Effect of lemon leaves on energy and C–N balances, methane emission, and milk performance in Murciano-Granadina dairy goats

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ABSTRACT: The objective of this experiment was to find out the effect of lemon leaves on energy and C–N balances, methane emission, and milk performance in dairy goats. Lemon leaves were used to replace alfalfa as forage in a diet for Murciano-Granadina goats. Ten Murciano-Granadina dairy goats (44.1 ± 4.47 kg of BW) in late lactation (185 ± 7.2 d) were selected in a crossover design experiment, where each goat received 2 treatments in 2 periods. One group was fed a mixed ration with 450 g of pelleted alfalfa per kilogram of DM (**ALF** diet) and, the other group replaced alfalfa with 450 g of pelleted lemon leaves per kilogram DM (**LEM** diet). The concentrate was pelleted, being the same for the two groups (forage to concentrate ratio was 45/55). The goats were allocated to individual metabolism cages. After 14 d of adaptation, feed intake, total fecal and urine output, and milk yield were recorded daily over a 5-d period. Then, gas exchange measurements were recorded individually by an open-circuit indirect calorimetry system using a head box. Higher dietary lipids in LEM diet reduced DMI (200 g/d) and energy intake (251 kJ/kg of $BW^{0.75}$), although no differences between treatments were observed for ME intake (998 kJ/kg of $BW^{0.75}$, on average) and oxidation of nutrients (64% and 25% for carbohydrates and fat oxidation, respectively, on heat production from oxidation basis). Greater ($P < 0.05$) milk fat values for C18:2n6t and CLA 9c11t + 9t11c were found in LEM compared with ALF diet. Goats fed LEM diet produced significantly fewer CH_4 emissions than ALF diet (18%). Likewise, the use of lemon leaves as forage reduced the amount of CH_4 in 2.7 g/kg of milk. Results suggest that lemon leaves are effective in reducing CH_4 emission without detrimental effect on milk yield.

Key words: goats, lemon leaves, methane emissions

J. Anim. Sci. 2018.96:1508–1518
doi: 10.1093/jas/sky028

INTRODUCTION

Many foods and beverage by-products resulting from processing and manufacturing end up as waste, most of which is burned, dumped into landfills, or used as compost, which leads to wasted resources, and possible environmental problems due to unsuitable disposal. Using residues of the crop and food processing industries to feed livestock has the advantage to obviating the need for costly waste management programs. One of these by-products is citrus feedstuffs (Bampidis and Robinson, 2006). The main residue generated in citrus cultivations is pruning waste. Yearly, Spain generates 1.87 million t of pruning waste (DM basis), of which approximately 50% is leaf and 50% wood (EFEagro, 2016). Within Spain, the Valencian Community is one of the world's oldest citrus production areas. Due to the high production of lemons in the Valencian Community,

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Received January 27, 2017.
Accepted February 15, 2018.

lemon leaves are an important pruning waste in this area. Additionally, lemon leaves could be beneficial to reduce methane emissions from ruminants' digestive tract due to the essential oils present in the leaves; oil has a negative effect on ruminant methane emissions (Knapp et al., 2014).

In our study, we use lemon leaves to feed lactating Murciano-Granadina dairy goats. Our aim was to find out the effects of including lemon leaves as forage in mixed diets upon intake, energy, C and nitrogen (N) balance, milk performance, and their potential to reduce methane emissions.

MATERIALS AND METHODS

The experimental procedures were approved by the Committee on Animal Use and Care at the Universitàt Politècnica de Valencia, Valencia (Spain) and follow the codes of practice for animals used in experimental works proposed by the EU (2003).

Lemon Leaves, Animals, and Diets

Lemon leaves go through a process of dehydration until reaching an approximate humidity of 14%, followed by a crushing/refining and pelletizing process.

Ten multiparous mature Murciano-Granadina dairy goats in late lactation were selected and divided into 2 homogenous groups of 5 goats based on similar BW (44.1 ± 4.47 kg of BW), milk production in previous lactations (650.6 ± 50 kg during 210 ± 30 d of lactation, on average), and milk yield at the beginning of the experiment (2110 ± 298.3 g/d, on average). Treatments consisted of two different mixed rations (Table 1). Goats were fed daily with 1 kg forage and 1.2 kg concentrate (the ratio forage and concentrate was 45/55). The concentrate and premix were mixed and pelleted, being the same for the 2 groups. The forage was different between groups; 1 group was fed with pelleted alfalfa (**ALF**

Table 1. Ingredients and chemical composition of the diets.

	FORAGES		CONCENTRATE	Diet[b]	
Item[a]	Alfalfa	Lemon leaves	Compound feed	ALF	LEM
Ingredients, g/kg DM					
Alfalfa pellet	1000			450	
Lemon leaves pellet		1000			450
Barley			350	193	193
Corn			309	170	170
Wheat bran			150	83	83
Soy meal (44% CP)			148	81	81
Calcium carbonate			22	12	12
Sodium chloride			11	6	6
Bypass fat			5	3	3
Premix			5	3	3
Chemical composition, % of DM					
DM	92	90	91	91	90
OM	83	91	92	88	92
Ash	17	9	8	12	8
CP	17	13	18	17	16
Ether extract	1	3	3	2	3
NDF	49	26	17	31	21
ADF	32	18	5	17	11
ADL	7	4	0	3	2
NFC	16	48	55	37	52
Starch	1	11	50	28	32
C	41	42	42	41	42
Nitrogen	3	2	3	3	3
Carbon:nitrogen	15	20	15	15	16
Gross energy, MJ/kg DM	16	17	17	16	17

[a]Bypass fat of palm fatty acid distillate. Provided by Norel Animal Nutrition, Norel S.A., Spain. Premix = provided by NACOOP S.A. España. Premix composition (ppm or IU per kilogram of premix): Se, 40 mg/kg; I, 250 mg/kg; Co, 80 mg/kg; Cu, 3,000 mg/kg; Fe, 6,000 mg/kg; Zn, 23,400 mg/kg; Mn, 29,000 mg/kg; S, 60,000 mg/kg; Mg, 60,000 mg/kg; vitamin A, 2,000,000 IU/kg; vitamin D3, 400,000 IU/kg; vitamin E, 2,000 ppm; nicotinic acid, 10,000 ppm; choline, 20,300 ppm. NFC = nonfibrous carbohydrate content: 100-(NDF+ash+CP+EE). EE = Ether extract.

[b]ALF = alfalfa; LEM = lemon leaves.

diet) and the other with pelleted lemon leaves (**LEM** diet).

Nutrient requirements followed the recommendation of Lachica and Aguilera (2003) and Calsamiglia et al. (2009) for goats in lactation. The chemical composition of alfalfa, lemon leaves, and the whole mixed diet (forage and concentrate) is reported in Table 1. Half the daily ration was offered at 0800 h and half at 1600 h. Goats had free access to water.

Experimental Schedule and Measurements

Apparent total tract digestibility, gas exchange, energy partitioning, C and N balance, oxidation of nutrients, and milk composition and yield were determined. The experiment was conducted in a crossover design in two 29-d periods. During the adaptation, goats were fed the experimental diets in pens for 7 d and then allocated to individual metabolism cages at thermoneutrality (20 to 23 °C determined by a Hobo probe, ONSET data loggers, Cape Cod, MA, USA) for another 7 d. Next, data on the feed offered and refused, and total fecal, urine, and milk output were recorded daily for each goat for a 5-d period, as well as BW at the beginning and end of the period. Feces were collected in wire-screen baskets placed under the floor of the metabolism crates and urine was collected through a funnel into plastic buckets containing 100 mL 10% (vol/vol) of H_2SO_4 to prevent microbial degradation and the loss of volatile ammonia-N (**NH_3-N**). Representative samples (10%) of diet, feces, and urine were collected over 5 consecutive days, stored at −20 °C, and pooled for chemical analysis. The goats were milked once daily at 0800 h with a portable milking machine (Flaco, model DL-170, J. Delgado S.A., Ciudad Real, Spain). The individual milk yield was measured and a sample of 10% was placed in a bottle and frozen until analysis. In addition, samples were collected into plastic vials and immediately taken to the Interprofessional Dairy Laboratory of the Valencia Community Region (LICOVAL, Valencia, Spain) for compositional analysis (DM, protein, fat, and lactose). Ruminal fluid samples were collected by stomach tube before the morning feeding on the last day of the apparent digestibility trial. Ruminal fluid pH was immediately determined using a Model 265A portable pH meter (Orion Research, Inc., Beverly, MA, USA). A ruminal fluid sample was acidified with 3 mL of 50% H_2SO_4 and frozen until later determination of NH_3-N. Samples for analysis of VFA were mixed with H_3PO_4 and kept frozen until analysis.

Gas exchange was measured for each goat during 24 h (5 goats per treatment) by an indirect calorimetric system based on a ventilated head-box designed for small ruminants. To this end, each digestibility-balance period included 10 d of gas exchange determinations in the crossover design. Fernández et al. (2012, 2015) described the mobile open-circuit respirometry system used for these measurements. The whole system was calibrated according to McLean and Tobin (1987), and calibration factors were calculated according to Brockway et al. (1971).

Chemical Analysis

Feed, feed refusal, and feces samples were first dried in a forced air oven at 55 °C for 48 h, and then ground to pass a 1-mm screen before analysis. Urine and milk were dried by lyophilization. Chemical analyses of the diet, refusals, and feces were conducted according to AOAC (2000) for DM (method 934.01), ash (method 942.05), and ether extract (**EE**, method 920.39). The DM of diets and feces was determined by oven-drying at 102 ± 2 °C for 24 h. Ash concentration was measured by incineration in an electric muffle furnace at 550 °C for 6 h. The EE was extracted with petroleum ether after acid hydrolysis to recover saponified fat (Soxtec System HT Tecator, Hillerød, Denmark; 1047 Hydrolyzing Unit and 1043 Extraction Unit). The NDF and ADF were measured in an ANKOM Fiber Analyzer (A220, ANKOM Technologies, Fairport, NY, USA) according to Mertens (2002) and AOAC (2000), respectively. The NDF was determined using sodium sulfite and α-amylase. The nonfibrous carbohydrate (**NFC**) content of diets was calculated by difference based on chemical analysis of individual feeds according to NRC (2001): NFC = 100 − NDF − ash − CP − EE. The gross energy (GE) content of the dried samples (feed, feces, urine, and milk) was analyzed by combustion in an adiabatic bomb calorimeter (Gallenkamp Autobomb; Loughborough, UK). Starch content was determined by enzymatic method (α-amylase obtained from Sigma-Aldrich, Steinheim, Germany) according to Batey (1982). The C and N were analyzed by the Dumas principle (TruSpec CN; LECO Corporation, St. Joseph, MI, USA). Multiplying N by a factor of 6.25 converted the results to CP.

Milk composition (DM, fat, protein, and lactose) was analyzed with an infrared analyzer (MilkoScan FT120 Foss Electric, Hillerød, Denmark). Fatty acids (**FA**) methyl esters of total

milk lipids were prepared directly as described in the work of O´Fallon et al. (2007) and analyzed in a Focus Gas Chromatograph (Thermo, Milan, Italy) equipped with a split/splitless injector and a flame ionization detector. A subset of milk samples were collected and analyzed for milk urea. Milk urea was analyzed using flow injection analyses following the manufacturer´s instructions (Foss Tecator AB, Höganäs, Sweden).

The NH_3-N content of ruminal fluid samples was analyzed by the Kjeldahl procedure (2300 Kjeltec Analyzer Unit Foss Tecator, Hillerød, Denmark). Determination of ruminal VFA was based on the method described by Jouany (1982) using a gas chromatograph (Fisons 8000 series; Fisons Instruments SpA, Milan, Italy) equipped with a split/splitless injector and flame ionization detector.

Calculations

The ME intake (**MEI**) was calculated as the difference between GE intake (**GEI**) and energy losses in feces, urine, and CH_4 (with an energy equivalent value of 39.5 kJ/liter CH_4; Brouwer, 1965).

The heat production (**HP**) was determined from measurements of O_2 consumption, CO_2 and CH_4 production, and urine N ($\mathbf{N_{urine}}$), using the following Brouwer equation (1965):

$$\mathrm{HP(kJ)} = 16.18 \times \mathrm{O_2} + 5.02 \times \mathrm{CO_2} - 2.17 \times \mathrm{CH_4} - 5.99 \times \mathrm{N_{urine}}$$

where gases were expressed in liter/d and N_{urine} in g/d. The body tissue energy ($\mathbf{RE_{body}}$) was calculated as MEI − HP − milk energy ($\mathbf{E_{milk}}$).

The energy associated with the oxidation of protein (**OXP**), carbohydrate (**OXCHO**), and fat (**OXF**) was calculated by the method of Brouwer (1958) and Chwalibog et al. (1997) for ruminants. The CO_2 production from oxidation ($\mathbf{CO_{2x}}$) was calculated as $CO_2 - (2 \times CH_4)$, according to Fahey and Berger (1988). The calculations were carried as follows:

$$\mathrm{OXP} = 6.25 \times \mathrm{N_{urine}} \times 18.42\,(\mathrm{kJ/g})$$

$$\mathrm{OXCHO} = (-2.968 \times \mathrm{O_2} + 4.174 \times \mathrm{CO_{2x}} - 2.446 \times \mathrm{N_{urine}}) \times 17.58\ (\mathrm{kJ/g})$$

$$\mathrm{OXF} = (1.719 \times \mathrm{O_2} - 1.719 \times \mathrm{CO_{2x}} - 1.963 \times \mathrm{N_{urine}}) \times 39.76\,(\mathrm{kJ/g})$$

Then, the HP from oxidation (**HPx**) was

$$\mathrm{HPx(kJ)} = 16.18 \times \mathrm{O_2} + 5.02 \times \mathrm{CO_{2x}} - 5.99 \times \mathrm{N_{urine}}$$

Again, gases were expressed in liter/d and N_{urine} in g/d. Heat of fermentation (**HPf**) was estimated subtracting HP from HPx. The nonprotein respiratory quotient from oxidation of nutrients (**RQnpx**) was determined according to Chwalibog et al. (1996) as RQnpx = $(CO_{2x} - (N_{urine} \times 6.25 \times 0.774)/O_2 - (N_{urine} \times 6.25 \times 0.957))$.

The efficiency of use of ME for lactation was calculated according to AFRC (1993). Energy lost from the body, indicating mobilization of body fat reserves in support of milk secretion, was assumed to be used for milk synthesis with an efficiency of 0.84, and the concomitant energy storage during lactation was taken to be 0.95 times the milk secretion efficiency. Consequently, the corrected milk energy was estimated as E_{milk} + (0.84 × negative energy retention) + (1.05 × positive energy retention). The efficiency of use of ME for milk production ($\mathbf{k_l}$) was calculated as corrected milk energy/(ME − $\mathbf{ME_m}$ [ME for maintenance]). The value of ME_m was obtained from the estimation of Aguilera et al. (1990) for Granadina goats from both positive and negative energy retentions (401 kJ/kg of $BW^{0.75}$). Net energy for lactation (NE_L) was computed as MEI × k_l. The milk FA atherogenicity index was calculated as C12:0 + 4 × C14:0 + C16:0/unsaturated FA (Ulbricht and Southgate, 1991). Methane conversion ratio, also called Ym factor, represents energy loss as CH_4 per unit of GEI.

Statistical Analysis

The effects of alfalfa substitution by lemon leaves on intake, digestibility, ruminal fermentation, milk performance, energy and C–N balances, and oxidation of nutrients were analyzed using the PROC MIXED of SAS (2001). The experiment was conducted as a crossover design: each goat received both treatments in 2 periods. Goat served as the experimental unit for all data. The model for the dependent variables included the fixed effect of diet and period, with goat as random effect. The following statistical model was used as follows: Y = μ + D + T + goat + ε, where Y is the dependent variable, μ is the overall mean, and D and T are the fixed effects of diet and period of time, respectively; goat is the random effect of goat; and ε is the random error. Least squares means are reported throughout and differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

There was no significant effect for the fixed effect period and its interaction with diet throughout the experiment, so the tables report only the effect of diet. The average value for the calibration factor of indirect calorimeter was 1.0043 ± 0.00126 ($n = 4$) and 0.9951 ± 0.00982 ($n = 4$) for O_2 and CO_2, respectively.

Table 1 shows the composition of the ingredients and mixed diets. Alfalfa pellets had a higher value of CP, fiber, and ash than lemon leaves pellets, whereas lemon pellets were highest in EE, starch, and NFC. These differences were kept in ALF and LEM mixed diets and, subsequently, C to N ratio was lower in ALF than LEM diet because it is greater in CP. LEM diet had 1 and 4 points more EE and starch than ALF diet, respectively, whereas ALF diet was 1 point higher in CP.

Table 2. Fatty acid profile from alfalfa and lemon leaves (mg/100 mg).

Fatty acids	Alfalfa	Lemon leaves
C4:0	0.0	0.0
C6:0	0.0	0.002
C8:0	0.0	0.003
C10:0	0.001	0.0
C11:0	0.0	0.0
C12:0	0.006	0.01
C13:0	0.124	0.126
C14:0	0.013	0.053
C14:1	0.0	0.0
C15:0	0.006	0.004
C16:0	0.252	0.484
C16:1	0.009	0.002
C17:0	0.007	0.018
C17:1	0.001	0.003
C18:0	0.044	0.075
C18:1n9t	0.0	0.0
C18:1n9c	0.058	0.201
C18:1n7	0.012	0.018
C18:2n6t	0.0	0.0
C18:2n6c	0.143	1.081
C20:0	0.014	0.014
C18:3n6	0.005	0.01
C20:1	0.0	0.0
C18:3n3	0.157	0.708
CLA 9c11t + 9t11c	0.0	0.0
C22:0	0.018	0.013
C20:3n6	0.005	0.00
C22:1n9	0.003	0.017
C20:3n3	0.008	0.006
C20:4n6	0.0	0.0
C24:0	0.018	0.016
C20:5n3 EPA[a]	0.002	0.002

[a]EPA = eicosapentaenoic acid.

FA profile from forages is shown in Table 2. No short chain FA was observed in either forage. However, a greater amount of linoleic (1.081 vs. 0.143) and linolenic acid (0.708 vs. 0.157) was found in lemon leaves than alfalfa, respectively.

Feed Intake, Digestibility, and Rumen Fermentation

Intake and total tract apparent digestibility of nutrients by dairy goats during mid-lactation are shown in Table 3. Although the total quantity of feed offered was 2.2 kg/d, the DMI was low and different ($P < 0.05$) between diets (1.7 ± 0.05 and 1.5 ± 0.05 kg/d for ALF and LEM, respectively). The low DMI associated with LEM diet could be due to the high fat content, as indicated by other authors supplementing rice bran oil in dairy cows (Lunsin et al., 2012). As the concentrate and forages were pelleted, consumption of concentrate and forage was recorded separately. So the real forage concentrate ratio was 35:65 and 33:67 for ALF and LEM diet, respectively, rather than the theoretical ratio proposed (45:55).

No differences were found in EE and ADF apparent total tract digestibility between diets. However, apparent digestibility coefficients of DM, OM, CP, NFC, and GE were higher ($P < 0.05$) in LEM than ALF diets, probably linked to the lower forage to concentrate ratio (33:67) of LEM diet. A significant effect ($P < 0.05$) was observed for NDF and ADL in ALF diet, with higher digestibility than LEM diet. Increasing lipid content in the diet decreases fiber degradability and reduces fermentable substrate. It is expected that high levels of lipid should inhibit fiber digestion, possibly by coating food particles and preventing bacterial attachment (Palmquist and Jenkins, 1980). Other authors, such as Villarreal et al. (2006) in beef cattle, reported that digestion of total diet DM and OM tended to increase linearly with increasing citrus by-products supplementation (pelleted citrus pulp). Similar values of OM and energy digestibility were observed with dried lemon by Madrid et al. (1996). These citrus by-products were not comparable with the lemon leaves used in this trial.

Results from rumen liquor samples are shown in Table 4. The average rumen pH never fell below 6.2, so the values obtained can be considered sufficiently high to maintain normal rumen fermentation (Ørskov and Fraser, 1975). Goats fed LEM diet presented lower NH_3-N (49%, $P < 0.05$) concentration compared with ALF. It seems that the greater CP content (Table 1) and DMI (Table 3) in ALF than LEM diet led to an excess of N in the

Table 3. Body weight, intake, and apparent digestibility coefficients of Murciano-Granadina goats (n = 10) during mid-lactation according to the type of diet.

	Diet[b]			
Item[a]	ALF	LEM	SEM	*P*-value
BW, kg	41.5	46.7	0.80	0.001
total DMI, kg/d	1.7	1.5	0.04	0.034
concentrate DMI, kg/d	1.1	1.0	0.01	0.057
forage DMI, kg/d	0.6	0.5	0.04	0.059
Real forage: concentrate ratio	35:65	33:67		
Digestibility, % of DM				
DM	61.1	68.6	1.37	0.007
OM	62.9	70.8	1.35	0.002
CP	66.0	73.1	1.24	0.003
Ether extract	67.9	72.6	1.30	0.069
NDF	32.5	18.4	3.41	0.037
ADF	33.5	25.9	3.11	0.227
ADL	31.0	10.0	3.89	0.005
NFC	91.1	93.3	0.43	0.007
GE	65.1	72.4	1.28	0.002

[a]NFC = nonfibrous carbohydrate content: 100-(NDF+ash+CP+EE).
[b]ALF = alfalfa; LEM = lemon leaves.

rumen. On the other hand, it is generally accepted that the utilization of NH_3-N for ruminal microbial protein synthesis increases when diets contain a greater amount of NFC, as LEM diet. No effect of treatment on VFA profile was observed. The lower (P = 0.103) total VFA in LEM might suggest partial inhibition of microbial synthesis by the lemon leaves' lipid content.

Energy Balance

Daily energy balance obtained with the two diets is listed in Table 5. The GEI was 15% greater (P < 0.05) for ALF than LEM diet. The lower DMI in LEM diet (Table 3) was followed by lower GEI, as the LEM diet is richer in unsaturated C18FA and these FA have a greater hypophagic effect (Allen et al., 2014). Energy losses in feces were higher (P < 0.05) for ALF than LEM diet, possibly related to the increase in fiber content and lower digestibility. Urine energy losses were 26% higher (P < 0.05) for LEM than for ALF diet. The ALF diets presented greater (P < 0.05) energy losses in CH_4 than LEM diet, indicating that increasing the level of lipids in diet reduced the CH_4 production, as suggested by Knapp (2014). No differences between treatments were observed for MEI (998 ± 29.5 kJ/kg of $BW^{0.75}$, on average), although our values were lower than other (1,254 kJ MEI/kg of $BW^{0.75}$ for Murciano-Granadina goats [Criscioni and Fernández, 2016] and 1,126 kJ MEI/kg of $BW^{0.75}$ for Saanen goats [Bava et al., 2001]), both at late lactation. Criscioni and Fernández (2016) used mixed diets with similar forage:concentrate ratio (35:65) than the present study. The concentrate and premix were pelleted, although the forage was alfalfa hay not pelleted. Bava et al. (2001) compared forage with nonforage diet (both not pelleted), and the higher MEI obtained was the average value of the two diets. The HP values were superior (P < 0.05) for ALF than LEM diet (39 kJ/kg of $BW^{0.75}$), which is associated with greater MEI. The HP value is in

Table 4. Rumen pH, NH_3-N, and VFA of Murciano-Granadina goats (n = 10) during mid-lactation according to the type of diet.

	Diet[b]			
Item[a]	ALF	LEM	SEM	*P*-value
Rumen pH	7.1	7.0	0.06	0.893
NH_3-N, mg/dL	20.5	10.4	2.52	0.041
Total VFA, mM	45.1	40.6	2.70	0.103
Individual VFA, mol/100 mol				
Acetic acid	56.1	54.4	1.02	0.457
Propionic acid	20.3	16.8	1.06	0.104
Iso-butyric acid	1.5	2.1	0.28	0.349
Butyric acid	17.7	18.4	1.38	0.825
Isovaleric acid	1.8	2.6	0.35	0.313
N-valeric acid	1.7	2.8	0.32	0.103
N-caproic acid	0.5	1.3	0.35	0.334
Heptanoic acid	0.4	1.7	0.52	0.246

[a]NH_3-N = ammonia nitrogen.
[b]ALF = Alfalfa; LEM = Lemon leaves.

Table 5. Daily energy partitioning (kJ/kg of $BW^{0.75}$) of Murciano-Granadina goats (n = 10) during mid-lactation according to the type of diet.

	Diet[b]			
Item[a]	ALF	LEM	SEM	*P*-value
GEI	1,713	1,462	41.8	<0.01
E_{feces}	598	407	25.5	<0.01
E_{urine}	28	38	2.2	0.02
$E_{methane}$	63	47	1.7	<0.01
MEI	1,025	970	29.4	0.36
E_{milk}	358	336	11.7	0.36
HP	649	610	10.5	<0.01
RE_{body}	18	24	27.2	0.52
Kl	0.60	0.63	0.008	0.02

[a]GEI = gross energy intake; E_{feces} = energy losses in feces; E_{urine} = energy losses in urine; $E_{methane}$ = energy losses in methane; MEI = metabolizable energy intake; E_{milk} = energy losses in milk; HP = heat production; RE_{body} = recovered energy in tissue (RE_{body} = MEI − HP − E_{milk}); kl = efficiency of use of ME for milk production.
[b]ALF = alfalfa; LEM = lemon leaves.

agreement with the range of some literature values with some similitude with our study; so Tovar-Luna et al. (2010) found an average value at late lactation of 680 kJ/kg of $BW^{0.75}$ for Alpine goats fed mixed diets with 60% of concentrate; here, the forage was ground alfalfa hay, and Criscioni and Fernández (2016) found average values of 640 kJ/kg of $BW^{0.75}$, as we described above. No differences between treatments were observed for milk energy (347 kJ/kg of $BW^{0.75}$, on average), and energy recovered in the body was positive and did not differ between diets (21 kJ RE_{body}/kg of $BW^{0.75}$, on average).

Table 5 showed significant differences ($P = 0.02$) for k_l between diets. The k_l was 5% greater in LEM compared with ALF. Although Aguilera et al. (1990) and Tovar-Luna et al. (2010) found similar values (0.67 and 0.63, respectively) to those in our study (0.62 on average), the 9% of reduction in DMI in LEM was accompanied with an increase of 10% in GE digestibility. Besides, energy waste in feces and methane was lower in LEM compared with ALF (32% and 25%, respectively). Therefore, the inclusion of lemon leaves in the diet increased kl. The NE_L was expressed as MJ/kg DM and the values obtained were 6.1 and 7.0 for ALF and LEM diets, respectively.

Table 6. Heat production (kJ/kg of $BW^{0.75}$) from oxidation and fermentation; daily oxidation (kJ/kg of $BW^{0.75}$) of protein, carbohydrate, and fat; and their contribution to the heat production from oxidation substrates (%) of Murciano-Granadina goats ($n = 10$) during mid-lactation according to the type of diet.

	Diet[b]			
Item[a]	ALF	LEM	SEM	*P*-value
HPx	639	602	10.3	<0.01
HPf	10	8	0.3	<0.01
OXP	51	96	6.2	<0.001
OXCHO	422	373	11.1	<0.01
OXF	166	138	9.4	0.07
OXP/HPx	8	16	1.1	<0.01
OXCHO/HPx	66	62	1.3	0.08
OXF/HPx	26	23	1.8	0.40
RQnpx	0.91	0.92	0.005	0.72

[a]HPx = heat production from oxidation of nutrients; HPf = heat production of fermentation [HPf = HP − HPx (Brouwer, 1958)]; OXP = heat production associated with the oxidation of protein; OXCHO = heat production associated with the oxidation of carbohydrates; OXF = heat production associated with the oxidation of fat; RQnpx = nonprotein respiratory quotient (unitless) from oxidation of nutrients {$[CO_2x - (N_{urine} \times 6.25 \times 0.774)]/[O_2 - (N_{urine} \times 6.25 \times 0.957)]$, where $CO_{2x} = CO_2$ from oxidation, and N_{urine} = N in urine}.

[b]ALF = alfalfa; LEM = lemon leaves.

Oxidation of Nutrients

The proportional contribution to HPx due to oxidation of nutrients is shown in Table 6. Significantly ($P < 0.05$) higher HPx and HPf were found in ALF than LEM diet, probably associated with the greater intake of forage (Table 3) and VFA production (Table 4).

Indirect calorimetry estimates only the total net loss of substrates (carbohydrates or lipids) and their conversion to CO_2 and CH_4, but does not consider any exchange or cycling that the substrate itself or its intermediates undergo along the biochemical pathways to complete oxidation (Derno et al., 2013). The OXP was higher (88%, $P < 0.05$) in LEM than ALF diet in agreement with the greater E_{urine} (Table 5) of the first, even when N_{intake} was 23% lower (Table 7) in LEM compared with ALF diet. The OXCHO was greater ($P < 0.05$) in ALF than LEM (12%), and no differences in OXF (152 ± 9.4 kJ/kg of $BW^{0.75}$, on average) were found. Dietary FA can reduce DMI and energy intake, as we mentioned above. Greater hypophagic effects of unsaturated C18FA compared with saturated C18FA diets were demonstrated (Allen et al., 2014), being consistent with the hepatic oxidation theory, as unsaturated FA are more rapidly oxidized than saturated FA, decreasing the palatability of diets and releasing satiety-inducing gut peptides. Table 2 shows higher unsaturated C18FA in lemon leaves than alfalfa. In spite of this, and the depression in feed intake with LEM diet, late lactating goats fed ad libitum seem to respond by maintaining a higher rate of OXCHO, and a slight lower rate of OXF (Chwalibog et al., 1997) as the RQnpx close to 1 indicated (0.91, on average).

The oxidation of nutrients was not significant when it was expressed as percentage of HPx, with the exception of OXP. Taking average figures, most of the HPx derived from OXCHO (64%, on average) followed by OXF (25%, on average). The greater OXCHO and lower OXF found in the two diets would be partly responsible for the positive-recovered tissue energy in the body (Table 5) and the feed intake (Derno et al., 2013) due that feed is available all the time on the feeder. These authors suggested that carbohydrate ingestion and oxidation appear to correlate with subsequent satiety and, since ruminant diets usually contain no very much fat, the contribution of OXF to satiety of mid-lactating cows is certainly much lower than the effect of OXCHO, although no information is available in dairy goats. No significant difference was observed

Table 7. Carbon and nitrogen balance (g/kg of $BW^{0.75}$) of Murciano-Granadina goats ($n = 10$) during mid-lactation according to the type of diet.

	Diet[b]			
Item[a]	ALF	LEM	SEM	*P*-value
C_{intake}	43.28	36.11	1.092	0.0006
C_{feces}	16.14	11.21	0.683	0.0001
C_{urine}	0.69	1.01	0.059	0.0066
C_{CO2}	17.71	15.80	0.283	0.0008
C_{CH4}	0.85	0.63	0.021	0.0007
C_{milk}	7.14	6.68	0.232	0.3342
$C_{retained\ body}$	0.75	0.78	0.663	0.9691
N_{intake}	2.90	2.24	0.078	0.0001
N_{feces}	0.92	0.61	0.042	0.0001
N_{urine}	0.41	0.71	0.054	0.0030
N_{milk}	0.62	0.54	0.018	0.0102
$N_{retained\ body}$	0.95	0.38	0.074	0.0011
$C{:}N_{intake}$	14.9	16.1	0.12	0.001
$C{:}N_{feces}$	17.6	18.4	0.36	0.140
$C{:}N_{urine}$	1.7	1.4	0.07	0.038
$C{:}N_{milk}$	11.5	12.4	0.21	0.007
$C{:}N_{retained\ body}$	0.8	2.1	2.33	0.842

[a]C_{intake} = C intake; C_{feces} = C losses in feces; C_{urine} = C losses in urine; C_{CO2} = C losses in CO_2; C_{CH4} = C losses in methane; C_{milk} = recovered C in milk; $C_{retained\ body}$ = recovered C in tissue; N_{intake} = N intake; N_{feces} = N losses in feces; N_{urine} = N losses in urine; N_{milk} = recovered N in milk; $N_{retained\ body}$ = recovered N in tissue.

[b]ALF = alfalfa; LEM = lemon leaves.

for RQnpx with values around 1 (0.91, on average), indicating predominance of OXCHO vs. OXF (Chwalibog et al., 1997).

Carbon and Nitrogen Balance

The daily C and N balance is shown in Table 7. Significant differences ($P < 0.05$) were observed in C intake, feces, urine, CO_2, and CH_4. Due to the greater DMI in ALF diet, the C_{intake} was also higher ($P < 0.05$) in ALF than LEM (17%). Higher values in C_{feces} were related to lower DM and OM digestibility in ALF than LEM diet. And the higher ($P < 0.05$) value of C_{urine} in LEM compared with ALF was associated with the reduction in MEI. The C_{CO2} was 11% greater in ALF than LEM ($P < 0.05$) and C_{CH4} a 26% greater in ALF ($P < 0.05$). The ratio C_{CH4} to C_{CO2} was 0.05 and 0.04 for ALF and LEM, respectively. This ratio describes the proportion of the C excreted as CH_4 (microbial fermentation of the feed) that is not metabolized to CO_2. The efficiency of milk C output regarding C ingested was 16% and 19% for ALF and LEM, respectively. Therefore, energetically goats fed LEM diet metabolize more feed carbon to milk production than goats fed ALF diet.

Significant differences ($P < 0.05$) were observed in N intake, feces, milk, and N retained in tissue with higher lower (23%, 34%, 13%, and 60%, respectively) values in LEM compared with ALF diet, whereas N_{urine} was 73% greater ($P < 0.01$). Lower N_{feces} was associated with the lower NH_3-N in LEM compared with ALF found in the rumen liquor and the greater N_{urine}, maybe related to the slightly lower ($P > 0.05$) MEI found. Kebreab et al. (2010) indicated increases in urinary N output in dairy cows when MEI reduces. The ratio between milk N output and N ingested presented an average value of 22%.

When C and N were expressed as C:N ratio, differences in C:N intake were observed. This supports the view that animals seek out the higher nitrogen diets. The reason animals will select the more palatable C3 plants is in their quest to maximize protein intake (Lauder, 2000). On the other hand, the lower C:N in urine for LEM diet showed us the higher excretion of urine N, probably as it was mentioned earlier associated with lower DMI (Table 3), because CP content of diets (Table 1) was very similar. The lower C:N in milk for ALF diet anticipates the higher milk protein content (Table 8).

Milk Production, Metabolites, and FA

Table 8 reports milk yield and milk composition of the goats during the experiment. No difference was found in milk yield between diets. Previous studies in our lab (same breed and late lactation) delivered higher milk yield (2.2 kg/d; Criscioni and Fernández, 2016) and slightly greater DMI (1.8 kg/d); goats were fed mixed diets using alfalfa as forage and forage:concentrate ratio of 35:65. Unfortunately, optimal dietary starch, NFC, and NDF reference values during late lactation of dairy goats have not been fully developed. So as we

Table 8. Daily milk production and composition of Murciano-Granadina goats ($n = 10$) during mid-lactation according to the type of diet.

	Diet[a]			
Item	ALF	LEM	SEM	*P*-value
Milk yield, kg/day	1.7	1.8	0.05	0.238
Chemical composition, %				
Dry matter	14.7	14.5	0.12	0.282
Fat	5.1	5.0	0.11	0.683
Protein	4.3	3.9	0.06	0.001
Lactose	4.6	4.8	0.03	0.001
Urea, mmol/L	8.6	7.2	0.45	0.131

[a]ALF = alfalfa; LEM = lemon leaves.

discussed previously, a greater DMI was expected in the present trial. Havlin and Robinson (2015), with another citrus by-product, found that milk production did not change in dairy cows fed a citrus extract. Lemon leaves had no effect on milk fat. The replacement of alfalfa with lemon leaves significantly increases ($P < 0.05$) the percentage of lactose in milk and decreases the protein. Higher DMI ($P < 0.05$; Table 3) and NH_3-N ($P < 0.05$; Table 4) in ALF led to higher milk protein and urea in milk (possibly due to the excess on NH_3-N in the rumen liquor). Milk lactose was greater in LEM than ALF diet, perhaps due to the higher starch content (Table 1) of LEM than ALF diet (van Knegsel et al., 2007).

Table 9. Fatty acid composition (g/100 g of identified fatty acids) of milk fat for goats fed the experimental diets ($n = 10$).

	Diet[b]			
Item[a]	ALF	LEM	SEM	*P*-value
C4:0	0.6	0.5	0.03	0.032
C6:0	1.7	1.4	0.06	0.015
C8:0	2.4	1.9	0.10	0.604
C10:0	9.2	7.9	0.37	0.061
C11:0	0.3	0.3	0.01	0.226
C12:0	4.9	4.2	0.27	0.234
C13:0	6.0	5.8	0.23	0.581
C14:0	8.1	7.5	0.32	0.360
C14:1	0.1	0.2	0.01	0.908
C15:0	0.7	0.7	0.04	0.494
C16:0	24.8	22.0	0.593	0.0225
C16:1	0.6	0.6	0.04	0.876
C17:0	0.3	0.3	0.02	0.233
C17:1	0.1	0.1	0.01	0.950
C18:0	3.3	3.5	0.31	0.789
C18:1n9t	0.8	1.2	0.21	0.361
C18:1n9c	8.8	8.4	0.57	0.784
C18:1n7	0.3	0.3	0.04	0.946
C18:2n6t	0.1	0.2	0.02	0.021
C18:2n6c	2.2	2.3	0.07	0.561
C20:0	0.1	0.1	0.00	0.075
C18:3n6	0.0	0.0	0.00	0.990
C20:1	0.0	0.0	0.00	0.444
C18:3n3	0.2	0.3	0.03	0.198
CLA 9c11t + 9t11c	0.2	0.6	0.07	0.028
C22:0	0.0	0.0	0.01	0.755
C20:4n6	0.2	0.2	0.01	0.743
Short-chain fatty acids	2.3	1.9	0.09	0.015
Medium-chain fatty acids	22.9	20.1	0.73	0.047
Long-chain fatty acids	51.2	48.6	1.23	0.319
SFA	62.5	56.1	1.67	0.047
MUFA	10.9	10.9	0.59	0.965
PUFA	3.0	3.5	0.16	0.101
AI	4.5	4.0	0.25	0.318

[a]CLA = conjugated linoleic acid; AI = atherogenicity index calculated as C12:0 + 4 × C14:0 + C16:0/unsaturated fatty acids (Ulbricht and Southgate, 1991).

[b]ALF = alfalfa; LEM = lemon leaves.

Effect of diet on the FA profile of milk fat is shown in Table 9. The FA with 16 or fewer C atoms derive from de novo synthesis, whereas those with 18C or more carbons atoms come from the diet or from lipid mobilization (Chilliard et al., 2003). Milk C15:0 and C17:0 are potential biomarkers of rumen function, as they are found in rumen bacterial lipids and may be partially synthesized endogenously from rumen substrates in the mammary gland (Vlaeminck et al., 2015). No differences were found in this study, showing appropriately performed with excess of NH_3-N in ALF diet (Table 4).

Short-chain FA contents were higher ($P < 0.05$) in ALF compared with LEM diet: C4:0 and C6:0 (derived from novo synthesis). Similar results were obtained by Fegeros et al. (1995) in lactating ewes fed another citrus by-product (dried citrus pulp). Higher values ($P < 0.05$) were found in C18:2n6t and CLA 9c11t + 9t11c in LEM compared with ALF diet; these higher values came from essential oil in lemon leaves. In our study, the C16:0 was 11% greater in ALF than LEM. Short, medium, and SFA were higher ($P < 0.05$) in ALF than LEM. Fievez et al. (2012) relate these increases in short and medium FA in milk with mammary gland de novo synthesis and greater rumen fermentation activity. No significant differences were found in MUFA, PUFA, and atherogenicity index.

Methane Emissions

The effects of diet on CH_4 emissions are shown in Table 10. Goats fed LEM diet produced significantly ($P < 0.05$) fewer CH_4 emissions than ALF diet. According to Johnson and Johnson (1995), fermentation of fibrous carbohydrates produces more CH_4 than fermentation of soluble sugars, which

Table 10. Methane emission of Murciano-Granadina goats ($n = 10$) during mid-lactation according to the type of diet.

	Diet[b]			
Item[a]	ALF	LEM	SEM	*P*-value
CH_4, g/d	18.2	15.0	0.32	0.001
Ym, %	3.7	3.3	0.10	0.026
CH_4/DMI, g/kg	10.9	9.9	0.29	0.079
CH_4/OMI, g/kg	12.3	10.8	0.33	0.018
CH_4/milk, g/kg	11.3	8.6	0.41	0.001

[a]Ym = methane energy/gross energy intake; DMI = dry matter intake; OMI = organic matter intake.

[b]ALF = alfalfa; LEM = lemon leaves.

in turn produce more CH_4 than fermentation of starch. Grainger and Beauchemin (2011) reported that increasing the level of starch and lipids, plus decreasing NDF and ADF in diet, reduces the CH_4 production, so these goats fed with LEM diet produced less CH_4 (higher content of starch, NFC, and fat, and lower in fiber).

Likewise, CH_4 output was positively correlated with milk C6:0 to C16:0 (Fievez et al. 2012), which result mainly from mammary de novo FA synthesis, based primarily on the use of acetate produced in the rumen during fiber digestion and these milk FA content was lower in LEM diet (Table 9).

In ruminant nutrition, decreased production of CH_4 represents an improvement in feed efficiency, as ruminants lose between 2% and 12% of their dietary GE as CH_4 (Johnson and Johnson, 1995). The Ym for both diets were 3.7% and 3.3% for ALF and LEM, respectively ($P < 0.05$). Regarding the LEM diet, the higher lipid content of lemon leaves (Tables 1 and 2) probably reduced methanogens and fiber degradation; lower NDF and ADL digestibility was observed in LEM diet (Table 3). Although CH_4 emission is most commonly expressed in the literature relative to GEI, the most meaningful expression is relative to DM or OM intakes. In the present work, when CH_4 was expressed related to OM intake or milk yield, differences were preserved. So the use of lemon leaves as forage reduced the amount of CH_4 in 2.7 g/kg of milk.

CONCLUSIONS

DMI and GEI decreased in LEM diet with no difference in MEI; RE_{body} was not affected, HP and CH_4 emissions decreased; OXP increased, and both OXF (slightly) and OXCHO decreased; milk yield was not affected but k_l was increased; milk protein was reduced and lactose increased, fat content was not affected; FA decreased in C16:0, increased in CLA and linoleic acid; short- and medium-chain and saturated FA decreased.

ACKNOWLEDGMENTS

This study was supported by HELIOTEC S.A., Vall de Uxó, Castellón de la Plana, Valencia, Spain, through LIFE Ecocitric Project, and LIFE LOW CARBON FEED PROJECT reference LIFE16/CCM/000088.

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