whittaker global beta-diversity were calculated using the PAST software, version 3 (Hammer, Harper and Ryan 2001). For multivariate analyses, environmental matrices (soil, straw and enzymes) were transformed via Box-Cox transformation (Box and Cox 1964). Nonmetric multidimensional scaling (NMDS) plots of principal coordinates analysis (PCoA) were used to compare taxonomic and functional potential structures, with Canoco software, version 5 (Lepš and Šmilauer 2005). In addition, we used the same software to generate a permutational multivariate analysis of variance (PERMANOVA) to test whether the clustering of community and functional structures were due selected sample categories (chronosequence and soil fraction) or random. Differences in microbial taxonomic and functional composition were calculated using two-sided Welch's t-test q-values (Welch 1934). Confidence intervals were calculated using the Newcombe–Wilson method (Newcombe 1998), corrected by the Benjamini–Hochberg false discovery rate (FDR) (Benjamini and Hochberg 1995). Both analyses were performed on the Statistical Analysis of Metagenomic Profiles (STAMP) software, version 3.0 (Parks and Beiko 2010). In order to identify the major environmental drivers of microbial structure patterns, a Distance-based Redundancy Analysis (db-RDA) of Euclidean distance matrices, with stepwise forward selection was performed, using the Canoco software, version 5 (Lepš and Šmilauer 2005). Spearman's correlation analysis was performed on the Vegan package from the R software, version 3.1.2 (R Development Core Team 2008), with the

Benjamini-Hochberg FDR correction. We inputted only significant ($P < 0.05$) and strong (Spearman's $\rho \geq |0.7|$) pairwise correlation data on the Gephi network exploration platform, version 0.8.2 (Bastian, Heymann and Jacomy 2009), in order to visualize the interactions among taxonomy, functions and environmental variables, through networks.

RESULTS

Sampling site attributes

We performed Tukey's honest significant difference (HSD) test for 54 variables, as described in the Materials and methods section. Variables that significantly changed along the chronosequence ($P < 0.05$) are shown in Table 1 (For details of the 54 variables evaluated, see supplementary Tables ST1–ST3 available online). Soil pH increased after 10-year no-till cultivation, with no difference between 10- and 20-year. The opposite effect was found for potential acidity [hydrogen (H) + aluminium (Al)], which decreased after 10 years, with no difference between 10- and 20-year. Cations saturation (V%) increased after 10-year cultivation, with no difference from 10- to 20-year. Similarly, calcium (Ca^{2+}) and magnesium (Mg^{2+}) increased with 10-year cultivation, with Ca^{2+} increasing even after 20-year no-till. Nitrogen (N)-total increased from 10- to 20-year no-till. We found the same pattern for phosphorus (P), soil organic carbon (SOC), soil organic matter (SOM) and clay contents. All the enzymes changed their activities along the chronosequence. Acid phosphatase activity (APA) decreased from 1- to 10-year and from 10- to 20-year no-till cultivation. Yet the β-glucosidase activity (BGA) presented the opposite behavior, increasing as the chronosequence advanced. Dehydrogenase activity (DA) increased from 1- to 10-year, with no difference from 10- to 20-year no-till. Fluorescein diacetate hydrolysis activity (FDA) and urease activity (UA) were higher in 10-year, compared with 1- and 20-year, no-till cropping. From all litter variables, only P-total and total organic carbon (TOC) changed, both increasing from 10- to 20-year no-till cropping.

Microbial community structure and composition in the soil–rhizosphere interface along the chronosequence

A total of 10.7 million sequences were obtained by high-throughput shotgun metagenomics, for 36 soil samples. Shannon's α-diversity did not vary across bulk soil and rhizosphere, but did across the chronosequence, with samples from 1-year being less diverse than samples from 10- and 20-year, with no differences between 10- and 20-year no-till (Fig. 1). Regardless of time, α-diversity was higher in bulk soil compared with the rhizosphere. Whittaker's global β-diversity decreased along the chronosequence, in both bulk soil and rhizosphere, indicating that the communities in the same fraction became more similar in both sides of the interface. Despite that, regardless of time, β-diversity was always higher in rhizosphere compared with bulk soil. Based on results that showed a clear reduction of β-diversity, in both bulk soil and the rhizosphere over time, we asked whether community structure presented distinct patterns across time and soil fractions. Taxonomic NMDS plot (Fig. 1 and supplementary Fig. S3 available online) revealed a separation of microbial communities from bulk soil and rhizosphere. Microbial community structure changed over time of no-till cropping, with separation of 1-year bulk soil samples from 10- and 20-year samples (PERMANOVA, $P < 0.001$), the same as found for 1-year

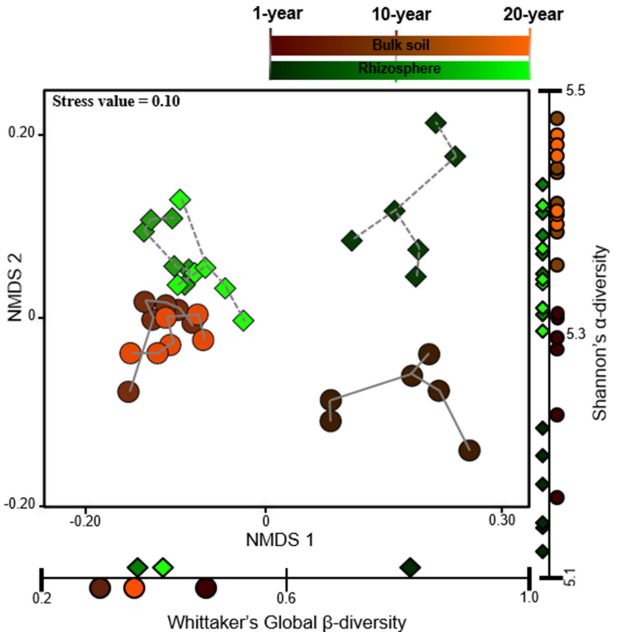

Figure 1. NMDS plot of PCoA, generated by Euclidean distance matrices, with 100 permutations, based on genus level. A vertical additional axis represented Shannon's α-diversity and a horizontal additional axis represented Whittaker's global β-diversity. Taxonomy was compared through Tukey's HSD, $P < 0.05$. Lines connecting samples represent the distance to the nearest neighbor, with Donelly edge correction. Stress value for NMDS plot is shown.

rhizosphere samples, which differed from 10- and 20-year samples (PERMANOVA, $P < 0.001$), with no differences between 10- and 20-year, in both bulk soil (PERMANOVA, $P = 0.38$) and rhizosphere (PERMANOVA, $P = 0.32$). The taxonomic variation from the whole set of samples was 41%.

We investigated possible shifts in microbial community composition (Fig. 2) through relative abundance of taxonomic profiles. From a total of 32 observed bacterial and archaeal phyla, 15 presented significant abundance differences between bulk soil and rhizosphere ($q \leq 0.05$) in 1-year, 19 in 10-year and 10 in 20-year no-till cropping (Fig. 2a). In almost all cases, relative abundances were higher in bulk soil than in rhizosphere, except for Proteobacteria and Bacteroidetes in 1-year, which presented higher abundance in rhizosphere. We also evaluated the microbial dynamics along the chronosequence. In bulk soil, 19 out of 30 bacterial phyla had significant ($q < 0.05$) changes in abundances after 20-year no-till cropping (Fig. 2b). Acidobacteria ($q < 0.001$) and Actinobacteria ($q < 0.05$) abundances decreased after 20-year no-till cropping, while 17 other phyla increased in abundance, markedly Proteobacteria ($q < 0.01$), Planctomycetes ($q < 0.001$), Gemmatimonadetes ($q < 0.001$), Bacteroidetes ($q < 0.01$), and both archaeal Euryarchaeota ($q < 0.001$) and Crenarchaeota ($q < 0.05$). Yet for rhizosphere, from 1- to 20-year no-till cropping, 16 bacterial phyla had changes in abundance (Fig. 2c). Proteobacteria ($q < 0.01$) and Acidobacteria ($q < 0.01$) decreased their abundances, while 14 other phyla increased, with emphasis on Planctomycetes ($q < 0.01$), Nitrospirae ($q < 0.001$), Gemmatimonadetes ($q < 0.01$), Firmicutes ($q < 0.05$), Cyanobacteria ($q < 0.05$), Aquificae and both archaeal Euryarchaeota and Crenarchaeota ($q < 0.001$). Owing the fact that Proteobacteria increased in bulk soil and decreased in rhizosphere after 20-year no-till cropping, we depicted its variability at class level (supplementary Fig. S4 available online). α- and β-Proteobacteria presented higher abundances in rhizosphere, while δ- and γ-Proteobacteria had

Table 1. Environmental variables that have significantly changed along the chronosequence. Tukey HSD test ($P < 0.05$). Letters represent significant differences. Standard deviation is shown.

Chronosequence	1-year	10-year	20-year
Soil physicochemical variable[a]			
Cations saturation	47.2 ± 8.1b	82.8 ± 5.2a	79.8 ± 2.5a
Ca^{2+}	15.3 ± 3.0c	29.0 ± 5.2b	35.7 ± 3.4a
Mg^{2+}	9.2 ± 1.8b	19.8 ± 3.4a	17.7 ± 2.0a
N-total	1311.3 ± 196.3b	1479.3 ± 237.4b	1564.5 ± 144.6a
P	14.0 ± 7.4b	13.2 ± 9.0b	37.7 ± 23.2a
pH_{KCl}	5.0 ± 0.2b	6.0 ± 0.1a	5.8 ± 0.2a
Potential acidity	28.7 ± 5.8a	10.7 ± 4.5b	13.8 ± 2.9b
SOC	20.5 ± 5.4b	23.0 ± 2.0b	26.5 ± 3.3a
SOM	35.5 ± 9.4b	39.5 ± 3.0b	46.0 ± 5.4a
Clay	298.2 ± 19.7b	293.5 ± 25.6b	321.0 ± 35.3a
Soil enzymatic activity			
Acid phosphatase	85.8 ± 8.4a	63.4 ± 8.4b	55.4 ± 8.3c
β-glycosidase	17.7 ± 6.0c	36.8 ± 8.5b	116.0 ± 18.6a
Dehydrogenase	2.4 ± 0.8b	5.1 ± 1.7a	5.6 ± 1.5a
FDA	83.6 ± 11.9b	107.9 ± 19.5a	85.3 ± 12.0b
Urease	9.4 ± 4.2b	18.4 ± 4.7a	9.0 ± 2.4b
Litter			
Straw P-total	0.17 ± 0.05b	0.18 ± 0.02ab	0.21 ± 0.04a
Straw TOC	24.3 ± 1.0b	23.4 ± 4.4ab	29.4 ± 10.6a

[a]From 54 explanatory variables measured ore calculated, only the ones that significantly changed along the chronosequence are shown.
Units: pH = -log[H+ mol L^{-1}]; potential acidity = [H+Al] mEq 100 g^{-1}; cations saturation = % of cations on cations exchange capability at pH 7; Ca^{2+} = $mmol_c$ dm^{-3}; Mg^{2+} = $mmol_c$ dm^{-3}; N-total = g dm^{-3}; P = mg dm^{-3}; SOC = g kg^{-1}; SOM = g kg^{-1}; clay = g kg^{-1}.
Soil enzymatic activities: APA = mg *p*-NP kg^{-1} h^{-1}; BGA = mg *p*-NP mg^{-1} h^{-1}; DA = μg TPF kg^{-1} h^{-1}; FDA hydrolysis = mg fluorescein kg^{-1} h^{-1}; UA = mg $N\text{-}NH_4^+$ kg^{-1} h^{-1}; Straw P-total = g kg^{-1}; Straw TOC = g kg^{-1}.

higher abundances in bulk soil. Looking at Proteobacteria phylum dynamics along the chronosequence, relative abundances of α-Proteobacteria reduced over time, while relative abundances of β-, δ- and γ-Proteobacteria increased, in both bulk soil and the rhizosphere. Despite that, α-Proteobacteria was always the most abundant class inside Proteobacteria, regardless of time or soil fraction, representing 53.7% of sequences within Proteobacteria and 23.5% of the total number of assigned sequences, followed by Betaproteobacteria, which represented 18.8% of sequences within Proteobacteria and 8.2% of the total.

Functional categories response in bulk soil and the rhizosphere over time

We performed an NMDS of functional categories structures at subsystems level 2. Functional structures of samples from 1-year bulk soil and 1-year rhizosphere were found clustered in a minor group, while samples from 10- and 20-year, from both bulk soil and the rhizosphere, were found clustered in a major group (supplementary Fig. S5 available online). Since the variation from the whole set of samples was only 4%, differences in functional structure were not significant (PERMANOVA, $P = 0.32$). We also investigated the possible changes in microbial functional categories along the chronosequence in bulk soil and rhizosphere. No significant changes were found in any functional category when comparing the relative abundances of bulk soil and rhizosphere metagenomic profiles, but were found for 'stress response' ($q < 0.05$), which was higher in rhizosphere 1-year, compared with bulk soil 1-year (supplementary Fig. S6 available online). Relative abundances of 10 functional categories were significantly altered in bulk soil from 1- to 20-year no-till cropping (Fig. 3a). Functions associated with 'protein metabolism', 'amino acids and derivatives', 'nucleosides and nucleotides metabolism', 'clustered-based subsystems' and 'iron acquisition and metabolism' increased from 1- to 20-year no-till cropping, while functions related to 'virulence, disease and defense', 'potassium metabolism', 'aromatic compounds metabolism', 'fatty acids, lipids and isoprenoids' and 'phages, prophages, transposable elements and plasmids' decreased. Otherwise, we found that, in rhizosphere, only functions associated with 'potassium metabolism' significantly changed from 1- to 20-year, decreasing from 0.5 to 0.3% (Fig. 3b). Since there was no variation from 1- to 10-year rhizosphere, we depicted the variation from 10- to 20-year and noticed that functions associated with 'virulence, disease and defense' and 'respiration' increased, while functions associated with 'clustered-based subsystem' decreased (Fig. 3c).

Relationship between soil microbial structures, functional potentials and environmental factors

Euclidean distance-based Redundancy Analysis (db-RDA) with stepwise forward selection (Fig. 4) of the 54 explanatory variables was performed (see supplementary Tables ST1–ST3 available online) in order to identify the main environmental drivers of the shifts in taxonomic and functional categories structures along the no-till cropping chronosequence for bulk soil and rhizosphere. The forward-selected variables on db-RDA explained together 35.5% of the total variation in soil microbial taxonomic composition, being 33.0% of the explanation on the first axis of RDA. Cations saturation (V%) was the main driver of the differences on community composition, explaining 31.5% of the total variation (pseudo-F = 15.6; $P_{adjusted} < 0.001$), followed by Al^{3+} concentration, which explained 4.0% (pseudo-F = 2.0; $P_{adjusted} = 0.044$) (Fig. 4a). Total variation across samples for taxonomic structures was 32%. From the total variation on soil functional subsystems structure, the forward-selected explanatory variables account for 16.8%. Cation saturation was, once more, the

Figure 2. Relative abundance and difference in mean proportions of soil bacterial and archaeal communities at phylum level, based on shotgun metagenomics. (**a**) Percentages of sequence reads are shown for soil and (brown) and rhizosphere (green) along the no-till cropping chronosequence. (**b**) Bulk soil mean proportions for comparisons between 1-year (dark brown) and 20-year (light brown) no-till. (**c**) Rhizosphere mean proportions for comparisons between 1-year (dark green) and 20-year (light green) no-till. Welch's t-test with corrected q-values calculated using the Benjamini–Hochberg FDR approach was performed for the significance levels: $*q \leq 0.05$, $**q \leq 0.01$, $***q \leq 0.001$. Only significantly altered taxa are shown ($q \leq 0.05$). Error bars show standard deviation of six replicates for each soil fraction and time.

major driver of the variation and explained 11.1% of the total variation (pseudo-F = 4.3; $P_{adjusted} < 0.001$), followed by aluminum saturation (m %), with 5.6% (pseudo-F = 2.2; $P_{adjusted} = 0.019$) (Fig. 4b). However, the total variation of functional structures across samples due to environmental factors was only 0.2%, which suggests no environmental selection in both no-till and rhizosphere functional potentials along the chronosequence.

In order to investigate co-occurrence patterns among taxa, functional categories and environmental variables, we calculated pairwise Spearman's correlation coefficients within bulk

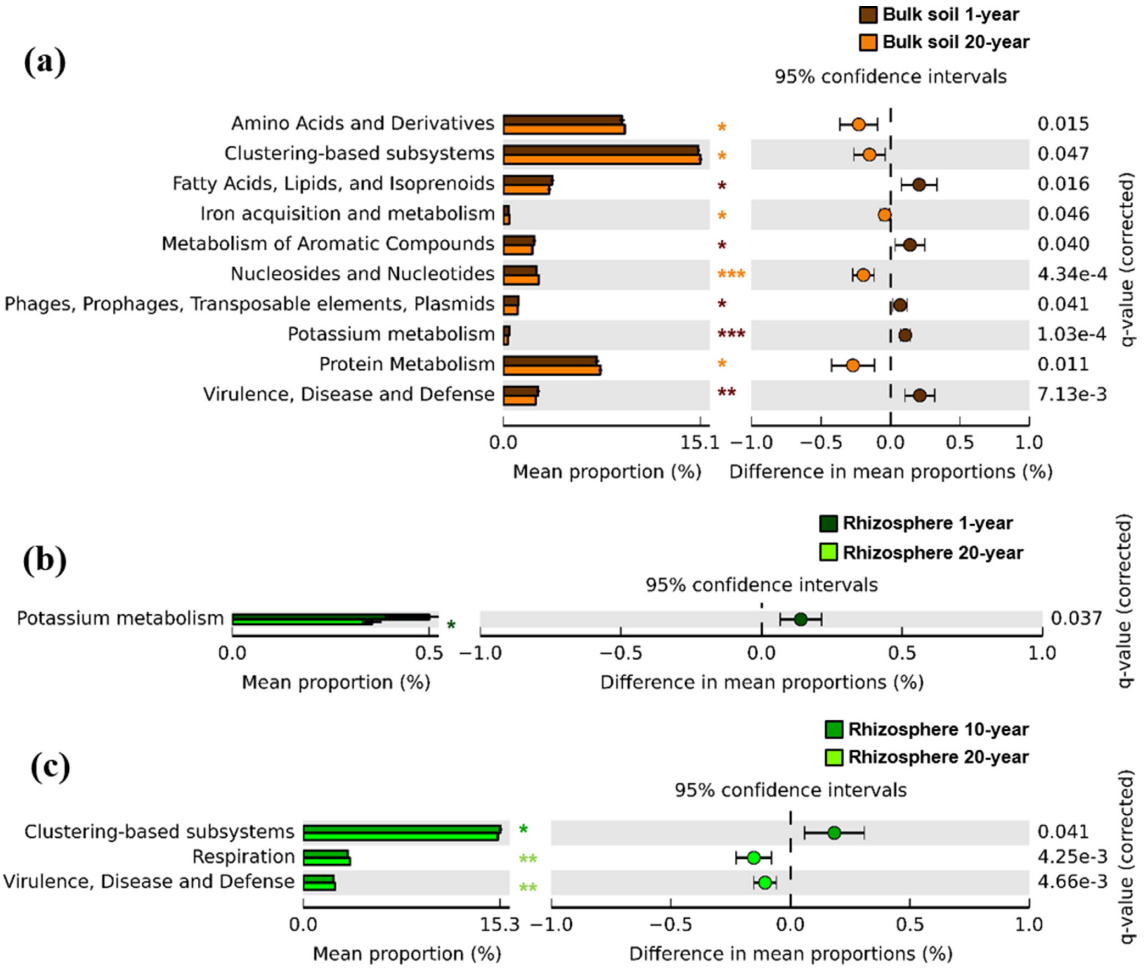

Figure 3. Difference in mean proportions of soil functional categories, at subsystems level 2, based on shotgun metagenomics, for (**a**) comparison between bulk soil 1- and 20-year, (**b**) comparison between rhizosphere 1- and 20-year and (**c**) comparison between rhizosphere 10- and 20-year no-till cropping. Samples were grouped according to soil fraction and time: bulk soil (from dark to light brown) and rhizosphere (from dark to light green).. Welch's t-test with corrected q-values calculated using the Benjamini–Hochberg FDR approach was performed for the significance levels: $*q \leq 0.05$, $**q \leq 0.01$, $***q \leq 0.001$. Error bars show standard deviation of six replicates for each soil fraction and time.

soil and rhizosphere, along the no-till cropping chronosequence. Resulting correlation matrices were used to generate visual networks of significant ($P < 0.05$) and strong (Spearman's $\rho \geq |0.7|$) connections (Fig. 5). Topological properties of the networks are shown in the supplementary Table ST4 available online. All networks presented modular structures (>0.4). The diameter of the network in bulk soil decreased in 20-year, when compared with 1- and 10-year no-till, while the rhizosphere network diameter increased from 1- to 10-year, with no differences between 10- and 20-year no-till over time. Clustering among neighbors in the network decreased in bulk soil and increased in rhizosphere as the chronosequence advanced. The number of active features increased along the chronosequence in bulk soil and decreased in rhizosphere. The number of strong and significant connections (Spearman's $\rho \geq |0.7|$; $P < 0.05$) increased along the chronosequence in both soil fractions. Interestingly, the number of strong and very significant connections ($P < 0.01$) behaved differently, increasing in bulk soil and decreasing in rhizosphere along the chronosequence. The number of positive connections increased from 1- to 10-year and decreased from 10- to 20-year no-till in bulk soil, while in rhizosphere these connections gradually increased along the chronosequence. The number of negative connections in bulk soil increased from 1- to 10-year and remained stable in 20-year. In the rhizosphere this number was higher in 10-year. The number of very significant negative connections increased constantly along the chronosequence in bulk soil and decreased in rhizosphere.

Looking into the networks, the taxonomic groups (represented by blue nodes) that presented larger number of high and significant Spearman's connections (represented by blue, red and green line vectors) in bulk soil were: α-, β-, γ- and ε-proteobacteria in 1-year; Nitrospirae, Actinobacteria, Spirochaetes, γ-, β- and α-Proteobacteria in 10-year; and Chloroflexi, Chlorobi and δ-Proteobacteria in 20-year no-till cropping. However, for rhizosphere the groups were: Deinococcus-Thermus, Chlorobi, Synergistetes and Actinobacteria in 1-year; Gemmatimonadetes, Chrenarchaeota and Verrucomicrobia in 10-year; and β- and δ-Proteobacteria in 20-year no-till. The functional categories (red nodes) with higher number of connections (red and green line vectors) in bulk soil were: 'cofactors, vitamins, prosthetic groups and pigments' in 1-year; 'cell wall and capsule', 'virulence and disease defense' and 'cell division and cell cycle' in 10-year; and 'protein metabolism', 'stress response', 'photosynthesis' and 'sulfur metabolism' in 20-year no-till cropping. Functional categories with most number of connections in rhizosphere were: 'dormancy and sporulation' and

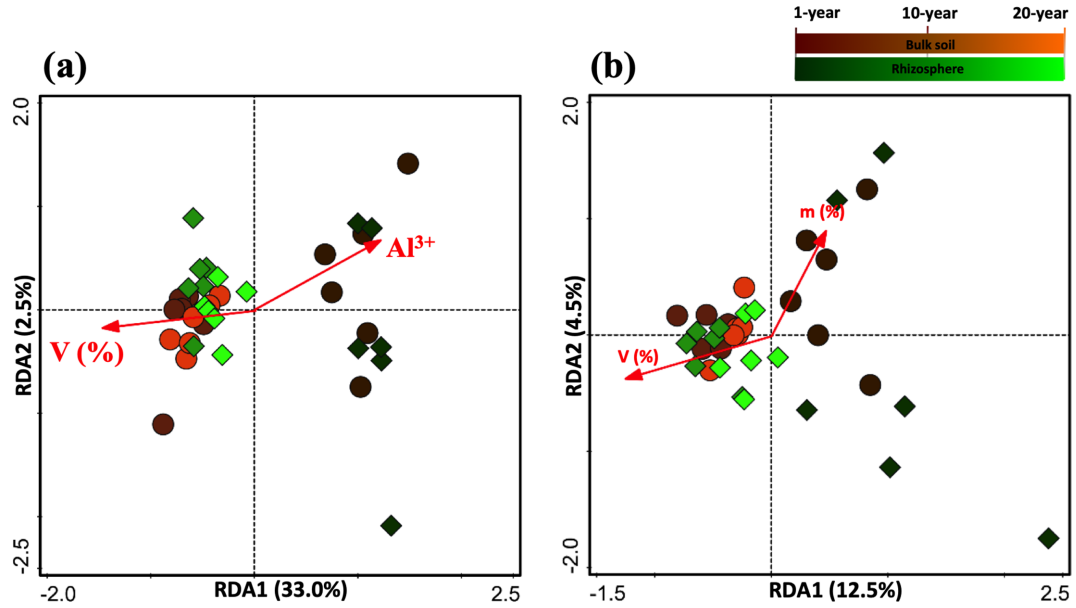

Figure 4. Environmental factors modulating soil microbial taxonomic structure in bulk soil and rhizosphere along the chronosequence. (**a**) Taxonomy at genus level based on M5nr database. (**b**) Functional profile based on Subsystem level 2. db-RDA with forward selection of explanatory variables generated from Euclidean distance matrices, with 1000 Monte-Carlo permutations and corrected by FDR. Samples were grouped according to soil fraction and time: bulk soil (from dark to light brown) and rhizosphere (from dark to light green)..

'RNA metabolism' in 1-year; 'phosphorus metabolism' and 'sulfur metabolism' in 10-year; and 'regulation and cell signaling', 'membrane transport' and 'phosphorus metabolism' in 20-year no-till cropping. The main environmental factors (green nodes) modulating the network configuration in bulk soil were: SOM and soil Al saturation in 1-year; soil Mg^{2+} concentration, soil pH and straw pH in 10-year; and straw P-total in 20-year. In rhizosphere, the highest number of connections were found to: straw Mg-total in 1-year; straw Kpotassium (-)total and soil zinc concentration in 10-year; and soil Al saturation and straw Mg-total in 20-year no-till cropping.

DISCUSSION

Local diversity, represented by Shannon's α-diversity, slightly increased after 10-year no-till, for both bulk soil and soybean rhizosphere. However, the number of shared taxa along samples also increased, resulting in a decrease of Whittaker's global β-diversity in the same period, representing a loss of microbial biodiversity from 1- to 10-year no-till. In addition, microbial communities of samples in the same fraction seemed to become more similar to each other as the chronosequence advanced. Although uncoupled patterns of α- and β-diversity are not a common observation, they are likely to occur when evaluating complex communities, such as microbial communities in soils (Rodrigues *et al.* 2013). Increases in α-diversity with decreases in β-diversity have been reported for both macro- and microorganisms in several areas with anthropogenic action, as a result of disturbances, leading to loss of biodiversity (Olden and Poff 2003; Smart *et al.* 2006; Hewitt, Thrush and Lohrer 2010).

Analyzing the shifts in the bacterial community along the chronosequence, we observed that the abundance of Proteobacteria was higher in rhizosphere than in bulk soil in 1-year no-till, with a general decrease in both soil fractions after 20-year no-till cropping. Proteobacteria is often the most prevalent phylum in soils (Spain, Krumholz and Elshahed 2009), regardless of geographic location (Reeve *et al.* 2015), soil type, or land use and management (Janssen 2006; Delgado-Baquerizo *et al.* 2018), although when depicting the variability within this phylum, some authors have found singularities in abundances of members from this phylum at more refined levels, in both bulk soil (Lavecchia *et al.* 2015; O'Brien *et al.* 2016) and rhizosphere (Reeve *et al.* 2015; Maarastawi *et al.* 2018). Our network analysis showed that members of this phylum, markedly α-Proteobacteria in bulk soil early successional stage (1-year) and β-Proteobacteria, from 1- to 20-year, in both bulk soil and rhizosphere, presented high numbers of significant correlations, indicating that they modulate the networks in no-till cropping, similar to that found for in other soils (Barberán *et al.* 2012; Ma *et al.* 2016). At a finer taxonomic level we found that α-Proteobacteria abundance was always higher in rhizosphere, compared with bulk soil, which is similar to other soil ecological studies (Fierer, Bradford and Jackson 2007; Mendes *et al.* 2014). Members of α-Proteobacteria represent the majority of taxa that inhabit the rhizosphere, occupying niches related to carbon, nitrogen and sulfur biogeochemical cycles (Remigi *et al.* 2016) or even acting as plant-growth-promoting bacteria (PGPB) (Brígido, Glick and Oliveira 2017). Interestingly, we noticed that the relative abundance of this class decreased along the chronosequence. Our data also revealed positive correlations of this class with ammonium and nitrate in rhizosphere and negative correlations with SOC and acid phosphatase activity in bulk soil. The β-Proteobacteria class also presented higher abundances in rhizosphere compared with bulk soil. Some representatives of this class, such as *Burkholderia* sp. and *Cupriavidus* sp., are now recognized as important players in the nitrogen cycle, with recent works demonstrating their ability to fix nitrogen in association with legumes (Bontemps *et al.* 2010) and also to inhabit and occupy niches in the rhizosphere of cultivated plants (Schlemper *et al.* 2017). We observed that the abundance of this class increased over time in long-term no-till. In a recent atlas study, it was demonstrated that β-Proteobacteria are ubiquitous in soils all over the globe, integrating the set of dominant phylotypes across continents and ecosystems (Delgado-Baquerizo *et al.* 2018). One

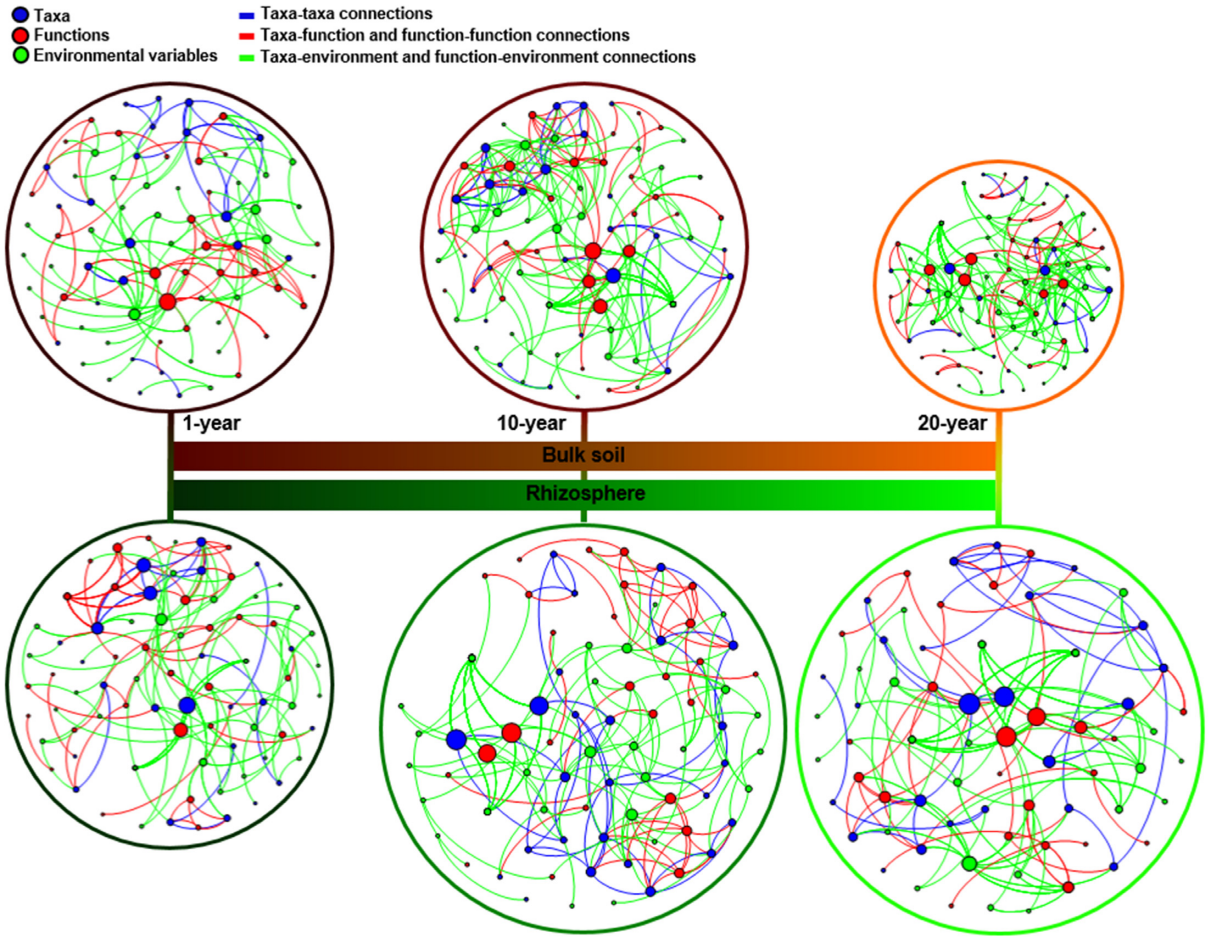

Figure 5. Network dynamics based on Spearman's pairwise correlation matrices. Taxonomic phylum level (blue nodes), except for Proteobacteria at class level, obtained from shotgun metagenomics. Functional categories at level 1 (red nodes) from the SEED database. Environmental variables (green nodes): soil physicochemical, litter and enzymes. The size of each node is proportional to its number of connections (degree). Each edge is colored according to the target: blue edges represent taxonomy–taxonomy connections, red edges represent taxonomy–function or function–function connections and green edges present taxonomy–environment or function–environment connections. Each edge represents a strong and significant correlation (Spearman's $\rho \geq |0.7|$; $P < 0.05$).

reason for the cosmopolitan distribution of β-Proteobacteria is the copiotrophic behavior of members from this class (Fierer, Bradford and Jackson 2007). Authors have argued that its highest relative abundances occur in soils with high carbon availability, naturally or amended, due to copiotrophic behavior. Our study corroborated this idea, as we found increases in SOC, SOM and straw organic carbon along a 20-year chronosequence, correlated to increases in β-Proteobacteria abundance for both bulk soil and soybean rhizosphere. In addition, lower abundances of this class in the early successional stage are correlated with high acid phosphatase activity in bulk soil and low β-glucosidase activity in rhizosphere.

Acidobacteria relative abundance decreased over time, for both bulk soil and rhizosphere. Acidobacteria has been highlighted as one of the most dominant phyla in natural ecosystems (Jesus *et al.* 2009; Rasche *et al.* 2011), such as tropical forests (Rodrigues *et al.* 2013; Mendes *et al.* 2015a). Besides, several studies have demonstrated that Acidobacteria is highly sensitive to both pH (Navarrete *et al.* 2013; Goss-Souza *et al.* 2017) and SOM (Eichorst, Breznak and Schmidt 2007), reducing its relative abundance with the increase of these variables in soils, showing its undoubtedly sensitivity to forest-to-agriculture and to soil management after land-use change. Additionally, we found Acidobacteria to be always more abundant in bulk soil compared with soybean rhizosphere, regardless of length of time of no-till cropping. Other works have demonstrated that, allied to bulk soil reductions in Acidobacteria with land-use change, this phylum also tends to decrease in the plant rhizosphere (Li *et al.* 2016). The abundance of Actinobacteria decreased in bulk soil after 20-year no-till cropping, while it remained stable in soybean rhizosphere after the same period. High abundances of this phylum have been noticed in forest soils and early successional stages of forest-to-agriculture conversion (Jesus *et al.* 2009), with lower percentages being found after long-term forest-to-agriculture conversion, mainly due increases in pH (Kuramae *et al.* 2012). Network analysis showed that Actinobacteria was the phylum that most correlated with environmental variables, markedly with SOC and Mg in bulk soil and with P and N-NO_3^- in rhizosphere. In order to investigate co-occurrence patterns of bacterial taxa, using 16S rRNA high-throughput sequencing Barberán *et al.* (2012) found high correlation between Acidobacteria and Actinobacteria through network analysis, indicating that they are likely to co-occur in soils. Thus, we argue that both phyla can be considered bioindicators of anthropogenic intervention in soils.

Bacteroidetes was overrepresented in soybean rhizosphere compared with bulk soil, in the early successional stage (1-year). As found for some α-Proteobacteria, this phylum includes some PGPB and cellulose-decomposing bacteria, which might be related to soybean cropping (Mendes *et al.* 2015c). Bacteroidetes also has increased abundance in bulk soil after 20-year no-till compared with 1-year, probably due SOC and SOM increases over time, leading to high positive correlation with carbon mineralization rates (Fierer, Bradford and Jackson 2007). Planctomycetes and Gemmatimonadetes increased their relative abundances in bulk soil and rhizosphere after 20-year no-till (same as found for Bacteroidetes). Interestingly, Bartram *et al.* (2014) found Planctomycetes phylum and its main representative class member, Planctomycetacia, to occur mainly in soils with pH ranging from 5.5 to 6, with consequent low abundance above and below this range. Mendes *et al.* (2014) found higher abundance of this phylum after 5-year no-till cropping compared with 1-year. We found a positive correlation between occurrence of Planctomycetes and P concentration in rhizosphere in 1-year and a negative correlation with nitrate concentration in 20-year, directly opposite to that found for Actinobacteria. Gemmatimonadetes has been identified as a cosmopolitan phylum, with high abundances in several land uses. Positive correlations of this taxa with carbon and N concentrations are also observed (Debruyn *et al.* 2011), which is the reason an increased abundance of Gemmatimonadetes was found in long-term no-till cropping (Goss-Souza *et al.* 2017). Interestingly, the authors made the unlikely finding that, after long-term conversion of forest to grassland, the relative abundance of this phylum decreased markedly. In addition, a co-occurrence pattern between this phylum and the above-mentioned Planctomycetes was noticed in long-term no-till. Besides abundance increases in both soil fractions over time, our network analysis showed that Gemmatimonadetes presented the highest number of correlations from all taxa in rhizosphere at 10-years. Thus, we argue that Gemmatimonadetes may play an important role in soybean rhizosphere microbiome, in transient forest-to-agriculture conversion.

Aquificae, Chlamydiae, Chloroflexi and Nitrospirae presented higher abundance in bulk soil compared with rhizosphere, in all chronosequence areas. Aquificae and Nitrospirae also presented increased relative abundance as the chronosequence advanced, for both soil bulk soil and rhizosphere. Aquificae and Chloroflexi have been associated with thermophilic chemoorganotrophic metabolism, by oxidizing hydrogen or reducing sulfur compounds with oxygen or nitrate as electrons acceptor, growing in high temperatures, which is a striking feature of Amazon tropical soils. The few cultured isolates from the Chloroflexi phylum presented several metabolic traits such as fermentation, nitrite oxidation, reductive dehalogenation and carbon anabolic pathways (Yamada *et al.* 2005; Fullerton and Moyer 2016). Similar to those, Chlamydiae has been related to decomposition of plant biomass and carbon incorporation in soils (Kanokratana *et al.* 2011). Thus, we deduced that higher abundances in bulk soil for those phyla and increases in long-term for Aquificae are related to increases in both SOC and SOM storage, following that found for β-Proteobacteria. Nitrospirae is related to N cycling in soil, mainly nitrite oxidation. Regarding this phylum, two important genera (*Nitrobacter* and *Nitrospira*) are highlighted, since they mediate nitrite oxidation, which is a crucial step in microbial nitrification metabolism in soils (Le Roux *et al.* 2016). *Nitrospira* has demonstrated the ability to convert urea into ammonia and carbon dioxide, thus interacting with urease-negative ammonia oxidizer bacteria (AOB) and archaea (AOA) by releasing ammonia to those taxa. In return, AOA and AOB give nitrite to *Nitrospira* as a result of ammonia-oxidizing metabolism (Koch *et al.* 2015). Network analysis showed a high number of correlations of Nitrospirae with taxa and functional categories linked to the N cycle. However, this phylum did not show significant correlations with any of the environmental variables, indicating that the mechanisms of selection for this phylum are yet to be discovered.

Long-term no-till cropping alters characteristics such as nutrient availability, potential acidity and SOM contents. Several studies have demonstrated a correlation between pH and microbial community assembly patterns (Reeve *et al.* 2010; Rousk *et al.* 2010; Bartram *et al.* 2014). We found that long-term no-till cropping in an eastern Amazon soil led to an increase in pH and cations saturation (supplementary Table ST1 available online), mainly due to liming via dolomite [$CaMg(CO_3)_2$], which is reflected in large increases in both Ca and Mg concentrations in soil solution. Together with K, Ca and Mg represent the main cation macronutrients for plant development, which resulted in higher cation saturation (V%) in long-term compared with 1-year no-till. Our network analysis showed that Mg presented a high number of significant correlations with taxa and functional categories in 10-year bulk soil and in 1- and 20-year rhizosphere. In addition, we found correlations between Mg and taxa abundances of Acidobacteria and Actinobacteria in bulk soil, corroborating a previous study in Amazon soils (Navarrete *et al.* 2013). The increase in pH also reduced Al concentration in soil solution, via precipitation, consequently reducing potential acidity over time. Reduction of acidity and Al^{3+} enable plants root system to develop better, enhancing nutrient and water uptake, which is reflected in plant productivity and straw quantity/quality for further cultivation. Al toxicity is found to impair several microbial taxa, acting as a constraining environmental factor and selecting microbial communities in soils (Jesus *et al.* 2009; He *et al.* 2012). Our network analysis demonstrated that, for bulk soil, Al saturation (1-year), soil pH and straw pH (10-year) were the variables that presented the highest number of correlations with taxa and functional categories.

It is well known that plants can have functional advantages by associating with microbes in the rhizosphere (Haney *et al.* 2015). These advantages range from enhanced immunity (Mendes, Garbeva and Raaijmakers 2013) and stress response (Castrillo *et al.* 2017), nutrient uptake (Sayer *et al.* 2017), growth and plant architecture improvement (Pérez-Jaramillo, Mendes and Raaijmakers 2016). Soil microorganisms are sensitive to environment disturbances, by altering taxa composition (trade-offs) and community structure, after disturbances, generally resulting in loss of microbial diversity. Thus, their assembly patterns are often used to predict and explain changes in ecosystems function due anthropogenic action, in several ecosystems (Nemergut, Shade and Violle 2014; Inkpen *et al.* 2017; Mendes *et al.* 2017a). However, there is no consensus about the role of disturbances on the consequent functional potential of those microbial communities (Allison and Martiny 2008; König *et al.* 2017), with both loss and maintenance of functional potential being found (Berga, Székely and Langenheder 2012; Shade *et al.* 2012). We found no significant differences in functional potential structures for both soil fractions and time after conversion. In addition, we did not find differences in functional potential abundances between bulk soil and soybean rhizosphere, except for functions related to 'stress response', which were more abundant in rhizosphere 1-year compared with bulk soil. Along the chronosequence, several functional categories presented shifts in their abundances in bulk soil as a result of

changes in soil physicochemical properties due to long-term no-till. However, only functions related to K metabolism varied in rhizosphere. Thus, we can deduce that the soybean root system acted as a filter, selecting microbial partners to inhabit the rhizosphere, in order to maintain important ecosystems functions, corroborating our central hypothesis.

In summary, our inferences have shown that long-term forest-to-agriculture conversion culminated in loss of microbial diversity, leading to biotic homogenization in both bulk soil and rhizosphere. However, functional potential remained stable in rhizosphere along the chronosequence, evidencing that the soybean root system selects microbial taxa via trade-offs, to keep functions in the rhizosphere microbiome over time. Further studies deciphering the ecological processes regulating plant–microbiome assembly and the causality nexus across the plant–microbiome–soil continuum may enable researchers to gain insights about plant bioengineering and soil microbiome modulation, with consequences for clean food production and ecosystem services resilience.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSEC Journal* online.

AUTHORS' CONTRIBUTIONS

All authors discussed the results and commented on the manuscript. DG-S and SMT designed the project. DG-S and CDB collected the soil samples. DG-S and CDB conducted the greenhouse mesocosm experiment. DG-S, CDB and LWM performed the molecular biology analyses. DG-S, JLMR and LWM analyzed the metadata. DG-S, LWM, JLMR and SMT wrote the manuscript.

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Conflicts of interest. None declared.

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