

Application of biofertilizer in degraded pasture modified C dynamics and improved forage yield in a short-term period at the tropical region

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Abstract

Recovery of degraded pastures improves the sustainability of meat and milk production systems. Humic acids (HA) and endophytic diazotrophic bacteria (EDB) are known to have bio-stimulating effects on several crops but have not been tested for the recovery of degraded pastures. The aim of the present study was to evaluate soil C and N dynamics, nutrient uptake and forage yield of *Brachiaria decumbens* following the foliar application of HA and EDB in a degraded pasture. A randomized block design with six replications was used. The four treatments were: HA, EDB, HA+EDB and Control. The subplots consisted of four evaluation times for soil characteristics: before treatment application (0) and 30, 60 and 120 days after treatment application (ATA). Soil total organic carbon (TOC), recalcitrant carbon (C_{recal}), labile carbon (C_{labil}), total nitrogen (TN) and C and N stocks were determined in the 0-5, 5-10, 10-20 and 20-40 cm of soil layers. Forage yield and leaf nutrient contents were evaluated at 30 days ATA. The application of the HA and EDB increased the TOC, C_{labil} , soil C stock ($\approx 18\%$), forage dry matter yield (16 to 52%) and nutrient uptake ($\approx 30\%$) after 30 days ATA. Our results showed that the application of HA combined with EDB may be a strategy for the recovery of degraded pastures in the tropical region.

Keywords: plant growth-promoting bacteria, humic substances, rhizodeposition, grassland, biofertilizer.

Abbreviations: HA_humic acids; EDB_endophytic diazotrophic bacteria; C_carbon; N_nitrogen; ATA_after treatment application; TOC_total organic carbon; C_{recal} recalcitrant carbon; C_{labil} labile carbon; TN_total nitrogen; ST_C soil carbon stock; ST_N soil nitrogen stock; SDMY_shoot dry matter yield; D_s soil density.

Introduction

Brazil is the world's largest producer and exporter of beef (IBGE 2017), and an estimated 90% of this production is pasture fed (ANUALPEC, 2015; Pedreira et al., 2015). Pastures are the most economical way for producing and supplying food to cattle. For this reason, beef production costs in Brazil are some of the lowest in the world (Ferraz and Felício, 2010; Dias-Filho, 2014).

Despite the advantages of extensive production on pasture, low investment and the use of inadequate pasture management practices have caused the decline of forage production and the beginning of pasture degradation. Low grazing capacity, plant cover and soil fertility, and decreased soil C and N stocks characterize degraded pastures (Cerri et al., 2004; Braz et al., 2013). Fifty to 70% of the pastures in Brazil are estimated to be degraded to some degree. For this reason, the expansion of pastures into native biomes to sustain animal production has become common (Dias-Filho, 2011). To avoid this, technologies are needed to increase production and recover degraded pastures in the tropical region.

Humic acids (HA) and endophytic diazotrophic bacteria (EDB) are known to have bio-stimulating effects on the development of several crops (Canellas and Olivares, 2014;

Baldotto and Baldotto, 2014). The application of HA and EDB resulted in higher corn (Canellas et al., 2015), sugarcane (Silva et al., 2017) and *Brachiaria decumbens* yield (Pinheiro et al., 2018). Plant physiological changes after the application of HA and EDB have been attributed to auxin-gibberellin-, ethylene- and cytokinin-like activities (Canellas et al., 2015; Olivares et al., 2017). The effects of HA and EDB most often reported for plants are on the root development (Olivares et al., 2017), namely lateral root production, root hair formation and root elongation (Conceição et al., 2008; Olivares et al., 2017). These effects improve the uptake of water and nutrients by the plant. Baldotto et al. (2009) observed higher nutrient uptake following HA application during pineapple acclimation. Piedade Melo et al. (2017) showed that the application of HA and EDB alleviated water stress in common beans by increasing their root size. Changes to roots increase the rhizosphere environment and interfere with root-soil interactions due to higher root exudation of organic acids and sugars (Puglisi et al., 2013; Nardi et al., 2017). These increases in the rhizosphere microbial community and may accelerate soil organic matter decomposition (Kuzakov, 2010). However, the high root density of grasses may increase rhizodeposition, thereby

maintaining organic matter in the soil (Kuzakov, 2002). Changes to roots due to the applications of HA and EDB may therefore change soil C and N dynamics and nutrient release for plant uptake. In this context, we tested the hypothesis that HA and EDB application, in the field experiment, may increase soil C and N stocks and nutrient uptake by *B. decumbens*. The aim of the present study was to evaluate soil C and N contents and *B. decumbens* growth and nutrient uptake at different times following HA and EDB application, in a pasture with simulated grazing.

Results

The soil TOC in the *B. decumbens* pastures with HA and EDB treatments are presented in Figure 1. For the surface soil layer (0-5 cm), no significant interactions between treatments and evaluation times were observed, but the soil TOC slightly increased and then decreased over time. Significant interactions between treatments were observed for the deeper soil layers. For the 5-10 cm soil layer, at 30 days ATA, treatment application resulted in a 10% increase in the soil TOC compared to the control (Figure 1b). The opposite effect was observed for deeper soil layers, with treatment application resulting in 11.7% decrease in soil TOC relative to the control for 10-20 cm soil layer, and 16.9% for 20-40 cm soil layer (Figure 1c-d).

For the 5-10 cm soil layer, the opposite to that observed at 30 days ATA was obtained at 60 and 120 days ATA, with the soil TOC being, on average, 10.4% lower for the treatments than for the control. At deeper soil layers (10-20 and 20-40 cm), the soil TOC increased with the HA+EDB treatment. This increase was 10.5% at 60 days ATA and 16.2% at 120 days ATA for the 10-20 cm soil layer, and 12.6% at 60 days ATA and 19.8% at 120 days ATA for 20-40 cm soil layer (Figure 1b, c and d).

Soil TN differs at the 0-5 and 20-40 cm soil layers (Figure 2a and d). The HA and EDB application significantly decreased the soil TN compared to the control for both soil layers. An average decrease of 16.1, 20.8 and 26.9% was observed in the soil TN compared to the control for the surface soil layer ($p < 0.05$), and a decrease of 13.9, 11.2 and 11.3% was observed for the 20-40 cm soil layer ($p < 0.10$), at 30, 60 and 120 days ATA, respectively. For all soil layers, the soil TN decreased over time (Figure 2).

Soil density was 1.13, 1.13 and 1.25 kg dm⁻³ at the 0-10, 10-20 and 20-40 cm soil layers, respectively. Soil C (ST_C) and N (ST_N) stocks are presented in Figure 3. The ST_C and ST_N were highest at the 20-40 cm soil layer. The application of HA+EDB increased the ST_C by 15.9 and 20.5% relative to the other treatments at the 10-20 and 20-40 cm soil layers, respectively. The soil ST_N decreased with the treatment application. The application of HA and EDB resulted in an average decrease of 13.9 and 11% in the soil ST_N compared to the control for the 0-5 and 20-40 cm soil layers, respectively.

A significant difference in soil C_{recal} between treatments was observed for all soil layers (Figure 4). At 30 days ATA, the treatments resulted in lower soil C_{recal} compared to the control (19.6, 10.5, 13.9 and 28.8% for the 0-5, 5-10, 10-20 and 20-40 cm soil layers, respectively). At 60 days ATA, a similar effect was observed for the superficial soil layers (0-5 and 5-10 cm), there were no differences among the treatments at the 10-20 cm soil layer. The EDB application resulted in a decrease of 29.1% in the soil C_{recal} at the 20-40

cm soil layer. For all soil layers and treatments, the soil C_{recal} decreased at 120 days ATA.

The soil C_{labil} was also significantly affected by the treatments for all soil layers (Figure 5). The treatments with HA and EDB induced a slight increase, followed by a decrease in C_{labil} for all soil layers. The EDB application increased the soil C_{labil} compared to the control, for all soil layers and evaluation times ($p < 0.05$). However, C_{labil} was on average 10.5 and 24.5% lower with the HA+EDB than with the EDB at the 10-20 and 20-40 cm soil layers, respectively. The shoot dry matter yield (SDMY) increased with the treatment application for all evaluation times (Figure 6). At 30 days ATA, the HA+EDB application resulted in a 52% increase in the SDMY compared to the control. The EDB and HA application resulted in 31% increase in the SDMY compared to the control. Similar results were obtained at 60 days ATA, with HA+EDB resulting in an increase of 41.2% in the SDMY, and the EDB and HA showing an increase of 23.5% compared to the control. No significant differences were obtained among the treatments at 120 days ATA. The SDMY was, on average, 16% higher for the tested treatments than for the control.

The mean leaf macro and micronutrient contents 30 days after application of HA and EDB to *B. decumbens* are presented in Table 3. The HA and EDB application increased the leaf N, P, K, Ca, Mg, Mn, Cu and Zn contents compared to the control. The HA and EDB application resulted in increases of 23, 29 and 80% in the N, P and Cu contents, respectively, compared to the control. The EDB application, alone or together with HA, resulted in higher plant N, P and Cu uptake. No significant differences in the leaf K, Mg and Mn contents were observed between the HA and EDB treatments, but HA and EDB application increased the leaf K, Mg and Mn contents (35, 23 and 31%, respectively) compared to the control. The leaf Ca contents were highest with HA+EDB, and the leaf Zn contents were highest with the HA applied alone.

Discussion

Foliar application of HA and EDB to *B. decumbens* changed the soil C dynamics (Figures 1, 3, 4 and 5). These results were observed for all experimental period ($p < 0.05$) and were more pronounced at the 10-40 cm soil layer. The highest percentage of effective roots of *B. decumbens* was observed in this soil layer (Cunha et al., 2010).

Several studies have reported that HA and EDB improve the root architecture (reviewed by Olivares et al., 2017). The rhizosphere is an environment where complex root-microorganism-soil interactions take place, and these interactions affect the soil C dynamics (Badri et al., 2009). The increase of rhizosphere, resulted in a higher release of organic compounds by plants through excretion or root death. These compounds increase the activity of the microorganisms involved in the C cycle, especially in the soil organic matter formation and decomposition (Kuzakov, 2010; Nardi et al., 2017). Our study shows changes in the soil TOC (Figure 1c-d), C_{recal} (Figure 4c-d) and C_{labil} (Figure 5c-d) contents 30 days ATA at the 10-40 cm soil layer. These changes may be related to the use of the soil C for microbial growth in the rhizosphere or in "hot spots" close to the roots. In addition, the compounds released by plants and higher microbial activity may have changed the soil C and promoted the transformation of C_{recal} into C_{labil} . Organo-

Table 1. Soil physical and chemical characterization of the 0-20 and 20-40 cm soil layers.

| Soil characteristics | Depth | |
|--------------------------------------------------------|---------|----------|
| | 0-20 cm | 20-40 cm |
| Chemical analysis | | |
| pH (H ₂ O) | 5.4 | 6.0 |
| P (mg dm ⁻³) | 8.6 | 1.6 |
| K (mg dm ⁻³) | 171.5 | 49.5 |
| Na (mg dm ⁻³) | 1.6 | 0.6 |
| Ca ²⁺ (cmol _c dm ⁻³) | 1.6 | 1.0 |
| Mg ²⁺ (cmol _c dm ⁻³) | 1.0 | 0.6 |
| Al ³⁺ (cmol _c dm ⁻³) | 0.1 | 0 |
| H+Al (cmol _c dm ⁻³) | 4.1 | 2.9 |
| SB (cmol _c dm ⁻³) | 3.1 | 1.7 |
| t (cmol _c dm ⁻³) | 3.1 | 1.7 |
| T (cmol _c dm ⁻³) | 7.1 | 4.6 |
| S (%) | 43.0 | 37.4 |
| m (%) | 1.9 | 0 |
| Granulometric analysis | | |
| Sand (g kg ⁻¹) | 498.6 | 508.2 |
| Silt (g kg ⁻¹) | 104.8 | 98.5 |
| Clay (g kg ⁻¹) | 396.6 | 393.4 |

pH: active acidity; P: phosphorus; K: potassium; Na: sodium; Ca: calcium; Mg: magnesium; Al: aluminium; H + Al: potential acidity; SB: sum of bases; t: effective cation exchange capacity; T: total cation exchange capacity; S: saturation of bases; m: aluminium saturation.

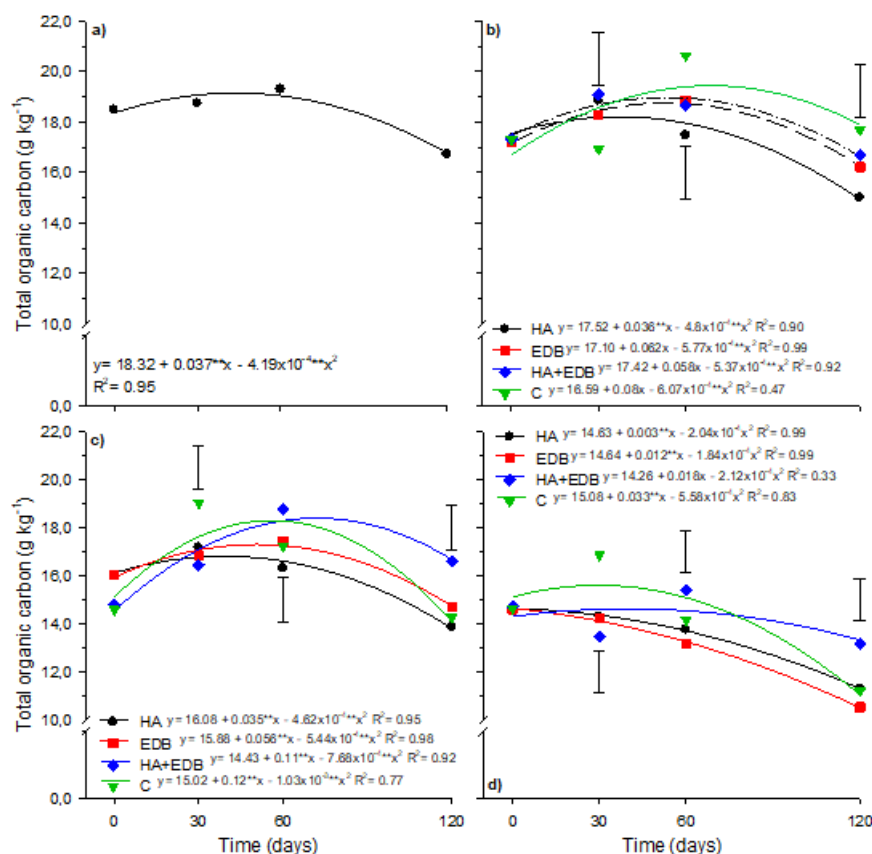


Fig 1. Soil total organic carbon concentrations for the 0-5 (a), 5-10 (b), 10-20 (c) and 20-40 cm (d) soil layers at different times after treatment application. HA: Humic acids; EDB: Endophytic diazotrophic bacteria; HA+EDB: Humic acids and endophytic diazotrophic bacteria; C: Control. ** significant according to the t-test ($p \leq 0.01$). Vertical bars indicate the least significant difference (LSD, $p \leq 0.05$) between treatments for each evaluation time.

Table 2. Mean composition of the vermicompost used to extract humic acids.

| pH | C | P | K ⁺ | Ca ²⁺ | Mg ²⁺ | Al ³⁺ | H ⁺ + Al ³⁺ | SB | CEC | BS |
|-----|--------------------|-----|---------------------|------------------|------------------|------------------------------------|-----------------------------------|------|------|----|
| | g kg ⁻¹ | --- | mg dm ⁻³ | --- | --- | cmol _c dm ⁻³ | --- | --- | --- | % |
| 7.1 | 67 | 952 | 4.9 | 22.2 | 8.9 | 0 | 2.2 | 45.1 | 47.2 | 96 |

pH in H₂O; SB: sum of exchangeable bases; CEC: cation exchange capacity; BS: base saturation.

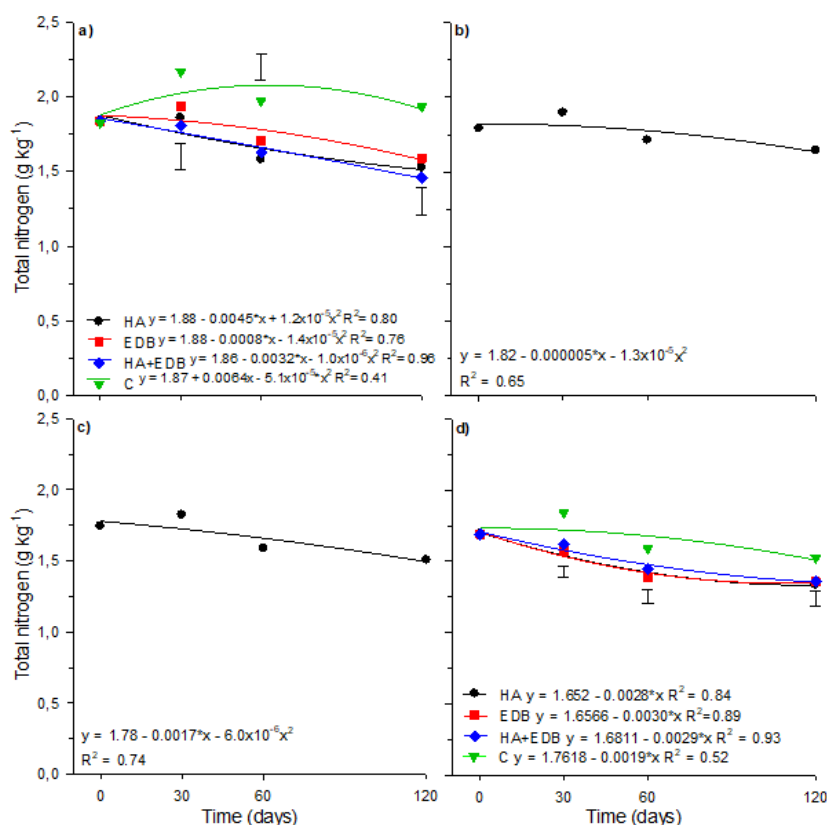


Fig 2. Soil total nitrogen concentrations for the 0-5 (a), 5-10 (b), 10-20 (c) and 20-40 cm (d) soil layers at different times after treatment application. HA: Humic acids; EDB: Endophytic diazotrophic bacteria; HA+EDB: Humic acids and endophytic diazotrophic bacteria; C: Control. * significant according to the t-test ($p \leq 0.05$). Vertical bars indicate the least significant difference (LSD, $p \leq 0.05$) between treatments for each evaluation time.

Table 3. Nutrient contents of *Brachiaria decumbens* 30 days after the application of the humic acids and endophytic diazotrophic bacteria.

| Treatments ⁽¹⁾ | Nutrient contents of <i>Brachiaria decumbens</i> ⁽²⁾ | | | | | | | | |
|---------------------------|-----------------------------------------------------------------|-------|--------|-------|-------|---------------------|--------|--------|---------|
| | Macronutrient | | | | | Micronutrient | | | |
| | N | P | K | Ca | Mg | Mn | Cu | Zn | Fe |
| | g kg ⁻¹ | | | | | mg kg ⁻¹ | | | |
| (-) | 33.15d | 1.03d | 9.98b | 3.20c | 3.66b | 124.95b | 10.00d | 9.00c | 316.91a |
| HA+EDB | 43.09a | 1.41b | 14.07a | 4.14a | 4.56a | 163.96a | 23.30a | 10.26b | 312.83a |
| EDB | 41.68b | 1.45a | 13.39a | 3.79b | 4.40a | 160.27a | 19.56b | 10.12b | 313.83a |
| HA | 37.62c | 1.14c | 12.54a | 3.59b | 4.51a | 167.50a | 11.15c | 11.42a | 314.86a |

⁽¹⁾ Treatments: (-): control; HA+EDB: humic acids and endophytic diazotrophic bacteria; HA: humic acids; EDB: endophytic diazotrophic bacteria. ⁽²⁾ Nutrient contents: N, P, K, Ca, Mg, Mn, Cu, Zn and Fe: nitrogen, phosphorus, potassium, calcium, magnesium, manganese, copper, zinc and iron, respectively. Letters indicate significant differences between treatments for nutrient contents, according to Tukey's test ($p \leq 0.05$).

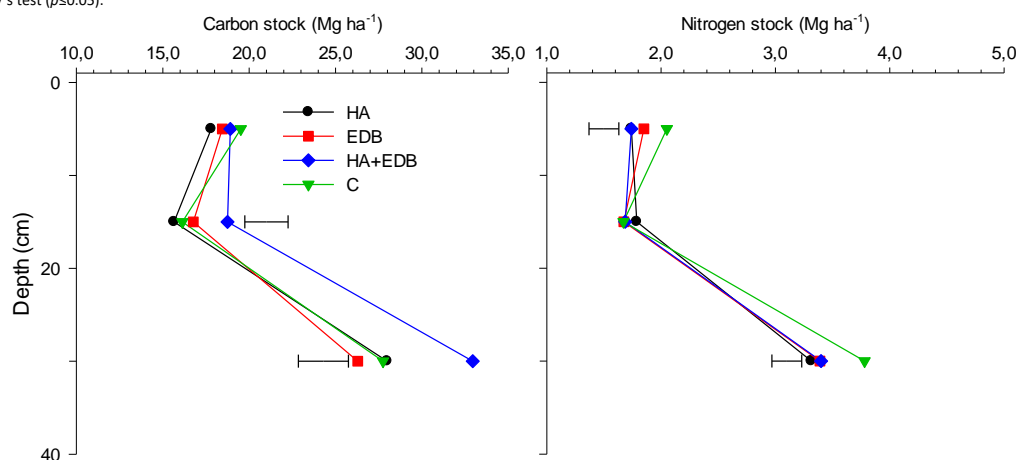


Fig 3. Carbon and nitrogen stocks 120 days after treatment application for the 0-10, 10-20 and 20-40 cm soil layers. HA: humic acids; EDB: endophytic diazotrophic bacteria; HA+EDB: Humic acids and endophytic diazotrophic bacteria; C: control. Horizontal bars indicate the least significant difference (LSD, $p \leq 0.05$) between treatments.

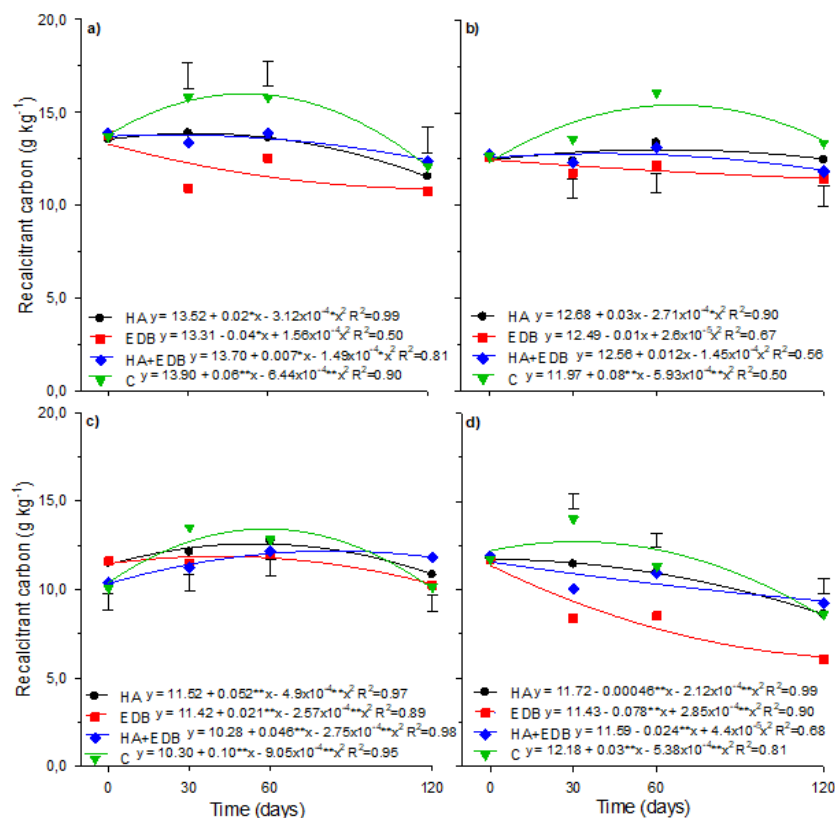


Fig 4. Soil recalcitrant organic carbon in the 0-5 (a), 5-10 (b), 10-20 (c) and 20-40 cm (d) soil layers at different times after treatment application. HA: Humic acids; EDB: Endophytic diazotrophic bacteria; HA+EDB: Humic acids and endophytic diazotrophic bacteria; C: Control. ** and * significant according to the t-test ($p \leq 0.01$ and $p \leq 0.05$). Vertical bars indicate the least significant difference (LSD, $p \leq 0.05$) between treatments for each evaluation time.

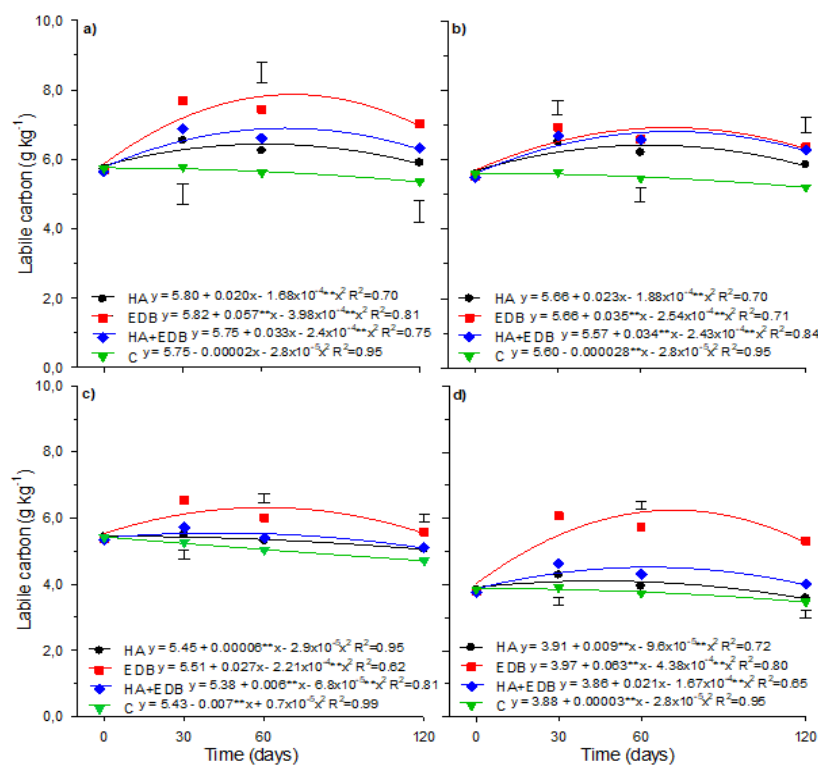


Fig 5. Soil labile organic carbon concentrations for the 0-5 (a), 5-10 (b), 10-20 (c) and 20-40 cm (d) soil layers at different times after treatment application. HA: Humic acids; EDB: Endophytic diazotrophic bacteria; HA+EDB: Humic acids and endophytic diazotrophic bacteria; C: Control. ** significant according to the t-test ($p \leq 0.01$). Vertical bars indicate the least significant difference (LSD, $p \leq 0.05$) between treatments for each evaluation time.

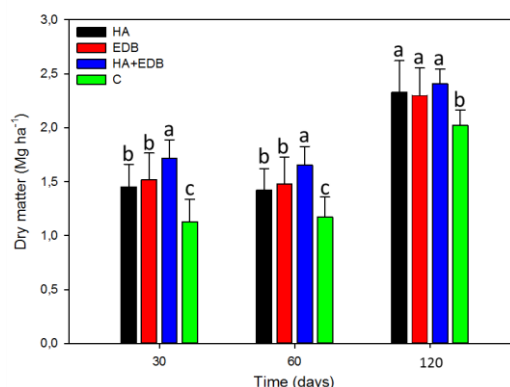


Fig 6. *Brachiaria decumbens* shoot dry matter yield for the different treatments at different times after treatment application. HA: Humic acids; EDB: Endophytic diazotrophic bacteria; HA+EDB: Humic acids and endophytic diazotrophic bacteria; C: Control. Vertical bars indicate the standard deviation. For each evaluation time, treatment means followed by different upper-case letters are significantly different according to Tukey's test ($p \leq 0.05$).

mineral associations are important mechanisms for soil C protection (Six et al., 2002). However, Keiluweit et al. (2015) showed that root exudates might release C from these organo-mineral associations, making the C readily available. The establishment of *B. decumbens* resulted in changes to the soil C dynamics at 30 days ATA. At 60 days ATA, the soil TOC was higher for treatment HA+EDB (Figure 1c-d). A slight increase in soil C_{recal} (Figure 4c-d) and a decrease in soil C_{labil} (Figure 5c-d) were observed. According to our hypothesis, the increase in the soil TOC was due to an increase in the roots and the slow decomposition of dead roots. As the C derived from dead roots has low lability, microorganisms become more efficient at decomposing the soil organic matter (Kuzakov, 2010). Labile organic compounds in soil organic matter and root exudates are easily decomposed and become substrates for microbial growth (Bais et al., 2006; Kuzakov, 2010; Philippot et al., 2013). This C dynamic explains the inversion of the soil C_{recal} and C_{labil} contents observed in the present study.

The response of the soil TOC to the application of EDB alone should be highlighted (Figure 1c-d). This treatment presented lower soil TOC but higher soil C_{labil} (Figure 5c-d) than the other treatments ($p < 0.05$). The increase in microbial activity in this treatment promoted the transformation of recalcitrant C into labile C. The EDB treatments generated an increase in the C content of the soil microbial biomass (data not shown). Furthermore, root exudates may increase the microbial activity in the rhizosphere, increasing the soil organic matter decomposition (Haichar et al., 2014).

Adequate pasture management may increase the soil ST_C (Braz et al., 2013). In our study, the HA+EDB application resulted in an increase in the ST_C in the soil layer where the grass effective roots are located (Figure 3). This treatment may have increased root mass in the soil layer, changing the C dynamics and increasing the soil C contents. In addition, the roots of *Brachiaria* species have a great potential to accumulate belowground C (Batlle-Bayer et al., 2010).

Soil N dynamics were different from the soil C dynamics. The HA and EDB application decreased the soil TN and ST_N . As previously discussed, the HA and EDB change the root architecture and may increase root exudation. Increased root exudation leads to higher N demand for microbial growth (Zhu et al., 2014). Soil organic matter decomposition and N mineralization in the rhizosphere may increase in

order to meet this increase in N demand (Koranda et al., 2011; Meier et al., 2017), resulting in higher amounts of soil N becoming available to plants due to the prompt turnover of microbial biomass (Kuzakov and Xu, 2013; Zhu et al., 2014). The low soil TN obtained after the HA and EDB application may therefore be related to the plant N uptake following soil organic matter decomposition by microorganisms. The use of HA and EDB together with adequate pasture management may therefore be an effective strategy to increase system sustainability.

The potential of HA and EDB application to increase *B. decumbens* forage yield was observed in the present study. The bio-stimulating effects of HA and EDB are related to their plant hormone-like effects and are responsible for plant growth and development (Canellas et al., 2015; Olivares et al., 2017). Notably, a synergistic effect between HA and EDB was observed in the present study, indicated by the higher SDMY observed for the HA+EDB treatment (Figure 6).

The application of HA and EDB increased plant uptake of all nutrients, except Fe. (Table 3). The main effects of HA and EDB are morpho-anatomical changes to root architecture, increasing lateral root and root hair formation (Canellas et al., 2015). In addition, HA and EDB also affect the root biochemistry, increasing the synthesis of plasma membrane H^+ -ATPases. This increase improves energy production for the secondary ion transport system and promotes root water and nutrient uptake (Canellas and Olivares, 2014; Lima et al., 2014).

In addition, the *Herbaspirillum* strains used in the present study are N-fixing and P-solubilizing (Olivares et al., 2017). Higher N and P contents were therefore observed with EDB application. The higher root growth and exudation may have promoted root colonization by *Herbaspirillum*. This enabled higher fixation of atmospheric N and solubilization of soil P compounds, resulting in higher root nutrient uptake. Similar effects were observed for the uptake of N (Canellas et al., 2013) and P in corn (Giro et al., 2016).

Materials and Methods

The study was carried out in a degraded pasture of palisade grass (*Brachiaria decumbens* Stapf cv. Basilisk) located in a farm in Alegre municipality, state of Espírito Santo, Brazil (20°45'49" S, 41°31'57" W; approximately 200 m altitude).

The region's climate is type Cfa according to the Köppen climate classification (Alvares et al., 2013), with hot and rainy summers and dry winters, 22°C mean annual temperature and 1200 mm mean annual rainfall. The soil has been classified as Typic Haplustox (Soil Survey Staff, 2010). The soil's physical and chemical characteristics are presented in Table 1.

The pasture was renewed 6 months before the beginning of the experiment. Liming and fertilization were performed based on the soil chemical analysis and crop requirements (Prezotti et al., 2007). One month before the beginning of the experiment, an area of 333 m² (37 m length and 9 m width) was selected, delimited and fenced. Twenty-four experimental units of 6 m², with a border of 1 m, were delimited within this area. Before the treatment application, the pasture was mowed using a mechanical mower to 10 cm above the ground to simulate grazing.

The experiment design adopted was randomized blocks with six replications and four treatments: HA (20 mg C L⁻¹) applied at 450 L ha⁻¹, EDB (EDB mix containing 10⁹ viable cells L⁻¹) applied at 350 L ha⁻¹, combined use of HA+EDB, and the control (C). Soil characteristics were evaluated at four times: before treatments application (0) and 30, 60 and 120 days after the treatment application (ATA). The treatments were applied to the leaves with a 20-L manual backpack sprayer (JACTO-PJH20), with the doses being adjusted to the experimental units.

The HAs were extracted from a vermicompost produced from pen manure and sugarcane filter cake (5:1, v/v) at the Centre for the Development of Biological Inputs for Agriculture (NUDIBA), Universidade Estadual do Norte Fluminense (State University of Northern Rio de Janeiro; UENF), Goytacazes Campus, state of Rio de Janeiro. The organic residues were mixed, and worms (*Eisenia foetida*) were added at a ratio of 5 kg of worms per m³ of organic residue. The vermicompost was air dried, sieved (2 mm) and chemically characterized (Table 2).

For HAs extraction, vermicompost was added to 0.5 mol L⁻¹ KOH (1:10, v:v), stirred for 6 h and left to stand for 12 h. The supernatant was collected, acidified to pH 1.5 with 1 mol L⁻¹ H₂SO₄ then extracted and discarded. The decanted material was centrifuged (2657 g, 20 min), and the supernatant was discarded. The HAs were titrated to pH 7.0 with 0.1 mol L⁻¹ KOH, placed in petri dishes, added to an oven at 45°C until constant weight was achieved. The C concentration of the HAs samples was quantified through wet oxidation of organic matter (Yeomans and Bremner, 1988).

A strain of *Herbaspirillum rubrisubalbicans* (HCC 101) and two strains of *Herbaspirillum seropedicae* (HIV 206, HIII 215) were used. The bacterial strains used have been deposited in the culture collection of the Laboratory of Cell and Tissue Biology of the Universidade Estadual do Norte Fluminense Darcy Ribeiro (State University of Northern Rio de Janeiro Darcy Ribeiro; LBCT/UENF). The inoculum was grown in DYGS liquid medium at 120 rpm and 30°C for 24 h, and the bacterial suspension was adjusted to 10⁹ cells per mL (Döbereiner et al., 1995).

For each experimental unit, three disturbed soil samples were collected from four soil layers (0-5, 5-10, 10-20 and 20-40 cm, with a Dutch auger) and combined to form a composite sample. The disturbed soil samples were air dried and sieved (2-mm diameter) to remove root pieces and stones. An undisturbed soil sample was also collected (0-10, 10-20 and 20-40 cm) for determination of soil density, using the volumetric ring method.

Soil samples (20 g) were ground and sieved (250 µm). The soil total organic carbon (TOC), total nitrogen (TN), labile C (C_{labil}) and recalcitrant C (C_{recal}) concentrations were determined for the 0-5, 5-10, 10-20 and 20-40 cm soil layers. The TOC was quantified through wet oxidation of the organic matter (Yeomans and Bremner, 1988). The TN was quantified by sulphuric acid digestion (Bremner and Mulvaney, 1982; Tedesco et al., 1995). Soil organic C fractions were determined according to Chan et al. (2001). Fraction 1 (very labile), C oxidized in acid medium (3 mol L⁻¹ H₂SO₄); Fraction 2 (labile), calculated as the difference between C oxidized in 6 and 3 mol L⁻¹ H₂SO₄; Fraction 3 (less labile), calculated as the difference between C oxidized in 9 and 6 mol L⁻¹ H₂SO₄; and Fraction 4 (non-labile), calculated as the difference between the TOC and C oxidized in 9 mol L⁻¹ H₂SO₄. The sum of fractions 1 and 2 correspond to C_{labil}; the sum of fractions 3 and 4, to C_{recal} (Chan et al., 2001).

Soil density (Ds) was determined and used to calculate soil C (ST_C) and N (ST_N) stocks (Mg ha⁻¹) for each soil layer according to $ST_C = TOC \times Ds \times e$ and $ST_N = TN \times Ds \times e$, where: ST_C,n are expressed in Mg ha⁻¹, TOC and TN are expressed in g kg⁻¹, Ds in kg dm⁻³, and e (soil layer thickness) in cm.

Shoot dry matter was determined 30, 60 and 120 days after treatment application. Three samples were collected randomly from each experimental unit, using a quadrant with 0.09 m² (0.3 x 0.3 m). The grass was cut to 10 cm aboveground to simulate grazing. The samples were oven dried at 65°C for 72 h, and used to determine the dry weight and the dry matter yield. Macro- (N, P, K, Ca and Mg) and micronutrient (Zn, Cu, Mn and Fe) concentrations were also measured. Leaf N content was determined by sulphuric acid digestion followed by Kjeldahl distillation. Leaf P, K, Ca, Mg, Zn, Cu, Mn and Fe contents were determined following nitric perchloric acid digestion according to Johnson and Ulrich (1959) and Malavolta and Moreira (1997).

Statistical analysis

The data were submitted to analysis of variance (ANOVA) and means between treatments were compared using the least significant difference of a Tukey test ($p \leq 0.05$). When significant differences were found, applied regression analysis was performed for the evaluation times. Models were chosen based on the significance of the regression coefficients, according to Student t-test ($p \leq 0.05$ and $p \leq 0.01$) and according to the coefficient of determination (R²). Statistical analyses were carried out with R program (R Core Team 2018).

Conclusions

The observed increase in forage production and the increase in soil ST_C showed the viability of HA and EDB application for the recovery of degraded pastures. Although N-fixing EDB were used in the present study, a decrease was observed in the soil ST_N. However, no N fertilization was applied, and animals were not allowed in the area during the experimental period. Thus, only N export from the system took place. Therefore, further studies of the use of this biotechnology, together with adequate pasture management, are needed. The most important factor in degraded pasture recovery is avoidance of the expansion of low production systems into native biomes. The present study may therefore result in strategies that help recover

degraded pastures, enabling the preservation of fragile ecosystems.

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Conflicts of Interest

The authors declare no conflicts of interest.

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