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Influence of silvopastoral systems on gastrointestinal nematode infection and immune response of Nellore heifers under tropical conditions

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ABSTRACT

Among the strategies for integrating crops, livestock, and forestry, silvopastoral systems must be highlighted due to their inherent microclimatic conditions, mainly in tropical countries such as Brazil, where cattle are frequently subjected to unfavorable thermal conditions. However, according to some studies, shading can potentially worsen herdś parasitism due to better microclimatic condition for the parasites. This study aimed to assess fecal egg count in Nellore heifers reared in two silvopastoral arrangements (pasture with single or triple tree rows), in a crop-livestock system, and open pasture. In the silvopastoral treatment composed of triple rows, lesser parasite burden means were found, with a peak infection in February/March and another in October. Regarding the effect of seasons over the year, there was an environmental influence on the egg counts, with higher averages during the late rainy season and the beginning of the dry season. An immunological investigation of animals from each group showed that cattle kept on the silvopastoral arrangements with either single or triple rows have significantly higher lymphocyte proliferation when stimulated with specific antigens than those kept on open pastures. Based on our results, it can be concluded that both silvopastoral systems were not considered as a risk factor for nematode egg counts in Nellore heifers. Indeed, the shadiest system promoted milder parasitism and higher immunological lymphocyte responses in animals.

1. Introduction

Flies, ticks and helminths are responsible for annual losses of more than 13 billion US dollars for livestock according to Grisi et al. (2014). Among those parasites, gastrointestinal nematodes frequently cause severe injuries to farm animals through its parasitic stage; such infections usually are associated with decreased weight gain, milk production, and feed conversion (Lopes et al., 2017). Regarding helminthoses control, chemical-based procedures have been used for decades (Geurden et al., 2015) although there are available other approaches. Furthermore, even though health protocols such as the strategic control proposed by Bianchin (1997) have been available for years, anthelmintic treatments without any criteria are common in many regions of Brazil. Consequently, the lack of information by farmers and inappropriate health management leads to drug resistance and economic losses in many farms (Grisi et al., 2014).

Cattle nematodes have a direct lifecycle with three larvae stages during the non-parasitic period. The third instar larvae (L3) survival is closely related to the local environmental conditions such as pasture composition and climate features (Faria et al., 2016). Based on that, integrated systems adoption could negatively affect the animal health if there was an interaction between the agent and the susceptible individual in a favorable environment, as usual found in the silvopastoral model. In the last years, those interactions have become more important due to livestock intensification in regions such as the Amazon (Gil et al., 2018), even though Nellore cattle, most common in that region, be considered resilient to parasites (Mwai et al., 2015; Zapa et al., 2021) due to cellular and humoral immunity (Piper et al., 2009).

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According to Oliveira et al. (2017) afforestation may be a risk factor for parasitosis. The reasons are pretty straightforward; the survival of free-living stages is mainly affected by radiation, temperature, and moisture, directly influencing the larval development rate. On the other hand, intensively farmed cattle managed under the full sun may have the immune system function compromised due to heat stress, reflected in a variation of cytokine profile and depression of the cell-mediated immunity (Peli et al., 2013). However, although there are many efforts to assess the physiological damage of heat-stressed cattle, there are no reports relating to the benefits of pastures arborization on bovine immunological parameters.

Concerning Nellore importance to the Brazilian livestock and the increased adoption of Integrated Systems by local farmers, the present study aimed to assess host-parasite dynamics through fecal egg count and cell-mediated immune response in Nellore heifers in four treatments, including an Open Pasture (OP), a Crop-Livestock system (CL), and two silvopastoral (SPS) arrangements, both based on *Brachiaria brizantha* cv. Marandu pastures associated with eucalyptus trees (*Eucalyptus urograndis* | H13 clone).

2. Materials and methods

The study was conducted according to ethical standards and approved by the Committee on Ethics in Animal Use of Embrapa Agrosilvopastoral (Protocol N_ 004/2016).

2.1. Study area

Experiments were carried out at Embrapa Agrossilvipastoril, Sinop/MT, Brazil (11_ 510 S, 55_ 350 W, the elevation of 370 m), and Amazon Biome (Fig. 1). The climate was classified according to the criteria of Köppen, as Am monsoon climate, which alternates between a rainy and a dry season (Alvares et al., 2014). The average annual temperature is 25.5º C, with 20.2º C minimum and 33.0º C maximum average temperatures. The typical annual relative air humidity is 70%, with annual precipitation of 2250 mm. The climatic data were obtained at Embrapa Agrossilvipastoril Meteorological Station (Campbell Scientific™). The experimental design was in randomized blocks, containing four treatments and four replicates (blocks). Blocks were adopted to reduce unexplained data variability due to variation in local conditions since few agronomic studies were also conducted in the same area focused on other variables (Magalhães et al., 2018).

2.2. Animals and experimental stocking systems

Ninety-six Nellore heifers were distributed in the experiment, including the four treatments: (a) livestock in an open pasture (OP) with *Brachiaria (syn. Urochloa) brizantha* (Hochst. Ex A. Rich.) Stapf. (palisade grass) cv Marandu; (b) silvopastoral system (single | SPSs), with eucalyptus trees (*Eucalyptus urograndis*, H13 clone) disposed into a single row (east-west direction with 3 m intra-row spacing trees) spaced 37 m apart, mixed with pasture Marandu, and overall density of 90 trees ha^{-1} (Magalhães et al., 2018); (c) silvopastoral system (triple | SPSt), also with eucalyptus trees but disposed into triple rows (east-west direction, 3.0 m intra-row and 3.5 m inter-row spacing) spaced 30 m apart, mixed with pasture Marandu, with an overall density of 135 trees ha^{-1} (Magalhães et al., 2018). This density was achieved after selective thinning removal of 50% of trees in 2016; (d) crop-livestock system (CL), where both components were rotated every two years. Before livestock started, from October 2018 to February 2019, soybean [*Glycine max* (L.) Merr.] was cultivated and then harvested. All systems were installed and evaluated in experimental plots of two hectares (100 ×200 m) each. Palisade grass was adopted in all systems due to its abundance as a monoculture pasture within our region (Carvalho et al., 2019).

Fig. 1. Experimental field of Embrapa Agrosilvopastoral, MT - Brazil.

The herd was composed of Nellore heifers (*Bos indicus indicus*) with an initial mean weight of 270 ± 36 kg and 14–16 months of age. In each system, weight gain was recorded every 28 days on 24 tracer animals (six heifers per block randomly distributed across treatments based on body weight). Three extra pastures were used as a reserved area, where animals (regulators) used only for adjustments in the stocking rate were kept throughout the experiment; no data was recorded from them. The individual weighing was performed after 16 h of fasting, including liquids, and the average daily weight gain (DWG) was expressed in g day^{-1}.

The study was carried out from February 2019 to February 2020, with animals set stocked with variable stocking rates, maintaining the pasture cover height at 30 cm (Gomes et al., 2020), assuming a variation of up to 15%. In February 2019, 50 kg N ha^{-1}, 50 kg K_2O, and 40 kg P_2O_5 ha^{-1} | urea, potassium chloride, and superphosphate, respectively, were applied to all paddocks. The animals had free access to water (ad libitum) and mineral supplements, set at 0.2% of body weight (mix for the dry season: NDT (min.) 530 g kg^{-1}; protein min. 300 g kg^{-1} | mix for the rainy season: NDT min. 720 g kg^{-1}; protein min. 200 g kg^{-1}); both formulas used came from Fortuna Nutriç ão Animal™. During the dry season, starting in June, corn silage was provided at 15 kg of animal^{-1} day^{-1}, ending offers on the first week of October.

2.3. Fecal egg count

The fecal samples were collected monthly directly from the rectum of all 96 tracer animals (24 heifers per treatment naturally infected) and taken to the laboratory to further analysis. There had been no cattle management in neither treatment for six months before the study started. Based on that information and in the absence of clinical symptoms involving tracer animals, there was no herd treatment with any anthelmintic previously the trial.

The technique of Gordon and Whitlock, modified by Ueno and Gonçalves (1998), was used to estimate the number of eggs per gram of feces | EPG. The preparation of the samples for examination was done by placing 2 g of feces in a sieve, adding 58 mL of hyper saturated NaCl solution. After trituration with a glass stick, the fecal suspension was homogenized with a pipette. The suspension was placed in a McMaster Chamber. After two minutes, the settled eggs were counted in the chambers using a 10x objective microscope. The total EPG was calculated by adding the number of eggs found in the two chambers and multiplying the sum by 100. One thousand and fifty-six samples of feces were gathered between February 2019 and 2020, covering both dry and rainy seasons.

2.4. Immunological measurements

To verify the immune status, 24 heifers from OP, SPSs, SPSt (eight per system/treatment) were immunized with a vaccine formulation containing four tick recombinant proteins (rAsKunitz, rAs8,9, rAsBasicTail, and rAsQuimera), according to Costa et al. (2021), while eight other animals from each system were kept as control. Animals were injected three times at a 28-day interval. Blood samples were collected from the animals 28 days after the last inoculation and submitted to a lymphocyte proliferation assay, according to Hawkins et al. (2007). Approximately 10 mL of blood was collected in EDTA tubes submitted to peripheral blood mononuclear cell (PBMC) isolation. These samples were diluted 1:1 with PBS and applied over a Ficoll-Paque™ Plus® (GEHeathcare) gradient (1:1:1). The mixture was centrifuged for 40 min at 600 g at room temperature. PBMCs were collected at the interface between the plasma and the Ficoll. Cells were washed three times by centrifugation with PBS and re-suspended in Roswell Park Memorial Institute (RPMI) 1640 Medium (Gibco). PBMCs were treated with CFSE (carboxyfluorescein diacetate succinimidyl ester) (Invitrogen) for proliferation assay (Quah and Parish, 2010). Briefly, cells were incubated with 2.5uM of CFSE at 37 ºC under 5% CO_2 for 10 min. Labeled cells were washed twice with RPMI with 10% fetal calf serum (FCS - Vitrocell) by centrifugation at 500 g for 10 min at 4 ºC. After CFSE staining cells were adjusted to 1×10^7 cells/mL in sterile RPMI-1640® with 10% FCS in culture plates in the presence of 10 μg/mL of the four recombinant antigens (antigen pool). Cell cultures were incubated for three days at 37ºC under 5% CO_2. Cell proliferation was acquired using a FACScan (Becton & Dickinson, San Jose, CA, USA). A minimum of 10, 000 lymphocyte events were acquired and the CFSE staining was analyzed in the lymphocyte population. Lymphocyte analysis was done by gating the population's classically-occupied region in size versus granularity plot (SSC x FSC). The analyses were performed using FlowJo 7.6.5 software (Tree Star Inc., Ashland, OR, USA).

2.5. *Statistical analysis*

The data was analyzed with a parametric structure using general linear mixed models (PROC MIXED | SAS® version 9.4 | Institute Inc., Cary, NC, USA). The treatments (OP, CL, SPSs, SPSt), seasons (rainy and dry), and their interaction were considered fixed effects. The repetition (block) was considered a random effect. Linear predictor and quantil-quantile plots of the residuals were used to verify homogeneity of variance and error normality. The variance and covariance matrix were selected using the Akaike information criterion (Wolfinger and O'Connell, 1993). Treatment means were estimated by "LSMEANS" and the multiple comparisons were made by the probability of difference ("PDIFF") using the t-test ($p < .05$).

3. Results

The study site follows the tropical climate features marked by high mean temperatures yearlong varying between 24 and 30ºC and annual precipitation distributed in rainy and dry periods that last approximately six months each (Fig. 2).

When the parasite burden was assessed over the year, it is possible to note a significant environmental influence on the EPG counts ($p < .0001$). Greater EPG averages marked the period of adaptation (first rainy season - RS1) followed by a gradual and significant ($p < .05$) reduction in EPG values in the subsequent seasons (Fig. 3). In general, four EPG peaks were observed during the experimental period, two of them during the rainy seasons (February/19 and May/20) and the others during the dry season (May/19 and July-August/19) (Fig. 3). As expected, (based on Fig. 1), the first peak had the highest EPG counts for all four systems when systems were analyzed separately. In addition to February-19, animals from SPSt and OP also had high EPGs in the third peak (August - 2019) while, for SPSs and CL, the third peak occurred earlier (July - 2019), and the second peak (May - 19) had higher EPGs than the third (Fig. 4).

Moreover, significantly lower mean egg counts ($p = .0154$) occurred in the SPSt (85.8), which along the whole experimental period, had a mean EPG at least 40% lower in comparison to the animals maintained on the other systems (OP | 137.9; SPSs | 133.8; CL | 169.4) (Fig. 5).

Concerning weight gain and its interactions, the CL system stands out due to its greater capacity to fatten the heifers ($p = .0435$) during the dry season, despite the worse gains, 490 versus 820 g/day (Table 1). Notably, the dry season influenced daily weight gain comparing the systems. However, during the second rain season (RS2) the high rainfall evened out the daily weight gain in all systems (Fig. 3).

Regarding the analysis of the immune response of animals from each

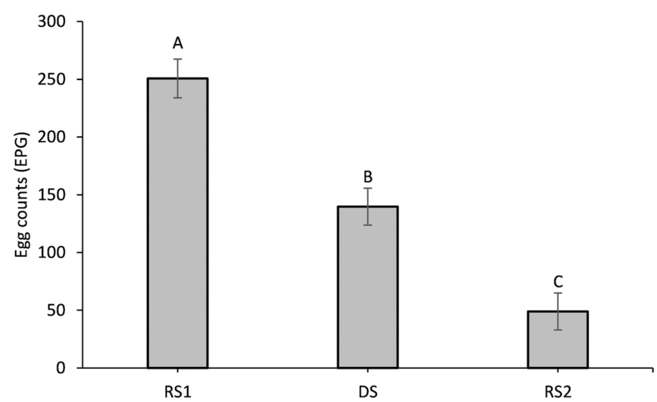

Fig. 3. Egg counting per gram of feces and seasonal influence. RS1 - the first rainy season, DS - Dry season, RS2 - Second rainy season. Data are means ± standard error.

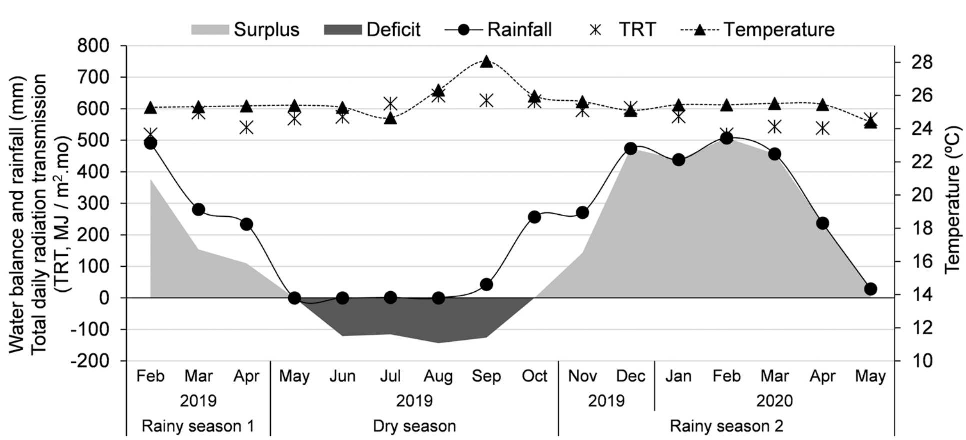

Fig. 2. Water balance (surplus and deficit), rainfall, total radiation transmission (TRT), and temperature during the seasons (rainy season 1, dry season, and rainy season 2) of the research site during the experimental period.

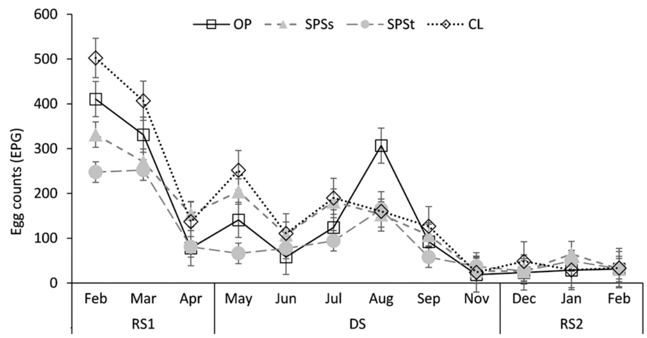

Fig. 4. Egg counting per gram of feces and the climatic influence in each system along the experimental period. OP - open pasture, SPSs - silvopastoral system with trees into single rows, SPSt - silvopastoral system with trees into triple rows, CL - crop-livestock system, RS1- the first rainy season, DS - Dry season, RS2- Second rainy season. Data are means ± standard error.

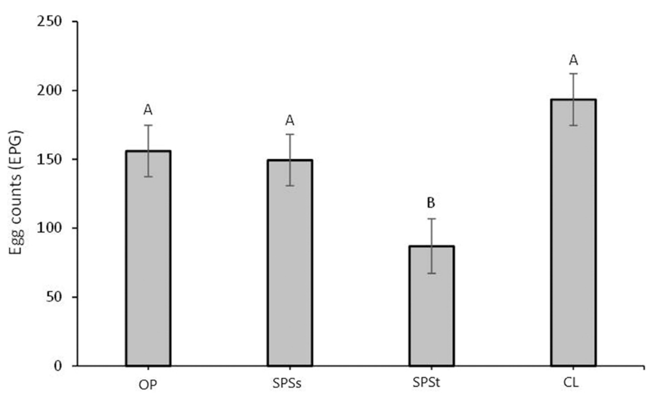

Fig. 5. Influence of systems over the EPG of the animals. Data are means* ± standard error. *Mean values of the whole period. OP - open pasture, SPSs - silvopastoral system with trees into single rows, SPSt - silvopastoral system with trees into triple rows, CL - crop-livestock system.

Table 1
Daily weight gain (g/day) in open pasture (OP), silvopastoral system with trees into single rows (SPSs), silvopastoral system with trees into triple rows (SPSt) and crop-livestock system (CL), and interaction with seasons (DS and RS2) in Sinop/MT, Brazil.

Season	OP	SPSs	SPSt	CL
DS	310^{Bb}	340^{Bb}	340^{Bb}	490^{Ba}
RS2	840^{Aa}	800^{Aa}	780^{Aa}	820^{Aa}
SE	64	57	57	57

DS: dry season; and RS2: rainy season 2.
Capital letters compare seasons within the system, and lowercase letters compare seasons by Student's t-test ($p < .05$).

group, cells from control and vaccinated animals were stimulated with a pool of our antigens and measured proliferation after three days. Fig. 5A shows the gating strategy used for flow cytometry analysis. PBMC's from vaccinated animals from all three systems proliferated after specific-antigen stimulation, as shown by the higher percentage of lymphocytes that proliferated at least once (Fig. 5B) and the reduced CFSE medium fluorescence intensity (MFI) (Fig. 5C) in vaccinated groups. Although a tendency for higher lymphocyte proliferation was observed in animals of the three systems, only vaccinated animals in OP and SPSt systems showed significant differences ($p < .05$) in comparison to controls (Fig. 6).

Vaccinated animals from the three groups were compared as well to assess which system enables a higher lymphocyte response. Results showed that vaccinated animals in SPSt and SPSs have significantly higher lymphocyte proliferation ($p < .05$) in comparison to vaccinated animals in the OP system, as demonstrated by both a higher percentage of cells that proliferated at least once and the lower CFSE MFI (Fig. 7).

4. Discussion

It is known that helminths have adverse effects on animal health and welfare, thereby posing significant economic losses to the livestock industry worldwide (Charlier et al., 2020). Therefore, numerous studies have been looking for the epidemiological effects associated with current livestock production models (Oliveira et al., 2017; Bello et al., 2020; Lopes et al., 2020a), as well as the interactions among variables associated with parasitoses (Faria et al., 2016; Lopes et al., 2020b), and the identification of risk factors (Villa-Mancera and Reynoso-Palomar, 2019), including environmental conditions attributable to the global climate changes (Kenyon et al., 2009).

In our study, it was verified that the environment influences egg counts. Visibly, the first rainy season was marked by higher averages due to previous parasitic burdens, probably from origin ranches' pastures. Regarding nutritional features of forage, protein levels and availability seem to be determinants of parasitism in young individuals. According to Soutello et al. (2002), nutritional requirements, when supplied, are essential to mitigate parasitism. In this sense, Gomes et al. (2021), assessing the nutritive value of Marandu palisade grass during the same long-term study, found crude protein levels varying from 147 ± 1 in the SSP to 128 ± 1 g kg^{-1} in OP, both superior to the recommended levels by Minson (1990).

Furthermore, as expected after an adaptation period with good feed management (Lopes et al., 2020b), egg counting got the lowest average by RS2, and the EPG peak predicted from Feb-20 was suppressed. However, although the higher EPG averages during the DS, the average remained under 150 eggs per gram of feces. Therefore, nutritional issues must be emphasized again. Although forage's nutritional status was not within our scope, outcomes such as those obtained by Gomes et al. (2021) corroborate our results, reinforcing the importance of feeding on the herd's parasitic burden.

Regarding system effects, SPSt negatively influenced the egg counts reducing them substantially, which was not seen in other studies, including Lopes et al. (2020a), although the study design was pretty similar. A reasonable explanation can be related to the herd profile. In this study, the assessed Nellore heifers had an initial mean weight of 270 ± 36 kg and were14 – 16 months old, against Nellore steers with an initial mean weight of 335 ± 14.5 kg and 16 ± 3 months age. The maturity of the animals seems to be determinant in this aspect, supporting the results found by Bianchin et al. (2007). Mendonça et al. (2014) did not find differences in helminth infestation between crossbred heifers grazed in SPS and OP, strengthening the premise that parasitism is influenced by factors such as breed, age, and management (Bianchin et al., 2007).

Based on that, some biotic factors must be addressed in our discussion. According to Magalhães et al. (2020), as shading increases, the mean values of the black globe-humidity index and radiant thermal load decrease naturally. The authors found milder microclimatic conditions in the SPSt than in OP, consequently, increasing the welfare of our heifers. That study was conducted in the same location, during the same period, and included the same systems (SPSt, SPSs, OP, and CL). That environmental setting is crucial to explaining the immunological parameters results obtained in this study. As shown in Figs. 6 and 7, there was a significant effect of the system on lymphocyte proliferation. Those animals in the SPSt treatment presented the most effective cell-mediated immunity and the lowest egg counts, corroborating our hypothesis that micro climatic conditions can influence host-parasite dynamics.

Peli et al. (2013) assessed stress through immune response under adverse environmental settings. Their results indicated a polarization of the body's response towards an antibody-like immunity, to the

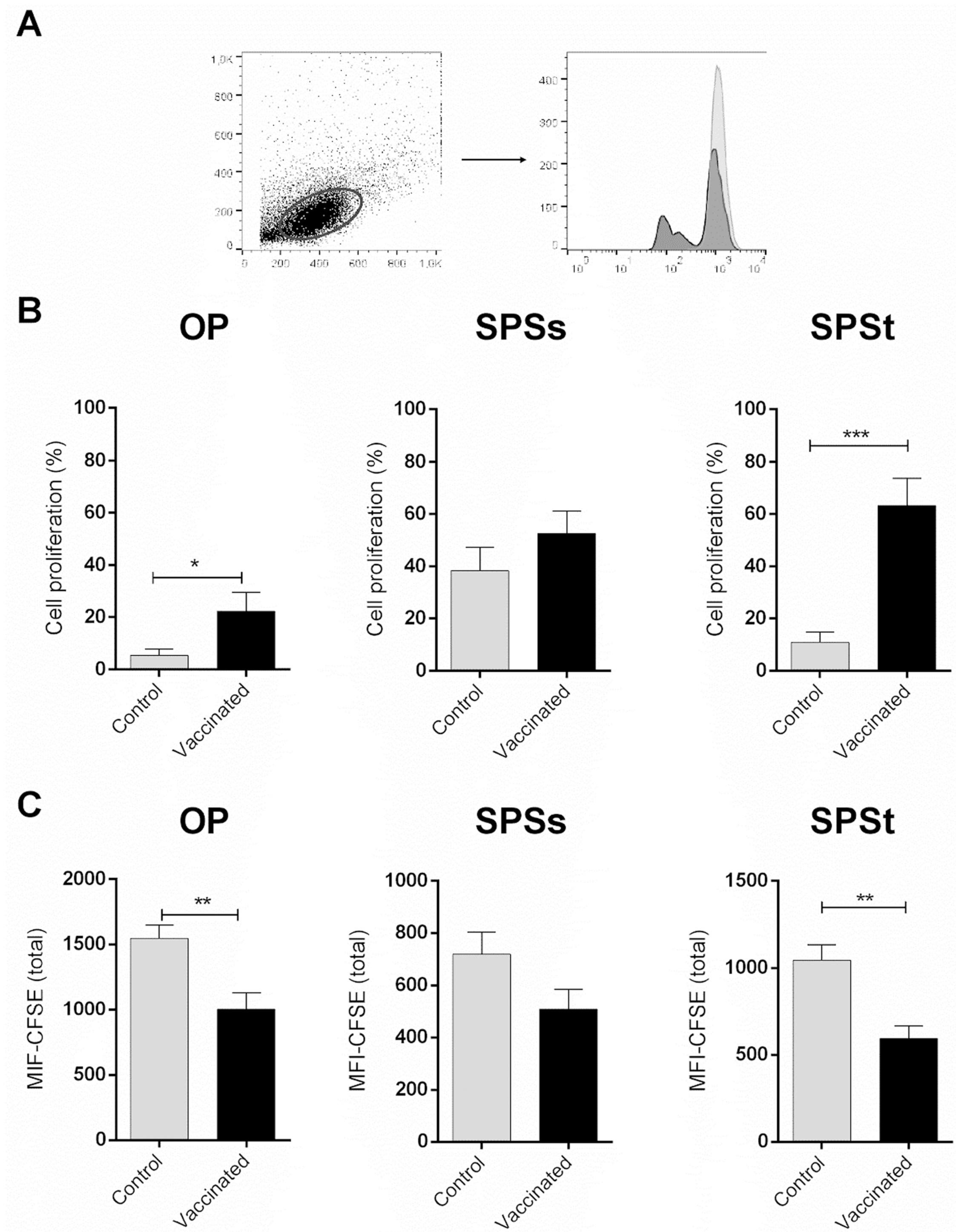

Fig. 6. Comparison of antigen-specific proliferation from PBMC from OP, SPSs, and SPSt animals. (A) Representative gating strategy showing lymphocyte population selection by size versus granularity (SSC x FSC) (left) and overlaid histogram showing medium fluorescence intensity of carboxyfluorescein diacetate succinimidyl ester (MFI-CFSE) of control (light grey) and vaccinated (dark) animals. Bar graphs show the percentage of lymphocytes proliferating at least once after stimuli with antigen pool (B) and MFI-CFSE (C). Graphs show different systems: livestock in open pasture (OP), silvopastoral system with trees into single rows (SPSs), and silvopastoral system with trees into triple rows (SPSt). Grey bars represent control animals and black bars vaccinated animals by Student's t-test, $*p < .05$, $* *p < .005$, $* **p < .0005$.

detriment of the cell-mediated response. Indeed, our results revealed lesser lymphocyte proliferation in animals from OP, reinforcing the theory that heat-stressed animals can be more vulnerable to nematoid infections due to the drop in the cell-mediated immune response. In fact, nematode infections generally elicit massive Th_2-like responses and large numbers of mast cells (Gasbarre et al., 2001), then interferences over that immunological response can be crucial to individuals.

Furthermore, although most vaccinated animals responded positively to the antigenic stimulus, our results showed better results in SPSt, superior to OP regarding the percentage of cell proliferation (Fig. 7). Besides, the drop in CFSE intensity after cell division supports that outcome, just as expected (Quah et al., 2007). All these findings

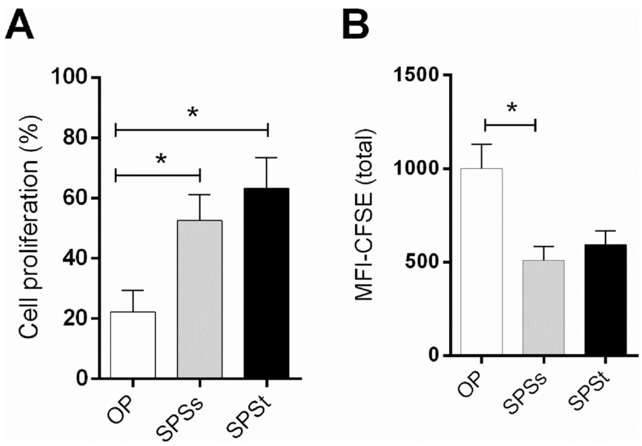

Fig. 7. Comparison of the cell-mediated response of vaccinated heifers according to the systems: A) Percentage of cells that proliferated at least once. B) medium fluorescence intensity of carboxyfluorescein diacetate succinimidyl ester (MIF-CFSE). Bars represent the different systems livestock in open pasture (OP), silvopastoral system with trees into single rows (SPSs), and silvopastoral system with trees into triple rows (SPSt) compared by Kruskal-Wallis followed by Dunn's. *$p < .05$.

reinforce the role of welfare in the immunological system.

Despite the antigen standards used in their study, Hu et al. (2007) conclusions corroborate that hypothesis, in which vaccines administration in cattle during periods of heat stress may not allow for the development of an optimum immune response, just like represented in Figs. 6 and 7 (A and B). All those findings highlight the necessity of new approaches considering immunological parameters and cattle welfare, whose heat stress is one of the most critical in the tropics.

Other trials have addressed the attenuation of adverse microclimatic conditions associated with the regulation of physiologic mediators. Lemes et al. (2021) assessed cortisol and heat-shock protein expression to characterize heat stress in females, of which heifers and cows grazing in SPS had lower cortisol levels, probably due to the milder environmental temperatures reached in that system. As highlighted by Bagath et al. (2019), heat stress has been reported to induce increased blood cortisol concentrations, which have been shown to inhibit the production of cytokines such as interleukin-4, interferon γ, and tumor necrosis factor-α. Ju et al. (2014) also associated cortisol secretion with immune suppression during chronic stress, a historically same condition we got in our experimental site (Magalhães et al., 2020). Although we do not have that scope, higher cortisol levels in heat-stressed animals could be a plausible explanation for our results since cell-mediated immunity is directly dependent on cytokine serum levels.

Beyond the health issue, herd performance strengthened the potential of integrated systems, according to our results. Besides the potential of SPS to improve animal welfare, the CL system promoted superior daily weight gain (g/day) in the DS, although that difference did not occur in the RS2. Silva et al. (2020) highlighted the CL integration as an option for sustainable intensification of the Amazon biome based on herbage accumulation of palisade grass and animal performance. Considering those results, the impact of two years of crop production management on DWG, particularly fertilization, on subsequent forage production, seems to be more significant, as Costa et al. (2015) mentioned in their manuscript.

5. Conclusion

February/March, and October are the most critical months in which there is a greater parasitic infestation. Besides, it can also be concluded that both silvopastoral systems were not considered as a risk factor for nematode egg counts in Nellore heifers. Indeed, the shadiest silvopastoral system promoted milder parasitism and higher lymphocyte proliferation in animals from that arrangement, then potentiating the cell-mediated immune response.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Luciano Bastos Lopes reports financial support was provided by Acrimat. Luciano Bastos Lopes reports administrative support was provided by Acrinorte.

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