Effect of Total Mixed Ration Composition on Fermentation and Efficiency of Ruminal Microbial Crude Protein Synthesis In Vitro

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ABSTRACT

The goal of this study was to identify dietary factors that affect fermentation and efficiency of microbial crude protein (CP_M) synthesis in the rumen in vitro. We used 16 total mixed, dairy cow rations with known digestibilities that varied in ingredient composition and nutrient content. Each ration was incubated in a Rusitec (n = 3) for 15 d, and fermentation of different fractions was assessed. Observed extents of fermentation in 24 h were 35 to 47% for organic matter, 25 to 60% for crude protein, 3 to 28% for neutral detergent fiber, and 31 to 45% for gross energy. Organic matter fermentation depended on the content of crude protein and neutral detergent fiber in the ration. We studied net synthesis of CP_M using an ¹⁵N dilution technique and found that 7 d of continuous ¹⁵N application are needed to achieve an ¹⁵N enrichment plateau in the N of isolated microbes in this type of study. The efficiency of CP_M synthesis was 141 to 286 g/kg of fermented organic matter or 4.9 to 11.1 g/MJ of metabolizable energy, and these ranges agree with those found in the literature. Multiple regressions to predict the efficiency of CP_M synthesis by diet data showed that crude protein was the only dietary chemical fraction that had a significant effect. Fat content and the inclusion rate of corn silage in the ration also tended to improve efficiency. We suggest that microbial need for preformed amino acids may explain the crude protein effect. A large part of the variation in efficiency of microbial activity still remains unexplained.

Key words: Rusitec, fermentation, efficiency, microbial yield

INTRODUCTION

The amount of microbial CP (CP_M) synthesized in the rumen plays a major role in providing the cow with amino acids for milk synthesis. Because fermentable energy limits CP_M synthesis (Hoover and Stokes, 1991)

and protein supply at the duodenum may limit milk protein synthesis (Clark, 1975; Wright et al., 1998), the prediction of the efficiency of CP_M synthesis is of value for feeding high-yielding dairy cows. If dietary factors were known to alter CP_M synthesis in the rumen, they could be used to influence or to predict microbial yield.

The mean of 69 observations reviewed by Stern and Hoover (1979) indicates that 169 g of CP_M/kg of apparently digested OM is synthesized with a range from 63 to 307 g. The efficiency of CP_M synthesis, determined with cannulated cows, is about 10.1 g of CP_M/MJ of ME (SD = 1.5 g) and 156 g of CP_M/kg of digestible OM (SD = 24 g), respectively, based on a recent review (Gesellschaft für Ernährungsphysiologie, 2001). In vitro investigations have shown similar results (Stokes et al., 1991; Bach et al., 1999).

Reasons for this wide range may originate from the methods of determination of CP_M (Stern and Hoover, 1979), the supply of energy and nitrogen (Veira et al., 1980; Kang-Meznarich and Broderick, 1981; Madsen and Hvelplund, 1988) and its synchronization (Herrera-Saldana et al., 1990; Aldrich et al., 1993; Sinclair et al., 1993), the availability of N (Siddons et al., 1985; Kajikawa et al., 2002), the supply of P (Petri et al., 1988), the feedstuffs (Hristov and Broderick, 1996; Sutton et al., 2000; Younge et al., 2004), or the management of feeding (Chamberlain and Thomas, 1979; Verbiè et al., 1999). The amount of fermentable OM is a dominant factor influencing CP_M synthesis in the rumen because it depends on the availability of N and carbohydrates (NRC, 2001).

The association between high levels of DMI and high amounts of concentrates has raised interest in feeding TMR to dairy cows. Such rations offer the opportunity to stabilize highly efficient ruminal digestion (Coenen, 1996), but systematic studies on factors that determine the efficiency of CP_M synthesis with TMR feeding have not been reported.

The purpose of this study was to determine the extent of fermentation of different nutrients from TMR and to derive equations that allow for an identification of factors influencing efficiency of CP_M synthesis. When such prediction equations are to be calculated mathematically, many data are generally needed, as is also

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Table 1. Composition of the 16 TMR (% of DM)

Ingredient	TMR															
	A ¹	B ^{1,2}	C	D	E	F	G	H	I	J	K ³	L ³	M	N	O	P ³
Corn silage	25.0	24.0	42.2	36.9	24.7		33.0	53.8	20.5	10.1	19.0	19.0	13.1	16.6	45.6	40.0
Grass silage	23.0	23.0	11.2	13.0	44.4	93.0	55.6	27.9	36.8	31.9	30.0		81.9	37.3	48.7	13.0
Beet pulp silage			4.7	6.8					6.1	6.0						
Alfalfa silage					13.6				14.0			29.0				
Corn cob silage	17.0	17.0									20.0	20.0				10.0
Brewers grains silage	7.0	7.0				7.0					4.0	4.0				8.0
Hay, straw											1.0	1.0	5.0		5.7	1.0
Grains (barley, wheat)	9.0	8.0			17.3				22.6	29.4	9.0	12.0		23.0		9.0
Soybean meal (SBM) ⁴	16.0									19.1				23.1		
Rapeseed meal (RSM) ⁴		17.0								3.5	4.0	4.0				10.0
SBM and RSM (protected)											12.0	10.0				8.0
Peas							11.4	18.3								
Farm mix ⁵			41.9	43.3												

¹Additional ingredient: 3% mineral and vitamin premix and propyleneglycol.

²Additional ingredient: 1% protected fat.

³Additional ingredient: 1.0% mineral and vitamin premix.

⁴Solvent extracted.

⁵Fish meal, corn, palm cake, rapeseed meal, soybean meal, barley, fat (protected), mineral and vitamin premix, and propyleneglycol.

a wide range in nutrient content. Such a data set, including CP_M synthesis, is difficult to obtain with dairy cows because these studies depend on cannulated cows and are prohibitively costly. In vitro methods provide an easier, less costly way to describe fundamental processes of rumen physiology and to define precisely the conditions used in experiments (Czerkawski and Breckenridge, 1985).

MATERIALS AND METHODS

Description and Preparation of Diets

Sixteen TMR were used (Table 1). They were composed to represent rations that are typical for the German feedstuff basis for dairy cows in different stages of lactation. Each TMR contained at least one silage. Corn silage, grass silage, or both were components in 15 of the 16 TMR. Silages from beet pulp, alfalfa, moist ensiled corn, and brewers' grains also were included. In most diets, the concentrates were barley or solvent-extracted meals from soybean and rapeseed. Two TMR contained peas. Ingredients were individually weighed into plastic bags, mixed, and stored at -18°C until further handling. Before the beginning of the experiment, the TMR were thawed, dried at 65°C for 24 h, and ground through a sieve with 1-mm pore size in a hammer mill.

The TMR showed a large variation in analyzed nutrient concentrations (Table 2). The concentrations (g/kg of DM) were from 874 to 947 for OM, 123 to 213 for CP, 159 to 282 for crude fiber, and 20.3 to 47.6 for ether extract. The NDF and ADF concentrations ranged from 290 to 552 and 163 to 314 g/kg of DM, respectively. All

rations had been previously investigated for nutrient digestibility (Boguhn et al., 2003; Bulang et al., 2004; Kluth et al., 2005) using wether sheep according to the standard method applied in Germany (Ausschuss für Bedarfsnormen, 1991). The energy value as calculated from digestible crude nutrients according to Gesellschaft für Ernährungsphysiologie (1995) ranged from 8.2 to 11.9 MJ/kg of DM for ME and from 4.8 to 7.4 MJ/kg of DM for NE_L (calculated according to van Es, 1978).

Experimental Procedure

The inocula for starting the incubation were obtained from 4 rumen-cannulated sheep (Schwarzköpfiges Fleischschaf). Rumen contents from all sheep were mixed and filtered through 2 layers of linen cloth. Sheep had been fed grass hay ad libitum and an additional 200 g of a concentrate mix and 10 g of a mineral mix/d. Alterations in the fermentation behavior were intentionally minimized by using the same ingredients over time and by using mixes from the donor animals.

The apparatus used was a modified rumen simulation technique (Rusitec) described by Czerkawski and Breckenridge (1977) in which 6 reaction vessels had a capacity of 800 mL each. Three vessels were used per ration. Each feed container inside the vessel contained 2 nylon bags (~100 × 50 mm, pore size = 100 µm, Fa. Linker Industrie-Technik GmbH, Kassel, Germany) filled with 15.0 g of the TMR that had been dried for 24 h at 65°C. At 24-h intervals, one bag was replaced with a new one so that each bag was incubated for 48 h. At the start of the system for the first 24 h, one bag was filled with pooled rumen solids (~60 g) instead of

Table 2. Chemical analysis of the 16 TMR (DM, g/kg; nutrients and detergent fibers, g/kg of DM) and energy value calculated from digestible crude nutrients (MJ/kg of DM)

TMR	DM _{original} ¹	DM _{dry} ²	OM	CP	Crude fiber	Ether extract	NDF	ADF	ME ³	NE _L ³
A	439	937	933	186	171	20.3	305	163	11.8	7.3
B	438	932	937	162	175	30.6	332	185	11.5	7.0
C	485	925	914	195	168	37.8	314	188	11.5	7.1
D	445	932	918	200	164	37.5	316	206	11.6	7.1
E	384	947	928	123	257	35.9	456	267	9.6	5.7
F	352	945	886	174	282	47.6	497	289	10.7	6.4
G	379	926	893	179	233	41.0	373	233	11.4	7.0
H	346	956	928	134	233	39.8	364	224	11.5	7.1
I	364	954	933	128	271	26.7	461	280	9.7	5.8
J	314	942	921	150	239	30.2	406	237	11.4	7.0
K	448	941	935	172	179	40.4	344	198	11.8	7.2
L	459	934	938	182	187	37.0	335	217	11.2	6.8
M	385	942	874	166	243	25.4	552	314	8.2	4.8
N	448	947	903	213	159	26.4	290	178	11.9	7.4
O	366	944	903	137	249	36.5	476	283	9.6	5.7
P	419	950	947	160	172	40.0	369	198	11.7	7.1
Mean	404	941	918	166	211	34.6	387	229	11.0	6.6
Minimum	314	925	874	123	159	20.3	290	163	8.2	4.8
Maximum	485	956	947	213	282	47.6	552	314	11.9	7.4

¹DM_{original} = dry matter of the original TMR.²DM_{dry} = dry matter of the TMR under study (dried at 65°C for 24 h and stored).³Calculated from digestible crude nutrients according to Gesellschaft für Ernährungsphysiologie (2001).

TMR. We mixed 400 mL each of strained rumen fluid and artificial saliva (McDougall, 1948) and placed the mixture into each reaction vessel initially. Artificial saliva was continuously infused at a rate of approximately 600 mL/d using an 8-channel peristaltic pump (Ole Dich Instrumentmakers ApS, Denmark). Vertical movement of the feed containers was ensured by an electric motor with 10 to 12 strokes/min. The effluent was collected in 1-L glass bottles standing inside an ice-cold water bath.

For determination of CP_M synthesis, ¹⁵N was used as a tracer for labeling the microbial fraction. Artificial saliva contained 0.7 mmol of NH₄⁺/L from NH₄Cl with a declared ¹⁵N excess of 10 atom% (Chemotrade Chemiehandelsgesellschaft mbH Leipzig, Germany).

The removed bags containing the feed residue were washed with 2 × 40 mL of artificial saliva and squeezed moderately; the liquid was then returned to its respective vessel. The feed residues were dried at 65°C for 24 h.

Sampling, Preparation, and Analyses

An 8-d period of collecting samples of the feed residues, the liquid effluent, and the microbes followed a 7-d period of adaptation. As determined during the course of the study, this adaptation was necessary to achieve a plateau in ¹⁵N enrichment of microbes isolated from liquid effluent. To describe the dynamic of ¹⁵N enrichment, samples of microbes were isolated for 2 TMR (O and P; Table 1) on each day of incubation

starting on d 1. Samples of the liquid effluent (320 mL) were taken daily, and the reference microbes were isolated by differential centrifugation according to Brandt and Rohr (1981; Suprafuge 22, Heraeus Instruments, Hanau, Germany). In brief, the suspension was centrifuged twice at 2,000 × *g* at 4°C for 5 min. Subsequently, the supernatant was centrifuged 3 times at 27,000 × *g* at 4°C for 15 min. In between, the microbes were resuspended with a saline solution (0.9% wt/vol). After the last centrifugation, the microbes were frozen at -18°C, freeze-dried, and crushed with pestle and mortar. For all other TMR, the microbes were isolated in the same way only in the period between d 7 to 15.

Microbes that were attached to the feed [solid-associated microbes (SAM)] were separated from the feed residues contained in the 2 nylon bags at the termination of the study, based on Minato and Suto (1978) and according to the method described by Carro and Miller (2002). The SAM were separated from the remaining fluid and further treated as described for the reference microbes.

The ¹⁵N enrichment of N in dry samples from NH₄Cl, feed residues, effluents, and isolated microbes was measured by emission spectrometry (NOI 7, Fischer Analysen Instrumente GmbH, Leipzig, Germany) after combustion in a coupled C/N analyzer (Vario EL, Elementar Analysensysteme GmbH, Hanau, Germany). To obtain a sufficiently high amount of N for the ¹⁵N analysis, ~1.0 mg of NH₄Cl, 15 to 30 mg of feed residues, and 2 to 5 mg of microbes were exactly weighed into tin capsules and made airtight prior to combustion. Thirty

milliliters of effluent was taken, and ammonia N was transferred into a sulfuric acid solution via NaOH. This solution was then desiccated by lyophilization, and up to 50 mg of the dry residue was weighed into the tin capsules for combustion. The amount of effluent N obtained this way was not sufficient to reliably measure the ^{15}N enrichment in this fraction for TMRA, B, and D.

The pooled samples of feed and feed residues were analyzed for DM, ash, CP, crude fiber, NDF, and ADF according to the official methods in Germany (Nauermann and Bassler, 1976). The gross energy was measured by a bomb calorimeter (C7000, Janka & Kunkel IKA, Analysentechnik). Nitrogen content in the liquid effluent after centrifugation was determined by a Kjeltac auto 1030 analyzer from Tecator AB (Höganäs, Sweden) without a preceding hydrolysis.

Calculations

The extent of fermentation of crude nutrients, NDF, ADF, or gross energy (F_x ; %) was determined as

$$F_x = \frac{\text{input of } x - \text{output of } x}{\text{input of } x} \times 100\% \quad [1]$$

where

input of x = input in the vessel with feed (g/d), and
output of x = output from the vessel with feed residue (g/d).

Fermentation of OM was also calculated after correction for OM originating from microbes attached to the feed residues. First, the amount of N originating from SAM [N_{SAM} (g/d)] was calculated as

$$N_{\text{SAM}} = \frac{{}^{15}\text{N}_{\text{FR}} \times N_{\text{FR}}}{{}^{15}\text{N}_{\text{SAM}}} \quad [2]$$

where

${}^{15}\text{N}_{\text{FR}}$ = ^{15}N enrichment determined in feed residues (%),

N_{FR} = amount of N in feed residues (g/d), and

${}^{15}\text{N}_{\text{SAM}}$ = ^{15}N enrichment in N of the SAM (%).

Second, the amount of OM originating from SAM in the feed residue (OM_{SAM} ; g/d) was calculated as

$$\text{OM}_{\text{SAM}} = \frac{N_{\text{SAM}}}{N} \times (100 - 12) - 0.93 \quad [3]$$

where

N_{SAM} = N originating from SAM (Equation 2; g/d),

N = analyzed N content of SAM (%) on an as-is basis,

12 = analyzed concentration of ash in SAM (%), and

0.93 = analyzed proportion of DM in the isolated SAM fraction.

Because of a small sample size, DM and ash content of SAM were analyzed in pooled samples of this fraction only.

The amount of microbial N (N_M in mg/d) leaving the vessel with effluent was calculated as

$$N_M = \frac{{}^{15}\text{N}_{\text{in}} - {}^{15}\text{N}_{\text{out}}}{{}^{15}\text{N}_{\text{Plateau}}} \quad [4]$$

where

${}^{15}\text{N}_{\text{in}}$ = ^{15}N amounts introduced into the vessel with buffer solution (calculated on the basis of daily buffer flow and analyzed ^{15}N content in NH_4Cl) and feed (assuming natural abundance = 0.3663% of total N; $\mu\text{g/d}$),

${}^{15}\text{N}_{\text{out}}$ = ^{15}N amounts leaving the vessel with liquid effluent and feed residues (except ^{15}N in reference microbes; $\mu\text{g/d}$), and

${}^{15}\text{N}_{\text{Plateau}}$ = ^{15}N in total N of the isolated reference microbes between d 7 and 15 (μg of ^{15}N /mg of N).

The amount of CP_M (mg/d) was calculated as $N_M \times 6.25$.

To describe the dynamics of ^{15}N enrichment in the microbial N fraction in the 2 experiments with continuous sampling of microbes, the following equation (GraphPad Prism 4.02 for Windows) was used:

$$y = a + \frac{(b - a)}{(1 + 10^{((\log_{50} e^c - x) \times s))}} \quad [5]$$

where

y = ^{15}N enrichment at given x (%),

x = time after onset of ^{15}N administration via the artificial saliva (d),

a = minimum level of ^{15}N enrichment (%),

b = plateau of ^{15}N enrichment (%),

c = one-half of the plateau value of ^{15}N enrichment (%), and

s = maximum slope.

In this equation, the expression $\log_{50}e^c$ describes the time needed to achieve one-half of the plateau response in ^{15}N enrichment.

The software package SAS for Windows (version 8.2, 1999–2001, SAS Inst., Inc., Cary, NC) was used for the statistical analysis. Multiple linear regressions were calculated with Proc Reg to identify factors that might influence CP_M synthesis in the rumen. Dietary concentrations of OM, CP, crude fiber, and ether extract, as well as inclusion rates of both corn and grass silage, were considered as variables with the aim of minimizing the average coefficient of variation. As a measure of the contribution of each regressor, the P values of partial correlation (P_p) according the users' guide of the SAS software were taken. The P_p described the influence of each factor on the target value. The global P value expressed the meaning of the sum of factors on the target value. Also, the values of r^2 and P will be given as measures for the goodness of fit of the complete equation.

RESULTS

Dynamic of ^{15}N Enrichment in Microbial N

With TMR O and P (Table 1), the course of ^{15}N enrichment in microbial N was studied during the entire experimental period. As shown in Figure 1, a plateau in ^{15}N enrichment of microbial N was achieved within 7 d after starting the ^{15}N dosage with both TMR. The course was different, however, for the 2 rations. With ration P, which contained more ingredients and a higher energy value than ration O, the plateau was higher and was achieved earlier than with ration O. Based on these observations, we decided to use pooled samples of microbes from d 7 onward in all other experiments and to consider the ^{15}N enrichment determined in these pooled samples as the respective plateau value.

Fermentation and CP_M Synthesis

Fermentation of OM ranged from 35 to 47% (Table 3). A wide range was also observed for CP (25 to 60%), crude fiber (10 to 30%), NDF (3 to 28%), ADF (6 to 23%), and gross energy (31 to 45%). The range in OM fermentation was not reduced when the amount of OM recovered in the nylon bags was corrected for OM from SAM (36 to 51%; Figure 2). The NH_3N content in the liquid effluent from the vessels varied among TMR from 45 to 207 mg/L (Table 4).

Organic matter fermentation was not significantly correlated with ether extract content of the diet ($r = 0.14$). The CP content in TMR increased OM fermentation ($r = 0.79$), and the NDF content decreased OM fermentation ($r = -0.76$; Figure 3). Organic matter fer-

Figure 1. ^{15}N enrichment of the microbes isolated from the effluent depending on the duration of ^{15}N application via the buffer solution. The equation is $y = a + \frac{(b-a)}{(1 + 10^{((\log_{50}e^c - x) \cdot s)})}$, where a = minimum of y , b = maximum of y , $c = 1/2$ maximum of y , and s = maximum slope.

mentation also decreased with increasing crude fiber content ($r = -0.66$). The following multiple regression was calculated based on the variables CP, NDF, and ether extract (EE) to predict OM fermentation ($R^2 = 0.80$; $\text{CV} = 4.7\%$):

Table 3. Fermented crude nutrients, NDF, ADF, and gross energy of the 16 TMR (%; n = 3)

TMR	OM		CP		CF		NDF		ADF		GE	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
A	43.8	0.9	34.7	2.1	25.7	0.5	3.1	5.4	7.9	0.9	41.9	7.2
B	40.5	0.4	34.4	0.3	21.1	1.0	8.0	1.8	5.5	4.2	36.7 ¹	
C	45.7	0.3	36.9	0.3	14.5	1.9	12.1	4.3	6.7	1.1	41.7 ¹	
D	44.9	1.2	39.8	0.7	11.6	1.5	8.1	2.6	9.8	3.8	38.8	13.3
E	35.7	0.8	41.2	1.0	9.6	1.1	3.9	3.6	5.9	1.5	35.1	1.3
F	40.3	0.1	52.5	0.8	30.0	1.1	27.8	0.6	23.4	0.4	39.6	0.8
G	46.2	1.0	59.8	0.9	22.6	0.7	22.4	2.2	20.5	0.6	44.9	4.1
H	42.2	0.8	59.9	0.3	20.9	2.0	13.6	2.0	13.6	4.4	40.4	2.0
I	34.9	0.2	37.5	6.0	23.2	3.5	8.3	2.5	14.1	4.2	31.4	5.0
J	38.2	0.5	36.3	2.0	29.1	3.5	15.6	1.9	12.6	1.3	36.2	1.9
K	41.3	0.7	25.2	1.1	22.9	1.0	14.0	1.1	16.1	0.6	38.8	1.0
L	39.6	0.5	38.5	1.8	13.8	2.3	12.7	1.5	7.9	5.3	37.6	2.3
M ²	36.1	0.5	45.2	1.4	10.8	2.3	14.0	1.0	7.2	4.7	35.0	2.1
N	47.3	1.3	49.5	1.0	13.1	2.8	22.3	0.9	19.3	1.1	45.4	2.5
O	38.4	0.5	55.5	1.1	12.9	1.2	17.3	0.8	16.1	1.2	37.4	0.2
P	41.0	1.0	34.4	1.2	18.0	3.2	24.9	0.9	17.9	2.1	38.7	2.2

¹n = 2.²d 7 to 12 only.

$$\begin{aligned}
 \text{OM}_{\text{fer}} = & 33.5716 \\
 & + 0.0754 \times \text{CP} \\
 & - 0.0227 \times \text{NDF} \\
 & + 0.1060 \times \text{EE}
 \end{aligned}
 \quad [6]$$

where

OM_{fer} = fermented OM (%) and CP, NDF, and EE are measured in g/kg of DM of TMR.

The amount of CP_M synthesized ranged from 677 to 1778 g/d (Table 5). For TMR A, B, and D (Table 1),

the CP_M synthesis could not be calculated because of a shortage in the amount of effluent N for isotope analysis. The correlation between fermented OM and the amount of CP_M synthesized/d was high ($r = 0.75$; Figure 4). However, the efficiency, expressed as grams of CP_M per kilogram of fermented OM or as grams of CP_M per megajoule of ME, ranged from 141 to 286 and 4.9 to 11.1, respectively (Table 5).

Regression Analysis

Multiple regressions shown in Table 6 revealed CP and ether extract content as the variables mainly determining the efficiency of CP_M synthesis expressed in

Figure 2. Comparison of OM fermentation determined either uncorrected (light bars) or under consideration of microbes associated with the feed residues taken from the nylon bags (shaded bars; mean $\pm$ SD). Rations K, L, M, and N are not included because the ¹⁵N enrichment in residues from the nylon bags could not be determined.

Table 4. Average NH₃N content in liquid effluent (mg/L; n = 3; means and SD)

TMR	NH ₃ N	
	Mean	SD
A	76.6	0.6
B	52.5	2.1
C	89.5	5.1
D	94.3	2.8
E	48.7	1.4
F	129.5	3.6
G	201.4	12.3
H	115.5	9.7
I	45.1	1.6
J	68.4	5.4
K	118.3	6.3
L	107.9	4.9
M ¹	129.7	9.5
N	206.8	9.9
O	122.0	5.0
P	67.2	3.7

¹d 7 to 12 only.

Figure 3. Relationship between fermented OM (OM_{fer}) and CP and NDF content of the 16 TMR.

relation to fermented OM (regression I). The P_p values were <0.01 and $=0.14$, respectively. The inclusion of OM and crude fiber content increased the coefficient of variation (regression II). When the dietary inclusion rate of corn silage was considered, the accuracy of the prediction improved, as judged by the coefficient of determination and the coefficient of variation (regression III). All attempts without consideration of ether extract led to higher coefficients of variation and lower coefficients of determination. When CP or corn silage inclusion rates were the considered variables alone or in combination, the accuracy of prediction was lower.

Regressions calculated to predict the efficiency of CP_M synthesis in relation to ME are shown in Table 7. Multiple regressions again revealed CP and ether extract contents as the main determinants for the prediction (regression IV; P_p values were 0.02 and 0.14, respectively), but the accuracy of predictions based on coefficient of determination and coefficient of variation was lower than that for regression I. Including OM and

Table 5. Microbial CP (CP_M) and efficiency of CP_M synthesis ($n = 3$)

TMR	CP_M		CP_M/OM_{fer}^1		CP_M/ME	
	Mean	SD	Mean	SD	Mean	SD
	— (mg/d) —		— (g/kg) —		— (g/MJ) —	
A ²						
B ²						
C ³						
D ²	1,778		286		11.1	
E	1,050	27	202	9.9	7.7	0.2
F	1,312	116	237	16.0	8.7	0.8
G	1,306	229	205	20.2	8.3	1.5
H	984	77	165	8.4	5.9	0.5
I	677	49	141	9.6	4.9	0.4
J	1,012	82	188	8.6	6.3	0.5
K	1,105	130	203	22.5	6.6	0.8
L	1,070	94	202	19.4	6.7	0.6
M	771	52	173	11.6	6.6	0.5
N	1,406	86	232	7.9	8.3	0.5
O	791	38	146	6.6	5.8	0.3
P	1,135	20	179	6.2	6.8	0.1
Overall mean	1,107		197		7.2	
Minimum	677		141		4.9	
Maximum	1,778		286		11.1	

¹ OM_{fer} = Microbial CP per OM fermented.

²Measurement of ^{15}N enrichment in effluent was not possible because of shortage in sample size.

³ $n = 2$.

crude fiber content in the regression did not reduce the error of estimate (regression V). When OM, CP, and ether extract contents were considered, the inclusion rate of corn silage reduced the error of estimate and substantially improved the goodness of fit (regression VI). The P_p value for the inclusion rate of corn silage was 0.15.

DISCUSSION

For the determination of CP_M synthesis, ^{15}N has often been used as a marker for labeling the ammonia

Figure 4. Relationship between fermented OM (OM_{fer}) and the amount of microbial CP (CP_M ; $n = 13$ TMR).

Table 6. Regressions calculated to predict the efficiency of microbial CP synthesis in relation to fermented OM (in g/kg, n = 13 TMR)

	Regression					
	I		II		III	
	β^1	P_p^2	β^1	P_p^2	β^1	P_p^2
Intercept	9.0243	0.86	-476.8325	0.45	-818.9876	0.25
OM ³			0.3736	0.49	0.7498	0.25
CP ³	0.8113	<0.01	1.2627	0.05	1.1428	0.07
Crude fiber ³			0.3641	0.33	0.3269	0.38
Ether extract ³	1.4261	0.14	1.1370	0.28	1.2240	0.25
CS ⁴					0.4720	0.28
P_{global}		0.01		0.07		0.09
R ²		0.62		0.68		0.74
CV, %		10.8		11.3		11.0

¹ β = Partial regression coefficient.² P_p = Partial P value.³Measured in g/kg of DM.⁴CS = Corn silage; measured in percentage of DM in TMR.

N pool. Several researchers have reported that ¹⁵N and also D-alanine (Voigt et al., 1991) or purine bases (Broderick and Merchen, 1992; Calsamiglia et al., 1996; Carro and Miller, 2002) are suitable markers for determination of CP_M synthesis. With regard to the in vitro approach that we used, we still had to investigate how much time was required to achieve steady-state ¹⁵N enrichment in microbial N after start of the ¹⁵N administration. Czerkawski and Breckenridge (1977) reported that the digestibility of DM remained constant after 4 to 6 d of incubation. We had assumed that the maximum ¹⁵N enrichment of microbes, using a continuous culture system, would be achieved 3 d after the ¹⁵N infusion started (Calsamiglia et al., 1996). It became obvious in the present study that 7 d of continuous ¹⁵N infusion were needed to achieve the

plateau in ¹⁵N enrichment of microbes in this Rusitec system supplied with TMR (Figure 1). Sampling microbes earlier would risk an overestimation of the CP_M synthesis.

The 2 TMR used for this comparison were very different in nutrient and energy contents, but basically, the course of ¹⁵N enrichment over time was similar between them. The time needed to achieve one-half of the response in ¹⁵N enrichment was about one-half of 1 d shorter for the higher quality TMR P than for TMR O. Also, the plateau was slightly higher for TMR P than for TMR O; however, the comparison suggests that the choice of TMR does not greatly affect the response to ¹⁵N administration. We concluded that a plateau in microbial N would be achieved for all other TMR after 7 d of ¹⁵N infusion.

Table 7. Regressions calculated to predict the efficiency of microbial CP synthesis in relation to ME (g/MJ; n = 13 TMR)

	Regression					
	IV		V		VI	
	β^1	P_p^2	β^1	P_p^2	β^1	P_p^2
Intercept	0.3583	0.87	7.1045	0.78	-4.1270	0.80
OM ³			-0.0092	0.68	0.0048	0.78
CP ³	0.0271	0.02	0.0298	0.21	0.0192	0.08
Crude fiber ³			0.0055	0.72		
Ether extract ³	0.0619	0.14	0.0626	0.16	0.0700	0.07
CS ⁴					0.0231	0.15
P_{global}		0.03		0.10		0.04
R ²		0.53		0.63		0.72
CV, %		12.7		12.8		11.1

¹ β = Partial regression coefficient.² P_p = Partial P value.³Measured in g/kg of DM.⁴CS = Corn silage; measured in percentage of DM of TMR.

Microbial growth depends on the amount and availability of nitrogen and energy (Stern and Hoover, 1979; Hoover and Stokes, 1991); thus, CP_M synthesis was expressed in relation to OM fermented in the rumen. The correlation between fermented OM and the amount of CP_M synthesized per day was high (Figure 4). However, in comparison with other *in vitro* investigations (Abel et al., 1990; Mansfield et al., 1994; Carro et al., 1995; Yang et al., 2002), the extent of nutrient fermentation was low in the present study. Different factors may be responsible for such low fermentation. The fermentation of OM may be depressed when N is deficient for the growth of microbes (Hespell and Bryant, 1979). In the present investigation, we found a trend toward a higher fermentation of OM with increased CP supply to the vessel (Figure 3). Because most rumen bacteria prefer to use ammonia as the source of N for growth (Pilgrim et al., 1970; Salter et al., 1979), the degradation of feed protein may become an important factor influencing the N supply of microbes in this system. Ammonia concentration in the liquid effluent (Table 4), however, was not significantly correlated with CP content ($r = 0.50$) or OM fermentation ($r = 0.55$). It is unlikely that the amount of ammonia limited the microbial activity because a maximum microbial growth was reported for an ammonia concentration in rumen liquid between 20 and 100 mg/L (Okorie et al., 1977; Slyter et al., 1979; Pisulewski et al., 1981). For the *in vitro* system, Satter and Slyter (1974) estimated that an increase in ammonia concentration beyond 50 mg of NH₃N/L in liquid effluent had no effect on the CP_M synthesis. Their data suggest, however, that the limiting concentration is closer to 20 mg of NH₃N/L. In our study, in no case was the concentration <45 mg of NH₃N/L (Table 4).

Furthermore, the relatively high pH values (6.9 to 7.3) and the low flow rate of artificial saliva (565 mL/d; SD = 24) are not in line with the observed low level of nutrient fermentation. Some researchers have suggested that a high pH promotes the degradation of OM (Grant and Weidner, 1992; Calsamiglia et al., 2002; Yang et al., 2002) and that the degradation of DM was not affected within a wide range of dilution rate (Czerkawski and Breckenridge, 1977). Even the chosen pore size of 100 μ m can be expected to have a positive effect on the degradation of DM and NDF when compared with 40 or 200 μ m (Carro et al., 1995).

Perhaps the postincubation washing and squeezing procedure contributed to the differences in fermentation rate compared with other studies; these procedures may affect the loss of small feed particles from the bag. Also, microbes attached to feed particles could cause an underestimation of fermentation; however, the correction of OM fermentation for SAM could also not ex-

plain the low level of OM fermentation. Figure 2 shows that the extent of fermentation of OM was only between 1.2 and 6.0% higher after correction for SAM. The percentage of OM in feed residues originating from SAM amounted to at least 2%.

High variation in efficiency of the CP_M synthesis has often been described both *in vivo* (Armstrong, 1980) and *in vitro* (Abel et al., 1990; Calsamiglia et al., 1995; Bach et al., 1999; Carro and Miller, 1999; Colombatto et al., 2003). Factors that contribute to this variation are the feedstuffs used, their chemical composition, and the amount of feed, as well as the microbial population in the rumen (Armstrong, 1980). Many interrelations make it difficult to systematically investigate single factors with regard to their effects on microbial activity. In the present study, it appears that CP content of TMR was an important factor determining the amount of CP_M per unit of fermented OM (Table 6). Both the N content in the ration and the availability of N and energy are decisive for CP_M synthesis. The use of TMR enables an optimization of ruminal digestion because different sources of N and carbohydrates with different kinetics of degradation are offered at the same time. Moreover, the TMR used in this work contained between 12 and 21% CP in DM. According to Kluth et al. (2000), in this range of dietary CP content, no effect on the N balance in the rumen on efficiency of CP_M synthesis can be expected. Therefore, we conclude that the content and availability of N were not the limiting factors in the present study. This conclusion is supported by the lack of correlation between ammonia concentration in the system and OM fermentation as discussed previously. Demeyer and Fievez (2004) argued that the synthesis of rumen bacterial protein may be limited by amino acid supply in the rumen at high production levels and that the optimization of bacterial protein production may require knowledge of the nature of the limiting free amino acids. Under *in vivo* conditions, free amino acids are generally contained in rumen fluid only in low concentrations (Wallace, 1996). Nevertheless, it turned out in *in vitro* studies that some AA are essential for bacteria (Atasoglu et al., 2001, 2004) and that the growth of microbes can be promoted by supplementation of peptides or AA (Maeng et al., 1975; Argyle and Baldwin, 1989; Griswold et al., 1996; Kajikawa et al., 2002). Along this line, the CP effect on efficiency of CP_M synthesis may be caused by the supply of certain (limiting) amino acids by the TMR.

The supplementation of lipids caused a decrease in ruminal degradation (Palmquist and Jenkins, 1980; Jenkins and Fotouhi, 1990). Abel et al. (1990) and Doreau et al. (1991) reported only small effects of fat supplementation on the fermentation of nutrients; however, the equations determined in this study indicate that

a higher content of EE increased the amount of OM fermented and also increased the efficiency of CP_M synthesis. This finding is in agreement with Jenkins and Fotouhi (1990), who reported that adding fat increased the efficiency of microbial yield.

Another factor that was identified as important for CP_M synthesis was the inclusion rate of corn silage in TMR (Table 6). In vivo studies have indicated that higher inclusion rates of corn silage in mixed rations increased the efficiency of CP_M synthesis, but the different inclusion rates were not taken into account (Givens and Rulquin, 2004). The efficiency in rations based on grass silage is reduced, probably as a consequence of rapid and asynchronous release of N relative to energy from carbohydrates (Siddons et al., 1985). In the present study, a significant monofactorial relationship between the efficiency of CP_M synthesis and the inclusion rate of corn silage was not observed ($r = -0.26$); however, the goodness of fit in multiple regression analysis could be improved by additional consideration of the maize silage inclusion rate in the regressions (Tables 6 and 7).

The efficiencies expressed in relation to ME as shown in Table 5 are on a lower level than data from studies with dairy cows (Gesellschaft für Ernährungsphysiologie, 2001), but they are difficult to interpret and to compare with in vivo data. First, CP_M was determined in vitro, and ME was calculated based on the digestibilities determined in standardized wether trials. Second, a comparison between the rumen and the Rusitec system must be made with care. The number of protozoa is extremely reduced in rumen simulation systems (Mansfield et al., 1995), and it is also possible that the bacterial population changes in comparison with the natural rumen (Slyter and Putnam, 1967). Feed is enclosed in nylon bags in the Rusitec, which may affect accessibility for microbes. Differences between fermentation in continuous culture and in vivo may also arise because of a lack of N recycling and absorption for products of metabolism, respectively (Mansfield et al., 1995); however, the degradation of OM, CP, and amino acids did not differ between dairy cows and a continuous rumen simulation system in the study of Hannah et al. (1986). Also, short-term studies with a modified, semicontinuous Rusitec in comparison with a natural rumen did not show significant differences in the fermentation, the concentration of short-chain fatty acids, and the number of microbes (Gizzi et al., 1998). Passage rates of fluid and feed, pH in the vessel, amount of feed per day, and the method of separation of microbes representing the total population are critical points in such comparisons and make it difficult to draw conclusions that apply to the feeding of cows.

CONCLUSIONS

In vitro fermentation of OM from TMR depends on the contents of CP and NDF in the ration. The CP_M synthesis correlated with OM fermentation. Regression analysis did not identify dietary factors that can clearly explain the high variation in the efficiency of CP_M synthesis; however, among the factors studied, CP and ether extract content as well as the inclusion rate of corn silage in the ration helped to explain part of the variation. When ¹⁵N is used as a marker to study microbial growth in a Rusitec system fed with TMR, 7 d of continuous ¹⁵N infusion are needed to achieve the steady-state enrichment in microbial N. Sampling of microbes for ¹⁵N analysis should not start prior to this time.

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