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Research Paper

Effective microorganisms: Microbial diversity and its effect on the growth of palisade grass

Microorganismos efectivos: Diversidad microbiana y su efecto sobre el crecimiento de Urochloa brizantha

LIDIANE FIGUEIREDO DOS SANTOS[1], MARLIANE DE CÁSSIA SOARES SILVA[2], ROGÉRIO DE PAULA LANA[3], NAYRON VILELA DIOGO[2], MARIA CATARINA MEGUMI KASUYA[2] AND KARINA GUIMARÃES RIBEIRO[3]

[1]*Departamento de Agroecologia, Universidade Federal de Viçosa, Viçosa, MG, Brazil.* posagroecologia.ufv.br
[2]*Departamento de Microbiologia, Universidade Federal de Viçosa, Viçosa, MG, Brazil.* dmb.ufv.br
[3]*Departamento de Zootecnia, Universidade Federal de Viçosa, Viçosa, MG, Brazil.* dzo.ufv.br

Abstract

Effective microorganisms (EM) are inoculants used by farmers on various crops, and the actual efficiency of EM and their composition have been widely discussed. The objective of this study was to analyze the profile of the microbial community in soils after applying 3 EM inoculants from different origins with and without manure and to determine the impacts on growth and chemical composition of *Urochloa brizantha* (palisade grass). We showed, by PCR-DGGE technique, that the community structure of the fungi and bacteria in soil differed with EMs from different sources and that adding manure to the soil also significantly altered the bacterial and fungal profile. We also found that adding manure to soil resulted in a pronounced increase in both dry matter yield and crude protein concentration in palisade grass, while benefits of applying EM were largely restricted to a farmer-produced inoculant, where CP% was increased and NDF% was reduced when applied along with manure.

Keywords: Crude protein, manure, neutral detergent fiber, PCR-DGGE, *Urochloa brizantha*.

Resumen

Los microorganismos efectivos (ME) son inoculantes utilizados por los agricultores en varios cultivos, siendo su eficacia y composición ampliamente discutidas. El objetivo del estudio, realizado en invernadero y laboratorio de la Universidad Federal de Viçosa, Minas Gerais, Brasil, fue analizar el perfil de la comunidad microbiana en el suelo después de la aplicación de 3 inoculantes de ME de diferentes orígenes, con y sin estiércol de bovinos, y determinar sus efectos en el crecimiento y la composición química del pasto *Urochloa brizantha* cv. Marandu. Mediante la técnica de PCR-DGGE se encontró que la composición de las comunidades de hongos y bacterias en el suelo fue diferente entre los ME de diferentes fuentes. La adición de estiércol al suelo igualmente alteró significativamente el perfil de estos microorganismos, dando como resultado un aumento tanto en la producción de materia seca como en la concentración de proteína cruda del pasto, mientras que los beneficios de la aplicación de ME estuvieron en gran medida restringidos a un inoculante no comercial. En este caso aumentó la concentración de proteína cruda en el pasto y la de fibra detergente neutro se redujo cuando el inoculante se aplicó en el tratamiento con estiércol.

Palabras clave: Estiércol, FDN, PCR-DGGE, proteína cruda.

Correspondence: Lidiane Figueiredo dos Santos, Departamento de Agroecologia, Universidade Federal de Viçosa, Av. Peter Henry Rolfe, s/n, Viçosa, CEP 36571 000 MG, Brazil.
E-mail: lidianefigueiredosantos@hotmail.com

Maria Catarina Megumi Kasuya, Departamento de Microbiologia, Universidade Federal de Viçosa, Av. Peter Henry Rolfe, s/n, Viçosa, CEP 36571 000 MG, Brazil.
E-mail: mkasuya@ufv.br

Introduction

Brazil is the world's largest producer and exporter of beef, generating 10.2 million tonnes and exporting 2.3 million tonnes per year (USDA 2019). Pastures occupy approximately 172.3 million hectares of the country and are the primary determinant of livestock production, since practically all beef production is supported by pasture, which is the most practical and economical way to feed animals (Dias-Filho 2014). However, the high level of pasture degradation is a major concern. Sowing of grasses in infertile soils combined with inadequate management has resulted in a reduction in nutritional value of forage and dry matter (DM) yields.

Calvo et al. (2014) and Santos et al. (2016) recommended beneficial microbial inoculation as a strategy for increasing grass productivity and producing healthier plants for feeding animals (Singh et al. 2011). Effective microorganisms (EM) to perform this task are formed by a mixture of microorganisms and their metabolites, including bacteria and fungi, isolated from fertile and vegetated soils, which coexist in a fermentative liquid medium enriched with some source of sugar (Bonfim et al. 2011). The technology was developed by Teruo Higa, who focused on the objective of optimizing the use of organic matter in natural agriculture, and EMs have been commercialized in several countries (Bonfim et al. 2011). However, some farmers produce household forms of EM in an endeavor to design more sustainable agroecosystems (Altieri 2002). These variable inoculants contain mixed cultures and are able to produce complex microbial combinations and fermentation metabolites (Bonfim et al. 2011).

EM suspensions can be more effective when inoculated with organic wastes, e.g. bovine manure, by accelerating the degradation process of substrates and releasing substances that may be useful for nutrition and growth of plants and other microorganisms (Daly and Stewart 1999). Recycling manure using EM may contribute to increased pasture productivity and reduced use of chemical fertilizers (Santos et al. 2019).

While effects on growth and chemical composition of other crops have been observed (Khaliq et al. 2006; Hu and Qi 2013; Santos et al. 2019), effects of EM on palisade grass (*Urochloa brizantha*) are unknown. Some failures from applying inoculants in soil and manure have caused concerns, and researchers have attributed these results to a number of factors, notably: genetic and physiological constitution of the inoculum used (Shin et al. 2017); competition between inoculated and native microorganisms; type of plant inoculated; soil pH, temperature and moisture; and substrate availability and quality (Van Veen et al. 1997). These characteristics can impact on the establishment of EM inoculants in soils and manures.

One way to evaluate the interaction between EM and soil microbial communities, and between EM and manure, is by diversity analysis using molecular techniques. Some of these techniques have been used in the study of effective microorganisms, including: denaturing gradient gel electrophoresis (DGGE) (Van Vliet et al. 2006; Shin et al. 2017); real-time polymerase chain reaction (qPCR) (Ahn et al. 2014); ribosomal intergenic spacer (RISA) analysis (Mayer et al. 2010); and sequencing (Gaggìa et al. 2013; Santos et al. 2020). Among these, PCR-DGGE is an efficient tool for monitoring changes in the microbial community structure in response to environmental changes and may contribute to the assessment of the practical potential of EM.

Despite the use of EM globally, both for agriculture and livestock, microbial profiles of the suspension and the system, where they are inoculated, are unclear and little is known about the effects of the application on tropical grasses. There is only a study showing the effect of EM on the germination of *Urochloa brizantha* seeds (Santos et al. 2020) and a study on the growth and quality of *Brachiaria humidicola* and *Pennisetum purpureum* when combined with chicken manure-based bokashi (Anis et al. 2019).

Thus, the objective of this study was to analyze the profiles of the microbial communities in 3 EM inoculants of different origins, as well as to determine their effectiveness for enhancing growth and chemical composition of palisade grass (*Urochloa brizantha* cv. Marandu), grown in soil with or without bovine manure.

Materials and Methods

Study site and experimental approach

The experiment was carried out in a greenhouse and at the Laboratory of Mycorrhizal Associations and Molecular Genetics of Microorganisms belonging to the Department of Microbiology/Institute of Applied Biotechnology for Agriculture and Livestock (BIOAGRO), and at the Laboratory of Forages/Animal Science Department, of the Federal University of Viçosa (UFV/MG), Viçosa, Minas Gerais, Brazil.

Microbial profile of inoculants by DGGE

Three EM inoculants were tested: EM1 - commercial EM®; and 2 inoculants produced by farmer families, i.e. EM2 - produced in Muriaé/MG; and EM3 - produced in Viçosa/MG. The manufacturer reported that the commercial EM® was composed of water, pasteurized cane molasses and natural microorganisms. The home-made EM2 and EM3 were prepared with microorganisms obtained from the forest in the Zona da Mata Atlântica, using cooked rice (700 g) placed under the soil litter as bait. After collection of the litter

microorganisms, the rice with microorganisms was placed in a bottle containing molasses (200 mL) and water (1,800 mL) and allowed to ferment at room temperature for 15 days, when the EM was ready for use (Bonfim et al. 2011).

For extraction of soil DNA, 350 mg of soil was used, employing the NucleoSpin® Soil Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. Three biological replicates of each type of EM were used. DGGE for fungi and bacteria was performed as follows:

The extracted DNA was amplified by PCR using the 18S rDNA and 16S rDNA genes. The primers NS1 (5'-GTAGTCATATGCTCTTTGC-3') (White et al. 1990) and EF3 (5'-TCCTCTAAATGACCAAGTTTG-3') (Smit et al. 1999) were used to amplify the 18S rDNA gene fragments, generating a 1,700 bp fragment. To obtain a smaller DNA fragment targeting the DGGE technique, a second PCR, the nested-PCR, was performed with the primers FR1-GC (5'-CCCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCA CGGGCCGAICCATTCAATCGGTAIT-3') and FF390 (5′-CGATAACGAACGAGACCT-3′) (Vainio and Hantula 2000) generating a fragment of 350 bp. For amplifying of 16S rDNA gene fragments, primers F27 (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Lane 1991) were used, yielding a 1,500 bp fragment. A nested-PCR reaction was also performed with primers U968-GC (CGCCCGGGGC-GCGCCCCGGGCGGGGCGGGGGCACGGGGGGAAC GCGAAGAACCTTAC) (Nübel et al. 1996) and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Lane 1991).

The PCR reaction consisted of mixing 20 ng of total DNA, 200 µM dNTP, 2 mM $MgCl_2$, 0.5 mg/mL of bovine serum albumin (BSA), 0.2 µM of each primer and 1.25 units of GoTaq® Flex DNA Polymerase (Promega, Madison, USA), totaling 50 µL of reaction. The Controls received purified water (MilliQ®) to replace the DNA in order to detect possible contaminants. The PCR amplifications were performed on a thermal cycler (Eppendorf Mastercycler ep Gradient) under the following conditions: an initial cycle at 94 °C for 4 min, 35 cycles at 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min and a final extension at 72 °C for 10 min. For nested-PCR, 1 µL of the product of the first PCR reaction was used with the same mixture and the same amplification conditions described in the first PCR reaction. To confirm the amplification of PCR and nested-PCR products, 5 µL were used to check by agarose gel electrophoresis (1.5% w/v, 80 V for 80 min), stained with ethidium bromide and visualized under UV light in a Molecular Imaging System (Loccus Biotecnologia L-Pix Chemi).

The fragments obtained by the nested-PCR technique in EM were analyzed with the DGGE (Van Diepeningen et al. 2005). A 15 µL aliquot of the nested-PCR reaction ranging from 150 to 200 ng of DNA was loaded on an 8% (w/v) polyacrylamide gel in 1X Tris-acetate-EDTA (TAE) buffer (Tris/acetic acid/EDTA, pH 8.0). The gel was prepared on a denaturation gradient ranging from 35 to 55%, the 100% denaturation condition consisting of 7 mol/L urea (Sigma, Cat # U5378) and 40% (v/v) formamide (Sigma, Cat # F9037). The gel was subjected to vertical electrophoresis at 100 V for 12 h at 60 °C. Then, the gel was stained for 40 min in a 1X solution of SYBR GOLD® (Sigma Aldrich), as recommended by the manufacturer, and the images were observed under UV light, captured and digitized by means of Molecular Imaging System (Loccus Biotecnologic L-Pix Chemi).

Greenhouse experiment

The soil used is classified as a Red-Yellow Latosol (Embrapa 2006) and the chemical analyses are shown in Table 1 (Defelipo and Ribeiro 1981; Claessen et al. 1997). A total of 8 kg of this soil was conditioned in each polyethylene pot with 7.5 L capacity. Fifty percent of pots received 90 g of dry manure (Table 1). The manure was mixed with the surface soil in the pot to a depth of 5 cm.

Table 1. Chemical properties of the soil and cattle manure used in the experiment.

Property	Value
Soil	
pH	4.30
Phosphorus (P) (mg/dm³)	0.80
Potassium (K) (mg/dm³)	27
Calcium (Ca^{2+}) ($cmol_c/dm^3$)	0.10
Magnesium (Mg^{2+}) ($cmol_c/dm^3$)	0.00
Aluminum (Al^{3+}) ($cmol_c/dm^3$)	0.30
Potential acidity (H+Al) ($cmol_c/dm^3$)	2.48
Sum of bases ($cmol_c/dm^3$)	0.17
Effective CEC[1] ($cmol_c/dm^3$)	0.47
Effective CEC at pH 7 ($cmol_c/dm^3$)	2.65
Base saturation (%)	6
Al saturation (%)	64
Organic matter (g/kg)	16.5
Manure (dry)	
pH	7.40
Phosphorus (P) (%)	1.61
Potassium (K) (%)	3.60
Calcium (Ca) (%)	3.40
Magnesium (Mg) (%)	0.82
Nitrogen (N) (%)	3.10
Organic carbon (%)	19.50
C:N (carbon:nitrogen ratio)	6.29

[1]CEC = Cation Exchange Capacity.

Seeds of palisade grass (*Urochloa brizantha* cv. Marandu) were superficially disinfected in 70% alcohol for 30 sec, followed by immersion in sodium hypochlorite for 10 min, and rinsed 4 times in deionized and sterilized water; excess moisture was removed by absorption on sterilized filter paper. Ten seeds were planted in each pot and soil in the pots was inoculated with EMs from the 3 sources where appropriate.

The EMs were inoculated on sowing day and again 30 and 60 days after sowing, by mixing 34 mL of EM inoculants with 66 mL of deionized water, in each application per pot, while Control pots received 100 mL of deionized water. After seed germination in sunlight, thinning was performed leaving 3 seedlings per pot. Pots were irrigated with fixed doses of deionized water. Plants were kept in the greenhouse for 100 days at a temperature ranging from 25 to 28 ºC, and the first harvest was performed 60 days after sowing, with a second harvest of regrowth 40 days later.

Agronomic and chemical characteristics

Length of the aerial part (LAP), i.e. distance from the apex of the largest leaf to the base of the plant, and diameter of the stems (DS) of palisade grass plants were determined at 60 and 100 days after sowing, with the aid of a rule. The number of leaves (NL) was also recorded at these times, considering only those leaves with more than 50% green area.

Harvested samples were dried at 55 ºC to constant weight in a greenhouse with forced-air ventilation. After drying, the samples were ground in a Wiley mill with a 1 mm sieve and dry matter (DM), neutral detergent fiber (NDF) and crude protein (CP) concentrations were determined (Detmann et al. 2012). Dry mass of the aerial part (DMAP) was determined at both 60 and 100 days, with dry mass of roots (DMR) only at 100 days.

Microbial profile in soil after EM inoculation

For evaluation of microbial diversity in the soil, composite soil samples from each treatment were collected from the surface soil in the pots (less than 5 cm) without disturbing plant roots. The samples were collected at the first application of inoculants and 30 days after the first application. The collected samples were placed in aluminum foil, transported to the laboratory on ice and stored at -20 ºC until analysis by DGGE.

Statistical analyses

The analyses were performed as a completely randomized experimental design and factorial scheme of treatments (2 × 4), i.e. with or without bovine manure, and with or without EM (EM1, EM2 and EM3), with 5 replications. The negative Control was the treatment without manure and EM. We also ran a second Control, i.e. with manure but without EM.

Agronomic and chemical data were subjected to analysis of variance (ANOVA) using program Assistat version 7.7, and Tukey's test was used to detect treatment differences at 5% significance level (Silva and Azevedo 2002).

Analysis of the microbial profile of the EM inoculants was performed using the Dice coefficient and the UPGMA method for construction of dendrograms, with the aid of the Bionumerics® version 6.0 program.

Results

Microbial profile of inoculant by DGGE

Analysis of 18S and 16S amplicons with the PCR-DGGE revealed the diversity of the fungi and bacteria in the EM inoculants from 3 sources (Figures 1 and 2).

In EM inoculants, 2 groups of fungi were identified: the first one had 60% similarity for groups EM2 and EM3, while the second group was formed by EM1 with 40% similarity (Figure 1). For bacteria, 1 group was formed by EM3 with 85% similarity (Figure 2).

Effects of manure and EM on the growth of palisade grass

There was no significant interaction between manure and EM inoculants ($P>0.05$). Manure showed a marked effect on all growth variables of palisade grass ($P<0.05$) at both harvests (Table 2). Applied in the absence of manure, EM2 increased: length of the aerial part of forage (LAP) relative to Control and other EMs; plus stem diameter (DS) and dry mass of the aerial part (DMAP) relative to EM1 at the first harvest. EM2 also increased LAP at the second harvest relative to EM3 ($P<0.05$) (Table 3) plus number of leaves and dry matter of roots (DMR) relative to other treatments but differences failed to reach significance.

In treatments that received manure, application of EM1 resulted in a reduction of 17.1% in diameter of the stem (DS) of palisade grass at the first harvest, relative to Control, but no other agronomic characteristics were affected and there was no effect on any of the measured characteristics at the second harvest ($P>0.05$) (Table 4).

Effect of EM on the chemical composition of palisade grass

Inoculation of soil with EM2 plus addition of manure reduced NDF concentration by 6% (4 units) relative to Control (Table 5). Crude protein concentration for EM2 was higher than for Control (15.4 vs. 8.3%) with the remaining treatments intermediate (Table 5).

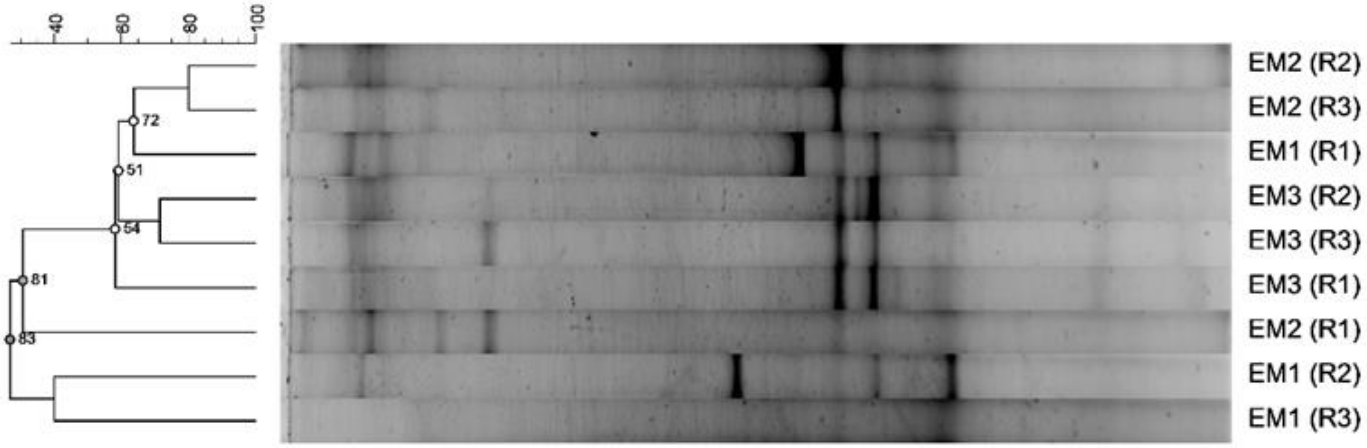

Figure 1. Cluster analysis, Dice-UPGMA, obtained by the DGGE bands profile of the 18S gene from the fungal communities of EM inoculants from 3 sources. R1, R2 and R3 = triplicate reactions.

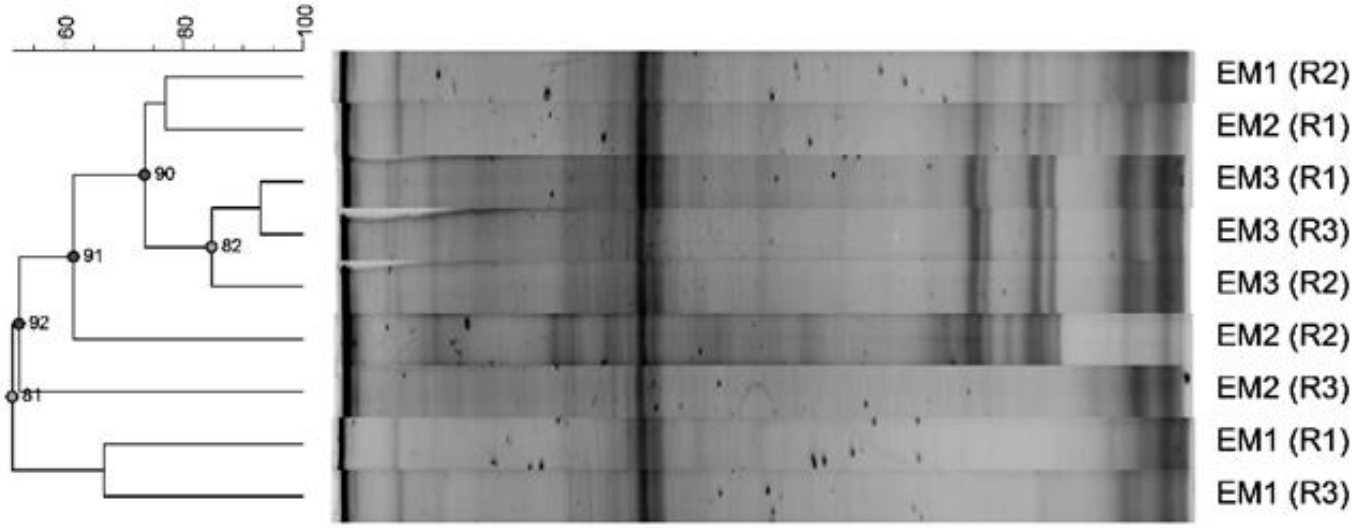

Figure 2. Cluster analysis, Dice-UPGMA, obtained by the DGGE bands profile of the 16S gene from the bacterial communities of EM inoculants from 3 sources. R1, R2 and R3 = triplicate reactions.

Table 2. Agronomic characteristics of palisade grass in the absence and presence of bovine manure at 60 and 100 days after sowing.

Parameter[1]	Harvest 1 (60 days)				Harvest 2 (100 days)			
	Without manure	With manure	s.e.	P-value	Without manure	With manure	s.e.	P-value
LAP (cm)	16.8	83.4	1.00	0.001	7.2	47.0	1.56	0.001
DS (cm)	1.98	2.26	0.10	0.048	0.22	2.03	0.05	0.001
NL	2.2	29.5	0.64	0.001	1.5	36.1	0.65	0.001
DMR (g)	-	-	-	-	0.2	18.1	0.52	0.001
DMAP (g)	0.2	20.6	0.61	0.001	0.1	10.4	0.29	0.001

[1]LAP = length of the aerial part; DS = diameter of the stem (tiller); NL = number of leaves; DMR = dry mass of roots; and DMAP = dry mass of the aerial part.

Table 3. Agronomic characteristics of palisade grass inoculated with 3 sources of effective microorganisms (EM) and grown in the absence of bovine manure, at 60 and 100 days after sowing.

Parameter[1]	Treatment				s.e.	P-value
	Control[2]	EM1	EM2	EM3		
Harvest 1 (60 days)						
LAP (cm)	15.8b[3]	15.3b	20.9a	15.1b	1.45	0.037
DS (cm)	2.0b	1.3c	2.6a	2.0b	0.14	0.001
NL	2.2	2.0	2.7	2.0	0.19	0.087
DMAP (g)	0.13ab	0.11b	0.26a	0.13ab	0.03	0.018
Harvest 2 (100 days)						
LAP (cm)	6.8ab	5.1ab	13.9a	3.1b	2.39	0.030
DS (cm)	0.13	0.10	0.23	0.40	0.10	0.173
NL	1.3	0.9	2.7	1.1	0.48	0.055
DMR (g)	0.11	0.07	0.39	0.08	0.09	0.054
DMAP (g)	0.08	0.03	0.28	0.03	0.08	0.110

[1]LAP = length of the aerial part; DS = diameter of the stem (tiller); NL = number of leaves; DMR = dry mass of roots; and DMAP = dry mass of the aerial part. [2]Control = without the addition of bovine manure and without EM. [3]Values within a row followed by the same letter are not significantly different at P≤0.05.

Table 4. Agronomic characteristics of palisade grass inoculated with 3 sources of effective microorganisms (EM) and grown in the presence of bovine manure, at 60 and 100 days after sowing.

Parameter[1]	Treatment				s.e.	P-value
	Control[2]	EM1	EM2	EM3		
Harvest 1 (60 days)						
LAP (cm)	82.1	85.6	81.4	84.3	2.28	0.539
DS (cm)	2.5a[3]	2.1b	2.2ab	2.3ab	0.09	0.019
NL	28.8	30.8	30.0	28.5	1.91	0.827
DMAP (g)	19.0	23.2	21.4	18.8	1.62	0.204
Harvest 2 (100 days)						
LAP (cm)	50.3	41.8	49.6	46.3	3.22	0.271
DS (cm)	2.0	2.1	2.0	2.0	0.09	0.697
NL	35.6	37.7	36.5	34.7	1.83	0.681
DMR (g)	16.9	18.0	18.7	18.7	1.54	0.815
DMAP (g)	10.7	9.3	11.5	10.3	0.79	0.276

[1]LAP = length of the aerial part; DS = diameter of the stem (tiller); NL = number of leaves; DMR = dry mass of roots; and DMAP = dry mass of the aerial part. [2]Control = without EM and with the addition of bovine manure. [3]Values within a row followed by the same letter are not significantly different at P≤0.05.

Table 5. Nutritive value characteristics of palisade grass inoculated with 3 sources of effective microorganisms (EM) and grown in the presence of bovine manure, at 60 days after sowing.

Parameter	Treatments				s.e.	P value
	Control[1]	EM1	EM2	EM3		
Neutral detergent fiber (%)	65.6a[2]	63.2ab	61.6b	64.4ab	0.79	0.018
Crude protein (%)	8.3b	10.0ab	15.4a	9.8ab	1.54	0.025

[1]Control = without EM and with the addition of bovine manure. [2]Values within a row followed by the same letter are not significantly different at P≤0.05.

Microbial profile in soil after EM inoculation

Fungal community. There were differences between treatments in fungal community profiles. The first cluster had 60% similarity and formed 3 subgroups (Figure 3). One subgroup, with 80% similarity, included fungal community profile of pure soil and soil 30 days after sowing which received manure, as well as samples after receiving the first dose of EM1 and again 30 days after sowing. The second subgroup presented approximately 85% similarity and included soil that received only manure and the one that received manure with the first application of EM1, EM2 or EM3. Finally, the third subgroup was formed by inoculating soil with EM1 and

EM2 plus manure and was collected 30 days after sowing with the first dose of EM, and had approximately 77% similarity. The second cluster, with approximately 55% similarity, was from soil samples without manure but inoculated with EM2 or EM3, after receiving the first dose of EM and 30 days after sowing (Figure 3).

Bacterial community. Cluster analysis of the soil bacterial community revealed 4 distinct groups (Figure 4). The first group had 75% similarity and included soil inoculated with EM2 or EM3 but without manure in both evaluated periods. The second group, with 78% similarity, occurred in soil that received manure with or without EM1, EM2 or EM3, all analyzed 30 days after sowing. The third cluster, with 88% similarity, included soil that received manure with or without EM1, EM2 or EM3 and analyzed just after sowing. The last group, with 82% similarity, was formed by the pure initial soil and soil that received the first dose of EM1 (Figure 4).

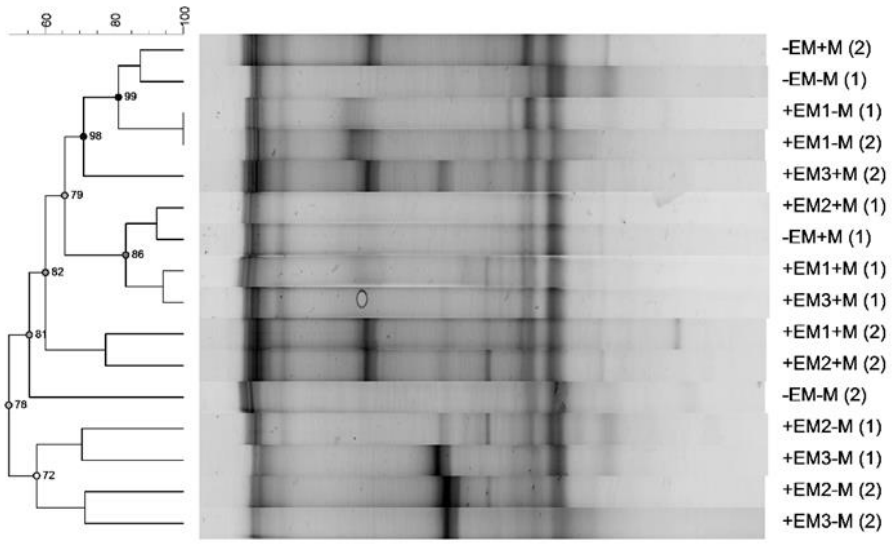

Figure 3. Grouping analysis, Dice-UPGMA, obtained by the DGGE bands profile of 18S gene from soil fungal communities that received EMs from 3 sources (EM1, EM2 or EM3) and bovine manure. +EM1, +EM2 and +EM3 = with EM; + M = with manure; -EM = without EM; -M = without manure; (1) = soil after receiving the first application of EM1, EM2 or EM3; and (2) = soil 30 days after the first application of EM1, EM2 or EM3.

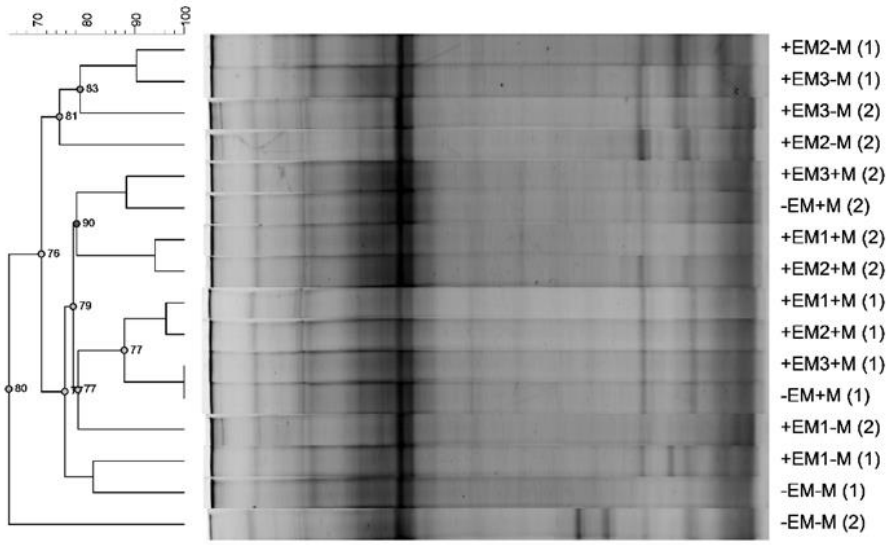

Figure 4. Grouping analysis, Dice-UPGMA, obtained by a DGGE bands profile of the 16S gene from soil bacterial communities that received EMs from 3 sources (EM1, EM2 or EM3) and bovine manure. +EM1, +EM2 and +EM3 = with EM; + M = with manure; -EM = without EM; -M = without manure; (1) = soil after receiving the first application of EM1, EM2 or EM3; and (2) = soil 30 days after the first application of EM1, EM2 or EM3.

Discussion

This study has shown that manure application had the major influence on growth and quality of palisade grass forage, with the only response from inoculating with EM in addition to manure application being a reduction in NDF concentration and an increase in CP% when EM2 was applied. A small (non-significant) response in growth was obtained from inoculating with EM2 alone and no response from inoculating with EM1 and EM3.

Addition of manure dramatically increased growth of palisade grass (about 100-fold; Table 2) when compared with Control without manure or with EM but no manure, which can be attributed to the acidity and low fertility of the soil used (Lana et al. 2016). Decreases in growth and crop production have been observed when cotton plants were cultivated with pure EM without manure (Khaliq et al. 2006), indicating that organic matter and/or nutrients in insufficient amounts for microorganisms and plants can influence plant development. Similar results were found with *Lolium perenne*, where the addition of EM plus manure did not affect plant biomass (Van Vliet et al. 2006). However, in our study even in the absence of manure, EM2 promoted the growth of palisade grass (Table 3), indicating possible differential microbial or biochemical composition of this inoculant. It is possible that metabolites produced by the group of microorganisms by the fermentative process of EM2, such as enzymes, hormones, vitamins and bioactive substances (Higa 2000), may have stimulated the growth of palisade grass. Similar results were found by Santos et al. (2020), where metabolites secreted by EM possibly stimulated the germination of palisade grass seeds.

The concentration of CP in palisade grass (Table 5) in this study was above that expected, considering that concentrations of 8.1–12.5% (13 to 20 g N/kg DM) are considered adequate for this species (Oliveira et al. 2007). This increase in CP concentration may be a function of nitrogen in the manure being mineralized by organisms in the EM2 inoculant. High levels of nitrogen in cotton were also found when EM and organic matter were combined (Khaliq et al. 2006). Similarly, application of EM increased N% in wheat (Hu and Qi 2013) and sunflower (Sharif et al. 2015), when this inoculant was associated with organic compounds and manure with rock phosphate. In *Brachiaria humidicola*, the application of bokashi-based chicken manure fermented with EM positively influenced the CP concentration (Anis et al. 2019). Conclusively, inoculation of EM in combination with organic materials may influence the protein concentration in plants. In this situation, EM acts as a 'potentiator' of organic matter, accelerating its decomposition and releasing its nutrients to the plants (Santos et al. 2019).

Positive effects from the combination of manure and EM2 inoculant may be associated with the low C:N ratio of manure (6.29). This low C:N ratio may have facilitated the decomposition of soil organic matter by the effective microorganisms, with increased N release to the plants and, consequently, greater protein synthesis (Santos et al. 2019).

It was of interest that inoculation with EM combined with manure addition promoted a reduction in NDF concentration in the palisade grass (Table 5). NDF concentration is a determinant of feed quality, since increase of less digestible fractions, such as cellulose, hemicellulose and lignin, may limit the ability of animals to digest forage (Van Soest 1994), i.e. the lower the NDF concentration in plants, the greater the capacity of animals to digest the forage. According to Van Soest (1994), NDF values above 60% reduce feed intake and, in this study, inoculation with EM2 maintained NDF levels at close to 60%, while NDF concentrations in other treatments were always above this value.

The PCR-DGGE technique revealed differences between EM2 and EM3 inoculants in relation to EM1, in terms of the communities of fungi and bacteria, which may be due to the procedure and locality where produced. It was of interest that EM2, manufactured by farmers, was the only product that produced useful responses while the commercial product was of no measurable benefit. The similarity between EM2 and EM3 may be related to the fact that these EMs are home-made and are produced from microorganisms captured in the Zona da Mata Atlântica of Minas Gerais. In contrast, EM1 is produced for commercial purposes, so the manufacturing process and consequently the composition may be different. Differences in the bacterial profile were also observed when EMs from stock inoculants were reactivated by different producers (Van Vliet et al. 2006; Shin et al. 2017), indicating that the preparation method for the EM and its multiplication can influence microorganism diversity. Santos et al. (2020), by sequencing EMs from different origins, found a greater number of operational taxonomic units (OTUs) shared between home-made EMs.

Band patterns obtained from the communities of fungi and bacteria in the soil under study revealed that inoculating with EMs and fertilizing with bovine manure resulted in changes in composition of the microbial community. The fungal community in soil fertilized with manure and inoculated with EM1 or EM2 (and evaluated 30 days after inoculation) was not grouped with samples from soil treated with manure alone (also evaluated 30 days after inoculation). In this case, combining EM (1 and 2) with manure changed the fungal profile in relation to the soil that received manure or pure EM.

However, clustering and similarity of the bacterial profiles of soil samples (Figure 4) that were treated with EM1, EM2 and EM3 and fertilized with manure, and those

in soil fertilized with manure but without any EM, indicated that the bacteria in manure were comparable with those captured in EM from forest soil. Similarities between the bacterial profiles of EMs and manure were also reported in another study (Van Vliet et al. 2006).

Conclusions

While inoculating with EM2 in the absence of manure produced a small increase in growth of palisade grass, these responses were negligible in comparison with the dramatic increases in growth following application of bovine manure. The reduction in NDF% and increase in CP% in forage when EM2 was applied in combination with manure indicates that there is some merit in adding EM2 to these pastures if manure is applied. Variation in bacterial and fungal populations in soils treated with the differing EMs indicates that there may be merit in examining further differing combinations of microorganisms in inoculants for adding to pastures to enhance the benefits obtained in this study.

Studies to better understand the effects of EM, including analysis with other plant species, and evaluation of the chemical composition of EMs seem warranted plus research to determine the quantity and quality of manure and/or organic matter to be added.

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References

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Ahn K; Lee KB; Kim YJ; Koo YM. 2014. Quantitative analysis of the three main genera in effective microorganisms using qPCR. Korean Journal of Chemical Engineering 31:849–854. doi: 10.1007/s11814-013-0274-6

Altieri M. 2002. Agroecologia: Bases científicas para uma agricultura sustentável. Editora Agropecuária, Guaíba, RS, Brazil.

Anis S; Kaunang C; Najoan M; Telleng M; Waleleng P. 2019. Utilization of organic fertilizer on growth, production and quality of *Brachiaria humidicola* and *Pennisetum purpureum* underneath coconuts based farming system. Scientific Papers: Series D, Animal Science 62:208–212. bit.ly/31z7AJ0

Bonfim FPG; Honório ICG; Reis IL; Pereira AJ; Souza DB. 2011. Caderno dos microrganismos eficientes (EM): Instruções práticas sobre uso ecológico e social do EM. 2nd Edn. Universidade Federal de Viçosa, Viçosa, MG, Brazil. glo.bo/3htO2vh

Calvo P; Nelson L; Kloepper JW. 2014. Agricultural uses of plant biostimulants. Plant and Soil 383:3–41. doi: 10.1007/s11104-014-2131-8

Claessen MEC; Barreto WO; Paula JL de; Duarte MN. 1997. Manual de métodos de análise de solos. 2nd Edn. Centro Nacional de Pesquisa de Solos, Rio de Janeiro, RJ, Brazil. bit.ly/3jcF0mG

Daly MJ; Stewart DPC. 1999. Influence of "effective microorganisms" (EM) on vegetable production and carbon mineralization - a preliminary investigation. Journal of Sustainable Agriculture 14:15–25. doi: 10.1300/J064v14n02_04

Defelipo BV; Ribeiro AC. 1981. Análise química do solo (metodologia). Boletim de Extensão UFV no. 29. Universidade Federal de Viçosa, Viçosa, MG, Brazil.

Detmann E; Souza MA; Valadares Filho SC; Queiroz AC; Berchielli TT; Saliba EOS; Cabral LS; Pina DS; Ladeira MM; Azevêdo JAG. 2012. Métodos para análise de alimentos. INCT - Ciência Animal. Suprema, Visconde do Rio Branco, MG, Brazil.

Dias-Filho MB. 2014. Diagnóstico das pastagens no Brasil. Documentos 402. Embrapa Amazônia Oriental, Belém, PA, Brazil. bit.ly/34zWWUq

Embrapa. 2006. Sistema brasileiro de classificação de solos. 2nd Edn. Embrapa Solos, Rio de Janeiro, RJ, Brazil. goo.gl/qx66bR

Gaggìa F; Baffoni L; Gioia DD; Accorsi M; Bosi S; Marotti I; Biavati B; Dinelli G. 2013. Inoculation with microorganisms of *Lolium perenne* L.: Evaluation of plant growth parameters and endophytic colonization of roots. New Biotechnology 30:695–704. doi: 10.1016/j.nbt.2013.04.006

Higa T. 2000. What is EM technology? EM World Journal 1:1–6.

Hu C; Qi Y. 2013. Long-term effective microorganisms application promote growth and increase yields and nutrition of wheat in China. European Journal of Agronomy 46:63–67. doi: 10.1016/j.eja.2012.12.003

Khaliq A; Abbasi MK; Hussain T. 2006. Effects of integrated use of organic and inorganic nutrient sources with effective microorganisms (EM) on seed cotton yield in Pakistan. Bioresource Technology 97:967–972. doi: 10.1016/j.biortech.2005.05.002

Lana RMQ; Lana AMQ; Reis GL; Lemes EM. 2016. Productivity and nutritive value of brachiaria forage intercropping with eucalyptus in a silvopastoral system in the Brazilian Cerrado biome. Australian Journal of Crop Science 10:654–659. doi: 10.21475/ajcs.2016.10.05.p7346

Lane DJ. 1991. 16S/23S rRNA sequencing. In: Stackebrandt E; Goodfellow M, eds. Nucleic acid techniques in bacterial systematics. Wiley, New York, USA. p. 115–175.

Mayer J; Scheid S; Widmer SSF; Fließbach A; Oberholzer HR. 2010. How effective are 'Effective microorganisms® (EM)'? Results from a field study in temperate climate. Applied Soil Ecology 46:230–239. doi: 10.1016/j.apsoil.2010.08.007

Nübel U; Engelen B; Felske A; Snaidr J; Wieshuber A; Amann RI; Ludwig W; Backhaus H. 1996. Sequence heterogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient

gel electrophoresis. Journal of Bacteriology 178:5636–5643. doi: 10.1128/jb.178.19.5636-5643.1996

Oliveira PPA; Marchesin W; Luz PHC; Herling VR. 2007. Guia de identificação de deficiências nutricionais em *Brachiaria brizantha* cv. Marandu. Comunicado Técnico 79. Embrapa Pecuária Sudeste, São Carlos, SP, Brazil. bit.ly/32pAP06

Santos LF dos; Pereira DS; Lana RP. 2019. Micro-organismos Eficientes (EM): Uma realidade presente em sistemas agroecológicos. In: Carmo DP; Pereira DS; Miguel ES; Lopes SO; Priore SE, ed. Pesquisa em agroecologia: Conquistas e perspectivas. Furnabe, Viçosa, MG, Brazil. p. 89–104. bit.ly/2Er3OZB

Santos LF dos; Lana RP; Silva MCS da; Veloso TGR; Kasuya MCM; Ribeiro KG. 2020. Effective microorganisms inoculant: Diversity and effect on the germination of palisade grass seeds. Anais da Academia Brasileira de Ciências 92:e20180426. 10.1590/0001-3765202020180426

Santos MP dos; Castro YO; Marques RC; Pereira DRM; Godoy MM de; Reges NPR. 2016. Importance of limestone and fertilization in traditional and alternative production of forage plants. Pubvet 10:1–12. (In Portuguese). doi: 10.22256/pubvet.v10n1.1-12

Sharif M; Khan M; Khan MA; Wahid F; Marwat KB; Khattak AM; Naseer M. 2015. Effect of rock phosphate and farmyard manure applied with effective microorganisms on the yield and nutrient uptake of wheat and sunflower crops. Pakistan Journal of Botany 47:219–226. bit.ly/31tbiE1

Shin K; Van Diepen G; Blok W; Van Bruggen AHC. 2017. Variability of Effective Micro-organisms (EM) in bokashi and soil and effects on soil-borne plant pathogens. Crop Protection 99:168–176. doi: 10.1016/j.cropro.2017.05.025

Silva FAS; Azevedo CAV de. 2002. Assistat computational program version for the Windows operating system. Revista Brasileira de Produtos Agroindustriais 4:71–78. (In Portuguese). bit.ly/3gAJe5J

Singh JS; Pandey VC; Singh DP. 2011. Efficient soil microorganisms: A new dimension for sustainable agriculture and environmental development. Agriculture, Ecosystems & Environment 140:339–353. doi: 10.1016/j.agee.2011.01.017

Smit E; Leeflang P; Glandorf B; Van Elsas JD; Wernars K. 1999. Analysis of fungal diversity in the wheat rhizosphere by sequencing of cloned PCR-amplified genes encoding 18S rRNA and temperature gradient gel electrophoresis. Applied and Environmental Microbiology 65:2614–2621. doi: 10.1128/AEM.65.6.2614-2621.1999

USDA (United States Department of Agriculture). 2019. Production, Supply and Distribution (PSD) Online Data. www.fas.usda.gov/psdonline

Vainio EJ; Hantula J. 2000. Direct analysis of wood-inhabiting fungi using denaturing gradient gel electrophoresis of amplified ribosomal DNA. Mycological Research 104:927–936. doi: 10.1017/S0953756200002471

Van Diepeningen AD; Vos OJ de; Zelenev VV; Semenov AM; Van Bruggen AHC. 2005. DGGE fragments oscillate with or counter to fluctuations of cultivable bacteria along wheat roots. Microbial Ecology 50:506–517. doi: 10.1007/s00248-005-0012-7

Van Soest PJ. 1994. Nutritional ecology of the ruminant. Cornell University Press, Ithaca, NY, USA. jstor.org/stable/10.7591/j.ctv5rf668

Van Veen JA; Van Overbeek LS; Van Elsas JD. 1997. Fate and activity of microorganisms introduced into soil. Microbiology and Molecular Biology Reviews 61:121–135. mmbr.asm.org/content/61/2/121

Van Vliet PCJ; Bloem J; Goede RGM de. 2006. Microbial diversity, nitrogen loss and grass production after addition of Effective Micro-organisms® (EM) to slurry manure. Applied Soil Ecology 32:188–198. doi: 10.1016/j.apsoil.2005.07.001

White TJ; Bruns T; Lee S; Taylor JW. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA; Gelfand DH; Sninsky JJ; White TJ, eds. PCR protocols: A guide to methods and applications. Academic Press, London, UK. p. 315–322. doi: 10.1016/B978-0-12-372180-8.50042-1

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