

**University of São Paulo
“Luiz de Queiroz” College of Agriculture**

**Association of tropical forage grasses in pastures: interactions between
sward characteristics and grazing animals**

Emanoella Karol Saraiva Otaviano

Thesis presented to obtain the degree of Doctor in
Science. Area: Animal Science and Pastures

**Piracicaba
2023**

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RESUMO

Associação de plantas forrageiras tropicais em pastagens: interações entre características do dossel e animais em pastejo

O aumento da diversidade de plantas forrageiras em pastagens, por meio do cultivo simultâneo de gramíneas forrageiras com características complementares, pode resultar em uso mais eficiente dos recursos, tornando o sistema mais resiliente e sustentável. Embora existam diversos estudos direcionados para misturas de espécies forrageiras de clima temperado, espécies anuais ou misturas de gramíneas com leguminosas, são escassas na literatura informações acerca de misturas formadas por gramíneas forrageiras de clima tropical. A hipótese geral do estudo foi que a associação de gramíneas forrageiras tropicais com diferentes características funcionais gera complementaridade espacial e temporal na exploração de recursos, resultando em melhoria do valor nutritivo da forragem ao longo do ano e melhor distribuição das folhas ao longo do dossel forrageiro, resultando em maiores taxas de consumo de forragem e ingestão de nutrientes. O objetivo geral foi identificar e descrever mudanças na estrutura do dossel forrageiro de três espécies de gramíneas forrageiras tropicais perenes cultivadas em monocultivo e em associação e as interações entre características do pasto e dos animais em pastejo, visando identificar características das misturas que poderiam proporcionar aumento do valor nutritivo e consumo de forragem. O experimento foi conduzido em Piracicaba, SP, Brasil, de Dezembro/2020 a Março/2022. Os tratamentos corresponderam a três gramíneas forrageiras tropicais: *Andropogon gayanus* cv. Planaltina, *Panicum maximum* cv. Massai e *Brachiaria brizantha* cv. BRS Piatã cultivadas em monocultivo e em associação (com base na mesma proporção de sementes puras viáveis). O manejo da desfolhação foi comum para todos os tratamentos, correspondendo a 35 cm de altura pré-corte e 17,5 cm de altura pós-corte. Os resultados indicaram que a associação foi composta principalmente pelo capim-massai ao longo de todo o período experimental. Entretanto, a participação percentual das espécies na mistura foi dinâmica e variou ao longo das estações do ano. Essas variações influenciaram a composição botânica e a distribuição vertical dos componentes morfológicos da massa de forragem, o que moldou o comportamento ingestivo dos animais em pastejo. O capim andropogon apresentou a menor densidade volumétrica de forragem entre as estações do ano e a maior presença de invasoras, o que resultou em menores taxas de consumo de forragem. Entretanto, foi o tratamento que apresentou maiores teores de proteína bruta na forragem ao longo do experimento. As diferenças na composição química entre os tratamentos ao longo das estações do ano influenciaram as diferenças nas variáveis de incubação *in vitro*, como produção de metano, cinética e parâmetros da fermentação ruminal. Apesar da associação não ter apresentado maior valor nutritivo e redução na emissão de metano, outros parâmetros foram superiores, como a concentração de nitrogênio amoniacal e degradabilidade *in vitro* da matéria seca e da fibra em detergente neutro. Como conclusão, a associação apresentou resultados similares ou superiores aos melhores monocultivos nas variáveis estudadas, o que revela resultados promissores para o uso da associação de gramíneas forrageiras tropicais, representando oportunidade para aumentar os serviços ecossistêmicos da pastagem por meio do aumento no número de espécies na área.

Palavras-chave: Comportamento ingestivo, Estrutura do dossel, Fermentação *in vitro*, Pastagens multiespecíficas, Valor nutritivo.

ABSTRACT

Association of tropical forage grasses in pastures: interactions between sward characteristics and grazing animals

Increasing forage plant diversity in pastures through simultaneous cultivation of forage grasses with complementary characteristics can result in more efficient use of resources, making the system more resilient and sustainable. Although there are several studies focusing on mixtures of forage species from temperate climate, annual species, or mixtures of grasses with legumes, there is little information in the literature regarding mixtures of tropical forage grasses. The general hypothesis of the study was that the association of tropical forage grasses with different functional characteristics generates spatial and temporal complementarity in the exploitation of resources, resulting in improved nutritional value of the forage throughout the year and better distribution of leaves along the vertical profile of the canopy, resulting in greater rates of herbage intake and nutrients. The general objective was to identify and to describe changes in canopy structure of three tropical perennial forage grass species cultivated in monoculture and in association. In addition, to understand the interactions between pasture characteristics and grazing animals, aiming at identifying characteristics of mixtures that can provide increased nutritional value and forage consumption. The experiment was conducted in Piracicaba, SP, Brazil, from December/2020 to March/2022. Treatments corresponded to three tropical forage grasses: *Andropogon gayanus* cv. Planaltina (Andropogon gambagrass), *Panicum maximum* cv. Massai (Massai guineagrass) and *Brachiaria brizantha* cv. BRS Piatã (Piatã palisadegrass) cultivated in monoculture and in association (based on the same proportion of viable pure seeds). Defoliation management was common to all treatments, corresponding to 35 cm pre-cut height and 17.5 cm post-cut height. The results indicated that the association was mainly comprised of Massai guineagrass throughout the experimental period. However, the proportion of species in the mixture was dynamic and varied throughout the seasons. These variations influenced the botanical composition and the vertical distribution of morphological components of the herbage mass, which shaped the ingestive behavior of the grazing animals. Andropogon gambagrass had the smallest herbage bulk density throughout the year and greater presence of weeds, which resulted in smaller rates of herbage intake. However, this treatment presented the greatest content of crude protein throughout the experiment. Differences in chemical composition among treatments throughout seasons of the year influenced differences in *in vitro* incubation variables, such as methane production, kinetics, and parameters of ruminal fermentation. Although the association did not present greater nutritional value or reduction in methane emissions, other parameters were superior, such as the concentration of ammonia nitrogen and *in vitro* degradability of dry matter and neutral detergent fiber. In conclusion, the association showed similar or superior results to the best monocultures for the studied variables, which reveals promising results for the use of association of tropical forage grasses. This represents an opportunity to enhance ecosystem services provided by pastures by increasing the number of species in the area.

Keywords: *In vitro* fermentation, Ingestive behavior, Multispecific pastures, Nutritive value, Sward structure.

1. GENERAL INTRODUCTION

Grasslands provide numerous ecosystem services, which are mainly controlled by management practices (Sollenberger et al., 2019). In recent years, sustainable intensification in grazing management has promoted increased productivity and mitigation of greenhouse gas emissions (Congio et al., 2019; Cunha et al., 2023). In this sense, grazing management based on canopy structures that optimize herbage intake with high nutritional value improves animal performance and has the potential to mitigate environmental impact and emission of greenhouse gases per unit of product produced (Congio et al., 2018; Zubieta et al., 2021). However, intra-annual climatic variations cause high variability in forage productivity and nutritional value throughout the seasons of the year (Habermann et al., 2019).

The multifunctionality of pastoral ecosystems demonstrates a positive correlation with biodiversity (Weigelt et al., 2009). Biodiversity in this context refers to the coexistence of various living organisms within an ecosystem, and its richness is established through the interactions of these organisms with the prevailing edaphic and climatic conditions (Nabinger et al., 2006). The equilibrium of ecosystems directly influences two essential aspects: the diversity of species within a specific area and the homogeneity in the distribution of individuals of these species. This homogeneous distribution fosters the complementarity of ecological niches and the multifunctionality of these ecosystems (Magurran, 2004).

Ecological niche refers to the function of a species within an ecosystem and is defined by a set of conditions, resources, and interactions required for the species to succeed (Miller and Spoolman, 2009). As result, increases in primary production from pastures with species diversity are associated with the effects of species complementarity (Fridley, 2001). This complementarity originates from at least two mechanisms (Loreau et al., 2001): Firstly, the differentiation of ecological niches, where each species has distinct resource and environmental requirements for survival, enabling the coexistence of species with varying requirements. Secondly, the species facilitation, a process by which a particular species creates favorable conditions for the survival and growth of others by enhancing resource availability or mitigating biotic and abiotic disturbances or stresses (Cardinale et al., 2006).

Increased species diversity in pastures offers numerous benefits by enhancing the three-dimensional occupation of space above and below the soil surface (Spehn et al., 2000; Tracy and Sanderson, 2004). This optimization enhances resource capture and utilization, thereby maximizing ecological processes (Naeem et al., 1994). According to Spehn et al. (2000), species complementarity leads to improved canopy architecture, resulting in greater

leaf area index and, consequently, greater interception of incident light. Similarly, Naeem et al. (1994) demonstrated that mixed pastures have greater consumption rates of carbon and nitrogen molecules, resulting in increased plant tissue productivity. These findings also suggested that mixed pastures provide greater soil cover, preserving soil moisture and contributing to enhance organic matter decomposition, and increase water and nutrient absorption. Consequently, in pastoral environments, increased forage productivity (Grace et al., 2018; Haughey et al., 2018; Sonkoly et al., 2019), greater production stability (Duchini et al., 2019; Gross et al., 2014), and improved nutritional value of the forage produced (Deak et al., 2009; Nobilly et al., 2013) can occur, along with environmental impact mitigation (Cardinale, 2011; Lange et al., 2015; Yang et al., 2019), and enhanced animal performance (Dillard et al., 2018; Jonker et al., 2019; Nieman et al., 2019). These benefits collectively characterize more resilient and resistant systems (Hector et al., 2010; Tilman and Downing, 1994) compared to monocultures. Many studies have shown that multispecies pastures, when subjected to periods of drought or low temperatures, maintain relatively stable forage production or exhibit less pronounced reductions compared to pastures composed of a single forage species (Nobilly et al., 2013; Sanderson et al., 2005; Volaire et al., 2014).

The choice of species to compose an association should consider, among other factors, the characteristics related to species complementarity. According to Cruz et al. (2002), grasses may be grouped based on their resource acquisition abilities, with more competitive species being more efficient at using resources and generating new growth. In fertile environments, these species have greater capacity to capture and use resources and exhibit faster tissue turnover than more conservative species, thereby possessing larger number of axillary buds capable of generating new tillers (Davies, 1974). Conservative species, on the other hand, have smaller specific leaf area, longer leaf longevity, and late flowering (Cruz et al., 2002). In this context, species with different competitive abilities (resource competitors vs. resource conservers) can coexist in fertile environments (Gross et al., 2007), as this condition reduces below-ground competition for resources (Wedin and Tilman, 1993). When combined, these species can reduce the seasonality of forage production due to their distinct flowering periods, favoring longer periods of forage availability throughout the year.

Despite the positive relationship between multifunctionality of pastoral ecosystems and species diversity (Weigelt et al., 2009), to increase forage production and stability, planting two or three species with complementary growth strategies may be more efficient than a complex mixture of 10 or more species randomly. This approach is most effective

when the associated species are adapted to environmental conditions and management practices (Tracy and Sanderson, 2004). In fertile environments, competition for light becomes the main limiting factor for species diversity (Borer et al., 2014; Eskelinen et al., 2022). However, frequent and non severe defoliation can maintain favorable conditions for development of populations of both competitive and conservative species. This reduction on the intensity of disturbances (e.g. defoliation) and stresses (e.g. competition for light) allows for these different species to coexist and persist (Borer et al., 2014; Duchini et al., 2018; Grime, 1977). There are few studies regarding the coexistence and persistence of tropical grass forages cultivated in association (multispecies pastures). The fact that hundreds of species coexist in the natural fields of Brazil, many of which perennial warm-season grasses (Boldrini, 2009), suggests that this system may also be viable for tropical species. Therefore, studies aiming at identifying the promising tropical grasses for pasture associations and ensuring their persistence and multifunctionality through adequate management practices are crucial.

Plant communities jointly regulate ecosystem processes and their agronomic and ecological responses to changes in the environment (Adair et al., 2019). In this context, pastures may be considered highly regulated systems, where any influence determines morphophysiological adaptations, modifying forage canopy structure and, consequently, patterns of plant growth and development. Understanding canopy structure and its implications to plant development, dynamics and agronomic performance is essential for managing the production system, given that canopy structure is more important than nutritional factors in regulating forage intake of grazing animals (Cunha et al., 2023; Da Silva et al., 2015; Hodgson et al., 1994; Poppi et al., 1987). Thus, describing changes in canopy structure when plants are grown in association allows for planning management practices that can maximize forage nutritive value and animal performance. Changes in canopy structure can cause changes in forage intake rate, primarily due to the presence and density of stem in the forage mass (Benvenuti et al., 2006; Drescher et al., 2006), and these changes occur mainly due to modifications in bite mass (Mezzalana et al., 2014). The intake rate of grazing animals is maximized when the ideal pre-grazing height is used for each forage species (Fonseca et al., 2012) because to maintain high ingestion rates, the pasture herbage mass must be comprised predominantly of leaves (Guzatti et al., 2017).

Enteric methane emissions are related to the nutritional value of forage and the canopy structure of pastures (Congio et al., 2018). In a recent meta-analysis, the authors concluded that sward structure plays a relatively more significant role in emissions compared

to nutritional value (Cunha et al., 2023). In this context, optimizing botanical composition and grazing management to ensure appropriate canopy structure of the grazing stratum, characterized by greater proportion of leaves relative to stems and dead material, can contribute to the intake of better-quality forage with superior nutritive value (Zubieta et al., 2021). There is limited existing literature on *in vitro* methane emissions in pastures composed of multiple grass species. Some studies involving mixtures of grasses and legumes have shown that methane emissions may not consistently decrease in these mixtures (Loza et al., 2021). This inconsistency may be attributed to the presence of tannins in legumes, which have anti-methanogenic properties but may also reduce forage digestibility (Lima et al., 2018).

Increasing biodiversity in pastures by cultivating forage grasses in association provides a series of benefits that can make the production system more sustainable (Nabinger et al., 2006; Pontes et al., 2012). Such benefits result from better use of the available environmental resources (Cardinale, 2011; Tilman et al., 1996), greater production stability in the face of intra-annual irregularities (Grace et al., 2018; Haughey et al., 2018), increased nutritive value of consumed forage (Nobilly et al., 2013) and greater animal performance (Schaub et al., 2020). This approach conditions the establishment of dynamic and multifunctional ecosystems (Weigelt et al., 2009), enabling the maintenance of productivity through increments of a holistic nature in the pastoral environment.

Despite the benefits of using multispecific pastures demonstrated in several studies with forage species from temperate climate (Duchini et al., 2019; Pontes et al., 2012; Scherer-Lorenzen et al., 2003; Spehn et al., 2000), there are few studies regarding perennial tropical forage species and their association in pastures. In Brazil, because of its edaphoclimatic conditions and seasonality of herbage production, the association of forage plants in pastures would be an interesting and strategic alternative to sustainable intensification of pastoral systems. Since pasture areas represent around 45% of the national territory (IBGE, 2017), increases in their productivity and sustainability have large potential impact on pasture-based production systems. In this context, the association of forage grasses has large potential for use, favoring its dissemination throughout tropical regions, particularly in areas where restoration of degraded pastures is necessary. Therefore, it is essential to understand how these plants respond when cultivated in association and their interaction with the environment and grazing animals. Additionally, these studies may help to develop protocols for selecting suitable forage plants for the mixtures and appropriate management strategies.

1.1. Hypothesis

The association of tropical forage grasses with different functional traits generates spatial and temporal complementarity in exploring ecological niches, resulting in more efficient resource use and greater productivity. This structural and phenological complementarity enhances the nutritional value of the forage throughout the year and improves leaf distribution within the sward canopy, resulting in greater rates of herbage and nutrient intake.

1.2. General objective

To identify and describe changes in canopy structure of three perennial tropical forage grasses cultivated as monocultures and in association and the interactions between sward characteristics and grazing animals, aiming at identifying characteristics of mixed pastures that may provide enhancement of herbage nutritional value and intake.

1.3. Specific objectives

Chapter 1: (i) to characterize the sward canopy structure of the monocultures and the association, and (ii) to investigate the interactions between these characteristics and the ingestive behavior of dairy heifers (Figure 1).

Chapter 2: to determine whether the nutritive value and in vitro gas production of the association among the three forage grass species differ from pastures cultivated as their monocultures (Figure 1).

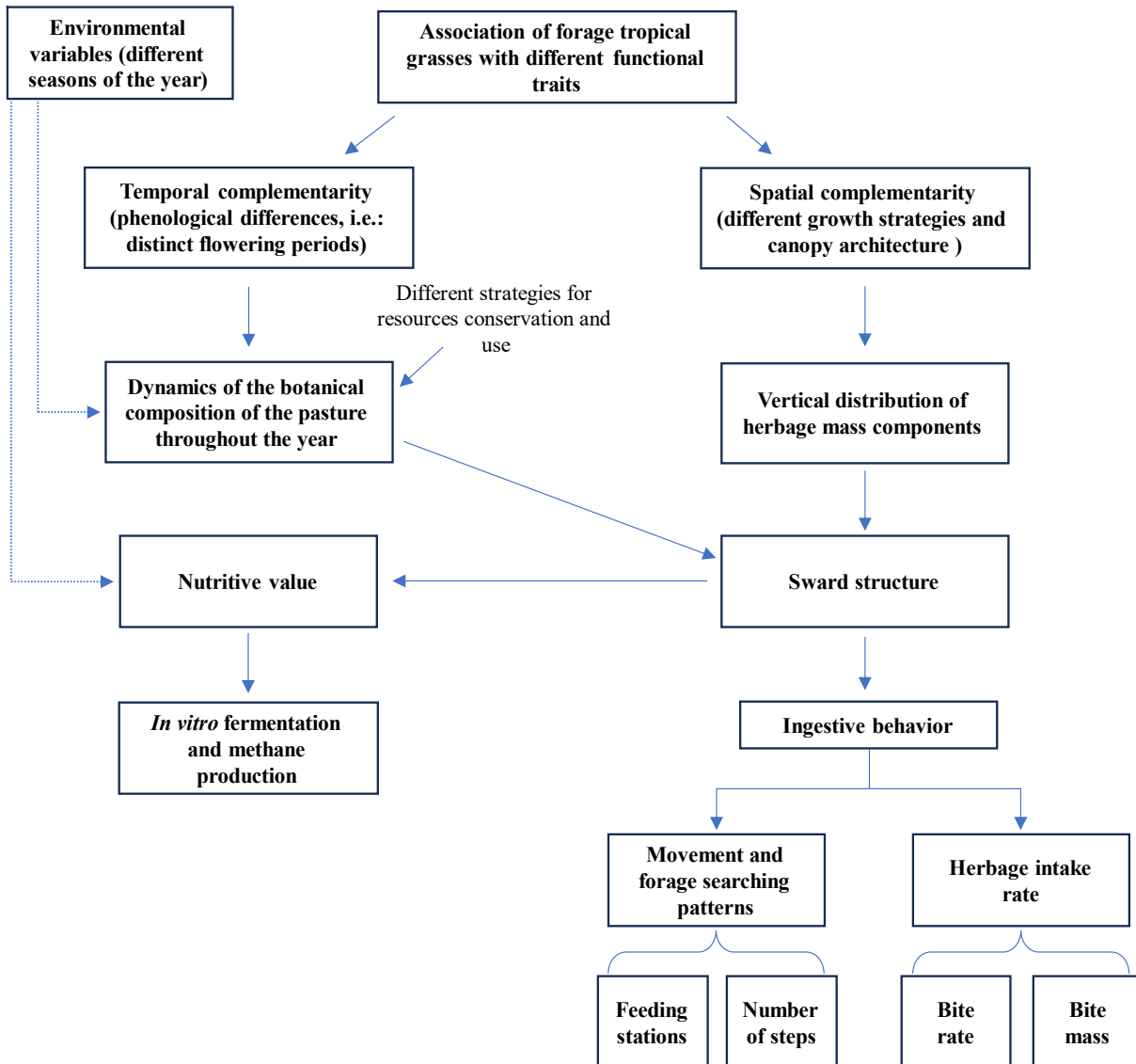


Figure 1. Conceptual model illustrating the relationships among the key variables investigated in the present study.

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2. SWARD STRUCTURE AND RATE OF HERBAGE INTAKE OF THREE TROPICAL FORAGE GRASSES CULTIVATED AS PURE OR MIXED STANDS

Abstract

Using forage grass species with complementary growth and resource-use strategies to increase forage plant diversity in pastures may be an alternative to traditional monocultures in tropical regions. The use of mixed swards has shown benefits in terms of efficient distribution of canopy leaf area, which may improve herbage intake rate of grazing animals. Hence, the objective of this study was to identify whether an association of three perennial tropical forage grasses could be an alternative to enhance herbage intake rate relative to their respective monocultures. Treatments corresponded to three perennial tropical forage grass species: *Andropogon gayanus* cv. Planaltina (Andropogon gambagrass); *Panicum maximum* cv. Massai (Massai guineagrass); and *Brachiaria brizantha* cv. BRS Piatã (Piatã palisadegrass) cultivated in monoculture and in association, with four replications. Defoliation management was common to all treatments and corresponded to a pre-cutting height of 35 cm and a post-cutting 17.5 cm. The botanical composition of the association was dynamic throughout the experimental period, with variations in the proportion of species across seasons of the year. The upper half of the canopy was comprised predominantly of leaves for all treatments. Sward structure of mixed stands allowed for high values of intake rate, being superior to the monocultures during Winter/Early Spring. Greater proportion of stems and dead material was present in the herbage mass during Autumn due to the climatic conditions and tropical grasses phenology. *Andropogon gambagrass* showed smaller rates of intake relative to the remaining treatments. The findings of this study indicate that it is possible to combine different tropical forage grass species without compromising sward structure and grazing animal responses compared to single grass species pastures. The choice of forage species for mixed pastures should consider their phenological cycle, growth, and resource use strategies aiming at achieving temporal complementarity and providing adequate grazing for animals throughout the year.

Keywords: Bite mass, Botanical composition, Feed stations, Ingestive behavior, Multispecific pastures

2.1. Introduction

Grasslands provide numerous ecosystem services when properly managed and play a crucial role in ensuring global food security (Boval and Dixon, 2012; Schaub et al., 2020). They cover a major part of the world's agricultural area (Hewins et al., 2018; Schaub et al., 2020) and are a valuable resource for pasture-based animal production systems. In recent years, the adoption of adequate grazing management strategies has contributed to sustainable intensification (Congio et al., 2018), along with increasing productivity and reducing the intensity of CH₄ and N₂O emissions (Congio et al., 2021; Cunha et al., 2023).

To achieve sustainable intensification of agriculture worldwide, it is crucial to develop resilient production systems capable of adapting to varying climatic conditions. This can be done by promoting biodiversity conservation and enhancing global food production

without causing harm to the environment (Royal Society, 2009). Increasing pasture biodiversity using forage grass species with complementary growth and resource use strategies may be an alternative to traditional monocultures in tropical regions (Medeiros-Neto et al., 2023). The use of mixed swards has shown benefits in terms of yield stability (Duchini et al., 2019; Lorenz et al., 2020), nutritive value (Sanderson, 2010), efficient distribution of sward leaf area (Naeem et al., 1994; Schmid and Niklaus, 2017; Williams et al., 2017), and animal performance (McCarthy et al., 2020; Roca-Fernández et al., 2016). More benefits can be obtained by mixing species belonging to different functional groups and with complementary growth characteristics (Deak et al., 2007). However, despite the benefits of using multispecific pastures demonstrated in several studies carried out in temperate climate regions (Duchini et al., 2019; Pontes et al., 2012; Scherer-Lorenzen et al., 2003; Spehn et al., 2000) information regarding perennial tropical forage species and their association is scarce or almost nonexistent in the literature.

Plant communities jointly regulate ecosystem processes, as well as their agronomic and ecological responses to environmental changes (Adair et al., 2019). In this context, pastures are considered highly regulated systems, where any influence determines morphophysiological adaptations of plants. These adaptations lead to modifications in forage canopy structure and, consequently, on patterns of plant growth and development (Pereira et al., 2014). Grazing management strategies based on canopy structures that optimize forage intake with high nutritive value improve animal performance and systems' productivity. It also has the potential to mitigate environmental impact and emission of greenhouse gases per unit of product produced (Congio et al., 2021, 2019; Zubieta et al., 2021).

Therefore, understanding of canopy structure characteristics is essential for pasture management, as it holds more significance than nutritional factors in regulating forage intake of grazing animals (Cunha et al., 2023; Da Silva et al., 2015; Hodgson et al., 1994; Poppi et al., 1987). Changes in forage canopy structure can cause changes in herbage intake rate, primarily due to the presence and density of stems in the herbage mass (Benvenuti et al., 2006; Drescher et al., 2006), and these changes occur mainly due to modifications in bite mass (Mezzalana et al., 2014). The intake rate of grazing animals is maximized when the ideal pre-grazing height is used for each forage species (Fonseca et al., 2012). In this sense, greater intake rates positively correlate to the prevalence of green leaves in the grazing stratum (Guzatti et al., 2017). Thus, describing changes in canopy structure and its interactions with grazing animals when plants are grown in association allows for management practices that maximize animal response.

Despite the substantial literature regarding increasing biodiversity in grasslands, the interactions, and responses of C₄ tropical forage grasses when mixed in the same area need to be better understood. We hypothesized that multispecific swards composed of grass species with complementary traits could lead to a better distribution of leaves within the canopy, influencing the ingestive behavior of grazing animals. Thus, this study aimed to identify whether an association of three perennial tropical forage grasses could be an alternative to enhance herbage intake rate relative to their respective monocultures.

2.2. Material and methods

The procedures for this study were approved by the Animal Ethics Committee of “Luiz de Queiroz” College of Agriculture (ESALQ/USP, Protocol No. 9581050121) from the University of São Paulo.

2.2.1. Study site

The experiment was carried out from December 2020 to March 2022 at Luiz de Queiroz College of Agriculture, University of São Paulo (ESALQ/USP), in Piracicaba, São Paulo, Brazil (22°42'34" S, 47°38'25" W, 546 m above sea level). According to the Köppen classification, the local climate is classified as Cwa (subtropical climate with dry Winter and hot Summer) (Alvares et al., 2013; Beck et al., 2018). Weather data were obtained from a meteorological station located approximately 2000 m from the experimental site (Figures S1 and S2, Supplementary material).

The soil is a Red Eutroferric Nitisol with a clayey texture (FAO, 2015). Average soil characteristics (0-20 cm layer) before the implementation of the experiment were phosphorus (P) = 49.5 mg dm⁻³ (ion-exchange resin extraction method); organic matter = 33.8 g dm⁻³; pH CaCl₂ = 4.50; potassium (K) = 3.45 mmolc dm⁻³; calcium (Ca) = 30.3 mmolc dm⁻³; magnesium (Mg) = 12.5 mmolc dm⁻³; hydrogen and aluminum (H + Al) = 72.5 mmolc dm⁻³; sum of bases = 46.0 mmolc dm⁻³; cation exchange capacity = 119 mmolc dm⁻³; base saturation = 39.0%. Sand = 358 g kg⁻¹, silt = 196 g kg⁻¹, and clay = 446 g kg⁻¹. These characteristics indicated the need to increase base saturation, which was carried out by using dolomitic limestone aiming at reaching 70% (Raij et al., 1997) during late Winter 2019. It was not necessary to apply any fertilizer during sowing because nutrient contents were adequate for the forage grass species to be cultivated (Raij et al., 1997).

2.2.2. Treatments, experimental design and establishment

Treatments consisted of three perennial tropical forage grass species: *Andropogon gayanus* cv. Planaltina (Andropogon gambagrass); *Panicum maximum* cv. Massai (Massai guineagrass); and *Brachiaria brizantha* cv. BRS Piatã (Piatã palisadegrass) cultivated in monoculture and in association. Treatments were assigned to experimental units (16 paddocks; 12 x 15 m each) according to a randomized complete block design with four replications.

For the choice of forage species that would compose the mixture, the criterion of different growth strategies (capture and conservation of resources) of plants, in addition to differences in architecture and growth habit (Loreau and Hector, 2001; Pontes et al., 2012), following a protocol similar to that described by Duchini et al. (2018, 2019). In this context, *Andropogon gambagrass* represents a conservative plant (low specific leaf area, high leaf longevity and caespitous growth), in addition to present mechanisms that grant it tolerance to insect attack, mainly spittlebugs (Pires, 2010); *Piatã palisadegrass* represents a competitive plant for resources (high specific leaf area and renewal of leaves and tillers), and *Massai guineagrass* represents an intermediate plant (intermediate specific leaf area and slower renewal of leaves and tillers when compared to *Piatã palisadegrass*, in addition to presenting semi-upright growth). In addition, these species are drought-tolerant and great productive capacity under intensive management (Fonseca and Martuscello, 2010).

Sowing was carried out manually in January 2020 at a rate of 300 pure viable seeds m², with 100 viable seeds from each species for the association, followed by compaction with a compactor roller weighing approximately 100 kg. Fertilization was performed 21 days after sowing using 40 kg N ha⁻¹ as ammonium nitrate. Paddocks were kept growing until reaching the pre-cutting target height of 35 cm when the first cut was made for standardization and definition of the post-cutting height of 17.5 cm.

2.2.3. Management and maintenance of experimental conditions

The pre-cutting height for each grass species was defined based on the 95% canopy light interception (LI) during regrowth criterion (Da Silva et al., 2015) and its flexibility range within which herbage accumulation is stable (Gomes, 2019; Sbrissia et al., 2018). This flexibility implies that a pre-cutting height up to 40% smaller than the corresponding canopy height at 95% LI may be used without reducing herbage accumulation. According to the 95% LI criterion, the maximum pre-cutting height for *Piatã palisadegrass*, *Andropogon*

gambagrass, and Massai guineagrass is 35, 50, and 55 cm, respectively (Barbosa et al., 2010; Crestani et al., 2017; Sousa et al., 2010). Considering the possibility of flexibilizing these management targets, it is possible to manage *Andropogon gamba* grass with up to 30 cm and Massai guinea grass with up to 33 cm of pre-cutting height. Therefore, the pre-cutting height of 35 cm was compatible with all three forage grass species cultivated. The post-cutting height was 17.5 cm, equivalent to a 50% defoliation severity, was sufficient to ensure high residual leaf area index (Giacomini et al., 2009; Silveira et al., 2013). This would allow for frequent, non-severe defoliations and favor growth and development of the associated species by reducing levels of disturbance (grazing severity) and stress (competition for light).

Canopy height was monitored every three days during regrowth, starting soon after each cut. As canopy height approached values close to the targeted pre-cutting height of 35 cm, the frequency of monitoring increased to daily until paddocks reached the targeted canopy height for defoliation. Measurements were taken using a sward stick along 40 points per paddock, distributed across four transect lines.

Nitrogen fertilization was performed only during the rainy periods of the year (October to March), always at post-cutting, using ammonium nitrate. The amount of nitrogen for each application was proportional to the cutting interval of each paddock (daily rate of 1.7 kg N ha⁻¹) and calculated to result in equal amounts of nitrogen applied to all paddocks at the end of each rainy season.

2.2.4. Measurements

2.2.4.1. Herbage mass

Herbage mass was measured once every season of the year (Summer I, Autumn, Winter/Early Spring, Late Spring, and Summer II) at the pre-cutting condition. Two samples were collected per paddock by harvesting all the herbage contained within two metallic frames (100 x 25 cm) at ground level from representative sites at the time of sampling (visual assessment of canopy height and herbage mass). After harvesting, herbage samples were taken to the laboratory, where a subsample was taken for manual separation of botanical and morphological components. For the monocultures, samples were separated into stems (stem + sheath), leaves (green leaf blade), seedheads, weeds, and dead material. For the association samples, each component was separated by grass species, except dead material, because of the difficulty of distinguishing this component for each grass species. After separation, all

samples were dried at 65 °C in a forced draught oven until constant weight, and data were used to calculate herbage mass (kg DM ha⁻¹).

2.2.4.2. Vertical structure of the sward and herbage bulk density

Assessment of the vertical structure of the sward was carried out once every season of the year (Summer I, Autumn, Winter/Early Spring, Late Spring, and Summer II). The vertical distribution of the morphological components (and species in the association) was measured at the pre-cutting condition using an inclined point quadrat with the graduated stem at a 32.5° angle (Laca and Lemaire, 2000; Wilson, 1960). The equipment was placed in areas that represented the average sward condition at the time of sampling (visual assessment of canopy height and herbage mass).

For each paddock, a minimum of 100 contacts was recorded. The structure (leaf blade, stem, seedheads, dead material, and weeds) and the touched species (in the association) were identified, and the contact height was recorded. The touched component was then carefully removed from the tip of the rod, and the process repeated until the rod's tip touched the ground. The last contact height reading of the rod was recorded as a reference for calculating the effective height of the previous contacts. Data were used to calculate the percentage of touches of morphological and botanical components distribution along 5-cm vertical canopy strata.

Herbage bulk density was calculated by dividing sward herbage mass by the corresponding average sward height and expressed in kg ha⁻¹. To estimate the distribution of the mass for each botanical and morphological component within each stratum, the mass of each component was multiplied by the respective percentage of touches in that component (Gomes et al., 2018; Homem et al., 2021), and the results presented as kg ha⁻¹ per stratum of 5 cm.

2.2.4.3. Ingestive behavior

Measurements of ingestive behavior were performed every season of the year (Summer I, Autumn, Winter/Early Spring, Late Spring, and Summer II) using three Holstein dairy heifers with an average body weight of 200 ± 30 kg. Two animals were used for actual - measurements and the third animal was used to maintain minimum group size (Silva et al., 2018). Animals were adapted to the experimental procedure approximately ten days before the beginning of the grazing trials. The evaluations were always carried out early in the

morning (between 6–8 am) or late in the afternoon (between 5–7 pm) when grazing activity of the animals is typically high (Gregorini, 2012). The animals were not deprived of food before grazing trials, as fasting could interfere with selectivity (Newman et al., 1994) and affect measurements of herbage intake rate (Greenwood and Demment, 1988). Each grazing trial lasted 45 minutes, the minimum time required to detect weight fluctuations on a 10 g precision electronic scale.

Short-term intake rate was determined using the double-weighing technique, according to Penning and Hooper (1985). Before the grazing trials, animals were equipped with bags for collecting urine and feces. Then, they were conducted to the paddocks, where they grazed for 45 minutes. After grazing, animals were maintained in an adjacent area, with no access to water and solids for the same grazing period, aiming to estimate the metabolic weight loss (H₂O evaporation and CO₂ and CH₄ production). All weights (pre- and post-grazing and post-metabolic losses) were determined using a 10 g precision electronic scale. The short-term intake rate was calculated using the equation:

$$STIR = DM \times \frac{(W2 - W1)}{t2 - t1} + \frac{(W3 - W4)}{t4 - t3} \times \frac{(t2 - t1)}{ET}$$

Where: STIR = short-term intake rate; DM = dry matter content of the forage; W1 and W2 = animal body weight before and after grazing (kg); t1 and t2 = pre and post-grazing time (min); W3 and W4 = animal body weight before and after metabolic losses (kg); t3 and t4 = pre and post metabolic losses time; and ET = eating time (min). The dry matter content was determined by collecting hand-plucked forage samples immediately after grazing. These samples were immediately weighed and placed into a forced draught oven at 65 °C until constant weight.

Bite rate (number of bites min⁻¹) was measured by a trained observer using a stopwatch and a manual counter. During grazing, readings of the time required for animals to take 20 bites were taken for each evaluated animal and used to calculate bite rate (Forbes and Hodgson, 1985). Bite mass (g DM bite⁻¹) was calculated by dividing the total amount of herbage consumed during the 45-min grazing trial by the total number of bites taken during the grazing trial.

2.2.4.4. Patterns of animal movement and forage searching

Measurements of animal movement and forage searching were carried out concomitantly to measurements of ingestive behavior. During the grazing trials, trained

observers recorded the time necessary for animals to encounter 10 feeding stations and the number of steps taken during this process. The feeding station was defined as the hypothetical semicircle in front of the animal that can be reached without moving its front paws (Ruyle and Dwyer, 1985). The steps were counted using the movement of the front paws as criterion. These data were used to calculate the following response variables: number of feeding stations per minute, number of steps between feeding stations, average movement rate, time per feeding station, and bites per feeding station (Gregorini et al., 2011).

2.2.5. Statistical analysis

Data were analyzed using the PROC GLIMMIX of SAS, version 9.4 (SAS Institute Inc.). Each paddock was considered as experimental unit. For evaluations relative to ingestive behavior and movement patterns of the grazing animals, data from the pair of test animals were averaged to obtain a single value of each variable per grazing session, which was then considered as the experimental unit. Treatments, seasons of the year, and their interactions were considered fixed effects, while block was treated as random effect. Seasons of the year were treated as repeated measures. Data were tested for normal distribution and homoscedasticity. The variance-covariance matrix was selected based on the Bayesian information criterion. Means were estimated using the LSMEANS procedure and compared using the probability of difference (PDIFF) with Student's t-test ($P < 0.05$).

2.3. Results

2.3.1. Herbage mass and bulk density

Herbage mass varied with treatment ($P = 0.0102$), season of the year ($P < 0.0001$), and with the treatment x season of the year interaction ($P = 0.0004$). During Summer I and Autumn, *Andropogon gambagrass* exhibited the smallest values, with no difference among the remaining treatments (Figure 1). During Winter/Early Spring, there was no difference among treatments. During Late Spring, *Piatã palisadegrass* and *Andropogon gambagrass* had greatest herbage mass. During Summer II, *Piatã palisadegrass* showed the greatest herbage mass. Overall, greater herbage mass values were recorded during Autumn, with minor differences among the remaining seasons of the year.

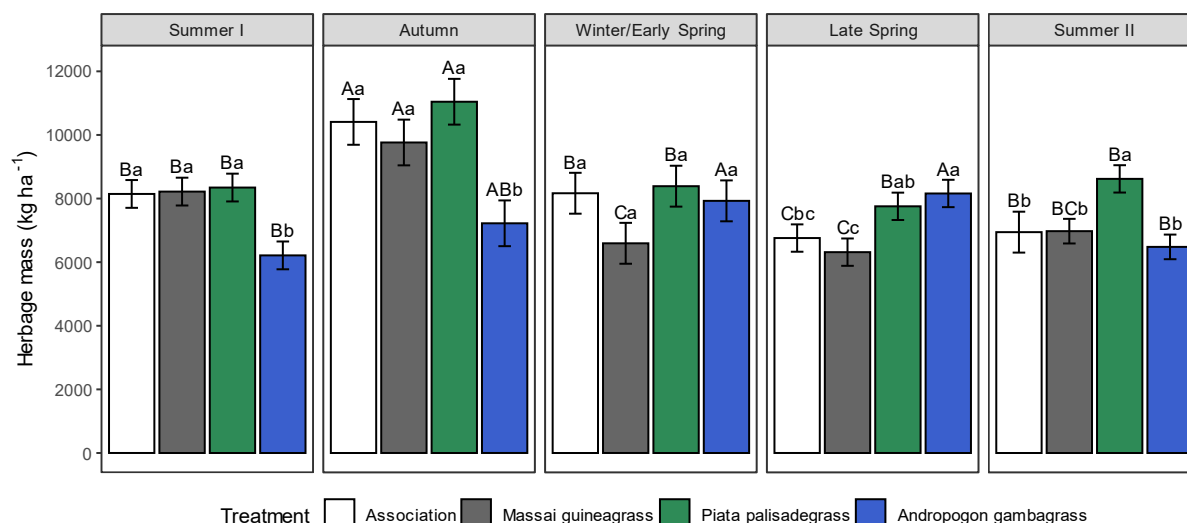


Figure 1. Herbage mass in pastures of *Andropogon gambagrass*, *Massai guineagrass*, and *Piatã palisadegrass* cultivated as monocultures and in association. Columns with the same lowercase letters within seasons of the year and treatments with the same uppercase letters among seasons of the year are not different ($p < 0.05$). Bar indicates the standard error of the mean.

Herbage bulk density varied with treatment ($P = 0.0028$), season of the year ($P < 0.0001$), and with the treatment \times season of the year interaction ($P < 0.0001$). Greatest values were recorded during Autumn, while smallest during Late Spring, with intermediate values during the remaining seasons of the year (Table 1). Overall, *Piatã palisadegrass* had greatest herbage bulk density, particularly during Autumn and Summer II. During Summer I and Autumn, the smallest values were recorded for *Andropogon gambagrass*. There was no difference among treatments during Winter/Early Spring. The Association generally showed similar values to *Massai guineagrass* across all seasons of the year.

Table 1. Herbage bulk density of *Andropogon gambagrass*, *Massai guineagrass*, and *Piatã palisadegrass* cultivated as monocultures and in association.

Season of the year	Association	Massai guineagrass	Piatã palisadegrass	Andropogon gambagrass	Mean
Summer I	231.8 BCa (12.68)	234.3 Aa (12.68)	237.0 BCa (12.68)	175.6 Bb (12.68)	219.7 BC (6.81)
Autumn	301.8 Ab (21.43)	279.2 Ab (21.43)	364.4 Aa (21.43)	209.4 ABc (21.43)	288.7 A (11.00)
Winter/Early Spring	232.7 Ba (18.43)	189.1 Ba (18.43)	238.7 BCa (18.43)	224.8 Aa (18.43)	221.3 B (9.55)
Late Spring	193.0 Dbc (12.02)	180.3 Bc (12.02)	220.9 Cab (12.02)	229.7 Aa (12.02)	206.0 C (6.51)
Summer II	199.5 CDb (11.28)	199.0 Bb (11.28)	251.3 Ba (12.24)	182.6 Bb (11.28)	208.1 BC (6.28)
Mean	231.8 b (8.26)	216.4 bc (8.26)	262.4 a (8.32)	204.4 c (8.26)	

Means followed by the same uppercase letters in columns and lowercase letters in rows are not different ($p < 0.05$). Values in parentheses correspond to the standard error of the mean.

Leaf bulk density varied with treatment ($P < 0.0001$), season of the year ($P < 0.0001$), and with the treatment x season of the year interaction ($P < 0.0001$). Greater values were recorded during Summer I, Autumn, and Summer II (Table 2). Piatã palisadegrass consistently showed greater leaf bulk density than the remaining treatments throughout the experimental period. Stem bulk density also varied with treatment ($P < 0.0001$), season of the year ($P < 0.0001$), and with the treatment x season of the year interaction ($P < 0.0001$). Similar to leaf bulk density, Piatã palisadegrass showed greater stem bulk density than the remaining treatments throughout the experimental period. Greatest values were recorded during Autumn. *Andropogon gambagrass* showed the smallest and relatively stable values throughout the experiment. Dead material bulk density varied with season of the year ($P < 0.0001$) and with the treatment x season of the year interaction ($P < 0.0001$). Greatest values were recorded during Autumn and Winter/Early Spring, while smallest were recorded during Summer I and Summer II. *Andropogon gambagrass* showed greater values during Late Spring and Summer II relative to the remaining seasons of the year. There was no difference among treatments during Summer I and Winter/Early Spring.

Table 2. Morphological components bulk density in pastures of *Andropogon gambagrass*, *Massai guineagrass*, and *Piatã palisadegrass* cultivated as monocultures and in association.

Season of the year	Treatments				Mean
	Association	Massai guineagrass	Piatã palisadegrass	Andropogon gambagrass	
Leaf bulk density (kg ha ⁻¹ cm ⁻¹)					
Summer I	86.2 Aa (3.73)	83.5 Aa (3.73)	86.3 Ba (3.73)	47.7 Cb (3.73)	75.9 A (1.87)
Autumn	60.1 BCb (7.62)	55.0 BCb (7.62)	106.6 Aa (7.62)	53.1 BCb (7.62)	68.7 AB (3.81)
Winter/Early Spring	57.11 BCb (3.98)	46.5 Cc (3.98)	86.4 Ba (3.98)	57.9 Bb (3.98)	62.0 B (1.99)
Late Spring	55.6 Cb (3.05)	64.6 Ba (3.05)	72.5 Ca (3.05)	68.3 Aa (3.05)	65.3 B (1.52)
Summer II	66.4 Bbc (4.19)	74.6 Aab (4.19)	80.3 BCa (4.66)	62.0 ABc (4.19)	70.8 A (2.16)
Mean	65.1 b (2.42)	64.8 b (2.42)	86.4 a (2.45)	57.8 b (2.42)	
Stem bulk density (kg ha ⁻¹ cm ⁻¹)					
Summer I	71.3 Ba (6.86)	73.6 Aa (6.86)	81.2 Ba (6.86)	30.3 Ab (6.86)	64.1 B (3.80)
Autumn	97.0 Aab (7.21)	79.4 Ab (7.82)	110.3 Aa (7.21)	35.5 Ac (7.21)	80.5 A (4.03)
Winter/Early Spring	42.6 Db (3.20)	33.49 Cc (3.20)	50.8 Da (3.20)	37.4 Abc (3.20)	41.1 D (2.29)
Late Spring	48.0 CDb (3.97)	45.2 Bb (3.97)	63.5 Ca (3.97)	41.5 Ab (3.97)	49.6 C (2.57)
Summer II	51.7 Cb (3.04)	51.6 Bb (3.04)	67.9 BCa (3.21)	35.2 Ab (3.04)	51.6 C (2.25)
Mean	62.1 b (3.10)	56.7 b (3.16)	74.8 a (3.11)	36.0 c (3.10)	
Dead material bulk density (kg ha ⁻¹ cm ⁻¹)					
Summer I	68.4 Ba (6.46)	69.9 Ba (4.46)	64.9 Ca (6.46)	61.9 Da (6.46)	66.3 C (3.23)
Autumn	123.1 Aa (10.82)	110.8 Aab (10.82)	137.8 Aa (10.82)	87.2 BCb (10.82)	114.7 A (5.41)
Winter/Early Spring	121.2 Aa (15.71)	107.9 Aa (15.71)	98.3 ABa (15.71)	101.5 ABa (15.71)	107.22 A (7.85)
Late Spring	77.7 Bb (8.81)	67.5 Bb (8.81)	80.5 BCb (8.81)	110.35 Aa (8.81)	84.0 B (4.40)
Summer II	69.6 Bb (6.01)	67.3 Bb (6.01)	92.4 Bb (6.69)	69.1 CDa (6.01)	74.6 C (3.10)
Mean	92.0 (5.10)	84.7 (5.10)	94.8 (5.13)	86.0 (5.10)	

Means followed by the same uppercase letters in columns and lowercase letters in rows are not different by the Student's *t*-test ($p < 0.05$). Values in parentheses correspond to the standard error of the mean.

2.3.2. Vertical distribution of herbage mass components and bulk density

Regarding the vertical distribution of morphological components, it was observed that regardless of treatment, the upper half of the canopy was mainly composed of green leaf blades, and the lower half was mainly composed of stem and dead material (Figure 2a). During Autumn, the percentage of seedheads increased in the strata above 20 cm relative to the remaining seasons of the year. Throughout the experiment, the Association showed variation in the vertical distribution of botanical components (Figure 2b). *Massai guineagrass* was dominant throughout the year, except during Winter/Early Spring when *Andropogon*

gambagrass and Piatã palisadegrass increased their percentage presence. However, although the percentage of Piatã palisadegrass remained stable after Winter/Early Spring, there was a reduction in the percentage of *Andropogon gambagrass* during Late Spring and Summer II.

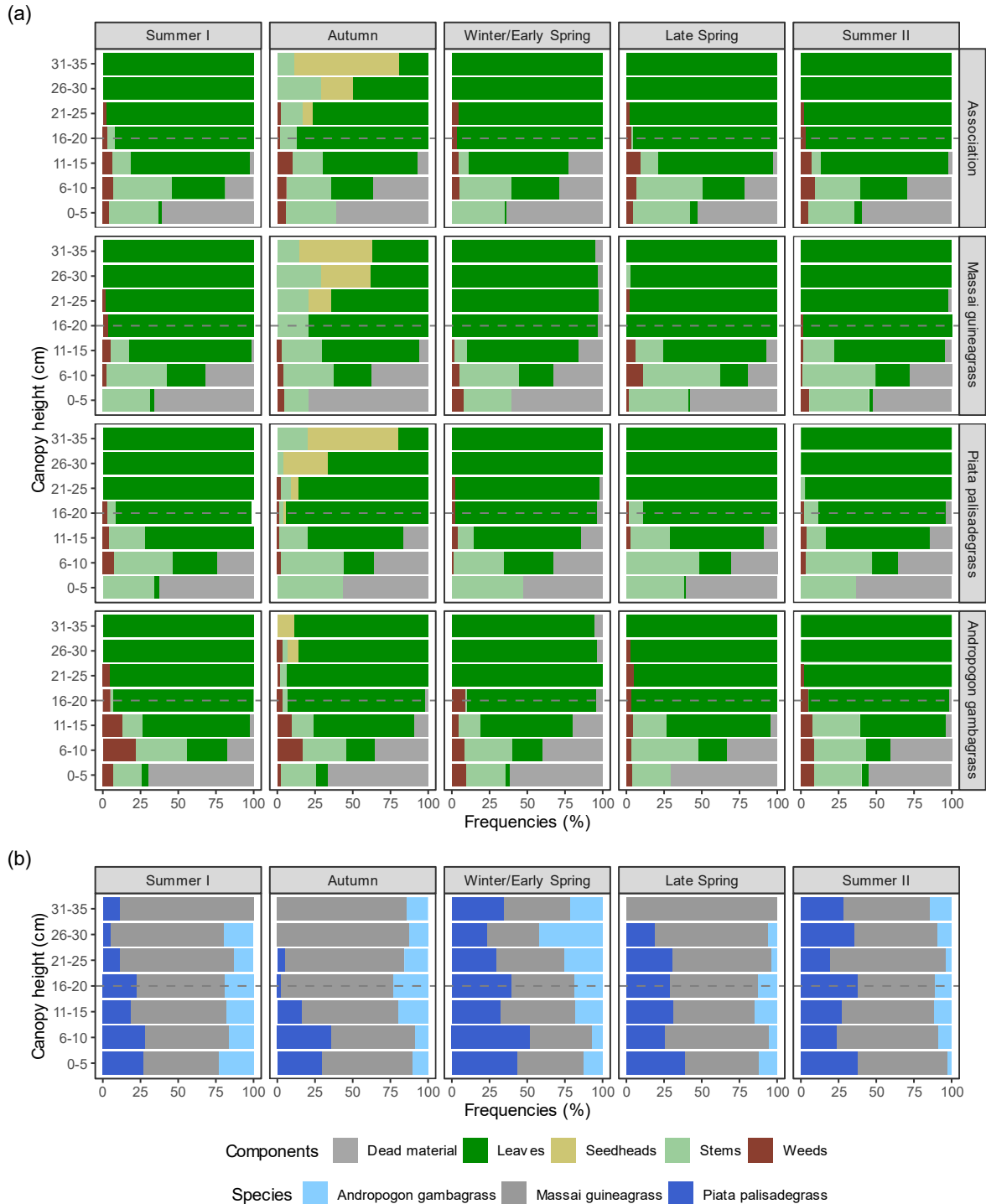


Figure 2. Vertical distribution (%) of canopy morphological (a) and botanical (b) components of *Andropogon gambagrass*, *Massai guineagrass*, and *Piatã palisadegrass* cultivated as monocultures and in association.

Generally, the lower half of the canopy was predominantly composed of dead material and stems, and the top half was composed of leaf blades for all treatments (Figure 3). The intermediate canopy strata (between 10 and 25 cm) had the greatest leaf density. During Autumn, there was an increase in stem and inflorescence density in the upper half of the canopy in Massai guineagrass and in the Association. *Andropogon gambagrass* showed the smallest morphological components bulk density during the entire experimental period, with greater presence of weeds relative to the remaining treatments.

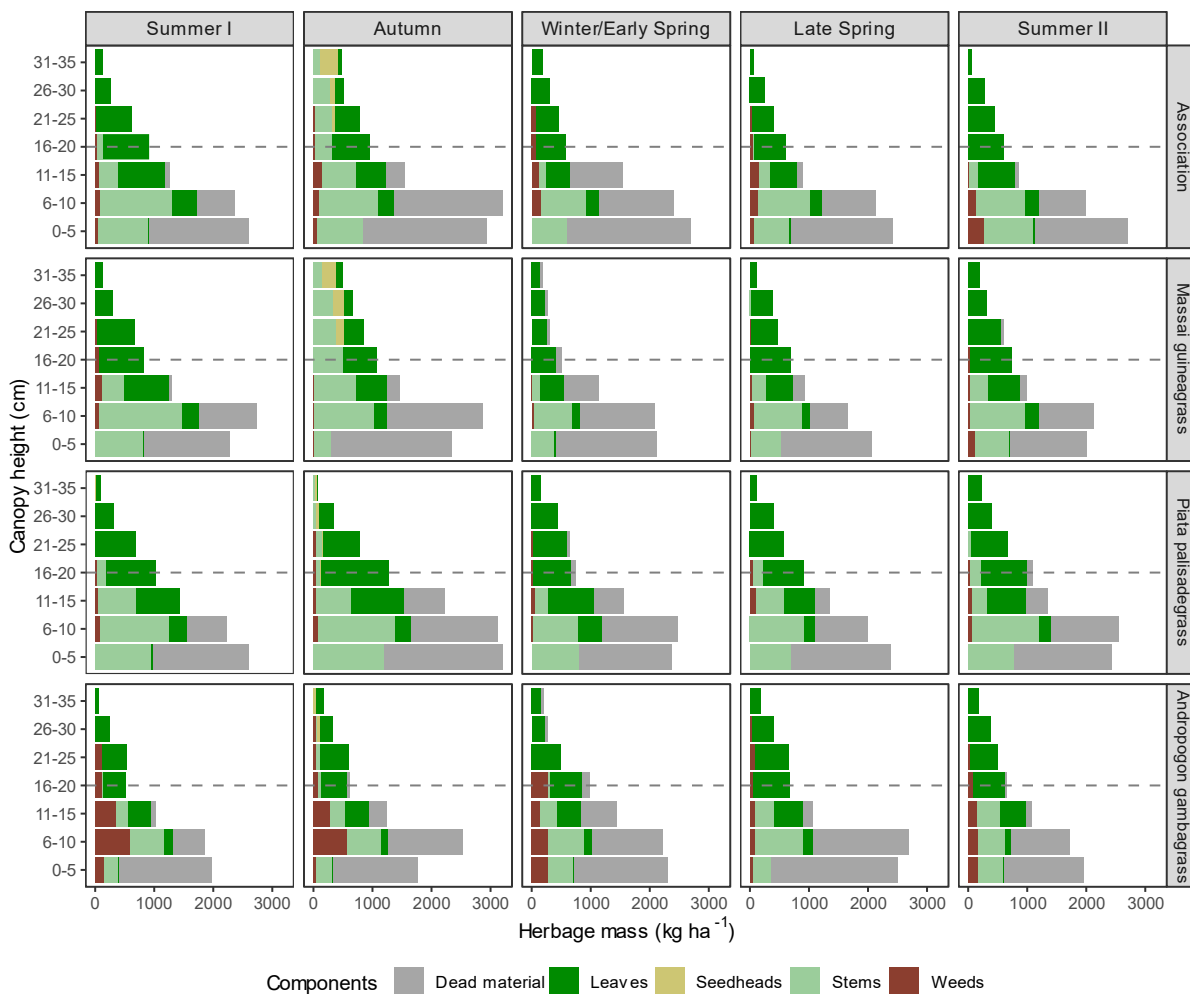


Figure 3. Vertical distribution of botanical and morphological components mass of *Andropogon gambagrass*, *Massai guineagrass*, and *Piatã palisadegrass* cultivated as monocultures and in association.

2.3.3. Ingestive behavior

Bite rate was influenced by treatment ($P=0.0001$), season of the year ($P<0.0001$), and treatment x season of the year interaction ($P=0.0009$). Greatest values were recorded during Summer I and Summer II, while smallest were recorded during Winter/Early Spring (Figure

4). Smaller bite rates were generally recorded for *Andropogon gambagrass* throughout the experimental period. In contrast, *Massai guineagrass* and *Piatã palisadegrass* showed greater bite rates throughout the year, except during Summer II for *Piatã palisadegrass*. The Association showed greater bite rate values during Summer I and Summer II than during Autumn, Winter/Early Spring and Late Spring.

Bite mass varied with treatment ($P=0.0140$), season of the year (<0.0001), and with the treatment x season of the year interaction ($P<0.0011$). Generally, the association and *Andropogon gambagrass* showed greater bite mass throughout the experiment, except during Summer II, when there was no difference among treatments. *Piatã palisadegrass* showed smaller bite mass than the remaining treatments during Summer I, Winter/Early Spring, and Late Spring, while *Massai guineagrass* showed smallest values during Autumn. Largest bite mass values were recorded during Summer I and Winter/Early Spring.

Herbage intake rate was influenced by treatment ($P=0.0015$), season of the year ($P<0.0001$), and treatment x season of the year interaction ($P<0.0001$). Generally, *Massai guinea grass* and the association showed greater intake rates than the other treatments throughout the experiment, except during Autumn, when greater values were recorded for *Piatã palisadegrass*, followed by the Association, and smaller values for *Massai guineagrass* and *Andropogon gambagrass*. During Summer I and Summer II, greater values were recorded for the Association and *Massai palisadegrass* relative to the remaining treatments. *Andropogon gambagrass* showed smaller intake rates than the other treatments, especially during Autumn, Winter/Early Spring, and Late Spring. *Piatã palisadegrass* and the Association showed greatest intake rates during Autumn and Summer I and Winter/Early Spring, respectively.

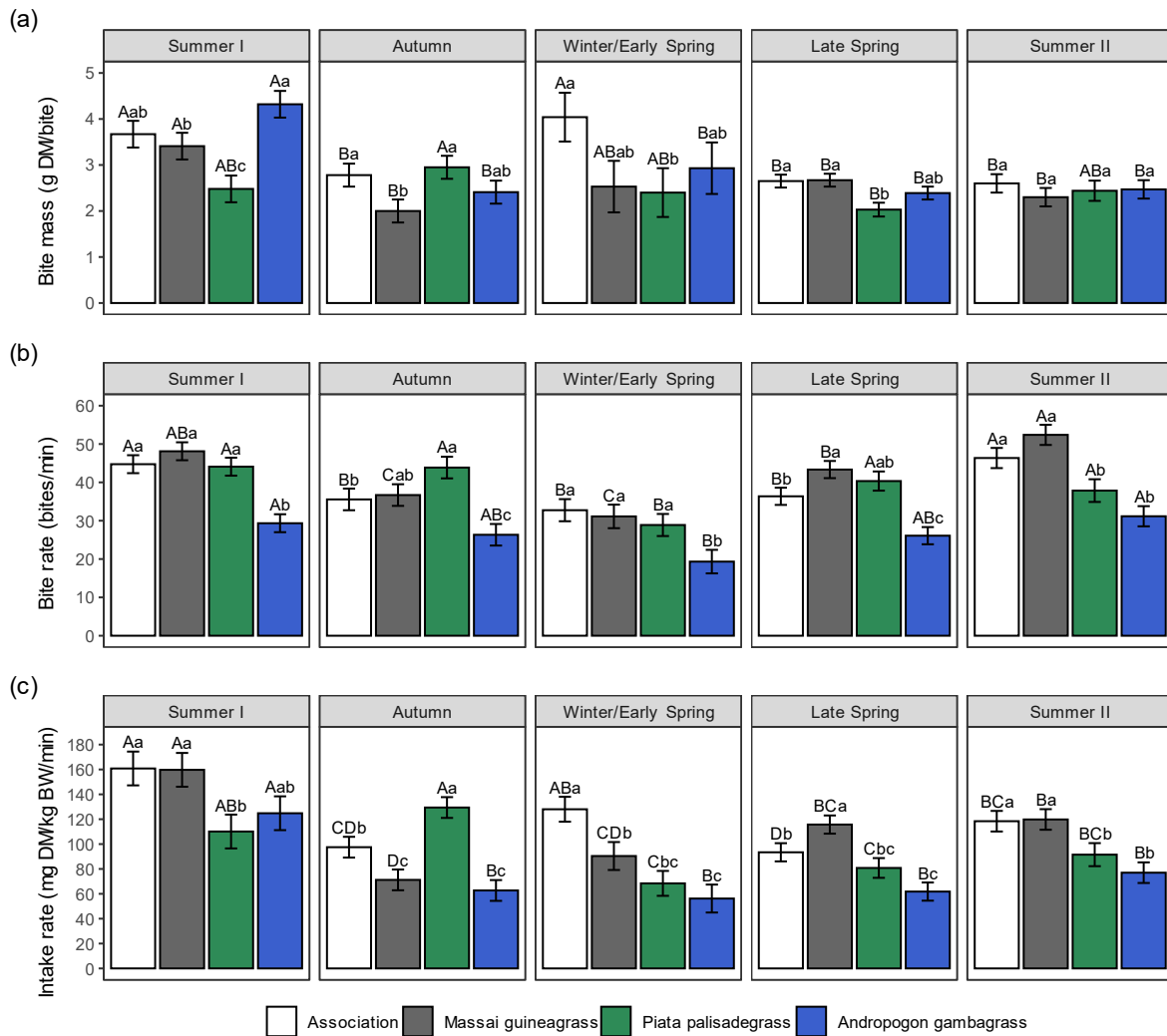


Figure 4. Bite mass (a), bite rate (b), and herbage intake rate (c) of dairy heifers grazing *Andropogon gambagrass*, *Massai guineagrass*, and *Piatã palisadegrass* cultivated as monocultures and in association. Columns with the same lowercase letters within seasons of the year and treatments with the same uppercase letters among seasons of the year are not different ($p < 0.05$). Bar indicates the standard error of the mean.

2.3.4. Movement and forage searching patterns

Data regarding movement and forage searching patterns are shown in Table 3. The number of feeding stations explored per minute varied with treatment ($P = 0.0073$) and season of the year ($P < 0.0001$). Overall, smaller numbers were recorded for *Andropogon gambagrass* relative to the remaining treatments. Greater values were recorded during Summer I, followed by intermediate values in Late Spring and smaller values during the remaining seasons of the year. The number of steps per feeding station was affected by treatment ($P < 0.0001$), season of the year ($P < 0.0001$), and treatment x season of the year interaction ($P = 0.0007$). Greatest values were recorded for *Andropogon gambagrass* throughout the experimental period, while

other treatments showed smaller values, except during Autumn when there was no difference between the Association and *Andropogon gambagrass*. Recorded values for *Massai guineagrass* were relatively stable throughout the experiment. *Piatã palisadegrass* and *Andropogon gambagrass* showed greater values during Winter/Early Spring and Late Spring. On the other hand, the Association showed greater values during Late Spring and smaller values during Summer I, with intermediate values recorded during the remaining seasons of the year.

The movement rate, characterized by the number of steps per minute, was only affected by season of the year ($P < 0.0001$), with greater values recorded during Summer I and Late Spring, followed by intermediate values during Autumn and Winter/Early Spring, and smaller values during Summer II. The time spent per feeding station was influenced by both treatment ($P = 0.0152$) and season of the year ($P < 0.0001$). Greatest values were recorded for *Andropogon gambagrass*, followed by *Piatã palisadegrass*, with smallest values recorded for *Massai guineagrass* and the Association. Over time, greatest values were recorded during Winter/Early Spring and Summer II, followed by Autumn and Late Spring, with smallest values recorded during Summer I.

The number of bites per feeding station varied with both treatment ($P = 0.0421$) and season of the year ($P < 0.0001$). Greatest values were recorded for *Piatã palisadegrass*, followed by intermediate values for *Massai guineagrass* and the Association, and smallest values for *Andropogon gambagrass*. Throughout the year, greatest values were recorded during Summer II, followed by intermediate values during Autumn and Winter/Early Spring, and smallest during Summer I and Late Spring.

Table 3. Movement and forage searching patterns of dairy heifers grazing *Andropogon gambagrass*, *Massai guineagrass*, and *Piatã palisadegrass* cultivated as monocultures and in association.

Season of the year	Treatments				Mean
	Association	Massai guineagrass	Piatã palisadegrass	Andropogon gambagrass	
Feeding stations min ⁻¹					
Summer I	7.2 (0.55)	6.7 (0.55)	5.8 (0.55)	5.4 (0.55)	6.3 A (0.28)
Autumn	4.5 (0.55)	4.1 (0.55)	5.4 (0.55)	4.0 (0.55)	4.5 C (0.28)
Winter/early Spring	4.4 (0.55)	5.0 (0.63)	4.1 (0.55)	3.0 (0.63)	4.1 C (0.30)
Late Spring	6.1 (0.55)	6.6 (0.55)	4.9 (0.63)	4.1 (0.55)	5.4 B (0.29)
Summer II	4.9 (0.55)	4.5 (0.55)	3.3 (0.63)	3.2 (0.55)	4.0 C (0.29)
Mean	5.4 a (0.26)	5.4 a (0.25)	4.7 a (0.27)	3.9 b (0.26)	
Steps between feeding stations					
Summer I	1.6 Bb (0.14)	1.5 Ab (0.14)	1.6 Bb (0.14)	2.3 BCa (0.14)	1.7 B (0.08)
Autumn	1.7 ABab (0.14)	1.6 Ab (0.14)	1.4 Bb (0.14)	2.0 Ca (0.14)	1.7 B (0.08)
Winter/early Spring	1.7 ABb (0.24)	1.6 Ab (0.26)	2.0 Ab (0.24)	3.1 Aa (0.26)	2.1 A (0.13)
Late Spring	1.8 Ab (0.09)	1.5 Ac (0.09)	1.9 Ab (0.10)	2.8 Aa (0.10)	2.0 A (0.06)
Summer II	1.4 ABb (0.22)	1.2 Ab (0.22)	1.6 Bb (0.23)	2.7 ABa (0.22)	1.7 B (0.12)
Mean	1.6 b (0.10)	1.5 b (0.10)	1.7 b (0.10)	2.8 a (0.10)	
Steps min ⁻¹					
Summer I	11.0 (1.39)	9.9 (1.39)	9.1 (1.39)	12.5 (1.39)	10.6 A (0.79)
Autumn	7.4 (1.11)	6.7 (1.11)	7.8 (1.11)	8.3 (1.11)	7.5 B (0.67)
Winter/early Spring	7.3 (1.29)	6.9 (1.34)	7.9 (1.29)	7.9 (1.34)	7.5 B (0.76)
Late Spring	11.1 (1.06)	9.7 (1.06)	8.3 (1.15)	10.2 (1.06)	9.8 A (0.66)
Summer II	6.7 (0.52)	5.6 (0.52)	4.8 (0.53)	8.0 (0.52)	6.3 C (0.46)
Mean	8.7 (0.70)	7.8 (0.71)	7.6 (0.71)	9.4 (0.71)	
Residence time per feeding station (seconds)					
Summer I	8.9 (1.11)	9.4 (1.11)	11.0 (1.11)	11.8 (1.11)	10.3 C (0.58)
Autumn	13.9 (0.99)	13.8 (1.09)	11.6 (0.99)	15.6 (0.99)	13.7 B (0.54)
Winter/early Spring	14.4 (2.02)	12.9 (2.16)	17.2 (2.02)	21.6 (2.16)	16.5 A (1.06)
Late Spring	10.5 (1.80)	9.6 (1.80)	13.8 (2.01)	16.4 (1.80)	12.6 B (0.94)
Summer II	13.0 (2.70)	13.7 (2.70)	22.5 (2.90)	22.6 (2.70)	17.9 A (1.39)
Mean	12.1 b (1.11)	11.9 b (1.13)	15.2 ab (1.15)	17.6 a (1.12)	
Bites per feeding station					
Summer I	6.5 (0.65)	7.5 (0.65)	8.0 (0.65)	5.5 (0.65)	6.9 C (0.32)
Autumn	8.2 (1.08)	9.8 (1.08)	8.4 (1.08)	6.9 (1.08)	8.3 B (0.54)
Winter/early Spring	7.8 (0.82)	6.7 (0.95)	7.4 (0.82)	6.5 (0.95)	7.1 BC (0.44)
Late Spring	6.1 (0.56)	6.8 (0.56)	8.5 (0.65)	6.7 (0.56)	7.0 C (0.29)
Summer II	9.9 (0.89)	12.1 (0.89)	12.3 (1.03)	10.7 (0.89)	11.2 A (0.46)
Mean	7.7 bc (0.37)	8.58 ab (0.38)	8.92 a (0.39)	7.23 c (0.38)	

Means followed by the same uppercase letters in columns and lowercase letters in rows are not different ($p < 0.05$). Values in parentheses correspond to the standard error of the mean.

2.4. Discussion

The distribution and arrangement of botanical and morphological components within the vertical profile of the canopy correspond to the sward structure (Laca and Lemaire, 2000).

This structure is described based on the characterization of canopy herbage mass and height, herbage bulk density, and vertical distribution of the botanical and morphological components. It significantly impacts herbage accumulation and pasture species dynamics (Gastal and Lemaire, 2015), as well as herbage intake and dynamics of the grazing process (Fonseca et al., 2012; Hodgson, 1990). The findings of this study demonstrated that the botanical composition of pastures cultivated in association was dynamic throughout the experiment, with variations in the proportion of species across seasons of the year (Figure 2b). The sward structure of mixed pastures allowed high intake rate values throughout the experimental period, being superior to the monocultures during Winter/Early Spring (Figure 4).

The control of the sward structure during the experiment was achieved by adopting defoliation management based on pre-defined height targets for pre- and post-cutting conditions, and the control of the targets was effective throughout the experiment (Supplementary Material, Figures S3a and S3b). The chosen defoliation heights were within the range of heights based on the 95% canopy light interception (LI) during regrowth criterium (Da Silva et al., 2015) that can be used without affecting forage accumulation when combined with lenient or moderate defoliation severity (Gomes, 2019; Sbrissia et al., 2018). In this scenario, maintenance of herbage accumulation and LI occur due to self-thinning mechanisms, which involve an established inverse relationship between tiller size and population density, as documented in the literature (Matthew et al., 1995; Sbrissia et al., 2018; Sbrissia and Da Silva, 2008). The pre-cutting target height was common for all treatments, and pastures reached values close to 95% LI throughout the experimental period (Supplementary Material, Figure S3c). In mixed swards, competition for light may be increased by the structural characteristics of the associated species (DeMalach et al., 2017), and defoliation management plays an important role in maintaining biodiversity. The defoliation management adopted (frequent defoliation with moderate severity) may have contributed to the species' coexistence in the association by reducing stress and disturbance levels (Duchini et al., 2023). Frequent defoliation allows for better control of sward structure (Da Silva et al., 2009) and maintenance of adequate light environment at ground level, reducing light competition and favoring the coexistence of species in the same area (Borer et al., 2014; Eskelinen et al., 2022).

Throughout the year, sward structure changes as seasons progress due to seasonal variation in climatic conditions determinant of plant growth (Pereira et al., 2010). It influences tiller population density (Caminha et al., 2010; Sbrissia et al., 2010) and

morphogenetic processes (Montagner et al., 2012; Sousa et al., 2011), consequently altering sward structure. It is well established that herbage bulk density increases towards the base of the canopy, both for temperate (Delagarde et al., 2000; Sanderson et al., 2006; Soder et al., 2009) and tropical grasses (Gomes et al., 2018; Pereira et al., 2010), as demonstrated in this study (Figure 3). In general, during Autumn greater values of herbage mass and total bulk density were recorded (Figure 1 and Table 1). However, these values occurred due to an increased stem and dead material percentage in the herbage mass relative to green leaves (Table 2). This fact reflects the phenological stage of the grass species since at that time of the year there was a reduction in air temperature and day length, which reduce leaf appearance and increase stem elongation, characteristic of the reproductive stage of tropical species (Barbosa et al., 2011; Rodrigues et al., 2011).

Regardless of treatment, it was observed that the upper half of the swards was composed predominantly of leaves, and the proportion of stems and dead material increased substantially towards the base of the canopy (Figure 2a), as reported previously for other tropical (Almeida et al., 2023; Geremia et al., 2022; Pereira et al., 2010) and temperate species (Brink and Soder, 2011; Gregorini et al., 2009). Despite the greater percentage of stems and seedheads at the top of the swards during Autumn, *Andropogon gambagrass* showed smaller percentage of these components relative to the remaining treatments, maintaining greater percentage of leaves in the upper strata of the canopy. This may have occurred due to the less pronounced flowering of *Andropogon gambagrass* relative to *Massai guineagrass*, which has a peak flowering in early Autumn. *Piatã palisadegrass* showed large percentage of stems and seedheads in the grazing stratum at that time of the year. However, herbage bulk density of these components was smaller compared to *Massai guineagrass* and the Association (Figure 3). The vertical distribution of morphological components mass during Autumn was similar for the Association and *Massai guineagrass*, consequence of the large presence of this species in the mixed swards. During the remaining seasons of the year, there was no significant difference in the vertical profile of the swards among treatments as a result of the defoliation management adopted that ensures predominance of leaves and small presence of stems in the grazing stratum (Da Silva et al., 2009; Zanini et al., 2012).

The differences in canopy structure, biomass allocation and phenology of the grass species in the mixture are examples of niche separation. It may allow species to coexist through better use of resources at different times of the year, increasing temporal stability of multispecific communities (Anten and Hirose, 1999; Haughey et al., 2018). Regarding the vertical distribution of botanical components in the Association throughout the year, it was

associated with changes in the percentage of species in the herbage mass as seasons of the year progressed. During Winter/Early Spring, the decrease in the percentage of Massai guineagrass was probably due to low temperatures below the base temperature, which is smaller than the other species in this study (Ahmed, 1990; Moreno et al., 2014; Silva et al., 2012) and the occurrence of frosts registered at the experiment site. The combination of these environmental conditions, along with the phenological stage, was critical for this grass species, as indicated by the smaller leaf bulk density relative to the remaining treatments (Table 2). The reduction in the percentage of Massai guineagrass allowed *Andropogon gambagrass* and *Piatã palisadegrass* to increase their proportion (Figure 2b) in the mixture.

The vertical arrangement of the morphological components in the canopy influences the grazing process and, consequently, herbage intake. Studies indicate that there is positive correlation between bite mass and mass of green leaves in the grazing stratum, which affects the amount of herbage consumed (Drescher et al., 2006). Conversely, an increase in the presence of stems in the grazing stratum leads to reduction in both bite size and intake rate (Benvenuti et al., 2006; Drescher et al., 2006; Fonseca et al., 2012; Geremia et al., 2018). In this sense, herbage intake rate may serve as an indicator of the quality of the grazing environment provided to animals (Carvalho et al., 2009; Da Silva et al., 2015; Geremia et al., 2022).

In general, smaller rates of herbage intake were observed during Autumn, Winter/Early Spring, and Late Spring relative to the Summers I and II (Figure 4c). This could be attributed to the smaller bulk density of leaves and greater bulk density of dead material at that time of the year (Table 2). These changes in sward structure are influenced by the climatic conditions that impact the growth patterns and biomass allocation of tropical grass species (Pereira et al., 2010). The predominance of leaves in the grazing stratum is the most important characteristic related to herbage intake (Sollenberger and Burns, 2001), as they are the morphological components with the best nutritional value and easy of prehension (Carvalho et al., 2009). The increase in stem and dead material percentage in the herbage mass negatively impacts sward structure. In this scenario, intake rate may decrease as animals tend to prioritize the consumption of leaves. This selective behavior may result in reduction of bite rate and, consequently, rate of herbage intake (Drescher et al., 2006; Fonseca et al., 2012; Geremia et al., 2018).

Additionally, *Andropogon gambagrass* exhibited smaller rates of intake relative to the other treatments (Figure 4). In a concomitant study in the same experimental area, Silva (2023) observed that *Andropogon gambagrass* showed greatest final leaf length and smallest

number of live leaves per tiller. This could have contributed to the decreased rate of herbage intake due to sparse leaf distribution in the grazing stratum, which may have led to smaller bite rate (Geremia et al., 2022). This indicates that the larger bite size on *Andropogon gambagrass* was not enough to compensate the smaller bite rate, resulting in reduced rate of herbage intake. Overall, herbage intake rate for the Association was consistently close to the greatest values recorded throughout the experiment. These results may have been influenced by the vertical distribution of botanical and morphological components of the herbage mass. During Autumn and Winter/Early Spring, changes in the botanical composition may have contributed to improving the distribution of leaves within the canopy of the Association relative to the monocultures, favoring greater rate of herbage intake. In mixed swards, animals can perform selective grazing (Soder et al., 2007), which can limit the intake rate due to increased time spent on foraging (Parsons et al., 1994). The reduction in intake rate was not observed in this study, which may be a result of the defoliation management adopted that favored large proportion of leaves in the grazing stratum for all three species.

In general, animals explored larger number of feeding stations in the Association, *Massai guineagrass* and *Piatã palisadegrass*, taking fewer steps between them and spending less time at each feeding station. This behavior is likely related to the ability of the animals to recognize grazing sites that favor herbage intake (Bailey et al., 1996). Considering that the grazing trials were carried out mostly during early morning and corresponded to single meals lasting 45 minutes, animals sought to recognize the grazing site aiming to maximize forage intake (Gregorini et al., 2011).

The findings described above indicate that it is possible to combine different tropical grass species without compromising sward structure and grazing animal responses compared to single grass species pastures. Particularly, during specific times of the year, the complementarity among species in the association can lead to more favorable canopy structure due to the greater proportion of leaves (Duchini et al., 2019), resulting in increased rate of herbage intake. Understanding how the dynamics of botanical composition impacts canopy structure is crucial for developing effective management strategies and selecting appropriate forage species to maximize animal herbage intake.

Considering that leaves are the morphological component with better nutritive value (Carvalho et al., 2009), the use of species and grazing management that promote greater presence of leaves in the grazing stratum enhances the nutritional value of the forage (Deak et al., 2007; Geremia et al., 2022). This, in turn, leads to high rates of herbage intake (Guzatti et al., 2017) and positively impacts animal performance (Congio et al., 2018; Gimenes et al.,

2011). This highlights the central role of choosing species and management practices as key components to establish mixed swards, aiming at improving ecosystem services and productivity benefits to pasture-based systems (Sollenberger et al., 2019).

2.5. Conclusions

The association of grass species provided rates of herbage intake similar to those of the monocultures throughout the experiment. However, during Winter/Early Spring, mixed swards allowed for greater rates of herbage intake relative to the remaining treatments, providing an adequate grazing environment during this challenging season of the year characterized by accentuated water deficit. The phenological cycle and strategies of growth and resource use must be considered when choosing forage species to compose mixed pastures, aiming at obtaining temporal complementarity and allowing for adequate grazing environment for the animals throughout the year.

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Supplementary material

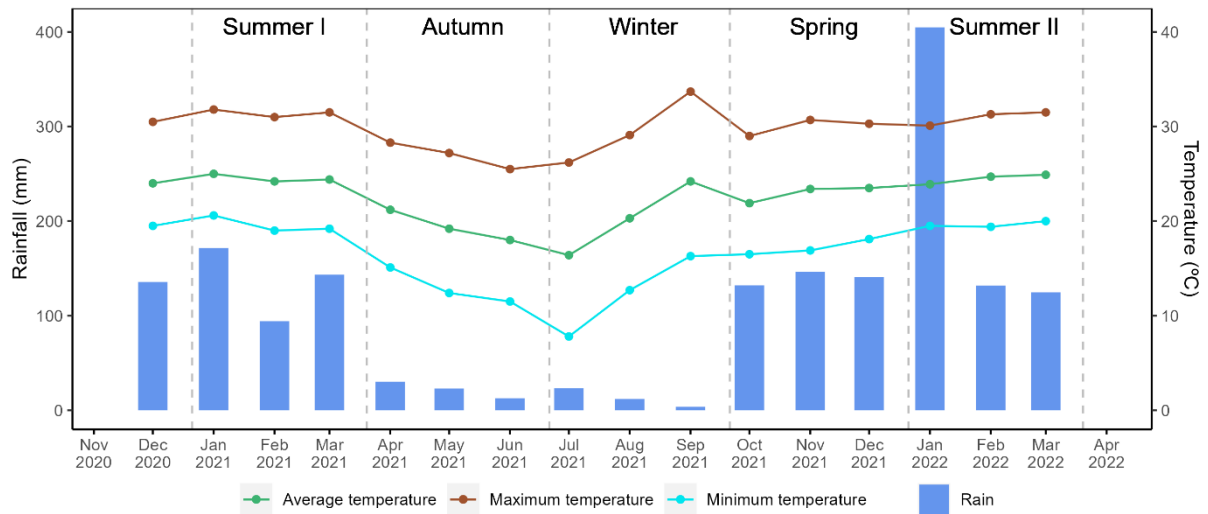


Figure S1. Monthly maximum, minimum and average air temperatures, and rainfall at the experimental site during the experimental period (from December 2020 to March 2022).

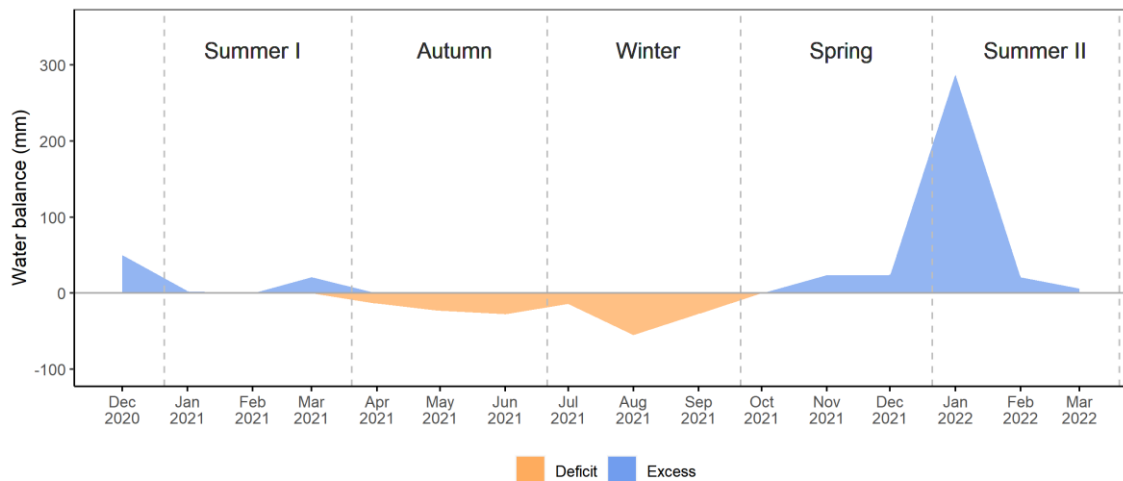


Figure S2. Monthly soil water balance at the experimental site during the experimental period (from December 2020 to March 2022), calculated according Thornthwaite & Mather (1955) using 50 mm of water storage capacity.

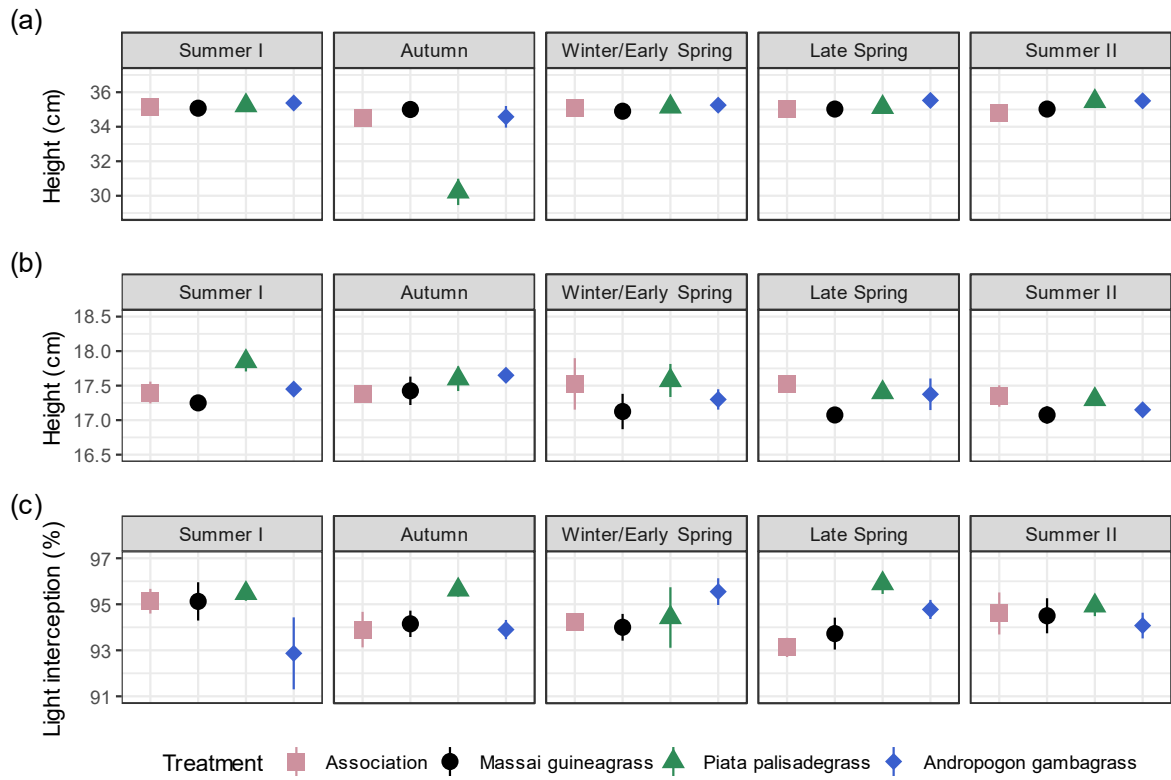


Figure S3. Pre-cutting height (a), post-cutting height (b), and light interception (c) during the experimental period (from December 2020 to March 2022; mean \pm standard error of the mean).

3. NUTRITIVE VALUE AND *IN VITRO* GAS PRODUCTION OF THREE TROPICAL FORAGE GRASSES CULTIVATED AS PURE OR MIXED STANDS

Abstract

Enteric methane from ruminants is one of the most important gases emitted by the livestock supply chain. The growing demand for food driven by the increase in world population poses challenges for food production chains to increase production with lesser environmental footprint. Grazing management has the potential to reduce methane intensity emissions and is a low-cost alternative to more sustainable pasture-based systems. In this context, the adoption of multispecific pastures may generate complementarity in the use of resources, providing several benefits. Studies have shown improvement in herbage nutritional value with the increase in the number of species comprising the pasture, which can improve dry matter degradation and rumen fermentation and reduce enteric methane emissions. Hence, the objective of this study was to evaluate whether the combination of three perennial tropical forage grasses could serve as an alternative to enhance herbage nutritive value year-round through species complementarity while also assessing its potential to decrease *in vitro* methane production compared to their individual monocultures. Treatments corresponded to three perennial tropical forage grass species: *Andropogon gayanus* cv. Planaltina (Andropogon gambagrass); *Panicum maximum* cv. Massai (Massai guineagrass); and *Brachiaria brizantha* cv. BRS Piatã (Piatã palisadegrass) cultivated in monoculture and in association, with four replications. Defoliation management was common to all treatments and corresponded to a pre-cutting height of 35 cm and a post-cutting 17.5 cm. The association presented chemical composition values similar to those of monocultures. Andropogon gambagrass showed the greatest values of crude protein content throughout the experimental period. The association showed greater ammoniacal nitrogen content and degradability of dry matter and neutral detergent fiber. Methane production showed inverse correlation with crude protein content and acetate:propionate ratio. The association did not reduce the methane production, showing similar values to those of monocultures. Differences in chemical composition among forage species influenced *in vitro* incubation variables.

Keywords: *In vitro* fermentation, Methane, Multispecific pastures, Rumen degradation, Sustainability

3.1. Introduction

The escalating global population presents challenges for the food supply chain, making it necessary to meet the demand for food through sustainable approaches (Leaver, 2011; Royal Society, 2009). These challenges include the adoption of strategies that improve productivity of pasture-based systems while minimizing environmental impact (Bizzuti et al., 2023). The livestock sector contributes with approximately 14.5% of total anthropogenic greenhouse gas (GHG) emissions, a substantial portion of which attributed to enteric methane (CH₄), a byproduct of ruminal fermentation (Gerber et al., 2013). This process not only results in release of CH₄ but also represents energy loss through inefficient fermentation, decreasing

the diet's energy utilization efficiency (Loza et al., 2021). Ruminant CH₄ emissions account for 44% of total GHG emissions from the livestock supply chain (Gerber et al., 2013). Within this context, it is necessary a comprehensive reassessment of management practices aiming at achieving sustainable livestock production and reducing environmental footprint associated with this important activity (Royal Society, 2009).

The search for sustainable and efficient solutions in pasture management has become increasingly relevant for animal production (Kemp and Michalk, 2007). Recent studies have shown that in pasture-based systems, proper grazing management allows for reducing the intensity of methane emissions by animals without affecting animal performance (Congio et al., 2021). This approach turns out to be a low-cost strategy to implement. In addition, sustainable intensification can improve soil health indicators in tropical regions (Damian et al., 2023). However, given the potential of tropical species, there is little information in the literature on methane emissions in well-managed pastures of tropical forage grasses when mixed in the same area.

The use of forage grasses represents a consolidated practice in pasture-based systems, particularly in tropical regions. In Brazil, most of the cattle are raised in pastures (Jank et al., 2014), which occupy approximately half of the arable land, covering around 159 million hectares (IBGE, 2017). Nevertheless, challenges persist in maintaining the nutritional value of pastures throughout the year (Habermann et al., 2019) and the environmental impact caused by methane production during enteric fermentation of ruminants (Moss et al., 2000). Research has indicated that the adoption of multispecific pastures can generate complementarity in the use of resources, providing several benefits. In temperate and subtropical environments, it has been observed improvement in herbage nutritional value with the increase in the number of species comprising the pasture (Nobilly et al., 2013), which can improve dry matter degradation and rumen fermentation (Santos et al., 2020). Other results demonstrate that increasing biodiversity can also increase production stability throughout the year (Duchini et al., 2019; Gross et al., 2014) and reduce environmental impact (Distel et al., 2020; Woodward et al., 2012). In this scenario, the association of tropical forage grass species may be an alternative to intensify the production system by increasing biodiversity, aiming at optimizing herbage productivity and nutritive value throughout the year, and potentially mitigating greenhouse gas emissions.

Despite the extensive literature on increasing biodiversity in grasslands, there is need for better understanding of the interactions and responses of tropical forage grasses when coexisting in the same area. Such insights are crucial for developing novel management

practices aiming at enhancing productivity, maintaining biodiversity, and minimizing environmental impact. Our hypothesis is that multispecific swards comprised of grass species with complementary traits can enhance herbage nutritive value throughout the year, thereby influencing the *in vitro* methane production and ruminal parameters. Hence, this study aimed at examining whether the combination of three perennial tropical forage grasses could serve as an alternative to enhance herbage nutritive value year-round through species complementarity while also assessing its potential to decrease *in vitro* methane production compared to their individual monocultures. Understanding the potential benefits of this approach could provide essential information to support the promotion of more sustainable pasture-based systems.

3.2. Material and methods

The procedures for this study were approved by the Animal Ethics Committee of “Luiz de Queiroz” College of Agriculture (ESALQ/USP, Protocol No. 9581050121) and Center for Nuclear Energy in Agriculture (CENA/USP, Protocol No. 0001/2021), both from the University of São Paulo.

3.2.1. Study site

The field experiment was carried out from December 2020 to March 2022 at Luiz de Queiroz College of Agriculture, University of São Paulo (ESALQ/USP), in Piracicaba, São Paulo, Brazil (22°42'34" S, 47°38'25" W, 546 m above sea level). According to the Köppen classification, the local climate is classified as Cwa (subtropical climate with dry Winter and hot Summer) (Alvares et al., 2013; Beck et al., 2018). Weather data were obtained from a meteorological station located approximately 2000 m from the experimental site (Figure S, Supplementary material).

The soil is a Red Eutroferic Nitisol with a clayey texture (FAO, 2015). Average soil characteristics (0-20 cm layer) before the implementation of the experiment were phosphorus (P) = 49.5 mg dm⁻³ (ion-exchange resin extraction method); organic matter = 33.8 g dm⁻³; pH CaCl₂ = 4.50; potassium (K) = 3.45 mmolc dm⁻³; calcium (Ca) = 30.3 mmolc dm⁻³; magnesium (Mg) = 12.5 mmolc dm⁻³; hydrogen and aluminum (H + Al) = 72.5 mmolc dm⁻³; sum of bases = 46.0 mmolc dm⁻³; cation exchange capacity = 119 mmolc dm⁻³; base saturation = 39.0%. Sand = 358 g kg⁻¹, silt = 196 g kg⁻¹, and clay = 446 g kg⁻¹. These characteristics indicated the need to increase base saturation, which was carried out by using

dolomitic limestone aiming at reaching $V_2 = 70\%$ (Raij et al., 1997) during late Winter 2019. It was not necessary to apply any fertilizer during sowing because nutrient contents were adequate for the forage grass species to be cultivated (Raij et al., 1997).

3.2.2. Treatments, experimental design and establishment

Treatments consisted of three perennial tropical forage grass species: *Andropogon gayanus* cv. Planaltina (Andropogon gambagrass); *Panicum maximum* cv. Massai (Massai guineagrass); and *Brachiaria brizantha* cv. BRS Piatã (Piatã palisadegrass) cultivated in monoculture and in association. Treatments were assigned to experimental units (16 paddocks; 12 x 15 m each) according to a randomized complete block design with four replications.

Sowing was carried out manually in January 2020 at a rate of 300 pure viable seeds m^{-2} , with 100 viable seeds from each species for the association, followed by compaction with a compactor roller weighing approximately 100 kg. Fertilization was performed 21 days after sowing using 40 kg N ha^{-1} as ammonium nitrate. Paddocks were kept growing until reaching the pre-cutting target height of 35 cm when the first cut was made for standardization and definition of the post-cutting height of 17.5 cm.

3.2.3. Management and maintenance of experimental conditions

The pre-cutting height for each grass species was defined based on the 95% canopy light interception (LI) during regrowth criterion (Da Silva et al., 2015) and its flexibility range within which herbage accumulation is stable (Gomes, 2019; Sbrissia et al., 2018). This flexibility implies that a pre-cutting height up to 40% smaller than the corresponding canopy height at 95% LI may be used without reducing herbage accumulation. According to the 95% LI criterion, the maximum pre-cutting height for Piatã palisadegrass, *Andropogon gambagrass*, and *Massai guineagrass* is 35, 50, and 55 cm, respectively (Barbosa et al., 2010; Crestani et al., 2017; Sousa et al., 2010). Considering the possibility of relaxing these management targets, it is possible to manage *Andropogon gamba* grass with up to 30 cm and *Massai guinea* grass with up to 33 cm of pre-cutting height. Therefore, the pre-cutting height of 35 cm was compatible with all three forage grass species cultivated. The post-cutting height of 17.5 cm, equivalent to a 50% defoliation severity, was sufficient to ensure high residual leaf area index (Giacomini et al., 2009; Silveira et al., 2013). This would allow for

frequent, non-severe defoliations and favor growth and development of the associated species by reducing levels of disturbance (grazing severity) and stress (competition for light).

Canopy height was monitored every three days during regrowth, starting soon after each cut. As canopy height approached values close to the targeted pre-cutting height of 35 cm, the frequency of monitoring increased to daily until paddocks reached the targeted canopy height for defoliation. Measurements were taken using a sward stick along 40 points per paddock, distributed across four transect lines.

Nitrogen fertilization was performed only during the rainy periods of the year (October to March), always at post-cutting, using ammonium nitrate. The amount of nitrogen for each application was proportional to the cutting interval of each paddock (daily rate of 1.7 kg N ha⁻¹) and calculated to result in equal amounts of nitrogen applied to all paddocks at the end of each rainy season.

3.2.4. Assessments

3.2.4.1. Nutritive value

Grazing trials were performed every season of the year (Summer I, Autumn, Winter/Early Spring, Late Spring, and Summer II) to measure ingestive behavior of the animals using three Holstein dairy heifers with an average body weight of 200 ± 30 kg. During all grazing trials, hand-plucked forage samples were collected, simulating grazing to estimate dry matter (DM) content and chemical composition. Samples were dried at 65 °C in a forced draught oven until constant weight. After drying, samples were ground in a Willey mill to pass through a 1-mm sieve in preparation for chemical analyses. Crude protein (CP) content was analyzed using a LECO FP-2000 nitrogen analyzer (Leco Instruments, Inc., St. Joseph, MI, USA). Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined according to Van Soest et al. (1991).

3.2.4.2. *In vitro* gas production and dry matter degradability

The same samples used for assessing the nutritive value were also used for evaluations regarding *in vitro* incubations. The *in vitro* incubation assay was carried out after the conclusion of the collection period, following the methodology described by Abdalla et al. (2012), Mauricio et al. (1999), and Theodorou et al. (1994), with adaptations. Dry substrate samples (approximately 1.0 g each) were weighed into Ankom® F57 filter bags. Each bag

was sealed and placed in 160 mL serum glass bottles with 25 mL of ruminal fluid and 50 mL of incubation medium (Bueno et al., 2005).

Ruminal content from three adult male Santa Inês sheep with rumen cannulas (body weight = 60 ± 2.5 kg) was used as the inoculum, as described previously by Abdalla et al. (2012). The donor animals had *ad libitum* access to mineral supplements, water, and Tifton hay (*Cynodon* spp.) with a concentrate mixture (soybean and corn). The ruminal fluid was collected prior to the morning feeding and maintained under anaerobic conditions at 39 °C until inoculation. Three separate inocula were prepared using a 50:50 solid:liquid ratio, as described by Bizzutti et al. (2023). Each sample was incubated within three different inocula. After inoculation, the bottles were sealed with rubber stoppers and placed in an forced draft oven at 39 °C for 24 h. Blank bottles (Ankom bags without substrate) were used to correct gas production originating from the ruminal fluid and incubation medium. Gas pressure was measured at regular intervals (2, 4, 8, 12, and 24 h) to calculate the total gas production using a pressure transducer and a datalogger (Press Data 800, LANA, CENA/USP). Gas volume produced was estimated using the following equation derived from linear regression ($n = 321$; $R^2 = 0.991$): $GP = 6.1432 \times psi + 0.0451$, where GP is the gas production (mL), and *psi* corresponds to the pressure measured with the transducer.

Following 24h of incubation, the bags were recovered from the bottles, and the fermentation was interrupted by immersing in cold water (4 °C). The solid residue was treated with a neutral detergent solution for one hour at 90 °C, followed by washing with hot water and acetone. This process aimed at evaluating the disappearance of the dry matter and fiber, utilizing the fiber analysis procedure (Tecnal TE-149, Piracicaba, Brazil) according to Van Soest et. al. (1991) and adapted by Mertens (2002). After drying, the bags were weighed and used to determine the truly degraded dry matter and NDF degradation.

3.2.4.3. Methane assessments

Following each gas pressure measurement, a subsample of gas (2 mL) was collected from each bottle and transferred to 10 mL tubes under vacuum conditions, resulting in the sample pool for the 0-24h interval. Methane (CH₄) concentration was determined on each bottle's composite sample using a gas chromatograph (Shimadzu GC 2014, Chiyodaku, Tokyo, Japan) equipped with flame ionization detector and a capillary HP-molesieve column (30 m × 0.53 mm × 25 μm). The temperatures of the column, injector, and flame ionization detector were 60°C, 200°C, and 240°C, respectively. Helium at 10 ml/min was the carrier gas. Methane concentration was determined by external calibration using an analytical curve (0,

30, 90, and 120 ml/L) prepared with pure CH₄ standard (Praxair Industrial Gases, Osasco, Brazil; purity 99.5 ml/L). The estimation of methane production was calculated according to Longo et al. (2006): $\text{CH}_4 \text{ (mL g}^{-1} \text{ DM)} = (\text{VGP} + \text{HS}) * \% \text{CH}_4$, where VGP is the volume gas produced (mL); HS is the bottle headspace (85 mL); %CH₄ is the percentage of methane in the sample; and CH₄ is the methane concentration (mL g⁻¹ DM).

3.2.5. Ammoniacal nitrogen and short-chain fatty acids

Ruminal fluid samples were collected from each bottle for analysis of ammoniacal nitrogen (NH₃-N) and short-chain fatty acids (SCFA). The concentration of NH₃-N was determined by the micro-Kjeldahl method, using sodium tetraborate solution (5%) according to Preston (1995). SCFA concentrations were determined through gas chromatography following the methodology described by Palmquist and Conrad (1971) and adapted by Lima et al. (2018).

3.2.6. Statistical analysis

For the determination of the *in vitro* fermentation kinetic parameters, the logistic model of Schofield et al. (1994) was selected based on the mean square prediction error (MSPE; Bibby and Toutenburg, 1977):

$$V(t) = V_f \exp \{1 + \exp [2 + k (\lambda - t)]\}$$

where $V(t)$ = accumulated volume at time t (ml); V_f = asymptotic cumulative gas volume (ml); k = fraction digestion rate (h⁻¹); λ = lag time (h); and t = incubation time (h).

Data were analyzed using PROC GLIMMIX in SAS, version 9.4 (SAS Institute, 2013). Treatments, seasons of the year, and their interactions were considered fixed effects, while blocks were treated as random effects. For the fermentation kinetic parameters estimates and other incubation variables, the inoculums were considered as random effects. Season of the year was considered repeated measure. The variance-covariance matrix was selected based on the Bayesian information criterion. Means were estimated using the LSMEANS procedure and compared by the probability of difference (PDIFF) with Student's t-test ($P < 0.05$). Pearson correlation matrix and principal components analysis were performed to evaluate the relationship among nutritive value and *in vitro* gas production parameters using R software (version 4.2.2 – R core team, 2022).

3.3. Results

3.3.1. Nutritive value of the hand-plucked forage samples

The concentration of crude protein (CP) varied with treatment ($P=0.0007$), season of the year ($P<0.0001$), and with the treatment x season of the year interaction ($P=0.0303$). *Andropogon gambagrass* usually showed greater CP levels throughout the experiment, except for Summer I (Figure 1a). *Piatã palisadegrass* showed smaller CP levels during Summer I and Autumn and showed similar values to the Association and Massai guineagrass during the remaining seasons of the year. Over time, greatest CP levels were recorded during Spring and Summer II, and smallest levels during Autumn for all treatments.

The content of neutral detergent fiber (NDF) varied with treatment ($P<0.0001$), season of the year ($P<0.0001$), and their interaction ($P=0.0008$). Massai guineagrass showed greater NDF levels throughout the experiment, with greater values recorded during Summer I and Autumn (Figure 1b). Except for Spring, *Piatã palisadegrass* showed the smallest NDF values throughout the experiment. Intermediate values were recorded for the Association and *Andropogon gambagrass*. The lowest NDF levels were recorded during Winter.

The acid detergent fiber (ADF) content was influenced by treatment ($P=0.0004$), season of the year ($P<0.0001$), and the treatment x season of the year interaction ($P=0.0001$). Smaller ADF values were recorded for *Piatã palisadegrass* during Summer I, Autumn, and Winter (Figure 1c), while Massai guineagrass showed greater ADF levels during Summer I, Autumn, Spring, and Summer II. The Association showed intermediate values throughout the experiment, except during Winter.

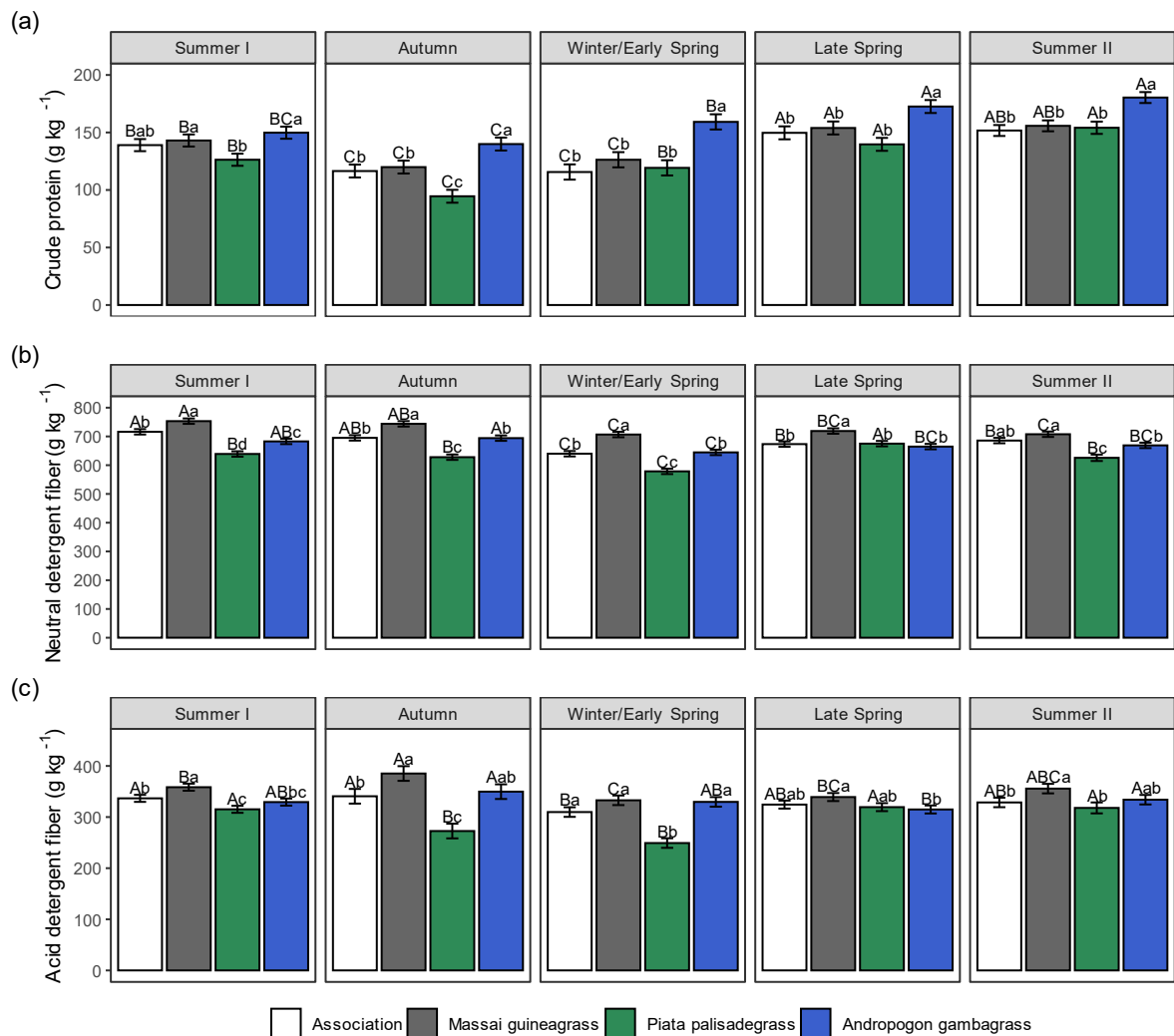


Figure 1. Crude protein (a), neutral detergent fiber (b), and acid detergent fiber content (c) in herbage samples of *Andropogon gambagrass*, *Massai guineagrass*, and *Piatã palisadegrass* cultivated as monocultures and in association. Columns with the same lowercase letters within seasons of the year and treatments with the same uppercase letters among seasons of the year are not different ($p < 0.05$). Bar indicates the standard error of the mean.

3.3.2. *In vitro* degradation and methane production

In vitro degradability of dry matter (IVDDM) varied with treatment ($P = 0.0003$), season of the year ($P < 0.0001$), and with the treatment x season of the year interaction ($P < 0.0001$). Greatest IVDDM values were recorded during Winter/Early Spring, Late Spring, and Summer II, while smallest were recorded during Summer I (Table 1). In general, Association and *Piatã palisadegrass* showed greater IVDDM throughout the experimental period relative to the remaining treatments.

In vitro degradability of neutral detergent fiber (IVDADF) was influenced by treatment ($P < 0.0001$), season of the year ($P < 0.0001$), and their interaction ($P < 0.0001$).

Greatest values of IVDNDF were recorded during Late Spring, while the smallest were recorded during Summer I with intermediate values during the remaining seasons of the year (Table 1). Generally, Massai guineagrass and the Association showed greater IVDNDF values compared to the remaining treatments throughout the experiment. Smaller IVDNDF values were recorded for Andropogon gambagrass throughout the experimental period, except during Summer II. Overall, Piatã palisadegrass showed intermediate values throughout the seasons of the year.

Table 1. *In vitro* degradability of dry matter and neutral detergent fiber of Andropogon gambagrass, Massai guineagrass, and Piatã palisadegrass cultivated as monocultures and in association.

Season of the year	Treatments				Mean
	Association	Massai guineagrass	Piatã palisadegrass	Andropogon gambagrass	
<i>In vitro</i> degradability of dry matter (g kg ⁻¹)					
Summer I	414.2 Cb (14.76)	371.7 Dc (14.76)	484.8 Ba (14.76)	362.4 Dc (14.94)	408.3 C (11.28)
Autumn	463.9 Ba (14.67)	413.0 Cb (14.67)	482.8 Ba (14.67)	394.6 Cb (14.71)	438.6 B (11.22)
Winter/Early Spring	494.6 ABab (17.85)	467.7 Bb (17.85)	525.3 Aa (17.85)	464.5 Bb (17.85)	488.0 (12.31) A
Late Spring	521.61 Aa (17.60)	510.3 Aab (17.60)	503.9 ABab (17.60)	472.4 Bb (17.60)	502.0 (12.25) A
Summer II	484.0 Bab (14.94)	462.2 Bb (14.45)	499.8 ABa (15.56)	505.1 Aa (14.45)	487.8 (11.36) A
Mean	475.7 b (12.38)	445.0 c (12.35)	499.3 a (12.41)	439.8 c (12.36)	
<i>In vitro</i> degradability of neutral detergent fiber (g kg ⁻¹)					
Summer I	177.1 Ca (18.05)	163.5 Da (18.05)	194.9 Ba (18.05)	68.3 Db (18.05)	151.0 E (15.04)
Autumn	226.0 Ba (15.84)	207.6 Ca (15.84)	178.3 Bb (15.84)	132.3 Cc (15.84)	186.0 D (14.43)
Winter/Early Spring	214.1 BCab (21.16)	247.1 Ba (21.16)	181.5 Bb (21.16)	184.8 Bb (21.16)	206.9 C (16.02)
Late Spring	289.3 Aab (21.88)	318.1 Aa (21.88)	265.1 Ab (21.88)	206.6 Bc (21.88)	269.8 A (16.28)
Summer II	252.2 Aa (16.99)	236.3 Ba (16.51)	203.8 Bb (17.32)	258.5 Aa (16.51)	237.7 B (14.76)
Mean	231.7 a (15.05)	234.5 a (15.03)	204.7 b (15.06)	170.1 c (15.03)	

Means followed by the same uppercase letters in columns and lowercase letters in rows are not different by the Student's t-test ($p < 0.05$). Values in parentheses correspond to the standard error of the mean.

After 24 hours of *in vitro* fermentation, the methane produced per unit of dry matter degraded (CH₄DMD) was affected by treatment ($P < 0.0001$), season of the year ($P < 0.0001$), and by the treatment x season of the year interaction ($P < 0.0001$). Greatest values of CH₄DMD were recorded during Winter/Early Spring, Late Spring, and Summer II (Table 2). There was no difference among treatments during Summer II. Piatã palisadegrass showed the greatest values of CH₄DMD during Summer I, Autumn, and Winter/Early Spring. The Association did

not differ from Piatã palisadegrass during Autumn and Winter/Early Spring and showed intermediate values during Summer I. During Late Spring, the greatest values of CH₄DMD were recorded for the Association and Massai guineagrass.

The methane produced per unit of neutral detergent fiber degraded (CH₄NDFD) was influenced by treatment (P<0.0001), season of the year (P<0.0001), and by the treatment x season of the year interaction (P<0.0001). Greatest values of CH₄NDFD were recorded during Late Spring and smallest values during Summer I, with intermediate values during the remaining seasons of the year. In general, the Association and Massai guineagrass showed the greatest values of CH₄NDFD, followed by Piatã palisadegrass and Andropogon gambagrass.

Table 2. Methane produced per unit of dry matter and neutral detergent fiber degraded from the *in vitro* gas production of Andropogon gambagrass, Massai guineagrass, and Piatã palisadegrass cultivated as monocultures and in association.

Season of the year	Treatments				Mean
	Association	Massai guineagrass	Piatã palisadegrass	Andropogon gambagrass	
Methane produced per unit of dry matter degraded (mL g ⁻¹ DMD)					
Summer I	1.76 Cb (0.28)	1.32 Dc (0.28)	2.61 Ca (0.28)	0.94 Dc (0.29)	1.66 C (0.27)
Autumn	2.39 Bab (0.30)	2.24 Cb (0.30)	2.82 Bca (0.30)	1.14 Dc (0.30)	2.15 B (0.27)
Winter/Early Spring	3.46 Aab (0.33)	2.93 Bbc (0.33)	3.99 Aa (0.33)	2.73 Bc (0.33)	3.28 A (0.28)
Late Spring	3.87 Aa (0.35)	3.90 Aa (0.35)	2.75 BCb (0.35)	2.09 Cb (0.35)	3.15 A (0.29)
Summer II	3.80 Aa (0.36)	3.22 ABa (0.36)	3.30 ABa (0.39)	3.39 Aa (0.36)	3.43 A (0.29)
Mean	3.06a (0.28)	2.72b (0.28)	3.09a (0.28)	2.06c (0.28)	
Methane produced per unit of neutral detergent fiber degraded (mL g ⁻¹ NDFD)					
Summer I	0.76 Db (0.15)	0.59 Db (0.15)	1.01 Ca (0.15)	0.19 Dc (0.16)	0.64 E (0.14)
Autumn	1.07 Ca (0.14)	1.13 Ca (0.14)	1.06 Bca (0.14)	0.44 Cb (0.14)	0.93 D (0.14)
Winter/Early Spring	1.41 Ba (0.19)	1.48 Ba (0.19)	1.29 ABab (0.19)	0.96 Bb (0.19)	1.28 C (0.15)
Late Spring	2.17 Aa (0.23)	2.46 Aa (0.23)	1.50 Ab (0.23)	0.97 Bb (0.23)	1.78 A (0.16)
Summer II	1.73 Ba (0.17)	1.57 Bab (0.17)	1.28 Abb (0.18)	1.63 Da (0.17)	1.55 B (0.14)
Mean	1.43 a (0.15)	1.45 a (0.15)	1.23 b (0.15)	0.84 c (0.15)	

Means followed by the same uppercase letters in columns and lowercase letters in rows are not different by the Student's t-test (p < 0.05). Values in parentheses correspond to the standard error of the mean.

3.3.3. Kinetic fermentative parameters

Data regarding kinetic parameters of fermentation during the 24-hours evaluation period are shown in Table 3. The cumulative gas volume was affected by treatment

($P < 0.0001$), season of the year ($P = 0.0017$), and by the treatment x season of the year interaction ($P < 0.0001$). Greatest values were recorded during Winter/Early Spring relative to the other seasons of the year (Table 3). Generally, Piatã palisadegrass showed the greatest values of gas volume, except during Late Spring, when greater values were recorded for the Association and Massai guineagrass. There was no difference among treatments during Summer II.

Digestion rate was only affected by treatment ($P < 0.0001$). Greater values were recorded for Piatã palisadegrass, followed by intermediate values for the Association and Massai guineagrass and smaller values for *Andropogon gambagrass* (Table 3). Lag time was influenced by both season of the year ($P < 0.0001$) and treatment x season of the year interaction ($P < 0.0001$). Smallest values were recorded during Autumn and greatest during Summer II, with intermediate values during the remaining seasons of the year. There was no difference among treatments during Autumn and Summer II. Piatã palisadegrass showed the greatest lag time values during Summer I and the smallest values during Winter/Early Spring. *Andropogon gambagrass* showed the smallest values during Late Spring.

Table 3. Kinetic fermentative parameters of the *in vitro* gas production of *Andropogon gambagrass*, *Massai guineagrass*, and *Piatã palisadegrass* cultivated as monocultures and in association.

Season of the year	Treatments				Mean
	Association	Massai guineagrass	Piatã palisadegrass	Andropogon gambagrass	
Asymptotic cumulative gas volume (ml g DM ⁻¹)					
Summer I	109.4 ABb (6.01)	100.3 Bb (6.01)	120.6 Aa (6.01)	90.2 Bc (6.03)	105.1 B (5.20)
Autumn	108.0 ABb (5.74)	106.9 ABb (5.74)	121.4 Aa (5.74)	87.8 Bc (5.74)	106.0 B (5.26)
Winter/Early Spring	115.1 Aa (5.88)	108.9 ABab (5.88)	117.0 Aa (5.88)	104.9 Ab (5.88)	111.5 A (5.29)
Late Spring	108.6 ABa (5.93)	109.5 Aa (5.93)	99.4 Bb (5.93)	91.2 Bb (5.93)	102.2 B (5.30)
Summer II	106.8 Ba (5.53)	107.9 ABa (5.53)	103.7 Ba (5.67)	105.9 Aa (5.67)	106.1 B (5.20)
Mean	109.6 ab (5.24)	106.7 b (5.24)	112.4 a (5.25)	96.0 c (5.24)	
Digestion rate (h ⁻¹)					
Summer I	0.058 (0.001)	0.060 (0.001)	0.063 (0.001)	0.061 (0.001)	0.060 (0.001)
Autumn	0.062 (0.001)	0.061 (0.001)	0.064 (0.001)	0.064 (0.001)	0.063 (0.001)
Winter/Early Spring	0.058 (0.001)	0.056 (0.001)	0.065 (0.001)	0.052 (0.001)	0.058 (0.001)
Late Spring	0.058 (0.001)	0.055 (0.001)	0.060 (0.001)	0.051 (0.001)	0.055 (0.001)
Summer II	0.056 (0.001)	0.053 (0.001)	0.062 (0.001)	0.053 (0.001)	0.056 (0.001)
Mean	0.058 b (0.001)	0.057 bc (0.001)	0.063 a (0.001)	0.056 c (0.001)	
Lag time (h)					
Summer I	0.38 Cb (0.11)	0.27 Cb (0.11)	0.78 Ba (0.11)	0.21 CDb (0.11)	0.41 C (0.083)
Autumn	0.30 Ca (0.10)	0.12 Ca (0.10)	0.31 Ca (0.10)	0.14 Da (0.10)	0.22 D (0.083)
Winter/Early Spring	0.92 Ba (0.13)	0.69 Ba (0.13)	0.33 Cb (0.13)	0.99 Ba (0.13)	0.74 C (0.094)
Late Spring	1.01 Ba (0.13)	0.96 Ba (0.14)	0.93 ABa (0.14)	0.43 Cb (0.13)	0.83 B (0.093)
Summer II	1.30 Aa (0.11)	1.33 Aa (0.11)	1.21 Aa (0.13)	1.34 Aa (0.11)	1.30 A (0.086)
Mean	0.78 (0.084)	0.67 (0.085)	0.71 (0.086)	0.62 (0.085)	

Means followed by the same uppercase letters in columns and lowercase letters in rows are not different by the Student's t-test ($p < 0.05$). Values in parentheses correspond to the standard error of the mean.

3.3.4. Ruminal parameters

Data regarding ruminal parameters are shown in Table 4. The ammoniacal nitrogen (NH₃-N) varied with treatment ($P=0.0333$), season of the year ($P<0.0001$), and with the treatment x season of the year interaction ($P=0.0442$). Greatest values of NH₃-N were recorded during Summer I and smallest during Winter/Early Spring, with intermediate values during the remaining seasons of the year (Table 4). There was no difference among treatments

during Summer I. Generally, the Association showed greatest values of $\text{NH}_3\text{-N}$ throughout the experimental period. *Andropogon gambagrass* showed values of $\text{NH}_3\text{-N}$ similar to the Association, except during Late Spring. On average, *Massai guineagrass* and *Piatã palisadegrass* showed the smallest $\text{NH}_3\text{-N}$ values throughout the seasons of the year.

Short-chain fatty acids (SCFA; acetate, propionate, butyrate, and valerate) were affected by treatment ($P=0.0002$), season of the year ($P<0.0001$), and their interaction ($P=0.0395$). Greatest values were recorded during Summer II and smallest during Winter/Early Spring and Late Spring. There was no difference among treatments during Winter/Early Spring and Summer II. On average, *Piatã palisadegrass* showed the greatest values of SCFA, while smallest values were recorded for *Andropogon gambagrass*.

The acetate:propionate ratio (A:P) was affected by treatment ($P<0.0001$), season of the year ($P<0.0001$), and their interaction ($P=0.0197$). In general, greatest values of A:P were recorded for *Andropogon gambagrass*, followed by *Massai guineagrass*, Association, and *Piatã palisadegrass* throughout the experimental period. Regarding seasons of the year, greatest values were recorded during Summer I and Late Spring, and smallest values during Winter/Early Spring and Summer II, with intermediate values during Autumn.

Table 4. Ruminal parameters of the *in vitro* gas production of *Andropogon gambagrass*, *Massai guineagrass*, and *Piatã palisadegrass* cultivated as monocultures and in association.

Season of the year	Treatments				Mean
	Association	Massai guineagrass	Piatã palisadegrass	Andropogon gambagrass	
Ammoniacal nitrogen (mg dL ⁻¹)					
Summer I	40.1 Aa (1.25)	39.0 Aa (1.25)	40.1 Aa (1.25)	39.8 Aa (1.25)	39.8 A (0.97)
Autumn	40.1 Aa (1.09)	37.0 Bb (1.09)	36.3 Bb (1.09)	39.3 Aa (1.09)	38.2 B (0.95)
Winter/Early Spring	27.8 Cab (1.11)	25.8 Db (1.11)	25.9 Db (1.11)	28.3 Ba (1.11)	26.9 E (0.93)
Late Spring	30.1 Ba (1.07)	26.6 Db (1.07)	27.7 Cb (1.07)	27.1 Bb (1.07)	27.85 D (0.92)
Summer II	30.1 Ba (1.23)	30.5 Ca (1.23)	27.3 CDb (1.33)	29.3 Bab (1.23)	29.5 C (0.98)
Mean	33.8 a (0.98)	31.8 b (0.98)	31.4 b (0.99)	32.7 ab (0.98)	
Short-chain fatty acids (mmol L ⁻¹)					
Summer I	100.8 ABb (2.64)	95.4 Bc (2.64)	108.1 Aa (2.64)	94.7 Bc (2.67)	99.8 B (2.36)
Autumn	102.4 ABb (2.71)	102.5 Ab (2.71)	108.7 Aa (2.71)	93.9 BCc (2.71)	101.9 B (2.37)
Winter/Early Spring	94.8 Ba (4.26)	89.1 Ba (4.26)	94.8 Ba (4.26)	86.0 CDa (4.26)	91.2 C (2.89)
Late Spring	82.0 Cb (3.43)	94.0 Ba (3.43)	95.1 Ba (3.43)	79.9 Db (3.43)	87.7 C (2.59)
Summer II	105.6 Aa (2.95)	106.5 Aa (2.95)	108.5 Aa (2.95)	103.7 Aa (3.18)	106.1 A (2.46)
Mean	97.1 b (2.47)	97.5 b (2.47)	103.0 a (2.48)	91.6 c (2.47)	
Acetate:propionate ratio					
Summer I	3.64 Ab (0.19)	4.08 Aa (0.19)	3.11 ABc (0.19)	4.27 Aa (0.19)	3.78 A (0.18)
Autumn	3.52 Ab (0.19)	3.77 Ba (0.19)	3.21 ABc (0.19)	3.89 Ba (0.19)	3.60 B (0.17)
Winter/Early Spring	3.17 Bab (0.21)	3.42 Ca (0.21)	2.99 Bb (0.21)	3.45 Ca (0.21)	3.26 C (0.18)
Late Spring	3.77 Ab (0.21)	3.91 ABb (0.21)	3.38 Ac (0.21)	4.38 Aa (0.21)	3.86 A (0.18)
Summer II	3.14 Bb (0.20)	3.36 Cab (0.20)	3.14 ABb (0.20)	3.55 Ca (0.20)	3.30 C (0.18)
Mean	3.45 c (0.18)	3.71 b (0.18)	3.16 d (0.18)	3.91 a (0.18)	

Means followed by the same uppercase letters in columns and lowercase letters in rows are not different by the Student's t-test ($p < 0.05$). Values in parentheses correspond to the standard error of the mean.

3.3.5. Relationship between nutritive value and gas production parameters

The results of the multivariate analysis conducted using a data set comprised of the main characteristics of nutritive value and parameters of the *in vitro* incubation are illustrated in Figure 2. The first two axes derived from the principal component analysis accounted for 79.9% of the total variation in the dataset (Table S1, Supplementary material). The first principal component (PC1) explained 56.4% of the total variance and represented a contrast

between dry matter digestibility and digestion rate *versus* fiber content (ADF and NDF). Piatã palisadegrass exhibited greater dry matter digestibility and digestion rate, while ADF and NDF content was primarily associated with Massai guineagrass. This component also showed that greater values of crude protein and acetate:propionate ratio were primarily associated with Andropogon gambagrass. The Association was positioned as intermediate between the other treatments. The second principal component (PC2) explained 23.5% of the total variance and revealed a relationship among methane production and *in vitro* degradability of NDF. Correlation data also showed that net methane production was inversely correlated with crude protein content and acetate:propionate ratio.

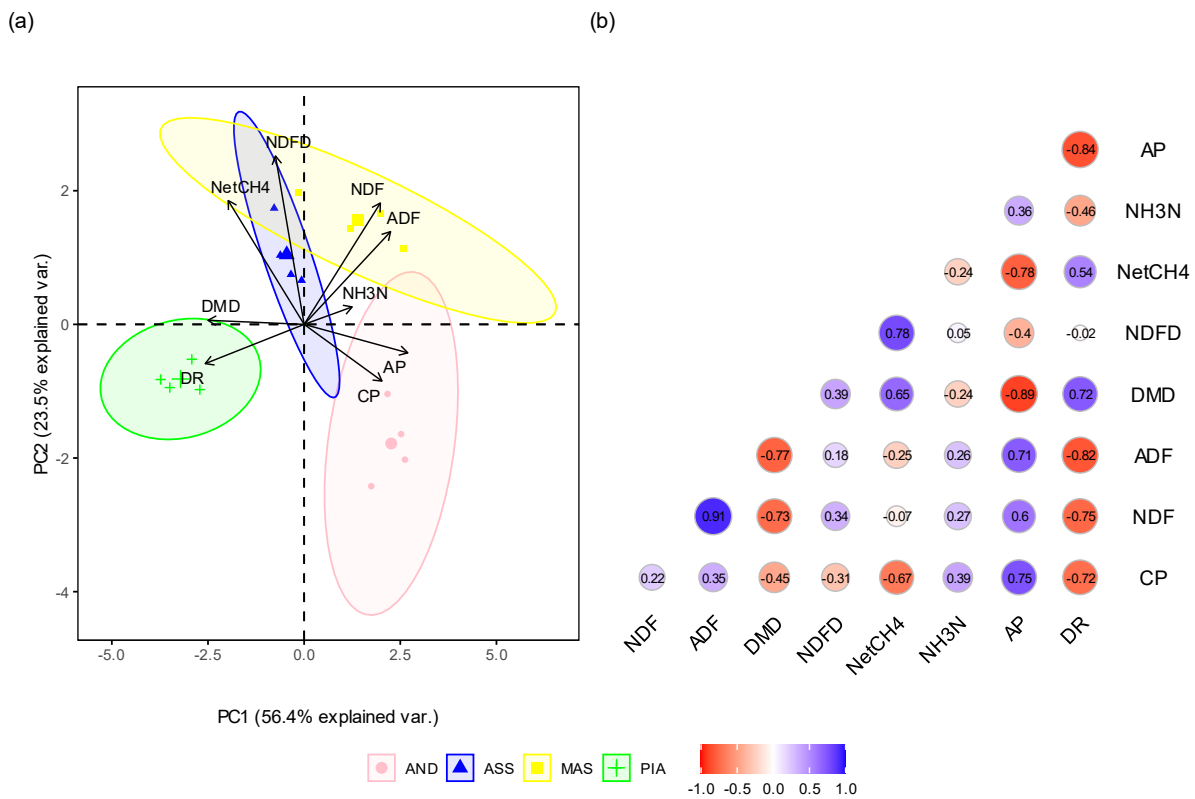


Figure 2. Principal components analysis (a) and Pearson correlation matrix (b) of nutritive value and *in vitro* incubation parameters of Andropogon gambagrass, Massai guineagrass and Piatã palisadegrass cultivated as monocultures and in association. The data correspond to the average of the five seasons of the year evaluated. CP: crude protein content; NDF: Neutral detergent fiber content; ADF: Acid detergent fiber content; DMD: *In vitro* degradability of dry matter; NDFD: *In vitro* degradability of NDF; NetCH₄: Methane produced; NH₃N: Ammoniacal nitrogen; AP: Acetate:propionate ratio; DR: Digestion rate.

3.4. Discussion

Management practices, botanical composition, and the prevailing edaphoclimatic conditions exert a significant influence on the morphogenetic and structural attributes of forage plants (Da Silva et al., 2019; Da Silva and Nascimento Júnior, 2007; Santos et al., 2020). These attributes, in turn, have a substantial impact on the chemical composition and digestibility of the forage, ultimately determining its nutritive value (Geremia et al., 2022). The nutritive value of forages has a significant relationship with *in vitro* incubation parameters, as reported in the literature (Bizzuti et al., 2023; Gemedá and Hassen, 2014; Marín et al., 2021). Information regarding the nutritional content of the consumed herbage enables the manager to draw inferences about the potential nutrient intake based on the botanical and morphological composition of the pasture, along with the herbage short-term intake rate data (Silva et al., 2018). In this study, the nutritive value and *in vitro* incubation parameters were different for the grass species and for their Association and also varied across seasons of the year. Despite the greater complexity of mixed swards, the Association showed satisfactory results regarding chemical composition and *in vitro* incubation parameters.

Nutrient intake by grazing animals is linked to the morphological composition and sward structure, as well as the chemical composition of the consumed forage (Geremia et al., 2022). These factors are, in turn, influenced by several other factors, including grazing management practices, botanical composition of the pasture, and season of the year. In this study, chemical composition varied across seasons of the year (Figure 1), consequence of the variable availability of growth factors which affects the phenological state of plant development (Pereira et al., 2010). The reduction in crude protein content was more pronounced during Autumn and Winter/Early Spring for all treatments as a result of climatic conditions during these times of the year. During Autumn, the reduction in air temperature and day length induces changes in the morphogenetic processes of the plants (Montagner et al., 2012; Sousa et al., 2011), affecting their morphological composition and, in consequence, their chemical composition (Da Silva et al., 2019). The greater proportion of stems and dead material in the herbage mass during these seasons provides an increase in fibrous content and a reduction in crude protein, negatively impacting the herbage's nutritional value. Greater acid detergent fiber content was observed in Massai guineagrass during this season as a reflection of the greater stem and dead material percentage in the herbage mass relative to green leaves, as shown in Chapter 2. Greater fiber content is related to reduction in crude protein content

and digestibility (Habermann et al., 2019), as shown for Massai guineagrass in this study (Figure 1; Table 1). *Andropogon gambagrass* had greater values of crude protein throughout the experimental period, varying from 140 to 180 g kg⁻¹. These values were greater than those reported in literature (Meale et al., 2011; Ribeiro Júnior et al., 2014; Silva et al., 2022). Despite being considered a plant adapted to marginal and low-fertility soils, it is possible that when cultivated in fertile soils, under nitrogen fertilization and adequate management, an improvement in nutritional value is noticed. Despite the rejection of the hypothesis that the association of tropical forage grasses would enhance the nutritional value of the herbage consumed by grazing animals, the nutritional value in mixed stands was always consistent with the greater values recorded for monocultures in this study.

Dietary crude protein for ruminants is required to favor microbial multiplication in the rumen, improving fermentation (Matthews et al., 2019). Crude protein concentrations below 70 g/kg may restrict microbial activity, which may affect the breakdown of cell wall components and, consequently, feed intake (Van Soest, 1994). In the present study, no recorded values were smaller than 9 g/kg for any of the treatments during the experimental period. In this context, adopting grazing management aiming at maintaining adequate sward structure is important to control the morphological composition of the canopy (Da Silva et al., 2015). This may prioritize the proportion of components with better nutritional value in the grazing stratum (Da Silva et al., 2019) and favor nutrient intake and animal performance (Savian et al., 2020). The defoliation management adopted was based on the 95% canopy light interception during regrowth criterion, which prevents the accumulation of fibrous components in forage mass during the vegetative stage of plant growth (Carnevali et al., 2006), having positive effects on chemical composition.

Short-chain fatty acids and ammoniacal nitrogen (NH₃-N) production may be used as indicators of degradation of carbohydrates and proteins (Lima et al., 2018). Ammoniacal nitrogen is required by the rumen microbiota (Pimentel et al., 2012) and increases in the concentration of NH₃-N during rumen fermentation are desirable to favor growth of fibrolytic rumen bacteria (Leng, 1990). The smallest values of NH₃-N were observed during Winter/Early Spring, concomitantly to the reduction in crude protein content in the forage (Table 4). Despite the Association did not show the greatest values of crude protein content, it presented the greatest values of NH₃-N throughout seasons of the year, not differing from *Andropogon gambagrass*. In this sense, mixing tropical grass species with contrasting morphological and phenological characteristics may result in more efficient degradation of the protein, favoring greater ruminal NH₃-N concentration. Increments in crude protein and

protein degradation *in vitro* have been shown to depress methane production (Singh et al., 2012). Although the multivariate analysis did not show a relationship between NH₃-N with other variables, the crude protein content showed a negative correlation with methane production (Figure 2). Andropogon gambagrass showed the smallest values of methane produced when expressed per unit of either dry matter or neutral detergent fiber degraded.

The primary source of energy for ruminants is the short-chain fatty acids, where the balance between acetate and propionate concentrations (A:P ratio) is determinant in influencing energy allocation and the presence of free hydrogen (H⁺) available for methanogenic organisms in the rumen. It is widely known that feeds that decrease acetate production and increase propionate are often associated with reducing ruminal methane production (Janssen, 2010). Despite the Association showing the smallest values of A:P ratio, this parameter was not related to reduction in methane production in this study (Table 2). The alternative pathway leading to increased propionate production is a more thermodynamically favorable alternative route to utilizing the H⁺ available in the rumen. When the A:P ratio is reduced, there is a decrease in acetate production, and consequently, less substrate is available for methane formation, ultimately resulting in improving the energy efficiency of the animal (Nogueira et al., 2020). In addition, the A:P ratio is related to the digestible neutral detergent fiber of feeds (Meale et al., 2011), and this relationship was observed in the Association and Massai guineagrass. Regarding the *in vitro* degradability of dry matter, the average values of the treatments varied from 439.8 to 499.3 g kg⁻¹. The greatest values were observed for Piatã palisadegrass, followed by the Association, and the smallest values for Massai guineagrass and Andropogon gambagrass. These results follow a similar pattern to the kinetic parameters of the gas volume produced and digestion rate. Greater values of potential gas produced and dry matter *in vitro* digestibility tend to have greater methane production per unit of dry matter fermented (Meale et al., 2011). In this context, Piatã palisadegrass and the Association had the greatest values recorded for these variables. While these treatments did not show reduction in methane production, it is imperative to conduct a comprehensive analysis of the factors influencing the efficiency of the production system. Such information is essential for the manager to make decisions effectively.

3.5. Conclusions

The association of grass species presented chemical composition values similar to monocultures and did not improve the nutritional value of the forage throughout the year.

Methane production was also not reduced in mixed pastures. However, other parameters were improved, such as NH₃-N and dry matter and neutral detergent fiber *in vitro* digestibility. Overall, all treatments presented high nutritional value, with variations throughout seasons of the year. Differences in chemical composition among forage species may have shaped the differences of *in vitro* incubation variables, such as methane production, kinetic and ruminal parameters.

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Supplementary material

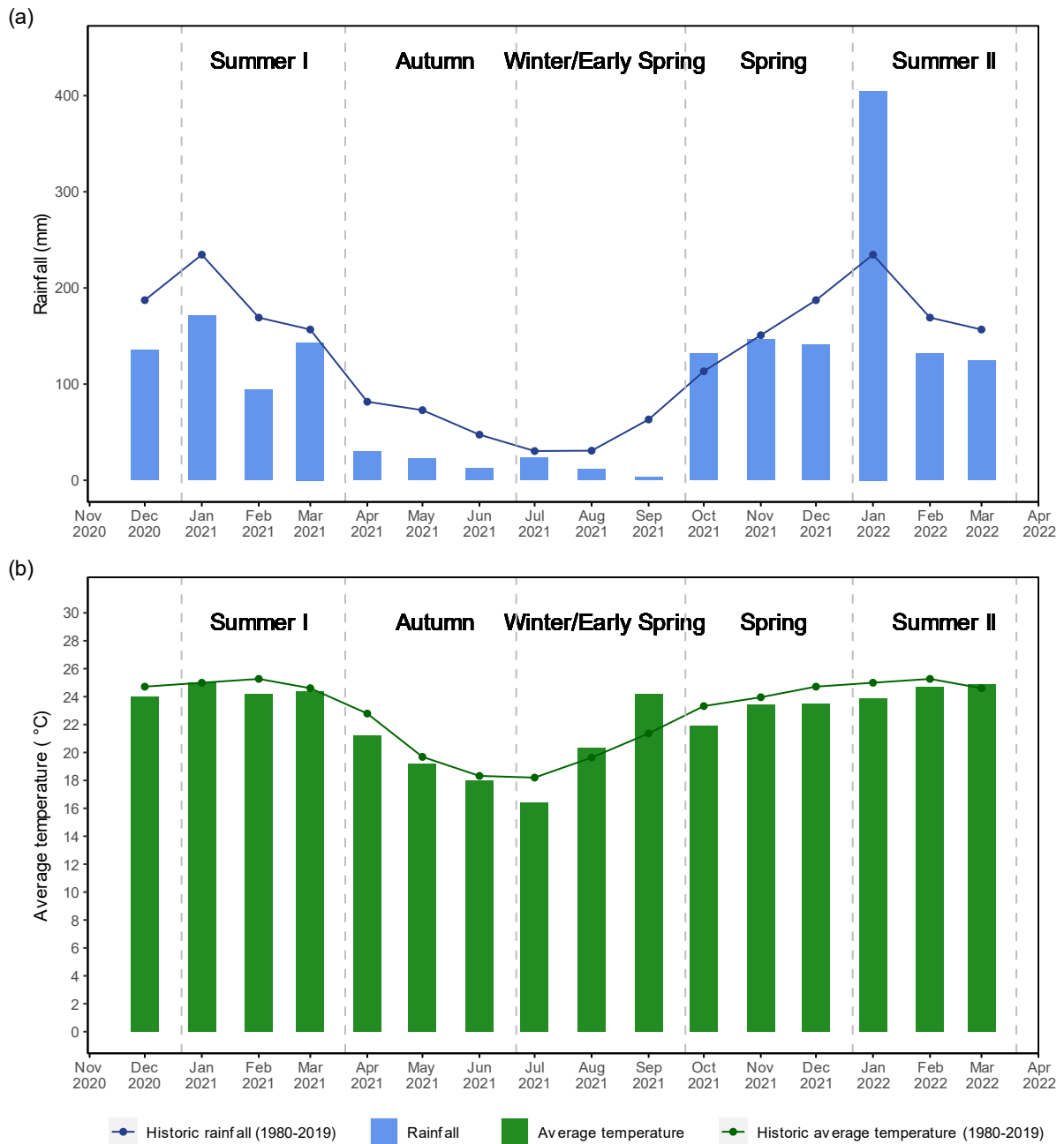


Figure S1. Monthly rainfall (a) and average air temperatures (b) at the experimental site during the experimental period (from December 2020 to March 2022) relative to historic weather data (1980-2019).

Table S1. Principal Component Analysis (PCA) performed on a subset of nutritive value and *in vitro* incubation parameters of *Andropogon gambagrass*, *Massai guineagrass*, and *Piatã palisadegrass* cultivated as monocultures and in association.

Principal Components	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9
CP	0.322	-0.209	0.377	0.614	0.455	0.095	0.212	0.265	0.013
NDF	0.316	0.449	-0.193	-0.117	0.222	-0.392	0.037	0.276	-0.604
ADF	0.358	0.343	-0.199	-0.012	-0.214	0.794	0.056	0.182	0.001
DMD	-0.399	0.015	0.344	0.310	-0.292	0.214	-0.276	-0.028	-0.645
NDFD	-0.119	0.623	0.224	0.281	-0.156	-0.233	-0.325	0.255	0.465
NetCH ₄	-0.315	0.458	0.028	0.025	0.565	0.214	0.178	-0.539	-0.004
NH ₃ N	0.199	0.064	0.765	-0.602	0.037	0.070	0.006	0.019	0.001
A:P	0.430	-0.105	-0.061	0.050	0.206	0.033	-0.784	-0.372	-0.007
DR	-0.410	-0.145	-0.141	-0.260	0.473	0.230	-0.347	0.568	0.035
Standard deviation	2.252	1.453	1.006	0.734	0.360	0.283	0.197	0.119	0.008
Proportion of variance	56.395	23.463	11.233	5.992	1.438	0.886	0.430	0.157	0.006
Cumulative proportion	56.395	79.859	91.093	97.085	98.523	99.410	99.841	99.999	100.000

CP: crude protein content; NDF: Neutral detergent fiber content; ADF: Acid detergent fiber content; DMD: *In vitro* degradability of dry matter; NDFD: *In vitro* degradability of NDF; NetCH₄: Methane produced; NH₃N: Ammoniacal nitrogen; A:P: Acetate:propionate ratio; DR: Digestion rate.

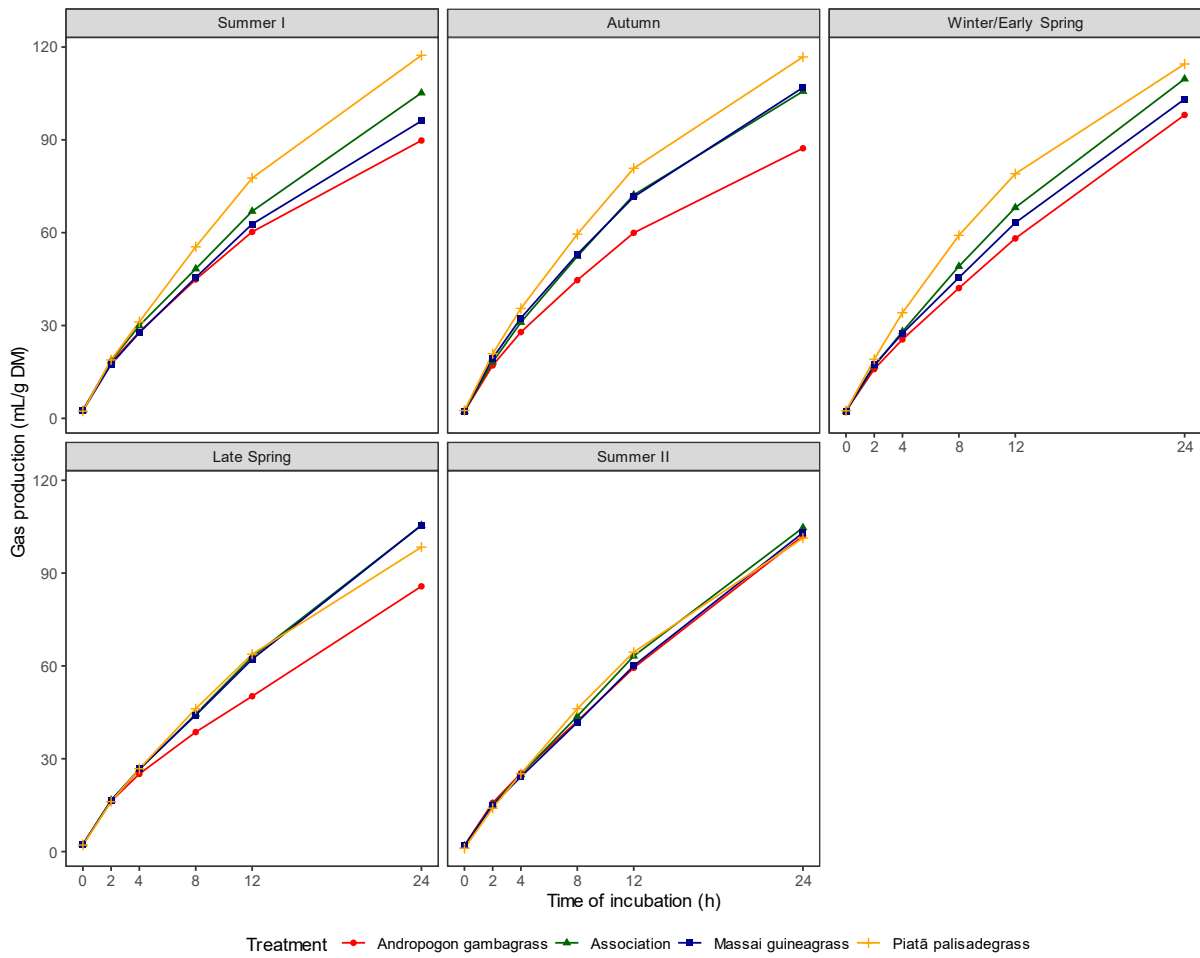


Figure S2. Cumulative gas production of *Andropogon gambagrass*, *Massai guineagrass*, and *Piatã palisadegrass* cultivated as monocultures and in association.

FINAL CONSIDERATIONS

The findings of this study revealed that the Association of tropical forage grass species has promising results based on the studied variables, representing an opportunity to increase ecosystem services by increasing the number of species in the pasture. Mixed swards showed performance as good as or better than the corresponding monocultures in providing greater forage intake rates while maintaining similar nutritional values and methane emissions. Since intake rate is related to daily intake and animal performance, these results may lead to improved animal performance and reduced environmental impacts.

Despite the inherent complexity associated with diverse swards, the results underscore their practical viability from a productive point of view. The dynamic variation in species proportions throughout the year highlights the complexity of the system. However, this variation may lead to greater buffering capacity during transitional periods, such as times of pronounced water deficit or reduced day length.

To further assess the feasibility of mixed-species pastures, future studies should be conducted under varying soil and climatic conditions within tropical regions. Additionally, gathering information about forage grasses available in these regions may help in selecting the most suitable species for mixing in different scenarios. Further, it is essential to evaluate appropriate management strategies, the compatibility of species within the mixtures, and methods for implementing these mixtures effectively. Conducting large-scale studies that encompass animal performance and economic viability will be crucial in analyzing production costs and optimizing productivity, aiming at combining the search for sustainable intensification of animal production systems in pastures and increased productivity.