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Effect of forage source and dietary rumen-undegradable protein on nutrient use and growth in dairy heifers

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ABSTRACT

Thirty-two Holstein heifers with mean (± standard error of the mean) age of 6.5 months (±0.12) and live weight (LW) of 166 kg (±1.6) were divided into four groups of eight animals to evaluate the effect of forage source and the concentrations of rumen-undegradable crude protein (RUP) in the diet with a 2 × 2 factorial design. As forage sources cowpea hay (*Vigna sinensis* L.) or pangola grass hay (*Digitaria decumbens* Stend) were added to the diet at 250 g/kg dry matter (DM). Also, 350 g/kg DM of chopped fresh King grass (*Pennisetum purpureum* Schum.) was included in all diets. The RUP proportions were 260 or 360 g/kg of total crude protein (CP). Heifers were housed in metabolism cages. The trial lasted ten weeks, with the first two weeks used for adaptation and the last week for data collection and sampling. Diets had a forage to concentrate ratio of 60:40 (on DM basis), and were offered as total mixed rations *ad libitum*. Fish meal or urea were used to produce varying concentrations of RUP at similar dietary CP content. Diet ingredients (i.e., hay, grass, and concentrate) and feces were sampled for nutrient analysis. Total collection of feces was performed to estimate apparent total tract nutrient digestibility (ATTD). Urinary purine derivative excretions were determined from urine spot samples to estimate rumen microbial protein synthesis. The effects of forage source, RUP, and its interactions were determined by general linear model analysis. Replacing dietary pangola grass hay with cowpea hay increased daily intakes of DM, organic matter, CP, acid detergent fiber (ADF), and digestible organic matter, and ATTD of DM, organic matter, neutral detergent fiber, and ADF ($P < 0.05$). Duodenal flow of RUP also increased when substituting cowpea hay for pangola grass hay ($P < 0.01$). Similarly, inclusion of cowpea hay in the diet improved live weight gain (LWG; $P < 0.05$), and decreased feed costs per kg of LWG ($P < 0.01$). The increase in RUP decreased rumen microbial protein synthesis ($P < 0.01$), but tended to increase LWG, feed conversion and nitrogen use efficiency, and feed costs per unit of LWG ($P < 0.10$). The use of cowpea hay in the diet of dairy heifers increases nutrient intake and ATTD, leading to improved LWG and lower feed costs per kilogram of LWG. Increasing RUP proportions may reduce rumen microbial protein synthesis, but may enhance LWG, feed conversion efficiency, and nitrogen use efficiency. There were no interactions between

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forage source and RUP proportion on intake and ATTD, but some synergistic effects were observed for the performance parameters.

1. Introduction

Improving dairy heifer growth can considerably advance their maturation and by this, prepone puberty and age at first conception, while, at the same time, assuring sufficient body condition and development stage at first calving with associated positive effects on milk performance, longevity, and thus recovery of the investments in dairy farms (Zanton and Heinrichs, 2005; Bayram et al., 2009). A nutrient and energy supply that meets the animals' requirements is crucial for successful heifer growth.

The use of grass hay to feed heifers is a common practice in areas with a marked dry season. Pangola grass (*Digitaria decumbens* Stend) is a commonly used grass in tropical cattle feeding. The concentrations of neutral detergent fiber (NDF) in fresh pangola grass have been reported as greater than 660 g/kg dry matter (DM; Corea Guillén et al., 2010a), whereas its crude protein (CP) concentrations might be less than 79 g/kg DM, with the nutritional value likely being even lower in the hay than in the fresh grass (Tikam et al., 2013), which limits considerable growth and pubertal development of replacement heifers.

Adding legumes to grass is a promising feeding strategy for dairy cattle (Corea Guillén et al., 2010b; Reiber et al., 2012), as they complement each other in providing fermentable energy and nitrogen (N) for rumen microbial growth and activity. Increases in animal performance in response to herbaceous legume feeding have been described for lactating cows (Wanapat et al., 2017), growing heifers (Waters et al., 2015), and bulls (Hossain et al., 2015), likely due to better nutrient use. For instance, in a similar environment the use of cowpea (*Vigna sinensis* L.) increased nutrient intake and digestibility in milking cows, enhancing overall nutrient use efficiency (Castro-Montoya et al., 2018; Corea et al., 2017).

The efficacy of the use of tropical legumes as forage could be limited by its protein characteristics, such as the proportions of rumen-degradable crude protein (RDP) or rumen-undegradable protein (RUP). It has been shown that excessive rumen CP degradation from alfalfa results in inefficient N utilization and depressed milk production in dairy cows (Broderick, 1985), while the addition of fish meal, a RUP source, to diets based on alfalfa silage increased milk and milk component yields (Broderick, 1992). Our hypothesis was that the addition of legume hay and higher concentrations of RUP in the diet will interact positively to increase live weight gain (LWG) by enhancing energy and amino acid supply as well as nutrient use efficiency in dairy heifers. Hence, this study evaluated the effects of substituting cowpea for pangola grass and their interactions with dietary RUP proportion on nutrient digestibility, synthesis of rumen microbial protein, N retention, and LWG in dairy heifers.

2. Material And Methods

2.1. Location

This study was conducted on the San Ramón dairy farm near Caluco in Sonsonate Department, El Salvador (13° 43′ N, 89° 42′ W at 379 m above sea level). The area is a tropical rainforest with an average daily ambient air temperature of 24.2 °C and 30-year mean annual rainfall of 2170 mm (MARN, 2019). The dry season occurs from November to April and the rainy season from May to October (MAG, 1993). The feeding trial was conducted during the dry season between February and April 2018.

2.2. Experimental design

Thirty-two Holstein Frisian heifers with an average (± standard error of the mean) age of 6.5 ± 0.12 months and live weight (LW) of 166 ± 1.6 kg at the beginning of the experiment were selected for normal health history as well as homogeneous ages and LW. Heifers were randomly distributed to four groups of eight animals. Each group received one of four different diets in a 2 × 2 factorial arrangement. Two forage sources were tested (i.e., cowpea hay (*Vigna sinensis* L.) or pangola grass hay (*Digitaria decumbens* Stend) offered at 250 g/kg of diet DM at two proportions of dietary RUP (i.e., 260 g/kg CP (RUP260) or 360 g/kg of CP (RUP360)).

The experimental procedures applied to animals were approved by the Research Council of the University of El Salvador. Heifers were housed in metabolic cages (1.8 m × 0.8 m) with individual feeders and water bowls in an iron-sheet-roofed open barn. The trial lasted ten weeks. The first two weeks were used for adaptation to diets, housing, and management. During the last week, considered the sampling period, offered feed, orts, and total feces were weighed and sampling of feed, orts, feces, and urine were performed.

2.3. Diets

Diets were formulated using CPM dairy V3.08, following the recommendations of the NRC (2001) for energy and protein supply to heifers weighing 175 kg with mean daily LWG of 0.8 kg/d (Table 2), and assuming a DM intake of individual heifers of 4.4 kg/d. Rations were mixed once a day in the morning and offered as total mixed ration (TMR) at 9:00 and 16:00 h. The diets had a forage to concentrate ratio of 60:40 (DM basis; Table 2) and were formulated to be isoenergetic and isonitrogenous. Besides the 250 g/kg diet DM of pangola grass hay or cowpea hay, 350 g/kg diet DM of fresh king grass (*Pennisetum purpureum* Schum.) was included as forage in all diets. All forages were chopped to 2 cm particle length with a mechanical chopper (Pecos 9004, Nogueira Máquinas Agrícolas, São Paulo, Brazil). The concentrate mixtures were formulated using corn grain meal, soybean meal, wheat bran, and sugarcane molasses as the main components and were prepared once a week. Fish meal or urea were used to achieve similar dietary N concentrations and to produce concentrates with the targeted concentrations of RUP. The heifers had access to clean drinking water and feed *ad libitum.*

2.4. Data collection and sampling

Animals were weighed at 09:00 h (before feeding) on two consecutive days every two weeks throughout the experiment using an animal scale (Gram Zebra, K3 8-3 T, Balanzas y Básculas Gram Precision, Tarragona, Spain). The rations were weighed individually for each animal. Feed refused was weighed on days 1 and 2 every week to adjust the amount of feed offered to yield refusals equivalent to about 10 % of offered feed (as-fed basis). Daily weights of feed offered and refused were recorded during the sampling week to estimate individual daily feed intake using an electronic scale (Defender 3000 Series D31P150BL, Ohaus, Parsippany, USA).

Samples of 0.5 kg fresh matter each of king grass, pangola grass hay, cowpea hay, concentrate mixtures, and offered and refused TMR were taken on one day every two weeks and during days 2, 4, and 6 of the sampling period and were stored at −20 °C for nutrient analysis.

Total feces excreted by each heifer was collected daily into a plastic container on days 1-6 of the sampling period and weighed to quantify daily fecal excretion. Additionally, spot urine and feces samples were collected at 7:00, 9:00, 11:00, 13:00, 15:00, and 17:00 h on days 1, 2, 3, 4, 5, and 6, respectively, of the sampling week. Fecal samples (200 g fresh matter each) were collected daily directly from the rectum. Perineal massaging was performed to initiate urination to take samples of about 0.8 L/animal during each sampling. A subsample of 100 mL of urine was taken and immediately mixed with approximately 1 mL of sulfuric acid (7.2 N) to reduce urine pH to below 3. Acidified urine was then filtered (filter paper Whatman # 42, Thomas Scientific, Swedesboro, USA) and 10 mL were diluted with distilled water at a ratio of 1:5, homogenized, and stored in 15-mL-aliquots. Additionally, 15 mL of the non-diluted, acidified urine were taken for N analysis. All urine and feces samples were stored frozen at −20 °C until analysis.

2.5. Laboratory analysis and calculations

At the end of the sampling period, samples of individual feeds, TMR offered and refused, feces, and urine were thawed. Samples of feces and urine were pooled by heifer and homogenized manually. One subsample of 20 g fresh matter per heifer of the pooled fresh feces was taken. All feed, orts, and feces samples were then dried in a forced-air oven (100-800, Memmert GmbH and Co. KG, Schwabach, Germany) at 60 °C for 72 h. Dried samples were ground to pass a 1-mm screen in a Wiley mill (Standard Model No 3, Arthur H. Thomas Company, Philadelphia, USA). Concentrations of DM were determined on dried feed, orts, and feces using a vacuum oven (Binder, VD 53, Tuttingen, Germany) at 105 °C for 5 h and 100 mm Hg (AOAC, 2005; method 934.01). The N concentrations were determined in dried feeds and TMR samples, in fresh feces, and in non-diluted urine samples by Kjeldahl procedure using digestion DK and distillation UDK 129 units (VELP Scientifica, Usmate, Italy). The CP concentrations in feed and feces were computed by multiplying N concentrations by 6.25 (AOAC, 2005; method 990.13). Concentrations of NDF (inclusive residual ash) and acid detergent fiber (ADF; inclusive residual ash) were determined using a heat-stable α-amylase in dried samples of feeds offered, refused TMR, and feces following the methods of Van Soest et al. (1991) using an Ankom 200 fiber analyzer (ANKOM technology, Macedon, New York, USA). Ash was analyzed in dried feed and fecal samples by combustion in a muffle furnace (Nabertherm L24/12/P320, Nabertherm GmbH, Bremen, Germany) at 600 °C for 2 h (AOAC, 2005; method 942.05).

Urine samples pooled by heifer were analyzed for creatinine and uric acid by colorimetry using kinetic tests (CREJ2 and UA2, Roche diagnostics, Mannheim, Germany) in a photometer (COBAS C 501 Module, Roche diagnostics, Mannheim, Germany), and for allantoin following the procedures described by Chen and Gomes (1992) using a spectrophotometer (Jenway 6305, Cole-Palmer, Stone Staffs, UK). All laboratory analyses were performed in duplicate and means of duplicate determinations with coefficients of variation > 10% were repeated.

Nutrient intakes (i.e., DM, organic matter (OM), N, CP, NDF, and ADF; in g or kg/animal and d) were calculated by subtracting the amount of nutrients in refused feed from the amount of nutrients contained in the offered feed. Apparent total tract digestibilities of DM, OM, CP, NDF, and ADF were estimated from the equation: ATTD (g/100 g ingested nutrient) = (Nutrient intake – Fecal nutrient excretion) * 100/Nutrient intake (all kg/animal and d).

Urine excretion (L/animal and d) was estimated from urinary creatinine concentrations assuming a constant creatinine excretion of 29 mg kg of LW as proposed by Chizzotti et al. (2008) for Holstein heifers. The N retention was estimated as the difference between the N intake and the excretion of N in feces and urine (all in g/d).

Urinary purine derivative (PD) excretions (mmol/animal and d) were obtained from the multiplication of urine volume (L/animal and d) by the concentrations of allantoin and uric acid (mmol/L). Ruminal microbial protein synthesis (MPS) was estimated as a function of absorbed microbial purines as proposed by Chen and Gomes (1992), calculated as the sum of daily uric acid and allantoin excretions, corrected for the fractions of endogenous PD in urine (0.385 mmol/kg$^{0.75}$ LW and d) and of absorbed microbial purine bases recovered in urine (0.85; Chen and Orskov (2004)).

The efficiency of the MPS (EMPS) was computed by dividing the MPS (g/animal and d) by the digestible organic matter intake (DOMI; kg/animal and d) or N intake (g/animal and d).

The metabolizable energy (ME) concentration of the diets were estimated from proximate nutrient concentrations and *in vitro* gas production (GP) determined in triplicate in two runs according to Menke et al. (1979) using the following equation:

$$ME = 1.242 + 0.146\ GP + 0.007\ CP + 0.0224\ CL;$$

where: ME = metabolizable energy in MJ/kg DM; GP = gas production in ml/200 mg of substrate dry mass during 24 h of incubation; CP = crude protein concentration of diet in g/kg DM; and CL = crude fat concentration of diet in g/kg DM.

The utilizable crude protein at the duodenum (uCP) is the sum of the duodenal flow of RUP and microbial CP (GfE, 2001). The dietary uCP concentration was estimated from the ammonium concentration in the rumen inoculum after 24 h of *in vitro* fermentation analyzed in triplicate during two runs according to the modified Hohenheim gas test (Edmunds et al., 2012). Rumen fluid of two dry Jersey cows fed a TMR comprised of (per kg of DM) corn silage 340 g, grass silage 300 g, grass hay 88 g, barley straw 12 g, and a concentrate mixture (i.e., soybean meal, soybean cake, dairy supplement, and a vitamin-mineral mixture) 260 g were used for the *in vitro* incubations. The rumen nitrogen balance (RNB) of the offered diet (g/kg DM), as an indicator for the RDP supply in relation to the N requirements of rumen microbes, was then obtained by subtracting the uCP supply from the dietary CP concentration (both in g/kg DM) and dividing the difference by 6.25 (GfE, 2001).

The RUP intake (g/animal and d) was estimated by multiplying the RUP proportion (g/kg CP) obtained from CPM dairy output by the animals' CP intake. The daily uCP supply was then calculated as the sum of the RUP intake and the rumen MPS (all in g/animal and d). The RNB (g/animal and d) was then estimated by subtracting the duodenal uCP flow from the animals' CP intake and dividing the results by 6.25 (both in g/animal and d).

Daily LWG (g/animal and d) was determined over the eight experimental weeks by subtracting the initial LW from the final LW and dividing the difference by the number of days between the end of the adaptation period and the end of the experiment. Feed conversion efficiency was estimated by dividing the daily LWG by the daily DM intake or DOMI of the respective animals (in g LWG per kg DM intake or DOMI). The N use efficiency (NUE) was obtained by dividing the LWG (g/d) by the daily N intake (g/d).

Feed costs per kg diet DM were calculated from the proportion of individual feeds in the diets and their respective prices obtained from the mean prices paid by the San Ramon farm over the course of the trial (E. Borja Letona, personal communication).

2.6. Statistical analysis

Statistical analyses were conducted using the software SAS 9.4 (SAS Institute Inc. Cary, North Carolina, USA). Data were analyzed using a general linear model in a factorial 2 × 2 arrangement with four treatments and eight replicates using the following model:

$$Y_{ij} = \mu + F_i + RUP_j + (F_i \times RUP_j) + e_{ij},$$

where Y_{ij} = dependent variable, μ = overall mean, F_i = effect of forage source, RUP_j = effect of RUP proportion, $(F_i \times RUP_j)$ = interaction of forage source and RUP proportion, and e_{ij} = residual error. Significance was declared for $P < 0.05$, whereas a tendency was declared at $0.05 \le P < 0.10$.

3. Results

The inclusion of cowpea hay in the diet increased ($P < 0.01$) daily intakes of DM, OM, CP, ADF, and digestible organic matter (DOM) compared to diets containing pangola grass hay. Moreover, daily DM intake per unit of LW increased when diets containing cowpea hay were fed ($P < 0.01$). However, NDF intake was similar in heifers consuming either forage source ($P = 0.61$; Table 3). There were no effects of dietary RUP proportion on nutrient intakes, except of a tendency ($P = 0.06$) for a greater NDF intake with RUP360 than RUP260. There were no interactions between forage source and RUP proportion on intakes, with the exception of a tendency ($P = 0.06$) for an interaction between forage and RUP for DOMI.

The ATTD of DM, OM, NDF, and ADF were greater ($P < 0.05$) in diets with cowpea hay compared to those containing pangola grass hay (Table 3), but no differences between forages were detected for ATTD of CP ($P = 0.64$). The proportion of RUP had no effects on ATTD of these nutrient fractions, except for ATTD of NDF, which tended to be greater in RUP360 than RUP260 ($P = 0.06$). No interactions between forage source and RUP proportion were observed for the ATTD of any nutrient ($P > 0.10$).

Nitrogen intake was greater with cowpea than with pangola grass hay ($P < 0.01$; Table 4); however, fecal and urinary N excretions and retained N were similar for both forage sources, irrespective of whether they were expressed in absolute terms or as proportion of daily N intake ($P > 0.10$). The proportion of RUP in the diet and the interactions between forage source and RUP proportion had no effects on N intake or any variables related to N balance ($P > 0.10$).

There were no effects of forage source or interactions of forage source and RUP on urinary PD excretion, PD to creatinine ratio, estimated rumen MPS, microbial N per unit of N intake, and EMPS (Table 5). However, these variables were lower for RUP360 than RUP260 ($P < 0.01$; Table 5).

The uCP supply was not affected by the forage source ($P = 0.62$) or by the RUP proportion ($P = 0.29$) in the diet. The RUP intake was greater for RUP360 than for RUP260 ($P < 0.01$) and for cowpea than for pangola grass hay diets ($P = 0.02$) and an interaction was observed between forage source and dietary RUP for RUP intake, while RNB was not affected by any of the evaluated effects ($P > 0.1$; Table 5).

The LWG was greater, whereas the feed cost per unit of LWG was lower with cowpea hay than with pangola grass hay ($P < 0.05$; Table 6). Increasing the RUP proportion from 260 to 360 g/kg CP tended to increase LWG, feed conversion efficiency, DOM conversion, NUE, and feed costs per unit of LWG ($P < 0.10$). No interactions between forage source and RUP proportion were detected for any of these variables.

4. Discussion

4.1. Nutrient intake and digestibility

Nutrient (i.e., DM, OM, CP, and ADF) intake increased in the present study when dietary pangola grass hay was replaced by hay of the tropical legume cowpea. In agreement with our findings, previous studies have reported increased nutrient intakes in response to the inclusion of herbaceous legumes in diets of lactating cows (Bernal et al., 2007; Castro-Montoya et al., 2018), heifers (Kariuki et al., 1998), and young bulls (Hossain et al., 2015) under tropical conditions, particularly when substituting herbaceous legumes for grasses of low or medium nutritional quality.

Because of generally low degradation rates, the NDF fraction is considered the primary dietary constituent associated with rumen fill (NRC, 2001) and thus voluntary feed intake. Hence, it has been proposed that, at an intake rate near to 10 g NDF/kg LW, DM intake in dairy heifers is regulated by dietary NDF concentrations (Hoffman et al., 2008; Hoffman and Kester, 2013). Daily NDF intakes in the present study were close to 16 g NDF/kg LW (Table 3), which is greater than those assumed for temperate regions (Hoffman et al., 2008; Hoffman and Kester, 2013) and reported for growing animals in tropical conditions, varying from 5.5 g (Lascano et al., 2012) or 8.6 g (Silva et al., 2018a) to 12-14 g (Machado et al., 2019) and 14 g NDF/kg LW (Rufino et al., 2016). Hence, the differences in DM intakes between forage sources were most likely due to the lower NDF concentration in cowpea hay (580 g/kg DM) versus pangola grass hay (683 g/kg DM) hay and thus in the respective diets (488 and 518 g/kg DM; Tables 1 and 2). Accordingly, daily NDF intakes per animal were similar for both, the cowpea hay and pangola grass hay diets (3.56 versus 3.58 kg NDF/animal and d; $P = 0.61$). Finally, the ATTD of DM, OM, NDF, and ADF were greater with cowpea than with pangola grass hay (Table 3), which could further explain the greater nutrient intakes for the cowpea than for the pangola grass hay diets.

Interestingly, despite these differences in ATTD, the ATTD of CP was similar for both forage sources. Reports in the literature about the effects of forage legumes feeding on CP digestibility vary mainly depending on the dietary treatments and animals used in experiments. For instance, substitution of cowpea hay for grass hay increased CP intake but not CP digestibility in dairy cows under poor conditions (Castro-Montoya et al., 2018) or in dual-purpose cows (Castro-Montoya et al., 2019), whereas in high-yielding dairy cows an increase in ATTD of CP but not in DM and CP intakes with cowpea hay was observed (Corea et al., 2017). Several studies found increases in both, intake and ATTD of CP (Foster et al., 2009; Hossain et al., 2015; Schnaider et al., 2014). However, Castro-Montoya and Dickhoefer (2018) suggested that, when compared with good-quality grass, ATTD of CP might also be lower for legumes. Indeed, the control diets in several of the studies with positive effects on CP digestibility were based on poor-quality forages or crop residues with greater proportions of fiber-bound CP than those in the evaluated legumes or did not contain any concentrate feed, which increased the scope for positive effects of legume feeding.

Moreover, in the present study, replacing pangola grass with cowpea hay was associated with additional changes in the dietary protein sources. According to the DM intakes of heifers for the proportions of CP coming from the individual feed ingredients, cowpea hay provided 278 g CP/d, corn 119 g CP/d, and soybean meal 146 g/d in the cowpea diets. In the pangola diets, pangola grass hay contributed to 96 g CP/d, corn 73 g CP/d, and soybean meal 301 g CP/d to total CP intake. Hence, relative to the pangola grass hay diet, CP from cowpea hay also replaced a portion of the CP from soybean meal, a good-quality protein source, which may also explain the lack of differences in ATTD of CP between diets containing the two forage sources.

Intakes and ATTD of DM, OM, CP, and ADF did not differ between the two levels of RUP. It is commonly expected that diets with low RDP supply may limit rumen microbial activity and therefore nutrient degradation. However, results of Zanton et al., (2007) who tested diets differing in rumen CP degradability did not show any effect on nutrient intake and digestibility. Similarly, other studies with dairy heifers (Gabler and Heinrichs, 2003; Silva et al., 2018a; Silva et al., 2018b) did not detect any effect of dietary RUP proportion on nutrient intake and digestibility at the same dietary CP level. Dietary RNB were only slightly negative or even positive, suggesting that N availability did not limit rumen microbial activity in the present study. Even temporal N scarcity in the rumen can partly be mitigated by physiological adaptations of the animal to avoid negative effects on rumen fermentation and thus nutrient digestibility (Zanton et al., 2007; Silva et al., 2018a).

Moreover, in the present study, intake and ATTD of NDF tended to increase with greater proportion of RUP. This observation is an apparent contradiction with previously proposed positive relation between RDP and nutrient digestibility. However, the concentrations of hemicelluloses and their proportions in total NDF were greater for RUP360 than RUP260 diets, suggesting a greater ruminal NDF degradability. Moreover, Reynal and Broderick (2005) also found an increase in ATTD of NDF with greater RUP proportions.

Table 1
Chemical composition (g/kg dry matter) of forages fed to the heifers (n = 6 samples).

Variable	Cowpea hay		Pangola grass hay		Fresh king grass	
	Mean	SEM[1]	Mean	SEM	Mean	SEM
Dry matter, g/kg fresh matter	880	10.9	897	11.9	179	7.71
Organic matter	880	3.01	898	3.68	861	7.78
Crude protein	151	4.01	55.5	1.60	62.1	1.46
Neutral detergent fiber	580	12.0	683	9.10	744	15.3
Acid detergent fiber	389	9.40	351	10.3	374	10.7
Hemicellulose	191	4.24	332	6.5	390	9.28

[1] Standard error of the mean.

Table 2
Ingredient and nutritional composition of experimental diets fed to the heifers (n = 6 samples).

	Pangola grass		Cowpea	
	RUP260	RUP360	RUP260	RUP360
Ingredients, g/kg DM				
Yellow corn meal	139	96	200	158
Wheat bran	82.5	82.5	82.6	82.5
Sugarcane molasses	67.3	67.2	67.3	67.3
By-pass fat[2]	14.3	14.3	0.0	0.0
Soybean meal	74.3	99.0	27.5	51.6
Fish meal	0.0	25.8	0.0	25.8
Urea	7.5	0.0	7.5	0.0
Mineral salt[3]	6.7	6.7	6.7	6.7
Calcium carbonate	4.4	4.4	4.4	4.4
Sodium chloride	4.4	4.4	4.4	4.4
Fresh king grass	350	350	350	350
Cowpea hay	0.0	0.0	250	250
Pangola grass hay	250	250	0.0	0.0
Nutritional composition[4], g/kg DM				
DM, g/kg fresh matter	371	379	377	372
Crude protein	126	126	127	129
Neutral detergent fiber	516	520	480	495
Acid detergent fiber	240	236	247	246
Hemicellulose	276	284	233	249
Utilizable crude protein[5]	130	130	127	126
Rumen nitrogen balance	−0.64	−0.64	0.0	0.48
RUP[5], g/kg crude protein	273	360	250	358
ME[6], MJ/kg DM	8.23	8.42	8.84	9.13

DM = Dry matter, ME = Metabolizable energy.
[1]Proportions of rumen-undegradable crude protein (RUP): 260 g/kg crude protein (RUP260) or 360 g/kg crude protein (RUP360).
[2] Lactomil®: palm oil =850 g/kg, calcium 80-96 g/kg, and net energy for lactation 24.18 MJ/kg (as-fed basis).
[3] NutroKel® (per kg DM): 8.5 g calcium, 5.6 g phosphorus, 1.6 g magnesium, 0.4 g sulfur, 80 mg copper, 40 mg iron, 241 mg zinc, 2.8 mg selenium, 5100 IU vitamin A, 4020 IU vitamin D, and 141 IU vitamin E.
[4] According to laboratory analysis.
[5] According to software CPM dairy V3.08 output.
[6] Estimated from proximate nutrient concentrations and gas production during *in vitro* fermentation (Menke *et. al.*,1979).

Table 3
Effect of replacement of pangola grass (*Digitaria decumbens* Stend) hay with cowpea (*Vigna sinensis* (L.)) hay, dietary rumen-ungradable crude protein (RUP) proportion[1], and their interactions on nutrient intake and apparent total tract nutrient digestibility in dairy heifers (Arithmetic means and standard error of the mean (SEM); n = 8 animals).

	Pangola		Cowpea			*P*-values		
	RUP260	RUP360	RUP260	RUP360	SEM	Forage	RUP	Forage × RUP
Nutrient intake (kg/d)								
Dry matter	6.88	7.00	7.39	7.36	0.073	<0.01	0.54	0.33
Organic matter	6.15	6.21	6.58	6.49	0.046	<0.01	0.71	0.26
Dry matter, g/kg live weight	31.4	31.6	33.5	32.7	0.03	<0.01	0.60	0.35
Crude protein	0.891	0.902	0.948	0.954	0.0607	<0.01	0.20	0.71
Neutral detergent fiber	3.54	3.61	3.51	3.60	0.021	0.61	0.06	0.95
Neutral detergent fiber, g/kg live weight	16.1	16.3	15.9	16.0	0.11	0.23	0.56	0.77
Acid detergent fiber	1.72	1.72	1.86	1.83	0.015	<0.01	0.58	0.45
Digestible organic matter	4.03	4.19	4.65	4.52	0.057	<0.01	0.80	0.06
Apparent total tract digestibility, g/100 g								
Dry matter	62.6	64.6	68.2	66.9	0.65	<0.01	0.71	0.13
Organic matter	65.5	67.6	70.8	69.7	0.59	<0.01	0.56	0.13
Crude protein	70.0	70.8	71.2	70.7	0.58	0.64	0.88	0.58
Neutral detergent fiber	54.2	57.5	58.7	60.8	0.79	0.01	0.06	0.64
Acid detergent fiber	47.8	51.1	54.7	55.4	0.98	<0.01	0.24	0.45

[1] Proportions of rumen-undegradable crude protein (RUP): 260 g/kg crude protein (RUP260) or 360 g/kg crude protein (RUP360).

Since these authors did not find any differences in ruminal NDF degradation, they suggested that this must have been due to differences in postruminal NDF digestibility. Finally, in the present study, also slight changes in NDF source occurred, as urea and corn were removed and replaced by fish meal and soybean meal to create RUP360 diets, which could also have contributed to the small changes observed in daily intake and ATTD of NDF.

The differences in nutritional characteristics of forage may interact with dietary RUP proportion to alter nutrient use; however, no

Table 4
Effect of replacement of pangola grass (*Digitaria decumbens* Stend) hay with cowpea (*Vigna sinensis* (L.)) hay, dietary rumen-ungradable crude protein (RUP) proportion[1], and their interactions on nitrogen (N) balance in dairy heifers (Arithmetic means and standard error of the mean (SEM); n = 8 animals).

	Pangola		Cowpea			*P*-values		
Variable	RUP260	RUP360	RUP260	RUP360	SEM	Forage	RUP	Forage x RUP
N intake, g/d	143	144	152	153	0.9	<0.01	0.20	0.71
Fecal N, g/d	42.8	42.1	43.7	44.7	0.88	0.33	0.92	0.64
Urinary N, g/d	74.8	73.9	79.9	77.9	1.96	0.26	0.72	0.90
Retained N, g/d	24.9	28.3	28.1	30.0	1.91	0.54	0.50	0.86
Fecal N, g/100 g N intake	30.0	29.2	28.8	29.3	0.58	0.64	0.88	0.58
Urinary N, g/100 g N intake	52.5	51.2	52.6	51.0	1.26	0.99	0.59	0.95
Retained N, g/100 g N intake	17.5	19.7	18.6	19.7	1.30	0.84	0.55	0.85

[1] Proportions RUP: 260 g/kg crude protein (RUP260) or 360 g/kg crude protein (RUP360).

Table 5
Effect of replacement of pangola grass (*Digitaria decumbens* Stend) hay with cowpea (*Vigna sinensis* (L.)) hay, dietary rumen-ungradable crude protein (RUP) proportion[1], and their interactions on indicators of ruminal nitrogen (N) turnover in dairy heifers (Arithmetic means and standard error of the mean (SEM); n = 8 animals).

	Pangola		Cowpea			*P*-values		
Variable	RUP260	RUP360	RUP260	RUP360	SEM	Forage	RUP	Forage x RUP
Urinary PD, mmol/d	147	127	156	124	4.48	0.69	<0.01	0.44
Microbial protein synthesis, g/d	668	562	716	545	24.1	0.71	<0.01	0.44
Microbial N, g/g N intake	0.75	0.62	0.76	0.57	0.027	0.62	<0.01	0.57
Microbial N, g/kg DOM intake	167	134	154	120	6.2	0.21	<0.01	0.96
PD to creatinine ratio (mmol/mmol)	2.6	2.2	2.7	2.1	0.29	0.88	<0.01	0.50
Utilizable crude protein, g/d	911	887	953	886	20.3	0.62	0.29	0.62
RUP intake, g/d	243	325	237	342	8.59	0.02	<0.01	<0.01
RNB, g/d	−3.20	2.42	−0.85	10.91	3.4	0.43	0.22	0.66

DM = dry matter, DOM = digestible organic matter, N = nitrogen, PD = purine derivatives, RNB = rumen nitrogen balance.
[1] Proportions of RUP: 260 g/kg crude protein (RUP260) or 360 g/kg crude protein (RUP360).

Table 6
Effect of replacement of pangola grass (*Digitaria decumbens* Stend) hay with cowpea (*Vigna sinensis* (L.)) hay, dietary rumen-ungradable crude protein (RUP) proportion[1], and their interactions on performance in dairy heifers (Arithmetic means and standard error of the mean (SEM); n = 8 animals).

	Pangola		Cowpea			*P*-values		
Variable	RUP260	RUP360	RUP260	RUP360	SEM	Forage	RUP	Forage × RUP
Initial LW at the beginning of experiment, kg/animal	176	175	173	172	1.60	0.27	0.61	0.65
Final LW at the end of the experiment, kg/animal	220	222	221	225	1.91	0.58	0.42	0.82
LWG, g/d	785	831	845	956	22.1	0.03	0.06	0.41
Feed conversion, g LWG/kg DM intake	114	118	114	130	2.7	0.26	0.06	0.29
DOM conversion, g LWG/kg DOM intake	195	199	182	212	1.9	0.97	0.09	0.20
N use efficiency, g LWG/g N intake	5.50	5.74	5.57	6.26	0.13	0.25	0.07	0.38
Feed cost, US$/kg LWG	2.65	3.00	2.49	2.57	0.066	0.01	0.07	0.26

DM = dry matter, DOM = digestible organic matter, LW = live weight, LWG = live weight gain, N = nitrogen.
[1] Proportions of RUP: 260 g/kg crude protein (RUP260) or 360 g/kg crude protein (RUP360).

interactions were observed between RUP proportion and forage source for nutrient intake and ATTD. Similarly, Zanton et al. (2007) found no differences in nutrient utilization when diets based on corn silage or grass hay at 700 g/kg DM of forage and varying in soluble protein and RUP were evaluated. The lack of interaction effects in the present study was probably due to the fact that the amount of substituted forage was only 250 g/kg DM and diets were balanced for similar metabolizable energy and CP concentrations and fed *ad libitum* (Table 3), suggesting that the dietary treatments were not contrasting enough to result in the expected interactions.

4.2. Nitrogen balance

Increases in DM intake due to cowpea substitution for pangola grass hay resulted in greater N intake with cowpea diets (144 versus 153 g N/d, $P < 0.01$, Table 4); however, there were no differences in N excretion. A similar observation was made in the study of Castro-Montoya and Dickhoefer (2018) who found that replacing grasses with legumes had no effects on N excretion and its partitioning between feces and urine. Despite similar ATTD of CP and greater N intake with cowpea than with pangola grass hay, daily fecal N excretion was similar for both forage sources (42.4 versus 44.2 g N/d for pangola grass and cowpea hay, respectively; Table 4).

Likewise, urinary N excretion was similar in cowpea and pangola grass hay diets. Studies have shown a greater partitioning of N excretion towards urine when alfalfa-based diets were compared to corn-silage-based diets due to the greater concentration of RDP found in this temperate legume (e.g., Wattiaux and Karg, 2004a; Wattiaux and Karg 2004b). Protein characteristics may differ between temperate and tropical legumes, an area worthy of further research. Yet, diets similar in metabolizable energy and CP concentration, RUP proportion (for each RUP level), and uCP supply were created for both forage sources by adjusting the ingredient composition of the concentrate mixture. Hence, it appears that the substitution of pangola grass hay by cowpea hay (at the inclusion levels of this study) did not cause major changes in rumen CP degradation dynamics and hence, N excretion. In this line, daily N retention, although numerically greater for cowpea, was not significantly different between treatments (Table 4), likely due to a high inter-animal variation for this variable.

The partitioning of ingested N into fecal, urinary, and retained N is affected by RDP supply (Hristov et al. 2004), with lower RUP proportions (i.e., greater RDP) increasing urinary N excretion and thus reducing N retention. However, N balance did not differ between RUP levels in the present study. Similarly, Lascano et al. (2012) found no differences in N excreted and retained in heifers under tropical conditions when two diets differing in soluble protein (460 g versus 250 g/kg CP) were compared. Silva et al. (2018a) fed diets varying from 380 to 570 g RUP/kg CP to Holstein heifers and observed tendencies of greater N retention and lower urinary N excretion at higher RUP proportions. Compared to the above-mentioned studies, differences in dietary RUP proportions in the present study (260 versus 360 g RUP/kg CP) may not have been large enough to induce significant differences in N balance. Besides, greater rumen MPS for RUP260 than RUP260 diets (see below) at least partly offset the effects of the lower RUP proportions on total postruminal CP flow, ruminal ammonium absorption, and thus urinary N excretion.

Rumen MPS and EMPS (both, in g microbial N per kg of N intake or per kg DOMI) were lower in diets with greater rather than lower RUP. Numerous studies have also shown that greater dietary RUP proportions can decrease MPS in milking cows and heifers (Santos et al., 1998; Silva et al., 2018a; Silva et al., 2018b). Authors of these studies suggested that limited RDP, low rumen energy availability, or a lack of synchrony between N and energy supply for microbial metabolism are possible causes for lower rumen MPS. Nevertheless, as mentioned above, RDP supply was likely not limiting rumen fermentation and MPS in the present study. Instead, RUP360 diets contained less corn meal, which reduced availability of fermentable carbohydrates for rumen microbes. Also, fish meal included in both RUP360 diets has been shown to depress microbial growth in cultured ruminal content due to its free fatty acids content (Hoover et al., 1989). Reasons for the observed differences in MPS in response to dietary RUP proportion are not completely clear and need further research; however, they did not impair nutrient intake and ATTD. Moreover, as increased RUP intake compensated for the lower rumen MPS for RUP360 than RUP260, duodenal uCP flow and also animal performance were similar for both RUP levels.

At RUP360, feeding cowpea hay slightly increased RUP intake (Table 5), likely a result of the greater CP intake when compared to the respective diet with pangola grass hay (Table 3).

4.3. Animal performance and feed conversion efficiency

Improved nutrition of dairy heifers will enhance their growth and can lead to conception and calving at adequate body development, increase subsequent milk yield and longevity, and fasten recovery of investments in dairy farms (Zanton and Heinrichs, 2005; Bayram et al., 2009). Substituting cowpea for pangola grass hay increased LWG of heifers in the present study. When legumes are added to grass or corn silage, they can complement each other by providing, respectively, RDP and fermentable carbohydrates for rumen MPS (Groff and Wu, 2005; Dewhurst, 2013). In this line, heifers fed diets with greater energy concentrations were shown to have greater LWG and feed conversion efficiencies (Beltrand et al., 1997). Cowpea has also been recommended as a source of CP and energy for feeding dairy cattle, because of its superior CP concentration and *in vitro* DM digestibility compared to Bahia grass (*Paspalum notatum* Flugge; Foster et al., 2009; Foster et al., 2011). The greater LWG found with cowpea diets in the present study might be related to factors such as 1) their greater metabolizable energy concentrations (Table 2), 2) greater intake and ATTD of OM (Table 3), and 3) greater RUP flow that likely increased amino acid supply to heifers (Table 5).

Tendencies for greater LWG, feed conversion efficiency, DOM conversion, and NUE were found with RUP360 compared to RUP260 (Table 6), which is also in line with the greater N retention observed for RUP360. The RUP contributes to duodenal uCP flow and it has been proposed that greater amounts of RUP than those recommended by NRC (2001) can improve performance in heifers. For instance, Tomlinson et al. (1997) showed that LWG and feed conversion efficiency improved linearly when dietary RUP proportion increased from 310 to 550 g/kg CP by using blood meal. Similarly, Silva et al. (2018b) found greater LWG and feed conversion efficiency in heifers fed diets with 510 g compared to 380 g and 440 g RUP/kg CP using fish meal as RUP source. Authors of both studies suggested that a greater flow of amino acids to the small intestine from RUP sources could explain these results, which in view of the lower rumen MPS and hence, duodenal flow of microbial protein was likely not the case in the present study. Additionally, the greater ATTD of NDF and dietary metabolizable energy concentrations for RUP360 than RUP260 likely contributed to the greater LWG and feed conversion efficiencies. Only tendencies were observed, because the difference in ingredient and nutritional composition between the diets of the two RUP levels were, however, small.

Feed cost per kg of LWG was lower for the cowpea hay than the pangola grass diet (Table 6). Most of this difference is due to greater LWG and the fact that cowpea substituted for part of the soybean meal, which is a more expensive source of dietary CP. Moreover, feed conversion efficiency tended to be greater with the cowpea than the pangola grass diets. Meanwhile, increasing dietary RUP proportion with fish meal, although a good quality RUP source (Santos et al., 1998), improved heifer performance variables but also increased feed cost per unit of LWG. An alternative source of RUP with a lower price compared with fish meal may help to increase LWG without increasing feed costs.

An interaction of forage source and RUP was not detected for performance variables (Table 6). However, when RUP proportion was increased from 260 to 360 g/kg CP, LWG increased by 46 and 111 g/animal and d (or 4 and 15 g/kg DM intake) for pangola grass and cowpea diets, respectively, suggesting that the greater ATTD of nutrients in the cowpea diets together with the greater dietary RUP proportions can synergistically increase animal performance. Total CP requirements are the sum of RDP required for rumen microbial growth and RUP required to supplement the microbial protein produced to support LWG (NRC, 2001). It has been proposed that excessive ruminal CP degradation may be the most limiting nutritional factor in higher-quality temperate legume forages (Broderick 1995), which is likely also the case for tropical legumes. Hence, growing dairy heifers can benefit from both, cowpea hay inclusion and greater RUP proportions in the diet, improving animal growth (Zanton and Heinrichs, 2005) and fertility. This may contribute to enhance NUE at individual animal and herd level and thus environmental sustainability of dairy farming in the Tropics (Powell, 2014).

5. Conclusions

Under the conditions similar to those in the present study, the use of cowpea hay in the diet of dairy heifers increases their feed intake, nutrient digestibility, and LWG, leading to lower feed cost per kg of LWG compared to the use of pangola grass hay. Increasing dietary RUP proportions from 260 to 360 g/kg CP reduces rumen MPS but may enhance LWG, feed conversion efficiency, and NUE. There are no interactions between forage source and dietary RUP proportion for nutrient intake and digestibility, but some synergistic effects may exist for animal performance parameters. Feeding legumes and/or increasing RUP proportions in diets of growing heifers can enhance heifer growth rates and may thereby contribute to reduced age at first calving in dairy farming in the Tropics.

Declaration of Competing Interest

There are no conflicts of interest.

CRediT authorship contribution statement

E.E. Corea: Conceptualization, Methodology, Investigation, Resources, Writing - original draft, Visualization, Project administration, Funding acquisition. **J. Castro-Montoya:** Conceptualization, Methodology, Formal analysis, Visualization, Writing - review & editing, Supervision. **M.V. Mendoza:** Investigation, Project administration, Funding acquisition. **F.M. López:** Investigation, Resources. **A. Martinez:** Investigation. **M.E. Alvarado:** Investigation. **C. Moreno:** Investigation. **G.A. Broderick:** Conceptualization, Validation, Visualization, Writing - review & editing. **U. Dickhoefer:** Resources, Formal analysis, Visualization, Writing - review & editing, Supervision.

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