CHAPTER III

EXPERIMENT 1: THE EFFECTS OF SOLUBLE PROTEIN AND CARBOHYDRATE ON *IN VITRO* FERMENTATION AND NUTRIENT DIGESTION

3.1 Experiment 1.1: The effects of soluble protein, sugar, and starch on *in vitro* digestibility of cellulose and rice straw

3.1.1 Introduction

Productivity of ruminants depends on adequate nutrition with respect to chemical composition and quality of feedstuffs, which is mainly reflected in voluntary intake and digestibility. Evaluation of feedstuffs for digestibility, and their ability to supply nutrients, is necessary for provision of a nutritionally balanced ration to the animal. Determination of digestibility of feedstuffs *in vivo* is time-consuming, laborious, and expensive, requires large quantities of feed and is unsuitable for large-scale feed evaluation (Stern et al., 1997; Getachew et al., 2005). Measurement of *in vitro* DM digestibility has been extensively used to analyze feeds, due to high degree of correlation to *in vivo* digestibility (Getachew et al., 2004). Compared to conventional methods, the newer ANKOM filter bag method (ANKOM Technology, Macedon, New York, USA) simplifies the measurement of *in vitro* digestibility by eliminating the requirement for filtering samples after digestion, which is often one of the most labor intensive steps in the conventional procedure. Several studies have also demonstrated that the ANKOM method produces comparable digestibility values to traditional *in vitro* procedures for many feeds (Holden, 1999; Mabjeesh et al., 2000; Wilman and Adesogan, 2000).

Sugar and starch are the main readily fermentable non-fiber carbohydrate (NFC) component and serve as the major source of carbohydrate in diets of lactating dairy cows and important substrate for rumen microbial growth. Supplementing with feedstuffs that contain high concentrations of rapidly fermentable NFC in cows consuming low-quality forage has been generally observed to depress forage fiber digestion (DelCurto et al., 1990; Sanson et al., 1990). This negative associative feed effect has been largely attributes to sub-optimal pH for cellulolytic bacteria and

microbial substrate preferences. Type of supplemental carbohydrate provided in conjunction with forage also has been suggested to be a factor that may impact the effect elicited on fiber digestion (Heldt et al., 1999; Fondevila et al., 2002). Although, sugars added to forage-based diets have been found to decrease fiber digestion (Huhtanen and Khalili, 1991; Heldt et al., 1999). Vallimont et al. (2004) reported that levels of sugar at 7.5% DM increased NDF digestion. Similar observations were reported by Broderick and Radloff (2004), when replacing high moisture shelled corn with dry molasses providing 2.6, 4.9, 7.4, or 10.0% total sugar. They found a quadratic response for digestibility of NDF and ADF. Heldt et al. (1999) indicated that supplementation with starch had a more negative effect on forage fiber digestion than did simple sugars (under conditions in which ruminally degradable protein supply was adequate). Therefore clarification of potential differences in NFC sources on fiber digestion is important and needed especially with world grain prices and more emphasis to feed cows higher fiber diets. Thus, the aim of this study was to elucidate the impact of N and type of NFC supplement on extent of *in vitro* DM digestibility of purified fiber (cellulose) and rice straw by using the ANKOM method.

3.1.2 Materials and Methods

1) Substrate preparation and experimental design

Feedstuffs used in this experiment were cellulose and rice straw. Rice straw was dried at 60°C and ground to pass a 1 mm mill screen using a Wiley mill. Sixteen substrates (Table 3.1) were used in a completely randomized design with 2 × 2 × 4 factorial arrangement, with 4 replications. The main effects included fiber source (cellulose or rice straw), soluble protein (no trypticase or added trypticase) and NFC (no NFC, added starch, sugar, or sugar plus starch). The source of N, trypticase (a pancreatic digest of casein) consisting of 11.76% of total N (73.5% of CP; HIMEDIA laboratories, Cat. No CR001), starch and sugar (sucrose) sources were cassava starch and sugarcane respectively. The chemical composition of fiber source used in this study, cellulose (cotton powder; HIMEDIA laboratories Cat.No RM126) contained 96.36 and 92.73% of NDF and ADF, respectively. Rice straw contained 76.0% and 50.9% of NDF and ADF, respectively.

Table 3.1 Ingredient used in experimental substrates

Substrate	Fiber		Nitrogen source (Trypticase), mg	NFC source, mg	
	Source	Level, mg		Starch	Sugar
Substrate 1	Cellulose	250	-	-	-
Substrate 2	Cellulose	250	-	100	-
Substrate 3	Cellulose	250	-	-	100
Substrate 4	Cellulose	250	-	50	50
Substrate 5	Cellulose	250	50	-	-
Substrate 6	Cellulose	250	50	100	-
Substrate 7	Cellulose	250	50	-	100
Substrate 8	Cellulose	250	50	50	50
Substrate 9	Rice straw	250	-	-	-
Substrate 10	Rice straw	250	-	100	-
Substrate 11	Rice straw	250	-	-	100
Substrate 12	Rice straw	250	-	50	50
Substrate 13	Rice straw	250	50	-	-
Substrate 14	Rice straw	250	50	100	-
Substrate 15	Rice straw	250	50	-	100
Substrate 16	Rice straw	250	50	50	50

The ANKOM bags (F57 filter bag; ANKOM Technology Corp.) were used. The bags had a pore size of 25 μm, 55 mm long and 50 mm wide at the top and tapered to a width of 25 mm at the bottom and made from polyester/polyethylene extruded filaments in a three-dimensional matrix to maximize the flux of solutions while minimizing particulate losses. Pre-rinsed F57 filter bags in acetone for three to five minutes and completely air-dried, weighed each bags and recorded weight (W_1). Set the balance to zero and weigh 0.25 g of cellulose or rice straw (W_2) directly into the filter bag plus N or NFC source as shown in Table 3.1 for each of four replicates. One empty bag was weighed and sealed as blank bag for use as correction factor (C_1), resulting in a total of 65 bags sample and placed 2 bags in 200 ml bottles (2 bags/ bottle) for incubation.

2) Buffer solutions and *in vitro* inoculant preparation

Two buffer solutions were prepared in advance and combined at the time of incubation (Robinson et al., 1999). Solution A consisted of 10.0 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of NaCl, 0.1 g of $CaCl_2 \cdot 2H_2O$, and 0.5 g of urea (reagent grade) dissolved in distilled water and made into 1 liter. Solution B consisted of 15.0 g of Na_2CO_3 and 1.0 g of $Na_2S \cdot 9H_2O$ dissolved in distilled water and made into 1 liter. In a separate beaker 1330.0 ml of solution A was measured, into which 266.0 ml of solution B was measured and added. The combined solution A/B was poured into digestion jars warmed to 39°C by placing them in ANKOM Daisy^{II} incubator set to 39 ± 0.5°C temperature and the jars were kept inside incubator for thirty minutes to allow the temperatures to equilibrate. During this equilibration time rumen inoculums were collected and prepared.

A mixed culture of rumen microorganisms from rumen fluid were obtained from dry cow offered rice straw *ad libitum* and fed 4 kg of concentrate diet twice daily was used as inoculant. Rumen fluid was collected into a thermos flask (filled with water heated to 39°C, and poured out just before filling it with inoculant) before the morning feeding via stomach tubing and strained through four layers of cheesecloth and maintained under a stream of CO_2 gas. A 400 ml volume of strained rumen fluid was added incubation jars that contained buffer solutions (1,596 ml) under CO_2 gas to maintain anaerobic conditions. Aliquot mixtures (1,996 ml) of buffer solution and rumen fluid were delivered to individual bottles that contained the sample bags (170 ml/ bottle). The bottles were purged with a stream of CO_2 gas for approximately 10 second prior to purged with screw cap and placing into the digestion jars. The ANKOM Daisy ^{II} incubator can be loaded with four jars that are reset at an 80° angle and rotate at 0.95 rpm by means of gear drives. The jars are fitted with a screw top with an inset gas release valve.

3) *In vitro* digestibility

Sealed digestion jars with samples and buffer solution were placed into ANKOM Daisy ^{II} incubator, following the procedure described in detail by Robinson et al. (1999). Incubations were conducted for 48 h to determine *in vitro* digestibility results. At the end of the 48 h incubation, jars were removed from the

incubation box, the incubation solution was drained out, and bags were rinsed thoroughly with cold tap water until the wash water was clear. For *in vitro* digestibility determination, bags were boiled in neutral detergent solution for 75 min at a temperature 70 - 90°C. Then, soaked twice in acetone for 5 min at each soaking and dried at 100°C for 24 h. recorded the post *in vitro* fermentation weight as W_3 and used the formula below to estimate digestibility:

$$\text{Cellulose or rice straw digestibility (\%)} = 100 - ((W_3 - (W_1 \times C_1)) \times 100 \div W_2)$$

Where:

W_1 = Bag tare weight

W_2 = Substrate weight

W_3 = Final bag weight after *in vitro* and sequential NDF determination

C_1 = Blank bag correction (final oven-dried weight ÷ original blank bag weight)

4) Chemical Analyses

The substrates were oven-dried until constant weight (55°C) ground to determine percentage of dry matter (DM) (AOAC, 1990). Crude protein (CP) obtained by total N determination using micro-Kjeldahl technique and a fix conversion factor (6.25) according to AOAC (1990). NDF were determined by using fiber analyzer (ANKOM$^{200/220}$, ANKOM Technology Corp., Fairport, NY, USA). Sodium sulfite and α-amylase were used for NDF analysis (Van Soest et al., 1991).

5) Statistical Analyses

All data was statistically analyzed by analysis of variance using the General Linear Model (GLM) procedure of statistical analysis system (SAS, 1996) according to a completely randomized design included as 2 × 2 × 4 factorial arrangement of treatments with two sources of fiber, two level of N and four NFC sources. Means response were examined for significant differences due to fiber, N, NFC source and interactions by the least squared means procedures of SAS.

Significance was declared at $P<0.05$ and considered to indicate a trend at $0.05 < P < 0.10$. The following model was used:

$$Y_{ijkl} = \mu + \alpha_i + \beta_j + \gamma_k + \alpha\beta_{ij} + \alpha\gamma_{ik} + \beta\gamma_{jk} + \alpha\beta\gamma_{ijk} + \varepsilon_{ijkl}$$

Where:

Y_{ijkl} = the measured variable,

μ = the overall mean,

α_i = the effect of fiber source (i= 1, 2),

β_j = the effect of N (j=1, 2),

γ_k = the effect of NFC source (k= 1, 2, 3, 4),

$\alpha\beta_{ij}$ = the effect of interaction term of fiber source and N,

$\alpha\gamma_{ik}$ = the effect of interaction term of fiber and NFC source,

$\beta\gamma_{jk}$ = the effect of interaction term of N and NFC source,

$\alpha\beta\gamma_{ijk}$ = the effect of interaction term of fiber source, N and NFC source,

ε_{ijkl} = residue error.

3.1.3 Results and Discussion

The *in vitro* dry matter digestibility (IVDMD) data for the various substrates in this study and statistical analysis are in Table 3.2 and Figure 3.1. The three-way interaction (fiber source × N × NFC source) as well as the fiber source × N and the fiber source × NFC source interaction were not significant for IVDMD. Only N × NFC source interaction was significant ($P<0.05$) for IVDMD after 48 h of incubation. It was found that supplementation of N plus NFC source improved IVDMD of cellulose and rice straw than supplemented with only NFC source averaged 69.9 and 62.4%, respectively. After 48 h of fermentation, the control (i.e., without N and NFC supplement) showed lower IVDMD than those substrate supplemented with N or NFC and N plus NFC (Table 3.2; Figure 3.1). Although the incubation buffer contained N (from urea) 152.9 N mg/l which was more than the recommended level as stated by Satter and Slyter (1974) determined with continuous culture fermentors. Dryhurst and Wood (1998) observed that the N should contain at least 80 mg N/l. Therefore, without N in substrate, microbes used ammonia (from

urea) as nitrogen source, and then digested fiber as energy source resulting in IVDMD of 59.7% for cellulose or 41.0% for rice straw. Addition of trypticase as a source of amino acid and peptides improved IVDMD of cellulose and rice straw ($P<0.01$) at 48 h post fermentation, IVDMD increased 9.7% compared with substrate with no trypticase supplement.

The higher level of IVDMD was found in cellulose as compared with rice straw based substrate, averaging 73.5 and 54.9% ($P<0.01$), respectively. Supplementation with NFC (starch, sugar or starch plus sugar) improved IVDMD of substrate compared with no NFC added ($P<0.01$; 66.1 vs. 58.5%). The sources of carbohydrates available may have been responsible for the microbial growth responses. However, IVDMD of substrate was lower when combination of starch and sugar added into substrate compared with addition of sugar alone ($P<0.01$). Supplementation of sugar, starch and sugar plus starch enhanced IVDMD of 10.4, 7.1 and 5.4%, respectively. This result indicated that the ratio of starch and sugar (50:50) may not be as suitable for enhancing microbial growth and IVDMD as compared to supplement starch or sugar by themselves.

The results of this study suggested that trypticase improved IVDMD by supplying either specific amino acids or branched chain fatty acids. Hall et al., (1954) reported partial hydrolysates of protein have stimulated rate of cellulose digestion. Difference between starch and sugar supplement, were too small to be of significance. Ribeioro et al. (2005) has reported addition of sucrose (4% of DM) were not affected fiber digestion of alfalfa hay in which concentration of sucrose in the hay was 6.8% of DM; however it tended to decrease organic matter (OM) digestibility.

Table 3.2 *In vitro* digestibility of cellulose and rice straw supplement with N and NFC at 48 h of incubation

Item	Fiber source (F) Cellulose/ Rice straw	Nitrogen source (N) No/ add	NFC source (NFC) No/Starch/ Sugar/Starch +sugar	IVDMD, %
Substrate 1	Cellulose	No	No	59.66 ^{de}
Substrate 2	Cellulose	No	Starch	68.46 ^{bc}
Substrate 3	Cellulose	No	Sugar	76.45 ^{ab}
Substrate 4	Cellulose	No	Starch+Sugar	68.38 ^{bc}
Substrate 5	Cellulose	add	No	79.33 ^{a}
Substrate 6	Cellulose	add	Starch	78.60 ^{a}
Substrate 7	Cellulose	add	Sugar	80.54 ^{a}
Substrate 8	Cellulose	add	Starch+Sugar	76.33 ^{ab}
Substrate 9	Rice straw	No	No	41.04 ^{g}
Substrate 10	Rice straw	No	Starch	53.04 ^{ef}
Substrate 11	Rice straw	No	Sugar	58.14 ^{de}
Substrate 12	Rice straw	No	Starch+Sugar	49.63 ^{ef}
Substrate 13	Rice straw	add	No	53.98 ^{def}
Substrate 14	Rice straw	add	Starch	62.19 ^{cd}
Substrate 15	Rice straw	add	Sugar	60.37 ^{cde}
Substrate 16	Rice straw	add	Starch+Sugar	61.14 ^{cde}
			SEM	2.63
			P-value	
			F	<0.01
			N	<0.01
			NFC	<0.01
			F*N*NFC	0.60
			F*N	0.57
			F*NFC	0.40
			N*NFC	0.02

[a,-g] Means within a row without a common superscript letter differ (P<0.05).

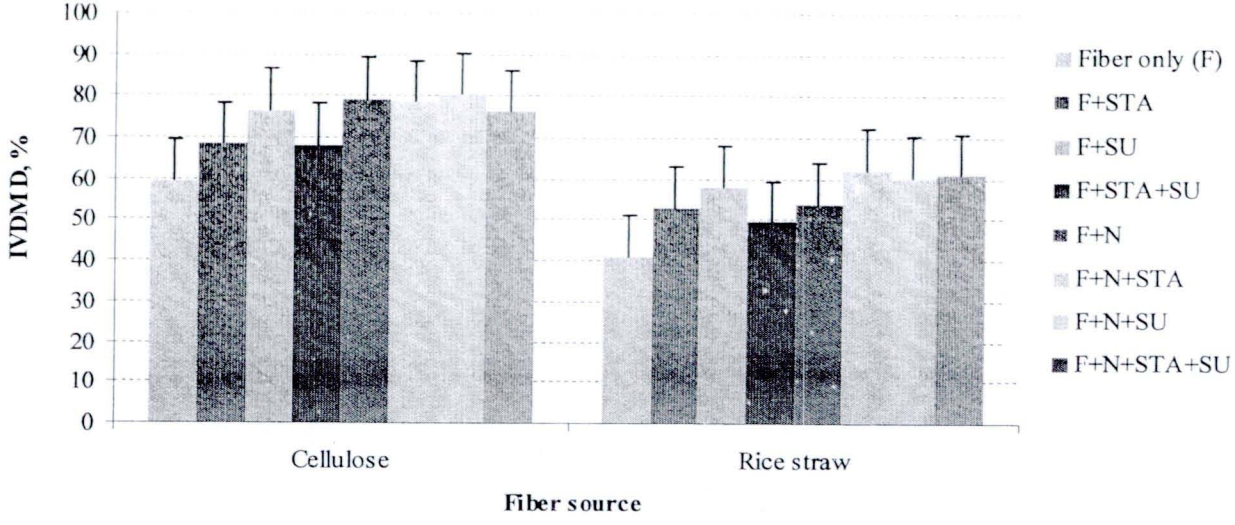

Figure 3.1 *In vitro* digestibility of cellulose and rice straw supplement with nitrogen (N) and NFC source at 48 h of incubation STA, starch; SU, sugar; STA+SU, starch plus sugar (50:50)

3.1.4 Conclusions

The addition of nitrogen (trypticase) improved digestibility of cellulose and rice straw at 48 h of incubation. Addition of starch, sugar or combination of starch and sugar increased IVDMD. Maximum digestibility was found when trypticase (14% of the DM) and sugar or starch as NFC source (42% of the DM) was added to the substrate together.

3.2 Experiment: 1.2 The effects of soluble protein and carbohydrate on *in vitro* digestibility of rice straw

3.2.1 Introduction

Energy availability from forages is limited by fiber manipulation such as harvesting time, maturation, preservation method because fiber is slowly and incompletely digested (Dwayne and Redfearn, 1997). A high negative correlation (r^2 - 0.76) has been established between dry matter (DM) intake and neutral detergent fiber (NDF) content of all forage diets (Hoover, 1986). Rice straw is one of largest by-products of Thai agriculture, and is a potentially useful resource for ruminant production in Thailand. However, its low nutritive value and poor digestibility when fed to animals, it necessary for cattle producers to adopt supplementary strategy for optimal performance (Broudiscou et al., 2003; Van Soest, 2006).

The studies by Belasco (1956) demonstrated that addition of non protein nitrogen compounds such as urea for short-term (24 h) semi-continuous fermentation of rumen contents greatly improved cellulose digestion. Maximum cellulose digestion occurred when ammonia nitrogen (NH_3-N) concentration reach 43 mg/dl. In several other studies it was found that the concentration of ammonia required for optimum microbial growth, or fermentation exceeded 6 mg/dl, the basal diet contain less than 6 % of CP but sufficient amount of readily fermentable carbohydrate to support a vigorous nonfibolytic population. It is suggested that under conditions of high fermentable carbohydrate and limited available protein, ammonia concentration required for optimum growth of cellulolytic organisms may be increased (Hoover, 1986). However, there is also evidence for improved forage intake and digestibility when supplemented with sugar; probably resulting from increased microbial growth in the rumen and consequently positive associative effects on forage utilization. (Broderick and Radloff., 2004; Vallimont et al.,

2004; Hristov et al., 2005). The objective of this study was to examine the effect of varying sugar levels as a proportion of NFC (sugar: starch) and soluble protein (urea:trypticase) on IVDMD of rice straw.

3.2.2 Materials and Methods

1) Substrate preparation and experimental design

The same procedures used as described in experiment 1.1 were followed in experiment 1.2 except that twenty five substrates were used in a completely randomized design with 5 × 5 factorial arrangement of treatment. The main effects include the source of soluble protein (urea:trypticase) and NFC (sugar:starch) (Table 3.3). Nutrient composition of rice straw and trypticase used in this study were similar in chemical composition as in experiment 1.1. Trypticase was replaced with urea in the ratios 0, 25, 50, 75, or 100% of total CP. NFC concentration in substrate was 42% of DM, starch and sugar source were cassava starch and cane sugar powder waste (sucrose), respectively. Starch concentration was replaced with sugar in the ratios 0, 25, 50, 70, or 100% of NFC.

The rice straw was dried at 60°C in forced air oven, ground to pass through 1 mm sieve in a Wiley mill and used for chemical analysis and *in vitro* DM digestibility study. Multi-layer polyethylene polyester cloth bag (F57 filter bag; ANKOM Technology Corp.) were used. F57 filter bags were pre-rinsed in acetone for three to five minutes and completely dried in a forced air oven at 60°C for 6 h and each bag weighted and recorded (W_1) before use. Ground rice straw samples were weighed (0.25 g per bag) and recorded (W_2) and weighed amounts of N and NFC sources added as presented in Table 3.3 for each of two replicates. One empty bag was weighed and sealed as blank bag for correction factor (C_1), resulting in a total of 51 bags substrates, and 2 bags were then placed into 200 ml bottles (2 bags/ bottle or 2 sample bag +1 correction factor bag/bottle).

Buffer solution and rumen fluid inoculant were prepared as described in experiment 1.1. Similarly the method for *in vitro* digestibility and chemical analysis of rice straw and trypticase were same as described in experiment 1.1. At the end of the 48 h incubation, jars were removed from the incubation box, the incubation solution was drained out, and bags were rinsed thoroughly with cold tap water until the wash water was clear. For *in vitro* digestibility determination, bags were boiled in

neutral detergent solution for 75 min at 70 - 90°C, then soaked twice in acetone for 5 min at each soaking and dried at 100°C for 24 h. Recorded the post *in vitro* weight as W_3 and used the formula below to estimate IVDMD:

$$\text{IVDMD (\%)} = 100 - ((W_3 - (W_1 \times C_1)) \times 100 \div W_2)$$

Where:

W_1 = Bag tare weight

W_2 = Substrate weight

W_3 = Final bag weight after *in vitro* and sequential NDF determination

C_1 = Blank bag correction (final oven-dried weight ÷ original blank bag weight)

2) Statistical Analyses

All data were analyzed by analysis of variance using the General Linear Model (GLM) procedure of statistical analysis system (SAS, 1996) according to a completely randomized design included as 5 × 5 factorial arrangement of treatments with five ratio of N and five ratio of NFC. Means response were examined for significant differences due to ratio of N and NFC and interactions by the least squared means procedures of SAS. Significance was declared at $P<0.05$ and considered to indicate a trend at $0.05<P<0.10$. The following model was used:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + \varepsilon_{ijk}$$

Where:

Y_{ijk} = the measured variable,

μ = the overall mean,

α_i = the effect of N ratio (i=1, 2, 3, 4, 5),

β_j = the effect of NFC ratio (j=1, 2, 3, 4, 5),

$\alpha\beta_{ij}$ = the interaction term of N and NFC level,

ε_{ijk} = residue error.

Table 3.3 Ingredient used in experimental substrates

Substrate	Rice straw, mg	Nitrogen source , % of CP in substrate		NFC source, % of total NFC in substrate	
		Trypticase	Urea	Sugar	starch
Substrate 1	250	100	-	-	100
Substrate 2	250	100	-	25	75
Substrate 3	250	100	-	50	50
Substrate 4	250	100	-	75	25
Substrate 5	250	100	-	100	-
Substrate 6	250	75	25	-	100
Substrate 7	250	75	25	25	75
Substrate 8	250	75	25	50	50
Substrate 9	250	75	25	75	25
Substrate 10	250	75	25	100	-
Substrate 11	250	50	50	-	100
Substrate 12	250	50	50	25	75
Substrate 13	250	50	50	50	50
Substrate 14	250	50	50	75	25
Substrate 15	250	50	50	100	-
Substrate 16	250	25	75	-	100
Substrate 17	250	25	75	25	75
Substrate 18	250	25	75	50	50
Substrate 19	250	25	75	75	25
Substrate 20	250	25	75	100	-
Substrate 21	250	-	100	-	100
Substrate 22	250	-	100	25	75
Substrate 23	250	-	100	50	50
Substrate 24	250	-	100	75	25
Substrate 25	250	-	100	100	-

All diets calculation provides protein and NFC of 14% and 42%, respectively.

3.2.3 Results and Discussion

There was a trend observed for N × NFC ratio interaction in IVDMD after 48 h of incubation ($P < 0.10$). It was observed that combining urea: trypticase (50:50) plus NFC (100% of sugar) had higher IVDMD compared to the others substrates (58.8%; Figure 3.2). Adding 100% of sugar or starch as NFC source in substrate enhances IVDMD when supplemented with soluble true protein as sole source of CP. Increasing urea level (up to 50% of CP) improved IVDMD, however, substrate that were supplemented with urea at 25 to 50% of CP, IVDMD were not significantly different ($P>0.05$) to those in which soluble true protein (trypticase) as sole nitrogen source were added (Table 3.4). The IVDMD was lowest in substrate that contained urea as a sole source of CP (52.9%; $P<0.05$), it was even lower than Maeng et al. (1976) who reported that the optimum ratio of non-protein N to amino acid N for microbial growth was 75% urea-N and 25 % amino acid-N. It is possible that carbohydrate source (glucose, soluble starch or cellobiose) was differing from this study.

IVDMD was not affected by varying sugar and starch concentration ($P>0.05$), average 55.4%, across substrate. In contrast, Vallimont et al. (2004) reported that inclusion of 7.5% of sucrose in the TMR containing 31-33% of non-structural carbohydrate (NSC) resulted in higher IVNDFD as compared with 2.5 and 5% of sucrose (66.1 vs. 58.5 and 59.4%, respectively) with incremental replacement of corn starch with sucrose in TMR (0, 2.5, 5 and 7.5% of DM) by using a dual-effluent continuous culture system. In Hoover et al. (2006) study, a dual flow continuous culture system was used to examine the effects of varying sugar (liquid sugar blend) as a proportion of sugar plus starch (corn grain and corn silage) reported addition of sugar (2.9 to 9.5%) to the low NSC (24% of DM) diet reduced fiber digestion; however, fiber digestion was either not affected or increased in the 28 and 33% of NSC diets when sugar was added. The results of present study indicated that diet with 42% NFC, IVDMD of rice straw (76% of NDF) may not respond to addition of concentrated sugar (100% of NFC) as well as supplementation with a less concentrated supplement containing a mixture of sugar and starch.

Table 3.4 The effects of varying soluble protein (urea:trypticase) and NFC (sugar:starch) on *in vitro* DM digestibility of rice straw

Item	Ratios of N source (urea: trypticase) (N)					Ratios of carbohydrates source (starch:sugar) (C)					SEM	P-value		
	0:100	25:75	50:50	75:25	100:0	100:0	75:25	50:50	25:75	0:100		N[c]	C	N*C
IVDMD	57.05[a]	56.48[a]	55.96[a]	54.41[b]	52.88[c]	55.07	54.98	55.28	55.37	56.09	0.40	<0.01	0.33	0.09

[a, b] Means within a row without a common superscript letter differ (P<0.05).

[c] Linear (P<0.01).

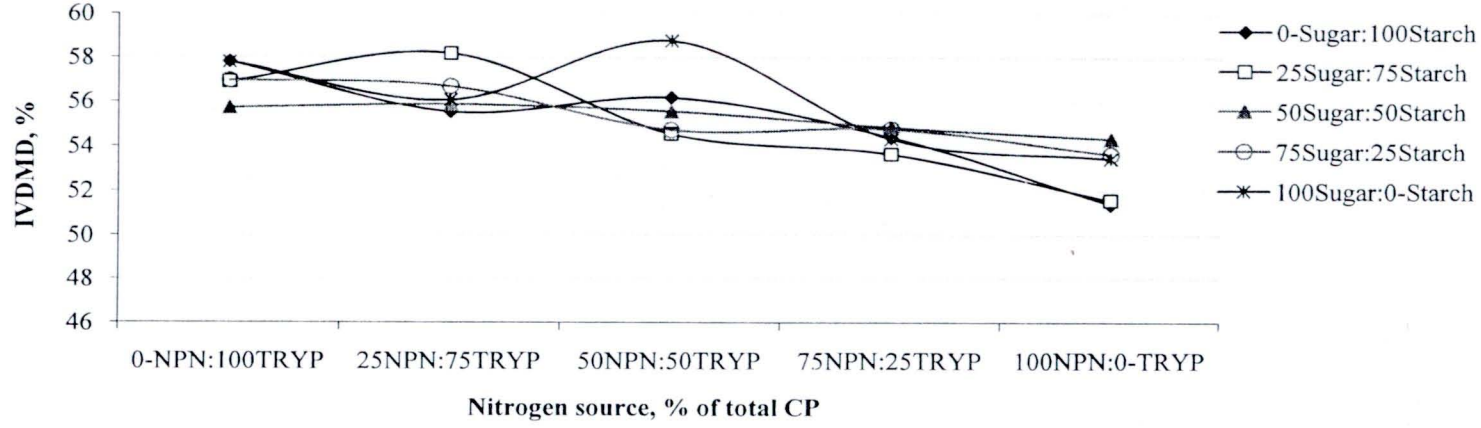

Figure 3.2 The effects of varying soluble protein (urea:trypticase) and NFC (sugar:starch) on *in vitro* DM digestibility of rice straw

3.2.4 Conclusions

The results showed that replacing true protein with NPN from urea at 75% or 100% of CP had negative effect on IVDMD of rice straw. Supplementation of NFC either in form of sugar or starch had positive effect on IVDMD. However, when the diet contained sugar or starch as sole source of NFC, supplementation with higher amount of true protein is required for enhanced IVDMD. A mixture of N and NFC source possibly provided a more synchronous sufficient nutrient balances to promote bacterial growth and enhanced IVDMD.

3.3 Experiment 1.3 The effects of soluble protein and carbohydrate on *in vitro* nutrient digestion and gas production

3.3.1 Introduction

The nutritive value of ruminant feed is determined by the concentrations of its chemical components, as well as their rate and extent of digestion. Determining the digestibility of feeds *in vivo* is laborious, expensive, requires large quantities of feed, and is largely unsuitable for single feedstuffs thereby making it unsuitable for routine feed evaluation. The *in vivo* method is also subject to errors associated with use of digesta flow rate markers, microbial markers, and inherent animal variation (Stern et al., 1997). Therefore i*n vitro* methods provide less expensive and more rapid alternatives.

There are a number of *in vitro* techniques available to evaluate the nutritive value of feeds at relatively low cost. Measurement of *in vitro* DM digestibility has been widely used to assess the nutritional quality of feeds, due to its high correlation with *in vivo* digestibility. Development of the ANKOM Daisy ^{II} incubator (ANKOM Technology Corp., Fairport, NY, USA), has allowed large numbers of feeds to be simultaneously assayed and greatly improved labor efficiency. The use of *in vitro* gas production technique to estimate digestion of feeds is based on measured relationships between the *in vivo* digestibility of feeds and *in vitro* gas production, in combination with the feed's chemical composition (Menke and Steingass, 1988). *In vitro* gas methods primarily measure digestion of soluble and insoluble carbohydrates (Menke and Steingass, 1988), and the amount of gas produced from a feed on incubation reflects production of volatile fatty acids (VFA),

which are a major source of energy for ruminants. Gas arises directly from microbial degradation of feeds, and indirectly from buffering of acids generated as a result of fermentation. Both *in vitro* gas production and the ANKOM devices can be used as rapid evaluation tools to assess nutritional quality of feeds. The results from experiment 1.2 found that true protein (trypticase) can be replaced with urea at 25 to 50% of CP; however Maeng et al. (1976) found that the optimum ratio of non-protein N to amino acid N for microbial growth was 75% and 25%, respectively. Moreover, also from the previous study it was found that starch can be replaced with 100 % of sugar. Therefore, a major aspect of the study is the containing interest in adding sugar and soluble protein in ruminant diet, to enhance nutrient digestion and gas production.

Based on the results of the experiment 1.2, the experiment 1.3 was conducted as a follow up with the objective of investigating a soluble protein and sugar fraction in total mixed rations (TMR), with increased soluble protein at 5 to 11% of DM and sugar at 8, 16, or 24% of DM on nutrient digestibility and gas production as well as the relationship between IVDMD and *in vitro* gas production.

3.3.2 Materials and Methods

1) Diets and experimental design

The experimental design was 3 × 3 factorial arrangement in completely randomized design. The main effects include soluble protein (low, 5%; medium, 8 %; or high, 11 % of DM) and sugar (low, 8%; medium, 16%; or high, 24% of DM) (Table 3.5). The levels of soluble nitrogen from urea were increased as 25, 50 and 75% of CP in TMR diets by using KCF 2006 Program (Pattarajinda and Duangjinda, 2006).

2) *In vitro* digestibility

The method for *in vitro* fermentation used was similar to technique described in experiment 1.1 except that 9 dietary treatments as TMR were used (Table 3.5). Ground TMR diet samples were weighed 0.5 g per bag and recorded the weight (W_2). The samples bags and blank bags were prepared for each of 5 sampling times, at 2, 4, 8, 24 and 48 h of incubation. The empty 5 bags were weighed and sealed as blank bag for correction factor (C_1) at each time point, giving a total of 95 bags at the beginning of incubation (2 replication /treatment + 5 blank bags). The sample bags and blank bags were placed into 200 ml bottle (2 bags/ bottle). Buffer solution and inoculant were prepared as described in experiment 1.1. The mixtures of buffer

solution and rumen fluid were divided among the bottles that contained the sample bags (170 ml/ bottle) and added into these bottles. The bottles were purged with CO_2 for approximately 10 second prior to purged with screw cap and placing into the digestion jars. The sample bags were incubated into the ANKOM Daisy ^{II} incubator to determine the *in vitro* digestibility for 2, 4, 8, 24, and 48 hours. Incubator was maintained at 39.5 ± 0.5°C. At the end of each sampling time of incubation, the bottles were removed from the chamber, the incubation solution was drained out, and bags were rinsed thoroughly in cold tap water until water became clear. For *in vitro* digestibility determination, bags were boiled in neutral detergent solution for 75 min. at 70 - 90°C, then, soaked twice in acetone for 5 min each and dried at 100°C for 24 h. The DM, NDF, and ADF digestibility were calculated as follow:

$$\text{IVDMD (\%)} = 100 - ((W_3 - (W_1 \times C_1)) \times 100 \div W_2)$$

$$\text{IVNDFD (\%)} = 100 - (\text{NDF remaining at t= 48 h} \div \text{NDF at t= 0 h}) \times 100$$

$$\text{IVADFD (\%)} = 100 - (\text{NDF remaining at t= 48 h} \div \text{NDF at t= 0 h}) \times 100$$

Where:

W_1 = Bag tare weight

W_2 = Substrate weight (as dry matter)

W_3 = Final bag weight after *in vitro* and sequential NDF determination

C_1 = Blank bag correction (final oven-dried weight ÷ original blank bag weight)

4) *In vitro* gas production

The *in vitro* gas production according to the procedure described by Makkar et al. (1995) was used to measure cumulative gas production from experimental substrate. The substrate samples used were similar to that used in the ANKOM method. Approximately 0.5 g on dry matter basis was transferred into a 50 ml serum bottles with four replicates. The bottles containing substrate were pre-warmed in hot air oven at 39°C for about 1 h prior to injecting 40 ml of rumen

fluid- media solution to each bottle. The media solution contained 1,095.0 ml of distilled water, 730.0 ml of buffer solution, 365.0 ml of macro-mineral, 0.23 ml of micro-mineral, 1.0 ml of resazurin, and 60 ml of freshly prepared reduction solution. The buffer solution contained 35.0 g of $NaHCO_3$ and 4.0 g of $(NH_4)HCO_3$ in 1 liter of distilled water. The macro-mineral solution contained 5.7 g of Na_2HPO_4, 6.2 g of KH_2PO_4, and 0.6 g of $MgSO_47H_2O$ in 1 liter of distilled water. The micro-mineral solution contains 13.2 g of $CaCl_22H_2O$, 10.0 g of $MnCl_24H_2O$, 1.0 g of $CoCl_26H_2O$, and 0.8 g of $FeCl_26H_2O$ in 100 ml of distilled water. To make resazurin solution, 100.0 mg of resazulin were dissolved in 100 ml of distilled water. The reducing agent was prepared fresh just before use, 3.7 ml of 1 *M*NaOH and 580.0 mg Na_2S7H_2O dissolved in 47.5 ml distilled water. The bottles were purged with CO_2 prior to plugging with rubbers stoppers, crimp sealed and incubated in hot air oven at 39°C. The rate of gas production was measured by recording amount of gas volume, at the end of each incubation period, using a 20 ml glass syringe connected to the incubation bottle with a 23 gauge, 1.5 inch needle. Amount of gas produced was recorded from 1 to 72 h (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 15, 18, 21, 24, 30, 36, 42, 48, 60, and 72 h) post incubation. After incubation periods, an amount of cumulative gas volume at 2, 4, 8, 12, 24, 48, and 72 h were fitted by using the equation $y = a+b\ [(1\text{-}Exp^{(-ct)}]$ (Ørskov and McDonald, 1979), where y is the volume of gas produced at time t, a the intercept which ideally reflects the fermentation of the soluble fraction (ml), b the gas volume at asymptote described the fermentation of the insoluble fraction (ml), and c the rate of gas production (%/h). Parameters b and c were estimated by an iterative least square method using a nonlinear regression procedure (SAS, 1996).

Rumen fluid inoculants were obtained from a dry cow that was fed a diet of rice straw *ad libitum* and fed 4 kg of concentrate twice daily. Rumen fluid was removed before the morning feeding under vacuum pressure via stomach tubing into a 2 liter (l) conical flask and transferred into two pre-warmed 1 l thermos flasks which were then strained through four layers of cheesecloth and flushed with CO_2.

5) Chemical Analyses

After drying in a forced-air oven at 60°C for 72 h, feed samples were ground through a 1-mm screen. Samples were analyzed for DM (AOAC, 1990); CP obtained by total nitrogen determination using the micro-Kjeldahl technique and fixed

conversion factor (6.25); EE, and ash (AOAC, 1990), SP (Krishnamoorth et al., 1982). ADF (AOAC, 1990) and NDF (Van Soest et al., 1991) were analyzed with a fiber analyzer (model 200, ANKOM Technology, Fairport, NY). Sodium sulfite and heat-stable α-amylase were used for NDF analysis; sugar (AOAC, 2000). The non-fiber carbohydrate (NFC) was calculated by subtracting CP, NDF, EE, and ash (Sniffen et al., 1992).

6) Statistical Analyses

All data obtained from the trials were analyzed by analysis of variance using the General Linear Model (GLM) procedure of statistical analysis system (SAS, 1996) according to a completely randomized design included as 3 × 3 factorial arrangement of treatments with three level of SP and three level of sugar. Means response were examined for significant differences due to SP and sugar level and their interactions by the least squared means procedures of SAS. The responses to SP and sugar level were also examined for linear and quadratic effects. The correlation between the IVDMD and gas production was analyzed by the PROC CORR procedure of SAS (SAS, 1996). Significance was declared at $P<0.05$ and considered to indicate a trend at $0.05<P<0.10$. The following model was used:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + \varepsilon_{ijk}$$

Where:

Y_{ijk} = the measured variable ,
μ = the overall mean,
α_i = the effect of SP level (i=1, 2, 3),
β_j = the effect of sugar level (j=1, 2, 3),
$\alpha\beta_{ij}$ = the effect of interaction term of SP and sugar level,
ε_{ijk} = residue error.

3.3.3 Results and Discussion

In this study, TMR diets were formulated to provide similar amounts of CP, average 14.0% CP, however, analyses ranged from 14.1-14.2%. Soluble protein (SP) increased at 5.4, 8.2, and 11.0% of the diet DM; sugar contained 9.1, 16.8, and 24.5% of DM for diet low, medium, and high-SP or sugar-level, respectively. Rice

straw was used as main source of fiber, contained 2.9, 76.0, or 50.9% of CP, NDF, or ADF, respectively. Additionally, soybean meal was reduced when increasing percentage of urea as nitrogen source (Table 3.5).

Table 3.5 Ingredient and chemical composition of experiment diets[a]

	L-SP			M-SP			H-SP		
Item	L-SU	M-SU	H-SU	L-SU	M-SU	H-SU	L-SU	M-SU	H-SU
Ingredients, %									
Rice straw	38.0	38.0	38.0	38.0	38.0	38.0	38.0	38.0	38.0
Cassava chips	30.4	21.4	12.4	39.2	30.2	21.2	48.0	39.0	30.0
Soybean meal	22.0	23.0	24.0	12.0	13.0	14.0	2.0	3.0	4.0
Cane sugar powder waste									
	8.0	16.0	24.0	8.0	16.0	24.0	8.0	16.0	24.0
Urea 46 % N	1.2	1.2	1.2	2.4	2.4	2.4	3.6	3.6	3.6
Vitamins-minerals									
	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Chemical composition, % of DM									
CP	14.18	14.16	14.13	14.21	14.19	14.17	14.24	14.22	14.20
SP	5.39	5.37	5.35	8.21	8.19	8.17	11.03	11.01	10.99
SP, % of CP	38.01	37.92	37.86	57.78	57.72	57.66	77.46	77.43	77.39
NPN, % of CP	24.33	24.36	24.42	48.55	48.62	48.69	72.68	72.78	72.89
EE	1.23	1.18	1.12	1.15	1.10	1.04	1.07	1.02	0.96
NDF	35.36	34.61	33.86	34.74	33.99	33.24	34.12	33.37	32.62
ADF	23.07	22.72	22.37	22.51	22.16	21.81	21.95	21.60	21.25
NFC	42.02	42.98	43.93	43.19	44.14	45.10	44.36	45.31	46.26
Ash	7.21	7.08	6.96	6.71	6.58	6.46	6.21	6.09	5.96
Total sugar	9.70	17.42	25.15	9.07	16.79	24.52	8.44	16.16	23.89
TDN	69.89	70.51	71.13	68.36	68.98	69.60	66.82	67.44	68.06
SP:sugar ratio	1:1.8	1:3.3	1:4.7	1:1.1	1:2.1	1:3.0	1:0.8	1:1.5	1:2.2

[a] L-SP-L-SU, low SP- low sugar; L-SP-M-SU, low SP-medium sugar; L-SP-H-SU, low SP- High sugar; M-SP-L-SU, medium SP-low sugar; M-SP-M-SU, medium SP- medium sugar; H-SP-L-SU, high SP-low sugar; H-SP-M-SU, high SP- high sugar; H-SP-H-SU, high SP-high sugar.

1) *In vitro* digestibility

There was a SP × sugar interaction that was statistically significant at 8 h of incubation ($P<0.05$) (Table 3.6), whereas, The SP × sugar interaction tended to be statistically significant for IVNDFD at 2 h, 8 h and 48 h of incubation ($P<0.10$). It was observed that IVDMD significantly increased (63.3%) for the diet medium in SP and high in sugar level; whereas, IVNDFD tended to increase at 2 h of incubation ($P<0.10$). The low- SP and low- sugar diet tended to increase IVADFD at 8 and 48 h of incubation ($P<0.10$). Differences in IVDMD, IVNDFD and IVADFD among dietary treatments were probably due to the differences in fiber and NFC levels among diets (Mandebvu et al., 2001). The ingredient combination in the diets was reported to have associative effect on diet digestibility (Blummel et al. 2003; Getachew et al., 2005).

At different time points of incubation the interaction was not significant, so these data are presented as means for SP (Table 3.7) and sugar (Table 3.8). With increasing SP level, there was linear increase in IVDMD at 2 and 8 h ($P<0.05$); however, there was quadratic response at 48 h of incubation. As level of SP increased, there was quadratic effect on IVNDFD at 2 ($P<0.01$) and 48 h ($P<0.05$), while at 4 h of incubation, there was linear increase ($P<0.01$). There was quadratic increase in IVADFD at 2 h ($P<0.10$) and 48 h ($P<0.01$). However, it was found lowest in IVADFD for medium SP level diet at 48 h of incubation ($P<0.01$) (Table 3.7 and Figure 3.3). These results indicated that at $\leq$ 8 h increased SP level improved microbial growth because of IVDMD, IVNDFD and IVADFD was increased.

It was significant increase in IVDMD ($P<0.05$) at 2, 4, 8 and 48 h of incubation when increasing sugar level in the diets (Table 3.8 and Figure 3.4). Increasing sugar level, caused linear increase in IVNDFD at 48 h of incubation ($P<0.05$). As level of sugar increased, there was quadratic increase in IVADFD at 8 h and 48 h of incubation ($P<0.05$). The results indicate that at 8 h of incubation, the effect of sugar might be due to a reduction in pH by its fermentation or the NFC-fermenting bacteria competing with the fiber-digesting bacteria for available N (Huhtanen and Khalili, 1991; Heldt et al., 1999). Hoover and Weimer (2007) found that sucrose was the predominant substrate at $\leq$ 8 h of fermentation and also yield of lactate tended to increase, linearly with increasing sucrose. Therefore, the quadratic

decrease in IVADFD of the TMR diet with increased sugar at 8 h in this study may be due to lowered pH and it negatively impacting celluolytic bacteria. According to Dryhurst and Wood (1998), when more rapidly fermentable carbohydrate was supplied, more N is required to meet bacterial growth requirement. Griswold et al. (1996) reported digestibility of ADF was increased by supplying true protein source compared to 100% of urea in an *in vitro* study. Although, in this study, the diet contained more N than recommended by Satter and Slyter (1974), the low SP (high true protein concentration) and low sugar level diet had higher IVNDFD and IVADFD (38.7 and 49.2%, respectively). Hoover et al. (2006) have reported that addition of sugar to the low (24% of DM) non-structural carbohydrate (NSC) diet reduce fiber digestion; however, fiber digestion was either not affected or increased in the 28 and 33% of NSC diets when sugar increased. The results from this study indicated that when the diet contained NFC at 42 to 46% of DM, supplementation of sugar had positive effect on IVNDFD and IVADFD in later hour of incubation. There was some interaction of soluble protein and sugar level indicating that availability of readily fermentable N and energy stimulates the activity of rumen microorganisms and enhances digestibility.

Table 3.6 The effects of increasing SP and sugar on *in vitro* digestibility of TMR diets at 2, 4, 8, 24, and 48 h of incubation

	L-SP			M-SP			H-SP				*P*-value		
	L-SU	M-SU	H-SU	L-SU	M-SU	H-SU	L-SU	M-SU	H-SU	SEM	SP	SU	SP*SU
IVDMD, %													
2 h	50.00	50.51	54.55	49.58	54.01	56.18	48.47	56.02	57.24	1.14	0.10	<0.01	0.12
4 h	53.83	51.51	56.57	53.92	57.69	58.43	53.00	58.33	58.02	1.24	0.06	0.01	0.11
8 h	58.43^{bcd}	55.88^{d}	58.09^{cd}	57.41^{cd}	58.41^{bcd}	63.26^{a}	57.24^{cd}	61.76^{ab}	60.76^{abc}	1.15	0.05	0.03	0.04
24 h	63.82	62.74	62.86	60.19	63.00	65.65	58.68	63.73	64.75	1.68	0.85	0.08	0.28
48 h	72.36	70.71	73.36	66.67	67.61	72.73	68.10	71.29	74.12	1.62	0.10	0.02	0.44
IVNDFD, %													
2 h	5.44	5.11	7.49	9.35	11.01	13.11	11.39	11.71	7.18	1.43	<0.01	0.87	0.09
4 h	10.04	6.57	10.05	13.82	10.42	16.22	12.86	14.55	14.13	1.63	0.01	0.18	0.41
8 h	19.21	10.70	13.47	16.52	15.55	19.00	15.51	15.75	15.66	3.05	0.61	0.48	0.56
24 h	26.62	22.72	15.86	21.58	20.29	23.94	19.36	19.87	20.29	2.31	0.50	0.44	0.10
48 h	38.65	35.81	37.83	27.26	25.50	33.67	26.74	32.12	36.45	2.49	<0.01	0.06	0.26
IVADFD, %													
2 h	12.91	7.72	8.35	5.60	9.14	12.47	14.07	15.28	12.68	2.09	0.03	0.96	0.12
4 h	17.83	9.25	12.36	11.29	10.61	16.85	18.28	17.75	13.91	2.65	0.23	0.42	0.19
8 h	26.85	11.08	13.57	18.51	13.59	18.45	20.61	18.49	18.17	2.34	0.48	0.01	0.06
24 h	30.41	21.83	14.42	19.69	19.85	20.71	21.10	20.29	19.69	3.13	0.67	0.16	0.15
48 h	49.15	36.08	45.68	24.66	22.39	36.59	22.91	31.33	38.46	3.63	<0.01	0.02	0.08

[a, b, c] Means within a row without a common superscript letter differ ($P<0.05$).

SP, soluble protein; SU, sugar; L-SP, low SP; M-SP, medium SP; H-SP, high SP; L-SU, low sugar; M-SU, medium sugar; H-SU, high sugar.

Table 3.7 The effects of SP level in TMR diets on *in vitro* digestibility with time of incubation

Item	Soluble protein level			*P*-value[1]	
	Low	Medium	High	L	Q
DM digestibility (IVDMD), %					
2 h	51.68	53.26	53.91	0.04	0.58
4 h	53.97	56.48	56.45	0.40	0.17
8 h	57.47	59.69	59.92	0.03	0.25
24 h	63.14	62.95	62.39	0.60	0.88
48 h	72.15	69.01	71.17	0.48	0.04
NDF digestibility (IVNDFD), %					
2 h	6.01	11.16	10.09	<0.01	0.01
4 h	8.89	13.49	13.85	<0.01	0.13
8 h	14.46	17.03	15.64	0.65	0.38
24 h	21.74	21.94	19.84	0.34	0.50
48 h	37.43	28.81	31.77	0.02	<0.01
ADF digestibility (IVADFD), %					
2 h	9.66	9.07	14.01	0.03	0.09
4 h	13.15	12.92	16.65	0.14	0.37
8 h	17.17	16.85	19.09	0.34	0.46
24 h	22.22	20.09	20.36	0.48	0.60
48 h	43.64	27.88	30.90	<0.01	<0.01

[1]L, linear; Q, quadratic.

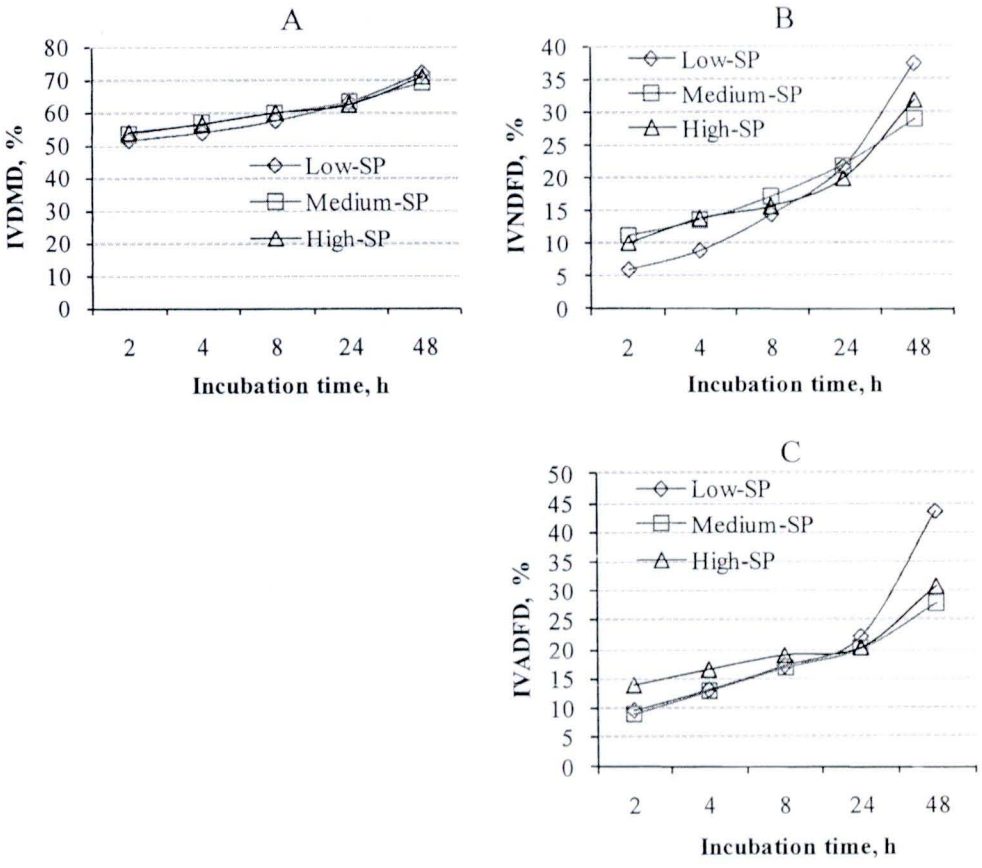

Figure 3.3 The effects of SP level in TMR diets on IVDMD (A), IVNDFD (B), IVADFD (C) with time of incubation

Table 3.8 The effects of sugar level in TMR diets on *in vitro* digestibility

Item	Sugar level			*P*-value[1]	
	Low	Medium	High	L	Q
DM digestibility (IVDMD), %					
2 h	49.35	53.51	55.99	<0.01	0.32
4 h	53.58	55.47	57.67	<0.01	0.83
8 h	57.70	58.69	60.70	0.01	0.54
24 h	60.70	63.16	64.42	0.03	0.68
48 h	69.05	69.87	73.41	<0.01	0.27
NDF digestibility (IVNDFD), %					
2 h	8.73	9.28	9.26	0.66	0.79
4 h	12.24	10.51	13.47	0.38	0.10
8 h	17.08	14.00	16.04	0.69	0.27
24 h	22.52	20.96	20.03	0.22	0.85
48 h	30.88	31.15	35.99	0.03	0.23
ADF digestibility (IVADFD), %					
2 h	10.86	10.71	11.17	0.86	0.84
4 h	15.80	12.54	14.37	0.53	0.25
8 h	21.99	14.39	16.73	0.02	0.01
24 h	23.74	20.66	18.27	0.06	0.88
48 h	32.24	29.94	40.24	0.02	0.04

[1]L, linear; Q, quadratic.

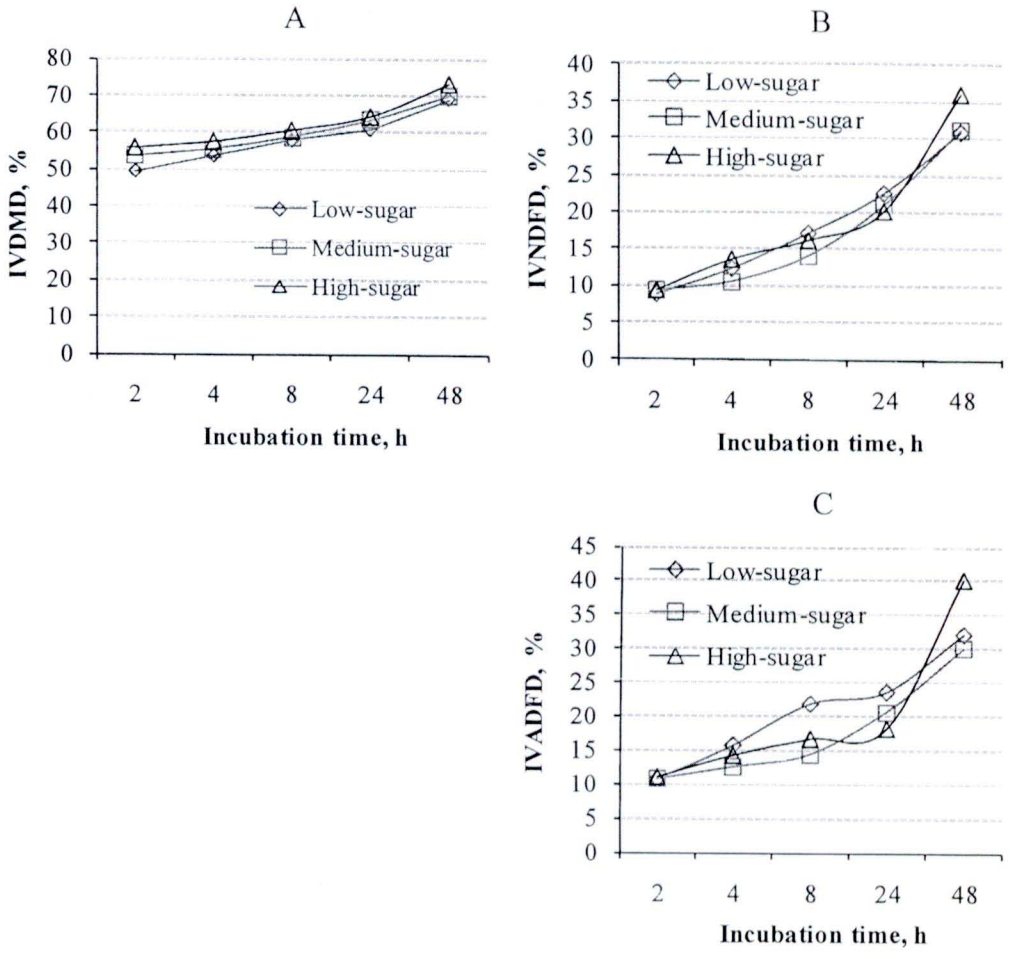

Figure 3.4 The effects of sugar level in TMR diets on IVDMD (A), IVNDFD (B), and IVADFD (C) with time of incubation

2) *In vitro* gas production

There were no interactions between SP and sugar levels on cumulative gas volume or kinetic of gas production (Table 3.9), so these data are presented as means for SP (Table 3.10) and sugar (Table 3.11). As SP level increased, there was linear increase in cumulative gas volume at 12 h ($P<0.01$) and 24 h ($P<0.05$) of incubation. The intercept (a) which ideally reflects the fermentation of the soluble fraction linearly decreased with increasing SP ($P<0.01$). However, it was not significant increase in potential of gas production with increasing SP level ($P>0.05$).

Positive associative effects on gas production were observed when sugar was increased, the effects were much stronger at early hours of incubation (4 and 8 h) compared to later hours (48 and 72 h). As level of sugar was increased,

there was linear increase in cumulative gas volume ($P<0.01$) at 4 and 8 h of incubation (Table 3.11). At increasing sugar levels, there was a linear increase in the intercept (a) ($P<0.01$) and rate of gas production ($P<0.05$), but potential extent of gas production and gas production from insoluble fraction expressed in ml. was linearly decreased with increased sugar level ($P<0.05$). This results are similar to Hall and Weimer (2007) who reported gas production from the insoluble fraction decreased linearly with increasing sucrose ($P<0.05$), suggesting a decrease in NDF fermentation. It is possible that gas production is a more sensitive tool for detecting differences in carbohydrate fermentation than is measurement of small quantities of unfermented diets.

Table 3.9 The effects of increasing SP and sugar on cumulative of gas volume in TMR diets[1]

Item	L-SP			M-SP			H-SP			SEM	*P*-value		
	L-SU	M-SU	H-SU	L-SU	M-SU	H-SU	L-SU	M-SU	H-SU		SP	SU	SP*SU
Gas volume, ml													
2 h	4.10	1.77	2.93	2.90	2.43	3.30	2.77	1.90	1.67	0.96	0.51	0.32	0.79
4 h	9.47	7.77	12.57	6.50	8.27	12.37	5.47	6.77	9.83	1.49	0.13	<0.01	0.76
8 h	21.43	21.30	28.90	17.57	23.83	30.27	17.43	22.10	29.67	2.26	0.88	<0.01	0.63
12 h	35.93	32.53	39.13	36.93	41.67	43.33	43.57	45.53	45.90	2.78	<0.01	0.22	0.60
24 h	65.17	46.03	50.13	54.77	64.70	57.93	71.43	62.60	61.67	6.32	0.11	0.35	0.27
48 h	85.23	58.27	60.87	68.90	81.97	72.83	87.67	76.37	74.63	8.89	0.31	0.30	0.29
72 h	98.73	66.43	71.03	77.33	92.03	82.23	95.37	82.93	80.67	10.01	0.64	0.29	0.25
Kinetic of gas production[2]													
a, ml	-4.29	-2.54	-0.95	-5.31	-5.34	-1.95	-9.67	-7.24	-5.28	0.81	<0.01	<0.01	0.58
b, ml	108.26	68.53	68.34	84.27	98.50	81.54	108.09	90.63	84.96	11.19	0.39	0.08	0.23
c, %/h	0.039	0.058	0.069	0.053	0.049	0.061	0.053	0.061	0.069	0.01	0.55	0.04	0.66
$\|a\|+b$, ml	112.55	71.08	69.28	89.58	103.84	83.65	117.77	97.87	90.23	11.31	0.19	0.04	0.22

[1] SP, soluble protein; SU, sugar; L-SP, low SP; M-SP, medium SP; H-SP, high SP; L-SU, low sugar; M-SU, medium sugar; H-SU, high sugar.

[2] Equation $y = a+b\ [(1-Exp^{(-ct)}]$ (Ørskov and McDonald, 1979); *a*, the intercept which ideally reflects the fermentation of the soluble fraction; *b*, the gas production from insoluble fraction; *c*, the rate of gas production; $|a|+b$, the potential gas production.

Table 3.10 The effects of SP level in TMR diets on *in vitro* cumulative of gas production and kinetic

Item	Soluble protein level			*P*-value[2]	
	Low	Medium	High	L	Q
Cumulative of gas production, ml					
2 h	2.93	2.88	2.11	0.31	0.61
4 h	9.93	9.04	7.36	0.05	0.71
8 h	23.88	23.89	23.07	0.67	0.80
12 h	35.87	40.64	45.00	<0.01	0.92
24 h	53.78	59.13	65.23	0.04	0.93
48 h	68.12	74.57	79.56	0.13	0.91
72 h	78.73	83.87	86.32	0.36	0.85
Kinetic of gas production[1]					
a, ml.	-2.59	-4.20	-7.40	<0.01	0.18
b, ml.	81.71	88.10	94.56	0.18	0.99
c, %/h	0.055	0.054	0.061	0.40	0.50
$\|a\|+b$, ml	83.30	62.36	101.96	0.07	0.92

[1] Equation $y = a+b\ [(1-Exp^{(-ct)}]$ (Ørskov and McDonald, 1979); *a*, the intercept which ideally reflects the fermentation of the soluble fraction; *b*, the gas production from insoluble fraction; *c*, the rate of gas production; $|a|+b$, the potential gas production.

[2] L, linear; Q, quadratic.

Table 3.11 The effects of sugar level in TMR diets on *in vitro* cumulative of gas production and kinetic

Item	Sugar level			*P*-value[2]	
	Low	Medium	High	L	Q
Cumulative of gas production, ml					
2 h	3.25	2.03	2.63	0.44	0.19
4 h	7.14	7.60	11.59	<0.01	0.11
8 h	18.81	22.41	29.61	<0.01	0.27
12 h	38.81	39.91	42.79	0.10	0.66
24 h	63.79	57.78	56.58	0.18	0.60
48 h	80.60	72.20	69.44	0.14	0.66
72 h	90.48	80.47	77.98	0.14	0.60
Kinetic of gas production[1]					
a, ml.	-6.43	-5.04	-2.72	<0.01	0.43
b, ml.	100.21	85.89	78.29	0.03	0.68
c, %/h	0.048	0.056	0.066	0.01	0.88
$\|a\|+b$, ml	106.6	90.93	81.06	0.01	0.72

[1] Equation $y = a+b\ [(1-Exp^{(-ct)}]$ (Ørskov and McDonald, 1979); *a*, the intercept which ideally reflects the fermentation of the soluble fraction; *b*, the gas production from insoluble fraction; *c*, the rate of gas production; $|a|+b$, the potential gas production.
[2]L, linear; Q, quadratic.

3) Relationship between IVDMD and gas production

Vogel et al. (1999) and Mabjeesh et al. (2000) reported high digestibility for the ANKOM method compared to traditional *in vitro* methods. However, Holden (1999) reported that comparison of methods of *in vitro* DM digestibility, the ANKOM method has resulted in similar results to Tilly and Terry procedures in determining digestibility of ten feeds (forage, grain and TMR). Blummel and Orskov (1993) reported gas measurement can be considered an estimate

of apparent rumen digestibility. Apori et al. (1998) also reported a positive correlation between 24 h gas production and 24 h IVDMD in browse leaves (r=0.70).

The relationship between IVDMD and gas production is shown in Figure 3.5. Positive correlation between IVDMD and cumulative gas production (r=0.82) is observed in present study. The results indicated positive correlation between IVDMD and gas production in diets with high level of soluble protein and sugar. Since truly digested substrates is metabolized into VFA, gases and microbial biomass. Gas production reflects the amount of substrate used for VFA and is indicative of quantitative VFA production. However, it has been shown that gas production is positively related to microbial protein synthesis (Krishnamoorthy et al, 1991). The *in vitro* digestibility and *in vitro* gas production were batch culture, in which, during the initial hours of incubation, substrate availability generally does not limit rate of microbial growth, whereas in later stages substrate is exhausted and inhibit microbial growth. The high correlations was observed between IVDMD and gas production technique indicate that both method are useful tool to evaluate the nutritive value of feeds

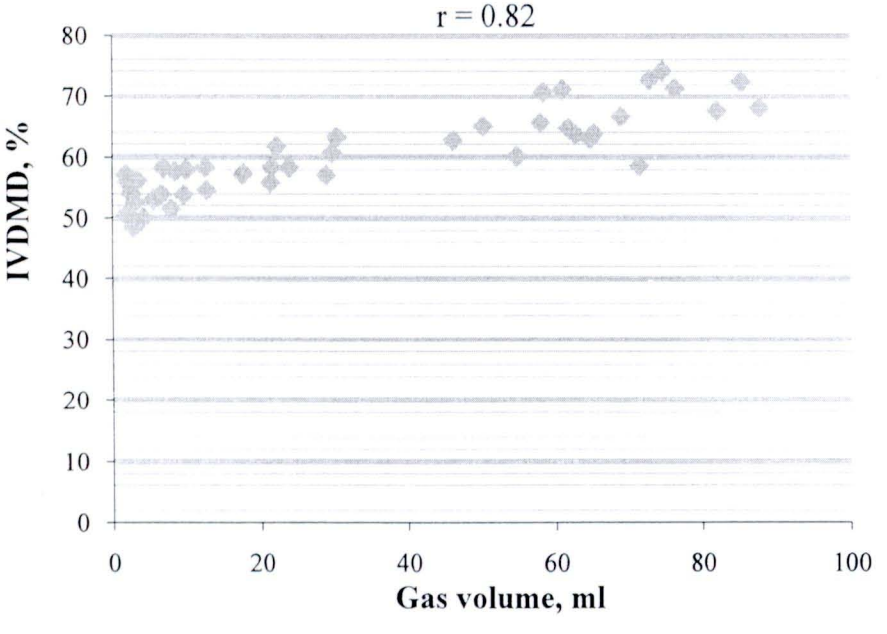

Figure 3.5 The correlation between IVDMD and gas production when increasing soluble protein and sugar in TMR diets

3.3.4 Conclusions

Increasing soluble protein (5 to 11% of the DM) and sugar level (8 to 24% of the DM) can alter digestibility and gas production. Increasing SP and sugar level had more positive effects on diets digestibility at ≤ 8 h of incubation, resulting in improved DM and fiber digestibility at 48 h of fermentation. The potential gas production increased with increasing SP level, however, the potential gas production and that from insoluble fraction decreased with increasing sugar. When the SP: sugar ratio is 1:1.8, or Low-SP (5% of the DM) and low sugar (8% of the DM) supplementation had a more positive effect on diet digestibility, gas production from the insoluble fraction. Where TMR diets contained high solubility nutrient, the high correlations was observed between IVDMD and *in vitro* gas production.