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*Article*

# Milk Production and Enteric Methane Emissions in Dairy Cows Grazing Annual Ryegrass Alone or Intercropped with Forage Legumes

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**Simple Summary:** This study explored whether adding forage legumes - common vetch and red clover - to annual ryegrass pastures, while reducing nitrogen fertilizer use, could improve milk production and lower methane emissions from grazing dairy cows. Twelve cows grazed either pure ryegrass or a mixture of ryegrass and legumes, and researchers measured their milk output, feeding behavior, and methane emissions. The legumes contributed only about 9% of the total pasture and slightly increased its protein content. However, cows grazing the mixed pastures had access to less available forage, resulting in lower milk production and reduced milk protein compared to cows grazing only ryegrass. There were no significant differences in daily methane emissions or methane produced per unit of milk between the two groups. These results suggest that simply reducing nitrogen fertilizer and adding a small amount of legumes may not be enough to improve productivity or reduce environmental impact. To make pasture-based dairy farming more sustainable, more effective strategies are needed to increase both the proportion of legumes and the total forage yield in mixed pastures.

**Abstract:** This study evaluated the effects of reduced nitrogen fertilization and the intercropping annual ryegrass (*Lolium multiflorum Lam.)* with forage legumes - common vetch (*Vicia sativa* L.) and red clover (*Trifolium pratense* L.) – on milk production and enteric methane emissions in grazing dairy cows. Twelve Holstein × Jersey cows were assigned to a crossover design involving two treatments: ryegrass monoculture (RG) or ryegrass - legume mixture (RG + Leg). Methane emissions were measured using GreenFeed systems; grazing behaviour, milk yield and composition, and organic matter digestibility were also assessed. Legume inclusion contributed ~9% of the pre-grazing biomass and modestly increased crude protein content of the sward. However, cows grazing RG + Leg pastures had lower herbage mass (-214 kg DM/ha), lower herbage allowance (-6 kg DM/cow/day) and produced less milk (-2.0 kg/day; $p < 0.05$) and milk protein (-88 g/day; $p < 0.01$) than cows on monoculture ryegrass. Energy-corrected milk (ECM), methane emissions (g/day and g/kg ECM), and grazing behaviour were not significantly affected by treatment. Organic matter digestibility showed a tendency to be higher in the monoculture system ($p = 0.067$). These results suggest that, under subtropical grazing conditions, reducing nitrogen fertilization combined with the modest inclusion of vetch and red clover does not mitigate enteric methane emissions nor enhance animal performance. The limited legume contribution to total forage biomass may explain the absence of expected benefits. Enhanced strategies to increase legume proportion in mixed swards are needed to unlock their potential for sustainable intensification of pasture-based dairy systems.

**Keywords:** methane; dairy cows; forage legumes; ryegrass; rotational grazing; digestibility; protein yield; pasture-based systems

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## 1. Introduction

Pasture-based dairy systems are increasingly recognized for their potential to offer a sustainable model for milk production, particularly when high-quality forages are available [1,2]. In such systems, maximizing forage utilization and quality is essential to support productive and environmentally efficient animals. Annual ryegrass (*Lolium multiflorum* Lam.) is widely used due to its high nutritive value [3] and productivity [4] in temperate and subtropical regions; however, it still requires significant nitrogen (N) inputs and may present limitations in crude protein (CP) concentration and digestibility, particularly in later growth stages [5,6].

The inclusion of forage legumes such as clovers and vetches in grass swards has been proposed as a strategy to improve herbage nutritive value and reduce environmental burdens [7]. Legumes contribute biological nitrogen fixation, which can reduce fertiliser use, and often exhibit higher protein content and lower fiber fractions [8]. Additionally, certain legumes may reduce enteric methane ($CH_4$) production by altering rumen fermentation kinetics or through the action of secondary metabolites [9].

Despite these potential advantages, successful incorporation of legumes into intensively managed pastures remains challenging. Factors such as interspecific competition [10], seasonal establishment constraints [11], and grazing pressure often limit legume contribution to sward biomass [12]. Consequently, the expected improvements in animal performance and emissions mitigation may not be realized under practical grazing scenarios.

This study aimed to investigate the effects reducing nitrogen fertilization and intercropping annual ryegrass with common vetch (*Vicia sativa* L.) and red clover (*Trifolium pratense* L.) on productive and environmental parameters in pasture-based systems for mid-lactation dairy cows. We hypothesized that the inclusion of legumes would enhance herbage quality, and reduce $CH_4$ intensity (g/kg ECM), without negatively affecting milk yield.

## 2. Materials and Methods

All procedures involving animals were approved by the Animal Ethics Committee of the State University of Santa Catarina (protocol number 6051180521).

### *2.1 Experimental Area and Treatments*

The experiment was conducted in Lages, Santa Catarina, Brazil (27°47'S; 50°18'W; 920 m altitude), from September to November 2023. A 4.2-ha experimental area was divided into two subplots (monoculture ryegrass: RG; ryegrass intercropped with legumes: RG + Leg), each subdivided into paddocks for adaptation and data collection.

Treatments involved annual ryegrass (*Lolium multiflorum* Lam.) either as a monoculture or intercropped with common vetch (*Vicia sativa* L.) and red clover (*Trifolium pratense* L.). Ryegrass was sown at 40 kg/ha and legumes at 6 kg/ha. Nitrogen application was 150 kg/ha for RG and 75 kg/ha for RG + Leg. Rotational grazing was employed (four days of occupation), targeting 40–50% defoliation. Twelve mid-lactation Holstein × Jersey cows were assigned to treatments using a crossover design (two 12-day periods: eight for adaptation, four for data collection). Cows were supplemented daily with 4 kg of ground sorghum (849 g DM/kg, 917 g OM/kg DM, 110 g CP/kg DM, 145 g NDF/kg DM and 52 g ADF/kg DM) and 160 g of a mineral mix.

### *2.2 Pasture Measurements*

Pasture height was measured before and after grazing in each period. In the first period, 100 height measurements per paddock were taken using a sward stick, and 200 compressed height readings were taken with a rising plate meter (Farmworks®, model F200, New Zealand). Pre-grazing herbage mass was estimated based on a calibration curve from the plate meter, developed using five sample points where compressed height was recorded and aboveground biomass was harvested from 0.1 $m^2$ quadrats. After cutting, the samples were weighed and dried in a forced-air oven at 60°C

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for 72 hours. Herbage mass (kg DM/ha) for the ryegrass-only and ryegrass-legume treatments was estimated using the following equations:

Equation 1 (monoculture ryegrass):

$$y = 142 - 340 \times \text{pre-grazing compressed height (cm)} \ (R^2 = 0.83) \quad (1)$$

Equation 2 (intercropped):

$$y = 215 - 1008 \times \text{pre-grazing compressed height (cm)} \ (R^2 = 0.89) \quad (2)$$

In the second period, due to a technical failure of the rising plate meter, pre-grazing forage mass was estimated by destructive sampling, using 20 quadrats (0.24 $m^2$ each; 0.8 × 0.3 m) per paddock. Samples were dried under the same conditions previously described for dry matter determination.

The chemical and botanical composition was assessed using samples collected the day before grazing in each period. At least 20 samples per paddock were harvested at ground level with hand shears, within a ~20 cm diameter, forming a composite sample. The samples were frozen at −20°C and, after thawing, split into two subsamples: one for botanical separation and the other cut at the average post-grazing tiller height, with the upper part used for chemical analysis of consumed forage. Post-grazing tiller height was measured on 160 tillers per paddock, using graduated ruler.

### *2.3 Animal Measurements*

Milk yield and composition were evaluated during the last four days of each period. Milk production was recorded at both daily milkings (7am and 3pm) using an electronic meter (Waikato Milking Systems, New Zealand). Milk samples from both milkings were taken for chemical composition analysis.

Methane emissions were measured using two GreenFeed systems (C-Lock Inc., Rapid City, South Dakota, USA). Each animal accessed the system on different days, specifically on days 9 and 11 of each period, ensuring samples were collected every 3 h over 24 h. On the second sampling day, feeding times were shifted 3 h earlier than on the previous day. Each cow accessed the GreenFeed at least once at the following times: 6:30am, 8:30am, 10:30am, 2:30pm, 3:30pm and 6:30pm.

Grazing behavior was visually and systematically recorded every five minutes on days 10 and 12 of each period, following the methodology of Penning [13]. Observations were conducted throughout the daytime grazing cycle, categorizing activities as grazing, ruminating, or idling. These data allowed estimation of the time and daily proportion spent on each activity.

Herbage digestibility was estimated using the chemical composition of feces and consumed forage. Fecal samples were collected after each milking for four consecutive days. After drying (72 h at 60°C), samples were ground (1 mm) for analysis. Organic matter digestibility (OMD, g/g OM) was estimated using the equation by Ribeiro-Filho et al. (2005):

$$OMD = 1.035 - 24.78/CPf - 0.00027 \times ADFf - 0.0571 \times CPh/CPf \quad (3)$$

Where CPf = fecal crude protein (g/kg OM), ADFf = fecal acid detergent fiber (g/kg OM), and CPh = forage crude protein (g/kg OM). Methane ($CH_4$) emissions were measured on two of the last four days of each period, on days different from those used for behavior observations.

### *2.4 Chemical Analyses*

Dry matter (DM) content was determined by drying at 105°C for 24 h. Ash content was obtained via combustion in a muffle furnace at 550°C for 4 h, and organic matter (OM) was calculated by mass difference. Total nitrogen (N) content was determined using the Dumas combustion method 968.06 [14] with a Leco FP 528 instrument (Leco Corporation, Saint Joseph, MI, USA). Crude protein (CP) was calculated by multiplying N content by 6.25. Neutral detergent fiber (NDF) was determined according to Mertens[15], with modifications: samples were weighed in filter bags and treated with neutral detergent in an ANKOM A220 system (ANKOM Technology, Macedon, NY, USA). This

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analysis included thermostable α-amylase and ash correction but excluded sodium sulfite. Acid detergent fiber (ADF) was analyzed according to AOAC Method 973.18 [14].

*2.5 Statistical Analysis*

Statistical analyses used ANOVA with treatment as fixed effect and period as random effects. Significance was set at $p < 0.05$; trends were noted when $0.05 \leq p < 0.10$.

## 3. Results

Pre-grazing herbage mass was higher in RG paddocks (1773 kg DM/ha) compared to RG + Leg (1549 kg DM/ha), with herbage allowance also greater in RG (51.7 vs. 45.8 kg DM/cow/day) (Table 1). Legumes accounted for ~9% of the DM in RG + Leg. Crude protein content was slightly higher in RG + Leg (178 vs. 172 g/kg DM), while NDF and ADF were also marginally greater.

**Table 1.** Herbage characteristics of annual ryegrass (Lolium multiflorum Lam.) alone or annual ryegrass + legumes.

| | RG | RG + Leg |
|---|---|---|
| *Pre-grazing herbage mass (kg DM/ha)* | 1773 | 1549 |
| Herbage allowance (kg DM/cow) | 51.7 | 45.8 |
| Pre-grazing herbage height (cm) | 32.4 | 28.2 |
| Post-grazing herbage height (cm) | 18.2 | 16.9 |
| Defoliation severity[1] | 0.44 | 0.40 |
| *Chemical composition (g/kg DM)* | | |
| DM[2] (g/kg fresh) | 144 | 160 |
| Organic matter | 897 | 901 |
| Crude protein | 172 | 178 |
| Neutral detergent fiber | 562 | 594 |
| Acid detergent fiber | 248 | 260 |
| *Botanical composition (g/kg DM)* | | |
| Ryegrass | | |
| Leaves | 434 | 398 |
| Stems | 357 | 342 |
| Legumes | 0.0 | 89.0 |
| Other species | 0.82 | 19.0 |
| Dead material | 205 | 151 |

[1]Defoliation severity: (pre-grazing herbage height – post-grazing herbage height) / pre-grazing herbage height.
[2] DM: dry matter.

**Table 2.** Methane emissions, milk yield, and milk composition of dairy cows grazing annual ryegrass (RG) or ryegrass + legumes (RG + Leg).

| | RG | RG + Leg | *rsd* | *P* < |
|---|---|---|---|---|
| Methane | | | | |
| g/day | 330 | 321 | 21.4 | 0.362 |
| g/kg ECM[1] | 11.0 | 10.6 | 1.01 | 0.377 |
| Milk production (kg/dia) | 31.9 | 29.9 | 1.23 | 0.040 |
| ECM production (kg/dia) | 30.9 | 30.0 | 1.66 | 0.393 |
| Milk fat (g/kg) | 38.5 | 40.7 | 3.44 | 0.282 |
| Milk prontein (g/kg) | 33.4 | 32.3 | 1.71 | 0.273 |

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| | | | | |
|---|---|---|---|---|
| Milk fat production (g/dia) | 1220 | 1213 | 90.6 | 0.893 |
| Milk protein production (g/dia) | 1053 | 965 | 21.6 | 0.001 |
| MUN[2] (mg/dL) | 5.9 | 6.1 | 1.18 | 0.732 |
| Live weight (kg) | 551 | 553 | 6.9 | 0.341 |
| Digestibility of OM | 0.807 | 0.803 | 0.0056 | 0.067 |

[1]Energy-corrected milk production, calculated as follows: kg of milk production × [37.6 × fat (g/kg) + 20.9 × protein (g/kg) + 948]/3,138 [16], [2]MUN: milk urea nitrogen. Rsd: residual standard deviation.

Grazing time averaged ~390 min/day with no major differences between treatments (Table 3). Cows grazed most after afternoon milking (4–8pm, 34%) followed by post-morning milking (8am–12pm, 28%). Organic matter digestibility showed a trend towards higher values in RG (0.807 vs. 0.803; p = 0.067).

**Table 3.** Grazing time of dairy cows grazing annual ryegrass (RG) or ryegrass + legumes (RG + Leg).

| | RG | RG + Leg | *rsd* | *P* < |
|---|---|---|---|---|
| Grazing time (min) | | | | |
| 5am - 8am | 60 | 54 | 6.4 | 0.170 |
| 8am - 12pm | 112 | 109 | 14.6 | 0.706 |
| 12pm - 4pm | 92 | 86 | 15.0 | 0.474 |
| 4pm - 8pm | 130 | 135 | 2.4 | 0.080 |
| Total | 394 | 384 | 25.9 | 0.541 |

Rsd: residual standard deviation.

## 4. Discussion

This study assessed the performance of dairy cows grazing ryegrass alone or intercropped with forage legumes under rotational management in a subtropical environment. Contrary to the hypothesis, intercropping with red clover and vetch did not improve milk yield or methane output. Instead, milk and protein yields were reduced in the mixed sward treatment, and methane emissions remained unchanged, suggesting limited functional impact of legume inclusion at the observed botanical composition.

The proportion of legumes in the intercropped pastures was approximately 9% of total dry matter. Although legumes slightly increased crude protein levels, they did not substantially alter neutral or acid detergent fibre fractions. The lower pre-grazing herbage mass and herbage allowance in the RG + Leg treatment suggest that legumes may have competed poorly with ryegrass, reducing total DM yield. Other studies reporting benefits from legume inclusion have typically achieved higher proportions (≥20%) in the sward, often through the use of more aggressive species or modified management practices [17,18].

The greater milk production in cows grazing monoculture ryegrass pastures, than cows on intercropped pastures, likely reflect higher herbage mass and herbage allowance in the monoculture treatment. Lower herbage mass in mixed swards, such as those containing white clover and perennial ryegrass, can lead to reduced herbage intake and milk yield. For instance, cows grazing on mixed swards with lower pre-grazing herbage mass had lower herbage intake and milk yield compared to those grazing on perennial ryegrass monocultures [19]. This suggests that the structure and availability of herbage in mixed swards can significantly impact intake and subsequent milk production. In the same way, it is well known that lower herbage allowance can decrease individual milk production [20,21].

The lack of difference in milk fat, ECM, and urea nitrogen concentrations suggests that the cows' metabolic responses were similar, but that reduced intake or forage quality limited output in the

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mixed treatment. The inclusion of legumes in grass swards generally improves the nutritional quality of the pasture, which can enhance milk production [22]. However, if the herbage mass is insufficient, the potential benefits of the mixed sward are not fully realized. Cows grazing on mixed swards with adequate herbage mass and allowance showed improved milk yield compared to those on monocultures, but this advantage diminishes with lower herbage mass [23,24].

The lack of effect of legume inclusion on daily $CH_4$ emissions and emission intensity may be partially explained by the fact that the methane-mitigating potential of legumes is often associated with the presence of tannins or other bioactive compounds [25]. In this study, however, neither red clover nor common vetch are known to contain appreciable levels of such secondary metabolites. Moreover, the limited legume biomass likely constrained any advantage in mixed swards. It has been shown that while legumes can reduce methane production due to their physical and chemical properties, this reduction is more related to the rate of passage and specific chemical interactions rather than a fundamental shift in fermentation pathways [25–27]

The tendency observed toward higher OM digestibility in the monoculture ryegrass pasture is in agreement with other experiments where including legumes decreased OM digestibility [28,29], which may reflect a combination of factors including lower sward maturity and greater post-grazing residue in the monoculture paddocks [30,31]. Additionally, legumes tend to have higher passage rates through the digestive system compared to other forages like grasses. This is due to their rapid fermentation and physical breakdown in the rumen, leading to lower digestibility [26]

Grazing behaviour patterns were broadly similar between treatments, indicating that short-term behavioural responses to changes in forage composition were minimal under the conditions tested. It is well known that factors such as herbage allowance grazing management practices, and specific structural characteristics of swards can play a role, but their effects are often context-dependent and may not lead to substantial changes in overall grazing behavior or animal performance [32,33]. Additionally, under high herbage allowance conditions, cows are able to graze selectively, which may reduce the influence of sward structure on grazing behavior [34]. In the present study, the daily herbage allowance exceeded 45 kg DM/cow, a level typically associated with lenient grazing management [35].

The limited benefits observed with legume inclusion are likely attributable to the low proportion of legumes in the sward. To achieve measurable gains in animal performance or environmental indicators, strategies that enhance legume establishment and persistence are essential. These may include species selection [34], adjusted sowing ratios [35], and grazing regimes [36] that favour legume competitiveness.

## 5. Conclusions

Reducing nitrogen fertilization and including red clover and common vetch in annual ryegrass pastures did not decrease enteric methane emissions or improve milk production under rotational grazing in a subtropical environment. Milk and protein yields were reduced in the intercropped treatment, likely due to lower herbage allowance and insufficient legume biomass. These findings suggest that the successful use of forage legumes for improving productivity and mitigating emissions in pasture-based dairy systems depends critically on achieving higher legume proportions in the sward. Further research is needed to optimise legume establishment and persistence under field conditions.

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**Institutional Review Board Statement:** The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by Ethic Committee on Animal Use of the University of Santa Catarina State (CEUA/UDESC), protocol number CEUA 6051180521 (June 25, 2021).

**Data Availability Statement:** Data is available upon request.

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## Abbreviations

The following abbreviations are used in this manuscript:

| | |
|---|---|
| DM | Dry matter |
| OM | Organic matter |
| CP | Crude protein |
| NDF | Neutral detergent fiber |
| ADF | Acid detergent fiber |
| OMD | Organic matter digestibility |
| MUN | Milk urea nitrogen |
| ECM | Energy-corrected milk production |

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