MILK PRODUCTION AND NITROGEN EXCRETION BY DAIRY COWS IN LATE LACTATION FED CUT PASTURES OF DIFFERENT RYEGRASS – CLOVER SPECIES IN SMALL-SCALE DAIRY SYSTEMS†

[PRODUCCIÓN DE LECHE Y EXCRECIÓN DE NITRÓGENO POR VACAS LECHERAS EN LACTACIÓN TARDÍA EN CORTE Y ACARREO DE PRADERA DE RYEGRASS-TRÉBOL EN SISTEMAS DE PRODUCCIÓN DE LECHE EN PEQUEÑA ESCALA]

Maria Danaee Celis-Alvarez[1], Carlos Manuel Arriaga-Jordán[1], Manuel González-Ronquillo[2], Aurora Sainz-Ramírez[1], Gonzalo Flores-Calvete[3] and Felipe López-Gonzalez[1*]

[1] Instituto de Ciencias Agropecuarias y Rurales (ICAR), Universidad Autónoma del Estado de México (UAEM), Campus UAEM El Cerrillo, El Cerrillo Piedras Blancas, 50090 Toluca, Estado de México, México. Email. flopezg@uaemex.mx
[2] Facultad de Medicina Veterinaria y Zootecnia. Universidad Autónoma del Estado de México (UAEM), Campus UAEM El Cerrillo, El Cerrillo Piedras Blancas, 50090 Toluca, Estado de México, México.
[3] Centro de Investigaciones Agrarias de Mabegondo (CIAM), Betanzos a Mesón do Vento, 15318 Mabegondo-Abegondo, La Coruña, Galicia, España.
**Corresponding author*

SUMMARY

Background: Nitrogen (N) plays an important role in dairy systems, both in production costs and as an indicator of environmental impact. **Objective:** The objective was to investigate the effects on milk production and nitrogen excretion by dairy cows fed cut pastures either of perennial ryegrass-red clover (PRG-RCL) or annual ryegrass-white clover (ARG-WCL), complemented with ground maize straw and of commercial concentrate. **Methodology:** Six dairy cows were used in a double cross-over on-farm experiment with 14-day experimental periods. Milk yield and composition, live weight, body condition score, chemical composition of feeds and N content in feces, urine, milk and blood were recorded over the last four days of each experimental period. **Results:** No differences were found ($P > 0.05$) for milk yield nor composition. Cows on ARG-WCL had a higher N intake and N balance than those on PRG-RCL ($P < 0.05$); also, there were higher levels of blood urea nitrogen (BUN) and urine urea nitrogen (UUN) when cows were fed ARG-WCL than PRG-RCL ($P < 0.05$). **Implications:** This work allows us to know the amount of nitrogen excreted in milk production in small-scale dairy systems. **Conclusions:** It is concluded that PRG-RCL may be a feeding strategy to reduce N excretion and achieve higher efficiency in N use for small-scale dairy systems.
Keywords: Nitrogen use efficiency; red clover; white clover; perennial ryegrass; annual ryegrass; Mexico.

RESUMEN

Antecedentes: El nitrógeno (N) juega un papel importante en los sistemas de producción de leche, tanto en los costos de producción como como indicador de impacto ambiental**. Objetivo:** El objetivo fue investigar los efectos sobre la producción de leche y la excreción de nitrógeno por vacas lecheras alimentadas con praderas de corte de ryegrass-trébol rojo perenne (PRG-RCL) o ryegrass-trébol blanco anual (ARG-WCL), complementado con 0.89 kg de MS. / vaca / día de paja de maíz molido y 4.07 ± 0.47 kg MS / vaca / día de concentrado comercial con 19% PC**. Metodología:** Se utilizaron seis vacas lecheras en un experimento doble reversible en finca con períodos experimentales de 14 días. La producción y composición de la leche, el peso vivo, la condición corporal, la composición química de los alimentos y el contenido de N en las heces, orina, leche y sangre se registraron durante los últimos cuatro días de cada período experimental. **Resultados:** No se encontraron diferencias (P> 0.05) para la producción ni la composición de la leche. Las vacas en ARG-WCL tuvieron una mayor ingesta de N y balance de N que aquellas en PRG-RCL (P <0.05); además, hubo niveles más altos de nitrógeno ureico en sangre (BUN) y nitrógeno ureico en orina (UUN) cuando las vacas fueron alimentadas con ARG-WCL que con PRG-RCL (P <0.05). **Implicaciones:** Este trabajo nos permite conocer la cantidad de nitrógeno excretado en la producción de leche en sistemas lecheros a pequeña escala. **Conclusiones:** Se concluye que PRG-RCL puede ser una estrategia de alimentación para reducir la excreción de N y lograr una mayor eficiencia en el uso de N para sistemas lecheros a pequeña escala.
Palabras clave: Eficiencia del uso de nitrógeno; trébol rojo; trébol blanco; ryegrass perenne; ryegrass anual; México.

† *Submitted September 28, 2020 – Accepted March 19, 2021.*
ISSN: 1870-0462.

INTRODUCTION

Nitrogen (N) utilization efficiency is 3.5 times smaller in livestock than in crops, while its excretion varies across livestock production systems (FAO, 2017). In the case of ruminants, the amount of N excreted ranges between 60% and 90%, via urine and faeces (Flachowsky and Lebzien, 2006). The growing concern regarding environmental pollution caused by animal production has contributed to an increased interest in improving the low efficiency of N retention of the animals without detriment to their production (Brito and Broderick, 2007); the above can be attained by means of improving feeding practices and using manure (Xiccato *et al*., 2005).

About 40% of all the cow milk produced at global level between 2012 and 2014 came from developing countries (FAO, 2016); of which 80% was supplied by small farmers (FAO, 2017). In Mexico, small-scale dairy systems (SSDS) supplied 37% of the national milk production (Hemme *et al*., 2007). The traditional feeding strategies used by small-scale dairy farmers in SSDS were described by Alfonso-Ávila *et al*. (2012) and Martínez-García *et al*. (2015) based, when irrigation is available, on perennial or annual ryegrass (*Lolium perenne* and *L. multiflorum*) pastures associated to white clover (*Trifolium repens*) traditionally managed under cut-and-carry systems.

SSDS in Mexico are heterogeneous in both technological and agro-ecological terms, so that there is an ample variation on the productivity of each farm (Camacho-Vera *et al*., 2017). In the central highlands of Mexico, small-scale dairy farms with access to some irrigation base the feeding strategies of their herds on small areas sown to temperate ryegrass/white clover cut-and-carry cultivated pastures (Fadul-Pacheco *et al*., 2013), similar to SSDS in southeast Asia (Moran, 2005). This herbage is a high quality component of diets (Martínez-García *et al*., 2015). In SSDS, a large amount of N may be excreted in feces and mostly urine, as the nutritional requirements of N are exceeded due to the large amount of commercial concentrates, with a high-protein content, supplied to the cows (Alfonso-Ávila *et al*., 2012; Martínez-García *et al*., 2015).

Associating legumes such as red clover (*Trifolium pratense*), and grass species has reported a reduction in N excretions (Bryant *et al*., 2017). Other studies mentioned that red clover contains polyphenol oxidase (PPO), which decreases N loss and positively influences on the efficiency in N use by ruminants (Eickler *et al*., 2011); this makes it an alternative legume to incorporate into ruminant feeding strategies.

It is important to have knowledge of N balance in production systems in order to produce an accurate quantification and be able to implement different feeding strategies that improve N retention (Xiccato *et al*., 2005; Pozo-Leyva *et al*., 2019). The use of red clover is not widespread among SSDS, though the use of white clover is.

The objective was to investigate the effects on milk production and nitrogen excretion by dairy cows fed cut pastures either of perennial ryegrass-red clover (PRG-RCL) or annual ryegrass-white clover (ARG-WCL), complemented with ground maize straw and of commercial concentrate.

MATERIALS AND METHODS

Study area

The experiment followed a participatory livestock research approach (Conroy, 2005), with an on-farm experiment. The farm is located in the municipality of Aculco, in the central highlands of Mexico at 20° 05’ 57” North and 99º 49’ 41” West; at a mean altitude of 2428 m); temperate sub-humid climate with summer rains and a mean annual temperature between 14 and 16°C; and 700-800 mm rainfall (INEGI, 2017). The mean temperature over the experiment was 17ºC, with minimum and maximum temperature means of 10ºC and 24º C, and 180 mm rainfall. The experiment lasted 42 days; from 16 August to 27 September 2019.

Pasture establishment and management

Two cut-and-carry pastures were utilized. Pasture 1, 0.5 ha (PRG-RCL), was established in March 2019 with a sowing rate of 28 kg/ha of perennial ryegrass (*Lolium perenne* cv. Tetragrain), 9 kg/ha of red clover (*Trifolium pratense* L. cv Emarwan) and 36 kg/ha of triticale (*X. Triticosale Witt.* cv Bicentenario) as a cover crop to prevent weed from growing and protect the red clover from late frosts; first growth, mainly of triticale, was cut with a forage harvester at 64 days post sowing. The pasture was left for one month to recover before cutting resumed following the conventional management in the area (Velarde-Guillen *et al*., 2019) till the start of the experiment. At 112 days post-sowing, 10 kg/ha red clover seed was broadcasted over the PRG-RCL pasture to increase herbage cover particularly in areas with low herbage present. The experiment started at 150 days post sowing of the PRG-RCL pasture. Fertilisation at sowing was 60 N-80 P-60 K kg/ha.

Pasture 2 was a 1.5 ha (ARG-WCL) plot already established for a number of years to annual ryegrass (*Lolium multiflorum*) left to head once a year to enable self-reseeding following traditional management in these systems, associated with white clover (*Trifolium repens* cv. Ladino), which tends to dominate grasses after some years (Velarde-Guillen *et al*., 2019).

Botanical composition

The botanical composition of the pastures was measured in three repetitions per pasture per day over the recording periods cutting an area of 0.16 m^2 (0.40 m x 0.40 m) each. Samples were manually separated in three component (grass, clover and forbs); the percentage contribution of such component was calculated from their corresponding fresh weights and dry matter contents.

Experimental design and cows

Six Holstein cows in the last third of lactation, 523 ± 46.5 kg mean live weight (BW), 214 ± 33 days in milk, and 15.4 ± 3.5 kg/cow/day initial mean milk yield (MY), were used in a double cross-over design with 14-day experimental periods; the first 10 days to adapt and 4 days for measurements (recording and sampling). Short experimental periods of 14 days are well established and accepted in feeding experiments with dairy cows (Miguel *et al*., 2014).

Cows were divided into two groups according to lactation days and pre-experimental milk production (Mulligan et al., 2004). The two groups were randomly assigned to each of the two possible treatment combinations following the sequences PRG-RCL ─ ARG-WCL ─ PRG-RCL (group 1) and ARG-WCL ─ PRG-RCL ─ ARG-WCL (group 2).

Treatments

Treatments were: PRG-RCL, perennial ryegrass associated with red clover and ARG-WCL, annual ryegrass associated with white clover under a conventional cut-and-carry system. The amount of pasture offered in troughs accounted for 2% of mean cow live weight (9.8 ± 1.8 kg DM/cow/day) + 4.07 ± 0.47 kg DM/cow/day commercial concentrate. Total intake was 13.87±2.27 kg DM/cow7day.

Animal variables

Milking was by hand at 05:00 and 17:00 h; and milk yield (MY) was recorded at both milking times. Cows were fed during milking. The chemical composition of milk (fat, protein and solids-not-fat) was determined with an ultrasound analyser (Lactoscan Model S), while milk urea nitrogen (MUN), by means of the colorimetric method (Chaney and Marbach,1962).

Fat-corrected milk (FCM) was calculated using the equation proposed by NRC (2001): 4% FCM (kg/cow/day) = 0.4 x MY kg + 15 x fat (kg/d); energy corrected milk (ECM) with 4.0% fat and 3.3% protein was calculated following the formula ECM (kg/cow/day) = [MY kg x (0.383 x fat% + 0.242 x protein% + 0.7832) /3.1138] (Ostergaard et al., 2003). N yield in milk (g/d) was calculated by CP in milk (g/d)/6.38 x MY (kg/cow/day) (AOAC, 1990).

Live weight (LW) and body condition score (BCS) were recorded at the end of each experimental period; LW by means of an electronic scale, while BCS, with a five-point (1 to 5) system (Wildman *et al*., 1982).

Commercial concentrate (CC) intake was defined on the day 1 of each period according to the metabolic live weight of each cow, and it was kept constant over the experimental period. Pasture intake (kg DM/cow a day) was determined on a daily basis recording the pasture offered and the amount refused by each cow.

Samples of 60 ml of urine were collected twice a day during each measurement period by means of vulvar stimulation; urine was acidified with 6N HCl (2ml) to prevent N from volatilising; then, the samples were frozen at -20ºC for later analyses. Additionally, samples of cow feces were collected *per rectum*, and frozen at -20ºC for later analyses. On the last day of each measurement period, blood samples of each cow were taken from the coccygeal vein in heparinised tubes 4 hours after morning milking; the samples were centrifuged, and the plasma stored at -20ºC.

Sample processing and laboratory analyses

Over the four days of each measurement period, daily samples of pasture of about 0.5 kg were collected and stored at -20ºC, and a single composite sample was prepared. A single sample of the each of the supplements (maize straw and concentrates) was collected per measurement period. The composite sample of pasture and supplements were dried for DM at 60ºC in a draught oven for 48 hours; and ground through a 2.0 mm sieve for chemical composition analysis. The samples were incinerated in a muffle at 550ºC to determine organic matter (OM), CP by Kjeldahl's method (N x 6.25), ether extract (EE) by means of AOAC protocols (1990). Neutral detergent fibre (NDF) and acid detergent fibre (ADF) with alpha-amylase and without ash correction, following Ankom (2019); and, the *in vitro* digestibility of dry matter (IVDMD) was processed by means of incubation with ruminal liquid using the micro-bags method in an Ankom Daisy II incubator following Ankom (2019). Starch content was determied with a commercial kit (product code: K-TSTA-100A, Megazyme, Bray, Ireland). The estimation of metabolisable energy (eEM) in food was carried out according to the equation described by Mackle et al. (1999): eEM (MJ kg DM) = 0.0156 x IVDMD - 0.535.

Urine samples were analysed by means of Kjeldahl's method, as described for food samples, for total nitrogen content. Urine urea nitrogen (UUN) was ascertained by means of spectrophotometry from a urine sample obtained on the last measurement day.

After drying at 55ºC for 72 hours, faeces samples were ground in a hammer mill, obtaining a single daily sample per cow from the mix of two same-weight aliquots. The total N content, ADF and NDF were calculated on the daily sampling of feces, as described for feed samples. Plasma samples were let to thaw at room temperature, homogenised and analysed to ascertain blood urea nitrogen (BUN), creatinine and uric acid by means of spectrophotometry.

Calculations

Total urine volume (TUV) was calculated from the LW of the cow using the equation TUV= (LW * 0.017) + 11.704, described by ASAE (2005).

Total dry matter excretion (DM_E) was from the regression equation by Nennich *et al.* (2005): DM_E (kg/cow/day DM) = MY (kg/cow/day) * 0.0874 + 5.6.

N urinary excretion for each cow was calculated as the urine volume multiplied by the mean N concentration in urine of each treatment; and N faecal excretion for each cow was calculated as faecal production multiplied by the mean N concentration in faces of each treatment.

N balance was calculated by N balance (g/d) = mean value of N consumed (g/d) – mean of the total N excreted in feces and urine (g/d) during each period (Totty *et al*., 2013).

Nitrogen use efficiency (NUE) in milk production was considered as use efficiency (%) = N excretion in milk / 100 g N ingested in diet (g/cow/day) (León *et al*., 2008).

Statistical analyses

The variables were analysed with a double cross-over design, according to the following model (Lawal, 2014): $Y_{ijkh} = \mu + S_i + C_{j(i)} + t_k + P_{h(i)} + e_{ijkl}$

Where: μ = general mean; S = fixed effect of the sequence, i = 1, 2 (PRG-RCL ─ ARG-WCL ─ PRG-RCL, ARG-WCL ─ PRG-RCL ─ ARG-WCL); C = random effect of the cows in the sequence, j = 1, 2, 3, 4, 5, 6; t = fixed effect of treatments, k = 1, 2 (PRG-RCL, ARG-WCL); $P_{h(i)}$ = random effect due to experimental period within treatment sequence, h = 1, 2, 3; e = residual error term. The data were analysed with ANOVA and a Tukey’s test was run if significant differences were detected ($P < 0.05$). Tukey´s test is used in this type of experimental designs (Lawal, 2014, pp. 474-483).

The chemical composition of the feeds was analysed with a completely randomised design by means of the model: $Y_i = \mu + Ti + e_i$.

Where: Y_i = response variable; μ = mean population; Ti = Effect of treatment; $e_=$ experimental error.

RESULTS

In terms of chemical composition, both pasture had a similar concentration of DM, OM, CP, NDF, ADF, eEM, IVDMD and starch, though PRG-RCL pasture had a 12-percent higher EE content than ARG-WCL (P<0.05) (Table 1). Over the three experimental periods, clover on average accounted for 38% of the total DM from ARG-WCL, and only 21% for that from PRG-RCL.

Table 2 shows the results of yield and chemical composition of milk. In which no significant differences are observed in the variables evaluated (P>0.05). Mean milk yield was 15.8 ± 3.6 kg/d, with an average chemical composition: 38.5 ± 3.8 g/kg fat; 28.9 ± 0.8 g/kg protein; and, 77.48 ± 1.7 g/kg solids-not-fat.

Table 1. Chemical composition (g /kg DM) of the ingredients used in dairy cow treatments and estimated metabolisable energy in (MJ/kg DM).

	DM	OM	CP	NDF	ADF	EE	IVDMD	eME	Starch
PRG-RCL	254	881	188	566	265	28[a]	755	11.24	35
ARG-WCL	278	890	223	586	281	25[b]	751	11.17	78
SEM	23.76	5.12	13.81	9.17	19.43	0.22*	19.49	0.31	17.67
P value	0.515	0.267	0.148	0.186	0.592	<0.05	0.883	0.883	0.168
MS	888	881	33	762	420	16	502	7.3	23
CC	887	916	199	238	65	ND	883	13.2	430

PRG-RCL: Perennial ryegrass-red clover; ARG-WCL: Annual ryegrass-white clover; MS: Maize straw; CC: Commercial concentrate; DM: Dry matter; OM: Organic matter; CP: Crude protein; NDF: Neutral detergent fibre; ADF: Acid detergent fibre; IVDMD : *in vitro* digestibility of dry matter; EE: Ethereal extract; eME: Estimated metabolisable energy; ND: Not determined; SEM: Standard error of the means. *$P < 0.05$; [a, b] $P < 0.05$.

Table 2. Production and chemical composition of milk in dairy cows fed two different pastures of perennial ryegrass-red clover vs annual ryegrass-white clover.

	Treatments			
	PRG-RCL	ARG-WCL	SEM	P value
Milk yield, kg/cow/day	15.6	15.9	0.27	0.070
4% FCM, kg/d	15.0	15.4	0.25	0.074
ECM, kg/d	16.0	16.4	0.18	0.084
Fat, g/kg	38.2	38.8	0.74	0.993
Fat, g/d	586.2	605.8	11.42	0.111
Protein, g/kg	29.03	28.79	0.23	0.623
Protein, g/d	450.7	454.8	8.19	0.112
SNF, g/kg	77.6	77.38	0.43	0.700
SNF, g/d	1204.7	1222.2	20.81	0.938
MY/DMI	1.07	1.04	0.02	0.349
FCM/DMI	1.01	1.02	0.02	0.778

PRG-RCL: Perennial ryegrass-red clover; ARG-WCL: Annual ryegrass-white clover; FCM: Fat-corrected milk 4%; ECM: Energy corrected milk (3.5% fat, 3.2% protein); SNF: Solids-non-fat; MY: Milk yield; DMI: Dry matter intake; SEM: Standard error of the means. *$P < 0.05$; $^{a, b}$ $P < 0.05$.

Table 3. Dry matter intake (kg DM/cow/day) in dairy cows fed two different pastures of perennial ryegrass-red clover v. annual ryegrass-white clover.

	Treatments			
	PRG-RCL	ARG-WCL	SEM	P value
DMI total, kg/d	14.7^{b}	14.9^{a}	0.02*	0.032
LW, kg	558.9	543.2	3.49	0.384
BCS, 1-5	2.3	2.3	0.02	0.427
Intake, kg DM/100 kg LW	2.62^{b}	2.73^{a}	0.05*	<0.05
Pasture, kg/d	9.7^{b}	10.0^{a}	0.24*	<0.05
Concentrate[1], kg/d	4.07	4.07	-	-
Maize straw, kg/d	0.89	0.89	-	-

PRG-RCL: Perennial ryegrass-red clover; ARG-WCL: Annual ryegrass-white clover; DMI: Dry matter intake; LW: Live weight; BCS: Body condition score; SEM: Standard error of the mean *$P < 0.05$; $^{a, b}$ $P < 0.05$. Concentrate[1]: Nutrisow 19% CP Trademark, ingredients as indicated on the label: ground and / or rolled cereal grains, cereal by-products, oilseed paste, by-products from cereal distillery, overfat fat, NNP, cane stump, vitamins; A, D3 and E, calcium carbonate, monodicalcium phosphate, magnesium oxide, sodium chloride, trace minerals, magnesium, zinc, copper, selenium and cobalt.

Dry mater intake (DMI) for ARG-WCL grass was significantly higher than for PRG-RCL ($P < 0.05$); thereby, total DMI and Intake in kg DM/100 kg LW showed differences between treatments ($P < 0.05$). Total feed intake on average was 14.8 ± 1.9 kg DM/cow/day (Table 3). There were no differences for variables LW and BCS ($P > 0.05$); being LW similar between treatments (551 ± 48 kg) and BCS remained at 2.4 ± 0.14.

Cows that were fed ARG-WCL had 15% higher N Intake (g/d) than those fed PRG-RCL ($P < 0.05$). However, no significant differences were observed ($P > 0.05$) in the percentage of N in urine and feces between treatments, nor in urinary N and N in feces ($P < 0.05$). As shown in Table 4, the diminution in N intake in PRG-RCL reduced N Balance per treatment ($P < 0.05$). There was a trend between the amount of N in feces / N intake ($P<0.051$) and the amount of N in urine / N intake ($P < 0.088$), being greater in PRG-RCL.

An NUE trend ($P < 0.065$) in PRG-RCL was evinced by the increase in N Intake (g/d) in diet and not in N in milk yield (g/d), which were not statistically different between treatments ($P > 0.05$). There were no significant differences ($P > 0.05$) in MUN content between treatments (Table 4).

There were statistical differences ($P < 0.05$) for UUN and toral ureic N excreted in urine, being significantly higher for PRG-RCL (Table 5). BUN contents were significantly different between treatments, being higher for PRG-RCL ($P < 0.05$).

DISCUSSION

Some of the factors that define the ratio of clover in a pasture under a cut-and-carry system are season, cutting time and age (Eriksen *et al*., 2012). In the present study, the clover ratio was higher for pasture ARG-WCL, which was established several years ago; this clearly shows the different crude protein contents in the two pastures.

Milk yield was 15.8 ±3.6 kg/cow/day; a lower value than those reported in other studies on small-scale milk production systems in the same area, assessing cut-and-carry pastures reported by Pincay-Figueroa *et al*. (2016) and Velarde-Guillen *et al*. (2019) with 17.8, and 19.2 kg/cow/day, respectively.

Johansen *et al*. (2017) did not find any effect on milk composition but did in milk production with red and white clover silages in Denmark with Holstein cows. Similar results are reported in the present study, where in terms of milk yield, there was a trend ($P < 0.070$) for higher milk yield in ARG-WCL.

N excretions in urine, feces and milk were lower than those reported by Spek *et al*. (2013) for lactating dairy cows in north-western Europe (212, 223 and 166 g/d) and North America (185, 159 and 133 g/d). Furthermore, a lower N excretion in feces in both treatments was noticed (144 g/d) in comparison with other authors (Spek, *et al*., 2013; Danes *et al*., 2013) in lactating cows.

Hristov *et al*. (2005) found a lower N excretion in feces (76%) in diets supplemented with fibre in comparison with starch; they attributed this difference in faecal N to the differences in the amounts of microbial N synthesised in the large

Table 4. Effect of the association of perennial ryegrass-red clover v. annual ryegrass white clover on nitrogen balance.

	Treatments			
	PRG-RCL	ARG-WCL	SEM	P value
Feces				
N in feces, %	2.04	2.11	0.06	0.685
OM, kg/d	3.11	3.22	0.09	0.081
NDF, kg/d	2.22	2.32	0.07	0.100
ADF, kg/d	1.26[b]	1.39[a]	0.06*	<0.05
N in feces/ N intake	0.34	0.30	0.01	0.051
Urine				
N in urine, %	0.74	0.77	0.05	0.389
N in urine/ N intake	0.38	0.33	0.02	0.088
Nitrogen Balance, g/d				
Intake N, g/d	423.6[b]	488.4[a]	15.63*	<0.01
Excreted, g/d				
N in feces	142.0	146.8	0.03	0.524
N urinary	158.2	161.5	14.61	0.411
N balance, g/d	123.4[b]	180.1[a]	15.86*	<0.05
Milk				
N in milk, g/d	4.54	4.51	0.03	0.703
N in milk yield, g/d	70.63	71.30	1.29	0.112
NUE %[1]	16.6	14.5	0.60	0.065
MUN, mg/dl	12.0	12.7	0.87	0.773

PRG-RCL: Perennial ryegrass-red clover; ARG-WCL: Annual ryegrass-white clover; DM: Dry matter; N: Nitrogen; OM: Organic matter; NDF: Neutral detergent fibre; ADF: Acid detergent fibre; MUN: Milk urea nitrogen; N in milk yield: milk yield true protein /6.38. NUE: Nitrogen use efficiency %[1]= N excretion in milk / 100 g of N ingested in the diet. SEM: Standard error of the means. *$P < 0.05$; [a, b] $P < 0.05$.

Table 5. Urine excretion of urea, uric acid and nitrogen and excretion of urea, creatinine and uric acid in blood in dairy cows fed two different pastures of perennial ryegrass-red clover v. annual ryegrass-white clover.

	Treatment			
	PRG-RCL	ARG-WCL	SEM	P value
Urine				
Urea, mg 100mL	22.35	25.74	1.84*	<0.05
Uric acid, m*M*/d	5.2	5.3	0.25	0.485
Urea nitrogen, g/d	104.1	120.0	0.86*	<0.05
Blood				
Urea, mg/dL	28.30	32.07	1.88*	<0.05
Creatinine, mg/dL	0.90	0.90	0.03	0.872
Uric acid, mg/dL	0.99	1.05	0.06	0.190

PRG-RCL: Perennial ryegrass-red clover; ARG-WCL: Annual ryegrass-white clover; SEM: Standard error of the means. *$P < 0.05$; [a, b] $P < 0.05$.

intestine and excreted in feces, and to the higher carbon: nitrogen ratio in feces from cows supplemented with fibre as well. As noticed in this present work, both treatments had a larger proportion of forage v. concentrate (72:28), and a larger N excretion in urine also. Almost 70% of the crude protein of the forages is degradable in rumen (RDP), the resulting ammonia may be utilized by rumen microorganisms to synthesise true microbial protein, or assimilated into the bloodstream to be absorbed, or recycled or transformed into urea and excreted mainly in urine (Pacheco and Waghorn, 2008).

ARG-WCL presented an excretion of faecal N only 3% higher regarding PRG-RCL; where the former treatment presented a higher percentage of clover in pasture composition, which may be due to the presence of PPO that the red clover has in comparation with the white clover (Eickler *et al*., 2011). Huhtanen *et al*. (2008) observed a larger production of faecal N in grass silages than in those in which the legume ratio was higher.

In a study that assessed various grass systems, Mc Auliffe *et al*. (2020) reported that the type of grass had an effect on urine and feces properties; for example, it was found that N content in herbage affects the concentration of faecal and urinary N.

Although there were no differences in urinary excretion comparing ARG-WCL with PRG-RCL, the first was the treatment with the largest crude protein content (20.0 % v. 18.2 %) and the largest total N excretion. A stronger effect on the excretion of urinary N is noticed in comparison with excretion in feces and milk when changes are effected on the total N intake of ruminants (Pacheco and Waghorn, 2008). A higher forage N content is attributed to harvesting at an early growing stage; therefore, large N excretions are produced (Wilkinson and Garnsworthy, 2017). Grabber (2009) noticed that early red clover cuts favourably decrease the content of non-degradable protein in rumen, fibres, and increase CP and RDP contents.

Spek *et al*. (2013) mentioned that diet and animal factors, as well as CP in diet and MUN are related to total N excretion in urine, and in combination, these two factors and dry matter intake, with UUN. Huntanen *et al*. (2008) related N excretion in urine to MUN. In the results of this study, it is noticed that a larger amount of CP in ARG-WCL expressed a larger total N excretion in urine (numerically) and higher UUN.

In a study on tropical grasses, Danes *et al*. (2013) reported higher BUN and MUN values and a linear decrease in NUE when PC content in diet increased. Brito and Broderick (2007) found an effect on the MUN and BUN levels for cows supplemented with various true proteins. In both treatments, a balance between MUN and BUN concentration was noticed, as well as large N excretions when N intake increases. The overproduction of ammonia in the rumen is the main reason for the transference of urea into the bloodstream (Pacheco and Waghorn, 2008).

Owing to the lactation stage of the cows, NUE for milk protein production was low in both treatments. In PRG-RCL, NUE was similar (15.6%) to the values reported by León *et al*. (2008) for cows grazing kikuyu. Systems with moderate milk production levels based on grazing, such as in New Zealand, usually exhibit a larger N intake in diet in virtue of the high CP content in the grass (Pacheco and Waghorn, 2008); or in Brazil, where because of a high level of protein supplementation (Danes *et al*., 2013), a negative effect on N efficiency in milk is noticed.

The increase of NUE in PRG-RCL was the result of lower N intake and not to N excretion in milk. This is consistent with the observations by Huhtanen *et al*. (2008), who reported that an increase in CP concentration in diet decreases NUE, not milk yield, which does not have a significant effect. Efficiency can only be improved via two channels: improving animal production increasing the content of true protein in milk; or reducing CP intake in diet (Pacheco and Waghorn, 2008). In both treatments, a balance between MUN and BUN values was noticed as well as larger N excretions when N intake increased.

CONCLUSIONS

Late lactating cows that produce about 15L/day under cut-and-carry systems of pastures cultivated with two legume varieties (RCL or WCL) with high CP contents in diet did not show any difference for animal variables; however, there was an effect in the concentration of BUN, UUN and total urea nitrogen in urine, indicating a higher NUE and lower total N excretion in PRG-RCL. Therefore, the ryegrass-red clover combination is used for feeding cows, because it reduces nitrogen excretion with the same results in terms of milk production.

From an environmental standpoint, high N excretions reflect a water or air pollution problem; hence, it is important to have a better management of CP concentration, from the chemical composition of the pasture to the inclusion of protein or energy ingredients in the commercial concentrates.

Acknowledgements
The authors are grateful to the farmer and his family for participating in this experiment; their privacy is ensured as their names are not disclosed.

Funding. This work was carried out with funding from PRODEP, through project 511-6/18-8981, and from National Council of Science and Technology of Mexico, CONACYT [Consejo Nacional de Ciencia y Tecnología], through a postgraduate grant for Maria Danaee Celis-Alvarez.

Conflict of interest. The authors declare there are no conflicts of interest whatsoever.

Compliance with ethical standards. This document reports an on-farm experiment carried out with a producer, who collaborated and was aware of the goals of the work at all times; whose privacy and that of his family are respected as their names are not revealed. The experimental procedures on the cows and the research with the farmer were carried out in accordance with the procedures accepted by the Autonomous University of the State of Mexico.

Data availability. Data are available with the corresponding author at: (flopezg@uaemex.mx) upon reasonable request.

REFERENCES

Alfonso-Ávila, A.R., Wattiaux, M.A., Espinoza-Ortega, A., Sánchez-Vera, E. and Arriaga-Jordán, C.M. 2012. Local feeding strategies and milk composition in small-scale dairy production systems during the rainy season in the highlands of Mexico. Tropical Animal Health and Production. 44: 637–644. https://doi.org/10.1007/s11250-011-9947-5

Ankom, 2019. Procedures for NDF, ADF, and *in vitro* Digestibility Ankom Technology Method. http://www.ankom.com. Accessed 10 Aug 2019.

AOAC, 1990. Official Methods of Analysis, 14th ed. (Association of Official Analytical Chemist, Washington)

ASAE, 2005. Manure production and characteristics, (American Society of Agriculture Engineers, St. Joseph, MI)

Brito, A.F. and Broderick, G.A. 2007. Effects of Different Protein Supplements on Milk Production and Nutrient Utilization in Lactating Dairy Cows. Journal of Dairy Science. 90: 1816-1827. https://doi.org/10.3168/jds.2006-558

Bryant, R.H., Miller, L.A., Greenwood, S.L. and Edwards, G.R. 2017. Milk yield and nitrogen excretion of dairy cows grazing binary and multispecies pastures. Grass and Forage Science. 72: 806-817. https://doi.org/10.1111/gfs.12274

Chaney, A.L. and Marbach, E.P. 1962. Modified reagents for determination of urea and ammonia. Clinical Chemistry 8: 130-132. https://doi.org/10.1093/clinchem/8.2.130

Conroy, C., 2005. Participatory Livestock Research. (ITDG Publishing, Bourton-on-Dunsmore, Warwickshire, UK).

Danes, M.A.C., Chagas, L.J., Pedroso, A.M. and Santos, F.A.P. 2013. Effect of protein supplementation on milk production and metabolism of dairy cows grazing tropical grass. Journal of Dairy Science. 96: 407-419. https://doi.org/10.3168/jds.2012-5607

Eickler B., Gierus, M., Kleen, J. and Taube, F. 2011. Specific polyphenol oxidase activity of red clover (*Trifolium pratense*) and its relation with forage quality in field experiments. Acta Agriculturae Scandinavica Section B - Soil and Plant Science. 61: 39-49. https://doi.org/10.1080/09064710903463626

Eriksen, J., Askegaard, M. and Søegaard, K. 2012. Complementary effects of red clover inclusion in ryegrass-white clover sward for grazing and cutting. Grass and Forage Science. 69: 241-250. https://doi.org/10.1111/gfs.12025

FAO, 2016. Food Outlook. Biannual report on global food markets. (Food and Agriculture Organization of the United Nations, Rome)

FAO, 2017. Smallholders in the value chain Food and Agriculture Organization of the United Nations of the Food and Agriculture Organization of the United Nations. http://www.fao.org/dairy-production-products/socio-economics/smallholders-in-the-value-chain/en/ Accessed 20 Nov 2017.

Flachowsky. G. and Lebzien, P. 2006. Possibilities for reduction of Nitrogen (N) excretion from ruminants and the need for further research – A. review. Landbauforschung Völkenrode. 56: 19-30. https://pdfs.semanticscholar.org/769c/a37d3030c4025e992580fa6bd34d41b69ffd.pdf

Grabber, J.H. 2009. Forage management effects on protein and fiber fractions, protein degradability, and dry matter yield of red clover conserved as silage. Animal Feed Science and Technology. 154: 284-291. 10.1016/j.anifeedsci.2009.09.011

Instituto Nacional de Estadística y Geografía - INEGI, 2017. Anuario estadístico y geográfico de México 2017. Available in: https://www.inegi.org.mx/app/biblioteca/ficha.html?upc=702825094706

Hemme, T. IFCN Dairy Team. and IFCN Researchers. 2007. IFCN Dairy Report 2007. (International Farm Comparison Network, Kiel).

Hristov, A.N., Ropp, J.K., Grandeen, K. L., Abedi, S., Etter, R. P., Melgar, A. and Foley, A.E. 2005. Effect of carbohydrate source on ammonia utilization in lactating dairy cows. Journal of Animal Science. 83: 408-421. https://doi.org/10.2527/2005.832408x

Huhtanen, P., Nousiainen, J.I., Rinne, M., Kytölä, K. and Khalili H. 2008. Utilization and Partition of Dietary Nitrogen in Dairy Cows Fed Grass Silage-Based Diets. Journal of Animal Science. 91: 3589-3599. https://doi.org/10.3168/jds.2008-1181

Johansen, M., Søegaard, K., Lund, P. and Weisbjerg, M.R. 2017. Digestibility and clover proportion determine milk production when silages of different grass and clover species are fed to dairy cows. Journal of Dairy Science. 100: 8861-8880. https://doi.org/10.3168/jds.2017-13401

Lawal, B. 2014. Applied Statistical Methods in Agriculture, Health and Life Sciences, (Springer International Publishing, Switzerland).

León, J.M., Mojica, J.E., Castro E.,Cárdenas E.A, Pabón M.L. and Carulla J.E. 2008. Balance de nitrógeno y fósforo de vacas lecheras en pastoreo con diferentes ofertas de kikuyo (*Pennisetum clandestinum*) suplementadas con ensilaje de avena (*Avena sativa*). Revista Colombiana de Ciencias Pecuarias. 21: 559-570. https://www.redalyc.org/pdf/2950/295023543004.pdf

Mackle, T.R., Bryant, A.M., Petch, S.F., Hill, J.P. and Auldist, M.J. 1999. Nutritional Influences on the Composition of Milk from Cows of Different Protein Phenotypes in New Zealand. Journal of Dairy Science. 82: 172-180. https://doi.org/10.3168/jds.S0022-0302(99)75221-5

McAuliffe, G.A., López-Aizpún, M., Blackwell, M.S.A., Castellano-Hinojosa, A., Darch, T., Evans, J., Horrocks, C., Le Cocq, K., Takahashi, T., Harris, P., Lee, M.R.F. and Cardenas, L. 2020. Elucidating three-way interactions between soil, pasture and animals that regulate nitrous oxide emissions from temperate grazing systems. Agriculture, Ecosystems and Environment. 300: 106978. https://doi.org/10.1016/j.agee.2020.106978

Martínez-García, C.G., Rayas-Amor, A., Anaya-Ortega, J.P., Martínez-Castañeda, F.E., Espinoza-Ortega, A., Prospero-Bernal, F. and Arriaga-Jordan, C.M. 2015. Performance of small-scale dairy farms in the highlands of central Mexico during the dry season under traditional feeding strategies. Tropical Animal Health and Production. 47: 331–337. https://doi.org/10.1007/s11250-014-0724-0

Miguel, M.F., Ribeiro-Filho, H.M.N., de Andrade, E.A., Moraes Genro, M.T. and Delagarde, R. 2014. Pasture intake and milk production of dairy cows grazing annual ryegrass with or without corn silage supplementation. Animal Production Science. 54: 1810-1816.

Mulligan, F.J., Dillon, P., Callan, J.J., Rath, M. and Mara, F.P.O. 2004. Supplementary Concentrate Type Affects Nitrogen Excretion of Grazing Dairy Cows. Journal of Dairy Science. 87: 3451-3460. https://doi.org/10.3168/jds.S0022-0302(04)73480-3

Nennich, T.D., Harrison, J.H., VanWieringen, L.M., Meyer, D., Heinrichs, A.J., Weiss, W.P., St-Pierre, N.R., Kincaid, R.L., Davidson, D.L. and Block E. 2005. Prediction of Manure and Nutrient Excretion from Dairy Cattle. Journal of Dairy Science. 88: 3721-3733. https://doi.org/10.3168/jds.S0022-0302(05)73058-7

NRC, 2001. Nutrient Requirements of Dairy Cattle Seventh (National Research Council National Academy Press, Washington).

Østergaard, S., Sørensen, J.T. and Houe, H. 2003. A stochastic model simulating milk fever in a dairy herd. Preventive Veterinary Medicine. 58:125–143. https://doi.org/10.1016/s0167-5877(03)00049-7

Pacheco, D. and Waghron. 2008. Dietary nitrogen-definitions, digestion, excretion and consequences of excess for grazing ruminants. Proceedings of the New Zealand Grassland Association. 70: 107-116. https://doi.org/10.33584/jnzg.2008.70.2738

Pincay-Figueroa, P.E., López-González, F., Velarde-Guillén, J., Heredia-Nava, D., Martínez-Castañeda, F.E., Vicente, M.F., Martínez-Fernández, A. and Arriaga-Jordán, C.M. 2016. Cut and carry vs. grazing of cultivated pastures in small-scale dairy systems in the central highlands of Mexico. Journal of Agriculture and Environment for International Development. 110: 349-363. https://doi.org/10.12895/jaeid.20162.496

Pozo-Leyva D., López-González F., Olea-Pérez R., Balderas-Hernández P. and Arriaga-Jordán, C.M. 2019. Nitrogen utilization efficiency in small-scale dairy systems in the highlands of central Mexico. Tropical

Animal Health and Production. 51: 1215–1223 https://doi.org/10.1007/s11250-019-01812-6

Spek, J.W., Dijkstra, J., van Duinkerken, G., Hendriks, W.H. and Bannink, A. 2013. Prediction of urinary nitrogen and urinary urea nitrogen excretion on by lactating dairy cattle in Northwestern Europe and North America: A meta-analysis. Journal of Dairy Science. 96: 4310-4322. https://doi.org/10.3168/jds.2012-6265.

Totty, V.K., Greenwood, S.L., Bryant, R.H and Edwards, G.R. 2013. Nitrogen partitioning and milk production of dairy cows grazing simple and diverse pastures. Journal of Dairy Science. 96: 141–149. https://doi.org/10.3168/jds.2012-5504

Velarde-Guillén, J., Estrada-Flores, J.G., Rayas-Amor, A.A., Vicente, F., Martínez-Fernández, A., Heredia-Nava, D., Celis-Alvarez, M.D., Aguirre-Ugarte, I.K., Galindo-González, E. and Arriaga-Jordán, C.M. 2019. Supplementation of dairy cows with commercial concentrate or ground maize grain under cut-and carry or grazing of cultivated pastures in small-scale systems in the highlands of central Mexico. Animal Production Science. 59: 368-375. https://doi.org/10.1071/AN15375

Wildman, E.E., Jones, G.M., Wagner, P.E., Boman, MY., Troutt, H.F. and Lesch, T.N. 1982. A dairy cow body condition scoring system and its relationship to selected production characteristics. Journal of Dairy Science. 65: 495-501. https://doi.org/10.3168/jds.S0022-0302(82)82223-6

Xiccato, G., Schiavon, S., Gallo, L., Bailoni L., and Bittante G. 2005. Nitrogen excretion in dairy cow, beef and veal cattle, pig, and rabbit farms in Northern Italy. Italian Journal of Animal Science. 4: 103-111. https://doi.org/10.4081/ijas.2005.3s.103