Productive performance and milk composition of dairy ewes supplemented with corn silage (*Zea mays L.*), sunflower (*Helianthus annuus*) silage, and their mixture

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SUPPLEMENTARY FILE

Material & methods

Roughages collection and conservation

The soil from the study site had a clay soil texture composed by 62% sand, 10% silt, and 28% clay. The land had a slope of 2 to 6%. The dominant rocks were of volcanic and clastic types. The soils were pelic vertisol type and haplic phaeozem and were characterized by being very compact and clayish. Likewise, soil had wide and deep cracks during drought season and showed a layer of tepetate between 10 and 50 cm of depth. Soils had 5.8 of pH, 23.8 cmol+kg^{-1} capacity of cationic interchange, 0.31 % of total nitrogen, 7.56 % of organic matter and 0.8 dS M^{-1} electrical conductivity (Vaca García *et al*., 2014).

A 2000 m^2 plot of corn was used and this plot was established on the 15th of April 2019. It was irrigated with side roll irrigation every 20 days and fertilized with 44 kg N/ha (44% N, FIMSA and ACIFEX,) and KCl 60 kg/ha, 60 days prior before harvest. A second plot of 2000 m^2 plot of sunflower was used (New Holland tractor, 3-5 cm).

For silage making, fresh corn and sunflower whole plant were chopped, placed, and compacted in 12 hermetically sealed plastic containers (100 × 120 cm) (n = 6), and *Pulque* (1 ml/kg FM) as an additive was used (Franco Martinez et al. 2020). Each container was kept in a dark room at 15 °C. After 60 days, silages were opened, and pH was determined (Conductronic model pH130).

Chemical Analysis

Silages were opened after 60 days and were used for ewes feeding. Three samples per container were taken from each treatment (n = 36 samples) for DM determination (Haigh and Hopkins, 1977). Samples were separately pooled and ground in a hammer mill with a 1-mm screen (Arthur Hill Thomas Co., Philadelphia, PA), and analyzed (three replicates) for dry matter (using a forced-air oven at 60 °C for 48 h; AOAC method 934.01), ash (incineration at 550 °C for 3 h;

942.05), nitrogen (Kjeldahl N; AOAC method 954.01), and ether extract (AOAC method 920.39) according to the AOAC (2015). Neutral detergent fiber (NDF, Van Soest *et al*., 1991), acid detergent fiber (ADF) and acid detergent lignin (ADL) (AOAC, 1997; 973.18) analyses were performed using an ANKOM200 Fiber Analyzer Unit (ANKOM Technology Corporation, Macedon, NY, USA). Neutral detergent fiber was assayed with alpha amylase. The non-fibrous carbohydrates (NFC) were calculated according to the equation proposed by Sniffen *et al*. (1992), NFC = 100 − (CP + EE + Ash + NDF), and adjusted in g/kg DM.

A second fresh silage subsample was used to assess pH (Conductronic model pH130, Puebla, Mexico), ammonia nitrogen (NH_3 -N) and volatile fatty acids (two replicates). The silage extract was obtained after homogenization in a stomacher device (model 400 circulator, Seward Inc., Bohemia, New York, USA) for 4 min, using 30 g of fresh sample and 270 g of distilled water. The measurement of NH_3 -N was performed using a specific electrode coupled to a multiparameter meter (Orion Star A214 pH/ISE benchtop meter, Thermo Scientific, Waltham, MA, USA) and concentrations of of lactic, acetic, and butyric acids according to Moon *et al*. (1981).

In vitro trial

Animal care and procedures for extraction of rumen inoculum were approved by the Ethics Committee for Animal Experimentation (Protocol ID UAEMex 4974/2020). Three dairy ewes (Suffolk × Texel; 84 ± 6 kg of live weight) were used to obtain rumen fluid for *in vitro* fermentation incubations. Sheep were fed a maintenance diet with 50:50 concentrate to roughage ration (DM contents was 62%) containing corn silage, sorghum grain, soybean meal, canola meal, wealth bran and mineral-vitamin premix at 08.00 and 16.00h. Diet and water were provided *ad libitum* throughout the trial. Ewes were adapted to the diet for 20 days. Rumen fluid was strained through four layers of cheesecloth and kept in a warm water bath at 39 °C. *In vitro* gas test was conducted according to the procedure described by Theodorou *et al*. (1994). Concentrate and silage samples were weighed (0.800 g DM). Each sample was analyzed in triplicate and incubated in glass flasks (125 ml) with 90 ml of buffer solution and 10 ml of ruminal fluid, and three incubation runs were performed. The buffer solution was prepared according to Menke & Steingass (1988), where 0.800 g DM of each ingredient and each diet mixture were incubated in glass bottles of 125 ml. Details on buffer solution composition have been described previously (Vargas-Bello-Pérez *et al*., 2020).

To determine ruminal fermentation kinetics, three incubation runs of 96 h were carried out. In each run, three glass flasks per sample were used. Also, three non-sample flasks in each run were considered as blank for correction of gas produced from previous particles left in rumen fluid. Rumen

fluid samples were extracted and filtered in a triple layer of cheesecloth gauze, and homogenized with CO_2 for 5 min. Then, filtered samples were mixed and used as inoculum. Flasks were incubated in a water bath at 39 °C. Gas volume was recorded at 0, 3, 6, 9, 12, 24, 36, 48, 72, and 96 h of incubation using a Delta pressure transducer (Model 8804 HD, Padova, Italy) at every reading time and gas production was corrected for blank incubations. At 96 h of incubation, samples were filtered, washed under tap water, and dried (65°C, 48 h) until analysis.

After *in vitro* incubation periods, dry matter dissapearance (IVDMD, mg/100 mg) was determined. Samples were filtered and dried (48 h, 60 °C) and then organic matter dissapearance (OMd, mg/100 mg) was determined (4h 550 °C). Gas yield production was determined at 24 h (GY24), with the gas volume (ml g/g DM) produced after 24 h of incubation divided by the amount of IVDMD (g) calculated as follows (Gonzalez Ronquillo *et al*., 1998): Gas production (GP24) = [(ml gas 24h / g DM) / g IVDMD].

Relative gas production (RGP, ml gas 96h/g IVDMD 96h) was calculated according to González-Ronquillo et al. (1998). Short chain fatty acids concentration (SCFA) was calculated according to Getachew et al. (2002) as: SCFA (mmol/200 mg DM) = 0.0222 GP - 0.00425. Where: GP is the 24 h net gas production (ml/200 mg DM).

Microbial biomass production (MP) was calculated according to Blümmel et al. (1997) as: MP (mg/g DM) = mg IVDMD - (ml gas × 2.2 mg/ml). Where 2.2 mg/ml is a stoichiometric factor, which expresses mg of C, H and O required for the SCFA gas associated with production of one ml of gas (Blümmel *et al*., 1997).

In vivo trial

The experimental protocol and implemented procedures were conducted in accordance with the guidelines of the National Council for Animal Control and Experimentation (Olaiz, 2015). This study was approved by the Ethics Committee on Animal Experimentation of the School of Veterinary Medicine and Animal Science of the Universidad Autónoma del Estado de Mexico (Protocol ID UAEMex 4974/2020).

Nine Suffolk × Texel dairy ewes were used [DIM=45 ± 6 d, BW=79.9 ± 10 kg, average daily milk yield=0.550 ± 0.14 kg (average ± SD)] were grouped in a replicated 3 × 3 Latin square design (n = 3), that included three 21-d periods of which 14 days were used for diet adaptation and the last 7 d for sample collection. Dietary treatments consisted of forage [Corn silage (CS), sunflower silage (SFS), or their 50:50 mixture (CS-SFS) and concentrate (30% corn grain and 70% soybean meal) supplemented with vitamins and minerals (Multitec of Malta®; Celaya; Mexico)]. Three different

diets consisting of 50/50 concentrate and corn silage, sunflower silage, or their mixture, formulated to be isocaloric (2.70 Mcal/kg metabolizable energy) and isonitrogenous (14% crude protein) and to meet NRC (2007) requirements of dairy ewes. All animals were fed 47 g/kg live weight $(LW)^{0.75}$ concentrate and *ad libitum* forage silage (Table 1S). Forage and concentrates were manually mixed in each individual trough and offered twice per day (0800h and 1600h), with free access to water. Animals were kept in a roofed pen with individual metabolic cages (1.0 × 1.2 × 1.2m) with slatted floor. The study lasted 63 days in which the first 14 days were used for diet adaptation and the last 7 d for sample collection during three consecutive periods.

The amounts of feed offered and refused, feces, urine, and milk during the last 7 days were recorded daily to determine nutrient intake, digestibility, and milk yield. Individual daily milk samples were taken at 16.00h, and individual daily samples of feed, orts, faces, and urine were takenat 08.00h. The collected feces were then well mixed, weighed, and a subsample was preserved at 20 °C until the next analysis. Feces samples were dried for 48 h at 65° C in a forced-air oven and then ground to pass through a 1-mm screen in a Wiley mill (Arthur H. Thomas Co., Philadelphia, PA) before analysis. The analytical procedures followed those described earlier in the chemical analysis section. The nutrient digestibility was measured based on the amount of nutrient consumed and excreted. Urine was collected in a sulphuric acid solution (10 %; pH < 3). Only 10 % of the total sample collected for feces and urine was used for analysis. Dry matter (DM) intake (kg/day), organic matter (OM), neutral detergent fiber (NDF) N intake, and N balance (excretion of feces, urine, and milk) were estimated and expressed as g/kg. Dry matter intake (DMI, g/d) and individual milk yields (kg/d) were recorded every day but only data from the last 7 days of each period were used for statistical analysis.

Calculations and statistical analysis

The accumulated gas volume of each sample was determined using the model proposed by France *et al*. (1993):

$$\mathbf{Y} = \boldsymbol{A}[\mathbf{1} - \boldsymbol{exp}\ (-\boldsymbol{B}(\boldsymbol{t} - \boldsymbol{T}) - \boldsymbol{cC}(\sqrt{\boldsymbol{t}} - \frac{\boldsymbol{A}}{\boldsymbol{T}}))]$$

Where: Y, is the cumulative gas production (mL); t, is the incubation time (h); A, is the asymptote curve (total gas produced, mL); B (h^{-1}), and C ($h^{-½}$) are the gas production constants; T, is the time of delay (h) that colonize the microorganisms to begin the fermentation.

Fat-corrected milk (FCM) was calculated at 3.5%, FCM (kg/d) = [milk (kg/d) × 0.432] + [fat kg/d) × 16.216], energy corrected milk (ECM) was calculated as, ECM = [milk (kg/d) × 0.327] + [fat (kg/d) × 12.86] + [protein (kg/d) × 7.65] (Tyrrell and Reid, 1965). The feed efficiency (FE) was calculated

using the following formula: FE = milk yield (kg/d)/dry matter intake (kg/d). Adjusted FE was calculated using the following formula = 3.5% FCM (kg/d)/dry matter intake (kg/d).

A completely randomized design was used for in vitro gas production parameters and in vitro microbial fermentation using the procedure of Statistical Analysis System 9.2 software (SAS, 2002) .

$Y_{ij} = \mu + T_{xi} + \varepsilon_{ij}$

Where Y_{ij} = is each observation of treatments it; μ is the general mean; $T_{x\ (i = 3)}$ is the treatment effect; and ε_{ij} is the experimental error.

In vivo data were analysed using a completely latin square design repeated 3 × 3, with the factors being the silage suplementation (n = 3) using the following equation:

$Y_{ij} = \mu + A_i + P_j + T_k + e_{ijkl}$

Where Y_{ij} is the dependent variable, μ is the general average, A_i is the animal, P_j is the period, T_k is the silage supplementation treatment and e_{ijkl} the error term.

The analyses were carried out by SAS (2002). For both in vitro and in vivo data, least square means (LSM) separation was performed using the PDIFF statement by Tukey's test (Steel *et al.*, 1997) and presented as LSM ± SEM. Significance was declared at $p \leq 0.05$ and trends at $p \leq 0.10$ and $p \geq 0.05$

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Table S1. Chemical composition (g/kg DM) of concentrate supplement, corn silage (CS), sunflower silage (SFS) and their mixture (CS-SFS) in sheep diets

Item	Diets			
	Concentrate[1]	CS	SFS	CS-SFS
Dry matter[2]	910.0	286.1	209.8	247.8
Chemical composition				
Organic matter	886	950	870	910
Crude protein	188.8	78.2	105.1	91.6
Ether Extract	79.4	51.9	108.1	88.6
NFC[3]	617.8	819.9	656.8	729.8
Neutral detergent fiber	229.1	615.4	554.6	571.2
Acid detergent fiber	87.0	439.0	493.5	450.3
Acid detergent lignin	25.2	68.0	122.2	86.7
ME, Kcal/kg DM	2873	2508	2600	2554
pH		4.0	4.6	4.3
NH_3 -N (g kg^{-1} Total N)[4]		112	121	116
Volaty fatty acids (mol/100 mol)				
Lactic acid		68.2	40.2	54.4
Acetic acid		13.0	13.9	13.5
Propionic acid		16.3	2.4	9.5
Butiric acid		2.6	1.2	2.0
Lactate/ acetate ratio		5.2:1	2.9:1	4.0:1

[1] Contained (g/kg of DM) = Sorghum grain 472, Soyabean meal 250, Canola meal 50, Wheat bran 160, Vitamin and trace mineral 68. Chemical composition (g/kg DM), Sorghum grain 970 OM, 80 g CP; 60 g NDF, 27 g ether extract; SBM 934 g OM, 440 g CP, 313 NDF, 24 g ether extract; Canola meal 924 g OM; 360 g CP, 278 g NDF, 35 g ether extract; Wheat bran contain 930 g OM, 170 g CP, 456 g NDF, 45 g ether extract and trace mineral and vitamin premix (Gold line Hitec-nutrition; Multitec Malta Cleyton; Celaya, Mexico) containing vitamin A (250,000 IU/kg), vitamin D (50,000 IU/kg), vitamin E (1,500 IU/kg), manganese (2.25 g/kg), calcium (120 g/kg), zinc (7.7 g/kg), phosphorus (20 g/kg), magnesium (20.5 g/kg), sodium (186 g/kg), iron (1.25 g/kg), sulfur (3 g/kg), copper (1.25 g/kg), cobalt (14 mg/kg), iodine (56 mg/kg) and selenium (10 mg/kg).

[2]Expressed of fresh matter

[3] Non-fibrous carbohydrates (NFC) were estimated according to the equation: NFC = 1000 − (NDF + CP + EE + Ash).

[4] NH_3 -N - ammonia nitrogen

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Table S2. *In vitro* rumen gas kinetics (mL gas/ g DM) and fermentation profile of in dairy ewes supplemented with corn silage (CS), sunflower (SF) and their mixture (CS-SFS).[1]

Item[2]	Diets				SEM[3]	*p*-value
	Concentrate	CS	SFS	CS-SFS		
In vitro gas kinetics						
A	257.06^{a}	223.22^{b}	118.20^{d}	171.05^{c}	6.165	0.0001
B	0.051^{a}	0.037^{b}	0.033^{b}	0.038^{b}	0.001	0.0004
C	-0.062^{b}	-0.043^{ab}	-0.036^{a}	-0.041^{ab}	0.004	0.0219
Lag time	1.69	1.69	1.44	1.36	0.253	0.7051
In vitro gas production, mL gas/g DM						
3h	9.33^{a}	5.33^{b}	3.67^{b}	6.33^{ab}	0.882	0.0112
6h	28.67^{a}	19.00^{b}	10.67^{c}	17.33^{bc}	1.554	0.0001
9h	57.00^{a}	38.67^{b}	19.00^{c}	31.00^{b}	2.505	0.0001
12h	62.67^{a}	62.67^{b}	30.00^{c}	48.67^{b}	3.266	0.0001
24h	158.67^{a}	113.33^{b}	55.33^{d}	86.00^{c}	4.368	0.0001
36h	191.67^{a}	143.67^{b}	72.67^{d}	110.67^{c}	4.910	0.0001
48h	218.67^{a}	170.00^{b}	86.33^{d}	132.67^{c}	5.809	0.0001
60h	235.67^{a}	187.67^{b}	96.00^{d}	145.67^{c}	6.076	0.0001
72h	247.33^{a}	201.33^{b}	103.33^{d}	155.33^{c}	6.405	0.0001
96h	215.67^{a}	215.67^{b}	111.67^{d}	163.00^{c}	5.744	0.0001
DMD96h	89.00^{a}	73.67^{b}	46.67^{d}	57.00^{c}	0.623	0.0001
RGP96h	289.33^{a}	292.33^{a}	239.33^{b}	285.33^{a}	17.061	0.0022
GP24h200	32.00^{a}	22.67^{b}	11.33^{d}	17.33^{c}	1.000	0.0001
GY24h500	79.33^{a}	56.33^{b}	27.33^{d}	43.00^{c}	2.160	0.0001
GY24h	177.67^{a}	153.33^{a}	118.00^{b}	150.67^{a}	26.151	0.0010
SCFA	25.00^{b}	11.00^{d}	29.00^{a}	20.00^{d}	1.356	0.0001
MCP	776.67^{a}	642.67^{b}	418.00^{d}	497.67^{c}	5.291	0.0001

Within row, different letters (a, b) indicate difference between diets ($p \leq 0.05$).

[1] Values are least-square means.

[2] A = total gas production (ml gas/g DM incubated); B = fermentation rate (h^{-1}); C = fermentation rate ($h^{-1/2}$); Lag time = the initial delay before gas production begins (h); DMD96 = DM degraded substrate (mg/g DM); GY24 = gas yield at 24 h (mL gas/g DMD); SCFA = short chain fatty acids (mmol/g DM); MCP = microbial CP production (mg/g DM).

[3] SEM = pooled standard error of the mean.

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Table S3. Intake and nutrient digestibility in dairy ewes supplemented with corn silage (CS), sunflower (SF) and their mixture (CS-SFS).[1]

Item[2]	Diets			SEM[3]	*p*-value
	CS	SFS	CS-SFS		
Intake (g/d)					
DMI, Concentrate	1384.52[a]	1200.54[b]	1247.37[ab]	43.248	0.0166
DMI, Silage	1181.97	1348.68	1209.42	84.176	0.3404
Ratio Concentrate:silage	0.46[b]	0.52[a]	0.49[ab]	0.015	0.0116
DMI, Total	2566.49	2549.23	2456.79	111.018	0.7565
OM intake	2390.57	2289.78	2249.97	101.369	0.6062
Fat intake	171.27[c]	241.11[a]	206.19[b]	9.647	0.0001
NDF intake	1044.58	1023.02	976.59	53.554	0.6612
ADF intake	639.34	770.02	653.12	40.535	0.0616
ADL intake	115.26[b]	195.06[a]	136.29[b]	8.475	0.0001
Digestibility (kg/kg)					
Dry matter	0.72[a]	0.69[ab]	0.63[b]	0.021	0.0357
Organic matter	0.74[a]	0.71[ab]	0.67[b]	0.019	0.0500
NDF	0.59	0.57	0.49	0.030	0.0926
ADF	0.54	0.55	0.43	0.035	0.0674
ADL	0.27[ab]	0.32[a]	0.14[b]	0.041	0.0138
Body Weight					
Body weight (BW), kg	91.00[a]	70.33[b]	78.33[ab]	3.680	0.0022
Metabolic $BW^{0.75}$	29.45	24.77	26.22		

Within row, different letters (a, b) indicate difference between diets ($p \leq 0.05$).
[1] Values are least-square means.
[2] Dry matter intake, DMI; natural detergent fiber, NDF; acid detergent fiber, ADF; Acid detergent lignin, ADL.
[3] SEM = pooled standard error of the mean.

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Table S4. Nitrogen balance in dairy ewes supplemented with corn silage (CS), sunflower (SF) and their mixture (CS-SFS).[1]

Item[2]	Diets			SEM[3]	*p* -value
	CS	SFS silage	CS-SFS		
N intake (g/d)	56.48	58.83	55.29	2.187	0.5164
Fecal N excretion (g/d)	19.30	15.41	19.54	1.331	0.0673
Urine N excretion (g/d)	32.49	34.49	32.10	1.216	0.3432
Milk N excretion (g/d)	5.38^{a}	2.81^{b}	4.42^{ab}	0.523	0.0069
N balance (g/d)	-0.697^{b}	6.102^{a}	-0.774^{b}	1.359	0.0017
Fecal N excretion (%)	34.22^{a}	26.26^{b}	35.45^{a}	2.109	0.0101
Urine N excretion (%)	9.67^{a}	4.78^{b}	8.30^{ab}	1.061	0.0097
Milk N excretion (%)	57.54^{b}	58.67^{a}	58.10^{ab}	0.237	0.0096

Within row, different letters (a, b) indicate difference between diets ($p \leq 0.05$).
[1] Values are least-square means.
[2] Nitrogen, N; nitrogen intake, N intake; nitrogen balance, N balance.
[3] SEM = pooled standard error of the mean.

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