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Study on the associative effects of different proportions of soybean pod, alfalfa and concentrate on the diets at different ratio of concentrate to roughage *in vitro*

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Abstract: This study aimed to determine the associative effects (AEs) of 28 feed combinations of concentrate/soybean pod/alfalfa at different concentrate-roughage ratios that were incubated for 72 h in single tubes (120 ml) which were added 30 ml rumen buffered fluid. The gas production (GP) at 0, 2, 4, 6, 9, 12, 24, 36, 48, 72 h was recorded. A single exponential equation was applied to calculate the GP parameters a (rapid GP), b (slow GP), a + b (GP potential) and c (rate constant of slow GP that can reflect the specific process of GP, rapid and slow GP and GP rate). The AEs were calculated by 72 h GP and weighted estimation value of each combination. After 72 h incubation, pH, volatile fatty acids (VFA) and ammonia nitrogen (NH_3–N), dry matter digestibility (DMD), organic matter digestibility (OMD) were determined the incubation fluid and residues. The single-factor AE index (SFAEI) and multiple-factor AE index (MFAEI) were computed. The results showed that the groups 50 : 50 : 0, 40 : 60 : 0, 60 : 20 : 20, 60 : 10 : 30, 50 : 30 : 20, 50 : 20 : 30, 40 : 50 : 10, 30 : 55 : 15, 30 : 40 : 30, 20 : 65 : 15, 20 : 50 : 30 had higher $GP_{72\ h}$, a, b, DMD, OMD, NH_3–N, in addition, higher AE of GP, DMD, OMD, total VFA and NH_3–N than those of the other groups ($P < 0.05$ or $P < 0.01$), especially the group 30 : 55 : 15 was optimal. In conclusion, *in vitro* data reveal reliable fermentability and the highest SFAEI and MFAEI occurred when concentrate, soybean pod and alfalfa were combined at the ratios of 50 : 50 : 0, 40 : 60 : 0, 60 : 20 : 20, 60 : 10 : 30, 50 : 30 : 20, 50 : 20 : 30, 40 : 50 : 10, 30 : 55 : 15, 30 : 40 : 30, 20 : 65 : 15, 20 : 50 : 30.

Keywords: soybean hull; combination effects; clover; commercial concentrate; *in vitro* incubation

Soybean (*Glycine max* Linn. Merr) is one of the most familiar oil crops and also a paramount source of high-quality protein feed all over the world. When the soybean matures, strings of soybeans are picked off and the pods are peeled. After the seeds are taken out, the remaining empty shells or skins are called the soybean pods. Soybean pod is a by-product of soybean crop, which has a high yield and is seldom used as feed in production and most of soybean pods are thrown away or burned as fertiliser sources. However, soybean pod can enhance immunity, prevent and cure cancer and promote the full play of the normal intestinal function of humans and animals (Li 2004). Nevertheless, soybean pod may have high crude fibre and low digestibility which might limit its application as feedstuffs. However, the utilization ratio of pods can be effectively improved by the rational combination of pods and high-quality legumes. The associative effect (AE) of the diet refers to the overall effect of the interac-

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tion between nutrients from different feed sources, including the interaction between nutritional factors and non-nutritional factors as well as various measures. The AE is due to the complementary effect between different kinds of feed after mixing in appropriate proportion, which makes the fermentation and gas production (GP) performance of mixed feed reach the optimum. In addition, these interactions can alter metabolic pathways in rumen and gastrointestinal tract. It has been confirmed that alfalfa (*Medicago sativa*) can enhance the utilization of low quality forage (Mosi and Butterworth 1985). Goetsch and Gipson (2014) reported that as to forage-based diets, in many instances, it is essential to add concentrate to the diet for animals to achieve ideal performance and with the amount and composition of the supplement depending on characteristics of the basal forage and the target animal.

The fermentation activity of milk thistle and crown daisy which were poor quality forage could be promoted by means of supplementing apple pomace or citrus pulp (Tagliapietra et al. 2015). Dolebo et al. (2017) found that the negative AE of goat feed could be predicted by the level of concentrate and the source of hay which indicated that metabolizable energy intake can be predicted exactly by using low or appropriate levels of concentrate in growing wethers.

Though there is a considerable amount of literature on feed associative effects, especially those between concentrate feeds and forage in ruminants have long been studied (Forbes et al. 1933; Mould 1982); however, the research on evaluating the AE of soybean pod added to the diets of ruminants is less common. The primary purpose of this study was to determine the associative effects of different proportions of soybean pod, concentrate and alfalfa at different concentrate-roughage (C : R) ratios by *in vitro* GP technology. It is expected that the results of this experiment can provide valuable reference and guidance for the scientific application of soybean pods in ruminants.

MATERIAL AND METHODS

Experimental design and experimental feedstuffs

There were 28 diet combinations in this experiment: concentrate/soybean pod/alfalfa ratios were 60 : 30 : 10, 60 : 20 : 20, 60 : 10 : 30, 50 : 40 : 10, 50 : 30 : 20, 50 : 20 : 30, 50 : 10 : 40, 40 : 50 : 10, 40 : 40 : 20, 40 : 30 : 30, 40 : 20 : 40, 40 : 10 : 50, 30 : 55 : 15, 30 : 40 : 30, 30 : 25 : 45, 30 : 10 : 60, 20 : 65 : 15, 20 : 50 : 30, 20 : 35 : 45, 20 : 20 : 60 when C : R was 60 : 40, 50 : 50, 40 : 60, 30 : 70, 20 : 80; also, concentrate/soybean pod/alfalfa ratio was 80 : 20 : 0, 60 : 40 : 0, 50 : 50 : 0, 40 : 60 : 0, 20 : 80 : 0 and three single feeds (concentrate, soybean pod and alfalfa) (Table 1). The 28 groups (the mixtures were made on as fed basis) mentioned above were incubated for 72 h in individual gas production tubes.

The three single feedstuffs were beforehand ground to 1 ml. The experimental scheme of this experiment was authorized by the Institute of Animal Use and Ethics, Gansu Agricultural University. The formula of the concentrate is as follows: corn 84.37%, soybean meal 7.92%, cottonseed meal 3.56%, salt 1.65% and premix 2.50%.

In vitro incubation process

Concentrate, soybean pod and alfalfa were dried and ground to 1-mm particles. Then concentrate, soybean pod and alfalfa, three single feedstuffs, and 25 diet combinations were incubated in the respective culture tubes (Häberle Labortechnik GmbH & Co. KG, Lonsee, Germany) with 20 replicates; moreover, six blanks were included for the correction of all of these fermentation parameters such as GP, dry matter digestibility (DMD), organic matter digestibility (OMD), ammonia nitrogen (NH_3–N) and volatile fatty acids (VFA) produced

Table 1. The ratios of ingredients of different combinations of diets

Composition			60:40	50:50	40:60	30:70	20:80
Concentrate : soybean pod : alfalfa	100:0:0	80:20:0	60:30:10	50:40:10	40:50:10	30:55:15	20:65:15
	0:100:0	60:40:0	60:20:20	50:30:20	40:40:20	30:40:30	20:50:30
	0:0:100	50:50:0	60:10:30	50:20:30	40:30:30	30:25:45	20:35:45
	–	40:60:0	–	50:10:40	40:20:40	30:10:60	20:20:60
	–	20:80:0	–	–	40:10:50	–	–

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from the microbial culture medium in each run, for 560 culture tubes of samples, 48 blank tubes with eight batch tests in total. On the day of *in vitro* incubation, three samples were weighed accurately according to various concentrations of diet combinations and recorded, afterwards, they were added to nylon bags (3 cm × 5 cm; pore size 40 ± 12 μm) with constant weight and placed at the bottom of the culture tube. According to Menke and Steingass (1988), each tube (120 ml) was filled with 10 ml of rumen fluid and 20 ml of microbial buffer solution in each culture tube (headspace of the tube = 90 ml) and placed vertically on a 77-hole inorganic glass bracket (this bracket was designed and manufactured by the reagent company) in an invariant temperature water bath at 38.5–39.5 °C. In addition, the preparation of microorganism buffer solution was done according to Menke and Steingass (1988). Rumen fluid supplied by five dairy cows aged about six years and weighing 535 ± 4.2 kg, fed wheat straw *ad libitum* and concentrates (chemical composition and nutritional level are listed in Table 2) 5 kg/day/head three times a day by means of a rumen fistula probe 2 h before morning feeding, was collected in two hot water bottles preheated to 38.5–39.5 °C, and fast delivered to the lab as soon as possible (not more than 20 min). Then, the rumen fluid was quickly filtered through four layers of gauze to remove the solid feed, afterwards, the filtered rumen fluid and the above-mentioned microbial buffer solution were well mixed at a 1 : 2 ratio.

All of these procedures were carried out under the conditions of anaerobic environment by means of continuous injection of carbon dioxide flow and were accomplished in half an hour or less. Gas collection tubes (Häberle Labortechnik GmbH & Co. KG, Lonsee, Germany) were used to determine actual GP at 10 different fermentation times (0, 2, 4, 6, 9, 12, 24, 36, 48, 72 h).

Computation of gas production

The accumulated GP curves (Orskov and Mc Donald 1979) were fitted to an exponential equation to estimate the dynamics of GP : GP as follows:

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

where:

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

According to the actual GP readings of three kinds of individual feedstuffs at 72 h, the expected GP of each feed combination was calculated according to the different proportion of three kinds of feedstuffs. All actual GP values must be corrected by blank GP data (blank treatment was set up to eliminate the errors caused by *in vitro* culture systems).

Determination of feed nutritional indices

For the twenty-five diet combinations, the "predicted GP" was calculated as the weighted value of the GP provided by concentrate, alfalfa and soybean pod incubated separately (it is assumed that there is no AE among concentrate, alfalfa and soybean pod). The three feedstuffs were determined in quadruplicate for dry matter (DM), crude protein (CP), ether extract (EE), crude fibre (CF) and crude ash (ASH) on the basis of the AOAC (2003). The content of CP was measured by the Kjeldahl method (No. 2001.14) by

Table 2. Ingredients and nutrient levels of the concentrate of the experimental diet for three Holstein-Friesian cows (%, DM basis)

	Corn (60.5)	Soybean meal (23.0)	Wheat bran (11.5)	$CaHPO_4$ (2.0)	NaCl (1.0)	Premix[1] (2.0)	Total (100.0)
Nutrient levels	DM	CP	NE_L(MJ/kg)[2]	NDF	ADF	Ca	P
	83.2	16.7	7.2	12.7	5.2	0.5	0.5

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 D_3 300 000 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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Danish FOSS using a Kjeldahl nitrogen analyser. The EE content was determined by Soxhlet extraction (No. 993.20). The ASH was analysed by a muffle furnace method and organic matter (OM) was calculated using the formula as follows: organic matter = dry matter – crude ash (Sandoval-Castro et al. 2002). Neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) were measured on the basis of the Van Soest method using an ANKOM A2000i Automated Fiber Analyzer (ANKOM Technology, Macedon, NY, USA), and results for both NDF and ADF are expressed as ash inclusive. Determination of crude fibre content was carried out by an acid and alkali washing treatment method.

Measurement of *in vitro* dry matter digestibility and *in vitro* organic matter digestibility

After the termination of incubation (72 h), the residue in tubes was filtrated through qualitative filter paper, the filtered residue was collected and put into the respective nylon bags, the residue and the nylon bags were dried in the oven at 105 °C until the weight of the residue with nylon bags was constant. The *in vitro* dry matter digestibility was calculated by determining the difference between the initial weight of the samples (about 0.200 0 g, DM basis) and the constant weight after drying (corrected by blank tube). The *in vitro* organic matter digestibility was accessed by the incineration of dry residues (550–580 °C) and it was also calculated by determining the difference between initial weights of feed mixtures and final constant weights (corrected by blank tube) of the residues which determined the ASH content of feed and residues, then the values of OM and *in vitro* organic matter digestibility were calculated according to the formula: organic matter = dry matter – crude ash (Sandoval-Castro et al. 2002).

Determination of pH, ammonia nitrogen and volatile fatty acids

After 72 h incubation, the samples of filtered microbial culture solution were collected to measure pH, ammonia nitrogen (NH_3–N) and volatile fatty acid (VFA) content. The pH was determined rapidly with a digital meter fitted with a glass electrode; then the culture medium was centrifuged at 5 000 × *g* for 10 min, 4 ml supernatant was collected and placed into a 5 ml centrifuge tube with lid with 1 ml of a 25% (weight/volume) metaphosphoric acid solution and frozen at –20 °C for VFA analysis by the operation of Lu et al. (1990). Total VFA (TVFA) concentrations and molar proportions of acetate, propionate and butyrate were determined in a gas chromatograph (GC-14; Shimadzu, Duisburg, Germany) using N_2 as a carrier gas at a flow rate of 50 ml/min and a glass column (2 m length × 2 mm diameter) packed with Chromosorb AW, 100 g/kg polyethylene glycol and 30 g/kg H_3PO_4 (Supelco Inc., Bellefonte, PA, USA). The temperature of the oven, injector port and detector port was 155, 185 and 190 °C, respectively. In addition, 3 ml supernatant was placed into a 5 ml centrifuge tube with lid with 2 ml of 3 M HCl and frozen at –20 °C for NH_3–N analysis according to Broderick and Kang (1980). Ruminal NH_3–N concentrations were determined using glutamate dehydrogenase (171-B; Sigma Chemical Co. St Louis, MO, USA) and a method adapted for use on a COBAS FARA II centrifugal analyser (Roche Diagnostic Systems, Montclair, NJ, USA).

Computation of associative effects

The concept and method of single-factor associative effects index (SFAEI) and multiple-factor associative effects index (MFAEI) were proposed by Wang (2011).

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

where:

SFAEI – single-factor associative effects index;

MV – measured value of each diet combination;

WEV – weighted estimate value, the MV of a kind of feed × the proportion of this feed in the combination + the MV of another kind of feed × the proportion of the other kind of feed in the combination;

MFAEI – the sum of each SFAEI. In this study, MFAEI = the sum of five SFAEI (AE of GP, DMD, OMD, NH_3–N and TVFA).

Statistical analysis

Experimental data were statistically analysed by one-way analysis of variance (ANOVA) using the SAS software v7.0 (SAS Institute 1996). The significance of differences between means for all

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treatments was analysed by Tukey-Kramer multiple comparisons. The differences between treatment means were considered to be significant at $P < 0.05$ and highly significant at $P < 0.01$. Furthermore, the differences between treatment means were considered to be tended to change at $0.05 < P < 0.10$. The results are shown as the mean and standard error of the means (SEM).

RESULTS

Nutrient levels and GP parameters of single feedstuff

Table 3 shows that CP content of soybean pod was the lowest, while its NDF and ADF contents were higher than in alfalfa. In contrast, the a, b, a + b and $GP_{72\ h}$ of soybean pod were the highest in alfalfa, concentrate and soybean pod, and the c value of soybean pod was equal with alfalfa.

Gas production$_{72\ h}$, a, b, c, DMD, OMD and NH_3–N and VFA of mixtures of feedstuff

Table 4 shows that the a, b, c, DMD, OMD and NH_3–N of groups 50 : 50 : 0, 40 : 60 : 0 (concentrate/soybean pod/alfalfa C : S : A, the same as below) were higher than in the other groups ($P < 0.05$). However, there was no difference between group 60 : 40 : 0 and the other four groups as to b, c, and DMD ($P > 0.05$). The a + b and $GP_{72\ h}$ of groups 50 : 50 : 0 and 40 : 60 : 0 were higher than in the other three groups ($P < 0.01$) and $GP_{72\ h}$ of group 80 : 20 : 0 was lower than in the other four groups ($P < 0.01$). Moreover, Table 5 indicates that propionic acid (PA), butyric acid (BA) and total volatile fatty acids (TVFA) of groups 50 : 50 : 0 and 40 : 60 : 0 were higher than in the other three groups ($P < 0.05$), acetic acid (AA) of groups 50 : 50 : 0 and 40 : 60 : 0 was higher than in group 80 : 20 : 0 ($P < 0.05$).

Table 4 and 5 demonstrate that the a, b, DMD, OMD, NH_3–N, AA, PA, TVFA and a + b, $GP_{72\ h}$ of groups 60 : 20 : 20 and 60 : 10 : 30 were higher than in group 60 : 30 : 10, respectively ($P < 0.05$ and $P < 0.01$). In addition, the b value of groups 50 : 40 : 10, 50 : 30 : 20 and 50 : 20 : 30 was higher than in group 50 : 10 : 40 ($P < 0.05$). Moreover, the a, DMD, OMD, NH_3–N, AA, PA, TVFA and a + b, $GP_{72\ h}$ of groups 50 : 30 : 20 and 50 : 20 : 30 were higher than in groups 50 : 40 : 10 and 50 : 10 : 40, respectively ($P < 0.05$ and $P < 0.01$) and the a + b, $GP_{72\ h}$ of group 50 : 40 : 10 were higher than in group 50 : 10 : 40 ($P < 0.01$).

It was indicated (in Table 4) that when the C : R ratio was 40 : 60, the a value of groups 40 : 50 : 10 and 40 : 40 : 20 was higher than in groups 40 : 30 : 30, 40 : 20 : 40 and 40 : 10 : 50 ($P < 0.01$) and the a value of group 40 : 30 : 30 was higher than in group 40 : 10 : 50 ($P < 0.01$). Furthermore, the b, a + b and $GP_{72\ h}$ of group 40 : 50 : 10 were higher than in the other four groups ($P < 0.05$) and those of groups of 40 : 40 : 20, 40 : 30 : 30 and 40 : 20 : 40 were higher than in group 40 : 10 : 50 ($P < 0.05$). Also, the c value of groups 40 : 50 : 10, 40 : 40 : 20, 40 : 30 : 30 was higher than in groups 40 : 20 : 40 and 40 : 10 : 50 ($P < 0.05$). Besides, the DMD of group 40 : 10 : 50 was lower than in the other four groups ($P < 0.05$). Further, the OMD of groups 40 : 50 : 10 and 40 : 40 : 20 was higher than in group 40 : 10 : 50 ($P < 0.05$). Additionally, the NH_3–N of group 40 : 50 : 10 was higher than in groups 40 : 20 : 40 and 40 : 10 : 50 ($P < 0.05$). Table 4 shows that the AA and BA of group 40 : 50 : 10 were higher than in groups 40 : 30 : 30, 40 : 20 : 40 and 40 : 10 : 50 ($P < 0.05$). Also, the PA and TVFA of groups 40 : 50 : 10 and 40 : 40 : 20 were higher than in the other three groups ($P < 0.05$).

Table 4 and 5 demonstrate that the a, c, AA, PA, TVFA and b, a + b, $GP_{72\ h}$ of groups 30 : 55 : 15 and 30 : 40 : 30

Table 3. Chemical composition and *in vitro* gas production parameters of single experimental feedstuffs

	Nutrient levels (%)								*In vitro* GP parameters (ml)				
	DM	OM	CP	EE	NDF	ADF	ADL	CF	a	b	c (%/h)	a + b	$GP_{72\ h}$
S	94.6	87.1	9.7	6.8	51.3	37.1	6.7	30.4	10.4	65.3	0.056	75.7	69.2
C	91.9	90.8	19.2	4.2	25.9	nd	nd	7.1	−9.8	57.9	0.121	48.1	42.5
A	94.9	91.1	18.5	6.6	32.2	24.1	nd	nd	−3.1	30.7	0.056	27.6	35.8

A = alfalfa; a = rapid gas production (GP); ADF = acid detergent fibre; ADL = acid detergent lignin; a + b = GP potential; b = slow GP; c = rate constant of slow GP; C = concentrate; CF = crude fibre; CP = crude protein; DM = dry matter; EE = ether extract; $GP_{72\ h}$ = GP at 72 h; nd = not determined; NDF = neutral detergent fibre; OM = organic matter; S = soybean pod

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Table 4. Gas production (GP) and fermentation parameters at 72 h when soybean pod was incubated with alfalfa and concentrate *in vitro*

C:S:A	GP parameters (ml)					Fermentation parameters (%)			
	a	b	c (%/h)	a + b	$GP_{72\,h}$	pH	DMD	OMD	NH_3–N
80:20:0	5.97^{b}	65.10^{b}	0.120^{b}	71.07^{B}	53.00^{C}	6.34	39.12^{b}	41.78^{b}	9.05^{b}
60:40:0	7.06^{b}	68.22^{ab}	0.158^{ab}	75.28^{B}	68.60^{B}	6.31	44.07^{ab}	45.69^{b}	11.36^{b}
50:50:0	12.03^{a}	75.29^{a}	0.179^{a}	87.37^{A}	78.92^{A}	6.22	51.55^{a}	52.98^{a}	17.29^{a}
40:60:0	11.17^{a}	74.93^{a}	0.190^{a}	86.10^{A}	79.25^{A}	6.15	50.40^{a}	53.02^{a}	16.49^{a}
20:80:0	6.40^{b}	62.11^{b}	0.115^{b}	68.51^{B}	65.50^{B}	6.41	40.05^{b}	41.91^{b}	8.88^{b}
P-value	0.039	0.030	0.040	0.004	0.002	0.463	0.026	0.030	0.021
SEM	1.224	1.411	1.208	1.207	1.442	0.358	1.034	1.188	1.179
60:30:10	7.38^{b}	71.22^{b}	0.125	78.60^{B}	76.85^{B}	6.29	43.59^{b}	44.66^{b}	12.88^{b}
60:20:20	11.48^{a}	76.86^{a}	0.149	88.34^{A}	86.44^{A}	6.11	52.38^{a}	54.02^{a}	18.68^{a}
60:10:30	12.43^{a}	77.75^{a}	0.160	90.18^{A}	87.89^{A}	6.15	54.11^{a}	54.85^{a}	19.97^{a}
P-value	0.040	0.024	0.439	0.003	0.005	0.510	0.029	0.032	0.028
SEM	1.087	1.621	0.050	1.333	1.442	0.146	1.501	1.880	0.442
50:40:10	9.05^{b}	72.40^{a}	0.143	81.45^{B}	79.32^{B}	6.35	43.59^{b}	45.18^{b}	13.46^{b}
50:30:20	10.19^{a}	77.78^{a}	0.167	87.97^{A}	86.09^{A}	6.22	52.38^{a}	53.96^{a}	19.88^{a}
50:20:30	11.36^{a}	78.03^{a}	0.159	89.39^{A}	88.45^{A}	6.19	54.11^{a}	55.08^{a}	20.48^{a}
50:10:40	8.71^{b}	63.35^{b}	0.147	72.06^{C}	69.40^{C}	6.11	44.95^{b}	46.88^{b}	11.39^{b}
P-value	0.042	0.029	0.435	0.005	0.004	0.421	0.030	0.036	0.025
SEM	1.253	1.468	0.029	1.408	1.585	0.202	1.459	1.742	0.780
40:50:10	14.59^{A}	75.42^{a}	0.185^{A}	90.01^{a}	88.78^{a}	6.23	52.91^{a}	54.88^{a}	18.92^{a}
40:40:20	14.38^{A}	59.10^{b}	0.176^{A}	73.48^{b}	71.84^{b}	6.14	51.62^{a}	53.76^{a}	15.18^{ab}
40:30:30	8.88^{B}	61.60^{b}	0.183^{A}	70.48^{b}	67.10^{b}	6.81	49.36^{a}	50.31^{ab}	14.89^{ab}
40:20:40	4.35^{BC}	62.98^{b}	0.103^{B}	67.33^{b}	66.17^{b}	6.76	47.94^{a}	49.25^{ab}	12.62^{b}
40:10:50	1.19^{C}	50.51^{c}	0.097^{B}	51.70^{c}	50.00^{c}	6.43	40.06^{b}	41.85^{b}	11.58^{b}
P-value	0.003	0.035	0.005	0.040	0.018	0.276	0.032	0.040	0.033
SEM	1.454	2.131	0.015	2.023	2.092	0.219	1.845	1.802	0.087
30:55:15	15.87^{a}	83.76^{A}	0.188^{a}	96.63^{A}	88.04^{A}	6.15	55.59^{a}	56.08^{a}	22.63^{A}
30:40:30	15.45^{a}	82.36^{A}	0.179^{a}	94.81^{A}	87.52^{A}	6.17	56.04^{a}	57.19^{a}	21.95^{A}
30:25:45	9.20^{b}	62.89^{B}	0.117^{b}	73.09^{B}	68.76^{B}	6.25	53.78^{a}	57.05^{a}	20.18^{A}
30:10:60	9.48^{b}	63.20^{B}	0.123^{b}	74.41^{B}	69.19^{B}	6.29	46.92^{b}	48.04^{b}	12.41^{B}
P-value	0.040	0.003	0.021	0.004	0.025	0.154	0.027	0.032	0.004
SEM	1.394	1.283	0.013	1.316	1.664	0.168	2.008	1.802	0.117
20:65:15	11.66^{a}	77.83^{A}	0.149	89.49^{A}	83.66^{A}	6.39	54.88^{a}	55.65^{a}	18.44^{a}
20:50:30	11.47^{a}	79.86^{A}	0.134	91.33^{A}	82.33^{A}	6.33	54.19^{a}	55.27^{a}	19.13^{a}
20:35:45	7.04^{b}	63.17^{B}	0.119	70.71^{B}	64.65^{B}	6.48	46.07^{b}	47.86^{b}	13.06^{b}
20:20:60	6.79^{b}	61.37^{B}	0.124	68.16^{B}	63.25^{B}	6.50	46.12^{b}	48.19^{b}	12.51^{b}
P-value	0.031	0.005	0.501	0.001	0.002	0.326	0.034	0.039	0.041
SEM	1.224	1.486	0.022	1.444	1.779	0.155	1.432	1.700	0.158

A = alfalfa; a = rapid GP; a + b = GP potential; b = slow GP; c = rate constant of slow GP; C = concentrate; DMD = dry matter digestibility; $GP_{72\,h}$ = GP at 72 h; NH_3–N = ammonia nitrogen (mg/dl); OMD = organic matter digestibility; S = soybean pod; SEM = standard error of the means

$^{a–c}$Means within a column differ at $P < 0.05$; $^{A–C}$means within a column differ at $P < 0.01$

Table 5. Volatile fatty acids at 72 h when soybean pod was incubated with alfalfa and concentrate *in vitro* (mmol/l)

C : S : A	Acetic acid	Propionic acid	Isobutyric acid	Butyric acid	Isovaleric acid	Valerianic acid	A/P	TVFA
80 : 20 : 0	74.90[b]	17.45[b]	1.26	5.14[b]	2.39	1.29	4.29	103.43[b]
60 : 40 : 0	78.26[ab]	19.05[b]	1.22	5.23[b]	2.48	1.35	4.11	108.59[b]
50 : 50 : 0	84.09[a]	23.98[a]	1.36	6.85[a]	2.57	1.46	3.51	120.31[a]
40 : 60 : 0	84.42[a]	24.78[a]	1.33	6.97[a]	2.50	1.52	3.41	121.52[a]
20 : 80 : 0	77.08[ab]	17.89[b]	1.21	5.19[b]	2.43	1.28	4.31	106.08[b]
P-value	0.037	0.035	0.135	0.043	0.220	0.370	0.152	0.036
SEM	1.449	1.034	1.420	1.124	0.658	1.490	0.236	1.023
60 : 30 : 10	80.12[b]	20.00[b]	1.35	6.11	2.39	1.37	4.01	111.34[b]
60 : 20 : 20	85.90[a]	26.44[a]	1.40	6.37	2.57	1.59	3.25	124.27[a]
60 : 10 : 30	86.11[a]	25.76[a]	1.43	6.45	2.60	1.70	3.34	124.05[a]
P-value	0.043	0.039	0.230	0.256	0.198	0.185	0.312	0.029
SEM	0.587	0.442	1.034	1.029	1.332	1.662	1.256	1.112
50 : 40 : 10	81.08[b]	21.44[b]	1.39	6.01	2.48	1.40	3.78	113.80[b]
50 : 30 : 20	87.52[a]	27.21[a]	1.42	6.54	2.60	1.63	3.22	126.92[a]
50 : 20 : 30	86.88[a]	26.82[a]	1.46	6.42	2.51	1.59	3.24	125.68[a]
50 : 10 : 40	82.47[b]	21.76[b]	1.33	6.10	2.49	1.31	3.79	115.46[b]
P-value	0.041	0.040	0.305	0.706	0.604	0.239	0.502	0.037
SEM	0.338	0.201	0.420	0.140	0.185	0.190	0.027	1.145
40 : 50 : 10	85.80[a]	26.91[a]	1.38	6.88[a]	2.90	1.49	3.19	125.36[a]
40 : 40 : 20	80.72[ab]	25.95[a]	1.19	6.48[ab]	2.79	1.44	3.11	118.57[a]
40 : 30 : 30	76.18[b]	20.19[b]	1.21	6.01[b]	2.57	1.34	3.77	107.50[b]
40 : 20 : 40	75.40[b]	20.80[b]	1.18	5.89[b]	2.48	1.36	3.63	107.11[b]
40 : 10 : 50	74.12[b]	20.29[b]	1.21	4.94[b]	2.33	1.29	3.65	104.18[b]
P-value	0.031	0.042	0.232	0.038	0.139	0.224	0.128	0.027
SEM	0.524	0.140	0.016	0.054	0.046	0.025	0.018	0.542
30 : 55 : 15	89.45[a]	27.76[a]	1.37	6.46	2.59	1.68	3.22	129.31[a]
30 : 40 : 30	87.58[a]	25.86[a]	1.38	6.98	2.36	1.69	3.39	125.85[a]
30 : 25 : 45	82.80[b]	22.34[b]	1.31	6.33	2.39	1.37	3.71	116.51[b]
30 : 10 : 60	83.53[b]	22.45[b]	1.30	6.24	2.62	1.34	3.72	117.48[b]
P-value	0.039	0.041	0.488	0.706	0.604	0.323	0.502	0.037
SEM	0.502	0.244	0.188	0.140	0.185	0.054	0.027	1.145
20 : 65 : 15	86.92[a]	25.89[a]	1.39	6.45	2.33	1.58	3.36	124.56[a]
20 : 50 : 30	86.28[a]	25.26[a]	1.34	6.33	2.40	1.54	3.42	123.15[a]
20 : 35 : 45	79.60[b]	21.55[b]	1.27	6.21	2.51	1.30	3.69	112.44[b]
20 : 20 : 60	78.93[b]	21.06[b]	1.28	6.19	2.50	1.32	3.75	111.28[b]
P-value	0.040	0.038	0.502	0.504	0.405	0.400	0.441	0.033
SEM	1.011	0.358	0.209	0.210	0.150	0.168	0.150	1.145

A = alfalfa; A/P = acetic acid to propionic acid ratio; C = concentrate; S = soybean pod; SEM = standard error of the means; TVFA = total volatile fatty acids

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

were higher than in groups 30 : 25 : 45 and 30 : 10 : 60, respectively ($P < 0.05$ and $P < 0.01$). Besides, the DMD, OMD and NH_3–N of groups 30 : 55 : 15, 30 : 40 : 30 and 30 : 25 : 45 were higher than in group 30 : 10 : 60, respectively ($P < 0.05$ and $P < 0.01$). In addition, the a value, DMD, OMD, NH_3–N, AA, PA, TVFA and

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b, a + b, $GP_{72\ h}$ of groups 20 : 65 : 15 and 20 : 50 : 30 were higher than in groups 20 : 35 : 45 and 20 : 20 : 60, respectively (P < 0.05 and P < 0.01). However, there were no significant differences in the c value, rumen pH and other VFAs and A/P (acetic to propionic acid ratio) between all groups (P > 0.05).

Associative effects of mixtures of feedstuffs

Table 6 indicates that the AE of $GP_{72\ h}$, DMD, OMD, TVFA, MFAEI and NH_3–N of groups 50 : 50 : 0 and 40 : 60 : 0 were higher than in groups 80 : 20 : 0, 60 : 40 : 0 and 20 : 80 : 0, respectively (P < 0.01 and P < 0.05) and the AE of DMD of group 60 : 40 : 0 was higher than in groups 80 : 20 : 0 and 20 : 80 : 0 (P < 0.01). Moreover, the AE of $GP_{72\ h}$, DMD, OMD, MFAEI and TVFA, NH_3–N of groups 60 : 20 : 20 and 60 : 10 : 30 were higher than in group 60 : 30 : 10, respectively (P < 0.01 and P < 0.05). Additionally, the AE of $GP_{72\ h}$, DMD, OMD, MFAEI and TVFA, NH_3–N of groups 50 : 30 : 20 and 50 : 20 : 30 were higher than in groups 50 : 40 : 10 and 50 : 10 : 40, respectively (P < 0.01 and P < 0.05) and the AE of $GP_{72\ h}$ and DMD of group 50 : 40 : 10 were higher than in group 50 : 10 : 40 (P < 0.01).

When the C : R ratio was 40 : 60, the AE of $GP_{72\ h}$ of group 40 : 50 : 10 was higher than in the other four groups (P < 0.05), and that of group 40 : 40 : 20 was higher than in the other three groups (P < 0.05), also, that of group 40 : 30 : 30 was higher than in groups 40 : 20 : 40 and 40 : 10 : 50 (P < 0.05). In addition, the AE of DMD of groups 40 : 50 : 10 and 40 : 40 : 20 were higher than in the other three groups (P < 0.01) and that of groups 40 : 30 : 30 and 40 : 20 : 40 was higher than in group 40 : 10 : 50 (P < 0.01). Furthermore, the AE of OMD of group 40 : 50 : 10 was higher than in the other four groups (P < 0.01) and that of group 40 : 40 : 20 was higher than in the other three groups (P < 0.01) and that of groups 40 : 30 : 30 and 40 : 20 : 40 was higher than in group 40 : 10 : 50 (P < 0.01). Besides, the AE of TVFA of groups 40 : 50 : 10 and 40 : 40 : 20 were higher than in the other three groups (P < 0.01) and that of group 40 : 30 : 30 was higher than in groups 40 : 20 : 40 and 40 : 10 : 50 (P < 0.01). Moreover, the AE of NH_3–N of group 40 : 50 : 10 was higher than in the other four groups (P < 0.05) and that of group 40 : 40 : 20 was higher than in the other three groups (P < 0.05). Afterwards, the MFAEI of group 40 : 50 : 10 was higher than in groups 40 : 30 : 30, 40 : 20 : 40 and 40 : 10 : 50 (P < 0.01) and that of groups 40 : 40 : 20 and 40 : 30 : 30 was higher than in group 40 : 10 : 50 (P < 0.01).

When the C : R ratio was 30 : 70, the AE of $GP_{72\ h}$, TVFA, NH_3–N and MFAEI of groups 30 : 55 : 15 and 30 : 40 : 30 were higher than those of groups 30 : 25 : 45 and 30 : 10 : 60 (P < 0.01) and the AE of TVFA and NH_3–N of group 30 : 25 : 45 was higher than those of group 30 : 10 : 60 (P < 0.01). In addition, the AE of DMD of groups 30 : 55 : 15 and 30 : 40 : 30 were higher than that of group 30 : 10 : 60 (P < 0.01). Besides, the AE of OMD of groups 30 : 55 : 15 and 30 : 40 : 30 were higher than those of groups 30 : 25 : 45 and 30 : 10 : 60 (P < 0.05) and the AE of OMD of groups 30 : 25 : 45 was higher than that of group 30 : 10 : 60 (P < 0.05).

When the C : R ratio was 20 : 80, the AE of $GP_{72\ h}$, DMD, OMD, TVFA, NH_3–N and MFAEI of groups 20 : 65 : 15 and 20 : 50 : 30 were higher than those of groups 20 : 35 : 45 and 20 : 20 : 60 (P < 0.01) and the AE of NH_3–N of group 20 : 35 : 45 was higher than that of group 20 : 20 : 60 (P < 0.01).

DISCUSSION

Gas production parameters of single feed

In this study, the concentrate (a = –9.8) had a longer lag time (LT) for GP than alfalfa (a = –3.1) while soybean pod (a = 10.4) had no LT of GP (Table 3). Aye Sandar et al. (2012) reported that LT of GP of corn was longer than in barley. In this study, the concentrate was composed of 84.37% corn. Accordingly, the concentrate had a longer LT which was consistent with Aye Sandar et al. (2012). Moreover, the a, b, a + b and $GP_{72\ h}$ of soybean pod were obviously higher than those in concentrate and alfalfa (Table 3), which demonstrated that soybean pod had superior performance of production gases to a great extent.

Gas production, DMD, OMD and rumen pH of mixtures of feedstuff

Gas production is a crucial indicator for forecasting feed digestibility of ruminants. The designs of mixture and single feed are quite useful methods to identify and research AE of mixed feed by an *in vitro* GP method. As an example, forage tree leaves and

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Table 6. Single-factor associative effects indices (SFAEI) and multiple-factor associative effects indices (MFAEI) after fermentation for 72 h when soybean pod was incubated with alfalfa and concentrate *in vitro* (%)

C:S:A	SFAEI					MFAEI
	AE of $GP_{72\,h}$	AE of DMD	AE of OMD	AE of TVFA	AE of NH_3–N	
80:20:0	73.99^{B}	16.06^{C}	19.58^{B}	15.01^{B}	11.34^{b}	135.98^{B}
60:40:0	78.58^{B}	25.54^{B}	21.02^{B}	19.01^{B}	12.09^{b}	156.24^{B}
50:50:0	101.09^{A}	41.87^{A}	48.33^{A}	46.40^{A}	30.19^{a}	267.88^{A}
40:60:0	99.52^{A}	41.05^{A}	46.90^{A}	44.89^{A}	28.65^{a}	261.01^{A}
20:80:0	75.44^{B}	14.00^{C}	20.55^{B}	16.72^{B}	11.22^{b}	137.93^{B}
P-value	0.003	0.001	0.002	0.003	0.026	< 0.001
SEM	1.544	1.352	1.387	1.559	1.430	1.408
60:30:10	78.90^{B}	20.11^{B}	22.12^{B}	18.08^{b}	12.02^{b}	151.23^{B}
60:20:20	103.45^{A}	42.86^{A}	49.12^{A}	47.70^{a}	29.11^{a}	272.24^{A}
60:10:30	100.23^{A}	43.19^{A}	47.97^{A}	46.55^{a}	28.54^{a}	266.48^{A}
P-value	0.002	0.005	0.004	0.019	0.016	< 0.001
SEM	1.124	0.986	1.336	1.550	1.450	1.660
50:40:10	82.88^{B}	29.38^{B}	23.86^{B}	20.17^{b}	13.68^{b}	169.97^{B}
50:30:20	107.22^{A}	44.06^{A}	51.49^{A}	50.47^{a}	31.55^{a}	284.79^{A}
50:20:30	102.94^{A}	43.67^{A}	50.56^{A}	49.28^{a}	29.88^{a}	276.33^{A}
50:10:40	61.22^{C}	13.41^{C}	21.77^{B}	18.55^{b}	10.01^{b}	124.96^{B}
P-value	< 0.001	0.003	0.003	0.014	0.021	< 0.001
SEM	1.880	1.005	1.189	1.224	1.340	1.802
40:50:10	112.45^{a}	46.66^{A}	53.54^{A}	55.89^{A}	32.55^{a}	301.09^{A}
40:40:20	103.03^{b}	44.74^{A}	41.58^{B}	52.68^{A}	21.06^{b}	263.09^{AB}
40:30:30	79.94^{c}	16.90^{B}	24.81^{C}	35.40^{B}	11.33^{c}	168.38^{B}
40:20:40	71.60^{d}	15.48^{B}	23.49^{C}	18.21^{C}	8.84^{c}	137.62^{BC}
40:10:50	69.05^{d}	4.95^{C}	13.05^{D}	17.49^{C}	9.02^{c}	113.56^{C}
P-value	0.013	0.002	0.001	0.002	0.034	< 0.001
SEM	1.712	1.044	1.348	1.544	1.644	2.012
30:55:15	135.79^{A}	43.95^{A}	56.77^{a}	58.02^{A}	37.55^{A}	332.08^{A}
30:40:30	143.15^{A}	44.04^{A}	55.89^{a}	59.54^{A}	38.89^{A}	341.51^{A}
30:25:45	94.91^{B}	26.38^{AB}	43.15^{b}	38.33^{B}	20.30^{B}	223.07^{B}
30:10:60	92.48^{B}	9.55^{B}	23.22^{c}	16.97^{C}	8.80^{C}	151.02^{B}
P-value	0.001	0.006	0.021	0.005	0.002	< 0.001
SEM	2.282	1.568	1.140	0.548	1.152	1.774
20:65:15	115.66^{A}	22.88^{A}	54.90^{A}	47.17^{A}	35.84^{A}	276.45^{A}
20:50:30	110.78^{A}	21.56^{A}	53.82^{A}	46.59^{A}	36.12^{A}	268.87^{A}
20:35:45	82.45^{B}	10.72^{B}	25.19^{B}	23.42^{B}	21.55^{B}	163.33^{B}
20:20:60	81.48^{B}	10.95^{B}	24.64^{B}	21.07^{B}	9.05^{C}	147.19^{B}
P-value	0.002	0.006	0.004	0.005	0.001	< 0.001
SEM	1.404	1.402	1.252	0.548	1.044	1.323

A = alfalfa; AE = associative effects; C = concentrate; DMD = dry matter digestibility; $GP_{72\,h}$ = GP at 72 h; NH_3–N = ammonia nitrogen; OMD = organic matter digestibility; S = soybean pod; SEM = standard error of the means; TVFA = total volatile fatty acids

$^{a–d}$Means within a column differ at $P < 0.05$; $^{A–C}$means within a column differ at $P < 0.01$

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concentrate were mixed which generated a positive AE on GP (Sandoval-Castro et al. 2002). Also, a positive AE on GP was produced when wheat straw was incubated with alfalfa (Tang et al. 2008). In addition, Haddad (2000) reported that the advantages of combined application with legume forage are the result of many synthetic factors which manifested that a positive AE is beneficial to straw utilization. Moreover, milk thistle and pure cellulose (two slowly fermentable fibres) were mixed as 25 : 75 or 75 : 25 with tomato peels without seeds, citrus pulp, and pectin (three rapidly fermentable fibres) *in vitro*, respectively, and GP of each combination was increased (Maccarana et al. 2013). There is a considerable amount of literature on AEs between forage and concentrate.

In this study, GP indices (a, b, a + b, $GP_{72\ h}$) and fermentation parameters (DMD, OMD) of groups 50 : 50 : 0, 40 : 60 : 0, 60 : 20 : 20, 60 : 10 : 30, 50 : 30 : 20, 50 : 20 : 30, 40 : 50 : 10, 30 : 55 : 15, 30 : 40 : 30, 20 : 65 : 15, 20 : 50 : 30 were higher than in the other groups in the same C : R ratio group (Table 4). Groups 30 : 55 : 15 and 30 : 40 : 30 were optimal among the above-mentioned 11 combinations as to the values of a, b, a + b, $GP_{72\ h}$, DMD, OMD, NH_3–N. Additionally, when alfalfa was not supplemented and the concentrate/soybean pod ratio was 50 : 50 or 40 : 60, the GP and fermentation indices were the best. When alfalfa was added and concentrate was 60 or 50 and roughage (soybean pod + alfalfa) was 40 or 50, groups 60 : 10 : 30 and 50 : 20 : 30 were optimal, followed by groups 60 : 20 : 20 and 50 : 30 : 20, which indicated that when concentrate was high enough, the requirements for soybean pod decreased (10 or 20). However, when concentrate continued to be reduced to 40, 30 or 20, it was found that the requirements for soybean pod increased to 50, 55 or 65. The reason for the results is that GP performance of soybean pod is superior to concentrate and alfalfa, and soybean pod can replace a part of concentrate to a certain extent.

The DMD and OMD have a strong positive correlation with GP, fermentation activity of microorganisms in rumen and feed digestibility (Menke and Steingass 1988). Also, alfalfa has a high effective degradation rate and suitable C : R ratio, which is beneficial to the strong activity of microorganisms (Gunun et al. 2013). It was obviously shown that soybean pod had better GP performance in this study (Table 3). Therefore, low concentrate (30), low alfalfa (15) and high soybean pod (55) have the best GP performance and fermentation characteristics. It should be the result of associative effects among the three feedstuffs.

It was demonstrated that the utilization of low-quality roughage is improved with alfalfa supplementation (Mosi and Butterworth 1985). Besides, a multitude of studies reported that poor-quality feeds incubated with alfalfa created AE on digestibility, intake and utilization (Haddad 2000). In addition, Zhang et al. (2010) studied corn starch supplemented to a rice straw-based diet and found that high levels of corn starch reduced cellulolytic bacteria populations and forage digestibility in lambs while appropriate levels of corn starch could effectively promote lamb growth. Additionally, positive AEs were generated on GP, DMD and OMD when spring pasture was incubated with corn and when autumn pasture was incubated with either corn or barley (Aye Sandar et al. 2012). Besides, Djamila and Rabah (2016) reported that the mixture of date palm leaves, *Aristida pungens* and *Astragalus gombiformis* was incubated *in vitro* and they found that with the increasing proportion of date palm leaves, the OMD was reduced. Moreover, AE was reported to occur when red clover and kikuyu grass silage were combined which contained proteolysis reduction and synergism (Guzatti et al. 2017). The above-mentioned studies demonstrated that the AE widely existed between different types of feeds such as between poor-quality feeds and alfalfa, between roughage and grain feeds, also, among different forage grasses, etc. The scope of this research lied in the AEs between poor-quality feeds (soybean pod) and alfalfa, between roughage (soybean pod and alfalfa) and grain feeds (concentrate), moreover, between soybean pod and concentrate.

The c value and rumen pH of all groups did not show a significant difference except the c values of groups 50 : 50 : 0, 40 : 60 : 0, 40 : 50 : 10, 40 : 40 : 20 and 40 : 30 : 30 that were higher than in the other groups (Table 4) which further proved that soybean pods could replace part of the concentrate to some extent and speed up GP. Additionally, rumen pH is an indispensable indicator to reflect rumen fermentation and environment changes. The normal rumen pH is about 6–7. When more VFA are generated than they are assimilated from the rumen, it leads to a reduction in rumen pH (Stritzler et al. 1998). In this experiment, the groups with higher GP tended to have lower pH, which was due to the higher easily fermentable substrates (Abdelhadi et al. 2005).

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Ammonia nitrogen and VFA of mixtures of feedstuffs

Ammonia nitrogen is a paramount indicator of reflecting feed protein degradation, rumen nitrogen metabolism and microbial protein synthesis. To ensure the normal synthesis of rumen microbial protein, it is indispensable to keep suitable NH_3–N. It was suggested that the appropriate range of ammonia nitrogen was 6.3–27.5 mg/dl (Calsamiglia et al. 2002). In the present experiment, the NH_3–N values were from 8.88 to 22.63 mg/dl that were all in the normal range (Table 4). The groups 50 : 50 : 0, 40 : 60 : 0, 60 : 20 : 20, 60 : 10 : 30, 50 : 30 : 20, 50 : 20 : 30, 40 : 50 : 10, 30 : 55 : 15, 30 : 40 : 30, 20 : 65 : 15, 20 : 50 : 30 were higher than the other groups in the same C : R ratio group (Table 3), especially the groups 30 : 55 : 15, 30 : 40 : 30 were the highest among them, and these 11 groups may accelerate the synchronous generation of energy and ammonia and the synthesis of microorganism protein in the rumen (Zhou et al. 2015).

The final products of rumen fermentation are VFA, NH_3–N, methane, carbon dioxide and other gases. The VFA supply most of energy sources for the maintenance and growth requirements of rumen microflora. In this study, AC, PC and TVFA of groups 50 : 50 : 0, 40 : 60 : 0, 60 : 20 : 20, 60 : 10 : 30, 50 : 30 : 20, 50 : 20 : 30, 40 : 50 : 10, 30 : 55 : 15, 30 : 40 : 30, 20 : 65 : 15, 20 : 50 : 30 were higher than in the other groups (Table 5). Among these 11 groups, the groups 60 : 20 : 20, 60 : 10 : 30, 50 : 30 : 20, 50 : 20 : 30, 30 : 55 : 15, 30 : 40 : 30, 20 : 65 : 15, 20 : 50 : 30 were better than the other three groups, also, the group 30 : 55 : 15 was optimal among the 11 groups mentioned above as for AA, PA and TVFA. The change trend of VFA is basically consistent with GP and fermentation parameters, so that AA, PA and TVFA of the group of low concentrate (30), low alfalfa (15) and high soybean pod (55) were optimal.

Digestible carbohydrate fermentation can generate VFA, and there is a positive correlation between acetic acid and $GP_{72\ h}$. The A/P ratio indicates the type of rumen fermentation to some extent, and A/P values in this study were all higher than three (Table 5), which inferred that the type of fermentation of all treatment groups was the AA fermentation type which is advantageous to promote the milk fat rate of ruminants. Taken together, the results demonstrated that the group 30 : 55 : 15 was optimal for rumen fermentation to produce VFA. It was reported that compared with soybean hulls, supplemental corn and tall fescue hay led to greater negative AE on OMD and lower NH_3–N (Fieser and Vanzant 2004). This report also indicated that soybean hulls or pods supplemented to the diets of ruminants were highly beneficial to promote AE of diets which was consistent with the present experiment. In addition, in this study, the amount of AA generated in all feed combinations was higher than that of PA (Table 5), which is due to the absorption rate of VFA by ruminants that is in the order of BA, PA and AA.

Associative effects of mixtures of feedstuffs

Indicators of AE include nutrient digestibility, utilization rate, animal growth performance and feed intake. Among these, energy or digestibility is the most commonly used index to measure AE. Gas production *in vitro* is highly correlated with OMD. However, it may be inaccurate to measure the nutritional value of feed and evaluate the AE by means of GP alone or one of any other indices because the mechanism of AE is quite complex. In addition, *in vitro* GP of feed is positively correlated with carbohydrate digestion and negatively related with feed protein. Accordingly, if the nutritive value of feed is measured by GP alone, it may be inaccurate, and the feed or combination with low GP and high protein production may be eliminated. Consequently, the accurate assessment of feed nutritive value should adopt a comprehensive index or a mathematical model. In the present study, the AE of soybean pod, alfalfa and concentrate was assessed by combining the $GP_{72\ h}$, DMD, OMD, VFA and NH_3–N as a comprehensive index.

Niderkorn et al. (2019) mixed rye grass and chicory in equal proportions which produced a synergy on voluntary intake and an improved N use efficiency likely due to complementarity in chemical composition, increased motivation to eat and faster ruminal particle breakdown. The effects of a mixture of aqueous and ethanol extracts of *Sapindus mukorossi* fruits, acetone extract of *Ficus bengalensis* leaves and *Eucalyptus globulus* essential oils on reducing *in vitro* methane production and modulating rumen fermentation were more obvious at quite low dose levels than their individual inclusion, thus, they showed positive AE (Singh et al. 2019). However, Grubjesic et al. (2019) suggested that in most cases, pelleting led to the greater effective ruminal deg-

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radation of crude protein and starch in some compound feeds than in compound feeds in mash form, probably due to undegraded finer feed particles which leave the bags. Afterwards, Grubjesic et al. (2020) concluded that when formulating compound feeds for cattle, single feed data for $GP_{24\ h}$, OMD, metabolizable energy (ME) and utilizable CP in the duodenum are additive, while those for intestinal digestibility of rumen undegraded protein are not, therefore, using CP fractions did not reliably predict the *in situ* ruminal CP degradation of compound feeds. The above four reports showed once again that the AE between feeds is widespread, but the mechanism of the AE between feeds is very complex. Some indicators between a single feed and its mixture are additive, some are not, the latter should be the result of the AE between feeds. This is in line with the result of the present study. The main aim of this study was to evaluate the additivity of single feeds in compound feeds made thereof.

It was found that the SFAEI and MFAEI of groups 50 : 50 : 0, 40 : 60 : 0, 60 : 20 : 20, 60 : 10 : 30, 50 : 30 : 20, 50 : 20 : 30, 40 : 50 : 10, 30 : 55 : 15, 30 : 40 : 30, 20 : 65 : 15, 20 : 50 : 30 were higher than in the other groups under the same C : R ratio group (Table 6). Especially, the MFAEI of groups 30 : 55 : 15 (332.08%) and 30 : 40 : 30 (341.51%) were the best among the 11 groups, followed by group 40 : 50 : 10 (301.09%), and the MFAEI of groups 50 : 30 : 20, 50 : 20 : 30, 20 : 65 : 15, 20 : 50 : 30, 60 : 20 : 20, 60 : 10 : 30, 50 : 50 : 0, 40 : 60 : 0 decreased in turn. It was demonstrated that when soybean pod was added to the diets, the low C : R ratio (30 : 70 or 40 : 60) was optimal compared to groups of either higher C : R ratio (60 : 40, 50 : 50) or lower C : R ratio (20 : 80) or groups that were not added alfalfa. The MFAEI of the 11 groups mentioned above were optimal. This may be due to the mutual supplementing of nutrients in these groups and increasing the fermentation rate of substrate promotes the feed digestibility. These groups had improved GP characteristics, rumen fermentation level and feed utilization after 72 h fermentation *in vitro* (Table 6).

CONCLUSION

Optimal SFAEI of $GP_{72\ h}$, DMD, OMD, NH_3–N and TVFA and MFAEI were obtained when the concentrate/soybean pod/alfalfa ratios were 50 : 50 : 0, 40 : 60 : 0, 60 : 20 : 20, 60 : 10 : 30, 50 : 30 : 20, 50 : 20 : 30, 40 : 50 : 10, 30 : 55 : 15, 30 : 40 : 30, 20 : 65 : 15, 20 : 50 : 30 under the respective C : R ratio group. Of all the 11 groups, the groups 30 : 55 : 15 and 30 : 40 : 30 were the best therein. Furthermore, *in vivo* studies on supplementation of a mixture should be conducted to find out its effect on rumen fermentation, animal health and animal performance, for adopting the soybean pod as a feedstuff for ruminants.

Conflict of interest

The authors declare no conflict of interest.

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