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Soil microbiological and enzymatic responses to nitrogen fertilization in crop-livestock systems with and without trees[1]

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ABSTRACT

Integrated crop-livestock systems can influence soil biological functioning. However, the effects of contrasting nitrogen (N) management histories and tree components remain poorly understood. This study aimed to evaluate the effects of N rates [90 (N90) and 180 (N180) kg ha^{-1}] on soil microbiological and enzymatic attributes in crop-livestock (CL) and crop-livestock with trees (CLT; *Eucalyptus dunnii*, *Schinus terebinthifolius*, and *Grevillea robusta*) systems. The experiment included winter pasture and corn and followed a 2 × 2 factorial design with three blocks. At the end of each crop cycle, microbial biomass carbon (MBC), microbial biomass nitrogen (MBN), microbial respiration (MR), metabolic quotient (qCO_2), arylsulfatase, β-glucosidase, acid phosphatase, and fluorescein diacetate (FDA) hydrolysis were evaluated. MBC showed a significant interaction between production system and N treatment, with lower values under N90 than under N180 in CLT at the end of both crop cycles. Arylsulfatase and FDA hydrolysis were greater in CL than in CLT at the end of the corn cycle, whereas acid phosphatase activity was greater in CL at the end of both corn and pasture cycles. At the end of the corn cycle, MBN and β-glucosidase were greater under N180 than under N90. MR and qCO_2 were not significantly affected. Because contrasting N rates were applied during pasture phases, responses at the end of the corn cycle should be interpreted in the context of long-term N management histories rather than as direct effects of differential N fertilization of corn. Production systems and N management histories were associated with distinct responses in soil microbial biomass and enzyme activities.

KEYWORDS: Soil biological functioning, nitrogen fertilization, nutrient cycling.

RESUMO

Respostas microbiológicas e enzimáticas do solo à fertilização nitrogenada em sistemas de integração lavoura-pecuária com e sem árvores

Os sistemas integrados de produção lavoura-pecuária podem influenciar o funcionamento biológico do solo. Entretanto, os efeitos de históricos contrastantes de manejo de nitrogênio (N) e de componentes arbóreos permanecem pouco compreendidos. Objetivou-se avaliar os efeitos de doses de N [90 (N90) e 180 (N180) kg ha^{-1}] sobre atributos microbiológicos e enzimáticos do solo em sistemas de integração lavoura-pecuária (ILP) e integração lavoura-pecuária com árvores (ILPF; *Eucalyptus dunnii*, *Schinus terebinthifolius* e *Grevillea robusta*). O experimento foi conduzido com pastagem de inverno e milho, em esquema fatorial 2 × 2, com três blocos. Ao final de cada ciclo de cultivo, avaliaram-se o carbono (CBM) e nitrogênio (NBM) da biomassa microbiana, respiração microbiana (RM), quociente metabólico (qCO_2), arilsulfatase, β-glicosidase, fosfatase ácida e hidrólise do diacetato de fluoresceína (FDA). CBM apresentou interação significativa entre sistema e tratamento de N, com menores valores sob N90 que sob N180 no ILPF após ambos os cultivos. Arilsulfatase e hidrólise de FDA foram maiores no ILP que no ILPF após o milho, enquanto a fosfatase ácida foi maior para ILP após ambos os cultivos. Após o milho, NBM e β-glicosidase foram maiores sob N180 que sob N90. RM e qCO_2 não foram significativamente afetados. Como as doses contrastantes de N foram aplicadas na pastagem, as respostas no milho devem ser interpretadas considerando-se o histórico de manejo de N em longo prazo, e não como efeitos diretos de adubação diferenciada do milho. Os sistemas e os históricos de manejo de N estiveram associados a respostas distintas da biomassa microbiana e das atividades enzimáticas.

PALAVRAS-CHAVE: Funcionamento biológico do solo, fertilização nitrogenada, ciclagem de nutrientes.

INTRODUCTION

The search for agricultural systems capable of maintaining productivity while preserving environmental quality has intensified in recent decades. In this context, integrated crop-livestock systems have emerged as important nature-based solutions, promoting greater resource-use efficiency and fostering

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ecological interactions that support both agricultural production and environmental sustainability (Oliveira et al. 2024, Nwaogu et al. 2025). By integrating crops, pastures, and livestock within the same production area, these systems diversify organic inputs, improve nutrient cycling, and help to maintain soil functioning, increasing the resilience and sustainability of agricultural systems (Carvalho et al. 2024).

As integrated systems become more complex, understanding how their components influence soil biological functioning becomes increasingly important. The inclusion of trees can modify environmental conditions within integrated systems, potentially affecting soil microbial activity and nutrient cycling (Zandona et al. 2019, Machineski et al. 2026). Studies evaluating the tree component in integrated production systems have reported contrasting responses of soil microbiological attributes. Microbial biomass, respiration, metabolic quotient, and enzyme activities may respond differently to the presence and arrangement of trees, with responses varying according to system configuration and management conditions (Oliveira et al. 2016, Assis et al. 2017, Santos et al. 2026). Therefore, the effects of tree components on soil biological functioning appear to be context-dependent and warrant further investigation, particularly under long-term integrated management conditions.

Soil microorganisms are key regulators of organic matter turnover and nutrient cycling, making biological attributes sensitive indicators of soil functioning and management effects. Changes in residue inputs, nutrient availability, and soil environmental conditions can significantly affect microbial biomass, respiration, and enzyme activities, reflecting shifts in nutrient cycling dynamics and soil biological quality (Lehmann et al. 2020). As soil biological activity plays a fundamental role in biomass decomposition, nutrient cycling, energy flow, and ecosystem functioning, biological indicators have been increasingly used to assess the impacts of agricultural management practices on soil functioning and sustainability (Cherubin et al. 2026).

Among the management practices used in integrated systems, nitrogen fertilization is one of the main factors regulating soil biological functioning (Wang et al. 2018). By influencing plant growth and biomass production, nitrogen availability directly affects the quantity and quality of organic residues returned to the soil, thereby modifying carbon inputs and nutrient availability for soil microorganisms (Smith et al. 2024). These changes can stimulate microbial growth, alter enzymatic activity, and affect nutrient cycling processes, ultimately influencing soil biological functioning (Dotaniya et al. 2019). In systems containing trees, these responses may also be influenced by changes in microenvironmental conditions, organic input, and root dynamics associated with the tree component.

Previous studies conducted in this long-term experiment have addressed different components of integrated production systems. Pontes et al. (2018a) evaluated corn yield and quality and the residual effects of N fertilization, whereas other studies assessed pasture productivity, animal performance, and methane emissions under contrasting tree and N treatments (Pontes et al. 2018b, Pontes et al. 2020).

The long-term economic performance of these systems was subsequently evaluated by Pontes et al. (2021). More recently, Menoncin et al. (2026) investigated specific components of the soil biota, focusing on arbuscular mycorrhizal fungi and glomalin-related soil proteins. However, the functional response of the soil microbial community, integrating microbial biomass, respiration, metabolic efficiency, and enzyme-mediated C, P, and S cycling, has not been addressed in this long-term experiment. Therefore, the novel contribution of the present study is the integrated assessment of microbial biomass C and N, microbial respiration, metabolic quotient, and enzyme activities associated with C, P, and S cycling under crop-livestock systems with and without trees and contrasting long-term N management histories. These biological attributes have not been previously evaluated together in this long-term experiment.

In this context, this study evaluated the effects of nitrogen fertilization rates on soil microbiological attributes and enzymatic activities following corn and winter pasture cycles in established crop-livestock (CL) and crop-livestock with trees (CLT) systems. We hypothesized that nitrogen availability and tree presence would differentially influence microbial biomass, metabolic efficiency, and enzymatic activity, resulting in distinct biological responses between the production systems.

MATERIAL AND METHODS

The long-term experiment was established in 2006 at the Instituto de Desenvolvimento Rural

do Paraná, in Ponta Grossa, Paraná state, Brazil (25º07′22″S; 50º03′01″W). The region's climate is humid subtropical, classified as Cfb according to the Köppen classification system. Monthly mean air temperature and total rainfall for 2018, the year in which the soil biological evaluations were conducted, are presented in Figure 1.

The experimental area comprises 13.07 ha, of which 6.09 ha are planted with trees in an integrated crop-livestock with trees system (CLT), whereas the remaining area is managed as an integrated crop-livestock system (CL). The area comprises an association of Cambisol and Ferralsol (Embrapa 2006), with a sandy texture and slopes ranging from 4 to 9 %. The experimental blocks were established to minimize the effects of spatial variation in soil type and slope, with one block predominantly located on Cambisol and the other two located in a transition between Cambisol and Ferralsol soils (Embrapa 2006). The spatial arrangement of the experimental units, treatments, and blocks is presented in Pontes et al. (2018a). Block was included in the statistical model to account for the main spatial heterogeneity associated with soil and topography. Soil chemical characterization was performed according to Pavan et al. (1992), using samples collected from the 0-20 cm layer after corn cultivation in March 2018 and after the winter pasture phase in October 2018, and is shown in Table 1.

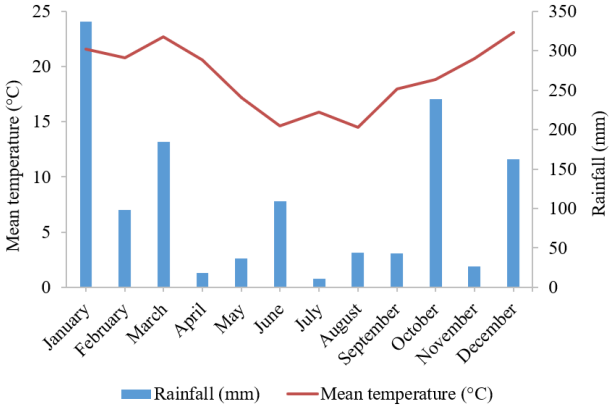


Figure 1. Monthly mean air temperature and total rainfall in 2018, when the soil biological evaluations were conducted. Mean monthly air temperature was calculated from the monthly minimum and maximum temperatures. Source: Sistema de Tecnologia e Monitoramento Ambiental do Paraná, Station 25135001, Ponta Grossa, Paraná state, Brazil.

The experiment was conducted in a randomized complete block design with three blocks, in a 2 × 2 factorial arrangement consisting of two integrated production systems [crop-livestock (CL) and integrated crop-livestock with trees (CLT) systems] and two N rates [90 (N90) and 180 (N180) kg ha^{-1}]. Each treatment combination was represented once within each block, resulting in three independent experimental replicates. Because of the large size of the experimental units, two spatially distinct subsamples were collected within each experimental unit to improve the representativeness of the sampling. The two subsamples were averaged to obtain a single value for each experimental unit, which was subsequently applied in the statistical analyses, using the model: $Y_{ijk} = \mu + B_k + S_i + N_j + (S \times N)_{ij} + \varepsilon_{ijk}$, where Y_{ijk} is the observed response in

Table 1. Soil chemical characterization (0-20cm depth) in integrated crop-livestock (CL) and integrated crop-livestock with trees (CLT) systems.

Treatment	pH	P	K^+	Ca^{2+}	Mg^{2+}	Al^{3+}	H + Al	SB	CEC	V	m
		mg dm^{-3}	cmol$_c$ dm^{-3}							%	
End of corn cycle											
CL N90	5.16	49.80	0.26	3.17	1.37	0.03	4.67	4.80	9.47	51.36	0.74
CL N180	5.20	53.40	0.21	3.22	1.39	0.06	4.66	4.82	9.48	51.59	1.45
CLT N90	5.09	51.90	0.18	2.47	0.97	0.02	3.96	3.61	7.58	47.74	0.59
CLT N180	4.09	58.60	0.16	2.59	0.97	0.07	3.92	3.72	7.64	47.88	2.78
End of pasture cycle											
CL N90	5.04	52.24	0.41	3.04	1.37	0.03	4.65	4.82	9.47	51.05	0.75
CL N180	5.05	37.66	0.36	2.86	1.44	0.05	4.65	4.67	9.32	49.9	1.35
CLT N90	5.12	81.78	0.31	2.59	1.19	0.02	4.24	4.08	8.32	49.19	0.74
CLT N180	5.09	61.59	0.24	2.59	1.16	0.04	4.35	3.98	8.32	47.89	1.06

pH: soil potential of hydrogen; SB: sum of bases; CEC: cation exchange capacity; V: base saturation; m: aluminum saturation. P and K^+ were extracted with Mehlich-1; Ca^{2+}, Mg^{2+}, and Al^{3+} were extracted with 1 mol L^{-1} KCl; pH was determined in 0.01 mol L^{-1} $CaCl_2$; H + Al was extracted with KCl and titrated with NaOH.

the experimental unit; μ is the overall mean; B_k is the effect of block; S_i is the effect of the system; N_j is the effect of the N rate; $(S \times N)_{ij}$ is the system x N rate interaction; and ε_{ijk} is the experimental error.

The N rates were selected to represent limiting (N90) and non-limiting (N180) N supply, based on the response of black oat (*Avena strigosa* Schreb. cv. IAPAR 61) to N fertilization reported by Alves (2002), as described by Pontes et al. (2018b). These contrasting N treatments were introduced into the experimental design in the 2009/2010 growing season and subsequently applied as urea during each stocking season, approximately 40 days after pasture establishment (Pontes et al. 2018b, Pontes et al. 2021). Thus, the experimental plots had a history of contrasting N management during the pasture phases before the soil sampling conducted in the present study.

In the CLT system, three tree species were planted in 2006, at the beginning of the experiment: eucalyptus (*Eucalyptus dunnii* Maiden), pink pepper (*Schinus terebinthifolius* Raddi), and silver oak (*Grevillea robusta* A. Cunn. ex R. Br.). The species were interspersed in the same rows running crosswise in relation to the slope, with a predominantly southwest-northeast direction at 3 × 14 m spacing (238 trees ha^{-1}). After thinning, the tree density at the time of sampling for this study was approximately 40 trees ha^{-1}.

In both systems, CL and CLT, soybean (*Glycine max*) or corn (*Zea mays*) was cultivated during the summer, whereas winter pastures consisted of black oat (*Avena strigosa* Schreb.) and ryegrass (*Lolium multiflorum* Lam.) under no-tillage and continuous stocking by beef cattle. Each experimental unit received three tester animals (permanent animals that remained throughout the experimental period) and a variable number of animals periodically adjusted using the put-and-take Mott & Lucas method, to maintain a target sward height of 20 cm (Moraes et al. 2014). The experimental animals were Purunã beef heifers aged between 8 and 10 months at the beginning of the stocking season. All procedures involving animals were approved by the Ethics Committee on Animal Use (CEUA) of the Iapar (Protocol nº 03/2017).

The winter pasture consisted of a mixture of black oat and ryegrass, sown on May 16, 2018, at seeding rates of 45 and 15 kg ha^{-1}, respectively, with a row spacing of 17 cm. The base fertilization used was 400 kg ha^{-1} of the 4-30-10 (N-P_2O_5-K_2O) formula, with 90 or 180 kg ha^{-1} of N applied as urea, as topdressing, and applied in a single application 40 days after the pasture was sown, constituting the two nitrogen fertilization doses evaluated. Corn (Balu 280 Pro) was sown on October 17, 2017, directly into the residual straw of the winter pasture. The row spacing was 0.80 m, with 6.0 seeds m^{-1}, resulting in an initial population of approximately 70,000 plants ha^{-1}. Base fertilization was 400 kg ha^{-1} of N-P_2O_5-K_2O in the 4-30-10 formula, and topdressing fertilization was done using a single dose of 250 kg ha^{-1} of urea in both systems. The contrasting N90 and N180 treatments were applied during the pasture phases, whereas the subsequent corn crop received the same N fertilization across treatments. Therefore, no differential N treatment was applied directly to corn.

In the winter of 2018, heifers weighing an average initial body weight of 225 ± 3.44 kg grazed the experimental area for 105 days (from July 5 to October 18, 2018).

Soil sampling was carried out twice: at the end of the corn cycle and at the end of the pasture phase (i.e., after the grazing period) in both production systems. The two sampling periods were analyzed separately, because they represented distinct phases of the production systems, and temporal comparisons between the corn and pasture phases were not an objective of this study. Within each experimental unit, two spatially distinct sampling points were established to improve sampling representativeness, particularly considering the large size and spatial variability of the experimental units. At each sampling point, a composite soil sample was collected from the 0-10 cm soil layer using a shovel. Each composite sample consisted of five subsamples, with one collected at the central point and four collected around it.

In the CLT system, the two sampling points within each experimental unit were positioned to account for different distances from the tree rows. Therefore, sampling was not restricted to areas immediately adjacent to the trees or to the center of the inter-row space, but was designed to better represent the spatial variability within each experimental unit. The two composite samples obtained from each experimental unit were considered subsamples of the same experimental unit rather than independent experimental replicates, and their values were averaged before statistical analysis, as previously

described. Soil samples were stored in plastic bags and transported in ice-cooled styrofoam boxes to the laboratory, where they were sieved through a 4-mm mesh and stored at 4 ºC for 7 days before microbiological and enzymatic analyses. Analyses were performed on field-moist soil without a pre-incubation step. Gravimetric soil moisture was determined by oven-drying a subsample at 105 ºC for 24 h, and the analytical results were corrected and expressed on an oven-dry soil mass basis.

Microbial biomass carbon (MBC) was determined using the fumigation-extraction method according to Vance et al. (1987). Soil samples were fumigated with ethanol-free chloroform for 18 h in the dark at approximately 25-28 ºC and subsequently extracted with 0.5 mol L^{-1} of K_2SO_4. MBC was calculated using a conversion coefficient of Kc = 0.33 (Sparling & West 1988), whereas microbial biomass nitrogen (MBN) was determined according to Brookes et al. (1985).

Microbial respiration was determined by incubating 50 g of field-moist soil in a hermetically sealed jar containing a beaker with 10 mL of 0.5 mol L^{-1} of NaOH to trap the CO_2 released during incubation. After 7 days (168 h), the remaining NaOH was quantified by titration with 0.5 mol L^{-1} of HCl using phenolphthalein as an indicator. The amount of C-CO_2 evolved during the incubation period was corrected for soil dry mass and incubation time, and microbial respiration was expressed as μg C-CO_2 g^{-1} of dry soil h^{-1}. The metabolic quotient (qCO_2) was calculated according to Anderson & Domsch (1993) as qCO_2 = MR/MBC, where MR is the microbial respiration rate (μg C-CO_2 g^{-1} of dry soil h^{-1}) and MBC is the microbial biomass carbon (μg C g^{-1} of dry soil). Thus, qCO_2 was expressed as μg C-CO_2 μg^{-1} of MBC h^{-1}. Arylsulfatase (EC 3.1.6.1), acid phosphatase (EC 3.1.3), and β-glucosidase (EC 3.2.1.21) activities were determined according to Tabatabai (1994). For each assay, 1 g of field-moist soil was incubated with the respective substrate for 1 h at 37 ºC. Arylsulfatase activity was determined using *p*-nitrophenyl sulfate in acetate buffer (pH 5.8), acid phosphatase activity using *p*-nitrophenyl phosphate in modified universal buffer (pH 6.5), and β-glucosidase activity using *p*-nitrophenyl-β-D-glucopyranoside in modified universal buffer (pH 6.0). Soil-specific controls without substrate were included for each enzymatic assay. The released *p*-nitrophenol (pNP) was quantified spectrophotometrically at 410 nm, and enzyme activities were corrected for soil dry mass and expressed as μg of pNP g^{-1} of dry soil h^{-1}. Microbial activity was also assessed by fluorescein diacetate (FDA) hydrolysis according to Schnürer & Rosswall (1982). The amount of released fluorescein was corrected for soil dry mass and incubation time and expressed as μg of fluorescein g^{-1} of dry soil h^{-1}. The evaluation of corn yield was performed 184 days after sowing. In the CLT system, three samples per plot were collected, corresponding to 5-m segments of corn row in three different positions between the tree rows: the central position, located between two tree rows; the position adjacent to the tree rows; and an intermediate position. Three random samples of 5-m corn rows were collected per plot in the CL system. Grain yield was calculated based on the total mass of grains harvested from all plants in each plot and/or position between the rows, adjusted to 13 % of moisture, and extrapolated to 1 ha.

Statistical analyses were performed using the R software version 4.3.2 (R Foundation for Statistical Computing, Vienna, Austria). Data were analyzed according to a randomized complete block design, in a 2 × 2 factorial arrangement, with three blocks. As previously described, the two subsamples collected within each experimental unit were averaged, and the resulting mean was used as a single observation for each experimental unit in the statistical analysis. The factors included production system (CL and CLT; DF = 1) and N rate (N90 and N180; DF = 1), with block included in the model (DF = 2). The production system × N rate interaction (DF = 1) was also tested. Analysis of variance (Anova) was performed separately for the corn and pasture sampling periods. The statistical model included the effects of block, production system, N rate, and production system × N rate interaction. Model residuals were examined to assess the assumptions of Anova, including normality using the Shapiro-Wilk test. When necessary, data transformations were applied to better meet model assumptions. When significant effects were detected, means were compared using the Tukey test.

RESULTS AND DISCUSSION

Mean values and standard deviations of soil microbiological and enzymatic attributes for each system × N treatment combination following the corn and pasture cycles are presented in Table 2, whereas the effects of production system, N treatment, and

their interaction are summarized by the analysis of variance in Table 3.

During the corn cycle, the production system significantly affected microbial biomass carbon (MBC), arylsulfatase, acid phosphatase, and fluorescein diacetate (FDA) hydrolysis, whereas N fertilization significantly influenced microbial biomass nitrogen (MBN) and β-glucosidase activity. During the pasture cycle, the production system significantly affected the acid phosphatase activity. A significant system × N interaction was observed for MBC, indicating that the response of microbial biomass to N treatment differed between CL and CLT.

The observed responses suggest that the N management and presence of trees were associated with differences in soil biological functioning. Tree components can influence soil microenvironmental conditions and resource distribution through processes such as shading, litter deposition, and root interactions, which may affect microbial metabolism and nutrient cycling (Zandona et al. 2019, Oliveira et al. 2024). Although these mechanisms were not directly evaluated in the present study, they may provide context for the contrasting biological responses observed between CL and CLT. These results reinforce the sensitivity of microbial and enzymatic attributes to management-related differences in integrated systems.

Microbial biomass carbon (MBC) was significantly lower under N90 than under the N180

Table 2. Soil microbiological and enzymatic attributes in crop-livestock (CL) and crop-livestock with trees (CLT) systems under two nitrogen treatments following corn and pasture cycles.

Treatments	MBC	MBN	MR	qCO_2	ARIL	FA	BETA	FDA
	μg g^{-1} dry soil		μg C-CO_2 g^{-1} dry soil h^{-1}	μg C-CO_2 $μg^{-1}$ MBC h^{-1}	μg pNP g^{-1} dry soil h^{-1}			μg fluorescein g^{-1} dry soil h^{-1}
End of corn cycle								
CL N90	282.9 ± 45	23.4 ± 15.7	0.50 ± 0.48	2.4 ± 2.0	31.6 ± 20.1	327.1 ± 147	94.4 ± 33	97.1 ± 37
CL N180	259.1 ± 67	34.8 ± 9.5	0.64 ± 0.22	2.6 ± 1.9	30.8 ± 13.3	305.4 ± 113	105.0 ± 24	91.9 ± 18
CLT N90	162.1 ± 38	21.9 ± 8.5	0.61 ± 0.37	4.3 ± 3.2	17.4 ± 6.3	222.2 ± 71	55.1 ± 19	80.9 ± 26
CLT N180	226.1 ± 72	24.7 ± 9.1	0.58 ± 0.33	3.1 ± 3.0	17.2 ± 7.8	232.5 ± 71	89.0 ± 17	69.6 ± 26
End of pasture cycle								
CL N90	257.1 ± 72	44.5 ± 16.5	0.95 ± 0.5	3.77 ± 2.7	29.0 ± 17.0	325.2 ± 111	106.6 ± 36	110.5 ± 23
CL N180	230.7 ± 73	39.0 ± 12.6	0.85 ± 0.6	3.97 ± 2.9	25.0 ± 11.1	297.0 ± 57	94.8 ± 21	103.3 ± 21
CLT N90	150.8 ± 35	34.8 ± 11.4	0.35 ± 0.3	2.79 ± 2.4	16.9 ± 4.8	229.7 ± 73	81.8 ± 21	97.4 ± 23
CLT N180	201.9 ± 26	31.8 ± 11.2	0.63 ± 0.6	3.24 ± 3.1	15.0 ± 7.0	223.3 ± 53	94.6 ± 30	100.5 ± 28

Values are presented as mean ± standard deviation (n = 3 experimental replicates). N90 and N180 represent N treatments of 90 and 180 kg ha^{-1}, respectively. MBC: microbial biomass carbon; MBN: microbial biomass nitrogen; MR: microbial respiration; qCO_2: metabolic quotient; ARIL: arylsulfatase activity; FA: acid phosphatase activity; BETA: β-glucosidase activity; FDA: fluorescein diacetate hydrolysis.

Table 3. F-ratio and statistical significance from the analyses of variance for soil microbiological and enzymatic attributes in crop-livestock (CL) and crop-livestock with trees (CLT) systems under two N treatments.

Source of variation	DF	MBC	MBN	MR	qCO_2	ARIL	FA	BETA	FDA
End of corn cycle									
System (S)	1	28.1*	ns	ns	ns	114.5**	106.8**	ns	19.4*
Dose (N)	1	ns	7.4**	ns	ns	ns	ns	13.2***	ns
Block	2	ns	ns	ns	ns	70.5*	157.4**	ns	25.1*
S x N	1	12.6***	ns	ns	ns	ns	ns	ns	ns
CV (%)		17.9	29.0	52.0	56.0	46.6	27.0	21.9	19.7
End of pasture cycle									
System (S)	1	ns	ns	ns	ns	ns	20.4*	ns	ns
Dose (N)	1	ns	ns	ns	ns	ns	ns	ns	ns
Block	2	ns	ns	ns	ns	ns	ns	ns	ns
S x N	1	11.45***	ns	ns	ns	ns	ns	ns	ns
CV (%)		15.6	24.7	43.7	44.8	32.1	20.5	17.9	13.8

CV: coefficient of variation. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns: not significant.

treatment in the CLT system at the end of both corn and pasture cycles (Figure 2). This response indicates that the effect of N treatment on microbial biomass depended on the production system. Previous studies conducted in the same long-term experiment documented reduced light availability and lower pasture productivity associated with tree presence (Pontes et al. 2018b, Pontes et al. 2020), suggesting that differences in plant productivity between CL and CLT may have contributed to the contrasting microbial responses.

Zandona et al. (2019), in a study also conducted in Ponta Grossa under a subtropical Cfb climate, evaluated soil microbiological attributes in a Dystrophic Red Latosol under full-sun pasture and a tree-based integrated system with *Eucalyptus dunnii*. The authors reported a lower microbial biomass N and acid phosphatase activity under the tree-based system, although microbial biomass C was not significantly affected by the production system. Their study included perennial warm-season grasses under contrasting N rates (0 and 300 kg ha^{-1} $year^{-1}$ of N), with soil sampled at 0-20 cm. The lower MBC under N90 in CLT may reflect a combined constraint on nitrogen and carbon availability for microbial growth. Lower pasture productivity previously reported in this system (Menoncin et al. 2026) may reduce plant-derived carbon inputs, whereas the higher N rate may alleviate microbial N limitation. At the end of the corn cycle, this response may be associated with the legacy of contrasting N management during previous pasture phases.

Under the higher nitrogen rate (N180), no significant differences in MBC were observed between the CL and CLT systems, indicating that the difference between systems observed under N90 was not maintained at the higher N rate. This response is consistent with the significant system × N interaction and indicates that the effect of N treatment on MBC depends on the production system. Previous findings from the same experimental area showed that higher N rates increased forage accumulation and affected arbuscular mycorrhizal fungal sporulation and glomalin-related soil proteins in CL and CLT systems (Menoncin et al. 2026), supporting the responsiveness of soil biological attributes to N management. However, the mechanisms underlying the MBC response observed here cannot be directly attributed to changes in plant growth, rhizosphere activity, or soil C inputs, as these processes were not directly measured in the present study.

Because the N90 and N180 treatments were imposed during the pasture phases, whereas the subsequent corn crop received the same N fertilization across treatments, the significant effects of N treatment detected at the end of the corn cycle should be interpreted as potential legacy effects of the contrasting N management history rather than as direct responses to differential N fertilization of corn. The contrasting N treatments had been part of the experimental design since the 2009/2010

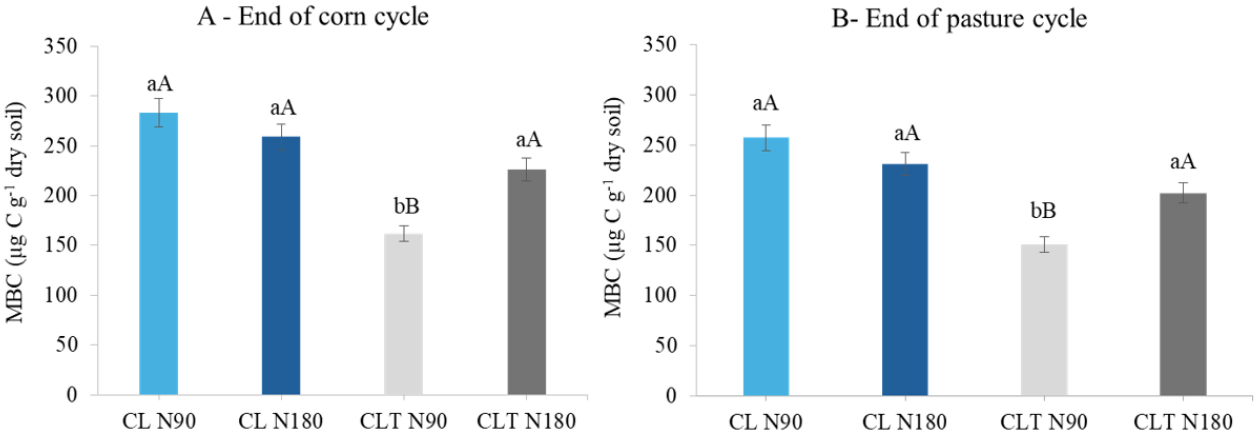


Figure 2. Microbial biomass carbon (MBC) in crop-livestock (CL) and crop-livestock with trees (CLT) systems under long-term contrasting N management histories of 90 (N90) and 180 (N180) kg ha^{-1}, evaluated at the end of corn (A) and pasture (B) cycles. Bars represent means ± standard error (n = 3). Different lowercase letters indicate significant differences between N treatments within each system, whereas different uppercase letters indicate significant differences between systems within each N treatment, according to the Tukey test ($p < 0.05$).

growing season (Pontes et al. 2021), indicating that the observed responses occurred within a long-term history of contrasting N management. Previous results from the same experiment also demonstrated residual effects of winter N fertilization on the subsequent corn crop (Pontes et al. 2018a). Microbial biomass nitrogen (MBN) increased significantly under the N180 treatment at the end of the corn cycle (Figure 3), indicating a greater amount of N associated with the microbial biomass in plots with a long-term history of higher N input. Because the contrasting N90 and N180 treatments were applied during the pasture phases, whereas the subsequent corn crop received the same N fertilization across treatments, this response should not be interpreted as a direct effect of recent N fertilization on corn. Instead, it may reflect legacy effects of the long-term contrasting N management during the pasture phases and the associated N cycling within the integrated system. In contrast, no significant differences in MBN were observed at the end of the pasture phase.

The microbial biomass C:N ratio was lower under N180 (8.2:1) than under N90 (9.8:1), indicating a relative increase in microbial biomass N under the long-term higher N treatment. According to Monteiro & Gama-Rodrigues (2004), microbial biomass accumulation depends on the capacity of the soil organic matter to meet microbial nutritional requirements. Soil microbial biomass commonly exhibits a C:N ratio of approximately 8:1 (Cleveland & Liptzin 2007); however, variation in this ratio may reflect changes in microbial community composition and nutrient availability. Therefore, the lower microbial C:N ratio under N180 is consistent with the greater MBN observed under this treatment, although the underlying mechanisms cannot be determined from the C:N ratio alone. The greater MBN under N180 was not accompanied by significant changes in microbial respiration (MR) or metabolic quotient (qCO_2). Thus, under the conditions evaluated, the higher N treatment was associated with greater microbial biomass N without detectable changes in these indicators of microbial metabolic activity. The absence of significant responses in MR and qCO_2 indicates that these attributes were less responsive to the contrasting N treatments than MBN during the corn cycle.

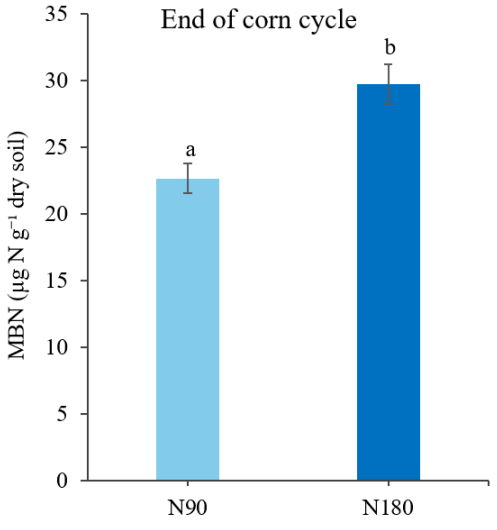


Figure 3. Microbial biomass nitrogen (MBN) at the end of corn cycle under long-term contrasting N management histories of 90 (N90) and 180 (N180) kg ha^{-1}. Values represent means across the crop-livestock and crop-livestock with trees systems because the system × N interaction was not significant. Bars represent means ± standard error. Different letters indicate significant differences between N treatments according to the Tukey test ($p < 0.05$).

The production system significantly influenced arylsulfatase, acid phosphatase, and FDA hydrolysis, with greater activities in CL than in CLT (Figure 4A). Arylsulfatase and FDA hydrolysis differed between systems at the end of the corn cycle, whereas the acid phosphatase activity was greater in CL at the end of both the corn and pasture cycles. These responses indicate differences in soil biological functioning between the two production systems. Previous studies conducted in the same long-term experiment documented differences in crop and pasture productivity between CL and CLT (Pontes et al. 2018b, Pontes et al. 2020, Menoncin et al. 2026), providing relevant context for the biological responses observed here. Greater arylsulfatase and acid phosphatase activities in CL indicate a higher potential for the enzymatic hydrolysis of organic S and P compounds, respectively, under the assay conditions. Greater FDA hydrolysis indicates higher overall hydrolytic activity. Together, these results suggest differences between the systems in the potential for biologically mediated hydrolysis of organic compounds under the assay conditions. Arylsulfatase activity was significantly greater in the CL system at the end of the corn cycle (Figure 4A), indicating differences between the production systems in an enzyme involved in the soil S cycling.

Arylsulfatase participates in the mineralization of organic sulfur compounds and is associated with organic matter turnover and decomposition processes (Balota et al. 2013). Nogueira & Melo (2003) reported positive correlations between arylsulfatase activity and total sulfur concentrations in agricultural soils, whereas Silva et al. (2015) associated greater enzymatic activity in integrated systems with higher *Brachiaria* biomass production and organic carbon inputs.

Although residual straw production did not differ significantly between systems during the evaluated period (Menoncin et al. 2026), greater corn and forage productivity has been reported under CL in this long-term experiment (Pontes et al. 2018a, Pontes et al. 2020, Menoncin et al. 2026). These findings provide agronomic context for the contrasting biological responses between systems. The greater arylsulfatase activity observed in CL indicates a higher potential for the enzymatic hydrolysis of organic sulfate esters under the assay conditions, highlighting a difference between the systems in an enzymatic process involved in soil S cycling.

Acid phosphatase activity was consistently greater in the CL system at the end of both the corn and pasture cycles (Figure 4A). Acid phosphatase plays a key role in the hydrolysis of organic P compounds and can originate from both plant roots and soil microorganisms (Tabatabai 1994, Balota et al. 2014). Therefore, the greater acid phosphatase activity observed in CL indicates differences between the production systems in an enzyme involved in organic P mineralization, but does not allow the relative contributions of plant roots and microorganisms to be distinguished. Galindo et al. (2020) also reported greater phosphatase activity in integrated production systems, demonstrating the responsiveness of this enzyme to agricultural management. Similarly, Zandona et al. (2019) reported lower acid phosphatase activity in a tree-based integrated system than in full-sun pasture. Despite differences in forage species, N rates, and sampling depth between the studies, both showed greater acid phosphatase activity in the system without trees, suggesting that this enzyme responds to differences in the configuration and management of integrated production systems.

β-glucosidase activity was significantly affected by N treatment, with greater activity under N180 than under N90 (Figure 4B). This enzyme participates in the final stages of cellulose degradation by hydrolyzing β-glucosides, releasing glucose that can be used for microbial energy metabolism and biomass synthesis (Tabatabai 1994, Dotaniya et al. 2019). Jian et al. (2016) and Chen et al. (2017) reported average increases of 11.2 and 11.1 %,

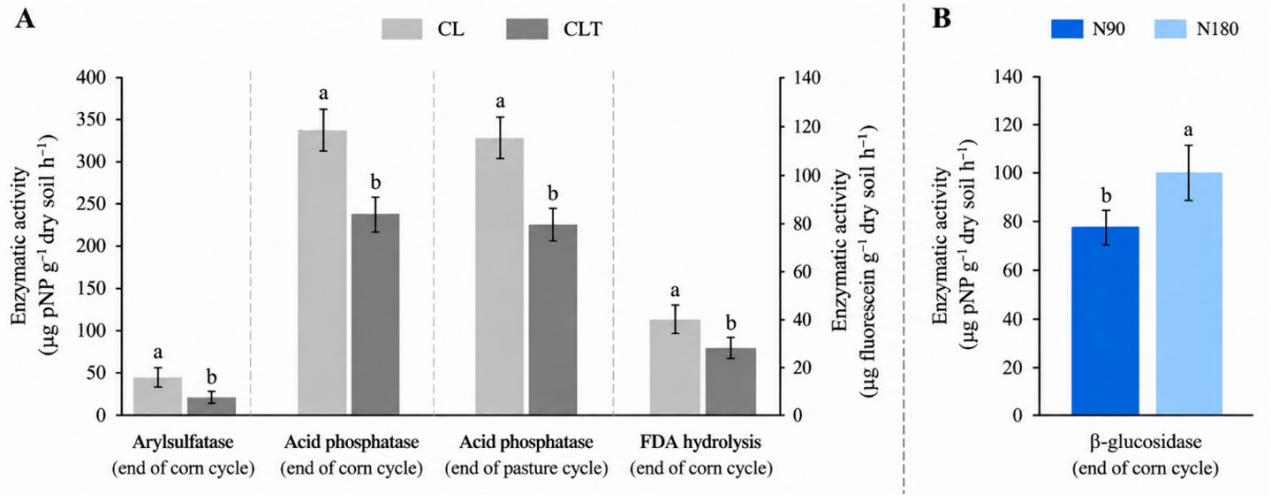


Figure 4. Soil enzymatic activities in crop-livestock (CL) and crop-livestock with trees (CLT) systems under long-term contrasting N management histories. A) arylsulfatase activity at the end of the corn cycle, acid phosphatase activity at the end of the corn and pasture cycles, and fluorescein diacetate (FDA) hydrolysis at the end of the corn cycle in CL and CLT systems; B) β-glucosidase activity at the end of the corn cycle under 90 (N90) and 180 (N180) kg ha^{-1} of N treatments. Enzyme activities are expressed as µg *p*-nitrophenol (pNP) g^{-1} of dry soil h^{-1}, except FDA hydrolysis, which is expressed as µg of fluorescein g^{-1} of dry soil h^{-1}. Bars represent means ± standard error (n = 3). Different letters within each enzyme indicate significant differences between production systems (A) or N treatments (B), according to the Tukey test ($p < 0.05$).

respectively, in β-glucosidase activity following N addition, consistent with the direction of the response observed here. One possible explanation is that greater N availability may alleviate microbial N limitation and favor investment in enzymes involved in C acquisition. In the present experiment, N90 and N180 were originally selected to represent limiting and non-limiting N supply for pasture production, respectively (Pontes et al. 2018b). Because corn received the same N fertilization across treatments, the greater β-glucosidase activity under N180 may reflect the legacy of contrasting N management during previous pasture phases. Franco et al. (2020) also reported greater β-glucosidase activity with increased plant diversity associated with grazing in an integrated crop-livestock system, further illustrating the responsiveness of this enzyme to agricultural management.

FDA hydrolysis was greater in the CL system at the end of the corn cycle (Figure 4A), indicating greater overall hydrolytic activity under the assay conditions. This assay reflects the combined activity of several hydrolytic enzymes and provides an integrative measure of soil microbial activity (Schnürer & Rosswall 1982). Its response was consistent with the greater arylsulfatase and acid phosphatase activities observed in CL after corn cultivation, showing that the differences between systems extended across enzymes with distinct substrates.

Taken together, the microbial biomass and enzymatic results show that the biological responses depended on the attribute evaluated, the production system, and the N management history. Differences between CL and CLT were evident in selected enzymatic activities, whereas higher MBN and β-glucosidase activity under N180 after corn cultivation indicated responses associated with contrasting N management during previous pasture phases. Greater corn yield and forage accumulation previously reported under CL in the same experiment (Pontes et al. 2018a, Pontes et al. 2020, Menoncin et al. 2026) complement this biological characterization by documenting agronomic differences between the systems.

At the end of the corn cycle, significant responses to production system or N treatment were observed in several microbiological and enzymatic attributes. At the end of the pasture phase, significant responses were restricted to MBC and acid phosphatase activity. These findings characterize the biological responses within each production phase, as the evaluation periods were analyzed separately and no direct statistical comparison between them was performed.

Neither MR nor qCO_2 showed significant responses to the management factors evaluated, whereas microbial biomass and selected enzyme activities did. Divergent responses among biological attributes were also reported by Jian et al. (2016), whose meta-analysis showed increases in several hydrolytic enzyme activities, but an average decrease in microbial biomass C under N fertilization. Although the response patterns differed from those observed here, their findings reinforce that microbial biomass and enzyme activities do not necessarily respond uniformly to N management. Together, these results highlight the value of combining microbial biomass, respiration, and enzymatic indicators to characterize soil biological functioning in integrated production systems.

CONCLUSIONS

1. The crop-livestock (CL) and crop-livestock with trees systems showed distinct soil biological responses under the specific conditions of this long-term experiment, with greater activities of selected enzymes observed in CL, whereas differences in microbial biomass carbon depended on the N treatment;
2. At the end of the corn cycle, microbial biomass nitrogen and β-glucosidase activity were greater in plots historically receiving 180 kg ha^{-1} of N than in those receiving 90 kg ha^{-1} of N during pasture phases, indicating responses associated with long-term contrasting N management histories;
3. Enzyme activities were more responsive to the management factors evaluated than microbial respiration and the metabolic quotient qCO_2, highlighting the value of using multiple biological indicators to assess soil biological functioning in integrated production systems.

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