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Evaluation of Associative Effects of Hybrid Penisetum and Chemically Treated Spent Mushroom (*Hypsizygus marmorens*) Substrate

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Abstract: Using the artificial rumen fermentation model, this project studies the associative effects of chemically treated spent mushroom (*Hypsizygus marmorens*) substrate and hybrid penisetum, the combination ratios of which were 0:100 (SMS0), 15:85 (SMS15), 30:70 (SMS30), 45:55 (SMS45) and 60:40 (SMS60). In addition, the *in vitro* Gas production (GAS), pH, *In Vitro* Organic Matter Digestibility (IVOMD), Microbial Crude Protein (MCP) and Total Volatile Fatty Acids (TVFA) generation at the 6, 12, 24, 48 and 72 h and associative effects composite index (MFAEI) were determined. The results showed the following: the MFAEI of SMS15 and SMS30 were positive and compared with the control, their *in vitro* gas production, MCP and TVFA increased by 3.22% ($p>0.05$), 27.05% ($p<0.01$), 10.68% ($p<0.05$) and 1.74% ($p>0.05$), 12.33% ($p<0.05$), 7.75% ($p>0.05$), respectively. There was a tendency of increasing IVOMD but the difference was not significant. The MFAEI of the remaining portfolios were negative. The results indicate that the optimal ratio of chemically treated spent mushroom (*Hypsizygus marmorens*) substrate combined with hybrid penisetum could significantly improve the overall level of fermentation of the mixed forage.

Key words: Spent mushroom (*Hypsizygus marmorens*) substrate, hybrid penisetum, artificial rumen fermentation, associative effects, microbial crude protein

INTRODUCTION

An interaction exists among feed. Forbes first pointed out the non-additive or associative effects of mixed feed in 1931, after which the associative effects of feed, especially the associative effects of crude feed have often been the focus of research (Niderkorn and Baumont, 2009; Robinson *et al*., 2001; Metzler-Zebeli *et al*., 2012; Cho *et al*., 2012). Gas production has been shown to be highly correlated with the nutrient digestibility value of the nutritive material (Campos *et al*., 2004) and *in vitro* gas production technology is simple thus the *in vitro* Gas Production Method has mostly been used to investigate the associative effects of feed. Wang (2003) investigated the associative effects of crude and mixed feed in 2003 for the first time using the comprehensive index (MFAEI) by means of the *in vitro* method. The index included the Gas production (GAS), *In Vitro* Degradation rate of Organic Matter (IVOMD), Microbial Protein Yield (MCP), Total Volatile Fatty Acid production (TVFA) and a number of indicators. Subsequently, many researchers have investigated the associative effects of different crude feeds by the *In Vitro* Gas Production Method and MFAEI and have made much progress (Cui *et al*., 2011; Yu *et al*., 2012). Fungus chaff is the medium produced after the cultivation of edible fungi and contains a great deal of edible fungus mycelium and abundant protein and other nutrients thus it is considered as a type of non-conventional feed resource (Wei *et al*., 2010). China is a major producer of edible fungus. According to the statistics of the Chinese Edible Fungi Association, the total output of edible fungi in China in 2011 reached 2.57×10^7 t. If the biological efficiency of edible fungus is 40%, then the total output of fungus chaff is about 6.42×10^7 t. At present, research regarding the associative effects of fungus chaff and pennisetum hybrid has not yet been reported. The present study investigated the associative effects of spent mushroom (*Hypsizygus marmorens*) substrate and pennisetum hybrid using artificial rumen fermentation technology to explore the reasonable collocation of the spent mushroom (*Hypsizygus marmorens*) substrate and pennisetum hybrid, thereby providing a theory basis for the scientific development of fungus bran feed resources.

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Table 1: Conventional ingredients of spent mushroom (*Hypsizygus marmorens*) substrate and hybrid penisetum (%)

Coarse material	DM	CP	EE	Ash	NDF	ADF	Ca	P
Chemical processing of *Hypsizygus marmoreus* fungus chaff (SMS)	33.82	11.60	2.10	11.98	63.39	55.85	2.01	0.34
Hybrid Pennisetum (HP)	24.82	10.22	2.76	8.57	67.35	46.38	0.42	0.23

MATERIALS AND METHODS

Experimental materials: The spent mushroom (*Hypsizygus marmorens*) substrate was provided by West Agriculture Co., Ltd. of Fujian Province. The fungus bran was fresh fungus chaff harvested once and was without mildew. The culture medium consisted of cottonseed hull (56-61%), corn cob (20%), wheat bran (12%), sawdust (5-10%), gypsum (1%) and lime (1%). The spent mushroom (*Hypsizygus marmorens*) substrate was treated with 5% NaOH+5% urea chemical treatment for 50 days at room temperature. *Pennisetum americanum* x *P. purpureum* was collected from the experimental base of Fujian Agriculture and Forestry University. The samples were made into dried samples which were then ground and treated with a 40 mesh sieve for further use.

Chemical composition analysis: The measurement of the Dried Materials (DM), Crude Protein (CP), Crude fat (EE), Ash (Ash), Ca and P of the spent mushroom (*Hypsizygus marmorens*) substrate and pennisetum hybrid were determined using the methods described by Yang (1993) while the determination of the Neutral Detergent Fiber (NDF) and Acid Detergent Fiber (ADF) was conducted by the method proposed by Van Soest *et al.* (1991). The routine nutritional ingredient contents are shown in Table 1.

Experimental design: A single factor experimental design was used in the present study. The artificial rumen fermentation substrate consisted of concentrate (0.12 g) and roughage (0.28 g) which were composed of the spent mushroom (*Hypsizygus marmorens*) substrate(5% NaOH +5% urea treated) and pennisetum hybrid. The experiment included five different treatments, namely the SMS0 group, SMS15 group, SMS30 group, SMS45 group and SMS60 group with the respective ratios between the spent mushroom (*Hypsizygus marmorens*) substrate and pennisetum hybrid being 0:100, 15:75, 30:70, 45:55 and 60:40. Each treatment was repeated three times. The gas production, pH, IDVOM, MCP and TVFA yield were measured in each group at 6, 12, 24, 48 and 72 h. The MFAEI was calculated and the associative effects of the chemically treated spent mushroom (*Hypsizygus marmorens*) substrate and pennisetum hybrid were evaluated.

Experimental animal feeding and management: Two Chinese Holstein rumen fistulated cows with body weights of 600-650 kg in late lactation were used to collect the rumen fluid. The roughage for the experimental cows was the hybrid pennisetum, carring out free choice feeding. Each cow was fed 6 kg concentrate and the composition and nutritional levels are shown in Table 2. The experimental cows were fed using tethered feeding, two times a day at 7:30 and 17:30 with free drinking.

Table 2: Composition and nutrient levels of concentrate (%)

Items	Values	Nutrient levels	Values
Corn	40.0	CP	18.20
Rapeseed meal	15.0	Ca	0.72
Wheat bran	22.0	P	0.55
DDGS	21.0	CF	4.00
$CaHPO_4$	1.0	EE	1.80
NaCl	0.5	-	-
Complex vitamin	0.5	-	-
Total	100.0	-	1.50

Design of gas generating device

Devices: The main subject was a constant temperature water bath shaker. A glass bottle with a volume of 100 mL was used as the culture bottle. The bottle mouth was equipped with a rubber stopper with a medical plastic three-way valve. The three-way valve was linked to single-use syringe with a volume of 30 mL and scale of 1 mL. The syringe was washed, then cleaned and dried before use. A small amount of vaseline was evenly coated around the piston cylinder before use to reduce friction and prevent leakage.

Buffer preparation: The buffer was prepared according to the method (Lu and Xie, 1991).

Rumen fluid collection and artificial rumen culture: A 0.4 g sample was weighed accurately and placed into the culture flask. The prepared buffer was added to the culture bottle 1 h before the experiment with a 40 mL buffer in each culture bottle. CO_2 was continuously introduced into the culture flask until the buffer became colorless with resazurin as an indicator. A rubber plug equipped with a three-way plastic valve was used to cover the bottle. The culture flask with buffer was placed on a thermostatic shaker until it was heated to 39°C for future use. Adequate rumen fluid was collected from the two ruminal fluid donor cows 2 h before feeding in the morning. The rumen fluid filtrated by the four layers of gauze was placed in the 39°C vacuum insulation cup filled with CO_2. Then, the rumen fluid was quickly distributed into the culture bottles with 20 mL of rumen fluid in each culture bottle. The culture bottle with rumen fluid was switched to syringe. Then, the oscillation switch was opened to begin the culture process.

Determination of indicators and analysis method: The gas productions were recorded at the time points of 6, 12, 24, 48 and 72 h and in the meantime the culture medium pH value of the culture flask was measured. The medium was transferred into a 100 mL large centrifugal tube without loss and centrifugation was conducted at 4000 r min^{-1} for 15 min. Then, the supernatant sample was prepared for the analysis of MCP and VFA. After centrifugation, the precipitation was transferred into a 30 mL crucible and dried at 105°C to a constant weight to determine the content of the dried materials. Then, the dried materials were transferred to a muffle furnace under 550-600°C ignition until reaching a constant weight to determine crude ash and the IVOMD was calculated. According to the experimental principles and methods, MCP was determined using the Purine Method of Makkar and Becker (1999). VFA was examined by the external standard method using Agilent 6890N type gas chromatograph (Sui, 2009). The gas production value at each time point in the different treatment groups was used in the gas production model formula of Orskov and McDonald (1979) in, then the gas production dynamic parameters were calculated.

Calculation of the associative effect value: The associative effects of the present study were evaluated according to the MFAEI described by Lu and Xie (1991). MFAEI was the addition value of each SFAEI value. The SFAEI equation was as follows:

$$SFAEI = \frac{\sum_{n=1}^{n} A_2 - A_1/2}{A_3}$$

Where:

A_1 = The value of each single index n at the different fermentation time points in the control group before combination

A_2 = The value of each single index n at the different fermentation time points in the experimental group after combination

A_3 = The average value of the total value of A_2 at n time points

n = The total number of the sampling time points

Data processing: The experimental data were analyzed by the one-way ANOVA variance analysis using the Statistical Software Program SPSS17.0. Duncan's multiple comparisons were used in the present multiple comparisons. The results are shown as mean±standard deviation.

RESULTS

Effects of different combination ratios on the pH value of the artificial rumen fermentation liquid: As demonstrated in Table 3, the pH values at each time point in the SMS0 group were the lowest. Except for those at 24 and 72 h, the pH values in the SMS60 group were significantly higher than those in the SMS0 group ($p<0.05$). There was no significant difference in the pH values in the remaining combined groups ($p>0.05$).

Effects of different combination ratios on artificial rumen fermentation gas production *in vitro* and gas production parameters: It can be analyzed from Table 4 that the *in vitro* fermentation cumulative gas production in each experimental group exhibited an increasing trend with the increase of the artificial rumen fermentation time in which there was a rapid fermentation process within the period of 6-48 h and the gas production rate slowed down after 48 h. There was no dramatic difference in the gas production in each group at the time point of 6 h ($p>0.05$). Except for those at 6 and 12 h, the cumulative gas production at each time point in the SMS15 and SMS30 groups were higher than those of the control group but the difference was not significant ($p>0.05$); the cumulative

Table 3: pH medium for mixture of spent mushroom (*Hypsizygus marmorens*) substrate and hybrid penisetum at different times in artificial rumen fermentation

Groups	6 h	12 h	24 h	48 h	72 h
SMS0	6.58±0.01^{b}	6.54±0.03^{b}	6.52±0.03	6.43±0.04^{c}	6.50±0.06
SMS15	6.60±0.03^{ab}	6.54±0.03^{b}	6.54±0.04	6.46±0.06^{bc}	6.53±0.04
SMS30	6.61±0.02^{ab}	6.56±0.01^{b}	6.57±0.04	6.50±0.04^{abc}	6.55±0.03
SMS45	6.63±0.03^{ab}	6.58±0.04^{ab}	6.57±0.03	6.54±0.04^{ab}	6.57±0.06
SMS60	6.65±0.04^{a}	6.63±0.04^{a}	6.59±0.04	6.56±0.01^{a}	6.58±0.03

Means in the same row with different lowercase letters are significantly different ($p<0.05$), means in the same row with different uppercase letters are very significantly different ($p<0.01$) and means unlabeled indicated or in the same row with the same letters show no significant difference ($p>0.05$)

Table 4: Gas production for mixture of spent mushroom (*Hypsizygus marmorens*) substrate and hybrid penisetum at different times in artificial rumen fermentation (mL)

Groups	6 h	12 h	24 h	48 h	72 h
SMS0	16.92±1.58	36.85±1.83^{aA}	53.35±2.37^{aA}	73.10±2.12^{aA}	77.65±2.05^{aA}
SMS15	16.54±2.43	33.76±1.62^{aA}	55.70±3.23^{aA}	75.20±3.35^{aA}	80.15±2.26^{aA}
SMS30	16.53±1.41	33.03±2.53^{aA}	55.70±3.45^{aA}	74.85±2.03^{aA}	79.00±2.34^{aA}
SMS45	15.78±1.72	28.80±2.75^{bB}	47.23±2.13^{bAB}	68.10±2.36^{bB}	69.00±1.22^{bB}
SMS60	15.26±1.46	27.85±2.06^{bB}	45.27±2.39^{bB}	66.13±4.43^{bB}	67.35±2.08^{bB}

Means in the same row with different lowercase letters are significantly different ($p<0.05$), means in the same row with different uppercase letters are very significantly different ($p<0.01$) and means unlabeled indicated or in the same row with the same letters show no significant difference ($p>0.05$)

Table 5: Gas parameters for mixture of spent mushroom (*Hypsizygus marmorens*) substrate and hybrid penisetum at different times in artificial rumen fermentation

Gas production parameters	SMS0	SMS15	SMS30	SMS45	SMS60
a (mL)	-4.20±1.25^{ab}	-6.79±1.70^{bc}	-7.41±2.50^{c}	-3.27±0.63^{a}	-3.10±0.93^{a}
b (mL)	83.92±0.38^{bB}	89.62±1.02^{aA}	89.15±0.73^{aA}	77.78±1.17^{cC}	75.01±0.79^{dD}
a+b (mL)	79.72±1.59^{aA}	82.84±2.60^{aA}	81.74±1.83^{aA}	74.51±1.80^{bB}	71.91±1.72^{bB}
c (mL h^{-1})	0.051±0.00^{a}	0.050±0.01^{a}	0.051±0.01^{a}	0.045±0.00^{b}	0.045±0.00^{b}

^{a}The rapid sequence of gas production; ^{b}The slow sequence of gas production; $^{a+b}$The potential sequence of gas production and ^{c}The rate of gas production

Table 6: OM digesting rate for mixture of spent mushroom (*Hypsizygus marmorens*) substrate and hybrid penisetum at different times in artificial rumen fermentation (%)

Groups	6 h	12 h	24 h	48 h	72 h
SMS0	33.78±1.26^{aA}	39.65±1.19^{aA}	49.35±0.79^{aA}	59.60±0.84^{aA}	63.09±1.31^{aA}
SMS15	33.17±1.00^{aA}	37.07±1.42^{bA}	47.58±0.94^{aA}	57.96±0.98^{abAB}	62.29±1.02^{aA}
SMS30	32.77±1.04^{abA}	36.72±0.96^{abA}	46.87±1.19 ^{aAB}	56.69±0.96^{bcAB}	61.46±1.41^{aAB}
SMS45	30.61±1.43^{bB}	31.97±1.31^{cB}	43.88±1.06^{bBC}	55.32±1.30^{cB}	58.46±1.08^{bBC}
SMS60	27.64±0.82^{cB}	31.53±1.09^{cB}	40.66±0.82^{cC}	52.14±1.35^{dC}	56.02±1.30^{cC}

Table 7: MCP generation for mixture of spent mushroom (*Hypsizygus marmorens*) substrate and hybrid penisetum at different times in artificial rumen fermentation (mg mL^{-1})

Groups	6 h	12 h	24 h	48 h	72 h
SMS0	2.68±0.25^{aA}	2.97±0.21^{aA}	2.93±0.26	3.24±0.15^{bB}	2.92±0.20^{cB}
SMS15	2.63±0.31^{aA}	2.70±0.12^{abA}	2.97±0.23	3.75±0.13^{aA}	3.71±0.18^{aA}
SMS30	2.32±0.14^{aAB}	2.48±0.28^{bAB}	2.98±0.14	3.92±0.13^{aA}	3.28±0.16^{bB}
SMS45	1.73±0.16^{bBC}	2.04±0.19^{cBC}	2.92±0.10	3.09±0.27^{bB}	2.20±0.09^{dC}
SMS60	1.42±0.25^{bC}	1.82±0.10^{cB}	2.58±0.25	3.02±0.15^{bB}	2.17±0.13^{dC}

Means in the same row with different lowercase letters are significantly different ($p<0.05$), means in the same row with different uppercase letters are very significantly different ($p<0.01$) and means unlabeled indicated or in the same row with the same letters show no significant difference ($p>0.05$)

gas productions at each time point in the SMS45 and SMS60 groups were either significantly ($p<0.05$) or very significantly ($p<0.01$) lower than those of the SMS0 group; the sequences of the cumulative gas production at 72 h were as follows, from high to low: SMS15>SMS30> SMS0>SMS45>SMS60.

Table 5 illustrates that the rapid gas production values in part a were all negative values indicating that there was a delay time of gas production in the early stages. Part b is the slow gas production sequence and the gas productions in the SMS15 and SMS30 groups were the highest and were significantly higher than those of the other groups ($p<0.01$); the orders of gas production in the remaining groups were as follows: SMS0>SMS45> SMS60 and the differences among these groups were very significant ($p<0.01$). The potential gas production was closely related to the *in vivo* digestibility (a+b). The potential gas production values were the highest in the SMS15 and SMS30 groups and the potential gas production value was higher in the SM50 group. There was no significant difference among the gas production values of these three groups ($p>0.05$) which were much higher than those of the SMS45 and SMS60 groups ($p<0.01$). In the gas production rate, the differences of the three groups (SMS0, SMS15 and SMS30) were not significant ($p>0.05$) but the gas production rates of the three groups were significantly higher than those of the SMS45 and SMS60 groups ($p<0.05$).

Effects of different combination ratios on the artificial rumen fermentation IVOMD: As demonstrated in Table 6, the IVOMD increased with the rise of the artificial rumen fermentation time. The degradation rate became slow after 48 h, the trend of which was the same as that of the gas production. The ratio of the spent mushroom (*Hypsizygus marmorens*) substrate in the experimental group increased with the increase of the chemical treatment while IVOMD decreased. The difference of the ratio of the spent mushroom (*Hypsizygus marmorens*) substrate between the SMS0 and SMS15 groups was not significant ($p>0.05$); the ratio of the spent mushroom (*Hypsizygus marmorens*) substrate at 12 and 48 h in the SMS30 group was dramatically lower than that in the SMS0 group ($p<0.05$); the IVOMD at each time point in the SMS45 and SMS60 groups were extremely significantly lower than those of the SMS0 group ($p<0.01$).

Effects of different combination ratios on ruminal fermentation production of MCP: Table 7 shows that the MCP productions in the SMS60 and SMS45 groups before 24 h were either significantly ($p<0.05$) or very significantly ($p<0.01$) lower than those of the SMS0 group. With the extension of the artificial rumen fermentation time, especially after 24 h, the MCP productions among all the groups became close to each other. The MCP productions in the SMS15 and SMS30

Table 8: TVFA generation for mixture of spent mushroom (*Hypsizygus marmorens*) substrate and hybrid penisetum at different times in artificial rumen fermentation (mm/L)

Groups	6 h	12 h	24 h	48 h	72 h
SMS0	28.65±1.53^{a}	35.95±1.66^{a}	43.37±1.78^{ab}	54.31±1.34^{bAB}	52.27±1.60^{bcAB}
SMS15	27.50±1.41^{ab}	34.10±1.27^{ab}	45.17±1.97^{a}	61.26±2.12^{aA}	57.85±2.23^{aA}
SMS30	27.19±1.09^{ab}	33.92±1.39^{ab}	44.37±1.56^{a}	59.77±1.62^{aA}	56.32±2.14^{abA}
SMS45	23.66±1.22^{b}	31.60±1.53^{b}	42.32±1.13^{ab}	49.85±2.05^{bB}	47.20±1.97^{cdB}
SMS60	23.48±1.27^{b}	29.84±1.28^{b}	38.86±1.53^{b}	49.53±1.46^{bB}	45.18±1.89^{dB}

Means in the same row with different lowercase letters are significantly different ($p<0.05$), means in the same row with different uppercase letters are very significantly different ($p<0.01$) and means unlabeled indicated or in the same row with the same letters show no significant difference ($p>0.05$)

Table 9: Different mixtures of the measured and estimated values of fermentation target *in vitro* of spent mushroom (*Hypsizygus marmorens*) substrate and hybrid penisetum

Items	SMS0	SMS15	SMS30	SMS45	SMS60
SFAEI					
GAS	0	0.0133	0.0048	-0.1214	-0.16230
IVDOM	0	-0.0335	-0.0504	-0.1241	-0.19620
MCP	0	0.0647	0.0160	-0.2304	-0.33880
TVFA	0	0.0502	0.0317	-0.1023	-0.14800
MFAEI	0	0.0947	0.0021	-0.5782	-0.84533

groups after 24 h increased sharply, being higher than those of the SMS0 group but the difference was not significant ($p<0.05$). The MCP productions in each group at 72 h were as follows: SMS15>SMS30>SMS0>SMS45> SMS60.

Effects of different combination ratios on TVFA production by artificial rumen fermentation: As exhibited in Table 8, the TVFA productions in the SMS15 and SMS30 groups at the time point of 24 h were higher than those of the SMS0 but the difference was not significant ($p>0.05$); at 48 h and 72 h, the TVFA productions in the SMS15 and SMS30 groups were significantly higher than those of the SMS0 group, in which the TVFA production attained significant levels at the time point of 48 h ($p<0.05$). The cumulative TVFA quantity order in the experimental group at 72 h was as follows: SMS15> SMS30>SMS0>SMS45>SMS60.

Comprehensive evaluation of the combined effects of the artificial rumen fermentation with the same combination ratio: According to the comprehensive index of the associative effects (MFAEI), the GAS, IVDOM, MCP and TVFA of the artificial rumen fermentation at 72 h were evaluated by conducting MFAEI and the associative effect value was obtained (Table 9). As shown in Table 9, associative effects were present in the collocation of the spent mushroom (*Hypsizygus marmorens*) substrate and pennisetum hybrid by chemical treatments in which the SMS15 and SMS30 groups showed positive associative effects while the SMS45 and SMS60 groups demonstrated negative associative effects.

DISCUSSION

pH value is an intuitive and important index for evaluating whether or not pH is normal for rumen fermentation; pH value is affected by dietary types, saliva secretion and acid-base substances formed in the rumen and is also the result of interaction of multiple factors. In the process of the artificial rumen fermentation, the pH value change is mainly affected by VFA generation and buffer effect. Prasad *et al.* (1994) found that the pH values ranging from 6.6-6.8 ensured an appropriate fiber digestion environment. When the pH value is lower than 6.4, the fiber digestibility decreases. The results in the present experiment show that the pH values in each group were within the range of 6.43-6.65 in which the pH value of the experimental group showed an increasing trend. Gas production is an index which comprehensively reflects the fermentation degree of feed and which shows the overall trend of rumen microbial activity. The results of Bodine *et al.* (2000) and Blummel and Becker (1997) demonstrated that gas production was closely related to *in vivo* digestibility and at the same time the high gas production contributed to the improvement of animal food intake. Liu *et al.* (2002) found that the gas production yields of the mixed ruminal fermentation with straw and ryegrass treated with ammonium bicarbonate at different ratios were higher than those of single fermentation with straw or ryegrass treated with ammonium bicarbonate and there were associative effects present in the gas production. The selected crude feed in the present study had a high content of cellulose and the ratio of the concentrated feed and crude feed was 3:7 thus having fewer soluble carbohydrates. Therefore, the gas production in each combination group in the present experiment all reached a peak at 48 h, after which they entered the slow gas production stage. In the early gas production stage (before 12 h), the gas productions of the SMS0 combination groups were higher than those of the other groups while those of the SMS15 and SMS30 groups in the middle and late stages were higher than those of the SMS0 group. The reason for this may be that the nitrogen and energy ratio in the SMS15 and SMS30 groups are more reasonable than those of the SMS0 group which is conducive to the growth of rumen microorganisms, thereby improving gas production. The degradation rate of the organic substances in the feed represents the transformation ability of feed by microorganisms. The high degradation rate shows better

rumen fermentation effects. In the meantime, the high degradation rate also shows that there is a large amount of carbohydrates in the feed that can be used by rumen microorganisms (Tang *et al*., 2008). The IVOMD in the SMS0 was the highest at 72 h indicating that the amount of available carbohydrates of the pennisetum hybrid was greater than that of the spent mushroom (*Hypsizygus marmorens*) substrate treated with chemicals leading to the high ratio of spent mushroom (*Hypsizygus marmorens*) substrate treated with chemicals in the diets and low levels of IVOMD. MCP synthesis requires a supply from a variety of nutrients and is affected by many factors, among which the most important are energy and nitrogen. Therefore, the MCP yield mainly depends on whether or not the degradation quantities of carbohydrate and protein match the degradation speed (Henning *et al*., 1991; Niderkorn *et al*., 2011). In the present study, the MCP yield of the SMS15 and SMS30 groups were the highest, suggesting that the energy nitrogen ratio of the combined diet and the degradation speed are helpful for the synthesis of MCP. Carbohydrates of diets produce acetic acid, propionic acid, butyric acid and other volatile acetic acids by means of rumen fermentation and degradation and these are major energy sources for ruminant animals. Gray *et al*. (1992) showed that 60-80% of the energy required by ruminant animals was provided by volatile fatty acids. The production of the volatile fatty acid was measured by the artificial rumen fermentation method which to some extent can reflect the energy value in the feed that can be used (Weimer *et al*., 2011). The experimental results showed that the TVFA production quantity in the SMS15 and SMS30 groups at 72 h was higher than those of the SMS0 group, exhibiting positive associative effects and this also consistent with the associative effects of the gas production and MCP yield. The concentration of the volatile fatty acid directly affects the rumen microbial activity. A large amount of ATP was produced in the process of production of the volatile fatty acids in the rumen microbial fermentation which not only can be used as energy for the rumen microorganisms but is also helpful for the synthesis of MCP (Feng, 2004).

CONCLUSION

The dietary associative effects were evaluated using the MFAEI described by Lu and Xie (1991). The results demonstrated that positive associative effects were shown in the combination of the spent mushroom (*Hypsizygus marmorens*) substrate and hybrid pennisetum treated with 15 and 30% chemicals which was consistent with the results of Sohane and Singh (2001).

Sohane and Singh (2001) found that addition of a certain amount of leguminous grass into two different types of rice straws significantly improved the feed intake and digestibility of the straw stalk, showing positive associative effects. It may seen from the analysis of SFAEI that MCP had the largest associative effect which is due to the fact that the chemical treatment of the spent mushroom (*Hypsizygus marmorens*) substrate increased the crude protein and non-protein nitrogen of the spent mushroom (*Hypsizygus marmorens*) substrate.

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