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Original Article

Physicochemical and microbiological quality of milk from cows subjected to two protein feeding strategies

Qualidade físico-química e microbiológica do leite de vacas submetidas a duas estratégias de alimentação proteica

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Abstract

The poor quality of pastures in tropical livestock systems limits milk production. Therefore, the use of local resources such *as Gliricidia sepium* (T1) and Tithonia diversifolia (T2) constitutes a sustainable strategy for improving protein intake, as well as providing environmental services by avoiding reliance on inputs with a high carbon footprint. With this objective, a study was conducted to evaluate the impact of strategic supplementation with T1 and T2 on the chemical composition and immune status of the mammary gland. To this end, using a two-factor treatment (A) × period (B) design, 10 cows were randomly assigned: group 1 received T1 in period 1 and T2 in period 2; group 2 followed the reverse sequence. Between treatment periods, a washout period was implemented to ensure the elimination of residual or carryover effects. Milk production, physicochemical composition, somatic cell count (SCC), and microbiological quality indicators were monitored for 21 days during each treatment period. No statistically significant differences were observed between treatments for milk components, including protein (3.42 vs. 3.33 ± 0.05%), lactose (5.13 vs. 4.96 ± 0.07%), and non-fat solids (9.47 vs. 9.42 ± 0.21%) (P > 0.05) for *Gliricidia* compared to *Tithonia.* SCC did not differ between treatments (P = 0.76), but differed between periods (P < 0.001), increasing from $\log_{10}$ 2.58 to 2.67 (≈3.86 × 10^5 vs. 4.80 × 10^5 cells/mL). In conclusion, supplementation with *Gliricidia sepium* and *Tithonia diversifolia* maintained milk composition and SCC within acceptable ranges, indicating that these forages are viable, low-cost and low-carbon protein alternatives for smallholder dairy systems without compromising milk quality or udder health.

Keywords: animal nutrition, milk quality, somatic cell count, small-scale farmers.

Resumo

A baixa qualidade das pastagens em sistemas de produção leiteira tropical limita a produção de leite. Portanto, o uso de recursos locais, como *Gliricidia sepium* (T1) e *Tithonia diversifolia* (T2) constitui uma estratégia sustentável para melhorar a ingestão de proteínas, além de fornecer serviços ambientais, evitando a dependência de insumos com alta pegada de carbono. Com esse objetivo, foi realizado um estudo para avaliar o impacto da suplementação estratégica com T1 e T2 na composição química e no estado imunológico da glândula mamária. Para tanto, utilizando um delineamento fatorial de tratamento (A) × período (B), 10 vacas foram aleatoriamente designadas: o grupo 1 recebeu T1 no período 1 e T2 no período 2; o grupo 2 seguiu a sequência inversa. Entre os períodos de tratamento, foi implementado um período de washout para garantir a eliminação de efeitos residuais ou de transferência. A produção de leite, a composição físico-química, a contagem de células somáticas (CCS) e os indicadores de qualidade microbiológica foram monitorados durante 21 dias em cada período de tratamento. Não foram observadas diferenças estatisticamente significativas entre os tratamentos para os componentes do leite, incluindo proteína (3,42 vs. 3,33 ± 0,05%), lactose (5,13 vs. 4,96 ± 0,07%) e sólidos não gordurosos (9,47 vs. 9,42 ± 0,21%) (P > 0,05) para *Gliricidia sepium* em comparação com *Tithonia diversifoli*a. A CCS não diferiu entre os tratamentos (P = 0,76), mas diferiu entre os períodos (P < 0,001), aumentando de $\log_{10}$ 2,58 para 2,67 (≈3,86 × 10^5 vs. 4,80 × 10^5 células/mL). Concluindo, a suplementação com *Gliricidia sepium* e *Tithonia diversifoli*a manteve a composição do leite e a CCS dentro de faixas aceitáveis, indicando que essas forrageiras são alternativas proteicas viáveis, de baixo custo e baixo carbono para sistemas leiteiros de pequenos produtores, sem comprometer a qualidade do leite ou a saúde do úbere.

Palavras-chave: nutrição animal, qualidade do leite, contagem de células somáticas, pequenos agricultores.

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1. Introduction

Several studies have established a close relationship between production levels, milk composition, and metabolic aspects when animals are kept and fed in efficient production systems (Biermann et al., 2017; Pulina et al., 2005). In parallel, changes in social factors (e.g., landscape values and animal welfare) (Moradi et al., 2021) this is why consumers around the world today are looking for products that come from more sustainable systems, with a focus on health and resilience to climate change (Elgersma et al., 2006; Hák et al., 2016). Therefore, the content and type of energy and protein in the diets of lactating cows (Ghaffari et al., 2025; Nichols et al., 2018) are important factors influencing nutrient transfer to milk components. Furthermore, ruminants exhibit highly complex interactions (Nichols et al., 2018). In this regard, studies conducted by Guamán Rivera et al. (2023) on tropical pastures confirm that rational grazing management could improve the crude protein content available for ruminants. However, González Marcillo et al. (2021) demonstrate significant seasonal variation in pasture quality (i.e., crude protein and cell wall content). Consequently, due to both intrinsic and extrinsic factors, the adoption of clean, low-carbon technologies is necessary (González-Quintero et al., 2021; Guamán-Rivera et al., 2025; Martínez-salinas et al., 2024) to meet the nutritional demands of animals within a framework of resilience to environmental changes. In this context, some studies have shown that feeding dairy cows with adequate levels of crude protein and/or bioactive compounds (phenols) (Bartłomiej et al., 2012; Li et al., 2014), results in improved mammary gland immune status (Gonzalo et al., 2002). For this reason, incorporating leguminous shrubs as a supplement is an alternative for increasing the production of grazing animals by substantially improving the composition of their feed components (González-Villalobos et al., 2002). In recent years, however, these production outcomes have been increasingly evaluated not only from a technical perspective but also in relation to broader social expectations, such as landscape preservation and animal welfare. As a result, consumers are demanding products derived from more sustainable systems, with greater attention to health and environmental resilience (Fraser et al., 2022; Horan et al., 2018). In this context, the nutritional composition of the diet becomes central, because the supply of energy and protein directly influences milk synthesis and composition. Therefore, the content and type of energy and protein in the diets of lactating cows are important factors influencing nutrient transfer to milk components.

Furthermore, from both an economic and a more ecological perspective (Akash-Hoque et al., 2022; Cheng et al., 2022), it is important to generate low-cost feed alternatives that do not require agrochemicals or fossil fuels. In a more local context, in Orellana, located in the northern region of Ecuador, livestock farming represents an important link in the economy of families, despite the low suitability of the soils for livestock use and high levels of environmental vulnerability. Orellana's agroecological constraints make it a relevant model for other tropical regions facing similar production limitations (GADPO, 2015). Therefore, livestock farming represents an important link in the economy of families (Peralta et al., 2024), despite the low suitability of the soils for livestock use, as well as high levels of environmental vulnerability due to their proximity to buffer zones and ecological transition areas (Bass et al., 2010; Lessmann et al., 2016). Tropical shrub legumes such as *Gliricidia sepium* and *Tithonia diversifolia* have emerged as climate-smart forage resources capable of simultaneously reducing the environmental footprint and enhancing the agroecological resilience of smallholder dairy systems (Guamán-Rivera et al., 2024; McGroddy et al., 2015; Wendling and Machado, 2021). Unlike conventional protein concentrates, which rely on energy-intensive manufacturing and long-distance transport, these multipurpose shrubs fix atmospheric nitrogen, improve soil structure, and sequester carbon in both aboveground biomass and root systems, thereby lowering the overall carbon intensity of milk production. Although *Gliricidia sepium* and *Tithonia diversifolia* have been proposed as alternative protein sources for dairy cattle in tropical areas, their comparative effects on milk physicochemical quality and mammary health under smallholder tropical conditions remain insufficiently documented. Despite all these limitations, it is crucial to investigate more sustainable production methods using local forage resources, which could represent environmentally friendly alternatives. We hypothesized that supplementation with *Gliricidia sepium* and *Tithonia diversifolia* would sustain milk quality while offering a low-cost, locally available, and environmentally friendly alternative to conventional concentrate Consequently, with this objective, a study was conducted to evaluate how the use of protein feeds from local resources could influence the physicochemical and microbiological quality of cow's milk.

2. Materials and Methods

2.1. Ethical issues

In accordance with international regulations, and in addition to being covered by international animal welfare protocols such as the International Council on Laboratory Animal Science (ICLAS), the Institute for Laboratory Animal Resources (ILAR), the University Federation for Animal Welfare (UFAW), the American Association for Laboratory Animal Science (AALAS), the Inter-Agency Research Animal Committee (IRAC), the Animal Welfare Guidelines (AWR), the Federation of South American Societies for Laboratory Animal Science (FESSACAL) and the Federation of European Laboratory Animal Science Associations (FELASA), activities have been carried out meticulously in accordance with these guidelines. Furthermore, we have also complied with national regulations set out in the Organic Law on Animal Health, as amended on 3 July 2017.

2.2. Animals and experimental procedures

For this study, a total of 10 multiparous cows of 3 breeds (**Jersey** = 3 animals; 490 ± 6 kg; **Holstein** = 3 animals, 530 ± 8 kg; and **J × Gyr cross** = 4 animals, 510 ± 5 kg) at mid-lactation (180 ± 6 DIM) were used. Mechanical milking was performed once daily (7:00 am) in a 2-stall milking parlor using a DeLaval milking machine of Spanish origin. The equipment also included a raised central milk line, silicone milking tubes (DeLaval SG-TF100), as well as equipment that automatically measures the quantity of milk produced (MM25SG, DeLaval, Tumba, Sweden). Consequently, the vacuum pressure was 40 kPa and the pulsation rate was 120/min, and a pulsation ratio of 50%. Therefore, the milking routine included manual connection of the cluster, mechanical milking, and automatic cluster disconnection (milk flow rate < 0.1 L/min or milking time > 3 min). As soon as milking was finished, she dipped her udder in an iodine-based disinfectant (P3-ioshield, Ecolab Hispan-Portuguesa, Barcelona, Spain). Additionally, somatic cell counts (SCC) prior to the start of the study were verified to be less than 200,000 cells/mL, and the absence of intramammary infections was confirmed by bacteriological analysis of the milk samples.

2.3. Experimental treatments

The study was conducted using a randomised two-period crossover (reversal) design with a washout period between treatments (Figure 1). Ten cows were randomly assigned to two treatment sequences: **T1** received *Gliricidia sepium* and T2 received *Tithonia diversifolia*. The ration was formulated with the following ingredients: King Grass, 65%; *Saccharum officinarum*, 5%; bypass fat, 0.3%; mineral salts, 0.1%; sodium bicarbonate, 0.05%; and *Zea mays* (CP, 14%, NFD, 33%). The experimental procedure consisted of a 7-day adaptation period. After treatment assignment, data were collected for 21 days, followed by a 7-day washout period, and finally, a second 21-day data collection period. According to AOAC (2000), the chemical composition for both shrubby forage are Gliricidia sepium contains between 20-30% dry matter, 10-12% ash, 14-20% crude protein, 35-45% NDF, 20-30% ADF, 2-4% ether extract, and 9-10 MJ/kg DM of metabolizable energy. In contrast, Tithonia diversifolia contains 18-25% dry matter, 10-15% ash, 20-25% crude protein, 30-40% NDF, 15-25% ADF, 2-4% ether extract, and 9-11 MJ/kg DM of metabolizable energy.

2.4. Milk measurements, sampling and analysis

2.4.1. Milk production

The milk produced by each individual animal, thanks to the automation provided by the milking equipment, was collected and the data uploaded daily to the AlPro 7.2 software (DeLaval); this data was subsequently analysed and updated weekly in spreadsheets to rule out inaccurate results.

2.4.2. Milk composition

Milk was collected in the required quantities (100 cm^3) from each animal for laboratory processing. The samples collected were stored at 4 °C with the addition of an antimicrobial tablet (Bronopol, Broad Spectrum Micro-tabs II, d&f Control Systems, San Ramon, CA) and stored at 4°C until analysis. Subsequently, milk composition (protein, fat, lactose, non-fat solids, density, and freezing point) was determined by infrared absorption. Somatic cell count (SCC) was measured using a Draminski device, which evaluates milk electrical conductivity/resistivity as an indirect indicator of udder health.

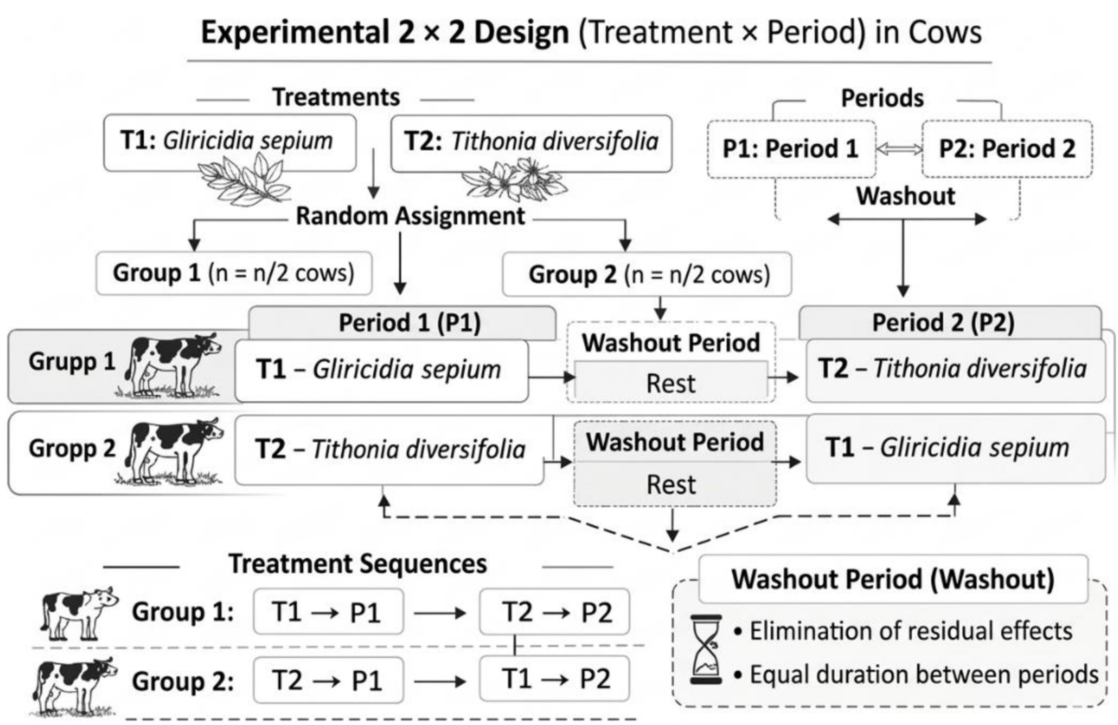

Figure 1. Two-factor experimental design (treatment × period).

2.5. Statistical analysis

All variables were recorded and entered, properly coded according to treatment and period. Subsequently, the data were analyzed using the PROC MIXED procedure for repeated measures in SAS v. 9.4 (SAS Inst. Inc.; Cary, North Carolina, USA). After verifying normality with the PROC UNIVARIATE procedure and using log10-transformed somatic cell data, a linear mixed model was applied. A first-order autoregressive (AR) covariance structure was selected based on information criteria (AIC and BIC) as it best fit the data. In our case, the statistical model included the following fixed effects: treatments (T1 vs. T2), period (1 and 2), and their interaction (T×P), while the random effects included animal (1-10) and residual error. Means were presented as least squares separated using the SAS PDIFF option and compared with Tukey's test. Differences in main effects were considered at a P-value < 0.05.

3. Results

Highly significant differences were observed for the effect of period (4.61 vs. 5.57 ± 0.29 kg/day; P < 0.001; Figure 2). However, the period × treatment interaction was not statistically significant (P = 0.29), while the treatment × week interaction was highly significant (P < 0.001). Although a moderate correlation with ambient temperature was observed (r = 0.58-0.63), this association did not reach statistical significance (P = 0.39-0.42). Regarding the chemical composition of the milk, except for lactose content, which showed a statistically significant trend when comparing *Gliricidia* vs. *Tithonia* (P = 0.08), cows that received the *Gliricidia* legume in their diet had a 3% increase (5.13 vs. 4.96 ± 0.07%; Table 1). No significant differences have been observed between treatments in the other chemical components of milk (P = 0.15-0.84; Table 1). While the fat content increased significantly between periods (Table 1) compared to the first period (1.97 vs. 3.52 ± 0.18%), similar results were observed for density (1033 vs. 1030 ± 0.66 g/mL) and freezing point (−0.6058 vs. −0.5295 ± 0.02). Despite this, these values are within the reference ranges for the species (Soder and Brito, 2023). On the other hand, no variation was observed between periods for protein content (3.37 ± 0.05%, on average) or lactose content (5.05 ± 0.07%, on average).

When interpreting the interaction between period × treatment, no significant difference was found in any of the milk components (P = 0.31-0.89; Table 1). However, when analyzing the treatment × week interaction, a significant difference (P = 0.002-0.007) was observed for the components (fat, lactose, and freezing point), but no significant differences were found for protein and density (P = 0.24 and P = 0.20, respectively). Regarding the effect of the triple interaction (period × treatment × week), no significant differences were found for the components (protein P = 0.26, lactose P = 0.41, and density P = 0.28), while fat content and freezing point showed a marked significant difference (P = 0.02-0.002, respectively).

The somatic cell count is shown in Table 1. The logarithmically transformed somatic cell count (SCC) was then analyzed using parametric methods. The data show that the SCC did not vary when comparing *Gliricidia* vs. *Tithonia* (2.6 vs. 2.52 Log10 ± 0.05 cells/mL), corresponding to 436,000 and 430,000 ± 0.001 cells/mL, respectively). However, in the period considered in this study, the SCC showed significant differences (P < 0.001). In this case, the SCC was higher in the second period than in the first (2.58 Log10, 386,000 cells/mL vs. 2.67 Log10, 480,000 cells/mL). In addition, all the interactions considered in this work (Table 1) showed a great influence on the SCC (P < 0.001 to 0.012; Figure 3), which will be discussed later.

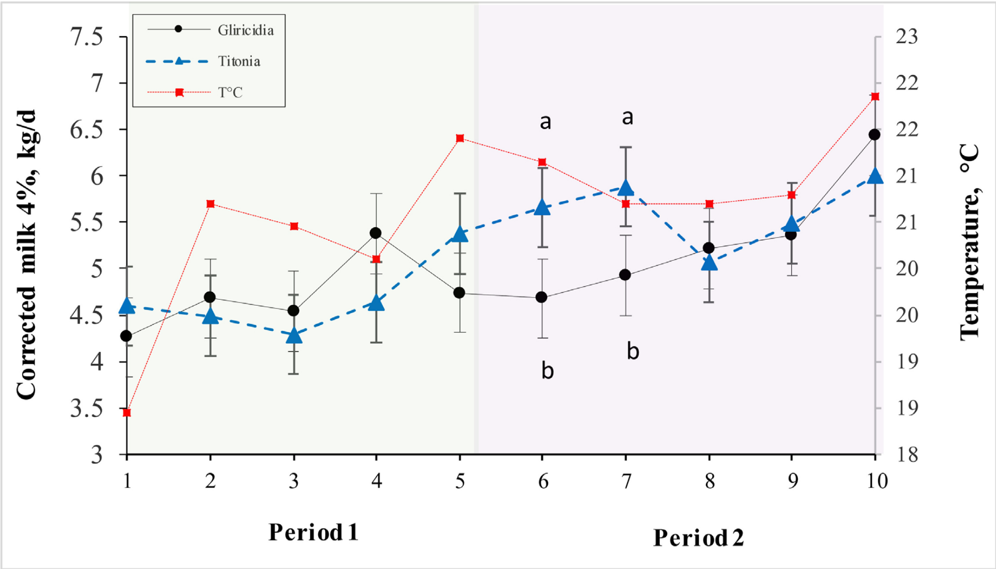

Figure 2. Temporal evolution of milk production corrected to 4% and ambient temperature under different forage treatments.

Table 1. Chemical composition of milk according to the main effects of the study on protein supplementation strategies.

Item	Treatment		Period		SEM	*P = value*				
	Gliricidia	***Tithonia***	**1**	**2**		**Treat**	**P**	**P × T**	**T × S**	**P × T × S**
Composition, %										
Fat	2.72	2.78	1.97[b]	3.52[a]	0.18	0.69	0.001	0.89	0.007	0.02
Protein	3.42	3.33	3.39	3.36	0.05	0.15	0.59	0.39	0.24	0.26
Lactose	5.13[x]	4.96[y]	5.12	4.97	0.07	0.08	0.16	0.58	0.03	0.41
Non-fat solids	9.47	9.42	9.65[a]	9.23[b]	0.21	0.720	0.004	0.296	0.370	0.48
Density (g/mL)	1031	1031	1033[a]	1030[b]	0.66	0.84	0.001	0.31	0.2	0.28
Cryoscopic point (°C)	−0.5790	−0.5563	−0.6058[a]	−0.5295[b]	0.02	0.43	0.009	0.41	0.002	0.002
SCC $_{log10}$[1], cells /mL	2.6	2.52	2.58	2.67	0.05	0.76	0.001	0.012	0.001	0.0001

[1]Data are presented logarithmically. [a-b]Values with different letters are significant differences at P < 0.05. [x-y]Values with different letters indicate statistical trends at P < 0.10. SEM = standard error of the mean; P = period; S = Week T= Treatment.

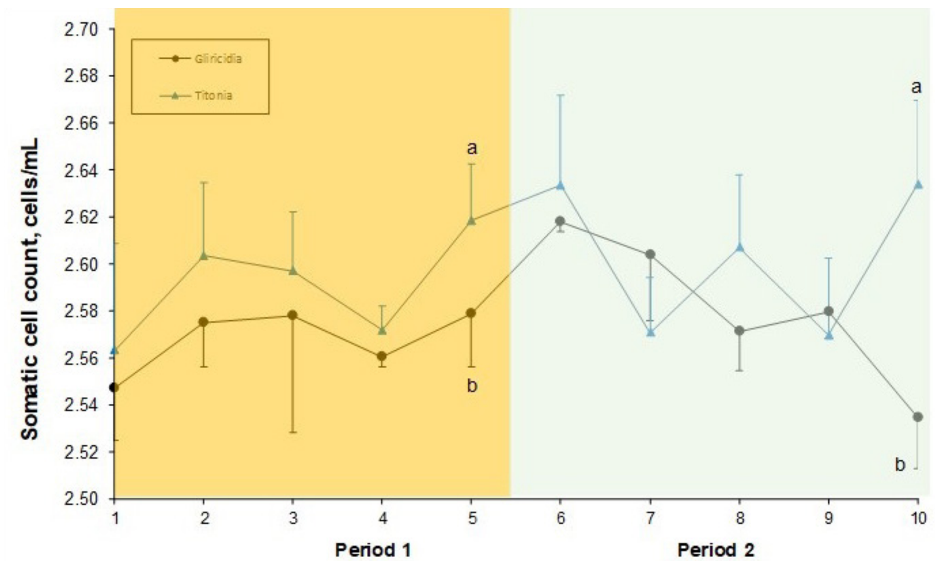

Figure 3. Somatic cells count as a function of the study period.

4. Discussion

The differences described in milk production are partly reflected in the chemical composition. In this regard, state that the inclusion of forage legume meals in the ration does not affect milk production on small farms. Unlike the study reported by Sharma and Ingalls (2022) which found higher fat percentages (3.99%) when 30% of the concentrated feed was replaced with *Gliricidia* spp., our study hypothesizes that a breed factor may have influenced the fat content, as there were no changes in live weight throughout the experiment. Cuevas et al. (2023) replaced 30% of the balanced feed with *Gliricidia* spp. and reported lower fat content than for a control group (2.93% vs. 2.38%). A study conducted on Holstein cows in which 15% of the concentrate was replaced with *Tithonia* spp. reported no changes in milk composition (Ribeiro et al., 2015). Razz and Clavero (1998) reported an inverse relationship between fat percentage and milk production, as well as with concentrate supply. Interestingly, our study, during the first period, showed fat content somewhat atypical compared to normal values (3.5% vs. 1.97%). By the second period, these values were within the reference range (Morton et al., 2016), indicating greater production of acetate in the rumen as the main precursor of fat in the mammary gland (Vranković et al., 2017).

On the other hand, although we are well aware that milk protein content can be influenced by several factors (genetics, diet, stage of lactation, and season) (Juozaitiene et al., 2006). our work reports normal reference protein values for both *Gliricidia* spp. (Sharma and Ingalls, 2022), and *Tithonia* spp. (3.08% and 3.98%, respectively)

(Ribeiro et al., 2015). This evidence leads us to believe that, in both groups, there was a symbiosis between energy and protein content in the rumen (Daniel et al., 2016). Lactose also plays an important role in milk synthesis in cows (Tyrrell and Reid, 1965). Lactose is the most abundant solid milk in bovine milk (Kobayashi et al., 2016). Lactose synthesis in mammals requires a large amount of dietary energy (Portnoy and Barbano, 2021). Several experimental studies replacing concentrate with *Gliricidia spp*. and *Tithonia spp*. in proportions ranging from 6% to 30% have not observed atypical variations in their values (Ribeiro et al., 2015; Sharma and Ingalls, 2022). Since lactose is the main osmotically active molecule in milk, its concentration regulates the movement of water into the alveolar lumen of the mammary gland, contributing to the total volume of milk produced (Portnoy and Barbano, 2021). Therefore, according to the reference values for lactose (range of 4.7-5%), these values fall within the range obtained in our study (4.97-5.3%). Consequently, this component, when found at recommended levels, is of fundamental importance, actively participating in the regulation intrinsically linked to homeostasis and osmotic balance, which allows for the correct functioning of the mammary gland and the consequent production of milk.

From a microbiological perspective, milk is one of the most nutritionally complete foods for human consumption (Siddiquee et al., 2014). From the perspective of food safety for the general public, great importance has been attached to the chemical composition as well as the microbiological characteristics of milk (Malek dos Reis et al., 2013). In dairy cattle, inflammation of the mammary gland (mastitis) is the main problem; this significantly reduces milk production, increases veterinary labour costs, leads to the use of antibiotics and, in some cases, the culling of the animals, resulting in financial losses for the farmer (Riva et al., 2022). Extensive scientific evidence has already revealed that improved immune responses can be modulated through nutrition (Alhussien and Dang, 2018; Hernàndez-Urzua and Alvarado-Navarro, 2001; Hoogland et al., 2015), which is why several research projects are currently underway in this field. In this study, a 3% increase in SCC was observed when comparing periods 1 vs. 2 (2.58 vs. 2.67 ± 0.001 cells/mL; $P < 0.001$), corresponding to an increase of approximately 94,000 cells/mL. Although some authors have expressed controversy regarding the use of the Draminski method for mastitis detection, several studies recommend its use as an alternative, easy-to-apply, and inexpensive method (Qolbaini et al., 2014). Therefore, The electrical conductivity and resistivity of milk have been used for over 40 years to indicate mastitis in milk and its positive correlation with somatic cell count (Qolbaini et al., 2014; Siddiquee et al., 2014). The SCC data, according to the correlation and regression analysis, indicate a strong influence of protein ($r = -0.46$; $P < 0.001$), lactose ($r = -0.59$; $P < 0.001$), and fat ($r = 0.81$; $P < 0.001$) content. These findings are supported by (Henningsson et al., 2005) reported that the electrical conductivity of milk depends on the type and concentration of ions, as these interact with the components (protein, fat and lactose) that give it its viscosity. Based on our results, we hypothesize that there was adequate synchrony between rumen-degradable protein (from *Gliricidia* and *Tithonia* spp.) and energy availability, in terms of the time required for its utilization by rumen bacteria. This allowed us to obtain a greater amount of microbial protein, which, in immunological terms, increased the somatic cell count (SCC) when using Draminski.

The integration of tropical shrub legumes into dairy rations aligns with mounting evidence supporting silvopastoral systems as viable strategies for enhancing both ecological resilience and nutritional efficiency in smallholder contexts. Research across Latin America has consistently demonstrated that incorporating non-conventional forage resources such as Gliricidia sepium and Tithonia diversifolia into grazing-based regimens not only buffers against seasonal forage deficits but also reduces reliance on imported, high-carbon-footprint concentrates (González-Quintero et al., 2021). These multipurpose shrubs contribute to improved soil fertility, enhanced carbon sequestration, and greater farm-level biodiversity, while simultaneously supplying rumen-degradable protein and bioactive polyphenols that modulate fermentation patterns (Fraser et al., 2022; Akash-Hoque et al., 2022). Field evaluations in silvopastoral environments have further shown that such feeding strategies maintain or improve milk yield and composition under climatic stressors (Baldassini et al., 2018; Guamán-Rivera et al., 2024; Montagnini et al., 2013), primarily by stabilizing energy-protein synchrony and mitigating heat-induced metabolic disruptions (Horan et al., 2018). Our findings corroborate this paradigm, as supplementation with *Gliricidia* and *Tithonia* sustained milk quality parameters and kept somatic cell counts within commercially acceptable thresholds, despite the inherent variability of tropical grazing systems. This underscores the potential of non-conventional, locally adapted forages to function as climate-smart nutritional tools that simultaneously address productivity, animal health, and environmental sustainability in resource-limited dairy operations.

5. Conclusions

Most components fall within the established parameter range. However, the study used cows of various breeds and crosses, but mostly female cattle with bone fragments and even high proportions of Indic genetics, which would partially explain the different fat and lactose values compared to those reported for Bos taurus breeds. Somatic cell count remained relatively stable across treatments, suggesting no evident adverse effect on udder health. We also noted that the Draminski method may produce higher SCC estimates, so the results should be interpreted with caution. For this reason, further immunological studies should be conducted to support these findings. Therefore, future research should include cytokine profiling, pathogen screening, and metabolomic analysis of milk to validate the immunomodulatory effects of phenolic-rich forages and to better understand their impact on mammary gland health.

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Data Availability Statement

Research data is only available upon request.

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