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Inclusion levels of white clover in annual ryegrass mixtures: characteristics of fresh and ensiled material

Níveis de inclusão do trevo-branco em misturas de azevém-anual: características da forragem verde e da silagem

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Abstract: Legumes can contribute to reducing the use of nitrogen fertilization, with improvements in the nutritional value of the diet. This study aimed to evaluate the effect of the inclusion of white clover in diets based on fresh ryegrass and for silage production at two developmental stages. The treatments consisted of four levels of white clover addition: 0, 200, 400, and 600 g/kg DM, when ryegrass was at the vegetative and flowering stages. The chemical composition did not vary with the level of white clover inclusion when annual ryegrass was in the vegetative stage, but crude protein levels increased, and NDF and ADF contents decreased with the inclusion of the legume when ryegrass was at the flowering stage. Including the legume at levels above 400 g/kg DM did not result in additional benefits for fiber reduction, both in fresh material and silage. The density of the ensiled material increased with legume inclusion regardless of the grass's developmental stage, while pH values decreased when the grass was at the flowering stage, regardless of the level of clover inclusion. Aerobic stability was maintained across all treatments up to seven days after silo opening, but the fermentation parameters evaluated could not be sufficient to explain this result. The inclusion of 400 g/kg DM of white clover in ryegrass-based diets at the flowering stage is a practice that can be recommended, including for silage production. However, the absence of organic acid measurements constrains the interpretation of the results.

Keywords: aerobic stability, chemical composition, *Lolium multiflorum*, silage, *Trifolium repens*.

Resumo: As leguminosas podem contribuir para a redução no uso de adubação nitrogenada, com melhorias no valor alimentar da dieta. Objetivou-se avaliar o efeito da inclusão de trevo-branco (*Trifolium repens*) em dietas à base de azevém-anual (*Lolium multiflorum*) na forragem e na silagem em dois estádios de desenvolvimento. Os tratamentos foram quatro níveis de adição de trevo-branco: 0, 200, 400 e 600 g/kg de MS, quando o azevém-anual se encontrava nos estádios vegetativo e em florescimento. A composição química não variou com o nível de inclusão do trevo quando o azevém estava em estádio vegetativo, mas os teores de proteína bruta aumentaram e os de FDN e FDA diminuíram com a inclusão da leguminosa no estádio de florescimento. A inclusão da leguminosa em mais de 400 g/kg de MS não contribuiu para a redução dos teores de fibra, tanto na forragem verde como ensilada. A densidade da silagem aumentou com a inclusão da leguminosa independentemente do estádio de desenvolvimento da gramínea, enquanto os valores de pH diminuíram no estádio de florescimento, sem influência do nível de inclusão do trevo. A estabilidade aeróbica se manteve em todos os tratamentos até sete dias após a abertura dos silos, mas os parâmetros de fermentação avaliados podem não ter sido suficientes para explicar esta resposta. A inclusão de 400 g/kg de MS de trevo-branco em dietas à base de azevém-anual no estádio de florescimento é uma prática que pode ser recomendada, inclusive para a confecção de silagem. Contudo, a ausência da determinação de ácidos orgânicos limita a interpretação dos resultados.

Palavras-chave: composição química, estabilidade aeróbica, *Lolium multiflorum*, silagem, *Trifolium repens*.

1. Introduction

Forage grasses generally undergo marked changes in chemical composition and nutritive value throughout their growth cycle, with increasing fiber levels and decreasing digestibility and crude protein (CP). In contrast, legumes show less variation in energy and protein contents across different developmental stages [(1)]. This stability is partly explained by their thinner cell walls compared with grasses [(2)].

The use of legumes in combination with grasses, either as as fresh forage [(3)] or in silage mixtures [(4,5)], can enhance the nutritional quality of diets, particularly during the later stages of grass development [(6)]. However, extensive degradation of CP during ensiling can limit the production of high-quality legume silages, which typically have high CP content [(7)]. This highlights the importance of further research to better understand the effects of legume inclusion in grass–legume mixtures at different growth stages.

We hypothesized that incorporating white clover (*Trifolium repens*) into annual ryegrass (*Lolium multiflorum*) pastures would improve forage quality without compromising silage fermentation and preservation, particularly when grasses are harvested at later stages of maturity. The objective of the present study was to determine the optimal level of white clover inclusion in annual ryegrass swards to improve forage and silage quality.

2. Material and methods

2.1 Treatments and ensiling

The experiment was conducted in Lages, SC, Brazil (27°48'57"S, 50°19'31"W; 916 m above sea level). Forage was harvested from two areas: one cultivated with white clover since 2021 and the other established with annual ryegrass in the year of evaluation (2022). Four levels of white clover inclusion in annual ryegrass were evaluated: 0, 200, 400, and 600 g/kg dry matter (DM).

Forages were harvested with a forage mower (Tarup®, model 501). The wilting process lasted for approximately 24 h, which was the time required to reach the desired dry matter content (300 g/kg DM). Wilting was carried out on a concrete floor, under direct sunlight and natural ventilation, with an average air temperature of 22 ± 3 °C and relative humidity of approximately 65%. The DM content during wilting was monitored by rapid measurement using a microwave oven. Prior to ensiling, the material was chopped in a stationary forage chopper (theoretical chop length: 10 mm). For chemical composition analysis of fresh forage, four independent samples were collected per treatment (n = 4). Each sample consisted of approximately 500 g of fresh forage composed of six to eight subsamples randomly selected from the chopped material. The remaining forage was treated with a commercial inoculant (Biotrato®) at a rate of 10 mg/kg fresh forage. Ensiling was performed in 3.8 L plastic buckets, with four replicates per treatment, to prepare the microsilos. Compaction was carried out using a manual press, and the microsilos were stored for 100 days in protected facilities, under an average ambient temperature of 20 ± 2 °C, natural ventilation, and no direct sunlight. The same procedure was applied at two ryegrass growth stages, vegetative and flowering, with collections carried out on September 5, 2022, and November 9, 2022. The average pasture height was approximately 25 cm and 40 cm during the vegetative and flowering stages, respectively.

2.2 Density and pH

Silage density was calculated as the ratio of silage mass to microsilo volume. The volume of each microsilo was measured individually by filling it with water at 20 °C and recording both the empty weight and weight when filled with silage. pH was determined by adding 100 mL of distilled water to 25 g of fresh silage. After homogenization, pH was measured using a digital pH meter (Del Lab, DL-PH).

2.3 Aerobic stability

To evaluate aerobic stability, silage was placed in 50 L plastic boxes (containing 4.3 kg of fresh matter), which were placed on a plastic grid to prevent direct heat exchange with the floor. A data logger (HOBO Temp/RH/2 External Channel Data Logger) was inserted into the center of the forage mass and programmed to record the temperature every 5 min for seven days. Aerobic stability was defined as the time until silage temperature rose 3 °C above ambient temperature [(8)].

2.4 Laboratory analyses

The samples were pre-dried in a forced-air oven (65 °C) for 72 h, ground in a Wiley mill with a 1 mm screen, and stored in plastic containers. The DM content was determined by drying at 105 °C for at least 16 h. Ash content was determined by combustion at 550 °C for 4 h, and organic matter (OM) content was calculated as the difference (OM = 100 – ash). CP was calculated from total nitrogen (CP = N × 6.25), quantified by the Dumas combustion method [(9)] using a LECO® FP528 analyzer. Neutral detergent fiber (NDF) was determined according to Mertens [(10)]. The samples were weighed in filter bags and treated with neutral detergent using an ANKOM A220 system (ANKOM Technology, NY, USA). This analysis included heat-stable α-amylase and residual ash, but no sodium sulfite. Acid detergent fiber (ADF) was analyzed according to AOAC Method 973.18 [(9)].

2.5 Statistical analysis

The data were subjected to analysis of variance (ANOVA) using the lme4 package in R. Assumptions of residual normality and homogeneity of variance were verified. The statistical model used was as follows:

$$Y_{ijk} = \mu + T_i + P_j + (T \times P)_{ij} + \varepsilon_{ijk}$$

where:

Y_{ijk} = observed value;

μ = overall mean;

T_i = fixed effect of legume inclusion level (i = 0, 200, 400, and 600 g/kg DM)

P_j = fixed effect of period (vegetative or flowering growth stage);

$(T \times P)_{ij}$ = interaction between legume inclusion level and period

ε_{ijk} = random error.

The means were compared using Tukey's test at a significance level of 5% ($P < 0.05$). Regression models were not fitted, as treatments were defined categorically, and the objective was not to test linear or polynomial responses to legume inclusion.

3. Results

The OM content decreased from 840 to 750 g/kg DM in fresh forage and from 820 to 747 g/kg DM in silage as the proportion of white clover increased when annual ryegrass was harvested at the vegetative growth stage (Figure 1). In contrast, when ryegrass was harvested at the flowering stage, the OM content did not vary with legume inclusion (treatment × period interaction, $P < 0.001$), with an average of 856 g/kg DM. Overall, OM content was higher at the flowering stage than at the vegetative stage in both fresh forage (915 vs. 796 g/kg DM) and silage (926 vs. 785 g/kg DM), regardless of the level of clover inclusion.

CP, NDF, and ADF contents were not affected by the level of white clover inclusion when annual ryegrass was at the vegetative stage, averaging 204, 589, and 355 g/kg DM, respectively, in fresh forage and 210, 511, and 341 g/kg DM in silage, respectively (Figure 2). However, when ryegrass was harvested at the flowering stage, the CP content in fresh forage increased by approximately 60 g/kg DM (106 vs. 165 g/kg DM), whereas NDF decreased by approximately 120 g/kg DM (663 vs. 531 g/kg DM), with increasing levels of white clover in the mixture (treatment × period interaction, $P < 0.001$). In silage, CP showed a similar increase (107 vs. 167 g/kg DM), whereas NDF decreased by approximately 20 g/kg DM (633 vs. 613 g/kg DM) (treatment × period interaction, $P < 0.001$). NDF and ADF levels were similar in the treatments with 400 and 600 g/kg DM of white clover, regardless of forage type (fresh or ensiled) or period (vegetative or flowering growth stages).

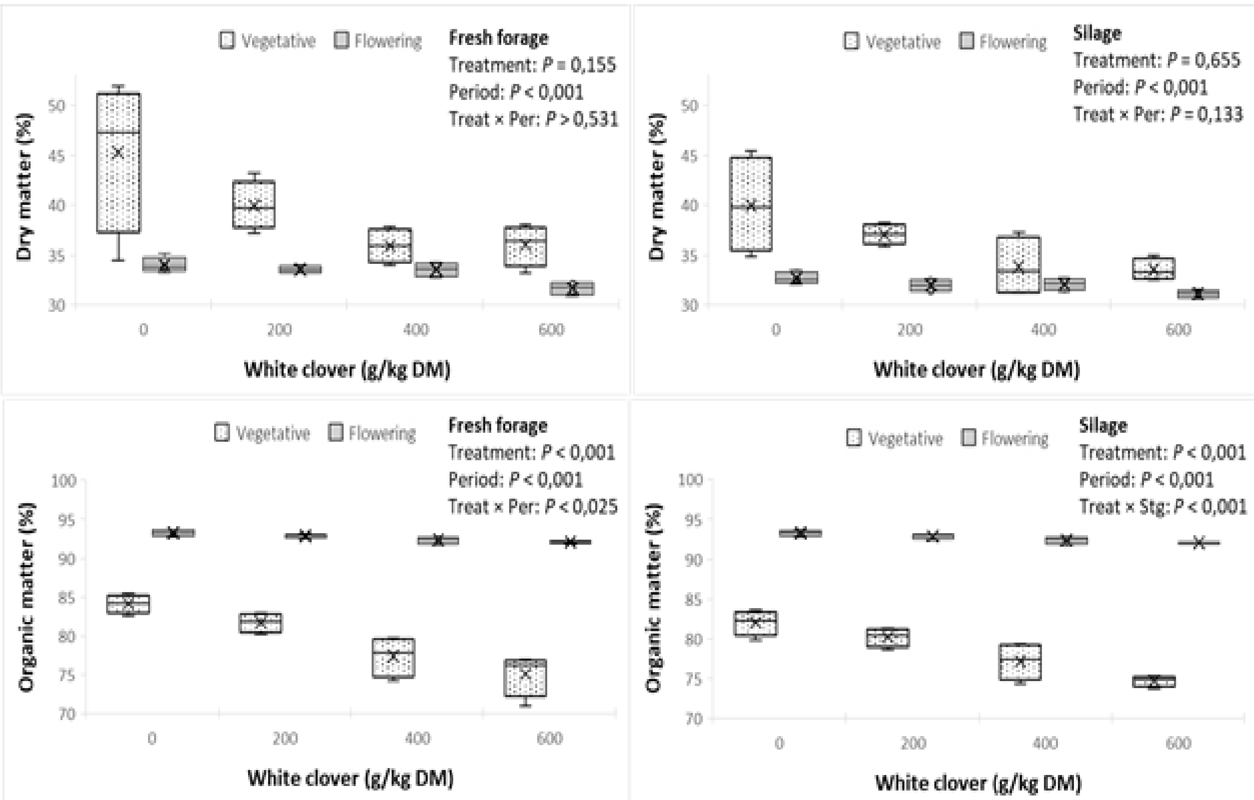

Figure 1. Effect of white clover inclusion in annual ryegrass mixtures on dry matter (DM) and organic matter (OM) contents of fresh and ensiled forage at two ryegrass developmental stages (vegetative and flowering).

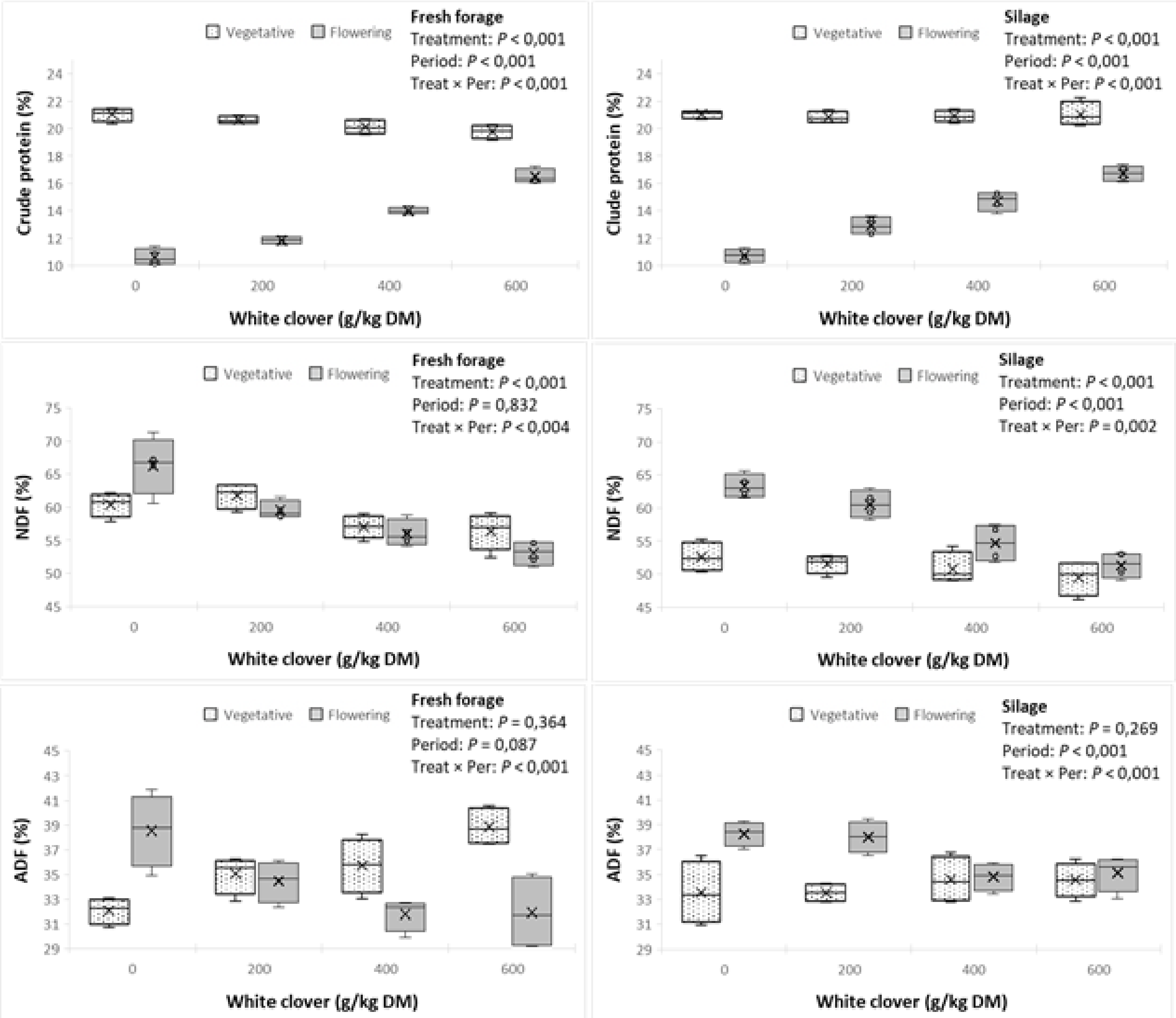

Figure 2. Effect of white clover inclusion in annual ryegrass mixtures on crude protein (CP), neutral detergent fiber (NDF), and acid detergent fiber (ADF) contents of fresh and ensiled forage at two ryegrass developmental stages (vegetative and flowering).

Silo density increased with white clover inclusion *(P* < 0.001) and was not affected by ryegrass growth stage (Figure 3). However, responses to clover supplementation above 400 g/kg DM were not evident. White clover inclusion did not affect silage pH, regardless of the ryegrass stage. Overall, silage pH was lower during the flowering stage (4.16) than during the vegetative stage (5.84; *P* < 0.001).

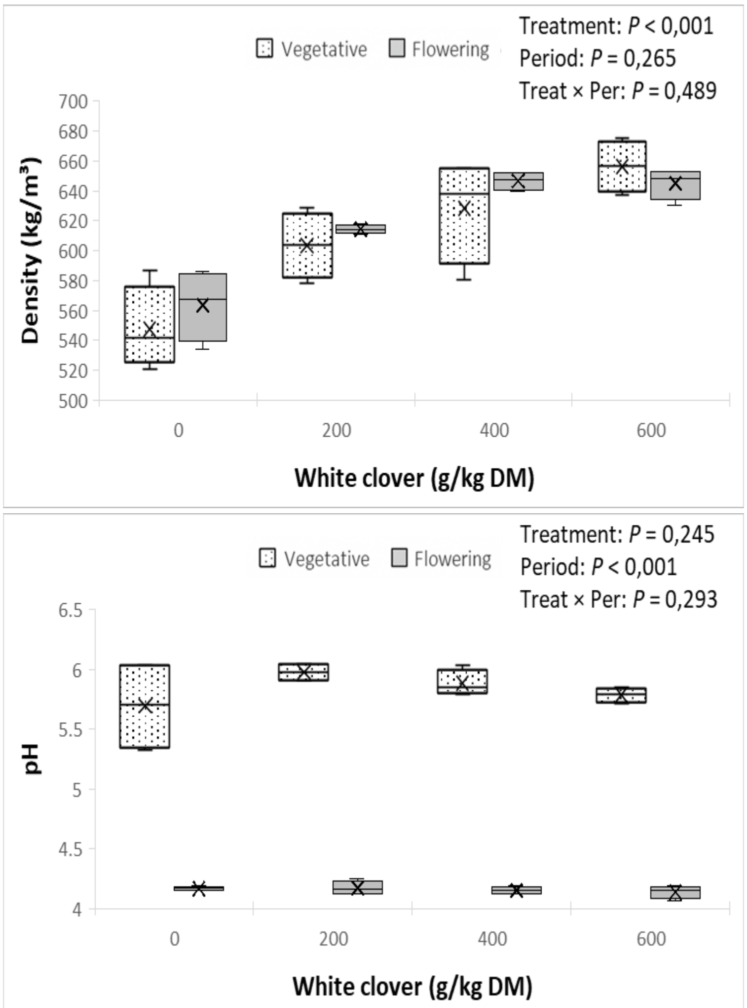

Figure 3. Effect of white clover inclusion in annual ryegrass mixtures on silage density and pH of fresh and ensiled forage at two ryegrass developmental stages (vegetative and flowering)..

Aerobic stability was maintained in all treatments for up to one week after silo opening, with greater stability observed in silages made from ryegrass harvested at the vegetative stage, regardless of legume inclusion (Figure 4). By the end of this period, the silage made from ryegrass harvested at the flowering stage showed a slight increase in temperature, although this was not sufficient to indicate the onset of aerobic deterioration.

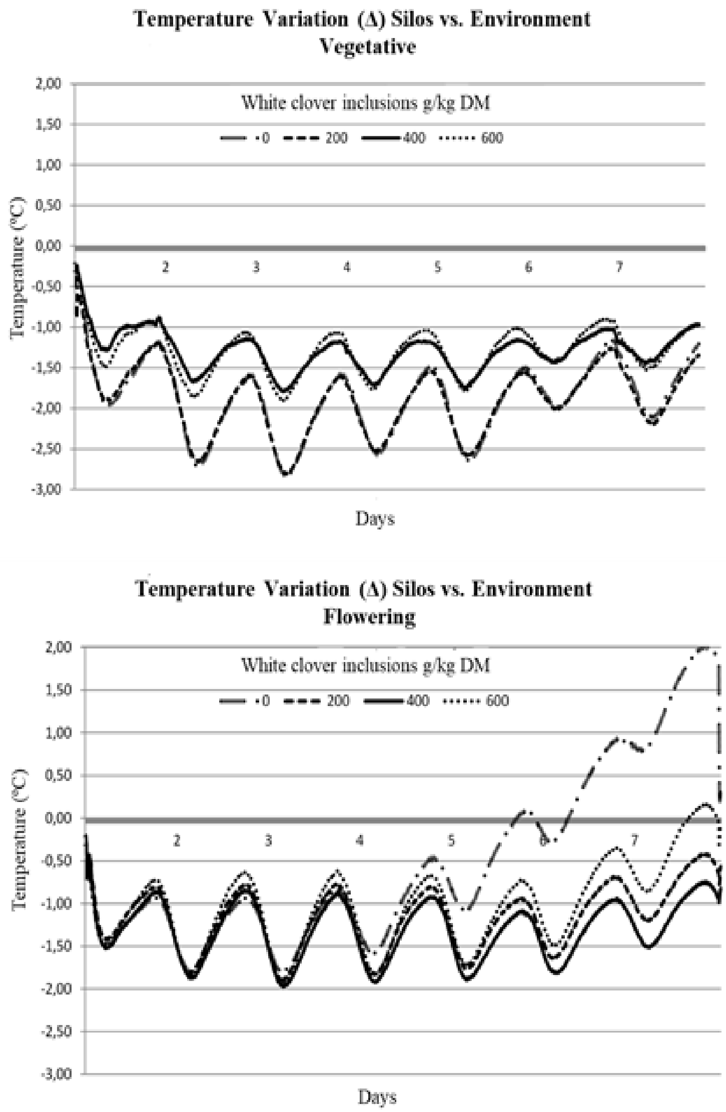

Figure 4. Effect of white clover inclusion in annual ryegrass mixtures on the aerobic stability of silage (Δt difference between silage mass temperature after silo opening and ambient temperature) at two ryegrass developmental stages (vegetative and flowering).

4. Discussion

4.1 Effect of white clover inclusion on the chemical composition of fresh forage and silage

The increase in CP and reduction in NDF with white clover inclusion when annual ryegrass was harvested at the flowering stage and the absence of such effects at the vegetative stage partially confirmed the hypothesis of the study. Legume inclusion proved to be less relevant for improving forage chemical composition when grasses already had a high nutritive value. Annual ryegrass at the vegetative stage is known to have a high nutritional quality, with a CP content above 200 g/kg DM and NDF values below 500 g/kg DM [11]. As development advances, CP decreases and NDF increases, whereas legumes exhibit smaller changes than pure grasses, particularly in NDF content [12]. In this study, the CP content in pure ryegrass decreased from 210 g/kg DM at the vegetative stage to 110 g/kg DM at flowering, whereas the NDF increased from 520 to approximately 650 g/kg DM. Conversely, at 400 g/kg DM white clover, the differences in CP content between silages produced at different stages were reduced to 50 g/kg DM (200 vs. 150 g/kg DM at the vegetative and flowering stages, respectively). At the same level of inclusion, the fiber content did not vary across growth stages, averaging 530 g NDF/kg DM. Silage DM content at both the vegetative (320 g/kg) and flowering (300 g/kg) stages fell within the recommended range (300–350 g/kg) for optimal fermentation and nutrient preservation [13]. Similarly, the average NDF content (511 and 576 g/kg DM) remained within the ideal range (500–600 g/kg DM), ensuring a balance between intake potential and digestibility [14].

The absence of additional effects above 400 g/kg DM indicates a threshold for the benefits of legume incorporation. These findings are consistent with those of Ebro *et al.* [15], who reported that the inclusion of up to 400 g/kg DM of four legumes (*Sesbania sesban, Medicago sativa, Lablab purpureus*, and *Vicia faba*) improved the nutritive value of silages based on two tropical grasses (*Cenchrus purpureus* and *Pennisetum pedicellatum*). Similarly, da Silva *et al.* [16] found that including 300 g/kg DM of the forage legume Stylosanthes in maize silage enhanced nutritional value without compromising fermentation.

4.2 Effect of white clover inclusion on silage density, pH, and aerobic stability

The increase in silage density with greater white clover inclusion can be explained by the lower resistance of clover particles to physical breakdown, thereby improving the chopping efficiency. This result was expected because the effect of particle size reduction on silage density is well established [17].

The lower pH observed at the flowering stage may be attributed to fermentable starch from grass seeds. These results partially align with the findings of Fluck *et al.* [18], who reported a reduction in pH from 4.3 (vegetative stage) to 3.7 (flowering stage) in pure ryegrass silages. In the present study, pH ranged from 4.0 to 4.5 at the flowering stage but exceeded 5.5 at the vegetative stage, with no influence of clover inclusion. Although the literature indicates that grass and grass–legume silages should ideally have a pH between 4.0 and 4.5, with slightly higher values possible in legume-containing silages [7,19], our results suggest that legume content did not significantly affect the final pH. The pH values observed in silages made from vegetative ryegrass exceeded the recommended maximum of 4.5, which is necessary to inhibit the growth of undesirable microorganisms such as *Clostridium* spp., *enterobacteria*, and *Listeria*, thereby ensuring silage quality [17]. These findings indicate that silages made from vegetative ryegrass, whether pure or mixed with legumes, require the addition of soluble carbohydrates, similar to elephant grass silage [20].

Aerobic stability was maintained for at least seven days after silo opening in all treatments, likely due to the relatively high silage density. According to Wilkinson & Davies [21], silages with densities below 210 kg DM/m^3 are more prone to aerobic deterioration after opening. In the present study, all treatments had average densities above 540 kg DM/m^3. These results are consistent with those of Jatkauskas and Vrotniakiene [22], who found that microsilos of red clover and perennial ryegrass (1:1 ratio), with or without inoculants, remained aerobically stable for at least eight days after opening.

It should be emphasized that organic acids such as lactic, acetic, and propionic acids play a key role in silage fermentation by lowering pH and inhibiting yeasts and molds, thereby improving aerobic stability [23,24]. Additionally, butyric acid, which results from undesirable *Clostridium* fermentation, can suppress fungal growth and increase aerobic stability after silo opening [25]. A limitation of this study is that the organic acid concentrations were not determined. Therefore, future studies should include detailed assessments of fermentation profiles, particularly organic acid production, in similar grass–legume mixtures.

5. Conclusion

Including up to 400 g/kg DM of white clover in annual ryegrass-based diets at the flowering stage is recommended for use as both fresh forage and silage, based on forage quality parameters. Although the absence of organic acid data is a limitation, the results indicate that legumes can enhance the quality of annual ryegrass silage harvested at the flowering stage.

Conflict of interest statement

The authors declare no conflict of interest.

Data availability statement

The full dataset supporting the findings of this study is available from the corresponding author upon request.

Author contribuitions

Conceptualization: H. M. N. Ribeiro–Filho Formal Analysis: C. M. Alves and R. Biasiolo. Funding acquisition: Ribeiro-Filho H. M. N. Investigation: C. M. Alves, R. Biasiolo, and M. I. Martini. Methodology: C. M. Alves, R. Biasiolo, J. L. P. Daniel, and M. I. Martini. Resources: H. M. N. Ribeiro-Filho. Supervision: H. M. N. Ribeiro and Filho. Validation: J. L. P. Daniel. Writing (original draft): C. M. Alves and R. Biasiolo. Writing (review & editing): J. L. P. Daniel and H. M. N. Ribeiro-Filho.

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