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The associative effects of sunflower straw, sunflower plate, sunflower seed shells associated with concentrate and alfalfa evaluated by using an *in vitro* gas production technique

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Abstract: This study aimed to evaluate the multiple-factor associative effects (AEs) of concentrate (C) to sunflower straw (SS) to alfalfa (A) ratios, concentrate to sunflower plate (SP) to alfalfa ratios, concentrate to sunflower seed shell (SSS) to alfalfa ratios at 40 : 60 : 0, 40 : 45 : 15, 40 : 30 : 30, 40 : 15 : 45, 40 : 0 : 60 and 30 : 70 : 0, 30 : 55 : 15, 30 : 40 : 30, 30 : 25 : 45, 30 : 10 : 60, 30 : 0 : 70, respectively, by using an *in vitro* gas production (GP) method. Thirty-three feed combinations and nine single feeds C, SS, A; C, SP, A; C, SSS, A were incubated respectively for 72 h in a GP tube. A total of 42 treatment combinations were tested, including 33 feed mix combinations [(5 + 6) × 3] and nine single raw materials, a total of 42 sample culture tubes. Each sample culture tube was repeated 20 times, totalling 840 (42 × 20) sample culture tubes. In this study, a total of 12 batches of *in vitro* culture tests were carried out. Seventy sample tubes and six blank tubes were cultured in each batch (eliminating systematic errors). A total of 912 sample culture tubes were used. The GP values of 0, 2, 4, 6, 9, 12, 24, 36, 48, 72 h were recorded. The GP parameters *a*, *b*, *c*, *a* + *b* were calculated by a single exponential equation. The AE values were calculated based on 72 h GP and weighted estimation value of 33 feed combinations. In the incubation fluid pH, ammonia nitrogen (NH_3–N) and volatile fatty acids (VFA) after 72 h incubation were determined and dry matter digestibility (DMD), organic matter digestibility (OMD) were measured in the residue. The single-factor associative effects index (SFAEI) and multiple-factor associative effects index were calculated. The results indicated that groups 40 : 45 : 15 (C:SS:A), 30 : 25 : 45 (C : SS : A), 40 : 60 : 0 (C : SP : A), 40 : 45 : 15 (C : SP : A), 30 : 40 : 30 (C : SP : A), 40 : 45 : 15 (C : SSS : A), 40 : 30 : 30 (C : SSS : A), 30 : 25 : 45 (C : SSS : A) and 30 : 10 : 60 (C : SSS : A) had higher *a*, *b*, GP_{72h}, NH_3–N, DMD, OMD, moreover, higher SFAEI (the AE of GP_{72h}, NH_3–N, total VFA, DMD and OMD) than other groups ($P < 0.05$). It was concluded that the optimal feed combinations occurred when concentrate/sunflower straw/alfalfa ratios were 40 : 45 : 15 and 30 : 25 : 45; concentrate/sunflower plate/alfalfa ratios were 40 : 60 : 0, 40 : 45 : 15 and 30 : 40 : 30; concentrate/sunflower seed shell/alfalfa ratios were 40 : 45 : 15, 40 : 30 : 30, 30 : 25 : 45 and 30 : 10 : 60.

Keywords: sunflower by-products; combination effects; concentrate; clover; *in vitro* gas production method

Sunflower (*Helianthus annuus L.*) is one of the most widespread cash crops and oil crops in the world and sunflower seeds are the most common leisure food in China. The by-products of sunflower include sunflower seed shells (SSS), sunflower straw (SS) and sunflower plate (SP) which have a high production

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volume but are rarely used as feed resource and most of sunflower by-products are discarded or burned as a source of fertilizer. As a matter of fact, the shell content of sunflower seeds is 22–40% and the composition of amino acids of the sunflower shell is similar to that of barley straw, while the quality of sunflower seed shell is much better. Additionally, the average daily gain of fattening sheep fed 50% sunflower seed hull pellets was about 10% higher than that of sheep fed barley straw pellets (Liu 1987).

The digestibility of sunflower by-products (SS, SP, SSS) may not be high, therefore feeding them alone cannot meet the nutritional needs of animals. However, these restrictions can be conquered by the positive combination effects of adding other feeds to low-quality roughage. The so-called associative effects (AE) between feeds refer to the digestibility or available energy of mixed feed or diet being unequal to the weighted sum of the digestibility or available energy values of feed contained in the diet (Doyle et al. 2005). And the AE result from the complementary action between different types of feed after mixing in a proper proportion, which makes the gas production (GP) and fermentation performance of mixed feed attain the best. Besides, the interaction between feeds can change rumen metabolic pathways. In addition, it has been proved that adding alfalfa (*Medicago sativa*) can enhance the utilization rate of inferior roughage (Mosi and Butterworth 1985). What's more, as to forage-based diets, concentrated feed is essential to reach the expected performance level (Goetsch and Gipson 2014). Consequently, concentrated feed must be considered.

Tagliapietra et al. (2014) reported that the fermentation performance of poor-quality forage such as milk thistle and crown daisy could be enhanced by combining these two forages with citrus pulp and apple pomace. Asrat et al. (2017) advanced a method for predicting negative AE by using a concentrate level and hay source for Boer goat wethers, and the method showed that using a low- or medium-level concentrate can precisely predict the intake of metabolic energy in growing wethers. In addition, Haddad and Nasr (2007) reported that in the high concentrate diet, at least 20% of barley diet DM is needed to replace corn diet DM in order to actively improve the production performance and feeding efficiency of growing lambs.

Although there are many reports about the feed combination effect, there are few studies on sunflower straw, sunflower plate and sunflower seed shells as a feed source. Given the wide distribution and easy availability of SS, SP, and SSS, this experiment hypothesizes that the optimal proportion of concentrate (C), sunflower by-products, and alfalfa (A) can promote AE. Therefore, the experiment aimed to determine single-factor associative effects index (SFAEI) and multiple-factor associative effects index (MFAEI) of mixtures of SS, C, A or SP, C, A or SSS, C, A on GP_{72h}, NH_3–N, total volatile fatty acids (VFA), DMD, OMD by *in vitro* GP technique.

MATERIAL AND METHODS

Experimental design and feeds

The purpose of this experiment was to investigate the effects of different ratios of sunflower straw (SS) to alfalfa (A) and concentrate (C) on the associative effects (AE) of diet, and the effects of different ratios of sunflower plate (SP) to A and C, and sunflower seed shells (SSS) to A and C on the associative effects of diet.

The C:SS:A ratios included 14 treatment groups with 20 replicates in each treatment group. These 14 treatment ratios of SS to C and A were 40 : 60 : 0, 40 : 45 : 15, 40 : 30 : 30, 40 : 15 : 45, 40 : 0 : 60 [when the concentrate-roughage ratio (C : R) was 40 : 60] and 30 : 70 : 0, 30 : 55 : 15, 30 : 40 : 30, 30 : 25 : 45, 30 : 10 : 60, 30 : 0 : 70 (when the C : R was 30 : 70), and 100 : 0 : 0, 0 : 100 : 0, 0 : 0 : 100 (namely, C, A and SS were fermented as a single feed), respectively. The ratio of sunflower plate to C and A and the ratio of treatment groups between sunflower seed shells, C and A were the same as those of sunflower straw mentioned above.

These 33 feed mix combinations [(5 + 6) × 3] and nine single feeds (C, SS, A; C, SP, A; C, SSS, A) were fermented for 72 h in single GP tubes. A total of 42 treatment combinations (Table 1) were tested. Each sample culture tube was repeated 20 times, totalling 840 sample culture tubes. In this study, a total of 12 batches of *in vitro* culture tests were carried out. Seventy sample tubes and six blank tubes were cultured in each batch (eliminating systematic errors). A total of 912 culture tubes were used in this experiment.

These nine single feeds (SS, A, C; SP, A, C; SSS, A, C) were pre-ground to 1 mm. The experimental plan of this study was approved by the Institute of Animal Use and Ethics of Gansu Agricultural

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Table 1. Forty-two treatment combinations of concentrate, sunflower by-products (SS, SP, SSS) and alfalfa

C:SS:A			C:SP:A			C:SSS:A		
100:0:0	40:60:0	30:70:0	100:0:0	40:60:0	30:70:0	100:0:0	40:60:0	30:70:0
0:100:0	40:45:15	30:55:15	0:100:0	40:45:15	30:55:15	0:100:0	40:45:15	30:55:15
0:0:100	40:30:30	30:40:30	0:0:100	40:30:30	30:40:30	0:0:100	40:30:30	30:40:30
–	40:15:45	30:25:45	–	40:15:45	30:25:45	–	40:15:45	30:25:45
–	40:0:60	30:10:60	–	40:0:60	30:10:60	–	40:0:60	30:10:60
–	–	30:0:70	–	–	30:0:70	–	–	30:0:70

A = alfalfa; C = concentrate; SP = sunflower plate; SS = sunflower straw; SSS = sunflower seed shells

University. The formula of the concentrate included corn 85.17%, soybean meal 6.63%, cottonseed meal 4.05%, salt 1.55% and premix 2.60%.

Gas production and fermentation process *in vitro*

These nine feeds (SS, A, C; SP, A, C; SSS, A, C) were dried and crushed to 1 mm (FZ102 Micro plant grinding machine; 1 400 r/min, Tianjin Taisite Instrument Co., Ltd, Tianjin, China). Then the above nine separate feeds, and 11 × 3 feed combinations (Table 1) were incubated in 100 ml glass gas collection tubes (Häberle Labortechnik GmbH & Co. KG, Lonsee, Germany) which were used to determine actual GP at 0, 2, 4, 6, 9, 12, 24, 36, 48, 72 hours. Furthermore, each sample culture tube was subjected to 20 replicates, a total of 840 [(11 × 3 + 9) × 20] sample tubes were tested in 12 batches (Table 1). Six blank tubes were made for each batch of the test to correct all gas production and fermentation parameters. Namely, the test was conducted for 840 culture tubes of samples, 72 blank tubes with 12 batch tests in total.

In the morning of the day of *in vitro* culture, accurately weighed 200 mg (DM) (Menke and Steingass 1988) and recorded the samples were cultured separately or in combination, then, they were added to the constant weight nylon bags (3 cm × 5 cm; pore size 40 ± 12 μm); afterwards, they were placed on the bottom of the culture tube. After the rumen fluid was filtered through four layers of gauze, 10 ml rumen fluid and 20 ml microbial buffer (Menke and Steingass 1988) were injected into each culture tube (100 ml), subsequently, the air in the tube was pushed out, and the gas production at 0 h was read, which was vertically placed on a 77-hole inorganic glass support of our own production in a 38.5–39.5 °C thermostatic water bath.

About 1 000 ml rumen fluid was provided by four Holstein-Friesian cows aged seven years and weighing 540 ± 4.0 kg, fed concentrates (Table 2) 4.5 kg/day/head and wheat straw *ad libitum* through a rumen fistula probe before morning feeding. The rumen fluid of 1 000 ml was gathered in thermos bottles preheated to 38.5–39.5 °C, and sent to the laboratory quickly (in 15 min); afterwards, the rumen fluid was filtered through four layers of gauze quickly and fully mixed with microbial buffer (Menke and Steingass 1988) at the ratio of 1 : 2 (10 ml rumen fluid filtered and 20 ml microbial buffer were mixed in each culture tube). The above procedures were completed in the shortest time (half an hour or less) under anaerobic environmental conditions (through continuous injection of carbon dioxide flow).

Table 2. Ingredients and nutrient levels of dietary concentrate for four cows (%, DM basis)

Ingredients	Corn	Soybean meal	Wheat bran	$CaHPO_4$	NaCl	Premix[1]	100.00
	61.00	22.50	11.50	2.00	1.00	2.00	
Nutrient levels	DM	CP	NE_L/(MJ/kg)[2]	NDF	ADF	Ca	P
	94.00	16.70	7.15	12.68	5.23	0.51	0.45

ADF = acid detergent fibre; CP = crude protein; DM = dry matter; NDF = neutral detergent fibre; NE_L = net energy of lactation

[1]1 kg of premix contained: vitamin A 650 000 IU, vitamin D3 300 000 IU, vitamin E 400 000 IU, Fe 500 mg, Cu 500 mg, Mn 1 000 mg, Zn 500 mg, Co 15 mg, Se 40 mg

[2]Estimated value: NE_L (Mcal/kg milk) = 0.351 2 + 0.096 2 × milk fat rate (Yang 2005)

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Calculation of gas production

The cumulative GP curve was fitted to an exponential equation (Orskov and McDonald 1979) to evaluate the kinetics of GP:

$$GP = a + b\,(1 - e^{-ct}) \quad (1)$$

where:
GP – gas production (ml);
a – rapid GP (ml);
b – slow GP (ml);
$a + b$ – potential GP (ml);
e – the base of the natural logarithm;
c – the rate constant of slow GP (%/h);
t – the time (2, 4, 6, 9, 12, 24, 36, 48, 72 h) since the beginning of fermentation culture (h).

Based on the actual GP readings of each individual feed for 72 h, the expected GP of each feed combination under different ratios of three feeds was calculated as the weighted value of the GP provided by concentrate, alfalfa and SS or SP or SSS incubated separately (it is assumed that there is no AE among concentrate, alfalfa, and sunflower by-products SS, SP, SSS). In order to eliminate the systematic error of *in vitro* culture, all actual GP values must be corrected by blank GP data.

Measurement of feed nutritional indicators

These five feeds were measured in quintuplicate for dry matter (DM), ether extract (EE), crude fibre (CF), crude protein (CP) and crude ash (ASH) based on the AOAC (2003). CP was determined by Danish FOSS using a Kjeldahl nitrogen analyser (No. 2001.14; Foss, Hilleroed, Denmark). In addition, EE was determined by Soxhlet extraction (No. 993.20). Besides, ASH was measured by a muffle furnace method and organic matter (OM) was computed: OM = DM – ASH (Sandoval-Castro et al. 2002). Lastly, CF was determined by the method of acid and alkali washing treatment.

Determination of *in vitro* DM digestibility and *in vitro* OM digestibility

After 72 h of incubation, the residue and nylon bag were soaked in ice water to stop fermentation, rinsed with distilled water several times until they were clean, and they were dried in an oven at 105 °C until the weight of the residue with nylon bag were constant. In addition, the *in vitro* dry matter digestibility (IVDMD) was computed by the difference between the initial weight of the sample (about 200 mg, dry basis) (Menke and Steingass 1988), the weight of the empty nylon bag and the constant weight of the dried residue and the nylon bag (corrected with blank tube). Besides, the *in vitro* organic matter digestibility (IVOMD) was evaluated by the incineration of dry residues (550–580 °C) and the crude ash content of feed and residue was calculated by measuring the difference between the initial weight of feed mixture and the final constant weight of residue (corrected with blank tube), then the values of OM and IVOMD were calculated according to: OM = DM – ASH (Sandoval-Castro et al. 2002).

Measurement of pH, volatile fatty acids and ammonia nitrogen

After 72 h incubation, the microbial culture solution was collected to determine pH, VFA and ammonia nitrogen (NH_3–N). The pH value was rapidly determined with a digital instrument equipped with a glass electrode (Sartorius AG PB-10; Beijing Saidoris Instrument System Co., Ltd, Beijing, China). Then at 5 000 × *g* centrifuge the culture solution for 10 min, collect 4 ml of supernatant and put it into 5 ml centrifuge tube, cover the centrifuge tube with 1 ml of 25% (weight/volume) metaphosphoric acid solution, and freeze at –20 °C according to Lu et al. (1990) for VFA analysis. Additionally, determination of the total VFA concentration and molar ratio of acetic acid, propionic acid and butyric acid was carried out in a gas chromatograph (GC-14; Shimadzu, Duisburg, Germany), N_2 was used as the carrier gas at a flow rate of 50 ml/min, and a glass column (2 m length × 2 mm diameter) filled with Chromosorb AW, 100 g/kg polyethylene glycol and 30 g/kg H_3PO_4 (Supelco Inc., Bellefonte, PA, USA). And the temperature of the detector port, syringe port and oven was 190 °C, 185 °C and 155 °C, respectively. Besides, according to Broderick and Kang (1980), 3 ml of supernatant was put into a 5 ml centrifuge tube with a cover, filled with 2 ml of 3 M HCl and frozen at –20 °C for NH_3–N analysis. Besides, the NH_3–N content

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was measured by the meaning of glutamate dehydrogenase (171-B; Sigma Chemical, St. Louis, MO, USA) and Cobas Fara II centrifugal analyser (Roche Diagnostic Systems, Montclair, NJ, USA).

Calculation of associative effects

Wang (2011) proposed a single-factor AE index (SFAEI) and a multiple-factor AE index (MFAEI):

$$SFAEI = (MV - WEV)/WEV \quad (2)$$

where:
SFAEI – single-factor AE index;
MV – measured value of each diet combination;
WEV – weighted estimate value, the MV of a type of feed × the proportion of this feed in the combination + the MV of another type of feed × the proportion of the other type of feed in the combination.

MFAEI is the sum of each SFAEI. In this experiment, MFAEI = the sum of five SFAEI (AE of GP_{72h}, total VFA, NH_3–N, DMD and OMD).

Statistical analysis

SAS software v7.0 (SAS Institute, Inc., Cary, NC, USA) was used to analyse the experimental data by one-way analysis of variance (ANOVA). Tukey-Kramer multiple comparisons analysed the significance of the mean difference between all treatment groups. The difference between treatments was considered significant when $P < 0.05$. The results are shown as the mean and standard error of the means (SEM).

RESULTS

Nutrient levels and GP parameters of single feed

The CP content of sunflower plate (SP) was higher than in sunflower straw (SS) and sunflower seed shells (SSS) ($P > 0.05$); however, lower than that of alfalfa and concentrate ($P < 0.05$) (Table 3). In addition, the CF content of SSS was greater than that of SS, SP, alfalfa and concentrate ($P < 0.05$). Additionally, the rapid GP (a) of five feeds were all negative, which indicated that there existed a gas production lag. What's more, the slow GP value (b), potential GP ($a + b$), and GP_{72h} of alfalfa were lower than those of SS, SP, SSS and concentrate ($P < 0.05$).

Gas production parameters, fermentation parameters and AE of feed mixtures

Table 4 indicates that as to sunflower straw (SS), the b, ($a + b$), GP_{72h} and NH_3–N of groups 40 : 45 : 15, 40 : 30 : 30 and 40 : 15 : 45 (C : SS : A) were significantly higher than in other groups ($P < 0.05$) and the DMD and OMD of groups 40 : 45 : 15 and 40 : 30 : 30 were higher than in the others ($P < 0.05$). However, there were insignificant differences between the five groups as to a and c ($P > 0.05$).

Table 3. Nutrient levels and gas production parameters of five single feeds

	Chemical composition (%)					*In vitro* GP parameters (ml)				
	DM	OM	CP	EE	CF	a	b	c (%/h)	$a + b$	GP_{72h}
SS	90.73	88.39	5.72^{b}	0.89^{b}	30.15^{b}	–9.22^{a}	48.24^{a}	0.164^{a}	39.02^{b}	37.83^{a}
SP	89.63	85.96	11.84^{ab}	2.13^{b}	12.48^{c}	–7.55^{a}	51.38^{a}	0.131^{a}	43.84^{b}	39.67^{a}
SSS	92.60	89.30	5.90^{b}	3.30^{ab}	58.10^{a}	–2.30^{b}	57.50^{a}	0.054^{b}	55.30^{a}	33.00^{a}
C	91.90	90.80	19.20^{a}	4.20^{ab}	7.90^{c}	–10.50^{a}	56.10^{a}	0.121^{a}	45.50^{b}	42.50^{a}
A	94.90	91.10	18.50^{a}	6.60^{a}	29.40^{b}	–2.90^{b}	29.60^{b}	0.056^{b}	26.70^{c}	15.80^{b}
P-value	0.132	0.140	0.039	0.033	0.021	0.042	0.025	0.023	0.030	0.029
SEM	1.055	2.103	2.022	1.410	1.090	1.598	2.007	0.552	1.910	1.446

a = rapid gas production (GP); A = alfalfa; $a + b$ = potential GP; b = slow GP; c = the rate constant of slow GP; C = concentrate; CF = crude fibre; CP = crude protein; DM = dry matter; EE = ether extract; GP_{72h} = gas production at 72 h; OM = organic matter; SP = sunflower plate; SS = sunflower straw; SSS = sunflower seed shells
[a–c]Means within a column differ at $P < 0.05$

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Table 4. Gas production and fermentation parameters when sunflower by-products (SS, SP, SSS) were incubated with concentrate and alfalfa *in vitro*

	Gas production parameters (ml)					Fermentation parameters			
	a	*b*	*c* (%/h)	*a* + *b*	GP_{72h}	pH	DMD (%)	OMD (%)	NH_3–N (mg/dl)
C:SS:A									
40:60:0	–7.19	40.29^{b}	0.105	33.10^{b}	30.96^{b}	6.97^{a}	18.15^{b}	20.88^{b}	10.25^{b}
40:45:15	–2.85	58.50^{a}	0.144	55.65^{a}	47.85^{a}	6.35^{b}	40.55^{a}	45.45^{a}	15.49^{a}
40:30:30	–2.19	57.89^{a}	0.132	55.70^{a}	46.65^{a}	6.41^{b}	41.85^{a}	43.58^{a}	16.08^{a}
40:15:45	–4.02	55.66^{a}	0.121	51.64^{a}	45.80^{a}	7.02^{a}	20.58^{b}	24.87^{b}	14.47^{a}
40:0:60	–5.29	41.19^{b}	0.109	35.90^{b}	31.55^{b}	7.09^{a}	20.40^{b}	23.58^{b}	11.50^{b}
P-value	0.141	0.034	0.465	0.030	0.041	0.045	0.034	0.037	0.039
SEM	0.846	1.005	0.085	1.082	1.554	0.065	1.404	1.440	0.102
30:70:0	–5.88^{b}	20.01^{b}	0.049^{b}	14.13^{b}	22.53^{b}	7.05^{a}	12.09^{c}	15.58^{c}	13.17^{b}
30:55:15	–5.95^{b}	32.14^{ab}	0.058^{b}	26.19^{b}	33.85^{b}	6.98^{a}	31.65^{b}	34.54^{b}	14.82^{b}
30:40:30	–1.89^{a}	51.02^{a}	0.149^{a}	50.93^{a}	37.48^{b}	6.95^{a}	44.88^{a}	48.59^{a}	17.45^{b}
30:25:45	–0.40^{a}	57.89^{a}	0.154^{a}	57.49^{a}	50.08^{a}	6.35^{b}	49.65^{a}	49.07^{a}	27.55^{a}
30:10:60	–1.08^{a}	52.85^{a}	0.145^{a}	51.77^{a}	45.48^{a}	6.31^{b}	46.95^{a}	45.54^{a}	26.31^{a}
30:0:70	–6.85^{b}	37.50^{ab}	0.059^{b}	30.65^{b}	35.67^{b}	6.97^{a}	32.85^{b}	36.89^{b}	11.93^{b}
P-value	0.029	0.003	0.035	0.004	0.020	0.042	0.035	0.038	0.006
SEM	0.550	1.505	0.028	1.108	1.042	0.067	1.285	1.452	0.105
C:SP:A									
40:60:0	7.17^{b}	74.93^{a}	0.202	82.10^{a}	76.25^{a}	6.07	50.40^{a}	53.59^{a}	16.09^{a}
40:45:15	14.38^{a}	59.10^{ab}	0.176	73.48^{ab}	71.84^{ab}	6.14	51.62^{a}	53.01^{a}	15.88^{a}
40:30:30	8.88^{b}	61.60^{ab}	0.183	70.48^{ab}	67.10^{b}	6.81	49.36^{a}	50.81^{a}	14.81^{ab}
40:15:45	4.35^{b}	62.98^{ab}	0.203	67.33^{b}	68.17^{b}	6.76	47.94^{a}	49.25^{a}	14.62^{ab}
40:0:60	–3.19^{c}	53.74^{b}	0.117	50.55^{c}	45.39^{c}	6.82	34.12^{b}	38.74^{b}	11.09^{b}
P-value	0.003	0.044	0.305	0.031	0.029	0.059	0.035	0.037	0.040
SEM	1.453	2.130	0.014	2.022	2.091	0.219	1.845	1.802	0.087
30:70:0	10.41^{a}	62.56^{b}	0.160	72.97^{b}	64.32^{b}	6.91^{a}	48.60^{b}	49.98^{b}	13.29^{b}
30:55:15	12.45^{a}	62.36^{b}	0.169	74.81^{b}	73.02^{ab}	6.34^{b}	56.04^{a}	57.19^{a}	19.95^{a}
30:40:30	12.87^{a}	83.76^{a}	0.168	96.63^{a}	78.04^{a}	6.15^{b}	55.59^{a}	56.08^{a}	20.63^{a}
30:25:45	10.20^{a}	62.89^{b}	0.198	73.09^{b}	66.76^{b}	6.25^{b}	53.78^{a}	57.05^{a}	21.18^{a}
30:10:60	11.21^{a}	69.20^{b}	0.183	80.41^{b}	69.19^{b}	7.16^{a}	46.92^{b}	48.04^{b}	12.41^{b}
30:0:70	–4.05^{b}	53.36^{c}	0.125	49.31^{c}	46.54^{c}	6.98^{a}	44.86^{b}	47.79^{b}	12.57^{b}
P-value	0.040	0.032	0.521	0.024	0.025	0.044	0.027	0.032	0.004
SEM	1.394	1.282	0.012	1.315	1.664	0.152	2.008	1.802	0.117
C:SSS:A									
40:60:0	–6.98	37.14^{b}	0.099	33.17^{b}	31.83^{b}	6.72^{ab}	11.17^{c}	23.59^{b}	11.07^{b}
40:45:15	–8.37	52.25^{ab}	0.152	59.62^{a}	40.50^{ab}	6.24^{b}	43.35^{a}	43.01^{a}	13.88^{ab}
40:30:30	–7.11	59.63^{a}	0.103	46.01^{ab}	47.00^{a}	6.84^{ab}	33.05^{ab}	39.11^{ab}	15.87^{a}
40:15:45	–6.01	53.11^{ab}	0.096	45.28^{ab}	42.75^{ab}	6.82^{ab}	35.97^{ab}	33.25^{ab}	15.62^{a}
40:0:60	–3.07	49.14^{ab}	0.115	46.07^{ab}	42.33^{ab}	6.96^{a}	21.78^{bc}	38.74^{ab}	13.49^{ab}
P-value	0.170	0.045	0.570	0.036	0.040	0.043	0.002	0.035	0.039
SEM	0.795	1.267	0.012	1.719	2.106	0.059	1.572	1.802	0.081
30:70:0	–3.95	26.67^{b}	0.062^{b}	22.72^{b}	21.33^{b}	6.70^{a}	11.18^{c}	19.98^{c}	14.28^{b}

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Table 4 to be continued

	Gas production parameters (ml)					Fermentation parameters			
	a	*b*	*c* (%/h)	*a* + *b*	GP_{72h}	pH	DMD (%)	OMD (%)	NH_3–N (mg/dl)
30:55:15	−4.31	37.07^{ab}	0.097^{ab}	32.76^{ab}	32.00^{ab}	6.76^{a}	41.87^{ab}	44.19^{ab}	12.91^{b}
30:40:30	−0.92	51.85^{a}	0.147^{a}	50.93^{a}	36.50^{ab}	6.73^{a}	45.53^{a}	46.08^{a}	16.68^{ab}
30:25:45	−3.00	55.54^{a}	0.104^{ab}	52.54^{a}	48.33^{a}	6.56^{b}	43.03^{ab}	47.05^{a}	26.58^{a}
30:10:60	−3.66	50.63^{a}	0.096^{ab}	46.96^{a}	42.00^{ab}	6.50^{b}	34.50^{b}	25.04^{c}	12.36^{b}
30:0:70	−4.03	51.20^{a}	0.114^{ab}	47.17^{a}	43.33^{ab}	6.51^{b}	16.95^{c}	38.79^{b}	12.77^{b}
P-value	0.728	0.004	0.031	0.003	0.018	0.045	0.035	0.039	< 0.001
SEM	0.638	1.898	0.008	1.032	1.666	0.058	2.514	1.882	0.118

a = rapid GP; A = alfalfa; *a* + *b* = potential GP; *b* = slow GP; *c* = rate constant of slow GP; C = concentrate; DMD = dry matter digestibility; GP_{72h} = GP at 72 h; NH_3–N = ammonia nitrogen (mg/dl); OMD = organic matter digestibility; SEM = standard error of the means; SP = sunflower plate; SS = sunflower straw; SSS = sunflower seed shells

$^{a–c}$Means within a column differ at $P < 0.05$

Moreover, Table 5 shows that acetic acid (AA), propionic acid (PA) and total VFA in groups 40:45:15 and 40:30:30 were significantly higher than in groups 40:60:0 and 40:0:60 ($P < 0.05$). Table 5 demonstrates that the AE of GP_{72h}, DMD, TVFA, MFAEI of groups 40:45:15, 40:30:30 was greater than in other groups ($P < 0.05$) and the AE of OMD of group 40:45:15 was higher than in other groups ($P < 0.05$). Furthermore, the AE of NH_3–N, OMD and MFAEI of group 40:45:15 was greater than in group 40:30:30 ($P < 0.05$). Additionally, it was indicated (in Table 4) when the C:R ratio was 30:70, the *a*, *b*, *c*, (*a* + *b*), DMD and OMD of groups 30:40:30, 30:25:45 and 30:10:60 (C:SS:A) were higher than in other groups ($P < 0.05$) and the GP_{72h} and NH_3–N of groups 30:25:45 and 30:10:60 were higher than in other groups ($P < 0.05$). Besides, Table 5 shows that the AA, PA, total VFA, the AE of GP_{72h}, DMD, OMD, NH_3–N, total VFA and MFAEI of groups 30:25:45 and 30:10:60 were higher than in other groups ($P < 0.05$) and the MFAEI of group 30:25:45 was higher than in group 30:10:60 ($P < 0.05$).

Table 4 demonstrates that as to sunflower plate (SP), the *b*, (*a* + *b*), GP_{72h} of group 40:60:0 (C:SP:A) were higher than in groups 40:15:45 and 40:0:60 ($P < 0.05$). Besides, the DMD, OMD and NH_3–N of groups 40:60:0 and 40:45:15 were greater than in group 40:0:60 ($P < 0.05$). In addition, the *a* value of group 40:45:15 was higher than in other groups ($P < 0.05$). Further, Table 5 shows that the AA, PA, total VFA, the SFAEI of five indices (GP_{72h}, NH_3–N, total VFA, DMD, OMD) and MFAEI of groups 40:60:0 and 40:45:15 (C:SP:A) were significantly higher than in other groups ($P < 0.05$). And there was no difference between group 40:30:30 and other groups in AA, total VFA, SFAEI of GP_{72h}, SFAEI of NH_3–N, SFAEI of total VFA and MFAEI ($P > 0.05$). In addition, Tables 4 and 5 demonstrate that when C:R was 30:70, the DMD, OMD, NH_3–N, AA, PA, total VFA and the AE of GP_{72h}, DMD, OMD, NH_3–N and total VFA of groups 30:55:15, 30:40:30 and 30:25:45 was higher than in other groups ($P < 0.05$). Besides, the *b*, (*a* + *b*), GP_{72h}, the AE of GP_{72h} and MFAEI of group 30:40:30 were higher than in other groups ($P < 0.05$).

Tables 4 and 5 indicate that as to sunflower seed shells (SSS), when C:R was 40:60, the GP_{72h}, *b*, NH_3–N and PA of group 40:30:30 (C:SSS:A) were higher than in other groups ($P < 0.05$). However, the (*a* + *b*), DMD, OMD of group 40:45:15 were higher than in other groups ($P < 0.05$). In addition, the acetic acid (AA), total VFA, the AE of DMD, OMD, GP_{72h}, NH_3–N, total VFA and MFAEI of groups 40:45:15 and 40:30:30 were higher than in other groups ($P < 0.05$). When C:R was 30:70, the GP_{72h}, *b*, NH_3–N of group 30:25:45 (C:SSS:A) were higher than in other groups ($P < 0.05$). However, the *c*, (*a* + *b*), DMD, OMD of groups 30:25:45 and 30:40:30 were higher than in other groups ($P < 0.05$). Besides, the AA, total VFA, the AE of GP_{72h}, total VFA, NH_3–N, DMD, OMD and MFAEI of groups 40:45:15 and 40:30:30 were higher than in other groups ($P < 0.05$). Nevertheless, the AA, PA, total VFA, the AE of GP_{72h}, total VFA, NH_3–N, DMD, OMD and MFAEI of groups 30:25:45 and 30:10:60 were all higher than in other groups ($P < 0.05$).

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Table 5. The VFA, SFAEI and MFAEI when sunflower by-products (SS, SP, SSS) were incubated with concentrate and alfalfa *in vitro*

	Acetic acid (mmol/l)	Propionic acid (mmol/l)	A/P	Total VFA (mmol/l)	SFAEI (%)					MFAEI (%)
					AE of GP_{72h}	AE of DMD	AE of OMD	AE of TVFA	AE of NH_3–N	
C:SS:A										
40:60:0	74.50^{b}	19.85^{b}	3.75	105.36^{b}	1.56^{c}	–2.95^{b}	0.65^{c}	–4.77^{b}	–8.63^{c}	–14.14^{e}
40:45:15	82.54^{a}	23.54^{a}	3.51	119.07^{a}	63.77^{a}	15.59^{a}	18.14^{a}	21.58^{b}	28.68^{a}	147.76^{a}
40:30:30	81.98^{a}	23.33^{a}	3.51	117.74^{a}	60.56^{a}	11.88^{a}	9.95^{b}	16.99^{a}	19.90^{b}	119.28^{b}
40:15:45	78.41^{ab}	21.90^{ab}	3.58	111.44^{ab}	38.56^{b}	7.46^{ab}	7.84^{b}	3.19^{b}	19.08^{b}	76.13^{c}
40:0:60	74.95^{b}	20.44^{b}	3.67	106.30^{b}	6.89^{c}	1.95^{b}	2.38^{c}	1.86^{b}	18.10^{b}	31.18^{d}
P-value	0.030	0.032	0.452	0.038	< 0.001	0.004	0.003	0.005	0.036	< 0.001
SEM	0.582	0.148	0.109	0.259	1.442	1.025	1.045	1.443	1.432	2.557
30:70:0	77.56^{b}	19.96^{b}	3.89	108.72^{b}	–10.87^{d}	–0.88^{b}	–2.94^{c}	–2.56^{b}	–6.08^{c}	–23.33^{e}
30:55:15	78.44^{b}	20.94^{b}	3.75	110.59^{b}	1.48^{d}	3.28^{b}	11.88^{b}	2.57^{b}	–1.55^{c}	10.66^{e}
30:40:30	83.96^{ab}	23.16^{b}	3.63	119.28^{a}	66.50^{b}	9.78^{b}	12.26^{b}	9.92^{ab}	8.51^{b}	106.97^{c}
30:25:45	88.89^{a}	23.45^{a}	3.79	125.19^{a}	95.56^{a}	38.01^{a}	38.58^{a}	20.27^{a}	28.45^{a}	220.87^{a}
30:10:60	87.68^{a}	23.57^{a}	3.72	123.81^{a}	89.96^{a}	31.52^{a}	32.92^{a}	18.65^{a}	21.50^{a}	194.55^{b}
30:0:70	77.90^{b}	20.68^{b}	3.77	109.40^{b}	20.01^{c}	8.44^{b}	13.28^{b}	4.23^{b}	10.03^{b}	55.99^{d}
P-value	0.035	0.041	0.352	0.030	< 0.001	0.006	0.018	0.002	0.019	< 0.001
SEM	0.608	0.277	0.112	1.146	2.108	1.540	1.275	0.448	1.028	1.985
C:SP:A										
40:60:0	84.42^{a}	24.78^{a}	3.55	121.57^{a}	114.00^{a}	13.28^{a}	11.96^{a}	23.95^{a}	24.65^{a}	187.84^{a}
40:45:15	83.80^{a}	23.91^{a}	3.39	120.26^{a}	98.03^{a}	14.14^{a}	10.58^{a}	24.68^{a}	26.06^{a}	173.49^{a}
40:30:30	80.72^{ab}	22.95^{a}	3.22	115.57^{ab}	79.94^{ab}	5.90^{b}	3.81^{b}	15.40^{ab}	20.12^{ab}	125.17^{ab}
40:15:45	76.18^{b}	20.19^{b}	3.37	107.50^{b}	71.60^{b}	4.48^{b}	3.10^{b}	5.21^{b}	13.84^{b}	98.23^{b}
40:0:60	75.40^{b}	20.80^{b}	3.72	107.11^{b}	68.63^{b}	1.18^{b}	1.93^{b}	1.87^{b}	14.97^{b}	88.58^{b}
P-value	0.031	0.037	0.662	0.039	0.029	0.004	0.005	0.002	0.034	< 0.001
SEM	0.524	0.140	0.018	0.542	1.712	1.044	1.348	1.544	1.644	2.012
30:70:0	81.64^{b}	23.02^{b}	3.55	116.10^{b}	47.07^{c}	–1.17^{b}	8.81^{b}	1.11^{b}	–3.41^{c}	52.41^{d}
30:55:15	87.58^{a}	25.86^{a}	3.39	125.85^{a}	95.79^{b}	19.95^{a}	36.77^{a}	17.02^{a}	16.55^{a}	186.08^{b}
30:40:30	89.45^{a}	27.76^{a}	3.22	130.31^{a}	143.15^{a}	22.04^{a}	35.89^{a}	19.54^{a}	18.89^{a}	239.51^{a}
30:25:45	88.80^{a}	26.34^{a}	3.37	127.98^{a}	94.91^{b}	20.38^{a}	33.15^{a}	18.33^{a}	17.30^{a}	184.07^{b}
30:10:60	83.53^{b}	22.45^{b}	3.72	117.48^{b}	92.48^{b}	9.55^{b}	13.22^{b}	5.97^{b}	13.80^{ab}	135.02^{c}
30:0:70	79.40^{b}	22.02^{b}	3.61	112.45^{b}	37.36^{c}	5.11^{b}	10.55^{b}	2.44^{b}	6.83^{b}	62.29^{d}
P-value	0.039	0.041	0.502	0.037	< 0.001	0.006	0.021	0.007	0.005	< 0.001
SEM	0.502	0.244	0.027	1.145	2.282	1.568	1.140	0.548	1.152	1.138
C:SSS:A										
40:60:0	78.42^{b}	22.87^{ab}	3.53	112.75^{ab}	–6.95^{c}	–1.88^{b}	0.79^{b}	–9.11^{b}	18.63^{b}	1.48^{d}
40:45:15	82.83^{a}	22.89^{ab}	3.62	118.27^{a}	66.05^{a}	11.14^{a}	5.07^{a}	19.90^{a}	26.56^{a}	128.72^{a}
40:30:30	82.72^{a}	23.18^{a}	3.57	118.20^{a}	61.86^{a}	6.97^{a}	6.96^{a}	14.54^{a}	29.69^{a}	120.02^{a}
40:15:45	77.28^{b}	22.50^{ab}	3.53	111.28^{ab}	35.18^{b}	4.31^{ab}	3.15^{ab}	1.18^{b}	21.88^{b}	64.34^{b}
40:0:60	76.40^{b}	21.86^{b}	3.49	109.03^{b}	10.06^{c}	0.98^{b}	1.93^{b}	–2.71^{b}	17.97^{b}	28.23^{c}
P-value	0.034	0.035	0.662	0.047	0.012	0.003	0.027	0.006	0.033	< 0.001
SEM	0.501	0.155	0.027	0.688	1.519	1.041	1.351	1.582	1.722	2.007

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Table 5 to be continued

	Acetic acid (mmol/l)	Propionic acid (mmol/l)	A/P	Total VFA (mmol/l)	SFAEI (%)					MFAEI (%)
					AE of GP_{72h}	AE of DMD	AE of OMD	AE of TVFA	AE of NH_3–N	
30:70:0	78.36^{b}	22.72^{b}	3.45	112.46^{b}	–14.49^{b}	–2.08^{c}	2.54^{c}	–4.11^{c}	–5.49^{c}	–23.63^{d}
30:55:15	77.58^{b}	22.90^{b}	3.39	111.22^{b}	–3.80^{b}	1.89^{c}	12.69^{b}	–1.02^{c}	–1.09^{c}	8.67^{d}
30:40:30	79.29^{b}	22.76^{b}	3.48	114.33^{b}	64.70^{a}	12.55^{b}	10.43^{b}	9.81^{b}	7.39^{b}	104.88^{b}
30:25:45	89.85^{a}	28.34^{a}	3.17	130.78^{a}	82.27^{a}	19.97^{a}	35.79^{a}	18.97^{a}	24.38^{a}	181.38^{a}
30:10:60	87.33^{a}	27.97^{a}	3.12	127.06^{a}	72.08^{a}	21.61^{a}	36.78^{a}	15.75^{a}	18.96^{ab}	165.18^{a}
30:0:70	78.40^{b}	22.98^{b}	3.41	112.12^{b}	18.99^{ab}	13.02^{b}	31.05^{ab}	5.43^{b}	10.83^{b}	79.32^{c}
P-value	0.036	0.042	0.875	0.030	< 0.001	0.024	0.019	0.031	0.026	< 0.001
SEM	0.633	0.278	0.033	1.146	2.005	1.613	1.029	0.675	1.113	1.134

A = alfalfa; AE = associative effects; A/P = acetic acid to propionic acid ratio; C = concentrate; DMD = dry matter digestibility; GP_{72h} = GP at 72 h; MFAEI = multiple-factor associative effects index; NH_3–N = ammonia nitrogen; OMD = organic matter digestibility; SEM = standard error of the means; SFAEI = single-factor associative effects index; SP = sunflower plate; SS = sunflower straw; SSS = sunflower seed shells; VFA = volatile fatty acids

[a–e]Means within a column with different superscripts differ at $P < 0.05$

DISCUSSION

GP parameters of single feeds

In this experiment, SS (–9.22), SP (–7.55), concentrate (–10.50) showed a longer lag time (LT) of GP than alfalfa (–2.90) and SSS (–2.30) (Table 3). Corn had a greater LT than barley *in vitro* (Aye Sandar et al. 2012). In this experiment, the concentrate consists of 83.20% corn. And the fitted values of *b*, *a* + *b* and GP_{72h} of SS, SP, SSS were higher or approximate than those for concentrate and alfalfa (Table 3). This indicated that sunflower straw, sunflower plate and sunflower seed shells had excellent GP performance to a great degree.

GP parameters of feed mixtures

Gas production is a crucial index that can forecast feed digestibility for ruminants. The AE of mixture feeds was highly estimated, and it was precisely an acceptable method by designing a mixture and single feed which were incubated respectively *in vitro*. Such as, positive AE on GP occurred when forage tree leaves were added to the concentrate (Sandoval-Castro et al. 2002). In addition, Haddad (2000) reported that the combination of legume forage and straw shows a positive AE, which is helpful in improving the utilization rate of straw, which is the result of the comprehensive action of many comprehensive factors. Furthermore, pure cellulose and milk thistle (two kinds of slow fermented fibre) were incubated as 75 : 25 or 25 : 75 with pectin, citrus pulp, and tomato peels without seeds (three kinds of fast fermented fibre) *in vitro*, respectively, the GP of all combinations were increased (Maccarana et al. 2013). Besides, there is still a lot of literature on AEs between concentrate and forage.

In the experiment, GP index [*a*, *b*, (*a* + *b*), GP_{72h}] and fermentation parameters (NH_3–N, DMD, OMD), AA, PA, total VFA, the AE of GP_{72h}, NH_3–N, total VFA, DMD, OMD and MFAEI in groups 40 : 45 : 15 (C : SS : A), 30 : 25 : 45 (C : SS : A), 40 : 60 : 0 (C : SP : A), 40 : 45 : 15 (C : SP : A), 30 : 40 : 30 (C : SP : A), 40 : 45 : 15 (C : SSS : A), 40 : 30 : 30 (C : SSS : A), 30 : 25 : 45 (C : SSS : A) and 30 : 10 : 60 (C : SSS : A) were higher than in other groups (Tables 4 and 5). These results mentioned above indicated that when the concentrate level was high such as 40 (C : R was 40 : 60), the demands for optimal associated effects on SS, SP or SSS increased to 45 or 60 [groups 40 : 45 : 15 (C : SS : A), 40 : 60 : 0 (C : SP : A), 40 : 45 : 15 (C : SP : A), 40 : 45 : 15 (C : SSS : A) presented the best MFAEI]. However, when C : R was 30 : 70, it was found that the requirements for optimal associated effects on SS, SP or SSS decreased to 25 or 10 [groups 30 : 25 : 45 (C : SS : A), 30 : 25 : 45 (C : SSS : A) and 30 : 10 : 60 (C : SSS : A) presented the best MFAEI]. Nevertheless, group 30 : 40 : 30 (C : SP : A) was an exception. The reason

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for the results is that the nutritive value (the crude protein of SP was higher than that in SS and SSS, while the CF content of SP was quite lower than that of SS and SSS) of sunflower plate is superior to that of sunflower seed shells and sunflower straw. In addition, there were optimal associative effects in groups 40 : 60 : 0 (C : SP : A), 40 : 45 : 15 (C : SP : A), 30 : 40 : 30 (C : SP : A) which need a small amount or even have no need for alfalfa. As to SS and SSS, groups 40 : 45 : 15 (C : SS : A), 30 : 25 : 45 (C : SS : A), 40 : 45 : 15 (C : SSS : A), 40 : 30 : 30 (C : SSS : A), 30 : 25 : 45 (C : SSS : A) and 30 : 10 : 60 (C : SSS : A) presented optimal AE that demonstrated that a higher level of concentrate (40) needs a higher level of SS/SSS (45 or 30) and a lower level of alfalfa (15 or 30), while a lower level of concentrate (30) needs a lower level of SS or SSS (25 or 10) and a higher level of alfalfa (45 or 60). To summarize, it showed that when the concentrate was higher, the amount of SS, SP, SSS increased; however, when the concentrate was reduced, the amount of alfalfa increased. Therefore, SS, SP and SSS can substitute a part of concentrate to a certain degree.

Rumen pH, DMD, OMD of feed mixtures

The rumen pH is a crucial index of rumen environmental changes. In general, rumen pH usually ranges from 6 to 7; too high or too low pH is disadvantageous to rumen fermentation. However, VFA can lower rumen pH for the reason that VFA are produced faster than they are assimilated from the rumen. Abdelhadi et al. (2005) found that the reduction of pH value is usually linear with fermentable carbohydrate intake. In the experiment, almost all combinations which had higher total VFA showed lower pH (Tables 4 and 5), which is consistent with the above reports.

Menke and Steingass (1988) reported that DMD and OMD were significantly positively correlated with GP, rumen microbial fermentation activity and feed digestibility. Besides, DMD and OMD were important indicators to determine the feed nutritional value. Gunun et al. (2013) reported that alfalfa had a higher efficacious degradation rate and appropriate concentrate-roughage ratio conducive to the vigorous microbe activity. In this study, there were higher AEs in groups 40 : 45 : 15 (C : SS : A), 40 : 60 : 0 (C : SP : A), 40 : 45 : 15 (C : SP : A) and 40 : 45 : 15 (C : SSS : A) which need a small amount or even have no need for alfalfa, which further proved that SS, SP and SSS themselves had good gas production and fermentation performance. To sum up, it should be the result of AE among the three feedstuffs.

Mosi and Butterworth (1985) reported that adding alfalfa can improve the utilization rate of low-quality roughage. Additionally, Haddad (2000) found that low-quality feeds cultured with alfalfa could generate AE on digestibility, utilization and intake. Besides, Aye Sandar et al. (2012) reported that there were positive AEs on DMD, OMD and GP when autumn pasture was cultured with barley or corn and when spring pasture was cultured with corn. Moreover, Guzatti et al. (2017) reported that AE was generated when Kikuyu grass silage and red clover were incubated in which protein hydrolysis, reduction, and synergy occurred. In summary, widespread associative effects existed among different kinds of feedstuffs (for example, grain feeds and roughage, low-quality feeds and alfalfa, among all kinds of forage grasses, etc.). This study aimed to investigate the AE among low-quality feeds (SS, SP, SSS), alfalfa and grain feeds (concentrate).

Ammonia nitrogen and VFA of feed mixtures

Ammonia nitrogen is an important indicator reflecting the degradation of feed protein, nitrogen metabolism in the rumen and the synthesis of microbial protein. And the proper level of NH_3–N must be maintained to guarantee microbial protein synthesis. Calsamiglia et al. (2002) stated that the appropriate range of NH_3–N was 6.3-27.5 mg/dl. In this experiment, the NH_3–N values were between 10.25 mg/dl and 27.55 mg/dl (Table 4). It is well known that VFA and NH_3–N are the main final products of rumen fermentation besides methane, carbon dioxide, etc. Moreover, VFA are the main source of energy for the normal maintenance and growth of rumen microflora of ruminants. The NH_3–N, PA, AA and total VFA in groups 40 : 45 : 15 (C : SS : A), 30 : 25 : 45 (C : SS : A), 40 : 60 : 0 (C : SP : A), 40 : 45 : 15 (C : SP : A), 30 : 40 : 30 (C : SP : A), 40 : 45 : 15 (C : SSS : A), 40 : 30 : 30 (C : SSS : A) and 30 : 25 : 45 (C : SSS : A) were significantly higher than in other groups (Tables 4 and 5), which demonstrated that these eight groups promoted the simultaneous production of rumen

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energy and ammonia and increased microbial protein synthesis (Zhou et al. 2015). The fermentation of digestible carbohydrates can produce VFA, and acetic acid is positively correlated with GP_{72h}. The A/P can reflect the type of fermentation in the rumen. The A/P ratio in the present experiment was higher than 3 (Table 5); it can be inferred that the fermentation type of each treatment group is acetic acid fermentation type that is helpful to improve the rate of milk fat for ruminants. Furthermore, in the experiment, the acetic acid yield in all combinations was much higher than that of propionic acid (Table 5). The absorption rate of VFA in ruminants was BA, PA and AA in the order from fast to slow. And these results are consistent with Copani et al. (2015) report.

Associative effects of feed mixtures

The evaluation indices of associative effects of feeds included nutrient digestibility, rate of utilization, energy, feed intake and growth performance of animals in which energy and digestibility are the most frequently used indices to evaluate AE. GP *in vitro* is quite relevant with DOM. Nevertheless, due to the complexity of the AE mechanism, it may be inaccurate to use GP alone or one of several indices to evaluate the feed nutritional value and assess AE. Besides, *in vitro* GP of feed was negatively relevant with feed protein and positively relevant with carbohydrate digestion. Therefore, if GP only determines the feed nutritional values, it may not be accurate, and the feed or combination with either low GP or high protein production may be eliminated. Therefore, the nutritional value of feed should be accurately evaluated by comprehensive indices, even by a mathematical model. In this study, the associative effects of sunflower by-products (SS, SP, SSS), concentrate and alfalfa were assessed by a comprehensive index which combined GP_{72h}, NH_3–N, VFA, DMD and OMD.

In most cases, granulation results in the more efficient degradation of crude protein and starch in the rumen in some compound feeds than in the crushed form of compound feed, which may be due to the departure of ungraded finer feed particles from the bag (Grubjesic et al. 2019). Afterwards, the single feed data on GP_{24h}, metabolic energy, OMD and available CP in the duodenum have additivity, however the data are not additive on intestinal digestibility of undegraded protein in the rumen, so the degradation of CP in compound feed in the rumen cannot be forecasted reliably by CP fractions (Grubjesic et al. 2020). Yuan and Wan (2019) reported that when peanut shell was combined with concentrate and alfalfa, the best combinations of single-factor and multiple-factor associative effects index were 30 : 10 : 60 and 40 : 30 : 30 (concentrate/peanut shell/alfalfa ratios). Furthermore, it was found that the optimal AE occurred when concentrate/soybean pod/alfalfa were 20 : 50 : 30, 20 : 65 : 15, 30 : 40 : 30, 30 : 55 : 15, 40 : 50 : 10, 50 : 20 : 30, 50 : 30 : 20, 60 : 10 : 30, 60 : 20 : 20, 40 : 60 : 0, 50 : 50 : 0 (Yuan et al. 2020). Singh et al. (2019) reported that the mixture of *Eucalyptus globulus* essential oils, acetone extract of *Ficus bengalensis* leaves and ethanol and aqueous extracts of *Sapindus mukorossi* fruits had more obvious effects on promoting rumen fermentation and reducing methane production *in vitro* at very low dose levels than their individual inclusion, meanwhile, they presented positive associative effects. Niderkorn et al. (2019) mixed chicory and ryegrass at an equal ratio. They found that the mixture greatly improved the nitrogen utilization efficiency and had a synergistic effect on the voluntary intake of animals. The reason may be that the complementarity of the chemical components of the two forages increased the motivation of animals to eat and helped the rumen particles decompose faster.

These six reports mentioned above suggested that the associative effects among different feeds exist widespread; however, the mechanism of the associative effects is quite intricate. Some indicators are additive among single feedstuff and their mixture, and others are not; the non-additivity is due to the AE produced among feeds. In this experiment, MFAEI of groups 40 : 45 : 15 (C : SS : A), 30 : 25 : 45 (C : SS : A), 40 : 60 : 0 (C : SP : A), 40 : 45 : 15 (C : SP : A), 30 : 40 : 30 (C : SP : A), 40 : 45 : 15 (C : SSS : A), 40 : 30 : 30 (C : SSS : A), 30 : 25 : 45 (C : SSS : A) and 30 : 10 : 60 (C : SSS : A) were optimal (Table 5). This is because the three feeds of the nine groups are nutritionally complementary to each other. The feed combination in this proportion improves the fermentation rate of the substrate and then promotes the feed digestibility. Therefore, after 72 h of *in vitro* fermentation, the GP performance, rumen fermentation level and feed utilization rate of the above groups were greatly improved.

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CONCLUSION

The optimum SFAEI of GP_{72h}, total VFA, NH_3–N, DMD, OMD and MFAEI were obtained when the concentrate/sunflower straw/alfalfa ratios were 40 : 45 : 15 and 30 : 25 : 45, concentrate/sunflower plate/alfalfa ratios were 40 : 60 : 0, 40 : 45 : 15 and 30 : 40 : 30, concentrate/sunflower seed shell/ alfalfa ratios were 40 : 45 : 15, 40 : 30 : 30, 30 : 25 : 45 and 30 : 10 : 60. However, in order to use these three sunflower by-products as feed for ruminants, *in vivo* studies of mixture supplementation should be carried out to figure out their effects on fermentation characteristics in the rumen and the health and performance of animals.

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Conflict of interest

The authors declare no conflict of interest.

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