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Comparative Study between Three-*Step In Vitro* and Modified Three-Step *In Vitro* Procedures in Small Intestinal Digestibility of Rumen Undegraded Protein

Yan Sumei[1*], Zhao Yanli[1], Guo Xiaoyu[1], Han Shuai[2], Zhu Xiaowei[2] and Zhang Fan[3]

[1]Department of Animal Science, Inner Mongolia Agricultural University, Inner Mongolia, China

[2]Department of Animal Science, Inner Mongolia Medical University, Hohhot, China

[3]Department of Animal Science, Tianjin Medical University Cancer Institute and Hospital, Tianjin, China

[*]**Corresponding author:** Yan Sumei, Department of Animal Science, Inner Mongolia Agricultural University, Inner Mongolia, China, Tel: 15222390529; E-mail: naruto1989@qq.com

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Abstract

The experiment was carried out using fistulated multiparous Chinese Holstein dairy cows in their lactation period to evaluate the nutritive value of local protein feed resources. Rumen Undegraded Protein (RUP) digestibility in eleven types of concentrates was estimated by Three Step *in vitro* Procedure（TSP）and Modified Three Step *in vitro* Procedure (MTSP) and correlation. There were eleven experimental feeds were chosen and air dried to investigate the effect of different growth period and variety on nutrition value and its RUP digestibility. In this experiment, the small intestinal digestibility of RUP by TSP in concentrated feed was about 65%, higher than in roughages, among them, SBM (soybean mean) had the highest percentage (79%) and corn had the lowest (65%), DDGS (distillers dried grains with solubles) and SFM (Sunflower Meal) were 70.9 and 74.9%, respectively. ASS (alfalfa mowed at squaring stage) had the highest small intestinal digestibility of RUP (55%) among roughages, and WCS (Whole-Plant Corn Silage) had the lowest (40.5%).

When small intestinal digestibility of RUP was determined using the MTSP method, exhibited similar results to the TSP method but values were generally higher, and there was a strong significant correlation between them (R_2=0.967, p< 0.01).

Keywords: Small intestinal digestibility; Original three-step *in vitro* procedure; Modified three-step *in vitro* procedure

Introduction

Many reports indicate that one of the most important nutritional limiting factors for ruminants is protein [1]. Recent trends in protein in small intestinal of ruminants are such that the Digestible Crude Protein (DCP). The DCP in small intestinal is supplied primarily by combinations of Microbial Protein (MCP), Rumen Undegraded Protein (RUP) and endogenous proteins [2]. Among them, RUP content varied from 30-50%, MCP contents are stable and endogenous proteins are a small proportion of three these. So the difference in RUP leads to composition variation in DCP. The availability of nitrogen in growing ruminants is very important, and the rate and extent of protein degradation in the rumen directly determine this indicator. Small intestinal digestibility of RUP has an important effect on milk production and growth performance. RUP supplementation in ruminant diets improves the effective use of protein. The supply of protein in the feed small intestine is obviously very important, and the digestibility of rumen undegraded protein in the small intestine can reflect the supply of protein in the small intestine [3]. Tests have shown that the digestibility of feed in small intestinal digestibility of ruminants is related to many factors change to change, like the feed type, processing technology and origin [4]. Therefore, it is particularly important to study different raw materials in small intestinal digestibility of rumen.

Over the past several decades, much of the research on small intestinal digestibility used mobile nylon bag and *in vitro* method [5,6]. The method of measurement has been to put the feed into a nylon bag of the rumen of the ruminant through a fistula and incubate it in the rumen to determine the degree of rumen degradation of the feed protein [7]. A three-step *in vitro* procedure is a way to measure the feed N which escapes rumen degradation and digestibility [8]. However, Cows should be equipped with ruminal and duodenal cannulas to determine the RUP of mobile nylon bag technique. The different physiological of experimental animal compared with normal mice is ineluctable. Calsamiglia and Stern expressed the view that the Three Step *in vitro* Procedure (TSP) which used comprehensive rumen nylon bag technique and *in vitro* method, and simulates the physiological conditions of ruminants, is easy to be standardized and is economical [8]. Gargallo developed a Modified Three Step *in vitro* Procedure (MTSP) to improve the TSP, and reported that the although the two methods are highly relevant R_2=0.98, P<0.001. MTSP requires less labor and time, reduces pollution and promotes commercial trials [9]. The aim at the present study was to contribute to the knowledge of the small intestinal digestibility RUP of different protein sources

by TSP and MTSP, and provide a theoretical basis of a simplified method to examine the small intestinal digestibility.

Materials and Methods

Experimental animals and design

Four Chinese Holstein cows, with an average BW of 550 (± 28 kgs), 20 (± 2.3 kgs) daily milk production and lactation days of 156 (± 8.2) at the start of the experiment, were used for the study. Total Mixed Ration (TMR) and forage was used in a concentrate ratio of 45:55 (Table 1). The cows were feeded libitum twice daily at 06:30 am and 04:30 pm. Fresh water was ensured during the experimental period. The feed was started 10 days before the experiment. In this experiment, eleven types of concentrates which can pass through a 2.5 mm sieve were prepared. The eleven types of concentrates were Corn, SBM (Soybean Mean), DDGS (Distillers Dried Grains with Solubles), SFM (Sunflower Meal), CS (Corn Stalk), WCS (Whole-Plant Corn Silage), CH (Corn Haylage), CW (Chinese wildrye), ASS (Alfalfa mowed at Squaring Stage), AFS (Full-Bloom Stage) and APS (pod stage). Three varieties of ASS, AFS and APS were chosen to investigate the effect of different growth period and variety on nutrition value and its RUP digestibility. It was tested in Hohhot to mo te zuo qi Xiao dan dam Ranch.

Table 1: Ingredients and nutritive value of experimental diets.

Ingredients	%	Nutritive value	%
Corn	25.36	DM	87.2
Soybean meal	6.56	NEL* (MJ/kg)	5.92
Sunflower cake	3.26	CP	10.65
Cottonseed meal	5.4	Ca	0.62
bran	1.69	P	0.37
Sodium bicarbonate	0.52	NDF	45.35
Calcium phosphate dibasic	0.85	ADF	27.83
Salt	0.52	ADL	6.21
Powder	0.39	AIA	1.37
Premix	0.45		
Corn silage	55		
Total	100		

DM=Dry Matter; CP=Crude Protein; NDF=Neutral Detergent Fiber; ADF=Acid Detergent Fiber; ADL=Acid Detergent Lignin; AIA=Acid Insoluble

Chemical analysis

The supplied sample was dried at 65°C for 5 hours to remove the initial moisture in the sample. The samples were analysed for Dry Matter (DM) according to the moisture measurement method. This experiment used the Kjeldahl method to determine crude protein, and used the methods of Van Soest et al. to determine Neutral Detergent Fiber (NDF) and Acid Detergent Fiber (ADF) and ADL [10,11].

Original TSP

There were three parallels per cow. Nylon bags were incubated one hour before morning feeding and were removed, washed and dried at 65°C to constant weight, CP level of the residue was measured, after the feedstuff was pre incubated in the rumen for 16 h and rinsed with tap water. The residue (15 mg N) was introduced into a 50 ml centrifuge tube and 10 ml of HCl (pH 1.9) containing 1 g/L pepsin (sigma P-7012). Incubate the tube for 1 hour of 38°C with constant rotation before 13.5 ml Trypsin solution (pH 7.8 phosphate buffers, 50 ppm Thymol, 3 g/L trypsin (sigma P-7545) were added and incubated for 24 h with constant rotation at 38°C. After 24 h, 3 ml 100% trichloroacetic acid was added and left for 15 min, before the supernatant was collected by centrifugation (10000 rpm, 15 min). Kjeldahl nitrogen method was used to analyze N content of the supernatant. Calculation formula as follows:

% Digestibility=(% TCA soluble N in, g/% TCA soluble out, g) × 100 % .

Modi ied TSP

The sample test also used the modified TSP. Briefly, 1 to 2 g of the residue were weighed into bags of duplicate in each sample of three times after incubated in the rumen for 16 h. And then the residue was sealed in a nylon bag (R510), and the nylon bag was placed in a Daisy II flask, and each bottle was allowed to hold 30 nylon bags. The solution to the culture flask was 2 L of a hydrochloric acid solution having a pH of 1.9 containing 1 g/L pepsin (P-7000, sigma). Incubate at 39°C for 1 h. After that, the nylon bag was taken out and rinsed, and 2 L of a trypsin solution (0.5 mol/L phosphate buffer, 50 ppm thymol, 3 g/L trypsin (P-7545, sigma)) was added, and cultured at 39°C for 24 h. After 24 hours, the nylon bag was taken out and rinsed, and the weight was constant at 65°C. The content of CP in the residue was determined.

Calculation formula for digestibility of RUP as follows:

% Digestibility=[(amount of RUP in, g,-amount of RUP put, g)/ amount of RUP in, g] × 100%.

Statistical analysis

The data was processed using the GLM program of SAS 9.0 (2002). Data analysis uses a completely randomized design paired t test. TSP method and MTSP method were treated as random effects in the experiment. Statistical evaluation of eleven kinds of feedstuff was calculated by variance test. The CORR procedure of SAS was utilized to examine the relationship between standardized TSP and MTSP method in small intestinal digestibility of RUP.

Results

Nutrients composition

The chemical composition of the feeds used in this study is presented in Table 2. Table 2 lists the chemical composition of the feed used. All 11 feeds Dry Matter (DM) was similar except for corn and SBM. NDF (75%) was the highest in CS and so was ADF (42.1%), while the lowest ADF (1.8%) was in corn, and SBM had the lowest NDF (15.3%). Ranking of CP from low to high be CS, CH, WCS, CW, corn, AH, DDGS, SFM and SBM. Three varieties of alfalfa mowed at squaring stage, full-bloom stage and pod stage were measured. Obviously the content of CP, reduced as the growth period extended; while the components content of NDF, ADF and ADL increased.

Table 2: The contents of DM, CP, NDF, ADF and ADL in raw feeds (%)

Feedstuff	DM	CP	NDF	ADF	ADL
Corn	85.54	8.69	15.57	1.78	0.13
SBM	87.49	43.15	15.34	6.37	0.93
DDGS	95.7	24.49	46.18	16.86	8.63
SFM	96.82	29.1	45.96	23.68	9.71
CS	95.2	3.84	75.01	42.12	4.38
WCS	94.27	4.69	69.63	41.37	4.34
CH	95.43	12.54	63.53	35.72	6.14
CW	94.85	8.62	66.58	38.55	5.24
ASS	95.14	10.58	65.06	37.14	5.69
AFS	95	9.6	65.82	37.84	5.47
APS	95	9.6	65.82	37.84	5.47

SBM=Soybean Mean; DDGS=Distillers Dried Grains with Soluble; SFM=Sunflower Meal; CS=Corn Stalk; WCS=Whole-Plant Corn Silage; CH=Chinese Wildrye; CW=Chinese Wildrye; ASS=Alfalfa Mowed at Squaring Stage; AFS= Full-Bloom Stage; APS=Pod Stage

The small intestinal digestibility of RUP

The small intestinal digestibility of RUP is presented in Table 3. In the TSP method, SBM and SFM were highly digested than others. The small intestine digestibility of RUP in concentrated feed is approximately over 65% and is higher than roughage. ASS has the highest RUP intestinal digestibility in roughage, about 55%, while WCS has the lowest intestinal digestibility at all, about 40.5%. This feature is also applicable in the MTSP method.

Table 3: The small intestinal digestibility of RUP.

Feedstuff	The TSP method	The MTSP method
Corn	65.38 ± 1.78^{c}	91.41 ± 0.21^{b}
SBM	79.01 ± 3.40^{a}	98.22 ± 0.24^{a}
DDGS	70.94 ± 2.68^{bc}	88.49 ± 1.26b
SFM	74.85 ± 2.13^{ab}	92.98 ± 0.56^{ab}
CS	48.89 ± 4.63^{ef}	63.47 ± 2.48^{de}
WCS	40.55 ± 0.90^{g}	55.01 ± 3.17^{f}
CH	43.57 ± 3.44^{gf}	57.50 ± 5.32^{ef}
CW	49.92 ± 2.46^{ef}	64.80 ± 2.04^{d}
ASS	58.51 ± 3.64^{d}	74.50 ± 2.26^{c}
AFS	52.68 ± 0.88d^{e}	67.45 ± 2.93^{d}
APS	52.84 ± 3.08^{de}	67.35 ± 3.10^{d}
Between differenent feeds	P<0.001	
SEM	57.92A	74.65B
TTEST	P<0.001	

DM=Dry Matter; CP=Crude Protein; NDF=Neutral Detergent Fiber; ADF=Acid Detergent Fiber; ADL=Acid Detergent Lignin; AIA=Acid Insoluble; A–B Means within a row with different superscripts differ significantly (P< 0.01).; a–g Means within a column with different superscripts differ significantly (P< 0.001).; TTEST=Paired T Test

Also the 11 kinds of feeds were analyzed by paired T test in different methods. All experimental values of MTSP are higher than TSP method and the difference was significant after the paired T test.

Correlation analysis

The Data analyses for the small intestinal digestibility of RUP showed that a high positive correlation existed between the TSP Method and the MTSP Method on small intestinal digestibility of RUP (Y=1.1864X+5.7186, R_2=0.967, p<0.0001). There had a good correlation between the TSP methods and MTSP methods for the different feedstuffs tested. But there was no significant correlation of measured results between the content of CP in feedstuffs after incubated 16 h in rumen and after intestinal digestion (Y=-0.014X2+0.2369X+0.1477 R_2=0.3350, p=0.1594).

Discussion

The CP content of SBM (43.15) in raw feeds obtained in this study was similar to reported by Yao Xue-bo etc and C. Promkot et al. [12]. The CP content of corn in this study was comparable to that reported by C. Mikolayunas etc [13]. While that of DDGS, was in the range reported by D.H. Kleinschmit [14,15]. Expressed that the high RUP sources that were compared with SBM were Distillers Dried Grains (DDG), DDGS, and Heated SBM (HSBM). The CP degradability *in vitro* pepsin-pancreatin digestibility of feedstuffs for ruminants had been reported a lot for recent years. They reached the same conclusion that small intestinal digestibility of RUP in concentrated feed is higher than for roughages. Used the TSP method to study small intestinal digestibility of DDGS from different producing areas after 12 h incubation in rumen and concluded 70.9% by TSP method in this test, and slightly higher by MTSP method. This may be caused by different time incubation in rumen. C. Promkot reported that small intestinal digestibilities of cassava hay, SBM and DDG were 70.4%, 79.8% and 71.7%. The NRC (2001) found small intestinal digestibility of RUP of DDGS, corn, SBM, CS, alfalfa meal, values were respectively 80, 90, 93, 70 and 75%. Under this test condition, TSP method result was slightly lower compared to NRC, while MTSP method was identical with NRC. High-protein, low-fiber feeds are easily digested and utilized by the small intestine [16]. The ruminal degradation rate of corn stalk protein is lower than that of corn stalk silage, which is consistent with this experiment [17].

Small intestinal digestibility of RUP measured by TSP in roughages was lower than concentrated feed. This may be due to the protein of forage being degraded in the rumen, and the remainder combined with the lignin which would have been difficult to be digested. The MTSP method also provided similar results, but the determination of the value was slightly higher than the TSP method. A possible explanation is that polypeptides and small peptides in the residues which after incubation in rumen, were precipitated by trichloroacetic acid. In this experiment, Data analyses for the small intestinal digestibility of RUP showed that a high positive correlation existed between the TSP Method and the MTSP Method on small intestinal digestibility of RUP ($Y=1.1864X+5.7186$, $R_2=0.967$, $p<0.0001$, Y=Small intestinal digestibility of RUP by TSP Method, X=Small intestinal digestibility of RUP by MTSP Method). In this experiment, Data analyses for the small intestinal digestibility of RUP showed that a high positive correlation existed between the TSP Method and the MTSP Method on small intestinal digestibility of RUP, and the results of the correlation test and the paired T test are consistent. Gargallo, etc. Expressed that $MTSP=1.37 \times TSP-15.45$ ($R_2=0.98$, $P<0.001$, n=14, Y=The protein contents after 16 h incubation in rumen (%), X=The protein contents after small intestine digestion by MTSP method (%)).

Reported that the RUP in 13 kinds of concentrates were measured by TSP and MTSP. The result is lower than the results of this test, which may be related to factors such as feed type and origin. It was reported that the improvement of the three-step *in vitro* measurement results was 0.8383 in comparison with the measurement results of the mobile nylon bag method [18].

Conclusion

Used the TSP method to study small intestinal digestibility of DDGS from different producing areas after 12 h incubation in rumen and concluded 70.94% by TSP method in this test, and slightly higher by MTSP method. This may be caused by different time incubation in rumen. C. Promkot reported that small intestinal digestibilities of cassava hay, SBM and DDG were 70.4, 79.8 and 71.7%.

Small intestinal digestibility of RUP in concentrated feed was higher than in roughages. SBM had the highest (79.0%). Small intestinal digestibility of RUP and WCS had the lowest (40.5%) by TSP. Small intestinal digestibility of RUP determined by MTSP was slightly higher than TSP, and there was a strong significant correlation between them. Compared with the TSP, the modifications could measure a maximum of 120 samples at one time, faster and more efficient. MTSP also avoids the use of trichloroacetic acid which is corrosive, toxic and pollutes the environment. So it facilitates assessment of feed digestibility RUP.

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Authors' contributions

Han Shuai (FG/ES) and these authors contributed equally to this work and should be considered co-first authors

Zhang Fan (FG/ES) and these authors contributed equally to this work and should be considered co-first authors

Yan Sumei (ES) and Corresponding author

Zhu Xiaowei (JY)

Zhao Yanli (MT)

Guo Xiaoyu (MT)

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Competing Interests

I hereby declare that the co-authors of this manuscript, familiar with its content, have given their consent to publish the manuscript in the presented form in the Animal Diseases and all authors declare that there is no conflict of interest.

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