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Including 8 hours of access to alfalfa in 1 or 2 grazing sessions in dairy cows fed a partial mixed ration: Effects on intake, behavior, digestion, and milk production and composition

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ABSTRACT

The aim of this study was to evaluate the inclusion of alfalfa grazing during 8 h continuous or partitioned in 2 separated sessions of 4 h after each milking, on nutrient intake, digestibility, ruminal fermentation, feeding behavior, milk production, milk composition, and milk fatty acid profile, in late-lactation cows fed a partial mixed ration (PMR). Twelve dairy cows (193 ± 83 d in milk, 584 ± 71 kg of body weight) were housed in individual outdoor pens and assigned to treatments according to a 3 × 3 Latin square design replicated 4 times. The treatments were as follows: (1) control (T0), cows were fed a total mixed ration (TMR) provided ad libitum 20.0% crude protein (CP), 32.2% neutral detergent fiber (NDF); (2) fed a diet combining a PMR which had the same ingredient composition as the TMR (60% of ad libitum intake) + 1 session of 8 h of pasture (T8), continuous grazing alfalfa (*Medicago sativa*; 20.6% CP, 35.8% NDF) after the p.m. milking; and (3) PMR (60% of ad libitum intake) + 2 daily sessions of 4 h of access to pasture after each milking (T4+4). The experiment lasted 57 d and was divided into 3 periods of 19 d each. The first 12 d of each period was used for diet adaptation, and the last 7 d was used for data collection. No differences among treatments were observed for any of the productive variables, feeding efficiency, or purine derivatives excretion. Cows in T0 had greater intake and apparent digestibility of dry matter, organic matter, and nonfibrous carbohydrates compared with T4+4 and T8. Compared with T0, alfalfa grazing increased the concentration of C18:1 *trans*-11 and decreased those of C16:0 and C17:0 in milk fat. Cows in T4+4 consumed 1.1 more kg DM/d of alfalfa and N provided by alfalfa in the diet was 3 percentage points higher compared with T8 cows (266 vs. 229 g/d, respectively). In addition, T4+4 cows had a greater daily range of ruminal pH than T8 (0.73 vs. 0.93), and the highest concentrations of NH_3-N were recorded during the a.m. grazing session while in T8 cows it occurred during the night. In conclusion, including 8 h of alfalfa grazing in T8 and T4+4 treatments allowed the substitution between 35.8 and 38.7% of the total dry matter intake (DMI) of a PMR (with a similar CP concentration to alfalfa) for pasture, maintaining milk solids production and increasing the C18:1 *trans*-11 of milk fat compared with a TMR in mid late–lactation cows. In an herbage plus PMR diet, splitting the 1 continuous grazing session of 8 h into 2 sessions of 4 h increased the proportion of energy and N provided by alfalfa pasture and reduced PMR intake, without modifying the total nutrient intake or productive performance of cows.

Key words: alfalfa, fresh forage, partial mixed ration, ruminal fermentation

INTRODUCTION

Feeding systems that use partial total mixed rations (**PMR**) with periods of access to grazing (hereinafter herbage plus PMR) for dairy cows can reduce feeding costs (Soriano et al., 2001; Tozer et al., 2003) and improve the milk fatty acids profile for human consumption (Chaudhry, 2008). Pasture herbage and fresh forage have high proportion of C18 n-3 (Elgersma et al., 2006). When part of the diet is herbage, there is an increased proportion in milk of certain fatty acids (e.g., 18:2 *cis*-9,*trans*-11, and C18:1 *trans*-11) considered beneficial for human health (Chilliard et al., 2007; Mendoza et al., 2016b; Grille et al., 2022). In addition, these systems have an increasingly better perception by consumers compared with TMR-based systems (Joubran et al., 2021). The use of herbage plus PMR for dairy cows is increasing in temperate regions that use grazing as a food resource (Wales and Kolver, 2017) because this strategy has better results

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than supplying forage and concentrates separately (Wales et al., 2013). However, increasing herbage in the diet could limit DMI and milk production. Pastorini et al. (2019), working with cows producing 30 kg/d of milk, consuming annual ryegrass (*Lolium multiflorum*) harvested and offered fresh combined with PMR (0 to 75%), observed that as the proportion of herbage increased, the DMI and milk production decreased. Even with only 16% (3.6 kg DM) of ryegrass harvested and offered fresh in the diet of mid-lactation dairy cows, producing above 30 kg/d of milk, Mendoza et al. (2016a,b) reported a decrease of 6.6% (2.5 kg/d) in milk production. In feeding systems using herbage plus PMR it is difficult to reach high intake and milk production when herbage inclusion in diets exceeds 30% of DMI (Wales et al., 2013; Wright et al., 2020). However, other studies included a higher proportion of herbage in the diet, and no differences in DMI or milk production were observed compared with cows fed a TMR. For example, Morales-Almaráz et al. (2010) included up to 37% of DMI of herbage in a diet of grazing mid-lactation dairy cows producing 34 kg/d of milk, without differences in DMI or milk production. In late-lactation cows producing less than 20 kg/d of milk, Dall-Orsoletta et al., (2016) included 46% of DMI of herbage in the diet, without detrimental effects on total DMI and production. Different factors may explain these contradictory results (i.e., the daily amount and composition of PMR offered; Soriano et al., 2001; Pastorini et al., 2019; Ribeiro-Filho et al., 2021), the herbage allowance and sward characteristics (Ison et al., 2020a,b), the quality and composition of the herbage grazed used, and the grazing management. Vibart et al. (2008), using different levels of inclusion of high- and low-quality herbages, reported no negative effects on DMI and milk production with the inclusion of up to 41% high-quality annual ryegrass with similar CP to that of the PMR of the diet, which was 16%. However, when annual ryegrass in a more advanced stage of growth and with a lower quality was used, milk production decreased linearly as its inclusion in the diet increased.

Most of the previous studies used cool-season grasses (*Poaceae*) as the herbage, such as annual ryegrass (Mendoza et al., 2016a; Pastorini et al., 2019), perennial ryegrass (*Lolium perenne* L.; Wright et al., 2020), or orchard grass (*Dactylis glomerate*; Soriano et al., 2001), while Morales-Almaráz et al. (2010) used forage mixtures (orchard grass, ryegrass, white clover, vetch) containing a high proportion of grasses. Information about the use of alfalfa (*Medicago sativa*) in direct grazing plus PMR is limited. It is well known that frothy bloat limits the practice of alfalfa grazing, and prophylactics and management techniques are necessary to reduce its incidence (Berg et al., 2000). In contrast, alfalfa could present more suitable conditions than grasses for inclusion in herbage plus PMR diets. Dairy cows have a natural preference for this species (Horadagoda et al., 2009; Buse et al., 2022), and alfalfa has a lower degradable fraction but a greater rate of NDF degradation (Tamminga, 1993). Kammes and Allen (2012a,b) observed that dairy cows fed alfalfa silage as the only forage source had shorter ruminal time retention, greater ruminal pH, and higher DM apparent digestibility compared with diets containing orchard grass silage. Finally, compared with grasses, legumes show less variation in their composition (particularly in NDF and water soluble carbohydrates concentration) and fermentation rate with maturation and throughout the day (Bargo et al., 2003; Cajarville et al., 2015; Elgersma and Søegaard, 2018).

A common management of dairy farms in some South American regions (Uruguay, Argentina, Chile, and the south of Brazil) is to graze dairy cows twice a day. The 2 grazing sessions can be performed on the same strip or changing the strip each time. The practice of grazing twice a day probably comes from a time when the systems were predominantly pastoral. It would be of interest to study if this management results in an increase in milk production due to the additional walking of the cows.

Bargo et al. (2002) using mixtures of grasses (60% in vitro digestibility) compared 2 feeding systems (herbage plus PMR and herbage plus concentrate) and observed higher herbage DMI in the system that used herbage plus PMR. The authors related the higher DMI with a longer grazing time, which in turn, was associated with an increase in the bites per day. In contrast, several authors reported an increase in the amount of herbage DMI of animals by increasing the number of grazing sessions, although most studies have been carried out with herbage as the sole or main component of the diet and on the same herbage allotment during the day (Kennedy et al., 2009; Clark et al., 2010). Dall-Orsoletta et al. (2016), in cows fed annual ryegrass plus PMR, reported that increasing the number of grazing sessions from 1 to 2 increased herbage DMI. At the same time, the intake of PMR decreased, and therefore total DMI and milk production did not change.

Splitting the grazing time into more sessions will also modify the sequence of feedstuffs offered, and the substrate pattern entering the rumen would also change. This would modify ruminal fermentation, which in turn, may have consequences on animal productivity (Robinson, 1989; Cabrita et al., 2006; Reynolds and Kristensen, 2008).

Thus, we hypothesized that splitting 1 session of 8 h of access to grazing into 2 sessions of 4 h would increase alfalfa intake by changing the eating behavior and digestive use of nutrients, which would also lead to an increase in the concentration of beneficial fatty acids for human consumption in the milk. This experiment aimed to evaluate changes in milk production and fatty acid profile, ruminal fermentation, feeding behavior, and digestion of including grazing in TMR-fed cows, in 1 or 2 daily sessions.

MATERIALS AND METHODS

Animals, Experimental Design, and Treatments

This experiment was performed from October to December (springtime) at the Experimental Dairy Farm of the Veterinary School (Facultad de Veterinaria, Universidad de la República), located in San José, Uruguay (S 34°40′, W 56°32′). The experiment was conducted following the regulations of the Bioethics Committee of the Veterinary Faculty (protocol number: CEUAFVET–351/16). Twelve Holstein cows (584 ± 71 kg, 193 ± 83 DIM, 3.2 ± 1.2 lactations) with a milk production record during the previous 305 d of lactation of 7,876 kg (SD = 642) were used. The cows were grazing a pasture legumes and grasses) and were supplemented with corn silage and concentrate with the rest of the herd. Before the beginning of the experiment (2 wk), the cows were fitted with a halter, tamed down, and adapted to the experimental facilities and manipulations that would be performed during the experiment. During this period, they grazed alfalfa and consumed a PMR. The cows were blocked by BW, previous milk production, days in milk, and parity and were allocated into 4 squares, and within each square, they were randomly assigned to treatment sequences (PROC PLAN of SAS 9.0, SAS Institute Inc.), according to the experimental design of 4 times replicated 3 × 3 Latin squares, balanced in the succession of treatments to minimize possible carryover effects. Three treatments (n = 12 per treatment) were evaluated: (1) a TMR provided ad libitum (**T0**, control treatment); (2) a PMR (60% DMI) plus 1 daily session (8 h) of grazing alfalfa (**T8**); and (3) a PMR (60% DMI) plus 2 daily sessions (4 h) of grazing alfalfa (**T4+4**). The sample size was calculated using the PROC POWER of SAS (version 9.1, SAS Institute Inc.) to detect a difference in 1 kg of milk produced with a type I error (α) = 0.05 and a power of 80% (Festing and Altman, 2002), based on previous studies by our group and carried out under similar conditions (Mendoza et al., 2016b; Pastorini et al., 2019; Pozo et al., 2022). Each period lasted 19 d and consisted of 12 d for adaptation followed by 7 d for data and sample collection. The length of periods was determined considering recommendations for digestion trials with cattle of previous authors (Machado et al., 2016), the re-establishment of ruminal fermentation pattern and the microbiome in dairy cows (Weimer et al., 2017) and previous research combining pastures with PMR in diets for dairy cows (Dall-Orsoletta et al., 2016; Mendoza et al., 2016a,b; Pastorini et al., 2019; Pozo et al., 2022). Experimental cows were milked twice daily at 0700 and 1800 h, as 1 group, with the total process (driving the cows to the milking parlor and milking) lasting 15 min. The potential ad libitum DMI was determined individually 10 d before the beginning of the experiment by offering increasing amounts of TMR until orts greater than 5% were obtained for 6 consecutive days. The TMR (Table 1) was prepared daily and was formulated to fulfill the requirements of cows with 600 kg of BW and producing 28 kg of milk/d as recommended by the NRC (2001), except for the CP concentration. This nutrient was calculated to match the alfalfa values to avoid an extra source of variation, and this led to the CP concentration of all diets exceeding NRC (2001) recommendations. When the cows were not grazing, they were housed in individual outdoor pens (2.0 × 4.0 m) with individual feeders and ad libitum access to water. The pens had natural shade and did not have supplemental lighting. Cows in T0 were fed daily amounts of TMR equivalent to the recorded individual potential ad libitum DMI, delivering half at 1000 h and the rest after the p.m. milking (1800 h). In T8 and T4+4, the PMR had the same ingredient composition as the TMR of T0, but it was supplied at 60% of the individual ad libitum DMI and offered once daily (1000 h for T8 and 1200 h for T4+4). Due to the fact that the treatments involved differences in animal handling, the experimenter could not be blinded with respect to which animal was receiving which treatment.

The alfalfa used for the experiment had a stand age of 2 years. The pastureland (2.5 ha) was seeded (20 kg/ha) with a commercially available cultivar (cv. Crioula) with intermediate fall dormancy. Based on the information from soil analysis, calcium and magnesium carbonate (lime) were applied for pH correction, and fertilized with Ca, P, S, K, and N, before seeding. Annual refertilizations were performed. The botanical composition of the pregrazing herbage mass was determined by cutting herbage samples to ground level before each period (n = 3). The herbage grazed by T4+4 and T8 was composed of 83.1% (±4.4%) alfalfa, 5.3% (±1.2%) red clover (*Trifolium pratense*), 4.1% (±1.9%), annual ryegrass (*Lolium multiflorum*), and 7.5% (±1.7%) dead

Table 1. Ingredient and nutrient composition (SD in parentheses) of TMR and alfalfa

Item	TMR	Alfalfa	CS	CG	SM
Ingredient of TMR (%)					
Corn silage (CS)	58.48				
Ground corn grain (CG)	14.62				
Solvent extracted soybean meal (SM)	24.37				
Urea	0.39				
Vitamin-mineral premix[1]	1.46				
Additive premix[2]	0.67				
Nutrient composition (% of DM, unless noted)					
DM (% of as fed)	46.7 (0.4)	18.4 (2.3)	36.1 (0.7)	88.1 (0.4)	88.8 (0.3)
OM	92.1 (0.2)	91.0 (0.3)	94.9 (0.8)	97.5 (0.2)	93.5 (0.1)
NDF	32.2 (0.8)	35.6 (1.7)	51.9 (1.5)	14.7 (0.1)	14.8 (0.3)
ADF	20.9 (0.4)	27.1 (0.8)	32.4 (0.8)	4.5 (0.1)	11.6 (0.1)
NFC	38.1 (0.9)	32.1 (1.7)	31.7 (1.6)	70.2 (0.3)	30.6 (0.6)
EE[3]	1.7 (0.1)	2.6 (0.0)	2.1 (0.0)	4.1 (0.0)	1.4 (0.0)
CP	20.0 (0.6)	20.6 (2.5)	9.0 (0.4)	8.5 (0.2)	46.7 (0.9)
NPN	2.1 (0.3)	4.3 (0.2)	—	—	—
SN[4]	4.2 (0.2)	9.8 (0.1)	—	—	—
NDIN	1.2 (0.2)	1.3 (0.1)	—	—	—
ADIN	0.8 (0.1)	0.9 (0.1)	—	—	—
NE_L (Mcal/kg of DM)	1.71	1.51	—	—	—
Particle size distribution (% as fed)					
19 mm	3.7 (0.7)				
8 and 19 mm	49.8 (0.4)				
1.8 and 8 mm	38.2 (1.8)				
1.8 mm	8.3 (0.2)				

[1]Provides (per kg of DM): 0.85 g of Cu; 2.6 g of Zn; 0.9 g of Se; 1.0 g of Mn; 23 mg of I; 3 mg of Co; 63,700 IU of vitamin A; 12,700 IU of vitamin D; 250 IU of vitamin E.

[2]Provides (per kg of DM): 2.4 g of sodium bicarbonate, 2.4 g of magnesium oxide, and 1.9 g of ethoxylated alcohols as surfactants (Bloker Premix, Phibro Animal Health de Argentina S.A).

[3]EE = ether extract.

[4]SN = soluble N.

material (average for the 3 periods). During the experiment, cows entered the pasture strip in a vegetative phenological stage (less than 8 nodes for alfalfa). The average forage availability for the 3 periods was 1.321 ± 399 kg DM/ha. Each cow was individually fenced within 1 individual daily strip, delimited with electric fences, and had ad libitum access to water. The strip size was adjusted daily to offer 14 kg/d of alfalfa DM per cow, above 5 cm from the ground, which was estimated for unrestricted intake, following Pérez-Prieto and Delagarde (2013), for systems using herbage plus PMR. In T8 cows grazed once daily after the p.m. milking between 1800 to 0200 h. In T4+4 cows grazed twice daily, in 2 sessions of 4 h after each milking (between 0800 to 1200 h and 1800 to 2200 h). The new daily pasture strip was assigned at 1800 h for both treatments, and cows in T4+4 returned to the same strip (without changing the allotment of grass).

The maximum daily distance walked by the cows between the alfalfa paddock, the outdoor free stalls, and the milking parlor was 300 m.

Body weight was measured at the beginning of the experiment and at the end of each period (4 times), between 0800 and 0900 h, with a digital scale. Meteorological data were obtained from the website of the Instituto Nacional de Investigación Agropecuaria (http://www.inia.uy/gras/Clima/Banco-datos-agroclimatico, last accessed March 3, 2022).

Dry Matter and Nutrient Intake and Digestion

The daily intake of TMR/PMR was measured and alfalfa forage was estimated from d 13 to 17 of each period. The intake of TMR/PMR was measured by weighing the amounts offered and refused (refusal measurement from d 14 to 18). The TMR/PMR orts of each cow in all treatments were weighed between 0930 h and 1000 h, and a composited sample by cow and period was frozen for subsequent composition analysis. The offered TMR/PMR sample was taken immediately after offering the feed at 1000 h.

The daily consumption of alfalfa forage was estimated for each grazing cow individually by fencing each cow within 1 daily strip. For alfalfa intake estimation, in each of the 8 individual daily strips (4 for T8 and 4 for T4+4), the difference between the forage mass before

and after grazing was measured by cutting 5 cm above the ground (Macoon et al., 2003). The pregrazing forage mass was determined at 1000 h by cutting a strip of 5 m^2 (0.5 m × 10 m) forage mass, which was weighed, and the strip area was adjusted to offer 14 kg of DM/cow (without considering the 5 m^2 cut) using the % DM from the previous day. In each strip per cow, the postgrazing forage mass was determined at 1200 h on the following day by cutting all the forage mass contained in 10% of the area, avoiding places with manure. All cuts were made with a mower (Toro CNB94, The Toro Company) 5 cm from the ground and weighed. Before grazing, daily separate samples (100–200 g) were taken for each cow strip from the offered herbage at 1000 h using the hand plucking procedure (Cook, 1964), which briefly consists of hand clipping samples of the forage offered to the animal, according to the observations of the previously grazed strips. The samples were immediately immersed in liquid N for all nutrient conservation. All samples were kept frozen at −20°C until further analysis.

The intake of DM, OM, CP, NDF, ADF, ether extract (**EE**), and NFC was calculated using data from samples taken by the hand plucking procedure described above, and PMR chemical composition.

Apparent total-tract nutrient digestibility was indirectly determined using the indigestible NDF (**iNDF**) as an internal marker (Huhtanen et al., 1994). On d 14 and 15 of each period, spot fecal samples were collected directly from the rectum of all cows at 0400 and 1600 h, 6 h before and after the main feeding session began. Approximately 200 g of the fecal samples were dried in a forced-air oven at 60°C for 72 h and ground to pass through a 1 mm screen. A composite sample per cow and period was obtained by mixing equal DM amounts from each sample and was analyzed for DM, ash, NDF, ADF, EE, and total N as described in the chemical analysis section. Fecal composite samples, as well as TMR and alfalfa offered and orts, were also analyzed for iNDF. Briefly, dried samples were ground to pass through a 2 mm screen, and 6 g samples were weighed into 22 × 10.5 cm nylon bags (Ankom Technology Corporation) with a pore size of 50 μm and a sample size-to-surface area ratio of 13 mg/cm^2 and were incubated for 288 consecutive h in the rumen of 2 nonlactating cows fed a standardized maintenance diet consisting of (DM basis) grass hay (60%), corn grain (25%), soybean meal (13%), and a mineral and vitamin mix (2%). Following the incubation, bags were rinsed with tap water for 15 min and dried in a forced-air oven at 60°C for 72 h, and the residues were analyzed for NDF and the iNDF concentration in the feces. Apparent total-tract digestibility coefficients for DM, OM, NDF, ADF, NFC, EE, and total N were calculated as follows: {[nutrient intake (g/d) − fecal nutrient output (g/d)]/nutrient intake (g/d)} × 100.

Feeding Behavior

On d 13 and 16 of each period, feeding behavior was recorded by scan-sampling of individual cows (Martin and Bateson 1993), by 4 trained observers every 5 min for 19 h (except from 0300 to 0700 h, and during each milking) as suggested by Hirata et al. (2002). For the observations, the cows were numbered with reflective paint and remained during the observations in their corresponding strip or pen. The observers were trained before starting the experiment to consistently recognize the feeding behaviors. For this experiment, feeding behaviors were defined as 'eating' (picking, grasping, chewing, or consuming PMR or herbage), 'ruminating' (chewing movements without feed in the mouth, regurgitation of feed, or both), and others (not showing any of the previous activities). The proportion of each feeding behavior per hour was calculated as a fraction of the total observations following Mendoza et al. (2018). Additionally, observations of treatments T8 and T4+4 during the 8 h of grazing access were analyzed separately (1800 h = h 0, start of PM grazing, and a new strip was assigned).

Ruminal Fermentation

The 12 cows were fitted with permanent ruminal catheters (K227 Koler, chest drainage probes, 150 cm long, 13.5 mm outer diameter; Kine Estetic). On d 18 of each period, samples of ruminal fluid of all cows (including those grazing) were taken hourly from 1000 to 2200 h, and at 0100, 0400, and 0700 h (16 samples). Ruminal fluid pH was immediately measured using a digital pH meter (EW-05991–36, Cole Parmer). The ruminal liquid was pressed through 2 layers of cheesecloth, and a 10 mL sample of ruminal fluid was preserved with 0.2 mL of a 6.6 M H_2SO_4 for NH_3-N analysis. Another 0.5 mL sample was preserved with 0.5 mL of 0.1 M $HC1O_4$ for VFA analysis; both were stored at −20°C until analysis. The NH_3-N concentration was determined by colorimetry using the phenol-hypochlorite reaction according to Weatherburn (1967) and a spectrophotometer (UNICO, 1200; United Products and Instruments Inc.). For VFA and lactate determination, only samples taken at h 7, 10, 14, 20, and 22 were analyzed. The samples were thawed at room temperature, centrifuged (10,000 × g at 4°C for 15 min), and analyzed using an HPLC (Dionex Ultimate 3000), as described by Adams et al. (1984), using an Acclaim Rezex Organic Acid H^+

column (8%) of 7.8 × 300 mm, adjusted at 210 nm. The ΣVFA concentration was calculated as the sum of the acetate, propionate, and butyrate concentrations.

Excretion of Urinary Purine Derivatives

On d 14 and 15 of each period, urine purine derivative excretion was determined according to Valadares et al. (1999). Urine spot samples (2/d) were collected by manual stimulation of the perineal zone from each cow at 0400 h and 1600 h (approximately 6 h before and after the beginning of the feeding bout). For each sample, 15 mL of urine was acidified with 60 mL of 0.072 *N* H_2SO_4 and stored at −20°C until analysis (Broderick et al., 2009). The urine samples were thawed at room temperature, and equal parts of each of the 4 samples were composited by cow, which was used for the analyses. A subsample of this urine composited sample was filtered through a paper filter (7.5 μm porosity) and analyzed for creatinine by a colorimetric method using a commercial kit (Labtest Diagnóstica S.A.). Another subsample of urine was centrifuged (10,000 × *g* for 15 min at 4°C) for uric acid and allantoin analysis as described by Balcells et al. (1992) using an HPLC (Dionex Ultimate 3000) with an AcclAim C18 of 205 nm, 5 μm, 4.6 × 250 mm column. The total daily excretion of purine derivatives (PD; mmol/d) was calculated from the concentration (mmol/L) of PD in urine and the total volume of urine excreted.

Milk Production and Composition

Milk production was determined from d 13 to 17 of each period in the 2 milkings, with a manual milk meter (Tru-test by Tru-test Limited). Individual representative milk samples were collected from the milk meter, at 4 consecutive milkings at d 14 and 15 of each period using bronopol as a preservative agent; the samples were used to determine the fat, protein, and lactose by infrared analysis (model 2000, Bentley Instruments Inc.).

Feed efficiency was calculated as solid corrected milk production (kg)/DMI (kg), calculated as solid corrected milk = 12.24 × fat production (kg/d) + 7.10 × protein production + 7.35 × lactose production (kg/d) − 0.0345 × milk production (kg/d; Tyrrell and Reid, 1965). Additional individual milk samples (2) were taken without preservatives on d 15 from each milking and stored at −20°C until analyzed for fatty acid composition. For fatty acid analysis, frozen milk samples were thawed at room temperature, and milk lipids were separated according to Feng et al. (2004). An aliquot of 50 mg of milk fat was dissolved in 100 μL of hexane, followed by esterification with 100 μL of 2 *N* potassium hydroxide in methanol to obtain the FAME which were separated and quantified using a GC–MS (Agilent 7890A GC System, Agilent Technologies Inc.) equipped with a 60 m column (250 μm i.d., 0.25 μm film thickness; Thermo Scientific Inc.). Helium was used as the carrier gas, with a flow rate of 1.0 mL/min. The injector temperature (split ratio of 100:1) was set to 250°C. The initial column temperature (40°C) was held for 0.5 min, increased at 25°C/min to 175°C and held for 10 min, then increased at 5°C/min to 210°C and held for 5 min, and finally increased at 5°C/min to 230°C and was held for 5 min. Fatty acids were identified by comparing their retention times with the following FAME standards: 37 components FAME mix (47885, Supelco, Bellefonte, PA), *trans*-11-octadienoic methyl ester (46905-U, Supelco), octadecadienoic acid conjugated methyl ester (05632, Sigma–Aldrich) and those stored in the National Institute of Standards and Technology (US Government Library). The Δ^9-desaturase index and the atherogenicity index were calculated as described by Kelsey et al. (2003) and Ulbricht and Southgate (1991), respectively.

N Balance Calculation

Daily N balance (NB) was calculated during d 14 and 15 of each period as NB (g/d) = N intake (g/d) − [fecal N output (g/d) + urine N output (g/d) + milk N output (g/d)]. The concentration of N in urine and feces was determined by the Kjeldahl method (AOAC, 1990; method 955.04). In the manure, N was calculated as N urine plus N fecal. For the determination of milk N secretion, milk samples were taken as described for the determination of milk composition, and daily milk N secretion was calculated as milk protein (g/d)/6.38 (NRC, 2001). The efficiency of utilization of feed N for milk production was calculated as (milk N output/N intake) × 100.

Chemical Analysis

The DM concentration was determined by drying at 105°C for at least 16 h (Method ID 934.01; AOAC, 1990). Ash was determined by combustion at 600°C for 3 h, and OM was determined by the mass difference (Method ID 942.05; AOAC, 1990). The total N concentration was assayed with the Kjeldahl method (Method ID 984.13; AOAC, 1990) and NPN, soluble N, NDIN, and ADIN (Licitra et al., 1996). The EE concentration was determined in a reflux system (Soxtherm 2000 S 306 M, Gerhardt) with ethyl ether at 180°C for 2 h (Method ID 920.39; AOAC, 1990). The NDF concentration was analyzed according to the procedures described by Mertens et al. (2002) using heat-stable

α-amylase and sodium sulfite. The ADF concentration was analyzed according to Method ID 973.18 of AOAC (AOAC, 1990). The NDF and ADF analyses were performed independently and were expressed exclusively as residual ash. The concentration of NFC was estimated as 100 − (% NDF + % CP + % EE + % ash). The NE_L concentration was calculated based on the chemical composition of the feedstuffs used according to NRC (2001). For the TMR samples, particle size distributions were assessed using the modified Penn State Particle Size Separator (Kononoff et al., 2003).

Statistical Analysis

All data were analyzed using SAS software version 9.0 (SAS Institute Inc.). Data were initially analyzed for outliers, and the normality of the residuals was checked using univariate procedures (PROC UNIVARIATE). No animal or data point was excluded from the statistical analysis. Data were analyzed using the PROC MIXED procedure with the following model:

$$Y_{ijkl} = \mu + S_i + C_j(S_i) + P_k + T_1 + e_{ijkl},$$

where Y_{ijkl} is the dependent variable, μ is the overall mean, S_i is the random effect of the square (i = 1 to 4), $C_j(S_i)$ is the random effect of cows nested within the square (j = 1 to 4), P_k is the random effect of the period (k = 1 to 3), T_l is the fixed effect of treatment (l = T0, T4+4, or T8), and e_{ijkl} is the residual error.

The data of the variables with repeated measurements over time in each period, such as ruminal pH, NH_3-N, VFA, lactate, and feeding behavior, were analyzed using the PROC MIXED procedure with the following model:

$$Y_{ijklm} = \mu + S_i + C_j(S_i) + P_k + T_1 + H_m + T_1 \times H_m + e_{ijklm},$$

where Y_{ijklm} is the dependent variable, μ is the overall mean, S_i is the random effect of the square (i = 1 to 4), $C_j(S_i)$ is the random effect of cows nested in the square (j = 1 to 4), P_k is the random effect of the period (k = 1 to 3), T_l is the fixed effect of treatment (l = T0, T4+4, or T8), H_m is the fixed effect of the hour of measurement, $T_l \times H_m$ is the fixed effect of the interaction between treatment and hour of measurement, and e_{ijklm} is the residual error. Period × cow interaction within a square was the subject of repeated measurements, and AR (1) was the covariance structure chosen for evenly spaced data (i.e., behavioral events; Littell et al., 1998), while spatial power [SP (POW)] was chosen for unevenly spaced data (i.e., ruminal pH, NH_3-N, lactate, and VFA). The treatment × period effect was tested in both models, but it was not significant and therefore was removed.

The effects of the treatments were tested by orthogonal contrasts to study alfalfa inclusion in the diet compared with the T0 treatment versus the average of the T4+4 and T8 treatments (T0 vs. T4+4 + T8) and to study the T4+4 treatment versus the T8 treatment (T4+4 vs. T8). Significant differences were declared at $P \leq 0.05$, and trends were discussed at $0.05 < P \leq 0.10$.

RESULTS

The mean temperatures at a 2-m height in periods 1, 2, and 3 were 20.5, 20.0, and 20.5°C, respectively. Sunrise occurred at 0539 h, and sunset occurred at 1917 h on the first day of period 1 and 0526 h and 1955 h, respectively, during the last day of period 3. There were no differences among periods for any of the variables studied. Cows with access to alfalfa grazing (T8 and T4+4) had lower DM, OM, and NFC intake and digestibility ($P < 0.05$), as well as lower energy intake ($P < 0.05$), than T0 cows (Table 2). Cows in the T4+4 treatment had higher alfalfa DMI but this treatment did not affect total nutrient intake or digestibility (Table 2). A treatment × hour interaction was observed for eating and ruminating activities (Table 3 and Figure 1). The proportion of eating was lower at 2100 and 2200 h in T0 cows, while the proportion of ruminating activity by those animals was greater at 2100 h and lower at 1400 h compared with T8 and T4+4 cows. In addition, T4+4 cows had a higher proportion of eating behavior from 0800 to 1300 h (except 1000 h, when there was no difference) and fewer ruminating behaviors from 0800 to 1400 h and 2300 h compared with the other 2 treatments. Cows in the T8 treatment had a higher proportion of eating activities from 2400 to 0100 h than did the cows in the other treatments (Table 3). Analyzing only the 8 h of grazing, the cows in T4+4 grazing sessions had a higher proportion of eating behaviors in h 4 and from h 6 to 8 and decreased the proportion of ruminating behavior in h 6 (Figure 2).

Daily ruminal pH (average, minimum, and maximum) was lower in T8 and T4+4 cows, while no treatment differences were observed in the Σ of VFA concentration. The proportion of propionate was higher in T8 than in T4+4, and this was the only difference observed among treatments on individual VFA (Table 4). The T4+4 treatment led to an increase in the maximum value and the range of ruminal pH compared with T8 (Table 4). A treatment × hour interaction was detected for lactate (Figure 3) and ruminal NH_3-N

Table 2. Dry matter intake, nutrient intake, and digestibility of dairy cows fed a TMR exclusively (T0) or partial mixed ration (PMR) plus 8 h access to alfalfa grazing in 1 (T8) or 2 (T4+4) daily sessions

	Treatment[1]				Contrast probability[2]	
Item	T0	T8	T4+4	SEM	T0 vs. (T8 and T4+4)	T8 vs. T4+4
DMI (kg/d)						
TMR	23.0	13.0	13.2	2.20	<0.001	0.870
Alfalfa	—	7.0	8.1	0.35	—	0.029
Total	23.0	20.0	21.3	2.03	0.024	0.252
Total (% BW)	4.0	3.5	3.7	0.29	0.020	0.196
Nutrient intake (kg/d)						
OM	21.2	18.3	19.5	1.88	0.020	0.256
NDF	7.4	6.7	7.1	0.65	0.125	0.218
ADF	4.8	4.6	4.9	0.42	0.910	0.169
EE	0.40	0.41	0.44	0.465	0.132	0.131
NFC	8.7	7.2	7.6	0.78	0.001	0.298
Digestibility (%)						
DM	68.8	65.3	64.6	2.16	0.046	0.616
OM	68.9	65.6	64.6	2.16	0.038	0.731
NDF	51.1	50.4	50.8	3.73	0.368	0.261
ADF	45.9	46.7	46.6	4.05	0.426	0.320
EE[3]	81.8	83.1	82.8	1.53	0.192	0.766
NE_L intake (Mcal/d)						
TMR	39.3	22.3	22.6	3.76	<0.001	0.866
Alfalfa	—	10.5	12.2	0.524	—	0.028
Total	39.3	32.8	34.8	3.51	0.003	0.296

[1]Treatments: T0 = 100% TMR; T4+4 = 60% PMR plus access to alfalfa pasture after a.m. and p.m. milking (between 0800 to 1200 h and 1800 to 2200 h); T8 = 60% PMR plus access to alfalfa pasture after p.m. milking (between 1800 to 0200 h).

[2]T0 versus (T8 and T4+4) = orthogonal contrast comparing T0 versus the average of T8 and T4+4; T8 versus T4+4 = orthogonal contrast comparing T8 versus T4+4.

[3]EE = ether extract.

(Figure 4) concentrations. The main differences among treatments occurred between 1000 and 1700 h, with the animals on T4+4 presenting higher values. For the latter, higher NH_3-N values were also observed at 2100 and 2200 h (Figure 4). Although PD concentration was higher for T0, only a tendency was observed for PD daily excretion (Table 5). Cows in T8 and T4+4 had lower N intake, digestibility, and g of N excreted by the urine compared with T0 cows, while fecal N excretion was similar. There was a tendency of a higher NB for

Table 3. Behavioral events throughout the day (except between 0300 to 0700 h, and milking) and only during 8 h grazing (h 0 = 1800 h) of dairy cows fed a TMR exclusively (T0) or partial mixed ration (PMR) plus 8 h access to alfalfa grazing in 1 (T8) or 2 (T4+4) daily sessions

	Treatment (Trt)[1]				Probability[2]				
Item	T0	T8	T4+4	SEM	Trt	H	Trt × H	T0 vs. (T8 and T4+4)	T8 vs. T4+4
Behavioral events (proportion of total observations on 19 h)									
Eating	0.26	0.28	0.32	0.027	<0.001	<0.001	0.003	0.020	0.011
Ruminating	0.37	0.33	0.32	0.023	0.049	<0.001	<0.001	0.015	0.733
Others	0.37	0.39	0.35	0.028	0.092	<0.001	0.063	0.863	0.030
Behavioral events during grazing (proportion of total observations on 8 h)									
Eating	—	0.324	0.46	0.090	<0.001	<0.001	<0.001	—	—
Ruminating	—	0.321	0.18	0.039	<0.001	<0.001	0	—	—
Others	—	0.35	0.35	0.052	0.970	<0.001	<0.001	—	—

[1]Treatments: T0 = 100% TMR; T4+4 = 60% PMR plus access to alfalfa pasture after a.m. and p.m. milking (between 0800 to 1200 h and 1800 to 2200 h); T8 = 60% PMR plus access to alfalfa pasture after p.m. milking (between 1800 to 0200 h).

[2]Probability of Trt = the main effect of treatment; H = effect of sampling hour; Trt × H = interaction between treatment and sampling hour; T0 versus (T8 and T4+4) = orthogonal contrast comparing T0 versus the average of T8 and T4+4; T8 versus T4+4 = orthogonal contrast comparing T8 versus T4+4.

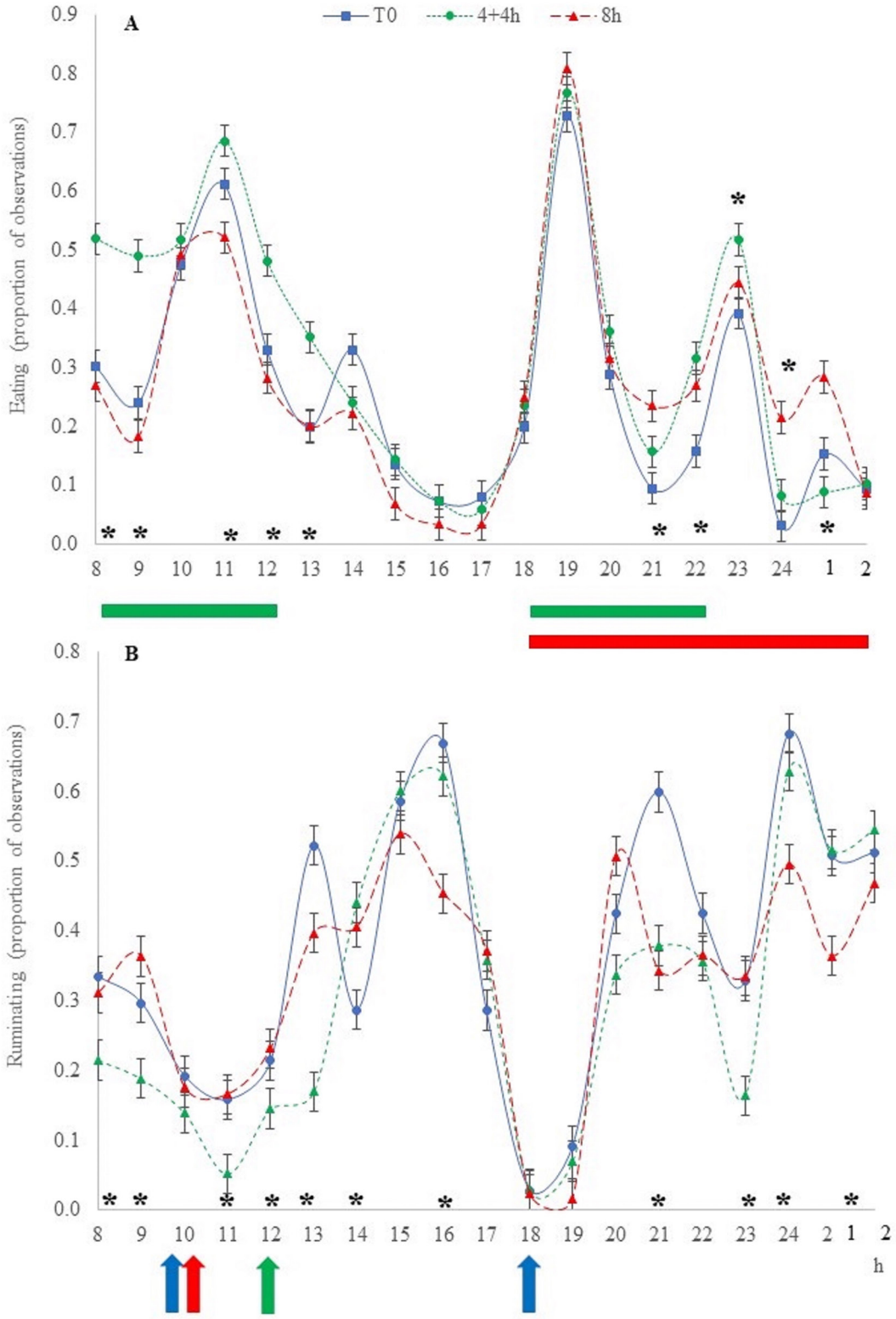

Figure 1. Eating (A) and ruminating (B) behavior events as a proportion of the total hourly observations during 19 h/d of dairy cows fed a TMR exclusively (T0) or partial mixed ration (PMR) plus 8 h access to alfalfa grazing in 1 (T8) versus 2 daily (T4+4) sessions. In each hour, at least 1 difference among treatments ($P \leq 0.05$) is indicated with an asterisk (*). Error bars represent the SEM. Green and red bars indicate the period when T4+4 and T8 had access to alfalfa grazing, respectively. Blue, red, and green arrows indicate the TMR delivery time in T0 or the PMR delivery time in T8 and T4+4, respectively.

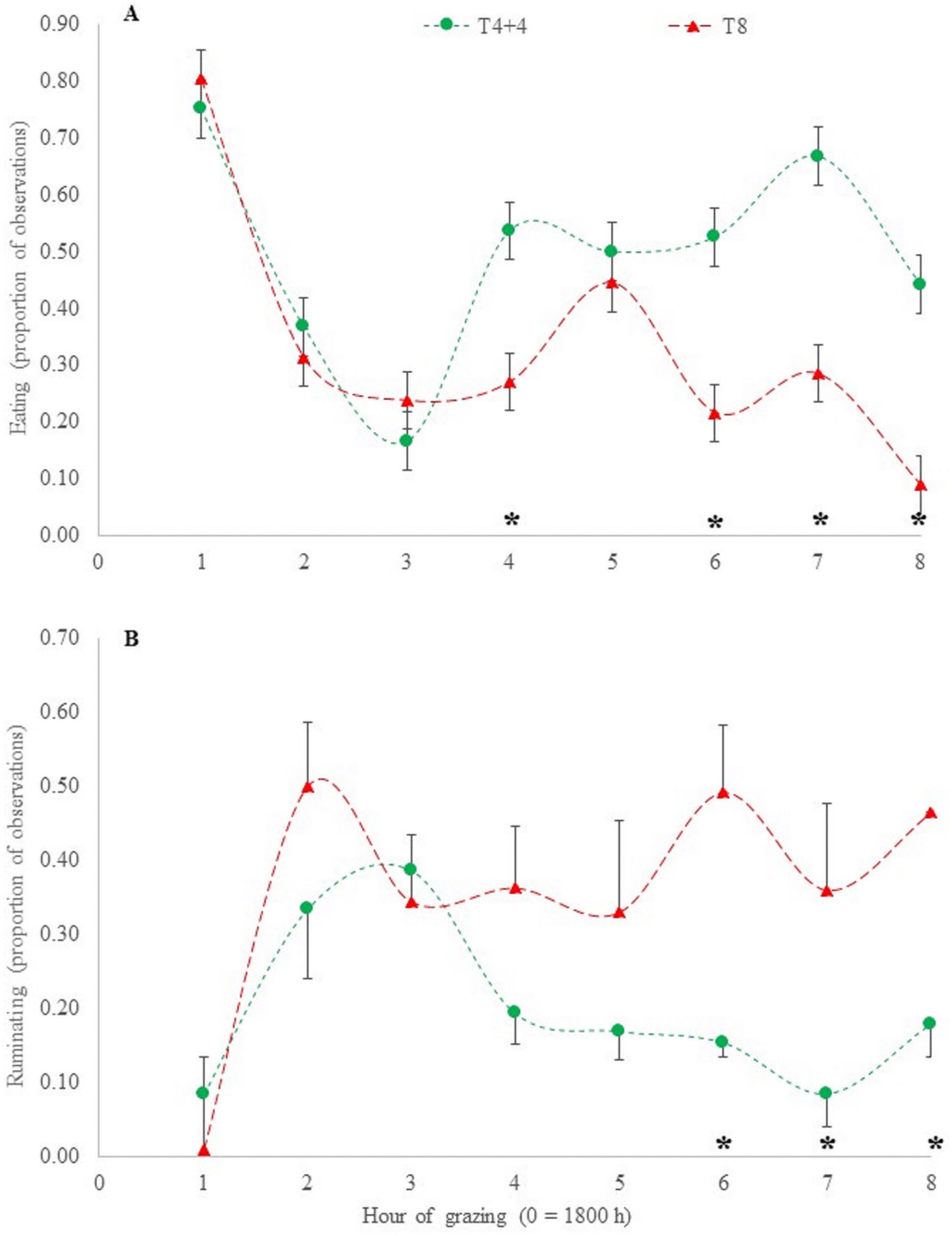

Figure 2. Eating (A) and ruminating (B) events as a proportion of the total hourly observations during the grazing time of dairy cows fed partial mixed ration plus 8 h access to alfalfa grazing in 1 (T8) versus 2 daily (T4+4) sessions. In each hour, at least 1 difference among treatments ($P \leq 0.05$) is indicated with an asterisk (*). Error bars represent the SEM.

T0 cows, both expressed in g/d, or as % of N ingested. Milk N secretion was similar between treatments. Cows on T4+4 had a higher N intake from alfalfa, and higher fecal N excretion compared with T8 cows (Table 6).

No differences among treatments were observed for any of the productive variables or feeding efficiency (Table 7). In both groups of cows with access to grazing (T8 and T4+4), the C18:1 *trans*-11 concentration was

Table 4. Ruminal H, VFA, and ammonia-N concentrations of dairy cows fed a TMR exclusively (T0) or partial mixed ration (PMR) plus 8 h access to alfalfa grazing in 1 (T8) or 2 (T4+4) daily sessions

	Treatment (Trt)[1]				Probability[2]				
Item	T0	T8	T4+4	SEM	Trt	H	Trt × H	T0 vs. (T8 and T4+4)	T8 vs. T4+4
pH									
Mean	6.29	6.19	6.16	0.083	0.004	<0.001	0.067	0.001	0.432
Maximum	6.73	6.48	6.69	0.055	0.001	—	—	0.007	0.001
Minimum	5.91	5.76	5.76	0.100	0.050	—	—	0.016	0.998
Range	0.81	0.73	0.93	0.077	0.034	—	—	0.788	0.011
VFA (mol/100 mol)									
Acetate	66.8	65.5	68.2	3.26	0.209	0.001	0.204	0.971	0.083
Propionate	22.0	23.5	21.2	1.05	0.137	0.014	0.227	0.716	0.047
Butyrate	11.2	11.2	10.5	2.56	0.191	<0.001	0.005	0.397	0.149
Σ^3 (m*M*)	108.9	111.1	112.6	15.20	0.833	0.011	0.394	0.605	0.836
Lactate (m*M*)	4.8	4.6	6.6	1.37	0.158	<0.001	0.029	0.364	0.070
Acetate:propionate	3.6	3.1	3.4	0.53	0.689	0.450	0.626	0.418	0.675
(Acetate+butyrate):propionate	4.2	3.6	3.9	0.51	0.609	0.549	0.589	0.357	0.618
NH_3-N (mg/100 mL)									
Mean	12.6	13.5	15.7	1.26	<0.001	<0.001	0.013	0.001	0.003
Maximum	24.5	25.4	27.1	3.38	0.712	—	—	0.546	0.584
Minimum	4.6	5.4	5.9	1.00	0.477	—	—	0.272	0.620
Range	19.9	20.1	21.0	2.80	0.922	—	—	0.803	0.759

[1]Treatments: T0 = 100% TMR; T4+4 = 60% PMR plus access to alfalfa pasture after a.m. and p.m. milking (between 0800 to 1200 h and 1800 to 2200 h); T8 = 60% PMR plus access to alfalfa pasture after p.m. milking (between 1800 to 0200 h).

[2]Probability of Trt = the main effect of treatment; H = effect of sampling hour; Trt × H = interaction between treatment and sampling hour; T0 versus (T8 and T4+4) = orthogonal contrast comparing T0 versus the average of T8 and T4+4; T8 versus T4+4 = orthogonal contrast comparing T8 versus T4+4.

[3]Σ = sum of acetate, propionate, and butyrate.

higher, and the C16:0 and C17:0 concentrations were lower, while C18:2 *cis*-9,*trans*-11 tended to be higher, compared with T0 cows (Table 8).

DISCUSSION

In the present study, alfalfa inclusion in the diet was 35 and 38% of the total DMI for cows in T8 and T4+4, respectively, while the total DMI was 10.2% lower than that in T0 cows. Total DMI reduction with herbage inclusion agrees with previous reports of authors working with grasses. For example, Civiero et al. (2021) observed a DMI reduction of 25% with the inclusion of a C4 grass (pearl millet, *Pennisetum glaucum*), while Bargo et al. (2002) and Pastorini et al. (2019), observed DMI reductions near to 30% when C3 grasses (ryegrass) were included. The lower DMI observed when cows consumed herbage plus PMR diets (T8 and T4+4) may be related to the final moisture concentration of the diets (Cabrera Estrada et al., 2004). The moisture in diets was 70 and 71% for T8 and T4+4, respectively, while the moisture in T0 was 53%. In this sense, according to Kellems et al. (1991), when the moisture concentration in the diet is above 50%, an increase of each percentage point causes DMI to decrease an equivalent to 0.02% of BW, which agrees with the values found in the present study (3 kg and 1.7 kg lower for T8 and T4+4, respectively, compared with T0). In turn, this lower DMI may be related to a lower intake rate of cows in grazing treatments: estimated average intake rates were 3.7, 2.9, and 2.8 kg DM/h for T0, T8, and T4+4, respectively. Another possible explanation for the lower DMI in the treatments T8 and T4+4 may be related to the higher DM and OM digestibility in T0 cows. The lower OM digestibility in diets with grazed herbage, in turn, may be a consequence of a decrease in the NFC consumed (1.3 kg on average) in T4+4 and T8 treatments. It is necessary to consider, however, that according to Morris et al. (2018), digestibility values could be overestimated due to the few spot samples used. Although the high ruminal distension determined by fiber fractions is considered the main cause of lower voluntary DMI in grazing cows (Allen et al., 2019), in this study, all diets had similar total NDF (%), forage NDF (%) and ADF/NDF ratios, which were consistent with the NASEM (2021) recommendation.

Alfalfa intake in T4+4 cows was 16% higher than in T8, but this did not lead to an increase in total DMI. This explains the similar milk production in T8 and T4+4 treatments, following previous reports in cows fed herbage plus PMR diets during mid-late lactation (Dall-Orsoletta et al., 2016). Our results suggest that under conditions similar to those of the present experiment (type of animals and diets), performing 2 grazing

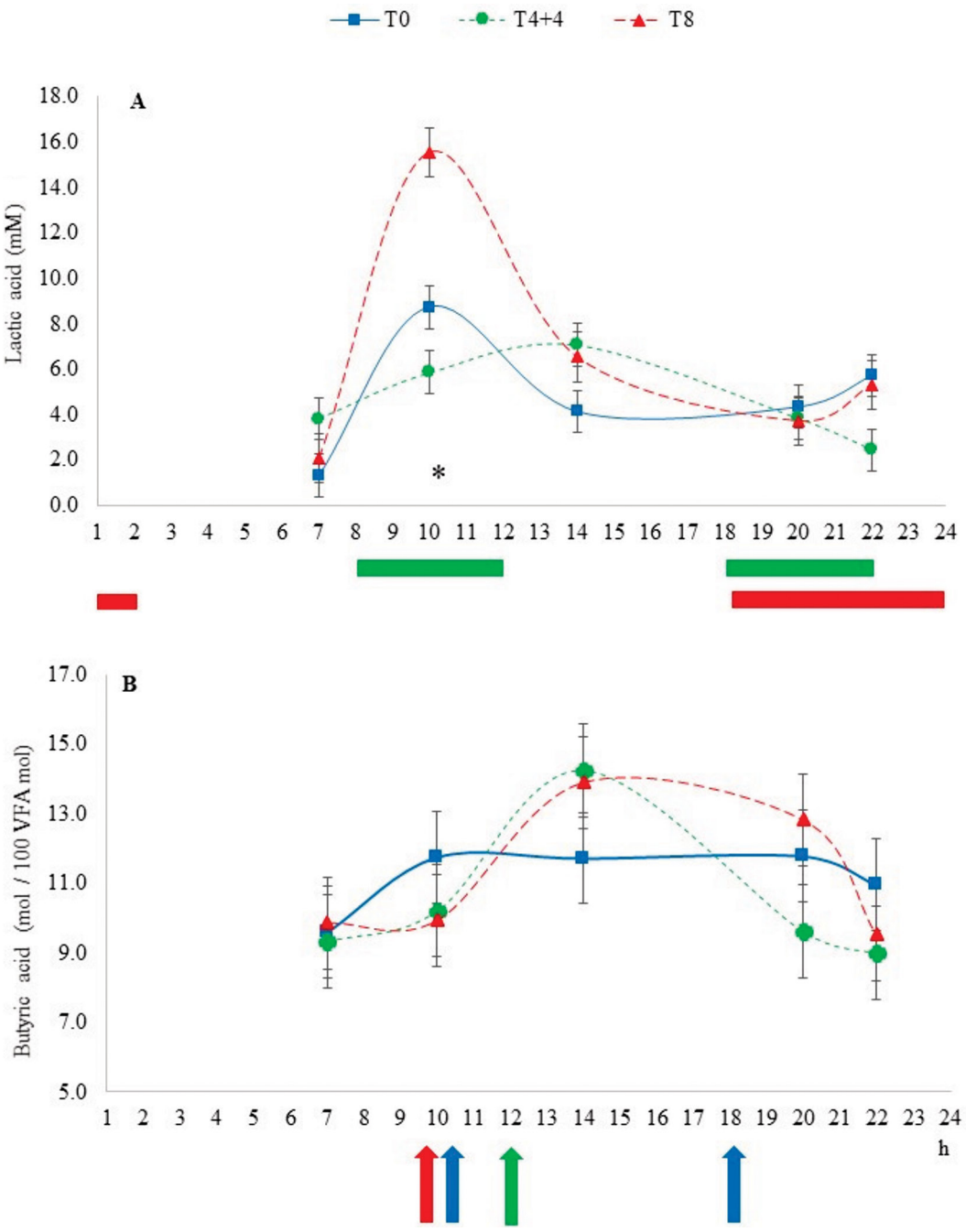

Figure 3. Ruminal lactate (A) and butyrate (B) concentration of dairy cows fed a TMR exclusively (T0) or partial mixed ration (PMR) plus 8 h access to alfalfa grazing in 1 (T8) versus 2 daily (T4+4) sessions. In each hour, at least 1 difference among treatments ($P \leq 0.05$) is indicated with an asterisk (*). Error bars represent the SEM. Green and red bars indicate the period when T4+4 and T8 had access to alfalfa grazing, respectively. Blue, red, and green arrows indicate the TMR delivery time in T0 or the PMR delivery time in T8 and T4+4, respectively.

sessions per day would not be justified, considering the higher labor and time spent by the cows during walking.

Although it cannot be discarded that intake at grazing may have been underestimated due to inaccuracies of the measurement method, the 14 kg DM/cow/d (5 cm from the ground) of alfalfa offered in this study may have limited pasture DMI. Cows in T8 and T4+4 treatments should consume an average of 9.2 kg DM of alfalfa/cow per day to match the total DMI of T0 cows. In herbage plus PMR diets and similar levels of PMR as used in this study, Ison et al. (2020a) reported that maximum alfalfa DMI is achieved with herbage

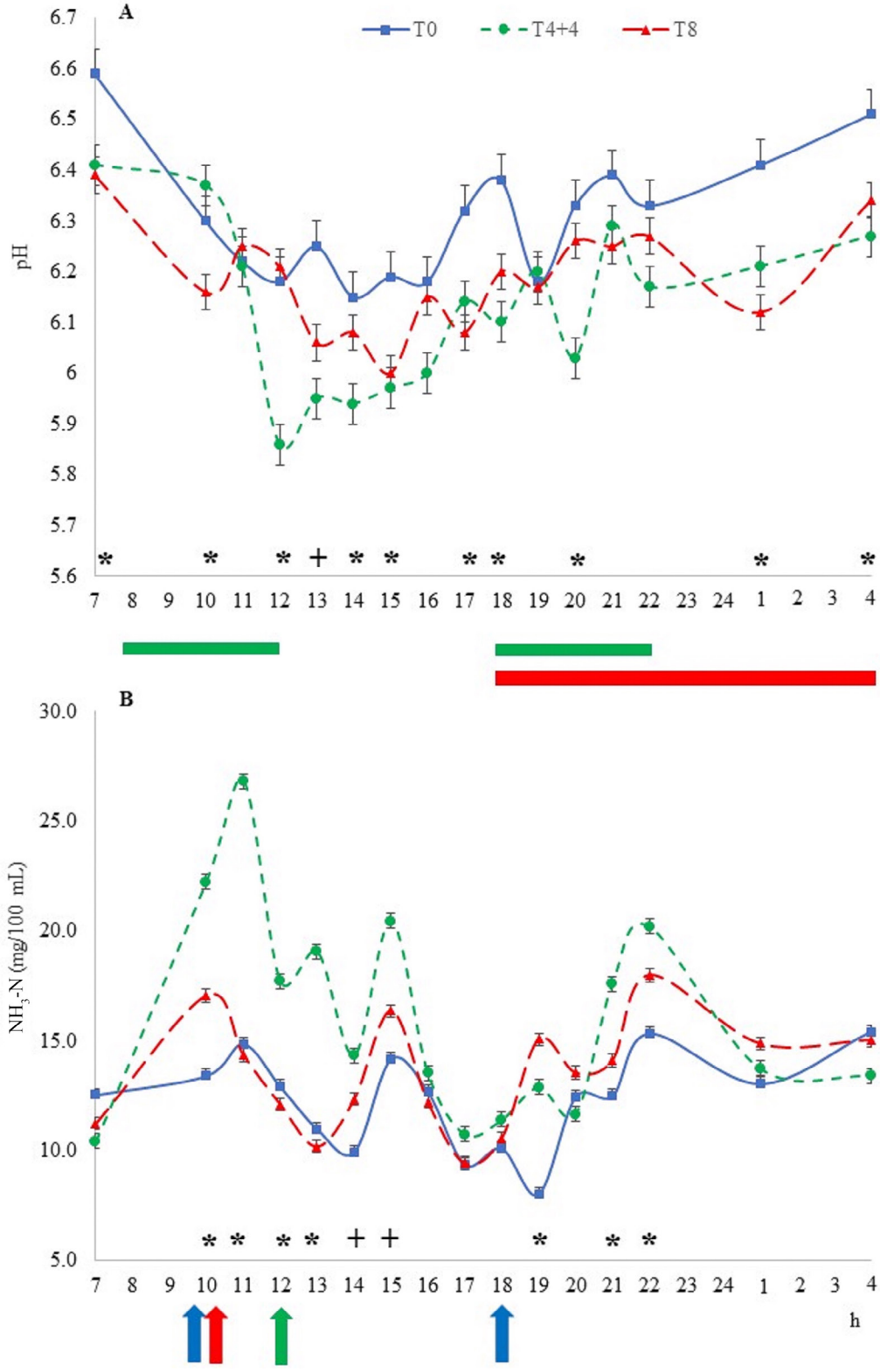

Figure 4. Ruminal pH (A) and NH_3-N concentrations (B) of dairy cows fed a TMR exclusively (T0) or partial mixed ration (PMR) plus 8 h access to alfalfa grazing in 1 (T8) versus 2 daily (T4+4) sessions. Asterisks (*) or crosses (+) at each hour indicate at least 1 difference among the treatments, $P \leq 0.05$ or $0.05 < P \leq 0.10$, respectively. Error bars represent SEM. Green and red bars indicate the period when T4+4 and T8 had access to alfalfa grazing, respectively. Blue, red, and green arrows indicate TMR delivery time in T0 or PMR delivery time in T8 and T4+4, respectively.

Table 5. Creatinine and purine derivatives (PD) of dairy cows fed a TMR exclusively (T0) or partial mixed ration (PMR) plus 8 h access to alfalfa grazing in 1 (T8) or 2 (T4+4) daily sessions

	Treatment[1]				Contrast probability[2]	
Item	T0	T8	T4+4	SEM	T0 vs. (T8 and T4+4)	T8 vs. T4+4
Creatinine (m*M*)	4.4	3.9	3.8	0.23	0.017	0.733
Alantoin (m*M*)	9.3	7.1	6.9	0.89	0.005	0.827
Uric acid (m*M*)	0.8	0.6	0.6	0.14	<0.001	0.384
PD[3] (m*M*)	10.1	7.7	7.4	0.91	0.003	0.780
PD:creatinine	2.3	2.0	2.0	0.15	0.069	0.852
PD (mmol/d)	352.7	311.0	306.0	22.56	0.089	0.863

[1]Treatments: T0 = 100% TMR; T4+4 = 60% PMR plus access to alfalfa pasture after a.m. and p.m. milking (between 0800 to 1200 h and 1800 to 2200 h); T8 = 60% PMR plus access to alfalfa pasture after p.m. milking (between 1800 to 0200 h).

[2]T0 versus (T8 and T4+4) = orthogonal contrast comparing T0 versus the average of T8 and T4+4; T8 versus T4+4 = orthogonal contrast comparing T8 versus T4+4.

[3]PD = purine derivatives.

allocations of 29 kg DM/cow/d, which is much greater than the values used in the study. Herbage DMI in the current experiment agrees with Gallardo et al. (2005) working with late-lactation cows, who observed an herbage alfalfa DMI of 7.7 kg/d in a total of 22.2 kg/d DMI and an herbage allowance similar to that used in the present experiment. The fact that the higher amount of nutrients consumed in favor of T0 was not reflected in a greater amount of milk or changes in feed efficiency, may be related to the late stage of lactation. Most likely, the greatest energy retained in T0 was diverted to body tissues. Although in this case the short duration of the treatments did not allow for the evaluation of differences in tissue deposition, the cows increased an average of 53 kg of BW during the experiment.

Table 6. Intake, digestibility, and N balance of dairy cows fed a TMR exclusively (T0) or partial mixed ration (PMR) plus 8 h access to alfalfa grazing in 1 (T8) or 2 (T4+4) daily sessions

	Treatment[1]				Contrast probability[2]	
Item	T0	T8	T4+4	SEM	T0 vs. (T8 and T4+4)	T8 vs. T4+4
N intake (g/d)						
TMR	735	417	423	70.3	<0.001	0.866
Alfalfa	—	229	266	11.5	—	0.028
Total	735	646	689	65.0	0.039	0.242
CP digestibility (%)	74.7	71.0	69.9	1.48	0.011	0.505
Urinary N excretion						
g/d	298	263	251	37.3	0.018	0.986
% of N intake	39.7	40.7	36.4	2.88	0.105	0.264
Fecal N excretion						
g/d	186	163	204	12.9	0.872	0.029
% of N intake	25.3	25.2	29.6	2.32	0.187	0.153
Manure N excretion						
g/d	484	425	455	41.8	0.051	0.182
% of N intake	65.9	65.8	66.0	2.54	0.616	0.980
Milk N excretion						
g/d	210	203	213	22.7	0.825	0.423
% of N intake	28.6	31.4	30.9	3.76	0.376	0.743
N balance						
g/d	41	17	21	19.05	0.067	0.527
% of N intake	5.6	2.6	3.1	2.64	0.094	0.482

[1]Treatments: T0 = 100% TMR; T4+4 = 60% PMR plus access to alfalfa pasture after a.m. and p.m. milking (between 0800 to 1200 h and 1800 to 2200 h); T8 = 60% PMR plus access to alfalfa pasture after p.m. milking (between 1800 to 0200 h).

[2]T0 versus (T8 and T4+4) = orthogonal contrast comparing T0 versus the average of T8 and T4+4; T8 versus T4+4 = orthogonal contrast comparing T8 versus T4+4.

Table 7. Milk production and composition of dairy cows fed a TMR exclusively (T0) or partial mixed ration (PMR) plus 8 h access to alfalfa grazing in 1 (T8) or 2 (T4+4) daily sessions

	Treatment[1]				Contrast probability[2]	
Item	T0	T8	T4+4	SEM	T0 vs. (T8 and T4+4)	T8 vs. T4+4
Milk (kg/d)	24.2	24.8	26.3	2.00	0.445	0.459
4.0% SCM[3]	25.6	25.7	26.7	2.18	0.699	0.600
Fat (%)	4.3	4.3	4.1	0.17	0.409	0.271
Fat (kg/d)	1.0	1.0	1.1	0.09	0.836	0.721
Protein (%)	3.75	3.58	3.55	0.096	0.095	0.849
Protein (kg/d)	0.9	0.9	0.9	0.08	0.810	0.541
Lactose (%)	4.8	4.8	4.8	0.05	0.648	0.526
Lactose (kg/d)	1.2	1.2	1.3	0.10	0.455	0.501
Feed efficiency[4]	1.16	1.30	1.28	0.143	0.196	0.833

[1]Treatments: T0 = 100% TMR; T4+4 = 60% PMR plus access to alfalfa pasture after a.m. and p.m. milking (between 0800 to 1200 h and 1800 to 2200 h); T8 = 60% PMR plus access to alfalfa pasture after p.m. milking (between 1800 to 0200 h).

[2]T0 versus (T8 and T4+4) = orthogonal contrast comparing T0 versus the average of T8 and T4+4; T8 versus T4+4 = orthogonal contrast comparing T8 versus T4+4.

[3]SCM = solid corrected milk (kg/d).

[4]Feed efficiency = 4.0% SCM production (kg/d)/DMI (kg/d).

The proportion of time that cows spent eating increased after each milking, as previously described by Orr et al. (2001). In T8 and T4+4 the cows had a higher eating time than T0, probably as a mechanism to achieve a nutrient intake according to their requirements. On the contrary, they spent less time ruminating than T0 cows, consistent with the higher time invested in eating activities (Kilgour, 2012). In T4+4 cows NE_L and N intake from alfalfa increased by 16% compared with T8 cows. This can be explained, at least in part, by a greater proportion of daylight grazing in the T4+4 treatment. According to Rook et al. (1994), approximately 80% of the time dedicated to grazing occurs during daylight. On the contrary, in the T8 treatment, the cows mostly grazed after sunset (75% of the time), which could have limited the intake of pasture in this treatment. Cows in T4+4 compensated for shorter rumination time during grazing by ruminating at other times of the day. The decrease in rumination during grazing, in T4+4 agrees with the report by Dall-Orsoletta et al. (2016). These authors analyzed feeding behavior in cows with 6 h of pasture access in 1 or 2 sessions using a more fibrous herbage than that of our experiment (54% vs. 35% NDF as % DM).

The days between samplings of the ruminal liquid between periods were 18. The time necessary for ruminal microbiome stabilization is still controversial. Some authors consider that 2 wk (de Menezes et al., 2011) or less (Machado et al., 2016; Weimer et al., 2017; Ricci et al., 2022) is enough, but others reported the need of using much longer periods (Clemmons et al., 2019). Given the above considerations, in this experiment, special care was taken to minimize differences in nutrient intake, despite the lower grain intake of cows fed herbage plus PMR. The calculated concentration of nutrients in the diets consumed by the cows in T0, T8, and T4+4 were 38.0, 36.0, and 35.7% of NFC; 32.0, 33.5, and 33.3% of NDF; 20.0, 20.4, and 20.3% of CP, respectively. Therefore, the changes observed in this experiment due to the treatments, particularly in ruminal pH and NH_3-N concentrations, seem to have been linked to the ingestive behavior. The lower ruminal pH in cows in T8 and T4+4 is probably related to decreased rumination time, which led to lower saliva production and liquid fraction flow reduction, compared with the T0 treatment (Welch and Smith., 1970; Rauch et al., 2012; Pérez-Ruchel et al., 2013). Moreover, although the alfalfa buffer capacity is greater than in other pasture forages such as grasses (Stepanova and Volovik, 2021), it might not have been enough to counteract the lower contribution of sodium bicarbonate and magnesium oxide in the PMR in T8 and T4+4 treatments. The fact that the ΣVFA concentrations among treatments were similar may reinforce the idea that there could be differences in the supply of buffer substances that led to a lower pH. Also, different fiber characteristics of the diets could affect fiber degradation dynamics, passage rate, and ruminal pH (White et al., 2017; Allen et al., 2019). For example, the alfalfa herbage and the corn silage used in TMR (its main source of fiber) had different ADF/NDF ratios (0.76 and 0.62, for herbage alfalfa and corn silage, respectively). In addition, splitting the grazing time into 2 sessions implied a greater fluctuation in ruminal pH, with more daily hours below 6, compared with T8. For the lactate concentration, the interaction

Table 8. Milk fatty acid (FA) profile and components of dairy cows fed a TMR exclusively (T0) or partial mixed ration (PMR) plus 8 h access to alfalfa grazing in 1 (T8) or 2 (T4+4) daily sessions

	Treatment[1]				Contrast probability[2]	
Item	T0	T8	T4+4	SEM	T0 vs. (T8 and T4+4)	T8 vs. T4+4
FA concentration (g/100 g of total FA)						
C6:0	1.33	1.78	1.55	0.195	0.179	0.419
C8:0	1.05	1.32	1.27	0.151	0.142	0.791
C10:0	2.96	3.57	3.67	0.387	0.094	0.815
C10:1 *trans*	0.29	0.32	0.33	0.054	0.349	0.945
C11:0	0.057	0.45	0.07	0.232	0.458	0.255
C12:0	4.08	3.93	4.50	0.393	0.749	0.268
C13:0	0.11	1.34	0.11	0.718	0.487	0.238
C14:0	13.53	12.67	13.74	0.824	0.732	0.332
C14:1 *cis*-9	1.32	1.24	1.14	0.142	0.263	0.462
C15:0	1.42	1.13	1.34	0.119	0.107	0.115
C16:0	40.61	37.82	37.60	1.713	0.019	0.865
C16:1 *cis*-9	2.02	1.93	1.86	0.140	0.418	0.680
C17:0	0.51	0.45	0.45	0.025	0.038	0.868
C17:1 *cis*	0.14	0.14	0.13	0.011	0.684	0.477
C18:0	7.77	8.14	8.18	0.820	0.507	0.948
C18:1 *trans*	0.14	0.09	0.13	0.025	0.221	0.240
C18:1 *cis*-9[3]	17.94	18.42	18.47	1.059	0.605	0.963
C18:1 *trans*-11 (TVA)[4]	0.99	1.25	1.59	0.323	0.032	0.132
C18:2	1.57	1.35	1.31	0.124	0.092	0.817
C18:2 *cis*-9,*trans*-11 (CLA)[5]	0.39	0.47	0.61	0.074	0.078	0.723
C18:3 *cis*-9,*cis*-12,*cis*-15[6]	0.20	0.26	0.25	0.039	0.301	0.817
C20:0	0.14	0.09	0.23	0.061	0.830	0.146
Summation by origin						
De novo (4:0–15:0)	27.49	29.54	29.12	2.007	0.334	0.848
Mixed origin (16:0+16:1)	42.64	39.76	39.46	1.780	0.016	0.825
Preformed (>17:0)	29.86	30.69	31.36	2.270	0.509	0.740
Summation by saturation						
SFA	74.92	74.48	74.13	1.460	0.620	0.805
MUFA	22.87	23.43	23.68	1.283	0.522	0.844
PUFA	2.20	2.07	2.14	0.205	0.636	0.762
UFA	25.07	25.51	25.83	1.458	0.632	0.828
Summation *trans* FA	1.84	2.13	2.63	0.418	0.037	0.093
Saturated:unsaturated ratio	3.07	2.99	2.97	0.247	0.656	0.908
n-6:n-3 ratio						
Δ9-desaturase index[7]	0.27	0.28	0.28	0.014	0.386	0.736
Atherogenicity index[8]	4.06	3.72	3.91	0.403	0.441	0.606

[1]Treatments: T0 = 100% TMR; T4+4 = 60% PMR plus access to alfalfa pasture after a.m. and p.m. milking (between 0800 to 1200 h and 1800 to 2200 h); T8 = 60% PMR plus access to alfalfa pasture after p.m. milking (between 1800 to 0200 h).

[2]T0 versus (T8 and T4+4) = orthogonal contrast comparing T0 versus the average of T8 and T4+4; T8 versus T4+4 = orthogonal contrast comparing T8 versus T4+4.

[3]Oleic acid.

[4]Vaccenic acid.

[5]Rumenic acid.

[6]Linolenic acid.

[7]Calculated as (14:1 *cis*-9 + 16:1 *cis*-9 + 18:1 *cis*-9 + 18:2 *cis*-9,*trans*-11)/(14:0 + 16:0 + 18:0 + 18:1 *trans*-11 + 14:1 *cis*-9 + 16:1 *cis*-9 + 18:1 *cis*-9 + 18:2 *cis*-9,*trans*-11).

[8]Calculated as (12:0 + 4 × 14:0 + 16:0)/(MUFA + PUFA).

treatment × hour shows a peak at the time of the main intake of PMR (at 1000 h).

As stated in the methodology section, TMR and alfalfa N concentrations exceeded NRC (2001) recommendations and were similar. Therefore, the higher DMI of T0 led to higher N intake in this treatment than when cows had access to grazing. The tendency to a higher NB observed for T0 is explained by the higher N intake and the similar manure excretions (expressed as a percentage of N ingested) among treatments. The higher fecal excretion in T4+4 treatment to T8 is explained by its higher N intake because CP digestibility was similar. Although ruminal concentrations of NH_3-N and % RDP (estimated using composition and intake obtained in this experiment, with NRC 2001 software) were 11 mg NH_3-N/dL and 12% RDP of DM, respectively, as suggested

for maximizing the flow of microbial protein (Reynal and Broderick, 2005), the PD excretion was lower than previous studies (Mendoza et al., 2016b; Pastorini et al., 2019), in line with the lactation stage of the cows of this experiment. We could consider the fact that 2 samples are few to predict urine excretion after Lee et al. (2019). However, both Mendoza et al. (2016b) and Pastorini et al. (2019) used the same sampling method as the present experiment. The entrance of nutrients to the rumen related to the ingestive behavior led to NH_3-N peaks in the rumen. It is known that ruminants can tap excess N by urea recycling and microbial glycogen reserves. Other authors reported that this process is not always efficient, particularly under conditions of high levels of N. Castillo et al. (2001) reported that above 400 g N/d supply, urinary N excretion increases with the amount and degradability of N ingested, leading to low N use efficiency. This is consistent with the results of our study and others (Mulligan et al., 2004; Colmenero and Broderick, 2006).

Even though cows in T0 had greater OM intake and NFC digestibility, only a tendency of higher PD excretion was found, which may be related to a possible lack of accuracy of the method used, or the fact that a portion of NFC was digested in the intestine, thus limiting the amount of fermentable OM in the rumen. In contrast, it was expected to find greater fecal N excretion due to higher metabolic N excretion linked to greater DMI in T0 (30 g/kg of DMI; NASEM, 2021). Similar to the ruminal pH results, ruminal concentrations of NH_3-N showed greater fluctuations in T4+4 compared with T8, surely associated with the different feeding behavioral patterns. In this sense, for cows in T4+4, many hours had passed because the ingestion of PMR (which had started 22 h earlier) when the cows entered the morning grazing session (0800 h), so NFC available in the rumen was the lowest daily amount. The NH_3-N peaks recorded when cows grazed in 2 sessions (T4+4) could then reflect a high N availability from herbage and lower use of it due to low NFC available from PMR. This could have limited the utilization of NH_3-N by ruminal microorganisms. However, grazing time did not modify PD excretion, or the NB, which is consistent with the absence of differences in OM intake and OM digestibility.

Previous studies reported that herbage inclusion in the diets of lactating dairy cows increased the proportion of unsaturated fatty acids. However, the effect of alfalfa inclusion in T8 and T4+4 treatments seems to have been low relative to other studies (Mendoza et al., 2016b; Grille et al., 2022; Pozo et al., 2022), especially considering that in this experiment herbage represented more than 35% of the diet. The lower effect of herbage DMI on the C18:2 *cis*-9,*trans*-11 concentration could be related to a lower concentration of C18:3 *cis*-9,*cis*-12,*cis*-15 acid in alfalfa than in grasses and up to 20% lower compared with annual ryegrass (Glasser et al., 2013).

CONCLUSIONS

Late-lactating dairy cows fed a restricted amount of PMR and grazing alfalfa during 8 h, had 10% lower DMI and lower nutrient digestibility compared with cows consuming only TMR ad libitum. Milk solids production and feed efficiency were similar in all groups, but the C18:1 *trans*-11 concentration of the milk fat was greater in cows on the herbage plus PMR diets. In the herbage plus PMR diets, 2 sessions of 4 h of alfalfa grazing instead of 1 session of 8 h, increased the proportion of nutrients from alfalfa in the diet but maintained the total nutrient intake and milk production. Considering the higher labor and time spent for a duplicated grazing session, this management is not justified under the conditions of the present experiment.

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