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Pigeon pea-mediated soil microbial shifts improve agroecosystem multifunctionality in long-term maize–palisade grass intercropping

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Abstract

Background Intercropping systems enhance agricultural sustainability by promoting ecosystem multifunctionality (EMF). This study examined the impact of adding pigeon pea (M + PG + PP) into a maize–palisade grass (M + PG) intercropping system under a no-till system (NTS) on soil microbial communities and ecosystem services. After five consecutive growing seasons, bulk soil samples from a soybean-based crop-livestock system were analyzed using metagenomics.

Results The inclusion of pigeon pea significantly improved the EMF index, with higher plant productivity and slightly enhanced outcomes in soil health, lamb meat productivity, and climate protection. The M + PG + PP treatment enriched *Bradyrhizobium* spp., which were positively correlated with soil health, plant productivity, and EMF index. Functional analysis indicated that M + PG + PP treatment enhanced nitrogen metabolism, biofilm formation, and exopolysaccharide (EPS) biosynthesis, improving soil fertility and microbial activity. Similarly, functional analysis of microbial plant growth-promoting traits revealed that the M + PG + PP treatment promoted microbial functions related to nitrogen and iron acquisition, sulfur assimilation, and plant colonization, all essential for plant growth and nutrient cycling. In contrast, the M + PG treatment primarily enhanced pathways related to competitive exclusion and phytohormone production.

Conclusions These findings highlight the importance of incorporating legumes such as pigeon pea into intercropping systems to optimize ecosystem services, enhance soil health, and promote long-term agricultural productivity and sustainability.

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Keywords *Cajanus cajan*, Soil fertility, Sustainable agriculture, Crop-livestock integration, Shotgun metagenomics, legume-*Bradyrhizobium* interaction

Introduction

Some intensive crop and livestock management practices can hinder agricultural sustainability by lowering grain yields, soil fertility, and pasture carrying capacity, potentially accelerating soil erosion and degradation [1]. In tropical regions, continuous cropping often leads to severe soil quality degradation, which undermines efforts to maintain or restore the productive potential of soils [2]. Globally, there is a growing interest in restoring degraded lands, reducing production costs, and intensifying year-round agricultural activity [3]. Conservation agriculture, particularly through integrated management systems, has proven effective in preserving natural resources and promoting sustainable use of tropical soils [4].

Integrated crop-livestock systems (ICLS), especially under a no-till system (NTS), offer a range of benefits, including food production (grains, meat, milk) and environmental services such as soil conservation, erosion control, and improved water and air quality [3, 5]. These systems also support biodiversity, reduce greenhouse gas (GHG) emissions, and lower herbicide use while boosting the rural economy and improving livelihoods [6, 7]. Their profitability and environmental advantages make ICLS under NTS a key strategy for achieving socio-economic and ecological sustainability [8, 9]. Complex crop-soil interactions in ICLS under NTS can help achieve dynamic equilibrium (steady-state) for long-term sustainability, with changes in soil properties, including microbial biomass and diversity, directly influencing crop productivity [10, 11]. Despite these benefits, there is a need for comprehensive studies on the role of ICLS under NTS in promoting specific microbial functions for sustainability in tropical regions. These functions include biological nitrogen fixation (BNF), carbon sequestration, nutrient cycling (e.g., phosphorus and potassium solubilization, sulfur assimilation, and iron acquisition), plant-microbe interaction, disease suppression, and microbial resilience to environmental stressors. Understanding how ICLS influences these microbial processes is essential for optimizing their benefits in agroecosystems [4, 12].

Maize–palisade grass intercropping is a widely adopted ICLS strategy in Brazilian agriculture, where palisade grass serves as both pasture and cover crop [13, 14]. According to the most recent survey conducted in Brazil (2020/2021 growing season), this system is adopted on approximately 17.4 million hectares by medium- to large-scale producers, with an average annual increase of nearly 1 million hectares [15]. Most of these areas are

located in major maize-producing regions of the Brazilian Cerrado (Central Brazil) and the southern and southeastern states, Mato Grosso, Mato Grosso do Sul, Goiás, Minas Gerais, São Paulo, Paraná, and Rio Grande do Sul, which together account for approximately 78% of Brazil's total maize grain (21 million hectares) production [16]. This system, particularly when rotated with soybean, improves nutrient use efficiency and increases residue inputs, intensifying tropical food production [17]. However, the reliance on inorganic nitrogen fertilizers in this system raises environmental concerns, including soil degradation, reduced microbial diversity, water contamination, ozone depletion, and altered nutrient cycling [18, 19]. The use of high-input fertilizers may select for fast-growing, copiotrophic microbial taxa while suppressing beneficial slow-growing microbes like oligotrophs, reducing overall soil functional diversity [20].

Integrating leguminous plants into maize–palisade grass intercropping has emerged as a promising solution to address these challenges. Legumes enhance nitrogen availability through BNF in symbiosis with diazotrophic bacteria, thereby reducing reliance on synthetic nitrogen fertilizers and promoting microbial diversity. Among these, pigeon pea (*Cajanus cajan*) stands out due to its distinctive agronomic and ecological characteristics. This deep-rooted, drought-tolerant crop has a long growth cycle and can access nutrients from deeper soil layers, making it well-suited for nutrient-depleted environments. Pigeon pea is capable of fixing between 40 and 250 kg N ha⁻¹ [21], contributing significantly to nitrogen enrichment and erosion control in degraded tropical soils. Compared to other legumes such as cowpea, groundnut, and soybean, pigeon pea demonstrates superior adaptability to marginal conditions—including drought, low soil fertility, and high temperatures—making it ideal for tropical and subtropical intercropping systems [22–24]. Furthermore, pigeon pea contributes to food and fodder production, supports the livelihoods of small-holder farmers, and enhances belowground biodiversity by fostering a beneficial soil microbial environment [24]. Despite these benefits, the role of pigeon pea in supporting other beneficial microbes in the soil of maize–palisade grass intercropping within a tropical ICLS under NTS remains underexplored.

Ecosystem multifunctionality (EMF) is an ecological framework used to evaluate how ecosystems maintain multiple functions and services simultaneously, such as nutrient cycling, productivity, carbon storage, and resilience to environmental stressors [25]. Previous studies have indicated that intercropping systems can enhance

EMF by promoting carbon cycling, enzyme activity, and microbial biomass [26], often driven by keystone microbial taxa [27]. However, the effects vary between regions [28] and have often been assessed with a narrow focus on individual or limited soil functions (e.g., enzyme activity, microbial biomass, or carbon cycling) [26–30], lacking a comprehensive approach that considers multiple EMF metrics. This limited scope hinders a holistic understanding of intercropping's impact on EMF in agroecosystems. A more comprehensive approach, integrating microbial, atmospheric, soil, crop yield, and meat production metrics, is needed to provide a clearer view of ecosystem health, which is critical for sustainable food production. Furthermore, most studies rely on amplicon sequencing [31–33], which only provides taxonomic insights and overlooks microbial functional potential, limiting our understanding of soil microbial contributions to EMF, particularly within maize–palisade grass intercropping systems.

In this study, we hypothesized that incorporating pigeon pea into a maize–palisade grass intercropping system would enhance EMF by fostering beneficial soil

microbial communities that support nutrient cycling and ecosystem services. To test this, we conducted a five-year field experiment on maize–palisade intercropping within a tropical ICLS under NTS. We quantified an EMF index related to soil health, climate protection, plant productivity, and animal productivity based on 133 measured variables. Additionally, we assessed soil microbial diversity and functional potential through shotgun metagenomic sequencing. Our findings provide valuable insights for sustainable agriculture, showing how legume integration can leverage microbial diversity to increase productivity and resilience in tropical agroecosystems.

Results

Long-term effects of intercropping systems on soil microbial and functional diversities

Although no statistically significant differences were observed, α -diversity analyses at the species and KEGG ortholog (KO) levels showed that the M + PG + PP treatment exhibited slightly higher Shannon index values than the M + PG treatment for both microbial taxonomy (Fig. 1a) and gene function (Fig. 1b). On the other hand,

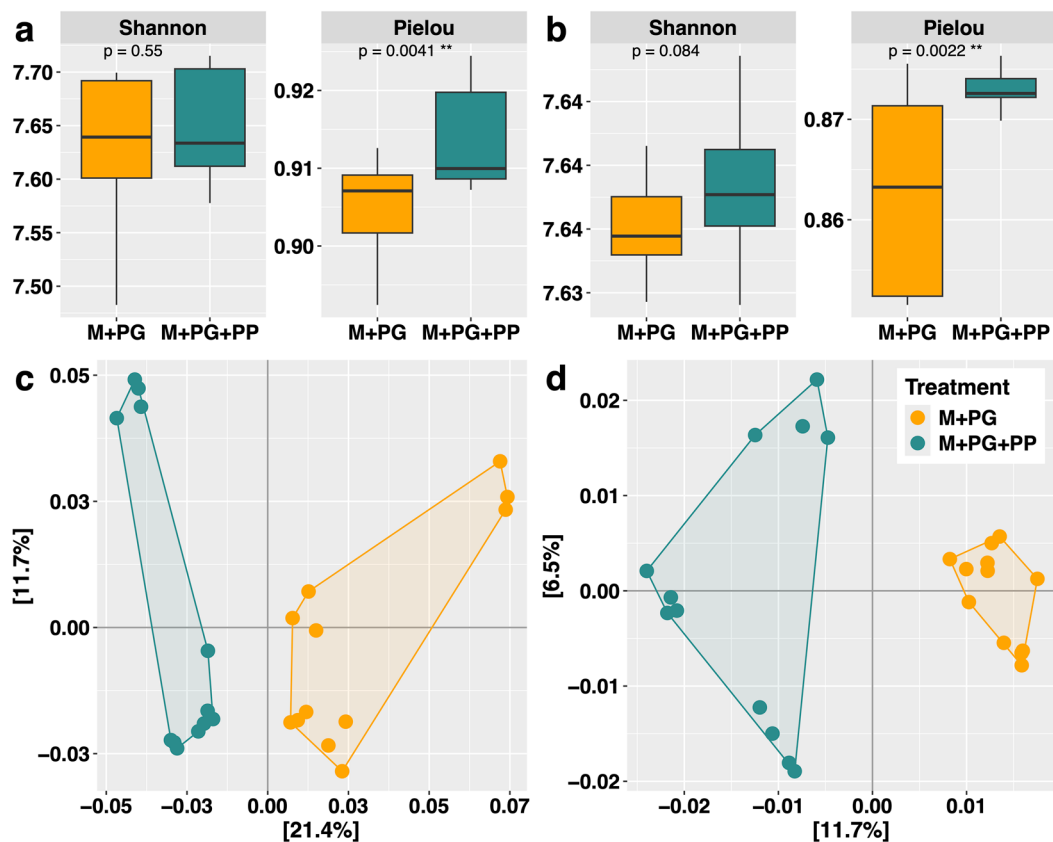


Fig. 1 Long-term effects of intercropping systems on soil microbial diversity. Alpha diversity of (a) microbial taxonomy and (b) KO functional potential. Statistically significant differences between treatments are indicated by * $P < 0.05$ and ** $P < 0.01$. Beta diversity of (c) microbial taxonomy and (d) KO functional potential across different treatments, visualized using unconstrained principal coordinate analysis (PCoA) based on the Bray–Curtis dissimilarity matrix

the Pielou index revealed that M+PG+PP treatment significantly enhanced the evenness of microbial communities (Fig. 1a) and gene functions (Fig. 1b). Principal coordinate analysis (PCoA) based on Bray–Curtis dissimilarity further illustrated the separation between the two treatments, with clear clustering observed in both taxonomic (Fig. 1c) and gene functional profiles (Fig. 1d). The first and second axes of the PCoA plots explained 21.4% and 11.7% of the variation in taxonomy and 11.7% and 6.5% of the variation in gene function, respectively. While these values indicated moderate explanatory power, the significant PERMANOVA results (taxonomy: $R^2 = 0.167$, $P = 0.001$; function: $R^2 = 0.108$, $P = 0.001$) confirmed that microbial communities differed significantly between treatments. However, a significant result from the BETA-DISPER test in gene function ($P = 0.001$) but not in taxonomic profiles ($P = 0.654$) suggested that observed differences in functional profiles might be partially influenced by heterogeneity within treatments rather than solely by treatment effects. These findings highlighted the pivotal role of pigeon pea in enhancing both microbial community diversity and functional potential.

Long-term effects of intercropping systems on soil microbial community composition

Taxonomic classification using Kraken2 and Bracken revealed that the majority of shotgun metagenomic reads were attributed to Bacteria (99.16%), followed by Archaea (0.83%) and Viruses (0.002%). At the phylum level, Actinomycetota dominated the microbial community across all samples, accounting for 52.51% of the total abundance, followed by Pseudomonadota (38.64%), Myxococcota (2.13%), Planctomycetota (1.62%), and several other phyla, each comprising less than 1% of the total abundance (Supplementary Fig. 1a). At the genus level, *Streptomyces* was the most abundant across samples (13.37%), followed by *Bradyrhizobium* (6.99%), *Nocardioideis* (3.25%), *Micromonospora* (2.33%), *Amycolatopsis* (2.13%), *Burkholderia* (1.95%), *Pseudomonas* (1.83%), *Mycobacterium* (1.80%), *Mycolicibacterium* (1.62%), *Microbacterium* (1.49%), *Conexibacter* (1.30%), *Sphingomonas* (1.27%), *Actinoplanes* (1.24%), and *Variovorax* (1.20%), with other genera each constituting less than 1% (Supplementary Fig. 1b).

To investigate further how the addition of pigeon pea (M+PG+PP treatment) affected the soil microbial community composition, differential abundance analysis was performed at the genus and species levels using LEfSe. This analysis identified 26 genera and 34 species enriched in M+PG+PP treatment, while 20 genera and 19 species were more abundant in M+PG treatment (Fig. 2). Notably, the genus *Bradyrhizobium* showed the highest LDA score in M+PG+PP treatment, with seven species significantly enriched. Several well-known plant

growth-promoting bacteria (PGPB) were also enriched in M+PG+PP treatment, including three species of *Streptomyces* and *Pseudomonas putida*. In contrast, the M+PG treatment enriched the genus *Mycobacterium*, with four species showing significant increases. Additionally, several PGPB were also identified in M+PG treatment, including two species of *Bradyrhizobium*, along with *Rhodopseudomonas palustris* and *Methylobacterium nodulans*. These findings highlighted the distinct effects of adding pigeon pea on intercropping systems on microbial community composition and the enrichment of plant-beneficial microbes.

Long-term effects of intercropping systems on microbial functional potential

To investigate the impact of adding pigeon pea (M+PG+PP treatment) on the functional potential of the soil microbial community, differential abundance analysis of KEGG ortholog (KO) was conducted using edgeR. A total of 1,509 KOs showed significant differences between the M+PG+PP and M+PG treatments, with 805 KOs enriched in the M+PG+PP treatment and 704 KOs enriched in the M+PG treatment (Fig. 3a). The genus *Bradyrhizobium* was the most prominent contributor to enriched KOs in M+PG+PP treatment, followed by *Solirubrobacter*, *Streptomyces*, *Rhizobacter*, and *Amycolatopsis*, among other genera with fewer than 50 coding sequences (CDSs) (Fig. 3b). Conversely, *Nocardioideis* was the top contributor to KOs enriched in M+PG treatment, followed by *Streptomyces*, *Bradyrhizobium*, *Solirubrobacter*, *Actinoplanes*, *Mycobacterium*, and additional genera with fewer than 60 CDSs (Fig. 3b). Notably, several genes related to phosphate (P) solubilization were found to enrich in M+PG+PP treatment, including 4-phytase (*appA*), C-P lyase core complex subunit PhnH (*PhnH*), and alkaline phosphatase D (*phoD*) (Supplementary Table 4).

Pathway enrichment analysis of significantly different KOs using MinPath further revealed enrichment of 71 and 61 KEGG pathways in M+PG+PP and M+PG treatments, respectively. Notably, several KEGG pathways were specifically enriched in M+PG+PP treatment, such as nitrogen (N) metabolism, carotenoid biosynthesis, lipopolysaccharide biosynthesis, exopolysaccharide biosynthesis, biosynthesis of various antibiotics (including vancomycin group antibiotics), flagellar assembly, and biofilm formation (Supplementary Fig. 2). In contrast, M+PG treatment was enriched in pathways related to fatty acid metabolism, including biosynthesis of unsaturated fatty acids, fatty acid degradation, and fatty acid biosynthesis. Additionally, pathways involved in the biosynthesis of siderophore group nonribosomal peptides, ansamycins, and biofilm formation were also specifically enriched in M+PG treatment (Supplementary Fig. 2).

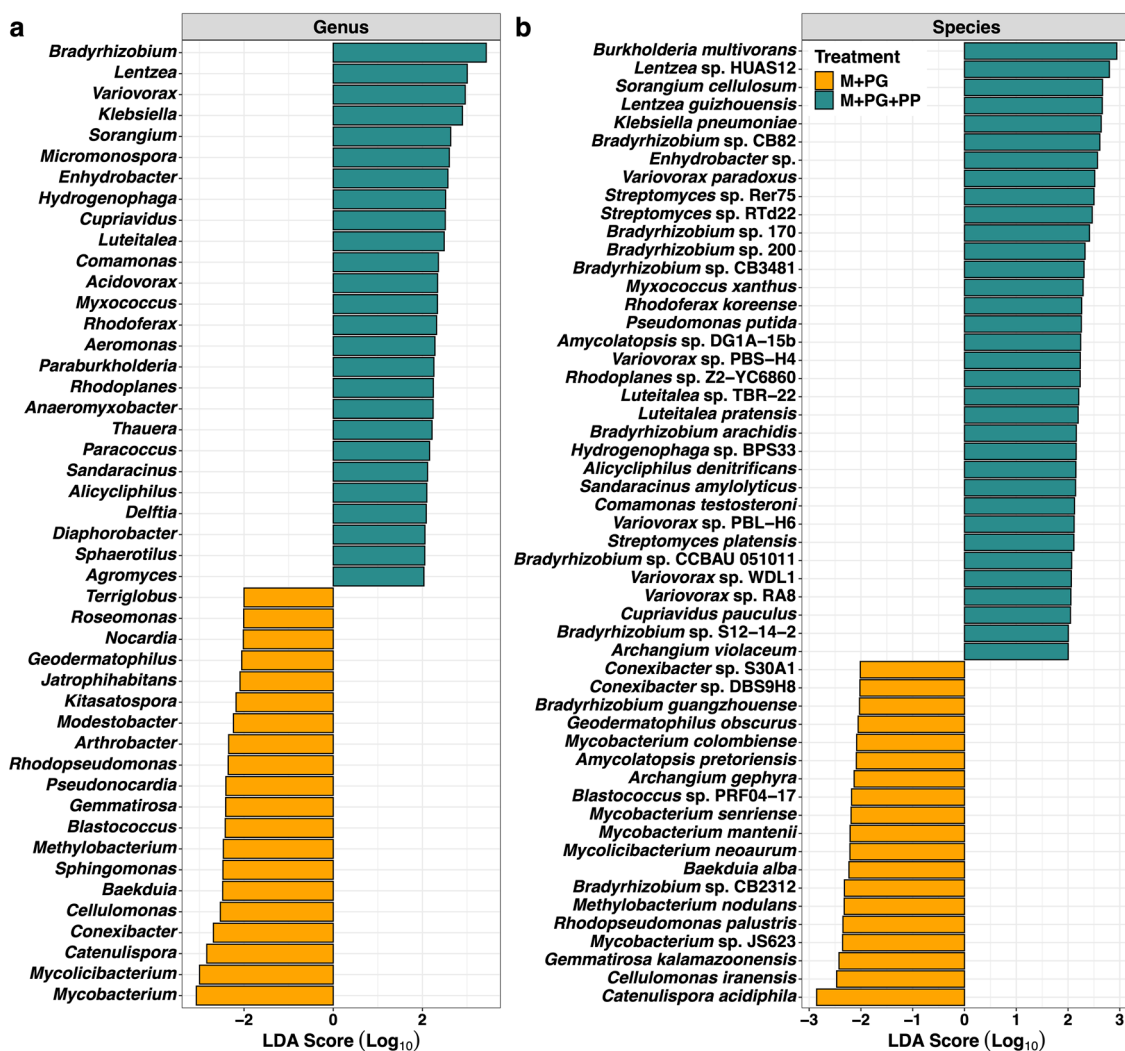


Fig. 2 Soil microbial taxonomic differences between treatments. Linear discriminant analysis (LDA) effect size (LEfSe) analysis of soil microbial taxonomic abundance between M + PG and M + PG + PP treatments at the (a) genus and (b) species levels. The LEfSe analysis was performed using an LDA score threshold of > 2.0 and a significance level of $P < 0.05$

These results underscored the distinct functional capabilities promoted by the intercropping system, with pigeon pea addition enhancing pathways associated with microbial growth, nutrient cycling, and antibiotic biosynthesis.

Long-term effects of intercropping systems on microbial plant growth-promoting traits

To assess the impact of adding pigeon pea (M + PG + PP treatment) on the plant growth-promoting potential of soil microbes, functional gene annotation was performed using the mgPGPT database, followed by differential abundance analysis with LEfSe. At functional subclass level 2, the M + PG + PP treatment significantly enriched functions related to plant colonization systems and bio-fertilization (Fig. 4). More specifically, at subclass level 3, the M + PG + PP treatment enhanced microbial functions related to nitrogen (N) acquisition, iron (Fe) acquisition,

and sulfur (S) assimilation (mineralization), all contributing to bio-fertilization. Additionally, the M + PG + PP treatment promoted colonization-related traits, including plant-derived substrate usage, motility (chemotaxis), surface attachment, and other colonization-related proteins (Fig. 4). In contrast, the M + PG treatment showed enrichment in functions related to competitive exclusion (CE) and phytohormone production (plant signaling), particularly the production of gibberellins and gamma-aminobutyric acid (GABA) (Fig. 4). Notably, phosphate (P) and potassium (K) solubilization were also enriched in the M + PG treatment (Fig. 4). P solubilization in M + PG treatment was associated with pathways involved in P transport, valeric acid biosynthesis, fumaric acid biosynthesis, propionic acid biosynthesis, citric acid biosynthesis, and acetic acid biosynthesis (Supplementary Fig. 3). Interestingly, P solubilization was also enriched

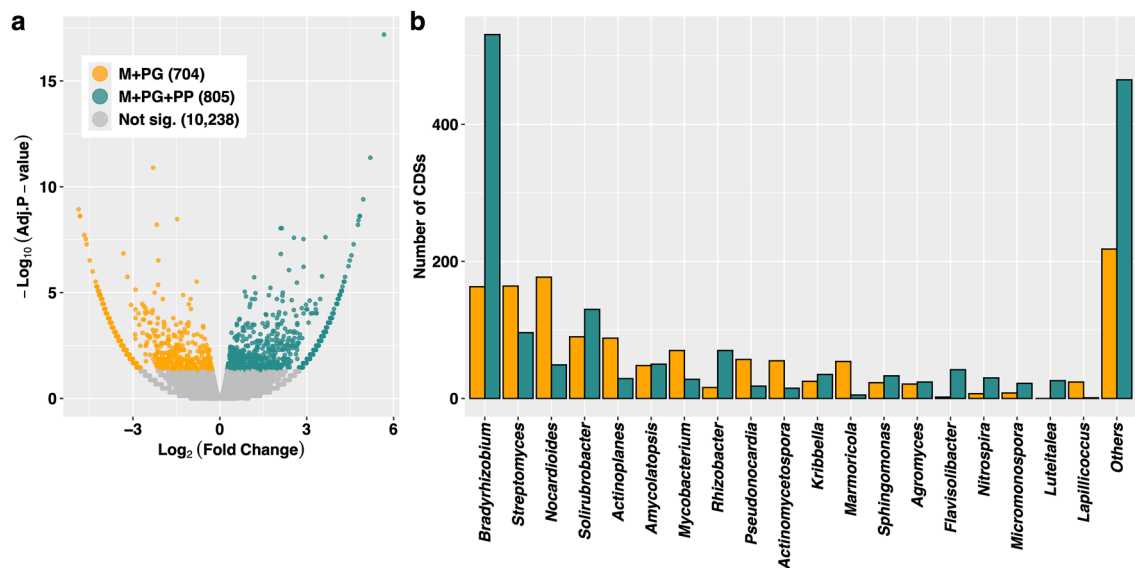


Fig. 3 Differential gene abundance analysis between treatments. **(a)** Differential abundance of soil microbial genes at the KO level. **(b)** Microbial genera contributing to differentially abundant genes. “Others” includes genera contributing fewer than 25 CDSs to the differential gene abundance

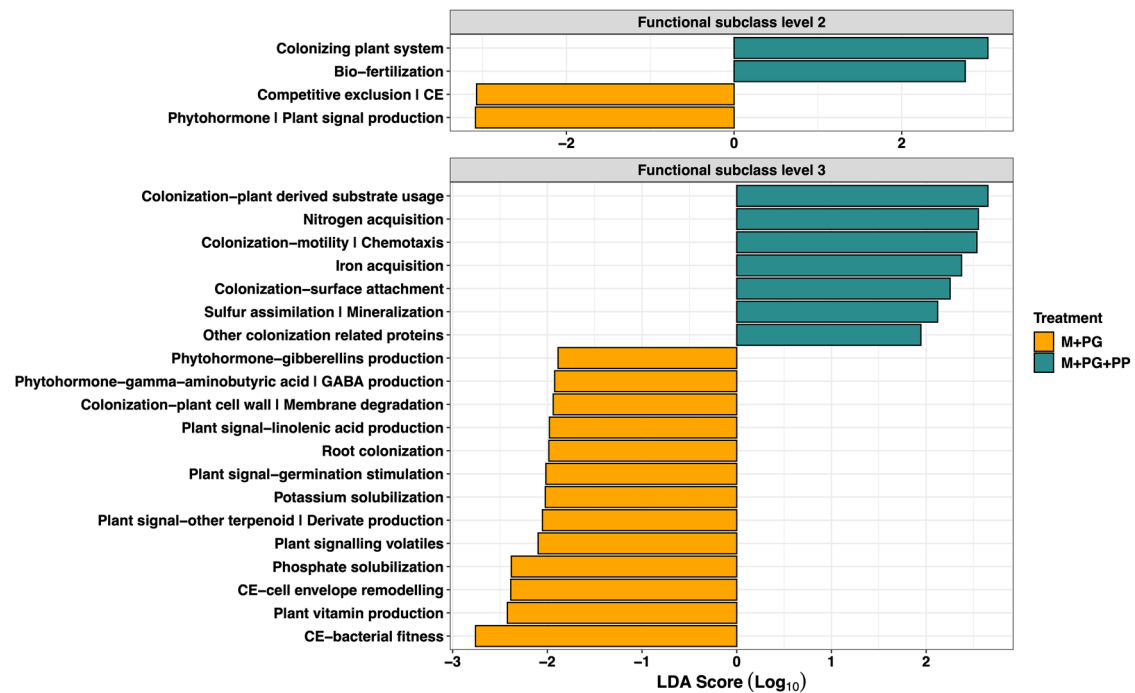


Fig. 4 Functional gene differences in soil microbial communities. Linear discriminant analysis (LDA) effect size (LEfSe) analysis of plant growth-promoting genes of soil microbial community between M+PG and M+PG+PP treatments at the functional subclass levels 2 and 3. The LEfSe analysis was performed using an LDA score threshold of > 1.5 and a significance level of $P < 0.05$

in the M+PG+PP treatment but was linked to different pathways, including D-gluconate biosynthesis, formic acid biosynthesis, the gluconic acid-PQQ pathway, alkaline phosphatase activity, and galactonic acid biosynthesis (Supplementary Fig. 3). These suggested distinct mechanisms of plant growth promotion between the two treatments.

Long-term effects of intercropping systems on ecosystem multifunctionality

The ecosystem multifunctionality (EMF) index of the maize-palisade grass intercropping system under NTS with the addition of pigeon pea (M+PG+PP treatment) was estimated using the averaging method, which assigned equal weight to each functional proxy

across four agroecosystem goods. The results showed that M+PG+PP treatment significantly promoted several functional proxies, such as soil microbial diversity (SoilDIV), nutritional quality of pasture in the off-season (Pasture), straw production and nutrient accumulation (Straw), leaf nutrient contents in soybean (SoybeanLEAF), leaf nutrient contents in maize (MaizeLEAF), and macronutrient accumulation in silage residues (SilageRES) (Supplementary Fig. 4). Overall, the M+PG+PP treatment improved multiple ecosystem goods, including soil health ($P>0.05$), plant productivity ($P<0.001$), lamb meat productivity ($P>0.05$), and climate protection ($P>0.05$), when compared to the M+PG treatment (Fig. 5). Notably, the EMF index was significantly higher in the M+PG+PP treatment than in the M+PG treatment, indicating an overall enhancement of ecosystem services (Fig. 5). Further analysis using Pearson

correlation revealed that most of the microbial species showing differential abundance in the M+PG+PP treatment was significantly positively correlated with plant productivity, soil health, and EMF index (Fig. 6). Among these, six out of seven species of *Bradyrhizobium* displayed a strong positive correlation with the EMF index, suggesting a key role for this genus in promoting multifunctionality in the intercropping system under NTS with the addition of pigeon pea.

Discussion

Impact on soil health and nutrient cycles

The findings of this study revealed that the addition of pigeon pea to the maize–palisade grass intercropping system under NTS positively influenced several key ecosystem functions, although not all effects were statistically significant. The M+PG+PP treatment showed slight

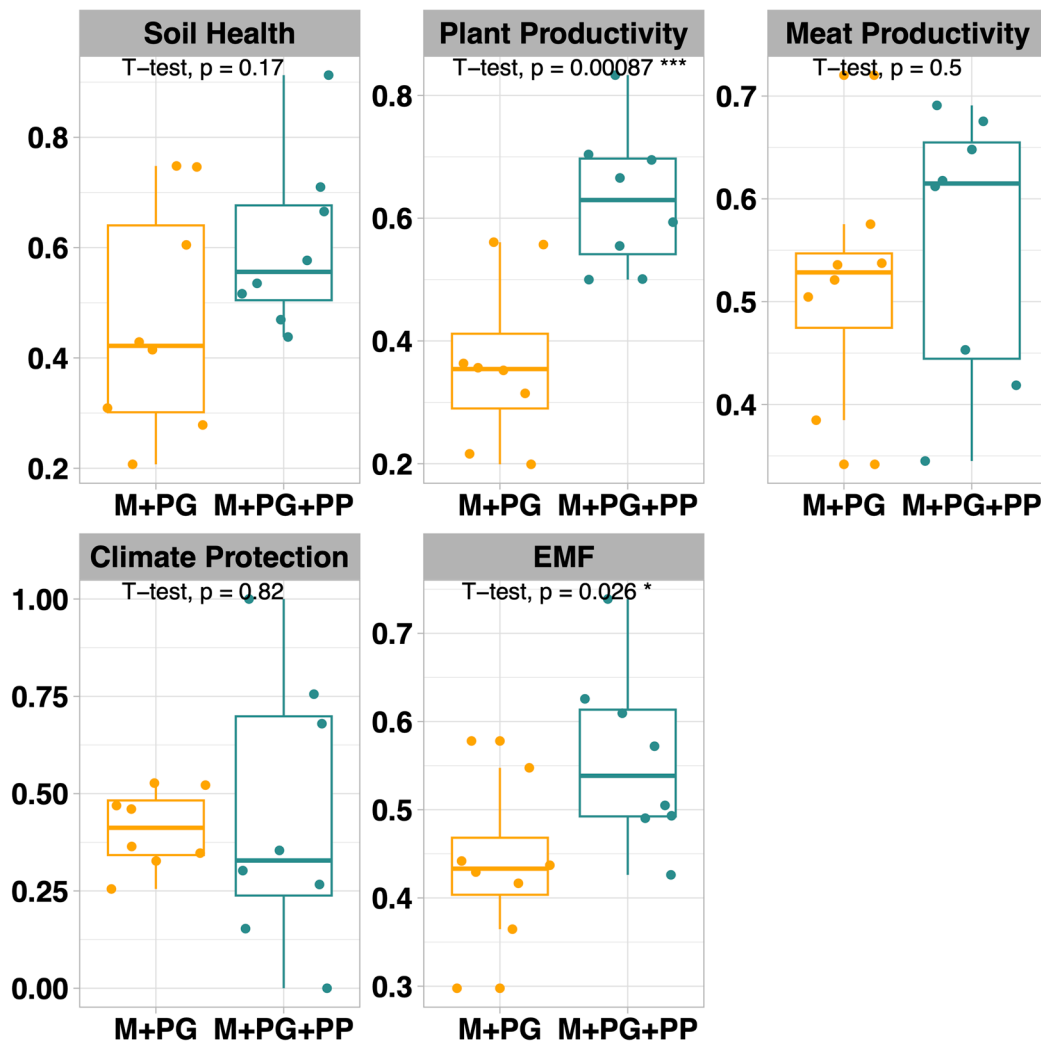


Fig. 5 Long-term effects of intercropping systems. Comparative impacts of intercropping systems on soil health, plant productivity, lamb meat productivity, climate protection, and ecosystem multifunctionality (EMF). Statistically significant differences between treatments are indicated by * $P<0.05$, ** $P<0.01$, and *** $P<0.001$

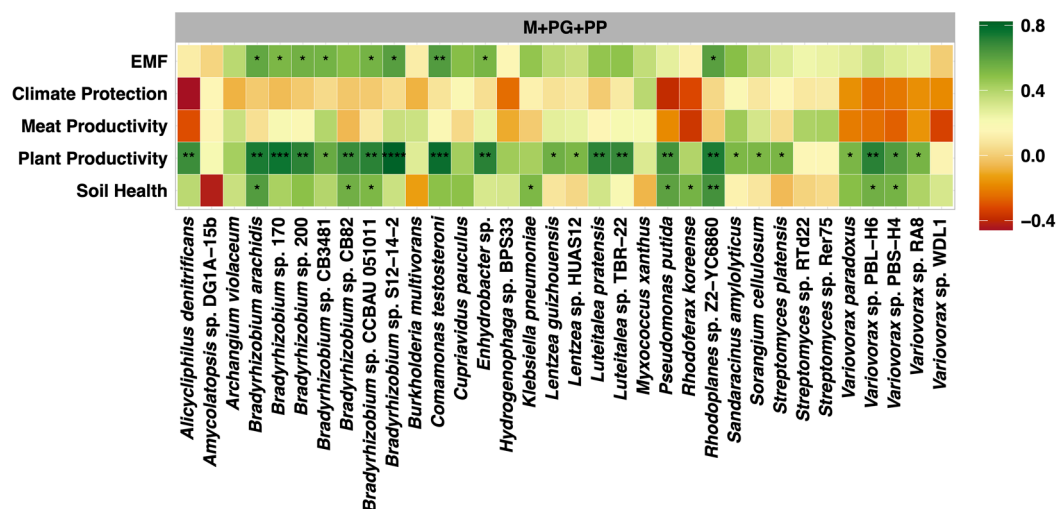


Fig. 6 Correlations between enriched microbial species and ecosystem multifunctionality. Pearson's correlation coefficients between microbial species significantly enriched in M + PG + PP treatment (identified via LEfSe analysis) and ecosystem multifunctionality metrics. Significance levels: *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$

improvements in soil health ($P > 0.05$) (Fig. 6), likely due to the enhanced microbial diversity and functional capacity, particularly the enrichment of microbial taxa associated with nitrogen fixation, phosphate solubilization, and exopolysaccharide (EPS) production. These findings are consistent with a previous study showing that pigeon pea intercropping enhances soil fertility by improving soil aggregation and organic phosphorus storage, while also increasing biological nitrogen fixation (BNF) efficiency [34]. Additionally, pigeon pea fallows have been reported to enhance soil biological and physical properties, leading to higher maize yields in degraded soils in South Africa [35]. Our results support these findings, as we observed an increased abundance of N_2 -fixing bacteria, particularly *Bradyrhizobium* spp., in the M + PG + PP treatment, which likely contributed to higher nitrogen (N) availability and soil fertility. Similarly, phosphate-solubilizing bacteria, such as *Streptomyces* spp. [36], were enriched, potentially increasing plant-available phosphorus and supporting nutrient uptake. In addition to nutrient cycling, soil aggregation and moisture retention are critical factors in sustaining soil health and productivity. The enrichment of EPS-producing bacteria, such as *Pseudomonas putida* [37] in the M + PG + PP treatment might have facilitated improved soil aggregation, enhancing soil structure and water dynamics. These microbial functions collectively promote greater microbial resilience and ecosystem multifunctionality, reinforcing the benefits of pigeon pea intercropping for sustainable agriculture.

The slightly higher richness index in the M + PG + PP treatment suggested that pigeon pea promoted a more diverse soil microbial community. This lack of significance in the richness might be attributed to environmental heterogeneity, transient microbial fluctuations,

and functional redundancy within the soil microbiome [38–40]. More importantly, the significant increase in the evenness index in the M + PG + PP treatment indicated a more balanced microbial community. This is in line with findings by Le Bagousse-Pinguet et al. (2021) [41], who emphasized the importance of evenness and the role of rare species in enhancing multifunctionality and reducing plant pathogen prevalence. The β -diversity analysis further showed that pigeon pea significantly altered the composition and functional potential of the soil microbial community, highlighting its role in selectively filtering microbial communities that might contribute to soil health.

The KO functional profile analysis revealed that pigeon pea enhanced several key soil ecosystem services. For instance, the enrichment of *appA*, *PhnH*, and *phoD* genes indicated improved P availability, which is essential for plant uptake. Organic P in the soil cannot be directly absorbed by plants and needs to be mineralized into inorganic P, a process facilitated by phosphatase, phytase, and C-P lyase produced by phosphate-solubilizing microbes (PSMs) [42–44]. Interestingly, while P solubilization was enriched in both treatments, it occurred through distinct microbial pathways (Supplementary Fig. 3). In the M + PG treatment, P solubilization was linked to organic acid biosynthesis, including valeric, fumaric, propionic, citric, and acetic acids. In contrast, the M + PG + PP treatment was associated with pathways like D-gluconate biosynthesis, the gluconic acid-PQQ pathway, and alkaline phosphatase activity. These differences suggested that the presence of pigeon pea altered the microbial mechanisms driving P availability. Additionally, the enrichment of the N metabolism pathway in M + PG + PP treatment indicated enhanced nutrient cycling, potentially improving

soil fertility. Moreover, the enrichment of lipopolysaccharide (LPS) and EPS biosynthesis pathways in M + PG + PP treatment further supports soil structure and moisture retention. EPS-producing microbes play a crucial role in soil aggregation, increasing porosity and stability, while their hygroscopic properties enhance water retention, reducing moisture loss and improving plant drought resilience [45–48]. These microbial-derived biopolymers contribute to the formation of stable soil aggregates, as reflected in the slightly higher total porosity (TP1 and TP2) observed in the M + PG + PP treatment (Supplementary Table 5). Improved porosity facilitates water infiltration and reduces surface runoff, while the hydrophilic properties of EPS contribute to organic matter retention, potentially explaining the slightly increased soil organic matter (SOM1, SOM2) in the M + PG + PP treatment (Supplementary Table 5). The enhanced aggregate stability, as indicated by the slightly higher aggregate stability index (ASI2; Supplementary Table 5), further supported the role of microbial EPS in improving soil resilience by reducing susceptibility to compaction and enhancing aeration.

Potential contributions to climate protection

Microbial-driven nutrient provision could naturally support plant growth, reducing the reliance on synthetic fertilizers, which are often linked to greenhouse gas (GHG) emissions [49–51]. By reducing fertilizer use, intercropping systems like M + PG + PP might contribute to climate change mitigation by lowering GHG emissions associated with fertilizer production and application. However, the slight improvement in climate protection observed in the M + PG + PP treatment might be influenced by fertilizer application in both treatments. Synthetic fertilizers, particularly N-based ones, are a significant source of nitrous oxide (N₂O) emissions [52]. Microbial-mediated N cycling can impact GHG fluxes, but the lack of statistically significant differences suggested that these positive effects might require longer observation periods or more sensitive metrics to detect substantial changes. Significant changes in soil carbon stocks and nitrogen cycling often take decades to become apparent [53, 54]. Given the five-year duration of this study, it is likely insufficient to capture long-term climate benefits. Future research should incorporate extended monitoring, including direct GHG flux measurements and soil organic carbon accumulation assessments, to better evaluate the potential of intercropping systems in mitigating climate change.

Impact on forage quality, yield, and lamb meat productivity

The inclusion of pigeon pea in the maize–palisade grass intercropping system under NTS demonstrated potential

benefits in enhancing forage quality and yield, which are important for improving lamb meat productivity. The results showed that the M + PG + PP treatment significantly increased several key functional proxies, including straw production and nutrient accumulation, leaf nutrient content in both maize and soybean, and macronutrient accumulation in silage residues (Supplementary Fig. 4). These improvements in forage quality likely contributed to a more nutrient-rich diet for lambs, which is essential for optimal growth and meat quality [55, 56]. These findings align with a previous study reporting that pigeon pea intercropping in tropical pastures significantly increased crude protein content and provided high-quality forage, particularly during the dry season [57]. The greater availability of essential nutrients in the feed, driven by enhanced soil nutrient cycling and better forage quality, indicated that the M + PG + PP treatment could provide long-term benefits for lamb production systems by improving feed efficiency and nutrient utilization. However, despite these improvements, lamb meat productivity did not show statistically significant differences ($P > 0.05$) compared to the M + PG treatment. This might be due to the lambs' nutritional needs already being sufficiently met in the M + PG treatment, suggesting a potential plateau in nutrient supply, where the added benefits of pigeon pea did not lead to further improvements in meat quality metrics [58].

Bradyrhizobium as a key driver of pigeon pea's beneficial impact

The increased plant productivity in the M + PG + PP treatment was not only due to enhanced N₂-fixation but also to the presence of microbial traits such as plant colonization, nutrient acquisition, and bio-fertilization. The M + PG + PP treatment enriched functions related to N acquisition, Fe acquisition, and S assimilation, all essential for promoting plant growth and soil fertility [59]. Additionally, traits linked to plant colonization, such as motility, surface attachment, and the utilization of plant-derived substrates, indicated that the microbial community in this system was well adapted for root colonization and symbiotic interactions [60–62]. By promoting the colonization of these beneficial microbes, the M + PG + PP treatment could enhance long-term soil health and sustainable plant productivity.

A major contributing factor to the increased plant productivity in the M + PG + PP treatment was the well-established symbiosis between pigeon pea and N₂-fixing bacteria, particularly *Rhizobium* and *Bradyrhizobium* [24, 63]. These bacteria play a central role in enriching soil N pools and promoting plant growth. Notably, seven species of *Bradyrhizobium* were enriched in the M + PG + PP treatment, along with other well-known plant growth-promoting bacteria (PGPB), such

as antimicrobial-producing *Streptomyces platensis* [64] and phytohormone-producing *Variovorax* sp. [65], and *Pseudomonas putida* [66]. The significant positive correlations between these microbes and plant productivity (Fig. 6) suggested that pigeon pea not only increased the abundance of N₂-fixing symbionts but also promoted the proliferation of PGPB with diverse functional traits. The increased N availability due to *Bradyrhizobium* activity likely contributed to improved crop performance while reducing the need for synthetic N fertilizers. In addition, *Bradyrhizobium* contributes to plant growth through the production of phytohormones such as indole-3-acetic acid (IAA), which can enhance root elongation and nutrient uptake [67]. Furthermore, our analysis revealed that *Bradyrhizobium*-enriched functions included genes associated with phosphate metabolism, particularly *phoD* and *phnH* genes, which are linked to organic phosphate mineralization. This suggested that *Bradyrhizobium* might contribute to phosphorus availability in intercropping systems [68], further supporting plant nutrient acquisition beyond nitrogen fixation alone.

Among the enriched microbes, *B. japonicum* was more abundant in the M + PG + PP treatment (Wilcoxon test, $P < 0.05$), despite being initially inoculated in both treatments. This indicated that pigeon pea played a pivotal role in supporting *Bradyrhizobium* persistence and proliferation, allowing it to thrive more effectively in the presence of pigeon pea compared to the maize–palisade grass intercropping alone. Legumes, including pigeon pea, are known to secrete root exudates rich in organic acids, flavonoids, and other signaling molecules that promote rhizobial colonization and survival [69–71]. These exudates act as chemical attractants, inducing nodulation genes in rhizobia and facilitating root-microbe interactions. While our findings are consistent with previous reports highlighting the role of legumes in shaping rhizobial communities [69–71], direct measurements of root exudate composition were not conducted in this study. Future research should integrate metabolomic profiling of root exudates to establish a direct mechanistic link between exudate composition and microbial community shifts in intercropping systems.

The ability of pigeon pea to support *Bradyrhizobium* populations is especially relevant for soybean cultivation, as soybean relies heavily on BNF to meet its high nutrient demands [70, 72]. Increased availability of *Bradyrhizobium* in the soil could enhance nodulation efficiency and optimize BNF, contributing to both higher crop productivity and the sustainability of the production system. These findings align with previous studies demonstrating the benefits of incorporating leguminous plants into intercropping systems to enhance crop yields and soil fertility [73, 74]. However, as this study focused specifically on pigeon pea, it remains unclear whether similar

microbial shifts would occur with other legumes. Different legume species shape their rhizobial associations through species-specific root exudates and signaling molecules [75, 76]. Future studies should explore a broader range of legumes to determine whether *Bradyrhizobium* enrichment is a generalizable phenomenon or unique to pigeon pea. While *Bradyrhizobium* plays a crucial role in nitrogen fixation, soil nutrient cycling depends on a diverse microbial community. Other beneficial microbes, such as phosphate-solubilizing *Pseudomonas* and *Bacillus*, sulfur-oxidizing bacteria, and arbuscular mycorrhizal fungi (AMF), contribute to soil fertility by enhancing nutrient availability and uptake. The co-occurrence of AMF and N₂-fixing bacteria further optimizes plant nutrition, highlighting the importance of microbial interactions in maintaining ecosystem productivity.

Ecosystem multifunctionality and long-term sustainability implications

The addition of pigeon pea in the intercropping system under NTS significantly enhanced the EMF index, highlighting the critical role of this legume in improving key ecological processes that are essential for long-term agricultural sustainability. The enrichment of N₂-fixing *Bradyrhizobium* spp. was a major factor contributing to the improved EMF in the M + PG + PP treatment. Notably, six out of seven *Bradyrhizobium* species showed strong positive correlations with the overall EMF index, demonstrating their importance in promoting ecosystem functions. Moreover, these species were also positively associated with soil health and plant productivity, of which increased soil health was translated into significantly higher crop yields in the M + PG + PP treatment. However, it is important to note that while these correlations suggest an association between *Bradyrhizobium* abundance and EMF, they do not establish a direct causal relationship. Other environmental and biotic factors, such as soil physicochemical properties, plant-microbe interactions, and microbial competition, may also contribute to the observed changes in ecosystem functions. Future studies employing experimental manipulations, such as controlled inoculations and isotope tracing, would be valuable in disentangling the direct contributions of *Bradyrhizobium* to EMF improvements. In contrast, the M + PG treatment, while still promoting certain beneficial traits like competitive exclusion (CE) and phytohormone production, lacked the multifunctional benefits provided by pigeon pea and its association with *Bradyrhizobium* spp. These findings highlight the importance of *Bradyrhizobium* in creating resilient and sustainable intercropping systems that enhance both soil health and crop productivity.

Although our study primarily focused on bacterial communities, we recognize the ecological significance

of archaea, fungi, and viruses in soil microbial ecosystems. However, their limited detection in our dataset may be attributed to both biological and methodological constraints. Soil metagenomes are typically dominated by bacterial DNA, making archaea, fungi, and viruses a minor fraction of the total sequence pool. Additionally, fungal genomes contain repetitive non-coding regions that complicate taxonomic classification in shotgun metagenomics, while standard DNA extraction methods may not efficiently lyse fungal and archaeal cell walls. Viral particles, with their small genomes and lack of conserved marker genes, are also often underrepresented. To improve the characterization of these microbial groups in future studies, we recommend employing targeted sequencing approaches, such as ITS amplicon sequencing for fungi, archaeal-specific 16S rRNA primers for archaea, and virome-enriched metagenomics or metatranscriptomics for viruses. Increasing sequencing depth and integrating metagenomics with metatranscriptomics or metabolomics could further enhance our understanding of their functional roles in intercropping systems.

Conclusions

In conclusion, this study demonstrated the significant benefits of incorporating pigeon pea into maize–palisade grass intercropping systems, particularly in enhancing soil health, plant productivity, lamb meat productivity, climate protection, and the EMF index. The M + PG + PP treatment, which included pigeon pea, promoted a more diverse and functionally resilient soil microbial community, leading to improved nutrient cycling, availability of essential nutrients, and overall soil fertility. The enrichment of N_2 -fixing *Bradyrhizobium* spp., along with other PGPB, played a crucial role in improving crop yields and EMF index in M + PG + PP treatment. Moreover, by creating a nutrient-rich environment, the inclusion of pigeon pea could serve as an effective method to boost the nodulation of subsequent rotational crops like soybean, particularly in poor soils with low nodulation capacity. In contrast, while the M + PG treatment provided some benefits, such as competitive exclusion and phytohormone production, it lacked the multifunctionality and broader ecosystem services observed in the M + PG + PP treatment. These findings support the potential of intercropping systems, particularly those incorporating legumes such as pigeon pea, as a viable strategy for enhancing soil health, boosting crop productivity, and contributing to long-term agricultural sustainability. At a broader scale, these results offer valuable insights for regions seeking to restore soil fertility, reduce reliance on chemical fertilizers, and improve food and feed production under variable climate conditions. Policymakers and practitioners can use this evidence to promote intercropping within agroecological transition frameworks, climate-smart

agriculture strategies, and incentive schemes rewarding ecosystem services. Ultimately, integrating legumes into intercropping systems represents a promising approach for achieving sustainable agriculture while minimizing environmental impacts.

Materials and methods

This study was conducted following the Ethics Committee on Animal Use (CEUA) of São Paulo State University (UNESP) at the College of Veterinary Medicine and Animal Science in Botucatu, São Paulo, Brazil, under protocol number 31/2014-CEUA.

Site description

The experiment took place in Botucatu, São Paulo State, Brazil (48° 25' 28" W, 22° 51' 01" S; 777 m above sea level) over five consecutive growing seasons: 2013–2014, 2014–2015, 2015–2016, 2016–2017, and 2017–2018. The soil at the site is classified as a clayey, kaolinitic, thermic Typic Haplorthox and consists of 630 g kg⁻¹ clay, 90 g kg⁻¹ silt, and 280 g kg⁻¹ sand. Prior to October 2013, the site had been used for the cultivation of annual crops and semi-perennial plants, following a no-till system (NTS). Over a four-year period, maize (*Zea mays* L.) was intercropped with palisade grass (*Urochloa brizantha*) in summer/autumn, and yellow oat (*Avena byzantina* 'São Carlos') was overseeded, allowing lamb grazing in winter/spring. Additionally, soybean [*Glycine max* (L.) Merr.] was intercropped with guinea grass (*Megathyrsus maximus*), with the pasture being harvested in winter/spring.

The climate in the region is classified as Cwa according to the Köppen system, which is characterized by tropical conditions, with dry winters and hot, rainy summers. The inclusion of climatic and edaphic information serves to contextualize the specific environmental conditions under which the experiment was conducted, thereby facilitating meaningful comparisons with other regions and studies. In addition, tropical conditions promote a direct impact on soil microbial activity and the performance of intercropping systems. Interannual variations in temperature and precipitation influenced water availability, organic matter mineralization, and, consequently, microbial diversity and crop development [77]. Long-term climate data (1955–2015) indicate average annual maximum and minimum temperatures of 26.1 °C and 15.3 °C, respectively, with an average annual rainfall of 1,359 mm. Meteorological data, including precipitation, temperature, and solar radiation, were recorded throughout the study period from 2013 to 2018 (Table 1).

Prior to starting the field experiment in September 2013, soil chemical characteristics were assessed at a depth of 0–0.20 m. The initial soil analysis revealed a pH (CaCl₂) of 5.1, total organic matter content of 38.1 g dm⁻³, resin-extractable phosphorus (P) of 12.2 mg kg⁻¹,

Table 1 Rainfall, maximum and minimum temperatures, and radiation receipt at botucatu, São Paulo, Brazil, during the study period and long-term averages

Climate characteristics		Month										
	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.
2013–2014												
Monthly rain, mm	45	65	74	116	104	99	72	1	26	19	96	37
Mean max. temp., °C	28.1	30.0	30.7	31.3	28.8	27.3	24.0	24.5	23.7	26.8	28.0	30.2
Mean min. temp., °C	17.3	19.0	19.7	20.4	18.8	16.7	13.9	13.5	11.7	11.5	12.5	13.4
2014–2015												
Monthly rain, mm	144	265	256	252	265	46	99	23	93	54	219	60
Mean max. temp., °C	28.1	28.6	31.7	28.4	27.1	27.0	23.4	23.5	22.6	26.7	27.3	28.7
Mean min. temp., °C	13.9	15.5	19.1	18.1	17.2	16.1	13.4	12.9	12.6	13.5	15.2	15.8
2015–2016												
Monthly rain, mm	186	299	492	367	134	29	146	127	0	86	0	160
Mean max. temp., °C	27.2	28.4	28.1	29.6	28.4	29.6	22.9	20.5	24.1	25.3	26.0	27.2
Mean min. temp., °C	15.7	18.3	18.2	18.4	17.9	16.0	13.1	11.8	12.8	12.7	14.5	14.4
2016–2017												
Monthly rain, mm	134	185	339	141	141	49	219	100	0	77	20	151
Mean max. temp., °C	27.9	27.9	26.4	29.8	28.0	26.2	24.1	22.9	22.7	23.9	29.2	28.0
Mean min. temp., °C	16.2	17.7	18.6	19.9	18.7	16.9	16.1	13.7	12.5	14.1	16.9	17.2
2017–2018												
Monthly rain, mm	201	185	269	104	139	54	23	9	7	117	70	133
Mean max. temp., °C	27.5	28.6	28.0	27.7	29.7	26.9	25.3	24.6	26.0	22.8	26.0	26.5
Mean min. temp., °C	16.8	19.3	18.8	18.3	19.9	17.6	15.1	15.0	14.1	12.8	15.3	16.9
2018–2019												
Monthly rain, mm	175	151	-	-	-	-	-	-	-	-	-	-
Mean max. temp., °C	27.2	29.8	-	-	-	-	-	-	-	-	-	-
Mean min. temp., °C	17.4	19.2	-	-	-	-	-	-	-	-	-	-
Long-term (60 year) avg.												
Monthly rain, mm	185	224	203	141	67	76	56	38	39	71	127	133
Mean max. temp., °C	27.2	28.1	28.0	28.0	27.0	24.0	23.0	23.0	25.0	26.2	26.7	27.2
Mean min. temp., °C	16.4	17.1	17.4	19.0	17.0	15.0	13.0	13.0	14.0	12.4	14.2	15.1
Radiation receipt, MJ m ⁻²	603	636	663	548	517	500	378	362	405	502	524	605

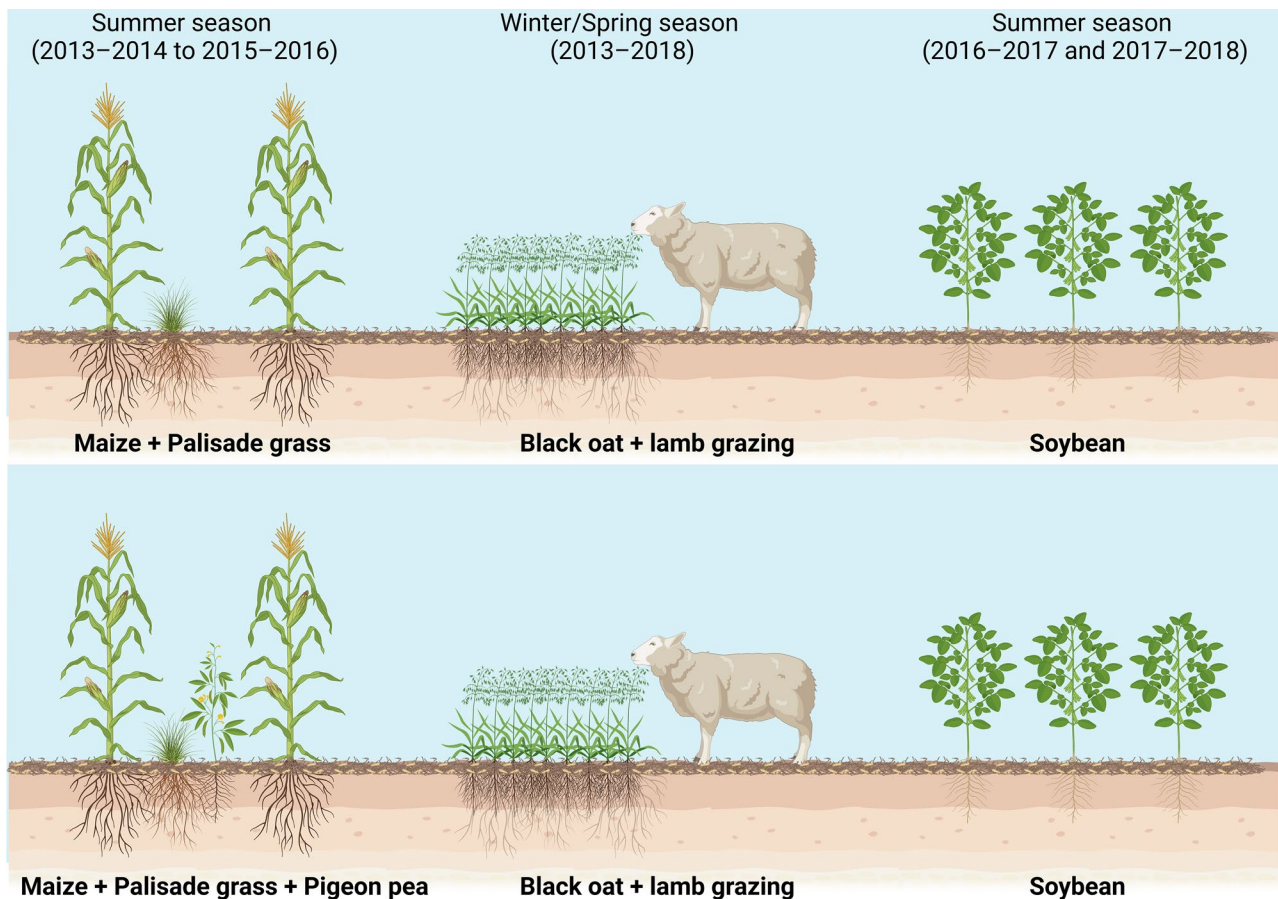


Fig. 7 Field experimental design and timeline. Schematic diagram of the field experimental design, illustrating intercropping systems, lamb grazing, and crop rotation over five consecutive growing seasons

and exchangeable potassium (K), calcium (Ca), magnesium (Mg), and total acidity (H + Al) at pH 7.0 of 1.0, 32, 16, and 41 mmol_c kg⁻¹, respectively. The base saturation was calculated at 54 mmol_c kg⁻¹. Organic matter was determined colorimetrically using sodium dichromate, and phosphorus, along with exchangeable Ca, Mg, and K, was extracted using ion exchange resins and quantified via atomic absorption spectrophotometry. Base saturation was calculated from the exchangeable base content, and total acidity was measured at pH 7.0 (H + Al).

Experimental design

The experiment was a completely randomized design as a function of soil fertility homogeneity, consisting of two intercropping systems and twelve replications. The treatments included two intercropping strategies implemented during the summer/autumn seasons: (i) maize intercropped with palisade grass (*Urochloa brizantha*), referred to as the M + PG treatment, and (ii) maize intercropped with palisade grass and pigeon pea (*Cajanus cajan*), referred to as the M + PG + PP treatment. The fourth growing season was considered a residual phase of the previous intercropping systems, during which

a uniform soybean crop was planted across all plots. Throughout the five consecutive growing seasons at the same site, black oat (*Avena strigosa*) was cultivated during each winter/spring season. For silage production, maize was cultivated in the 2013–2014, 2014–2015, and 2015–2016 seasons, whereas soybean was grown in the 2016–2017 and 2017–2018 seasons. Each experimental plot measured 25 m in length and 9 m in width, totaling 225 m² per plot.

This experimental setup allowed for the evaluation of the residual effects of intercropping systems on subsequent crops, as well as the assessment of the overall system's impact on soil and crop performance over time. A schematic representation of the intercropping systems, lamb grazing, and crop rotation is provided in Fig. 7.

Tillage and crop management

Corn hybrid 2B587 PowerCore (Dow AgroSciences, Indianapolis, Indiana, USA) with early relative maturity was sown in all crop systems on 16 Dec. 2013, 16 Dec. 2014, and 14 Dec. 2015 at a 4-cm depth, with a row spacing of 0.45 m and a density of 80,000 seeds ha⁻¹, using no-till seeding (Semeato, model Personale Drill 13, Passo

Fundo, Rio Grande do Sul, Brazil). When intercropped with corn, palisade grass was simultaneously sown at 12.0 kg ha^{-1} (pure live seed = 50%). Forage seeds were mixed with basic fertilizer [78, 79] and sown at depths of 0.08 m below the soil surface, as described by Crusciol et al. (2012) [80]. Pigeon pea was first sown to a depth of 0.04 m using the same no-till seeding (15 seeds per m) (approximately $35 \text{ kg of seeds ha}^{-1}$); then, the corn + palisade grass combination was sown. Therefore, pigeon pea emerged between the rows of corn + palisade grass. For all crop systems and in both growing seasons, basic fertilization in the sowing furrows was 36 kg ha^{-1} of N as urea, 126 kg ha^{-1} of P_2O_5 as triple superphosphate, and 72 kg ha^{-1} of K_2O as potassium chloride.

Corn seedling emergence occurred at 11, 7, and 4 days after sowing (27 Dec. 2013, 23 Dec. 2014, and 18 Dec. 2015, respectively). Differences were due to the absence of rain after sowing in the growing seasons (Table 1). Pigeon pea seedlings emerged at 17, 12, and 9 days after sowing (02 Jan. 2014, 28 Dec. 2014, and 23 Dec. 2015, respectively). Grass seedlings emerged at 25, 22, and 21 days after sowing (10 Jan. 2014, 07 Jan. 2015, and 04 Jan. 2016, respectively). In all growing seasons, due to the large amount of straw on the soil surface, there was no emergence of annual broadleaf weeds, and the application of herbicide during post-emergence of the corn crop was not necessary. Furthermore, it was not necessary to soften the initial growth of palisade grass with an herbicide sub-dose due to the different emergence times of corn and grass seedlings.

On 15 Jan. 2014, 13 Jan. 2015, and 13 Jan. 2016, when corn had four expanded leaves (V_4 stage), mineral fertilizer was broadcast with no incorporation at 150 kg ha^{-1} of N as urea, 38 kg ha^{-1} of P_2O_5 as triple superphosphate, and 150 kg ha^{-1} of K_2O as potassium chloride.

On 05 May 2014, 22 Apr. 2015, and 13 Apr. 2016, black oat was oversown in two modalities: (a) seeds were planted in line to a depth of 3 cm, with 0.17 m row spacing and 65 kg ha^{-1} pure live seed density, using no-till seeding (Semeato, model Personale Drill 13, Passo Fundo, Rio Grande do Sul, Brazil); (b) seeds were broadcast (manually) with 120 kg ha^{-1} pure live seed density, with superficial incorporation using a disc harrow (fully closed discs for minimum ground disturbance). In both oversown modalities, black oat seedlings emerged at 24, 13, and 20 days after sowing (29 May 2014, 05 May 2015, and 03 May 2016, respectively).

In the first growing season, 48 (four crop systems $\times$ twelve lambs/crop system) uncastrated male crossbred Dorper, Texel and Ile de France lambs, with a mean age of three months and an initial live weight of $27.0 \pm 3.2 \text{ kg}$, were used. In the second growing season, 48 (four crop systems $\times$ twelve lamb/crop systems) uncastrated male crossbred Poll Dorset and Corriedale lambs with a

mean age of three months and an initial live weight of $24.4 \pm 3.4 \text{ kg}$, were used; 16 (four crop systems $\times$ four lambs/crop system) additional lambs were used to adjust the animal stocking rate. In the third growing season, 48 (four crop systems $\times$ twelve lambs/crop system) uncastrated male crossbred Dorper and Texel lambs with a mean age of three months and an initial live weight of $26.4 \pm 3.5 \text{ kg}$ were used. The lambs were blocked based on weight variation and randomly allocated to crop systems.

On 17 Sep. 2016, 2.0 Mg ha^{-1} of dolomitic lime ($\text{CaCO}_3\text{-MgCO}_3$) with 28% CaO and 20% MgO was broadcast onto the soil surface, following the recommendation of Crusciol et al. (2016) [81]. On 02 Dec. 2014, 16 Nov. 2015, and 10 Nov. 2016, pasture composed of the remaining forage grasses and weeds was sprayed with glyphosate ($1.44 \text{ kg acid-equivalent ha}^{-1}$) and 2,4-D amine ($1.34 \text{ kg active ingredient ha}^{-1}$). On 17 Dec. 2014, 04 Dec. 2015, and 25 Nov. 2016, any weed regrowth was sprayed with glyphosate ($1.08 \text{ kg acid-equivalent ha}^{-1}$), and on 02 Dec. 2016, mulch was sprayed with paraquat ($0.4 \text{ kg active ingredient ha}^{-1}$). All herbicide applications used a spray volume of 200 L ha^{-1} .

The soybean cultivar 'AS 3610 IPRO – INTACTA RR2 PRO' (super early cycle, maturity group 6.1 and indeterminate growth rate) was sown on 06 Dec. 2016 at a 4-cm depth, with a row spacing of 0.45 m and a density of $350,000 \text{ seeds ha}^{-1}$ using no-till seeding (Semeato, model Personale Drill 13, Passo Fundo, Rio Grande do Sul, Brazil). The fungicide carboxin + thiram and the insecticide thiamethoxam were applied to the soybean seeds at a dose of 60 and 120 g of active ingredient (a.i.) to 100 kg of seeds, respectively. The soybean seeds were inoculated with *Bradyrhizobium japonicum* (SEMIA 5079 – CPAC 15 and SEMIA 5080 – CPAC 7) at $\sim 1,200 \text{ cells seed}^{-1}$. All soybean crop systems were fertilized in furrows, with 7 kg ha^{-1} of N as urea, 70 kg ha^{-1} of P_2O_5 as triple superphosphate, and 70 kg ha^{-1} of K_2O as potassium chloride. In all crop systems, guinea grass cv. Aruana was intercropped with soybean and planted at 15 kg ha^{-1} (pure live seed = 32%). Forage seeds were mixed with basic fertilizer [78, 79] and sown at depths of 0.06 m below the soil surface, as described by Crusciol et al. (2012) [80]. Soybean and guinea grass seedlings emerged at 6 and 15 d after sowing, respectively (12 Dec. 2017 and 21 Dec. 2017, respectively). The herbicide glyphosate ($0.54 \text{ kg acid-equivalent ha}^{-1}$) was applied 7 days after the emergence of soybean seedlings. All the crop systems were sidedressed 23 days after soybean emergence with 90 kg ha^{-1} of K_2O as potassium chloride, with incorporation using a row crop cultivator for no-till systems (Tatu Marchesan, model CPD, Matão, São Paulo, Brazil).

Soybeans were cultivated according to crop needs. The application of phytosanitary products was as follows: insecticide thiamethoxam + lambda-cyhalothrin

(21 and 16 g of a.i. ha⁻¹, respectively) during the V₄ stage; the fungicide trifloxystrobin + prothioconazole (30 and 35 g of a.i. ha⁻¹, respectively) and insecticide thiamethoxam + lambda-cyhalothrin (28 and 21 g of a.i. ha⁻¹, respectively) during the R₁ stage; the fungicide trifloxystrobin + prothioconazole (38 and 44 g of a.i. ha⁻¹, respectively) and insecticide acephate (600 g of a.i. ha⁻¹) during the R₄ stage; and fungicide azoxystrobin + benzovindiflupyr (60 and 30 g of a.i. ha⁻¹, respectively) and insecticide thiamethoxam + lambda-cyhalothrin (28 and 21 g of a.i. ha⁻¹, respectively) during the R₆ stage. All fungicide and insecticide applications used a spray volume of 200 L ha⁻¹ and adjuvant mineral oil (65 g ha⁻¹).

Finally, we assembled a descriptive table (Supplementary Table 1) summarizing all agronomic information for each crop over time, including cultivar/hybrid, seeding date, row spacing, basal and topdressing fertilization, and harvest date.

Lamb grazing management

During the off-season each year, 48 uncastrated male lambs, approximately three months old with an initial live weight of 25.0 ± 3.0 kg, were used in the experiment. The lambs were stratified based on weight and then randomly assigned to different crop systems. A rotational grazing system was employed, using a semi-feedlot approach. Each paddock was grazed for three days, followed by a 33-day rest period, resulting in 12 paddocks per treatment and two grazing cycles per season.

The lambs grazed on pasture from 6:00 AM to 6:00 PM daily, with unrestricted access to water and shade. At night, from 6:00 PM to 6:00 AM, they were confined to a covered pen where they were provided supplemental feed consisting of maize or soybean silage produced from the same crop system. All crop systems used the same concentrate mixture composed of ground maize, soybean meal, a vitamin-mineral supplement, limestone, and urea.

Dry matter intake (DMI) was estimated at 4.35% of live body weight, based on a projected average daily gain (ADG) of 200 g per lamb. Pasture made up approximately 30% of the lambs' total DMI, with the remainder provided by maize and soybean silage and concentrate, formulated according to their nutritional needs. The diet was balanced using the Small Ruminant Nutrition System (SRNS), based on the Cornell Net Carbohydrate and Protein System (2000), which is designed for sheep.

Throughout the grazing cycles, the lambs were weighed individually every 18 days using a mobile electronic scale to adjust their feed intake and monitor weight gain. Final body weights were recorded in September at the end of each growing season. The average daily gain (ADG) was calculated as the difference between the final and initial weights during the grazing cycle, divided by the

number of days in each cycle. At the conclusion of the experiment, the lambs were transported to a commercial slaughterhouse for processing.

Sampling and analyses

Several parameters were assessed to evaluate the performance of integrated crop-livestock systems. Briefly, leaf nutrient concentrations in maize and soybean were measured by collecting leaves at the full flowering and full bloom stages, respectively. Silage and grain yields were determined by harvesting whole maize plants and other crops at the maturing stage, dried, and analyzed for nutrient content and biomass yield. Pasture forage mass during lamb grazing cycles was measured by sampling three 1.0 m² areas per plot down to ground level, followed by bromatological analysis for crude protein, fiber content, and digestibility. Lamb meat quality was evaluated by measuring crude protein, ether extract, pH, cooking weight loss, shear force, color, and fatty acid composition. Mulch biomass and nutrient content were calculated by drying field residues and analyzing their nutrient concentrations. Soil emissions of CO₂, CH₄, and N₂O were quantified using closed chambers and gas chromatography, with soil temperature, moisture, and environmental factors recorded during each measurement. Soil properties, including fertility (C, N, and organic matter) and physical characteristics, such as bulk density, porosity, and aggregate stability, were also assessed. Soil collected during the flowering stage of the last soybean growing season was used for DNA extraction, sequencing, and bioinformatics analysis. Root systems were sampled, washed, and digitized to measure structural traits. Comprehensive evaluations of plants, soil, forage, and animal outputs provided an integrated assessment of agroecosystem performance and sustainability. Detailed methodologies for sampling and analysis are provided in the Supplementary Methods.

Soil DNA extraction, shotgun metagenome sequencing, and data processing

Total soil DNA was extracted from 0.25 g of each soil sample using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany), following the manufacturer's protocol without any modifications. The quality and concentration of DNA were assessed using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and 1% sodium boric acid agarose gel electrophoresis for visual confirmation. Subsequently, shotgun metagenomic libraries were prepared using the NextSeq 500/550 Mid Output v2 kit (300 cycles; Illumina, San Diego, CA, USA) and sequenced on the Illumina NextSeq 500 platform (paired-end, 2 × 150 bp).

Sequence quality was initially assessed using FastQC v.0.12.0 [82], followed by quality filtering with Trimmomatic v.0.39 [83] to remove low-quality reads.

High-quality reads were then taxonomically classified using Kraken2 v.2.1.3 [84] and Bracken v.2.9 [85], generating read-based taxonomic profiles. Subsequently, reads from all samples were concatenated and subjected to metagenome co-assembly using MEGAHIT v.1.2.9 [86]. Taxonomic classification at the contig level was performed using CAT v.6.0.1 [87], retaining only contigs classified at least at the kingdom level for Bacteria, Archaea, Viruses, and Fungi. Coding sequences (CDSs) were predicted with MetaProdigal v.2.6.3 [88], and gene functional annotation was carried out using the KEGG database through GhostKOALA [89] and the mgP-GPT database [90] with DIAMOND v.2.1.9 [91]. Prior to downstream analyses, both microbial taxonomy and gene abundance tables were normalized using rarefying (640,000 reads per sample) and transcript per million (TPM) methods.

Ecosystem multifunctionality (EMF)

Ecosystem multifunctionality (EMF) index was calculated following the method outlined by Wittwer et al. (2021) [92]. In brief, each ecological function indicator was standardized using Z-scores. Indicators representing undesirable environmental effects, such as high fiber content (NDF, ADF, LIG), which reduces forage digestibility, and greenhouse gas emissions (CO_2 , CH_4 , N_2O), were inverted by multiplying by -1 to ensure that higher values consistently reflected more desirable outcomes. All agroecosystem proxies were further scaled within a range between 0 and 1. Some variables were used directly as proxies for specific functions, while others were combined (averaged) into composite variables when they contributed to the same function. A total of 18 ecosystem function proxies were derived from the 133 measured variables (Supplementary Table 2), and the mean value of these proxies was calculated to represent the EMF index for each sample. The model framework used for evaluating ecosystem multifunctionality (EMF) in this study is provided in Supplementary Table 3. Additionally, detailed concept and framework are provided in the Supplementary Methods.

Statistical analyses

To evaluate within-sample (α) diversity, Shannon and Pielou indices were calculated for both microbial taxonomic profiles (species level) and functional genes (KEGG Ortholog). Shannon's index accounts for both richness and evenness, while Pielou's index specifically reflects evenness. Differences in α -diversity between treatments were assessed using independent t-tests, following confirmation of data normality (Shapiro–Wilk test) and homogeneity of variances (Levene's test). Between-sample (β) diversity was examined for both microbial taxonomy and gene functions (KEGG

Ortholog) using principal coordinate analysis (PCoA) based on the Bray–Curtis dissimilarity index. Group differences were tested using PERMANOVA with 999 permutations, and group dispersion homogeneity was evaluated using BETADISPER.

To identify differentially abundant microbial taxa (genus and species level) and functional traits (plant growth-promoting traits at subclass levels 2–4), LEfSe v.1.1.2 [93] was utilized. Microbial species enriched in the M + PG + PP treatment were further correlated with ecosystem function proxies and EMF index using Pearson correlation. Additionally, differential abundance of microbial genes at the KEGG Ortholog (KO) level was performed using the R package edgeR v.4.2.1 [94]. Significant KOs were then subjected to metabolic pathway reconstruction and enrichment analysis via MinPath v.1.6 [95].

All statistical analyses were performed in R v.4.4.1 [96], primarily using the package vegan v.2.6.8 [97], and visualized with ggplot2 v.3.5.1 [98], unless otherwise specified.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s40793-025-00727-0>.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

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Author contributions

E.E.K. and N.R.C. conceptualized and designed the research; N.R.C. conducted the experiment; A.N.K. performed data analysis and drafted the initial manuscript; E.E.K., A.N.K., and J.W.B. critically revised the manuscript; C.A.C.C., J.C.C., L.G.M., and J.W.B. performed soil sampling and chemical analysis; C.M.P., C.C., A.M.C., D.M.S., and P.R.L.M. managed animal husbandry and performed meat quality analyses; I.V.C. oversaw plant and intercropping management; J.W.B. carried out DNA extractions. All authors reviewed and approved the final manuscript.

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Data availability

All sequencing data generated in this study have been submitted to the European Nucleotide Archive (ENA) database under accession number PRJEB31659. The scripts for analyses are available at https://github.com/Nurudinkhoiry/Pigeon_pea_intercropping.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent to publication

Not applicable.

Competing interests

The authors declare no competing interests.

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