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Co-inoculation maintains yields and lowers N_2O emission by affecting rhizosphere bacterial diversity in common bean grown on a C-rich clayey Ferralsol

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ABSTRACT

Despite being a N-fixing legume, common bean (*Phaseolus vulgaris*) cultivation using diazotrophic bacteria is not commonly adopted by Brazilian farmers, who instead rely on mineral nitrogen sources like urea, increasing environmental and economic costs. Diversified systems that enhance soil quality may improve the crop's ability to depend solely on biological nitrogen fixation. This study investigated how co-inoculation with *Rhizobium* spp. and *Azospirillum brasilense* (+I or -I), with or without mineral N (+N or -N), affects N_2O–N fluxes, yield, and rhizospheric community of common bean. A trial was established within a 20-year-old integrated crop-livestock system (ICLS) over the 2019/2020 (Y1) and 2021/2022 (Y2) crop years. Soil and plant variables were assessed throughout both years. N_2O–N fluxes were measured using manual static chambers targeting days following co-inoculation and N fertilization applications, while the 16S rRNA microbiome composition was assessed by metabarcoding samples collected during flowering. Yields (~3000 kg ha^{-1}) were relatively high and similar among treatments. Co-inoculation alone (+I-N) led to reduced N_2O–N fluxes (yearly average of 43.8 ± 16.0 μg/m^2/h). Co-inoculation reduced the N_2O emission intensity of common bean; however, its effectiveness was limited when applied in conjunction with urea. Regardless of year and co-inoculation, N_2O–N fluxes remained high when N fertilizer was used (averaging 108.3 ± 30.3 μg/m^2/h). Microbial diversity was generally lower under N fertilization, with shifts in the abundance of nitrogen-related functional groups, particularly in the second year. Despite seasonal variations, results indicate that co-inoculation can mitigate N_2O emission while maintaining crop yield and soil organic matter (~4 %) in this biodiverse ICLS system.

1. Introduction

In Central Brazil, particularly in the State of Goiás, common bean (*Phaseolus vulgaris* L.) cultivation is ubiquitous and occurs along the year in three growing seasons: first harvest season with sowing between November and December, a second harvest season from January to February, and a third irrigated harvest with sowing from May to June. Most soils used for common bean cultivation are naturally dystrophic (base saturation lower than 50 %) Ferralsols, Acrisols, or Lixisols of clay or medium texture and liming is recommended to ameliorate soil acidity; to increase supply of Ca^{2+} and/or Mg^{2+}; and to improve nitrogen (N), phosphorus (P) and potassium (K) use efficiency by annual grain crops (Oliveira et al., 2023). In Brazil, due to the biochemical dynamics of N in soil, with many sources of addition and loss pathways in the soil-plant system, there is yet no soil analysis test suitable to measure soil N availability to crop plants to help recommend fertilizer rates. Nitrogen requirement is commonly established via crop-response curve showing yields versus N rates (Fageria and Baligar, 2005), which is a laborious

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Table 1
Crop rotation for biodiversity intensification in integrated crop livestock system (ICLS) on an eight-hectare paddock at Embrapa experimental farm, in Santo Antonio de Goiás, Brazil.

Crop year	Summer		Winter
	October to January	January to April	May to September
2019/2020 (Y1)	Common bean	Rice + Brachiaria grass	Brachiaria grass
2020/2021	Brachiaria grass		
2021/2022 (Y2)	Common bean	Rice + Brachiaria grass	

Common bean: *Phaseolus vulgaris* cv. BRS FC104; Rice: *Oryza sativa* cv. BRS A501 CL; Brachiaria grass: *Urochloa brizantha* cv. BRS Paiaguás. When in consortium, rice and brachiaria grass were cultivated using the Clearfield system (CL™). Y1 and Y2 stand for years 1 and 2 when common bean was cultivated as the main crop in the ICLS.

and time-consuming method not suitable for routine fertilizer recommendation before annual crops are established on a farm parcel.

Between 2013 and 2022, in the State of Goiás, the yearly area of common bean cultivation in the first harvest covered approximately 118,000 ha with an average yield of 2.2 Mg ha^{-1} (Embrapa, 2023). It is generally acknowledged that N fertilizer use is low in the first harvest in the State of Goiás, although it is recommended to apply 20 kg N ha^{-1} at planting and between 40 and 80 kg ha^{-1} for top dressing at the V4 plant stage depending on the expected yields (Carvalho et al., 2019). However, the N use efficiency by common beans varies between 50 % and 75 % depending on environmental factors such as microbial activity, available carbon, and soil aeration as well as the fertilizer amount, placement and timing (Carvalho et al., 2019; Peoples et al., 2004). Globally, approximately 1.2 % and 14.4 % of the N applied as fertilizer is lost as nitrous oxide (N_2O) and volatilized ammonia (NH_3), respectively. Despite its low proportion, N_2O is a powerful long-lived greenhouse gas that remains in the atmosphere for 114 years (Allen et al., 2016).

Nitrous oxide (N_2O) is one of the three major greenhouse gases (GHG), alongside carbon dioxide (CO_2) and methane (CH_4). These emissions contribute to climate change and further exacerbate environmental challenges associated with agricultural practices. Of those, N_2O is of particular concern in agriculture, given that agricultural soils account for 70 % of all global emissions (Tian et al., 2020). The general understanding is that soil N_2O emissions are dependent on N_2O-producing microbes present in soil environment (Bateman and Baggs, 2005; Gao et al., 2018; Xun et al., 2022). Synthetic N fertilization exacerbates the problem by introducing the substrate for the process: the longer N from fertilizers is available in the soil, the greater the losses via N_2O emission (Smith et al., 1997). The default value for emission factor has been set at 1 percent of the N applied to soils (IPCC, 2019).

Legume crops, including common beans, can establish symbiotic relationships with specific rhizobia. These rhizobia assist in converting atmospheric dinitrogen (N_2) gas into ammonia (NH_3) (Sadowsky et al., 2013). This process, known as biological nitrogen fixation (BNF), enhances the sustainability of legume-based agricultural and natural systems, proving particularly valuable in regions with limited access to chemical inputs. Notably, more than 250 million hectares worldwide are dedicated to the cultivation of crop and forage legumes. Forage and fodder legumes are responsible for fixing approximately 12–25 Tg N $year^{-1}$, while pulses and oilseeds contribute over 21 Tg N $year^{-1}$ (Thies, 2021). N fertilization may also affect which microorganisms are able to survive and thrive in the rhizosphere. For example, excessive inorganic N seems to hamper rhizobial N fixation and nodulation with a negative relationship between soil N levels and the presence of *Rhizobium* spp. in common bean rhizospheres (Reinprecht et al., 2020; Castellano-Hinojosa et al., 2021; Pias et al., 2022). While some investigations have examined the impact of management practices, such as the co-inoculation with *Rhizobium* and *Azospirillum* and inorganic N fertilization (Hungria et al., 2013; Messias et al., 2023), limited research has focused on understanding the effect of co-inoculation on plant and soil responses under more diversified systems, such as crop-livestock integration (ICLS).

In Brazil, mixed farming systems such as ICLS is promoted as a strategy to reduce GHG per kilogram of beef produced (Bogaerts et al., 2017; Cardoso et al., 2016) and has driven governmental support as part of an intended contribution to reduce GHG emissions by 35 % up to 2025 and 50 % up to 2030 (MAPA, 2021). The State of Goiás has the sixth largest pasture area in Brazil, covering 13.8 million ha, in which 25.63 % is under severe degradation (UFG-LAPIG, 2024). As restoration with planted pasture alone is hardly cost effective due to the need of heavy soil liming for acidity control, application of reasonable amounts of P and K fertilizers and the use of quality seeds, beef production benefits alone do not outweigh the expenses (Martha Jr. et al., 2011). Integrating crop and livestock production has advantages over specialized livestock systems. It improves soil properties; reduces degradation; and boosts yield, economic value, and farm competitiveness (Lemaire et al., 2014; Martha Jr. et al., 2011; Cortner et al., 2019). ICLS is part of the Brazilian Agricultural Policy for Climate Adaptation and Low Carbon Emission – ABC+ 2020–2030 (MAPA, 2021). This program promotes the adoption of crop, livestock and forestry integration and no-tillage systems, BNF, and other science-based practices to ensure greater yields and resilience of farming systems and to provide effective control of GHG emissions in Brazilian agriculture.

We hypothesized that co-inoculation of *Rhizobium spp.* and *Azospirillum brasilense* would enhance biological nitrogen fixation, thereby reducing the need for synthetic N fertilizers and mitigating N_2O emission in common bean production under an integrated crop-livestock system (ICLS). To test this, we evaluated N losses via N2O–N fluxes, as well as plant- and soil-related variables, including bacterial communities and their biological activity in the rhizosphere of common bean cultivated on a Ferralsol in the Brazilian Cerrado, a neo-tropical savanna.

2. Materials and methods

2.1. Study area and environmental conditions

The field trial was conducted from October 2019 to January 2022, on a typical clayey (clay ~ 623 g kg^{-1}) Ferralsol (Katohypergeric Rhodic Pantoferritic; IUSS Working Group WRB, 2022) of the Brazilian savanna in a slightly rolling and homogeneous area of 7.5 ha named Paddock 4 (P4). In the Brazilian savanna (Cerrado), the seasonality of climate is remarkable, divided in a rainy season (800–1800 mm), between October and April, and a dry season from May to September. That configures a tropical zone type Aw according to the Köppen climate classification (Beck et al., 2023). P4 is under a crop-livestock integration system (ICLS), located at Embrapa Rice and Beans, in Santo Antônio de Goiás, Goiás State, Brazil (16°29'17"S, 49°17'57"W, 804 m.a.s.l.). Oliveira et al. (2022) has described soil characteristics and management history of ICLS in P4.

Since 2004/2005, P4 has been cultivated only under no-tillage with a crop-livestock rotation where the pasture (brachiaria grass; *Urochloa brizantha* cv. BRS Paiaguás) phase remained continuously for 3 years. During this phase, cattle was fed with the forage produced in the plot. After 3 years, the grass is controlled with herbicide and the crop phase (sorghum, millet, corn, aerobic rice, or soybean) is introduced on the

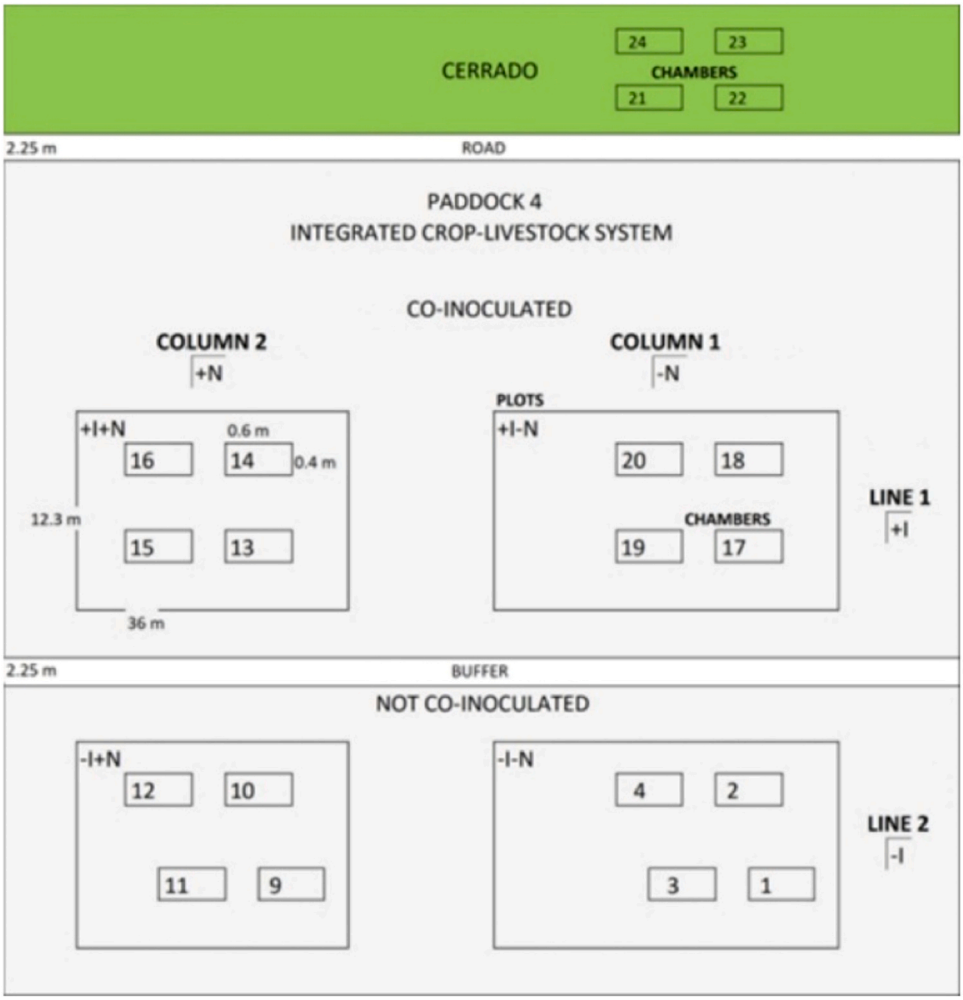

Fig. 1. Design of the field trial established in an integrated crop-livestock system to test the effects of co-inoculation (+I, -I) and mineral N fertilization (+N, -N) on the yield of common bean, N_2O–N fluxes from soil, soil microbiome and other soil- and plant-related variables. A native forest of the Brazilian savanna (Cerrado) was also monitored as a reference for a non-anthropized system. Management of Paddock 4 is described in Oliveira et al. (2022).

mulch, remaining throughout the rainy season of the year. This phase is kept for 2–3 years with rotating crops, followed by another pasture phase.

2.2. Experimental design and treatment implementation

A short-cycle (70 days) common bean cultivar (BRS FC104) was planted after 3.5 years of the pasture phase in the ICLS system. Four treatments, combining the presence or absence of co-inoculation (introduced in the area for the first time) and N fertilization (urea), were implemented at the beginning of the rainy season (end of October) in 2019 and monitored over two consecutive crop years: 2019/2020 (Year 1, Y1) and 2021/2022 (Year 2, Y2), as described in Table 1. In Y1, common bean was sown on October 23, 2019 and harvested on January 11, 2020 and in Y2 common bean was sown on October 26, 2021 and harvested on January 15, 2022. Between Y1 and Y2, throughout the pasture phase in the ICLS, P4 received 1.5 tonne ha^{-1} of liming (CaO 30 %, MgO 17 %, SiO_2 3 %) on September 24, 2020, and N fertilization with urea (50 kg N ha^{-1}) via topdressing on January 11, 2021.

The trial was conducted in two lines (1, 2) within the ICLS, separated by a buffer area, where co-inoculation was applied (+I) or not (-I). Within each line there were two plots of 36 × 13 m. Plots were in columns (2, 1) where synthetic N fertilizer was applied as top dressing (+N) or not (-N), as represented in Fig. 1. An adjacent area of native vegetation, a semi-deciduous seasonal forest, was monitored as reference for non-anthropized soil. On November 22, 2019, four soil samples in each plot were collected at the 0–10 cm soil depth using a hand auger to determine soil properties at time zero. On January 11, 2022, the analysis were repeated with new soil samples, now collected at the 0–20 cm soil depth, to gauge treatment effects after 2 years of implementation. Samples were air dried, passed through a 2-mm sieve, thoroughly homogenised, and divided into sub-samples for analyses of soil fertility indices (exchangeable K, Ca, Mg, Na, Al, pH, and available P [Mehlich I]), including soil organic matter, which was calculated from soil organic C determined by the dichromate method (Teixeira et al., 2017).

Co-inoculation of a mix of three species of *Rhizobium* (*R. topicii, R. freirei and R. leucaneae*) was applied via seed treatment (20,000 bacterial units per ha) before sowing and 300 mL ha^{-1} (10,000 bacterial units per ha) of *A. brasilense* was applied via pulverization at 15 days after sowing (DAS). Treatments with synthetic N fertilization consisted of urea applied as topdressing at 1 DAS (20 kg N ha^{-1}) and 27 DAS (80 kg N ha^{-1}) in Y1 and at 13 DAS (20 kg N ha^{-1}) and 30 DAS (120 kg N ha^{-1}) in Y2. At sowing, all treatments received phosphate and potassium in the formula 00–20–20 (N-P-K): 250 kg ha^{-1} in Y1 (50 kg P_2O_5 ha^{-1} and 50 kg K_2O ha^{-1}) and 190 kg ha^{-1} in Y2 (38 kg P_2O_5 ha^{-1} and 38 kg K_2O ha^{-1}). Treatments without co-inoculation were sown first.

2.3. N_2O–N fluxes, soil- and plant-related variables

Measurements of N_2O–N fluxes were taken using 20 manual static

Table 2
Soil chemical properties within the 0–10 cm soil depth before the crop phase in an integrated crop-livestock system with common bean (*Phaseolus vulgaris* cv. BRS FC104) and a native forest (Cerrado) on a clayey Ferralsol of the Brazilian savanna.

Treatment	pH (H_2O)	Ca^{2+}	Mg^{2+}	Al^{3+}	P	K	Cu	Zn	Fe	Mn	SOM (gkg^{-1})
		——($mmol_c\ dm^{-3}$) —			——————————($mg\ dm^{-3}$) ——————————						
+I-N	5.4*	19.3*	12.8*	1.0*	5.2	168	1.6*	4.8*	61*	42*	41
+I+N	5.2*	18.1*	11.8*	1.3*	5.8	143	1.5*	3.8*	52*	42*	42
-I+N	5.0*	20.3*	11.6*	1.5*	6.9*	152	1.5*	3.5*	43*	53*	41
-I-N	5.4*	30.1*	12.4*	0.5*	3.4	335*	1.6*	4.8*	45*	47*	39
Cerrado	4.4	0.4	1.03	9.3	0.8	48	2.1	0.6	138	25	41
StdError	0.1	5.3	1.6	0.3	2.8	79	0.1	0.5	4	3	2

Treatments include common bean cultivated with and without co-inoculation (+I, -I) and mineral N fertilizer (+N, -N). Means are statistically different from soil under native Cerrado vegetation (* $p \leq 0.05$). Standard error (*StdError*, n = 4) given by Dunnett test. SOM: soil organic matter.

chambers, 4 each for Cerrado, +I+N, +I-N, -I+N, -I-N, as shown in Fig. 1. The manual static chamber consisted of a metal base (0.4-m width x 0.6-m length) and a metal cap (0.15-m height) fixed on it during sampling. When closed, the chamber had a total volume of 36 L. The outside of the chambers was coated with reflective paint to keep the temperature inside the chamber as stable as possible during sampling. Air and soil temperatures (at 0–5 cm depth) and soil samples (at 0–10 cm depth for moisture and mineral N) were taken simultaneously during air sampling as shown in Figure S1. In Y1, average temperatures of air and soil were 32.1 ºC and 26.4 ºC, respectively, and soil moisture was around 23.6 % with total precipitation of 105.8 mm along 41 DAS. In Y2, average temperatures of air and soil were 28.5 ºC and 25.8 ºC, respectively, and soil moisture was around 26.8 % with total precipitation of 232.1 mm along 34 DAS.

Gas samples were taken between 9:00 and 11:00 a.m., as recommended by Alves et al. (2012). Gases accumulated in the static chamber in 0, 15 and 30 min were collected using 60 mL syringes and immediately transferred into 20 mL glass headspaces with chlorobutyl seals. Gas samples were analyzed by gas chromatography with an Ni^{63} containing electron capture detector (ECD), model GC-2014 (Schimadzu Corporation, Kyoto, Japan). Fluxes of N2O–N ($\mu g\ m^{-2}\ h^{-1}$) were calculated based on Rochette et al. (2004) and according to criteria established by HM function as in Ramos et al. (2024).

Soil moisture, ammonium (NH_4–N) and nitrate (NO_3–N) availability were determined from 100-g soil samples, collected at the 0–10 cm soil depth simultaneously with gas sampling. Around 10 g of soil were weighed before and after drying for 24 h at 105 ºC to determine soil moisture. Available ammonium and nitrate were determined by shaking 20 g of soil with 60 mL of 1 mol L^{-1} KCl for 60 min, according to Mulvaney (1996). Extraction was followed by determination through flow injection analysis (Ocean Optics. Inc., Dunedin. FL. USA); the result was given in mg L^{-1} and calculated in mg kg^{-1} of dry soil.

Fluxes were measured in 6–7 consecutive days following events of co-inoculation and N fertilization, then were measured weekly, spread across 41 days after sowing - DAS - common bean from October 28 to December 3 in 2019 (Y1) and 34 DAS, between October 27 and November 29 in 2021 (Y2), as shown in Figure S2. For precise analysis of repeated measurements in time, crop seasons were divided into periods within years. In Y1, period 1 corresponds to six consecutive days right after sowing with co-inoculation of seeds and first N fertilization (PER 1); period 2 to six consecutive days after *A. brasilense* application (PER 2); and period 3 to seven consecutive days right after second N fertilization (PER 3). In Y2, period 1 corresponds to six consecutive days right after sowing with inoculation of seeds (PER 1); period 2 to six consecutive days after *A. brasilense* application and first N fertilization (PER 2); and period 3 to seven consecutive days right after second N fertilization (PER 3).

To account for potential random effects related to repeated measurements taken within the same localization (relative to manual static chambers), analysis of N_2O–N fluxes was performed separately considering repeated measurements within periods of each year (PER 1, PER 2, PER 3) and for the entire crop year (Y1 and Y2). Analyses were performed using the linear mixed model procedure (Proc Mixed) of the SAS software (SAS Institute Inc., Cary, NC, USA, version 2011), as follows:

$$y = X\beta + Zu + e,\ u \sim N(0, G),\ e \sim N(0, R) \quad (1)$$

where: β = vector of fixed effects parameters (treatments 1–5 of the replication 1–4); u = vector of random effects of latent variables associated to location of a chamber (repeated measurements taken in chambers 1–20); e = random error vector associate to each measurement *y*. Conditional residual analysis of data is shown in Figure S3. Conditional residual analysis reduces standard deviation of data analysis from 51.09 to 48.28 in Y1 and from 94.09 to 86.54 in Y2. Dunnett test was applied to contrast co-inoculated treatment (+I-N) averages with the other treatments within periods and years.

Total N_2O emission were estimated summing hourly N_2O–N fluxes measured from static chambers and calculated according to HM function (Hutchinson and Mosier, 1981; Ramos et al., 2024). Therefore, the estimation of total emission represents the limited amount of N_2O-N fluxes measured along 41 and 34 DAS common bean (for Y1 and Y2, respectively), targeting periods following co-inoculation and N fertilization, when most of N_2O-N fluxes were expected to be detected due to the effect of treatments. The emission intensity (EI) was calculated considering the emission of N_2O in CO_2 equivalent (CO_2 eq) per kg of grain produced, using the global warming potential of N_2O–N, which is 273 (100 years) times higher than of CO_2 (IPCC, 2023). The treatment -I-N was used as reference of N lost via N_2O–N fluxes from the soil without co-inoculation and N fertilization. Therefore, the emission factor (EF) of the fertilizer was calculated as the difference between the amount of total N_2O emission in treatments with N fertilizer (-I+N, +I+N) and treatment -I-N, expressed as percentage of total amount of N fertilizer applied.

Grain yield of common beans was determined on January 10 in both 2020 and 2022. Number and weight of nodules per plant, weight of roots and above-ground plant parts were determined at the flowering stage of common beans, on November 22, 2019, and November 25, 2021, following the protocol adopted by Souza and Ferreira (2017).

The statistical analysis for total N_2O emission, and plant- and soil-related variables measured once every crop year was carried out considering position of plots (in a column and row) within the ICLS as a random effect (Fig. 1). Analysis of soil nitrate and ammonium availability, SOM, soil pH, grain yield, total emission and EI were performed considering both crop years as a repetition in time. Analysis for nodulation was done within each crop year, separately. Analyses were performed using the linear mixed model procedure (Proc Mixed) of the SAS software (SAS Institute Inc., Cary, NC, USA, version 2011), as follows:

$$y = X\beta + (c + d) + e,\ c + d \sim N(0, G),\ e \sim N(0, R) \quad (2)$$

where: β = vector of fixed effects parameters (treatments 1–5 of the replicate 1–4); c and d are the potential random effects related to location of a plot in a column (2, 1) and in a line (1, 2) within the ICLS; e = random error associate to each measurement *y*. Dunnett test was

Table 3
N_2O emission and soil variables in an integrated crop-livestock system with common bean (*Phaseolus vulgaris* cv. BRS FC104) and a native forest (Cerrado) on a clayey Ferralsol of the Brazilian savanna in crop years 2019/2020 (Y1) and 2021/2022 (Y2).

Treatment	SOM ($g\ kg^{-1}$)	pH (H_2O)	NO_3–N ($mg\ kg^{-1}$)	NH_4–N ($mg\ kg^{-1}$)	Emission ($kg\ ha^{-1}$)	EI ($g\ CO_2eq\ kg^{-1}$)	EF (%)
				Y1			
+I-N	42.3	5.4	14.97	5.52	0.208	22.52	ND
+I+N	40.5	5.2**	28.72**	16.40**	0.337*	32.34	0.01
-I+N	42.1	5.1**	31.39**	15.58**	0.404**	37.96**	0.06
-I-N	40.1	5.5	27.47**	5.67	0.406**	38.15**	ND
Cerrado	41.9	4.4**	6.51**	5.27	0.068**	ND	ND
SdError	1.4	0.1	3.92	2.71	0.062	6.72	0.04
				Y2			
+I-N	34.2	5.8	5.01	7.18	0.192	16.88	ND
+I+N	33.0	5.5*	9.03**	17.04*	0.595**	56.22**	0.42
-I+N	33.9	5.8	10.08**	20.22**	0.424	38.81	0.22
-I-N	34.3	6.4**	3.48	5.38	0.361	35.22	ND
Cerrado	32.9	4.5**	1.96*	3.82	0.151	ND	ND
SdError	1.7	0.1	1.66	5.12	0.136	14.53	0.21
				Y1 +Y2			
+I-N	38.2	5.6	9.99	6.35	0.199	19.70	ND
+I+N	36.7	5.3	18.87*	16.72**	0.466**	44.28**	0.21
-I+N	38.0	5.4	20.73**	17.90**	0.414**	38.39**	0.14
-I-N	37.2	5.9*	15.48	5.53	0.383**	36.68**	ND
Cerrado	37.4	4.5**	4.24	4.54	0.109	ND	ND
SdError	2.3	0.2	5.08	2.75	0.077	8.11	0.13

Treatments include common bean cultivated with and without co-inoculation (+I, -I) and mineral N fertilizer (+N, -N). SOM: soil organic matter and soil pH within the 0–20 cm soil depth; Emission: integration of N_2O–N fluxes per crop season; EI: emission intensity, GWP 100 years (IPCC, 2023), per unit of grain produced; EF: emission factor for the total amount of synthetic N applied. Means are statistically different from treatment *+I-N* according to Dunnett test and *p*-values: ** $0.01 \leq p \leq 0.05$; * $0.05 \leq p \leq 0.10$. *SdError*: standard error of means ($n = 4$). ND: not determined.

Table 4
Pearson correlation coefficients among means of N_2O emission and soil variables, in crop years 2019/2020 (Y1) and 2021/2022 (Y2), under an integrated crop-livestock system cultivated with common bean (*Phaseolus vulgaris* cv. BRS FC104), including and not measurements taken in a native forest (Cerrado) on a clayey Ferralsol of the Brazilian savanna*.

	Including Cerrado				Excluding Cerrado			
	NO_3–N	NH_4–N	pH	SOM	NO_3–N	NH_4–N	pH	SOM
Y1								
R^2	**0.65**	0.36	−0.24	−0.17	**0.83**	**0.49**	**0.55**	−0.18
p-value	0.0064	0.1649	0.3643	0.5386	< 0.0001	0.0271	0.0113	0.4571
N	16	16	16	16	20	20	20	20
Y2								
R^2	0.35	0.16	−0.25	−0.07	**0.49**	0.29	0.17	0.05
p-value	0.1862	0.5647	0.3537	0.8049	0.0274	0.2197	0.4814	0.8428
N	16	16	16	16	20	20	19	20

* According to data presented in Table 3. Bold values highlight statistically significant R^2.

applied to contrast the effect of treatment +I-N with the other treatments. Pearson correlation coefficients (R^2) among N_2O total emission and plant- and soil-related variables were determined for each crop year (Y1 and Y2). Data of native Forest was used or not to check for correlations in a non-anthropized system.

2.4. Microbiome in the common bean rhizosphere

Soil samples were collected during flowering (R6 phenological stage: 42 and 38 DAS in Y1 and Y2, respectively) and stored at −80 °C until DNA extraction, with the sampling authorization legally granted in Brazil and registered with SISGen under number A62993F. DNA was extracted from 250 mg of soil per sample using the PowerSoil® DNA Isolation Kit following the manufacturer's protocol (Qiagen, Doraville, CA). The region V3–V4 of the 16S rRNA gene was amplified and sequenced with Illumina MiSeq (2 × 300 cycles run) using the pair of primers 341 F (CCTAYGGGRBGCASCAG) and 806 R (GGACTACNNGGGTATCTAAT). The Illumina demultiplexed paired-end sequenced dataset was processed with the DADA2 package (Callahan et al., 2016) to correct for amplicon errors, to identify chimeras, and to merge paired-end reads. In more detail, the sequencing data files containing forward reads (fnFs) and reverse reads (fnRs) were processed by truncating reads at the first instance of a quality score lower than 2. After truncation, reads with more than 2 errors were discarded. Following error inference, the dereplication step was performed using the derepFastq function on the filtered forward (filtFs) and reverse (filtRs) reads. The denoised forward and reverse reads were then merged and chimeric sequences were removed using the "consensus" method. Then, taxonomic ids were assigned to the amplicon sequence variants (ASVs) against the SILVA 138 SSU database (Quast et al., 2012).

For the ecological diversity metrics, non-rhizosphere soil (bulk soil; BSL) was added as an internal control. Prior to all analyses, taxa with a prevalence lower than 5 % (i.e., taxa with a non-zero count in less than 5 % of the samples) were trimmed. Then, we performed an exploratory ordination looking for outlier samples, removing three samples from our dataset. Community level differences in relative abundance, alpha and beta diversity were analyzed using Phyloseq (McMurdie and Holmes, 2013) and Vegan (Oksanen et al., 2024) in R (version 3.2.0; R Core Team, 2020). First, the top 10 taxa at the phylum, family, and genus level were plotted for each treatment within a year.

For alpha diversity, we calculated the Observed richness (number of ASVs/sample) and the Shannon diversity index. Observed richness is calculated considering only the number of identified ASV and is a measure of species richness while the Shannon index also considers the relative abundance of these ASV in the samples (thus also being a measure of species evenness). Data were tested for normality (Shapiro-Wilk test; $p > 0.05$) and homogeneity of variances (Bartlett's test; $p > 0.05$) prior to ANOVA in the R environment with default parameters (R Core Team, 2020). When ANOVA was significant, a Dunnett test was applied to contrast +I-N means with the other treatments for each alfa diversity metric within a crop year. For beta diversity, principal coordinates analysis (PCoA) plots were created and between sample differences were tested using a permutational multivariate ANOVA, both using Bray-Curtis's dissimilarity. For the PERMANOVA, different models were fitted to find out what factors explained the difference between a) bulk soil and rhizosphere and b) the different treatments using both years combined or separately.

The linear discriminant analysis effect size (LEfSe) algorithm was used to identify discriminating features at the genus level that were significant both statistically and biologically (biomarker discovery) using the MicrobiomeMarker package (Cao et al., 2022). For this analysis, the cutoff value for both the Kruskal-Wallis and Wilcoxon tests were set as $p = 0.05$ and the linear discriminant analysis (LDA) effect size > 2.5 was set as the threshold. The functional abundance in the samples was estimated using the FAPROTAX algorithm (Sansupa et al., 2021) as implemented in the microeco package (Liu et al., 2021). A

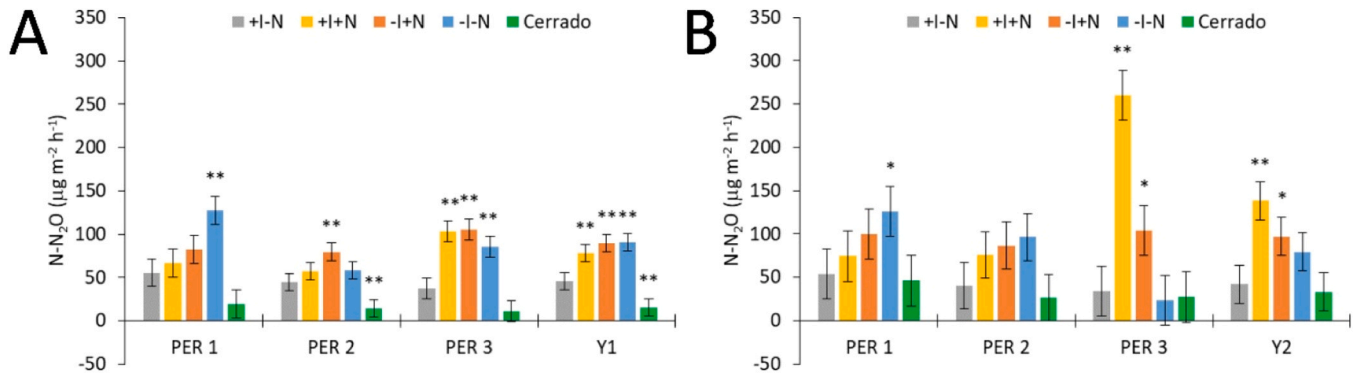

Fig. 2. Average nitrous oxide fluxes (N_2O–N) from soil (µg m^{-2} h^{-1}) in an integrated crop-livestock system with common bean (*Phaseolus vulgaris*) BRS FC104 cultivated with and without co-inoculation (+I, -I) and mineral N fertilizer (+N, -N), and in a native forest (Cerrado) on a clayey Ferralsol of the Brazilian savanna. Average within periods (PER 1, PER 2 and PER 3) in years 2019/20 (Y1: A) and 2021/22 (Y2: B). In Y1: 6 days after sowing with co-inoculation of seeds and N fertilization (PER 1); 6 days after *Azospirillum brasilense* application (PER 2); and 7 days after N fertilization (PER 3). In Y2: 6 days after sowing with co-inoculation of seeds (PER 1); 6 days after *Azospirillum brasilense* application and N fertilization (PER 2); 7 days after N fertilization (PER 3). Means are statistically different from treatment +I-N according to Dunnett test and *p*-values: ** $0.01 \leq p \leq 0.05$; * $0.05 \leq p \leq 0.10$. Error bars are standard error of means ($n = 4$). Raw data of N_2O-N fluxes shown in Fig. S2. Residual analysis of raw data shown in Fig. S3.

Kruskal–Wallis H test was initially conducted to identify variables showing potential associations with treatments ($p \leq 0.1$). Subsequently, the variables found to be significant in the Kruskal-Wallis H test were submitted to Dunn's test ($p \leq 0.1$) to perform pairwise comparisons between treatment groups. Since the primary focus was on comparisons with a control group, Dunn's test results were filtered to include only comparisons between the control and each treatment. Adjustments for multiple comparisons were made using the Benjamini-Hochberg method to control the false discovery rate. The Kruskal-Wallis H test was performed using base R, while the Dunn's test was performed with the Dunn test package (Dinno and Dinno, 2017).

3. Results

3.1. Soil chemical properties and nitrogen dynamics

All soil chemical properties within the 0–10 cm soil depth under common bean cultivation in the integrated crop-livestock system (ICLS) were statistically different, in all treatments, from the native forest (Cerrado), except for SOM (Table 2). At sowing, fertilization with the same amount of P and K to all treatments within the ICLS was done to adjust them to the same initial baseline. Significant differences of SOM within the 0–20 cm soil depth were also not observed throughout crop years in the ICLS (Table 3).

Considering both years, the nitrate (NO_3–N) and ammonium (NH_4–N) available in soil was significantly higher in treatments with N fertilization i.e., +I+N and -I+N (18.87, 20.73 ± 5.1 mg NO_3–N kg^{-1}; and 16.72, 17.90 ± 2.8 mg NH_4–N kg^{-1}, respectively) than in the treatment with co-inoculation only (+I-N; 9.99 ± 5.1 mg NO_3–N kg^{-1} and 6.35 ± 2.8 mg NH_4–N kg^{-1}). The concentration of soil NO_3–N and NH_4–N was positively correlated to total N_2O emission in Y1 (R^2: 0.83 and 0.49, respectively), including Cerrado (Table 4).

In Y1, average hourly N_2O–N fluxes were significantly lower in +I-N (45.48 ± 10 µg m^{-2} h^{-1}) than in all other treatments, including -I-N (90.48 ± 10 µg m^{-2} h^{-1}) (Fig. 2A). The highest N_2O–N fluxes were observed for -I-N (127.44 ± 16 µg m^{-2} h^{-1}) in PER 1, right after sowing common beans. In PER 2 and PER 3, -I+N had the highest N_2O–N fluxes (79.71 ± 10 and 105.56 ± 12 µg m^{-2} h^{-1}, respectively). At the end of Y1, only the native Cerrado soil had lower N_2O–N fluxes (14.84 ± 10 µg m^{-2} h^{-1}) than +I-N (45.48 ± 10 µg m^{-2} h^{-1}).

In PER 1 of Y2, average hourly N_2O–N fluxes were significantly higher in -I-N (126.30 ± 29 µg m^{-2} h^{-1}) than +I-N (53.75 ± 29 µg m^{-2} h^{-1}). In Y2, the effect of N fertilization on enhancing N_2O–N fluxes was more evident, especially in PER 3, when fluxes were significantly higher in +I+N and -I+N (260.18 and 140.38 ± 29 µg m^{-2} h^{-1}, respectively) than in +I-N (33.90 ± 29 µg m^{-2} h^{-1}). For the same period, fluxes in +I-N (33.90 ± 29 µg m^{-2} h^{-1}) were equivalent to -I-N (23.74 ± 29 µg m^{-2} h^{-1}) and Cerrado (27.16 ± 29 µg m^{-2} h^{-1}).

Finally, regardless of the period, treatments with N fertilization had significantly higher fluxes (+I+N: 138.60; -I+N: 97.04 ± 22 µg m^{-2} h^{-1}) than +I-N (42.15 ± 22 µg m^{-2} h^{-1}) in Y2 (Fig. 2B). In this crop year, N_2O–N fluxes were positively correlated with soil NO_3–N (Table 4). Considering both years, emission intensity (EI; Table 3) was significantly higher in +N treatments (+I+N: 44.28 and -I+N: 38.39 ± 8.1 g CO_2eq kg^{-1}) and -I-N (36.68 ± 8.1 g CO_2eq kg^{-1}) than in +I-N (19.70 ± 8.1 g CO_2eq kg^{-1}).

3.2. Common bean growth, yield, and nodulation

The treatments with co-inoculation (+I-N, +I+N) increased the number and weight of nodules in Y1 (Table 5). The number and weight of nodules (9 ± 7 and 0.004 ± 0.005 g $plant^{-1}$, respectively), root mass (0.29 ± 0.02 g $plant^{-1}$) and above-ground plant weight (3.63 ± 0.91 g $plant^{-1}$) in -I-N were significantly lower than in +I-N. Differences in grain yield of common bean were only observed in Y1 (Table 5).

Similarly, in Y2, the number and weight of nodules were significantly higher in +I-N (55 ± 10, 0.101 ± 0.009) than in +I+N (36 ± 10, 0.036 ± 0.009) and -I+N (33 ± 10, 0.031 ± 0.009). On the other hand, the weight of roots and plants were significantly higher in treatments with N fertilization (Table 5).

3.3. Microbiome in the common bean rhizosphere

After prevalence filtering and outlier removal, we found 8111 ASVs that were assigned to 484 different genera across all samples. Visually, there was little difference between treatments and between treatments and the bulk soil at the phylum level within a year, with Actinobacteriota and Proteobacteriota being the prevalent phyla. At the family level, no clear difference was found in the first year, while we observed an increased number of ASVs assigned to Rhizobiaceae in treatments that received N fertilization in second year (-I+N and +I+N; Fig. 3A). When visually comparing the bulk soil and the treatments, the abundance of Gemmatimonadaceae was higher in BSL than the treatments in Y1, while the treatments had higher abundance of Rhizobiaceae, especially in +N treatments, in Y2. At the genus level, the top 10 genus accounted for ~40 % of all counts in Y1 and Y2, except for +N treatments, where it accounted for ~55 % (Fig. 3B). That was due to an increase in counts assigned to closely related genera belonging to the

Table 5
Plant variables of common bean (*Phaseolus vulgaris* cv. BRS FC104) cultivated in an integrated crop-livestock system on a clayey Ferralsol of the Brazilian savanna in crop years 2019/2020 (Y1) and 2021/2022 (Y2).

Treatment	Y1					Y2				
	NN	WN	WR	WP	GY	NN	WN	WR	WP	GY
+I-N	28	0.016	0.53	5.49	2861	55	0.101	0.15	1.59	3596
+I+N	27	0.009	0.38**	5.07	3334*	36*	0.036**	0.26**	2.52*	3319
-I+N	7**	0.001**	0.35**	4.19	3280	33*	0.031**	0.23*	2.65*	3452
-I-N	9**	0.004**	0.29**	3.63*	3322*	36*	0.093	0.18	2.03	3116
StdError	7	0.005	0.02	0.91	258	10	0.009	0.04	0.48	306

Treatments include common bean cultivated with and without co-inoculation (+I, -I) and mineral N fertilizer (+N, -N). NN: number of nodules per plant; WN: weight of nodules per plant (g); WR: weight of roots per plant (g); WP: plant aerial part weight (g); GY: grain yield (kg ha^{-1}). *StdError*: standard error of means (n = 4). Means are statistically different from treatment +I-N according to Dunnett test and *p*-values:
* $0.05 \leq p \leq 0.10$.
** $0.01 \leq p \leq 0.05$;

family Rhizobiaceae (*Allorhizobium*, *Neorhizobium*, *Pararhizobium*, *Rhizobium*) with no detriment to the other top 10 genera (in comparison to -N treatments).

Two measures of alpha diversity were calculated: observed richness and Shannon index. All treatments had similar observed richness in both years. In contrast, both treatments that received nitrogen fertilization (-I+N and +I+N) had lower Shannon index values than -I-N ($p \leq 0.05$), indicating some taxa are more prevalent in these treatments (Fig. 3C).

In the principal coordinate analysis with Bray-Curtis dissimilarities, samples were clearly separated by year (Fig. 4A). Within each year, bulk soil samples are grouped together and separated from samples at the rhizosphere of common bean. In Y1, the most divergent treatment was -I+N, but it was not possible to see a trend regarding any of the factors (Fig. 4B). In Y2, there is a clear separation between -N and +N treatments, but not between -I and +I treatments (Fig. 4C).

Then, we fitted a model to find out which factors contribute to differences between treatments. For Y1, co-inoculation and the interaction between co-inoculation and N fertilization had $p = 0.097$ and $p = 0.014$, respectively. For Y2, N fertilization had $p < 0.001$. Combining both years, the factors that explained part of the variance were N fertilization, year and their interaction (Table 6).

The LEFSE analysis found 13 marker genera for the first year and 46 in the second year, including 4 genera belonging to the Rhizobiaceae family (*Allorhizobium, Neorhizobium, Pararhizobium, Rhizobium*) that was enriched in -I+N (LDA = 5.37) and *Bradyrhizobium,* enriched in +I-N (LDA = 3.97) (Fig. 5).

The number of FAPROTAX functions predicted were 44 and 45 for Y1 and Y2, respectively. For easier comparison, only appropriate i.e., functions related to the N cycle, and significant functions according to a Kruskal-Wallis H test are shown (Fig. 6). A comparison between both years shows divergent results, with -I+N having the lowest relative abundance of species with the N fixation function in Y1 and the second highest in Y2. Y1 had only two significant FAPROTAX functions, namely ureolysis and nitrogen fixation. Dunn's test showed that the relative abundance of both functions in -I+N was significantly lower than +I-N. On the other hand, Y2 had several exclusive, significant denitrification-related functions. In all of them, -N treatments had higher relative abundance than +N treatments, except for nitrogen fixation.

4. Discussion

In this study, we investigated the effects of co-inoculation and N fertilization on N_2O–N fluxes, soil- and plant-related variables, including bacterial community in the rhizosphere of common bean plants under an integrated crop-livestock system (ICLS). Conducting a field trial on ICLS was crucial to evaluate the impact of co-inoculation under conditions that incorporate technologies aimed at improving soil quality and resource use efficiency. Our focus was to study how co-inoculation alone (+I-N) would compare with treatments receiving nitrogen fertilization, while -I-N was added as a negative control. Our hypothesis was that co-inoculation could reduce the reliance on intensive N_2O–N emitting fertilizers, such as urea, which is one of the most common sources of N (55 %) and accounts for 6 % of total emission from the Brazilian agriculture sector, with a historical consumption of 6 million tonnes in 2021 (Alencar et al., 2023). Besides the effect of co-inoculation, we also tested N fertilization, as there are studies reporting that early N supplementation is necessary to kick start the growth of common bean plants before the root nodules are properly formed (Soares et al., 2016; Zoffoli et al., 2021).

Soil chemical properties within the 0–10 cm soil depth under ICLS were statistically different from those under the native forest (Cerrado), except for soil organic matter (SOM), which was equivalent in the ICLS treatments and the native Ferralsol of Cerrado at field trial establishment (Table 2) and throughout crop years of common bean under the ICLS within the 0–20 cm soil depth (Table 3). SOM is a fundamental indicator of soil quality and food security (Lal, 2004). The similar SOM

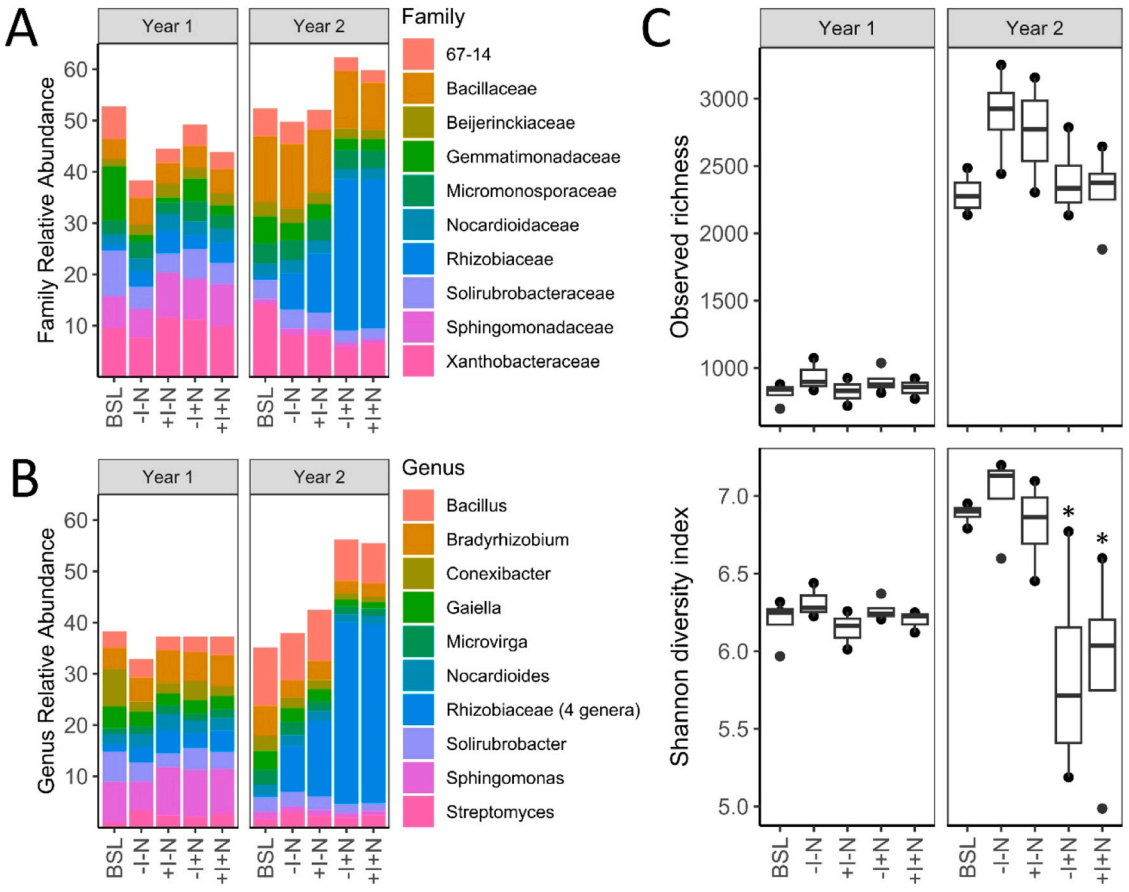

Fig. 3. Relative Abundance (%) of 16S (A) families and (B) genera, and alpha diversity metrics (C; observed richness and Shannon diversity index) in the rhizosphere microbiome of common bean (*Phaseolus vulgaris*) BRS FC104 cultivated in an integrated crop-livestock system with and without co-inoculation (+I, -I) and N fertilization (+N, -N), in crop years 2019/2020 (Year 1) and 2021/2022 (Year 2). Non-rhizosphere soil (bulk soil; BSL) was used as an internal control. In C, boxes headed by an asterisk are statistically different from treatment +I-N according to Dunnett test ($p \leq 0.05$).

levels in soils under ICLS and native Cerrado shows the benefit of intensifying plant diversity in agroecosystems, whether forages and crops are grown in the same space simultaneously or at different times of the year. As shown in Oliveira et al. (2022), the soil under the ICLS required 20 years to increase soil organic C at a rate of 800 kg ha^{-1} year^{-1} within the 0–10 cm soil depth. This aligns with global initiatives, such as the "4 per 1000" Initiative, which emphasize the role of agricultural soils in carbon sequestration and their potential to mitigate climate change (Minasny et al., 2017). Our findings show that ICLS can contribute to these objectives by maintaining SOM in quality levels and reducing dependency on mineral N from fertilizer to keep up common bean yield, offering sustainable pathways for food security and climate resilience.

N fertilization was influential in the composition of the root microbiome according to the PERMANOVA. We found that N fertilization increased the abundance of *Rhizobium* and related genera in detriment to several genera in Y2, according to the LEFSE analysis (Fig. 5). Our result conforms with Sepp et al. (2023) who, working with environmental DNA (eDNA) from 327 spatially distinct soil samples, found that rhizobia richness was negatively associated with the non-N-fixing prokaryotic community. The same authors also showed that two soil variables have distinct effects on different N-fixing groups, with intermediate soil pH levels (~ 5.5) favoring rhizobia richness and intermediate values of soil total N content (~ −1 [ln(N)]) favoring total N-fixers and Cyanobacteria. Under the condition of our study, soil pH was around 5.0 in Y1, and around 6.0 in Y2 (Table 3). In both crop years (Y1 +Y2), +I-N had significantly higher pH (5.6) than +I+N (5.3). Castellano-Hinojosa et al. (2021) found that *Rhizobium* spp. presence in the rhizosphere of common beans were negatively correlated with total N in the soil. At concentrations above 100 kg N ha^{-1}, the contribution for yield of rhizobial N fixation becomes negligible (Pias et al., 2022), with reduced symbiotic N fixation in common bean (Reinprecht et al., 2020). The same effect was observed in this study, especially in Y2, when the effect of N fertilization enhancing N_2O–N fluxes was more evident (Fig. 2), regardless co-inoculation, decreasing number and weight of nodules, although no significant differences on common bean grain yield was observed (Table 5).

The nitrogen cycle is a complex process that encompasses several steps: atmospheric N_2 is fixed into ammonia (NH_3) by free-living and symbiotic bacteria and archaea (diazotrophs) in a process called N fixation. In the soil, NH_3 may be converted into ammonium (NH_4^+) which can be oxidized to nitrate (NO_3^-), in a process called nitrification. Then, nitrite (NO_2^-) and NO_3^- may be reduced during the denitrification process, generating the gaseous forms of nitrogen, especially nitrous oxide (N_2O) and nitrogen (N_2), depending on soil aeration (Signor and Cerri, 2013). However, N_2O release is not limited to nitrification. In fact, multiple biological pathways, namely ammonia oxidation, nitrifier denitrification, nitrite oxidation, heterotrophic denitrification, anaerobic ammonium oxidation and dissimilatory nitrate reduction to ammonium (Wrage et al., 2001; Hu et al., 2015), may produce it. N_2O-N fluxes is particularly dominated by intermittent nitrification and denitrification processes in soil (Hu et al., 2015). A straight comparison between years Y1 and Y2 shows divergent results for N fixation. In Y1, the only different treatment was -I+N, with a lower relative abundance

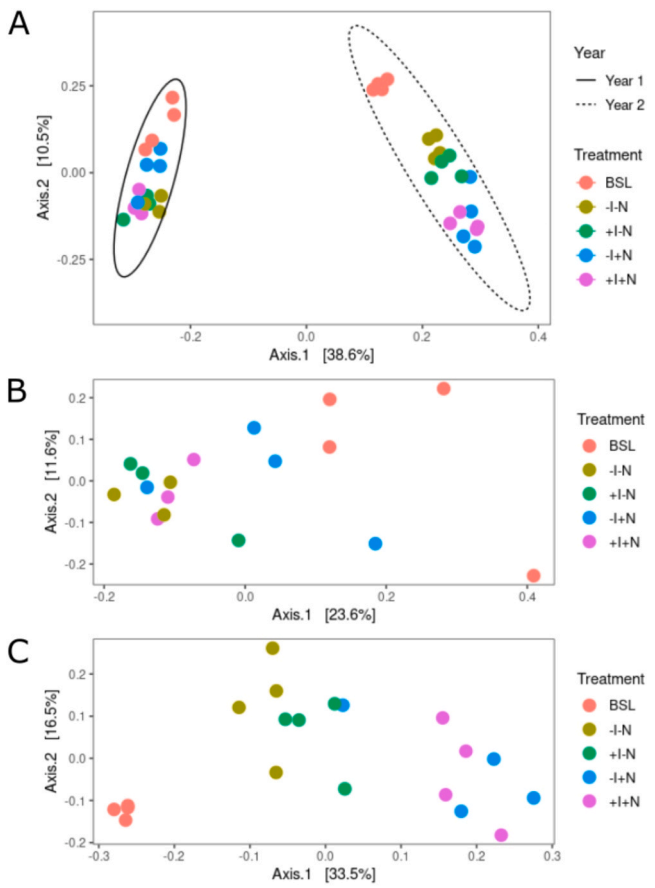

Fig. 4. Principal Coordinate Analysis (PCoA) with Bray-Curtis dissimilarity of the 16S rhizosphere microbiome of common bean (*Phaseolus vulgaris*) BRS FC104 cultivated in an integrated crop-livestock system with and without co-inoculation (+I, -I) and N fertilization (+N, -N), in crop years 2019/2020 (Y1) and 2021/2022 (Y2). Both crop years combined (A), Y1 (B), and Y2 (C). Non-rhizosphere soil (bulk soil; BSL) was used as an internal control.

of species with predicted genes for N fixation. In Y2, -I-N was the treatment with the lowest relative abundance, followed by -I+N, +I-N and +I+N (Fig. 6). All cropping conditions being equal, we can only hypothesize that environmental conditions (Fig. S1) may have played a part in these results and that consecutive years of experiment in the same area resulted in cumulative effects.

Co-inoculation clearly inhibited N_2O-N fluxes in Y1 when N fertilization was not applied (+I-N), as can be seen by comparing it to -I-N (Fig. 2). In the ICLS system, which is rich in SOM, N_2O fluxes were stimulated. This often occurs when soil has high organic nitrogen, especially ammonium, which ignites soil processes at the start of the rainy season or after sowing (Carvalho et al., 2013). There were higher levels of available ammonium in +N soils, regardless of crop year (Table 3). However, levels were especially high in Y1, where N_2O-N fluxes were positively correlated with the available NH_4–N (Table 4). Urea, in the presence of water, is hydrolyzed by the urease enzyme and converted into ammonium (NH_4^+) (Zaman and Blennerhassett, 2010). In the soil, ammonium (NH_4^+) is converted into nitrate (NO_3^-) in the nitrification process, which is mediated by ammonia-oxidizing Archaea and Bacteria and nitrite-oxidizing Bacteria (Francis et al., 2005; Muck et al., 2019). Once again, we found divergent results for [NH_4^+] and [NO_3^-] for each year. In Y1, there was a greater NO_3^-/NH_4^+ ratio than in Y2. Nitrification is a very dynamic process, influenced by various factors, including soil matrix, water status, aeration, temperature and pH (Sahrawat, 2008). One factor that may explain this divergence in magnitude between crop years is soil moisture, which was higher in Y2 (26.8 %) than in Y1 (23.6 %). Although soil nitrification is known to increase with an increase in soil moisture, it seems there is a ceiling. Meng et al. (2020) reported nitrification increased when soil water content increased up to 27.03 % and decreased when soil moisture > 27.03 %. Consistent with their results, the soil water content in Y1 was lower than 25 % throughout the crop year, whereas it was equivalent to 27 % in Y2. Carvalho et al. (2016) has also reported how discontinuity of water filling pore space (WFPS) can increase the nitrification/denitrification process in soil and therefore increase N_2O–N fluxes detection when common bean was cultivated under no-tillage and pivot irrigation. On the other hand, when WFPS is continuously high, denitrification can be dominant and then N_2O are overcome by not detectable N_2 fluxes. In fact, in our study, the magnitude of N_2O emission was higher in Y2 than in Y1 (Table 3, Fig. S2), including the treatment under native savanna (Cerrado), showing that intermittent nitrification/denitrification process must have been dominant in Y2 than in Y1. The emission factor for N_2O lost to atmosphere due to application of synthetic N fertilization, regardless of co-inoculation, was around 0.1–0.4 %, lower than that preconized by IPCC of 1 % (IPCC, 2019).

An increased amount of ammonium and nitrate in the soil has been proved to have a positive correlation with N_2O release by the soil nitrifier community (Avrahami et al., 2002), but this was due to physiological shifts rather than changes in the community structure of ammonia oxidizers. An in-vitro study by Liu et al. (2016) found that nitrification is a primary driver of N_2O production in several agricultural land-use scenarios under aerobic conditions, although their study focused on monocots (sugar cane, pasture, and cereals). Analyzing each year separately, we did not find quantitative differences in the relative abundance of nitrifiers among treatments in any of the crop years (Fig. 6). We did find, however, an increase in several denitrification-related functions in -N treatments in Y2, which may be related to the intermittent soil moisture content, as Y2 was a rainier crop

Table 6
Permutation Multivariate Analysis of Variance (PERMANOVA) and Permutation-based Test of Multivariate Homogeneity (Permutest) for the effects of the experimental factors and their interaction on the 16S rhizosphere microbiome structure of common bean (*Phaseolus vulgaris* cv. BRS FC104) cultivated under an integrated crop-livestock system on a Ferralsol of the Brazilian savanna in crop years 2019/2020 (Y1) and 2021/2022 (Y2).

Comparison	Year	Permutest *p*-value	PERMANOVA *p*-values			
			Crop Year[1]	Type[2]	Nitrogen[3]	Co-inoculation[4]
Bulk soil x Rhizosphere	Y1 +Y2	0.235	< 0.001*	< 0.001*	NA	NA
	Y1	0.894	NA	< 0.001	NA	NA
	Y2	0.001	NA	< 0.001	NA	NA
Between treatments	Y1 + Y2	0.922	< 0.001*	NA	0.032*	0.241
	Y1	0.128	NA	NA	0.150*	0.114*
	Y2	0.988	NA	NA	< 0.001	0.172

Non-rhizosphere soil (bulk soil) was used as an internal control (Bulk soil x Rhizosphere). NA: factor not used in PERMANOVA analysis.[1]Crop year: Y1 (2019–2020); Y2 (2021–2022). [2]Type: Bulk soil; Rhizosphere. [3]Nitrogen: treatments with and without synthetic N fertilization. [4]Co-inoculation: treatments with and without co-inoculation.

* Factors whose interaction were significant ($p < 0.05$).

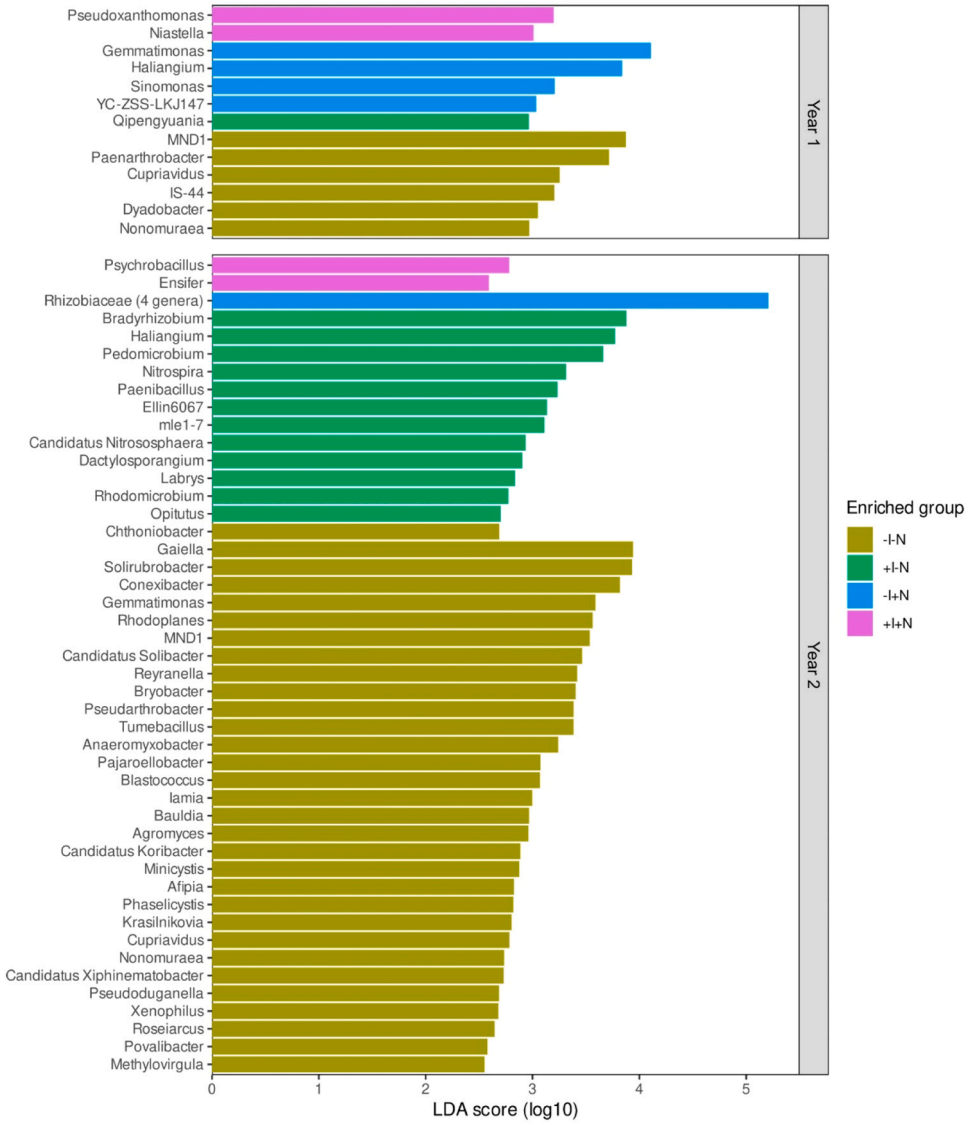

Fig. 5. Linear discriminant analysis (LDA) score of differentially abundant genera in the 16S rhizosphere microbiome of common bean (*Phaseolus vulgaris*) BRS FC104 cultivated under an integrated crop-livestock system with and without co-inoculation (+I, -I) and N fertilization (+N, -N), in crop years 2019/2020 (Y1) and 2021/2022 (Y2). Only genera with $p \leq 0.05$ and LDA ≥ 2.5 are shown. Rhizobiaceae (4 genera) includes the genera *Allorhizobium*. *Neorhizobium*. *Pararhizobium* and *Rhizobium*.

year. A cross-site study with five different soils did not find any effect of successive N inputs on the denitrification potential (Qu et al., 2014). The authors found that denitrification rates in these soils are controlled primarily by the same factors that affect their oxic respiration rates. Nevertheless, the annual input of organic material is a significant factor, which aligns with our study reporting high soil organic carbon under the ICLS. Our data shows that soil moisture and N fertilization increased N_2O-N fluxes in Y2 due to higher denitrification rates with increased soil moisture (Tan et al., 2018).

Considering the high C content of the studied Ferralsol in an ICLS of the Brazilian savanna, we found that co-inoculation reduced N_2O–N fluxes when no synthetic N fertilization was applied while maintained grain yield of common beans, along two crop years. Meanwhile, N fertilization reduced nodulation, had no effect on yield, and enhanced N_2O emission. Our study clearly demonstrates that it is possible to produce common bean yielding 3 Mg ha^{-1}, more than the national average (1.1 Mg ha^{-1}) in Brazil in 2023/24 (Embrapa, 2023), without application of urea in ICLS. This result is achievable by the exclusive use of co-inoculation in the ICLS, emitting two-fold less N_2O to the atmosphere, showcasing that the synergy of technologies (cultivars, microorganisms, integrated systems, no-tillage) is a feasible solution for low C agriculture in cropping systems of the Brazilian savanna. While the potential of ICLS to mitigate greenhouse gas emissions merits further exploration across diverse climatic and soil conditions, our study highlights the system's feasibility. By reducing synthetic N dependency while maintaining productivity, ICLS aligns well with sustainable intensification goals, such as those outlined in the previously mentioned "4 per 1000" Initiative and Brazilian Public Policy ABC+ 2020–2030.

CRediT authorship contribution statement

de Britto Siqueira Matheus Mentone: Investigation. **Schulman Pablo:** Writing – review & editing, Writing – original draft, Formal analysis, Data curation. **de Araujo Wilker Alves:** Investigation. **Hirose**

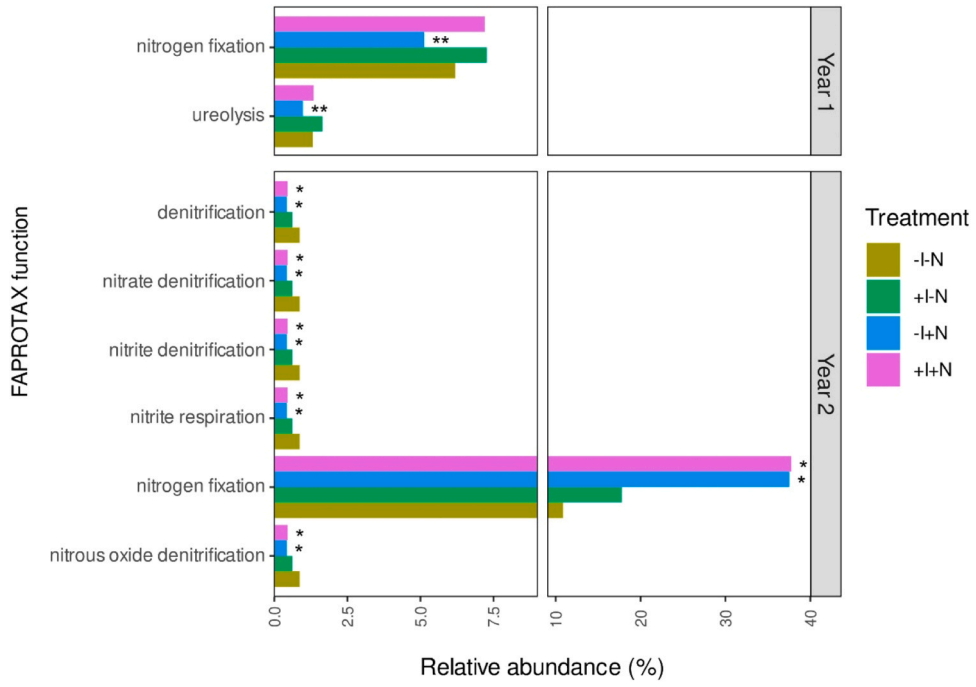

Fig. 6. Relative abundance (%) of differentially (Kruskal-Wallis H test; $p \leq 0.1$) abundant nitrogen-related predicted FAPROTAX functions in the 16S rhizosphere microbiome of common bean (*Phaseolus vulgaris*) BRS FC104 cultivated under an integrated crop-livestock system with and without co-inoculation (+I, -I) and N fertilization (+N, -N), in crop years 2019/2020 (Y1) and 2021/2022 (Y2). For each FAPROTAX function, means are statistically different from treatment +I-N according to Dunn's test and p-values: ** $0.01 \leq p \leq 0.05$; * $0.05 \leq p \leq 0.10$.

Edson: Investigation, Data curation. **Mendes Rodrigo:** Writing – original draft, Visualization, Validation, Methodology. **de Oliveira Borba Tereza Cristina:** Supervision, Formal analysis, Data curation. **Ferraresi Tatiana Maris:** Methodology, Investigation, Formal analysis. **de Brito Ferreira Enderson Petrônio:** Writing – review & editing, Methodology, Investigation, Data curation, Conceptualization. **Machado Pedro Luiz Oliveira de Almeida:** Writing – review & editing, Writing – original draft, Resources, Project administration. **Madari Beáta Emőke:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **de Oliveira Morais Pedro Augusto:** Supervision, Methodology, Investigation, Formal analysis. **de Mello Raquel Neves:** Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Rodrigues da Silva Ryan:** Investigation. **de Melo Carvalho Márcia Thaís:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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Declaration of Competing Interest

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

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.soilad.2025.100046.

Data availability

Data will be made available on request.

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