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Studies on the relationship between the synchronization index and the microbial protein synthesis in the rumen of dairy cows

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Abstract

A total of eight Holstein cows equipped with ruminal and duodenal cannulae were used to evaluate the suitability of the synchronization index (SI) for optimizing microbial protein (MP) synthesis in the rumen in two experiments. In a preliminary study, degradation characteristics of the feedstuffs were estimated by the nylon bag technique and the quantities of organic matter (OM) and nitrogen (N) degraded per 4 h interval were calculated as difference between the cumulative amounts of OM and N degraded at successive intervals. The relationship between the sum of degraded N and the sum of

Abbreviations: ADFom, acid detergent fiber (ash free); CN, maize grain; CP, crude protein; CS, maize silage; DLG, Deutsche Landwirtschaftsgesellschaft; DM(I), dry matter (intake); FOM, fermented organic matter (OMI − (OM at the duodenum − microbial OM)); FS, feeding sequence; GfE, Gesellschaft für Ernährungsphysiologie; GH, grass hay; GS, grass silage; ME, metabolizable energy; MP, microbial protein; MU, milk urea; N, nitrogen; NAN, non-ammonia-N; NIRS, near infra-red reflectance spectroscopy; NDFom, neutral detergent fiber (ash free); NEL, net energy lactation; OM(I), organic matter (intake); PE, peas; RNB, ruminal N balance (N intake − (uCP/6.25)); SBM, soyabean meal; SCFA, short chain fatty acids; SI, synchronization index; uCP, utilizable CP at the duodenum ((NAN − endogenes N) × 6.25)); UDP, undegraded feed protein; WH, wheat grain

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degraded OM from the different feeds at each interval were used to calculate the SI. In the first *in vivo* experiment equal amounts of maize silage, grass hay, wheat grain and peas + urea and in the second experiment grass silage, maize grain and soyabean meal were fed to seven and five cows, respectively, four times daily in three different sequences (FS): in FS-A equal amounts of the protein and energy sources were offered at each feeding time; FS-B: first and third feeding time were offered only the energy sources, second and fourth feeding time only the protein sources; FS-C: separate feeding of energy and protein sources as in "FS-B", but sequence was reversed. Mean feed intake was 14.2 and 17.0 kg DM in the first and second experiment, respectively.

The ratio of N/OM release varied during the course of the day in dependence on the feeding sequences for both experiments. The variations of the ratios and the calculated SI were higher in the first compared to the second experiment. The SI was 0.76, 0.52, and 0.82 for FS-A, FS-B, and FS-C in the first experiment, respectively. In the second experiment, indices were 0.85, 0.90, and 0.72 for FS-A, FS-B, and FS-C, respectively.

The means of rumen pH, ammonia-N, and total short chain fatty acids were not influenced by feeding sequences in both experiments.

For both experiments, flow of non-ammonia-N at the duodenum and efficiency of microbial protein (MP) synthesis tended to be highest in FS-B where energy sources were fed at the first feeding time. This was significant only in the second experiment (187, 245, and 197 g microbial CP/kg fermented OM (FOM) for FS-A, FS-B, and FS-C, respectively). Microbial CP synthesis was not consistent with the SI parameters. Only in the second experiment, the highest SI corresponded with the highest microbial CP synthesis. The lowest SI in the first (0.52, FS-B) and second experiment (0.72, FS-C) was not associated with the lowest efficiencies of microbial CP synthesis (163 and 187 g microbial CP/kg FOM, FS-A in Experiments 1 and 2, respectively).

Keywords: Synchronization; Synchronization index; Rumen; Feeding sequence; Microbial protein synthesis

1. Introduction

The strong relationship between animal production efficiency and environment pollution has created a challenge for animal nutritionists in most countries. Livestock may affect the environment through excretion of nitrogen (N), phosphorus and other chemical components produced during their metabolism. These contaminants are the result of inefficiencies in the processes of digestion and metabolism.

In dairy cows, inefficiency of N utilization is largely due to the losses of N in urine and faeces (Castillo et al., 2001; Van Soest, 1994). Losses of N in urine are mainly caused by an oversupply of crude protein and/or an imbalance in the supply of amino acids. The amount of N excreted in faeces, however, is reported to be relatively constant in proportion to dry matter intake (DMI) at about 7.5 g N/kg DMI (Castillo et al., 2001) or 6 g N/kg DMI (Van Soest, 1994). In some cases, an increase of N in faeces might be the result of high amounts of undegradable feed protein (UDP) or undigested microbial N. Moreover, in normal practical rations for dairy cows true digestibility of UDP and of microbial protein (MP) are normally high, suggesting that reduction of N excretion in the faeces is not a promising way for substantially reducing N losses from ruminants. Therefore, a more promising way to reduce N output is by reducing urinary N excretion (Castillo et al., 2001; Monteils et al., 2002). A possible means of achieving this is by improving the efficiency of MP synthesis through

synchronizing energy and protein supply in the rumen (Sinclair et al., 1993). A number of experiments (*e.g.* Herrera-Saldana et al., 1990; Newbold and Rust, 1992; Henning et al., 1993; Kolver et al., 1998; Shabi et al., 1998; Keller et al., 2002) have been performed to test this hypothesis with varying results.

Most of synchronization studies have been conducted by exchanging feed ingredients of the diets. However, this method cannot distinguish between the effects of synchronization and of individual feedstuffs (Dewhurst et al., 2000). The objectives of the present experiments were therefore firstly to alter the release of energy and nitrogen in the rumen by feeding the same energy and N-yielding feedstuffs in different sequences, secondly to calculate the synchronization index (SI) from *in sacco* experiments, and thirdly to test if this index is related to N flow at the duodenum and to the MP synthesis in the rumen.

2. Materials and methods

2.1. In sacco experiment

2.1.1. Incubation procedure

The purpose of the *in sacco* experiment was to determine the degradation characteristics of each feedstuff used in the *in vivo* experiments. Ruminal dry matter (DM), organic matter (OM) and crude protein (CP) degradabilities of feedstuffs were estimated on the basis of the disappearance of DM, OM, and CP using the polyester bag technique as described by Ørskov et al. (1980).

Four non-lactating, rumen cannulated cows weighing approximately 550 ± 24 kg each were used for the *in sacco* experiment. The cows were offered twice daily (at 5:30 and at 15:30 h) 4 kg (DM) grass silage and 3.5 kg concentrate pellets (250 g soyabean meal, 270 g wheat grain, 200 g barley grain, 240 g sugar beet pulp, 20 g soyabean oil, and 20 g vitamin–mineral pre-mixture per kg DM). Water was freely available.

Maize silage (CS), grass hay (GH), grass silage (GS), wheat grain (WH), maize grain (CN), peas (PE), and soyabean meal (SBM) from the same batches as used in the *in vivo* experiments were incubated in the bags. The samples of CS and GS were freeze dried before being milled. Samples were milled through a 3 mm sieve (RETSCH-Mühle, Typ. SK, Nr. 9516) and about 4 g were allotted per bag. The bags, made of polyester with a pore size of 53 ± 10 μm (Bar Diamond, Idaho, USA) had a size of 10 cm × 20 cm. Seven bags were attached with plastic bands onto a 30 cm long polyvinyl chloride rod and inserted into the ventral sac of the rumen of each cow via the rumen cannulae. Each incubation started at 05:30 h immediately before feeding. CN, WH, PE, and SBM were incubated for 0, 2, 4, 8, 16, 24, and 48 h, with CS, GS, and GH for 72 and 96 h additionally. Immediately after removing the bags from the rumen, they were gently washed under cold tap water to wash off feed particles adhering to the outside of the bags. Finally, all the bags were washed in a domestic washing machine (Foron, VA, 861 Electronic) for 20 min with cold water. Zero hour bags were not incubated but washed in the same way as the incubated bags. The bags, together with their contents were dried at 100 °C for 48 h and weighed to estimate the DM residues. Contents of the bags were removed and stored in plastic bottles at 5 °C until ash and N analyses were conducted.

2.1.2. Estimation of rumen degradation characteristics

The percent disappearance of DM, OM and CP was calculated as the difference between the feed and the residues after incubation in the rumen. Degradation data obtained were then fitted by the mathematical model of Ørskov et al. (1980) using the PROC NLIN (SAS® Release 8.01) program:

$$P = a + b(1 - e^{-c(t-t_l)})$$

where P is the degraded feed at time 't'; a the soluble fraction; b the insoluble but potentially degradable fraction; c the constant rate of degradation of b; t the time (h); t_l is the lag phase (h).

The effective degradabilities of OM and CP were calculated using the parameters a, b, c, and rumen outflow rates of 0.05 and 0.08 h^{-1} by:

$$P = a + \left(\frac{bc}{c+k}\right)$$

where P is the effective degradability of DM, OM, and CP and k is the estimated rumen outflow rate (h^{-1}).

2.1.3. Calculation of the synchronization index (SI)

The SI for the diets in the *in vivo* experiments was calculated from the degradation characteristics of OM and CP of the feed components from the *in sacco* experiments. Contrary to the experimental protocol of Sinclair et al. (1993) who quantified the feed degradation hourly, in the present experiment the OM and CP degradation of feed were calculated for six 4-h intervals, *viz.* 05:30–09:30, 09:30–13:30, 13:30–17:30, 17:30–21:30, 21:30–01:30, and 01:30–05:30 h. Intervals of 4 h were chosen, because it seemed unrealistic to assume that the cows will consume the whole meal within a shorter time without feeding them at extremely restricted levels.

Urea was assumed to be totally degraded within the first 4 h interval after feeding. The SI was calculated based on the following formula:

$$\text{Index} = \frac{25 - \sum_{1-6}\sqrt{(25 - \text{release of } N/\text{OM per 4 h})}/6}{25}$$

where 25 g N/kg OM truly digested in the rumen was assumed to be the optimal ratio for maximum efficiency of microbial protein production (Sinclair et al., 1993).

The quantity of OM and N from each feed component degraded per interval was calculated as the cumulative amount degraded (P) at successive intervals. This was summed for all feeds and used for calculating SI. An index of 1.0 represents perfect synchrony, whilst values <1.0 indicate the degree of asynchrony.

2.2. In vivo experiments

2.2.1. Animals

A total of eight multiparious lactating German Holstein cows fitted with cannulae in the rumen and in the proximal duodenum were used in two experiments (seven of them in the first and five in the second experiment). The rubber rumen cannula with an internal diameter

Table 1
Chemical composition and nutritive value of feedstuffs

	Feedstuffs						
	CS	GH	GS	WH	PE[a]	CN	SBM
DM (g/kg)	305	889	576	870	880	867	878
OM (g/kg DM)	955	915	912	982	972	983	927
CP (g/kg DM)	82	110	149	124	210	106	475
EE (g/kg DM)	25	21	23	11	7	21	12
NDFom (g/kg DM)	431	499	483	138[b]	158[b]	108[b]	176
ADFom (g/kg DM)	229	269	287	33	76	36	114
RNB (g/kg DM)[c]	−8	−1	+3	−7	+5	−10	+37
uCP (g/kg DM)[c]	132	125	135	169	182	164	296
UDP (g/kg DM)[c]	21	22	23	25	32	53	166
ME (MJ/kg DM)[c]	10.6	8.8	10.0	13.3	13.4	12.8	13.6
NEL (MJ/kg DM)[c]	6.4	5.1	6.0	8.5	8.6	8.2	8.5

CS, maize silage; GH, grass hay; GS, grass silage; WH, wheat grain; PE, peas; CN, maize grain; SBM, soyabean meal. EE, ether extract; NDFom, neutral detergent fiber (ash free); ADFom, acid detergent fiber (ash free); RNB, ruminal nitrogen balance ([CP − uCP]:6.25); uCP, utilizable CP (microbial CP + UDP); UDP, undegradable feed CP; ME, metabolizable energy; NEL, net energy lactation.

[a] Without additional urea.

[b] Measurements included the use of heat stable amylase (aNDFom).

[c] Calculations based on the analyses (Table 1) and on UDP and digestibility values for nutrients from DLG-tables (DLG, 1997) according to the GfE (2001) formulas.

of 10 cm was produced by Bar Diamond Idaho USA. The T-shaped duodenal cannula was made of PVC with internal diameter of 2 cm (MEDVET, Laatzen, Germany). The cows were in mid and late lactation, yielding 15–28 kg milk/day with an initial body weight of 559 ± 36 kg. All the cows were tethered in a tie stall. They had individual feeding facilities and free access to water.

2.2.2. *Feeding*

In two experiments the concentrate portion of the ration was given in three different sequences (FS) to achieve different SIs:

FS-A: energy and protein source together.
FS-B: energy source first followed by protein source.
FS-C: protein source first followed by energy source.

2.2.2.1. First experiment. In the first experiment, the diet offered was formulated to provide per day 14.2 kg DM, 102.4 MJ net energy lactation (NEL), and 2124 g utilizable CP (uCP: MP + UDP at the duodenum, calculated according to GfE (2001) from ME and UDP intake and tabulated UDP (DLG, 1997)) consisting of CS, GH, WH, and PE. The chemical composition and nutritive value of feedstuffs are presented in Table 1. The grass hay offered to the seven cows consisted mainly of perennial ryegrass (*Lolium Perenne*). Fifty-four grams (54 g) of urea were added to each kilogram of peas in order to balance ruminal available N (RNB) without increasing DM, energy, or UDP intake. The diet was offered in three differ-

Table 2
The arrangement of feeds offered (kg DM/day) in the three different feeding sequences (FS) for each feeding time in the first and second experiment

	Feeding times (h)			
	05:30	09:30	13:30	17:30
First experiment				
FS-A				
Maize silage	3.32	–	3.32	–
Grass hay	–	0.60	–	0.60
Wheat grain	0.99	0.99	0.99	0.99
Peas	0.59	0.59	0.59	0.59
Minerals	0.100	–	–	0.100
FS-B				
Maize silage	3.32	–	3.32	–
Grass hay	–	0.60	–	0.60
Wheat grain	1.98	–	1.98	–
Peas	–	1.18	–	1.18
Minerals	0.100	–	–	0.100
FS-C				
Maize silage	3.32	–	3.32	–
Grass hay	–	0.60	–	0.60
Wheat grain	–	1.98	–	1.98
Peas	1.18	–	1.18	–
Minerals	0.100	–	–	0.100
Second experiment				
FS-A				
Grass silage	5.15	–	5.15	–
Soyabean meal	0.375	0.375	0.375	0.375
Maize grain	1.30	1.30	1.30	1.30
Minerals	0.100	–	–	0.100
FS-B				
Grass silage	5.15	–	5.15	–
Soyabean meal	–	0.75	–	0.75
Maize grain	2.60	–	2.60	–
Minerals	0.100	–	–	0.100
FS-C				
Grass silage	5.15	–	5.15	–
Soyabean meal	0.75	–	0.75	–
Maize grain	–	2.60	–	2.60
Minerals	0.100	–	–	0.100

ent FS to create differences in the synchronicity calculated from the OM and N degradation characteristics of the single feedstuffs. PE and WH were given together in equal amounts at each feeding time in FS-A whilst in the FS-B WH was offered to the cows at the first and third feeding times and PE was offered at the second and fourth feeding times. In the FS-C, PE was offered to the cows at the first and third feeding times and WH was offered at second and fourth feeding times. Table 2 shows the feeding pattern for the first and the second experiment.

2.2.2.2. Second experiment. During the second experiment five cows received GS, CN, and SBM. The diet offered was formulated to provide daily 17.0 kg DM, 116.7 MJ NEL, and 2687 g uCP. In FS-A, CN and SBM were given together in equal amounts at each feeding time. In FS-B and FS-C, CN and SBM were given according to Table 2. Grass silage for all three feeding sequences was given twice daily at 5:30 and 13:30 h.

2.2.3. Design of the experiment

Both *in vivo* experiments consisted of three 19-day periods each and were arranged according to an incomplete Latin square design. Fourteen days were used for feed adaptation for each period. At the 9th and 11th day of the adaptation rumen fluid was sampled via the rumen cannulae. Adaptation time was assumed to be long enough because diet composition was constant within each experiment and only feeding sequence changed. After each adaptation spot-sampling of duodenal digesta was taken at 2 h intervals from Monday to Saturday (from day 14 to 19) according to Rohr et al. (1979), resulting in total in 60 samples per animal and treatment. Change over between the first and the second experiment took 4 weeks.

2.2.4. Measurements, sampling, and analysis

The amount of feed offered and refusals were weighed and recorded daily. In each period on the 9th day at 0, 1.5, 3, 4.5, 6 and 7.5 h and at the 11th day at 0, 0.5, 2, 3.5, 5, 6.5, and 8 h postmorning feeding 100 ml of rumen fluid was sampled through the rumen cannulae using a hand vacuum pump from four animals per treatment (five animals in the first experiment for FS-B). Rumen pH was measured immediately after sampling. Ammonia content was determined according to a modified Conway method (Voigt and Steger, 1967). Rumen samples collected on the 9th day were used to determine the concentration of short chain fatty acids (SCFA). The SCFA were determined by gas chromatography after centrifugation at 5000 rpm for 20 min, addition of 1.5 ml H_3PO_4 (2.92 mol/l), 0.5 ml formic acid (conc.) and a small drop of $HgCl_2$ (conc.) per 10 ml supernatant, and a second centrifugation.

To estimate digesta flow, 20 g Cr_2O_3 mixed with 80 g wheat flour was given in two portions per day into the rumen beginning 10 days before duodenal digesta collection periods and in four portions per day during the collection periods (Rohr et al., 1979). In comparative studies, this spot-sampling procedure has shown only small differences in flow as compared to total collection (Rohr et al., 1984).

The chemical composition of feedstuffs, feed residues, and nylon bag residues was determined according to the official methods of the "Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten" (VDLUFA, Bassler, 1976: DM, Section 3.1: ash, Section 8.1; CP, Section 4.1.1; ether extract, Section 5.1.1; NDF, Section 6.5.1: ADF, Section 6.5.2). ADF and NDF were expressed on an ash free basis (ADFom and NDFom). Only NDF measurements on the starch rich components wheat grain, maize grain, and peas included the use of heat stable amylase (aNDFom). Cr_2O_3 was analyzed by atomic absorption spectrophotometry according to Williams et al. (1962). The microbial N portion of NAN in the duodenal samples was estimated using near infra-red reflectance spectroscopy (NIRS) according to Lebzien and Paul (1997). Lebzien and Paul (1997) calibrated and validated this method using samples from duodenally fistulated cows receiving diets consisting of a wide range of basic feedstuffs and concentrates. Forage-to-concentrate ratio

varied between 100:0 and 39:61, digestible OM intakes ranged from 4.7 to 13.3 kg/day and concentrates were given twice to four times daily. The portion of microbial nitrogen in the duodenal samples was estimated by ^{15}N measurements and NIRS. No effect of ration composition on the precision of the NIRS method could be stated.

Duodenal samples on days 17 and 18 were analyzed for ammonia content by the same method as the rumen fluid.

Milk yield was measured and recorded twice daily at 6:30 and 16:30 h using Fullwood milk meter (Fullwood Flow processor MK1, UK). The proportional samples from consecutive morning and evening milking were taken twice at the beginning and at the end of the collection period. Evening milk samples were kept refrigerated (approximately 5 °C) until the next morning when morning milk samples were being recorded and collected. All milk samples were then analyzed for urea content (MU) by an infra-red analyzer (MILKOSCAN FT 6500, Foss Electrics®, after partial least squares (PLS) calibration (DIN 9622).

ME, NEL as well as RNB, uCP, and UDP were calculated on the basis of the analyses (Table 1) and UDP and digestibility values from DLG-tables (DLG, 1997) according to the GfE (2001) formulaes.

2.2.5. Statistical analysis

Analyses of variance of nutrient intakes and duodenal flow parameters was carried out according to PROC GLM (SAS® System for Windows release 8.01) with the following model:

$$Y_{ij} = \mu + \mathrm{FS}_i + \mathrm{Cow}_j + \varepsilon_{ij}$$

where Y_{ij} is the measured response variables; μ the overall mean; FS_i the effect of feeding sequences i; Cow_j the effect of the animal j; ε_{ij} is the error term.

Data for pH, ammonia-N, and SCFA were also analyzed according to PROC MIXED (SAS®) with the following model:

$$Y_{ijk} = \mu + \mathrm{FS}_i + \mathrm{Cow}_j + \mathrm{Time}_k + \varepsilon_{ijk} + (\mathrm{Time} \times \mathrm{FS})_{ik}$$

where Y_{ijk} is the measured response variables; μ the overall mean; FS_i the effect of feeding sequences i; Cow_j the effect of the animal j; Time_k the effect of time k; ε_{ijk} is the error term.

"Effect of time" and "effect of feeding sequences" were used as fixed terms.

Differences between means were reported only if the Tukey-test (SAS®) for treatments was significant at $P<0.05$. Tables with rumen pH, ammonia-N, and SCFA have been constructed by using the least squares (LS)-means. All other means have been calculated by using arithmetic means of the original values.

3. Results and discussion

3.1. In sacco degradation parameters and synchronization index (SI)

The OM and CP degradation kinetics are presented in Table 3. As expected, wheat had the highest effective degradability of OM and the peas the highest degradability of CP. Rate of degradation (c) of OM as well as of CP was highest for the wheat. The lowest

Table 3
Organic matter (OM) and crude protein (CP) degradation kinetics of the feedstuffs from the *in sacco* trial

Feedstuff	*a*	*b*	*c*	*a* + *b*	Lag time (h)	Effective degradability	
						$k = 0.05\ h^{-1}$	$k = 0.08^{-1}$
Grass silage							
OM	0.30	0.55	0.025	0.84	0.9	0.48	0.43
CP	0.56	0.26	0.047	0.82	7.6	0.69	0.66
Maize silage							
OM	0.52	0.35	0.086	0.87	12.5	0.74	0.70
CP	0.27	0.67	0.055	0.85	3.7	0.63	0.55
Grass hay							
OM	0.27	0.61	0.051	0.87	2.8	0.57	0.50
CP	0.15	0.71	0.062	0.86	2.9	0.54	0.46
Peas							
OM	0.47	0.53	0.096	1.00	3.4	0.82	0.76
CP	0.70	0.30	0.078	1.00	1.7	0.88	0.85
Soyabeans							
OM	0.29	0.71	0.059	1.00	1.3	0.68	0.59
CP	0.15	0.85	0.061	1.00	0.9	0.62	0.52
Maize grain							
OM	0.19	0.81	0.048	1.00	0.9	0.59	0.50
CP	0.03	0.97	0.037	1.00	1.5	0.45	0.34
Wheat grain							
OM	0.49	0.45	0.212	0.94	0.5	0.86	0.82
CP	0.35	0.65	0.183	1.00	3.2	0.86	0.80

a, soluble fraction; *b*, insoluble but potentially degradable fraction; *c*, constant rate of degradation of *b*; *k*, rumen passage rate.

rates were measured for the grass silage (OM) and for the maize grain (CP). Potential degradability varied between 0.82 for the grass silage protein and 1.00 for OM of the peas and the soyabeans as well as CP of the peas, the soyabeans, the maize grain and the wheat grain.

The ratio of available N/kg FOM calculated from the *in sacco* experiments and the amount and pattern of feeding in the *in vivo* experiments during each 4 h interval are shown in Fig. 1. Optimal N:OM ratio for microbial protein synthesis in the rumen was assumed to be 25 g N/kg FOM. In the first experiment, FS-C had less variation of the ratio N:OM than FS-A and FS-B, therefore the SI of FS-C was higher than with FS-A and FS-B. The biggest variation occurred in FS-B where the differences between the ratio of N:OM and the optimal value were about 20 g/kg. These variations were not so large in the FS-A and FS-C where it amounted to only 5 g/kg for most of the intervals. In the second experiment, the variations in N:OM to the optimal value were quite different to those seen in the first experiment. The biggest deviation in the ratio N:OM from the optimal value occurred at 7:30 and 15:30 h in the FS-C which accounted for nearly 15 g/kg (Fig. 1). Small differences were found in the FS-B where the highest deviation of the ratio N:OM from the optimal value was about 5 g/kg. As a result, the SI of FS-B (0.90) was found to be the most optimal among all three

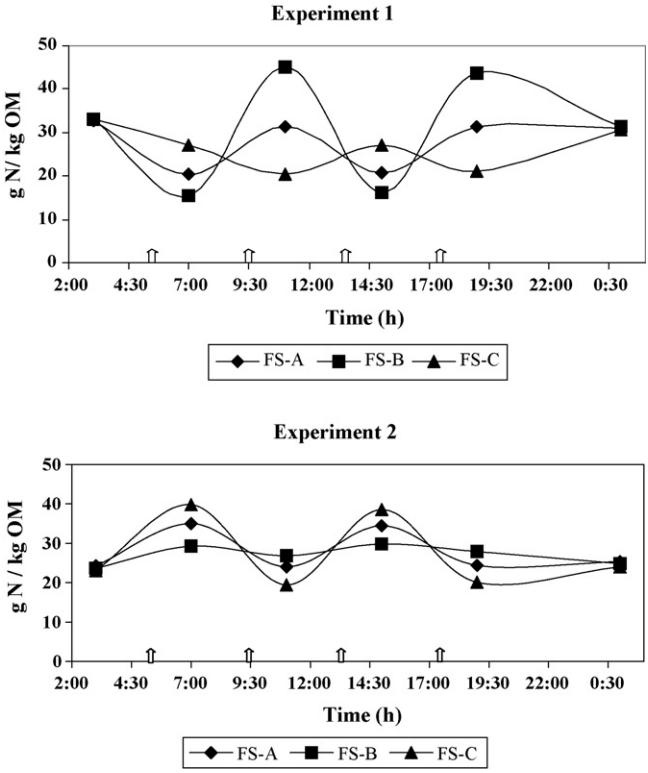

Fig. 1. Relationship of N to OM degraded in the rumen at the different times calculated using intake from the *in vivo* and degradation characteristics from the *in sacco* experiments (arrows on the axis indicate feeding times).

feeding sequences in this experiment. The synchronization indices calculated on the basis of the ratio between N:OM available in the rumen in Experiment 1 were 0.76, 0.52, and 0.82 and in Experiment 2: 0.85, 0.90, and 0.72 for FS-A, -B, and -C, respectively.

3.2. In vivo experiments

The *in vivo* experiments were conducted with a total of eight cows: seven in the first and five in the second experiment. In the first experiment, one cow had to be removed because of mastitis in the first period and two cows started their dry period at the end of the experiment, so that their feed intake decreased considerably, with the consequence that FS-A and FS-C were only studied on six or five cows, respectively. In the second experiment, FS-A and FS-C were investigated on four cows and FS-B on five cows. The reasons were mastitis or claw problems.

3.2.1. Intake

As intended, DM intake (DMI) ranged from 14.0 to 14.4 kg/day and 16.9 to 17.0 kg/day for the first and the second experiment, respectively (Table 4). The mean CP intake was 140

Table 4
Mean daily intake of the cows during duodenal collection periods

	DM (kg/day)	OM (kg/day)	CP (g/day)	NDFom (g/day)	ADFom (g/day)	ME[a] (MJ/day)	uCP[b] (g/day)
First experiment							
FS-A	14.4	13.9	2045	4445	2191	168.8	2102
FS-B	14.0	13.5	1936	4344	2157	163.8	2060
FS-C	14.1	13.6	2007	4371	2153	165.5	2118
Second experiment							
FS-A	17.0	15.9	2802	5802	3310	190.7	2686
FS-B	16.9	15.7	2774	5708	3254	188.5	2660
FS-C	17.0	15.9	2802	5802	3310	190.7	2686

[a] ME calculation based on the analyses (Table 1) and on digestibility values from DLG-tables (DLG, 1997) according to the GfE (2001) formulas.
[b] uCP (utilizable CP at the duodenum): MP + UDP, calculation based on the analyses (Table 1) and on UDP and digestibility values from DLG-tables (DLG, 1997) according to the GfE (2001) formulas.

and 165 g/kg DMI for the first and second experiment, respectively. The uCP intake which was calculated from DMI, nutrient analyses, and digestibility of OM and degradability of CP taken from DLG (1997) ranged from 2060 to 2118 g/day and from 2660 to 2686 g/day for the first and second experiment, respectively (Table 4).

The mean ME (MJ/day) and NEL (MJ/day) intakes were 166.0 ± 2.5; 102.6 ± 1.58 and 190.0 ± 1.2; 116.2 ± 0.8 for the first and second experiments, respectively.

3.2.2. *Rumen parameters*

Diurnal variation in rumen pH for the different feeding sequences is presented in Fig. 2. The mean of rumen pH was not influenced by feeding sequence in both experiments (Experiment 1: 6.43 ± 0.4, 6.37 ± 0.4, and 6.33 ± 0.5; Experiment 2: 6.53 ± 0.2, 6.54 ± 0.2, and 6.56 ± 6.56 for FS-A, FS-B, and FS-C, respectively). Pattern of rumen pH during the course of the day was different between experiments but differences between sequences were small ($P>0.05$) and more pronounced in the first compared with the second experiment. A treatment × time effect could not be proved statistically.

The mean rumen ammonia-N concentration (resulting from CP degradation and/or N use by the microbes) was also not affected by FS in both experiments (Experiment 1: 10.62 ± 7.04, 6.75 ± 5.03, and 9.10 ± 8.69; Experiment 2: 13.51 ± 8.10, 12.96 ± 7.08, and 14.94 ± 8.55 mg NH_3-N/100 ml for FS-A, FS-B, and FS-C, respectively). Mean rumen ammonia-N concentrations in all feeding sequences were above the minimum requirement of 5 mg/100 ml for maximal microbial growth suggested by Satter and Slyter (1974). However, for some sampling times rumen ammonia-N concentrations were lower, especially before feeding times at 05:30, 09:00, 12:00, 13:00, and 13:30 h (Fig. 3).

Rumen ammonia-N concentration increased dramatically just after feeding the peas + urea in the first experiment (FS-C: 05:30 h, FS-B: 09:30 h). All urea and more than 0.70 of N in the peas were soluble (Table 3). According to this there was a significant ($P<0.05$) time × treatment interaction in Experiment 1. The feeding of soyabean meal in the second experiment resulted in a more consistent increase in rumen ammonia-N concentration and followed the same course among feeding sequences. A treatment × time effect

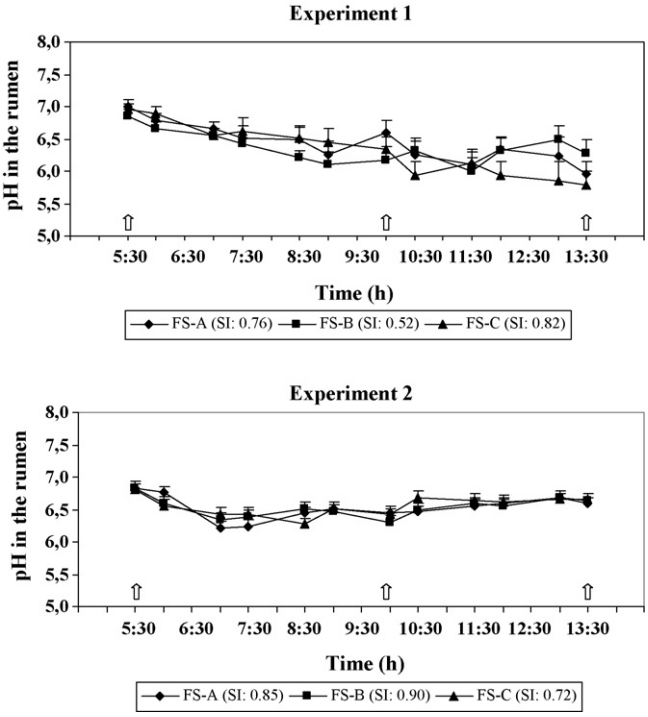

Fig. 2. Mean course of pH in the rumen of cows fed different feeding sequences (FS) ($n = 4$). Arrows on the axis indicate feeding times (SI, synchronization index).

could not be proved statistically. Daily mean rumen ammonia-N concentrations were lower in FS-B than in the other feeding sequences in both experiments (Fig. 3).

Considering that during the 8 h of rumen sampling (05:30–13:30 h), cows in each FS were fed the same amount of energy and protein from the same sources, and the CP degradability was the same, it may be expected that N released in the rumen would be the same for all feeding sequences within experiment. Thus, if it is assumed that the N utilization by microbes was the same over the day, then the same mean concentrations of rumen ammonia-N should result. In fact, the mean rumen ammonia-N concentrations were different among the various feeding sequences. Lower rumen ammonia-N concentration was found in FS-B in both experiments which might be the result of more effective ammonia-N utilization for MP synthesis. It may be that feeding energy sources in advance of protein sources stimulated the uptake of rumen ammonia-N which was already available in the rumen and resulted in a further decrease of ammonia-N concentration. Low rumen ammonia-N concentrations stimulate transfer of blood urea across the rumen wall into the rumen (Nolan, 1993) where it can be utilized by the microbes. This effect is not to be taken into consideration by the SI and will thus limit the value of using the SI. Increasing recycling of N from the blood will reduce blood urea-concentration resulting in lower concentrations of urea in the milk (Lebzien et al., 2006). This was indicated also by the numerically higher microbial protein

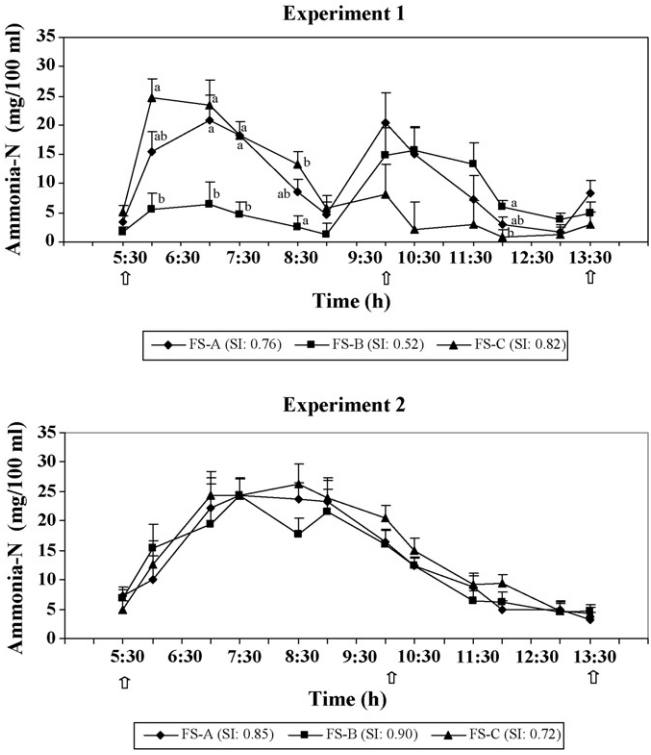

Fig. 3. Mean course of ammonia-N concentration (mg/100 ml) in the rumen of cows fed different feeding sequences (FS) ($n = 4$). Arrows on the axis indicate feeding times (values with different letters within experiment differ significantly) (SI, synchronization index).

synthesis (Table 7) and the slightly lower milk urea concentrations estimated in FS-B than in FS-A or FS-C (Table 7) in both experiments. However, these differences were significant only in the second experiment for the efficiency of microbial protein (MP) synthesis (g MP/kg FOM and g MP/MJ ME) with higher values for FS-B than FS-A and FS-C. In the first experiment, milk urea concentration was significantly lower for FS-B than for FS-A. Low rumen ammonia-N concentrations at a constant feed protein intake could also be due to less degradable N sources (Kolver et al., 1998; Shabi et al., 1998). In the present experiment, however, the amount and degradability of protein was the same in all feeding sequences within the experiments, therefore the lower mean rumen ammonia-N concentration found in FS-B is likely to be related to factors that promote use of ammonia by microbes, such as synchronization of energy and protein supply in the rumen and/or feeding sequence of concentrates *per se*.

Feeding sequence did not affect the SCFA concentration in the rumen (P>0.05), neither at each sampling time (Fig. 4) nor on average (Experiment 1: 96.5 ± 4.4, 106.5 ± 3.6, and 109.0 ± 4.1, Experiment 2: 98.4 ± 2.5, 100.3 ± 2.5, and 105.5 ± 2.3 mmol SCFA/l for FS-A, FS-B, and FS-C, respectively), in both experiments. A treatment × time effect could

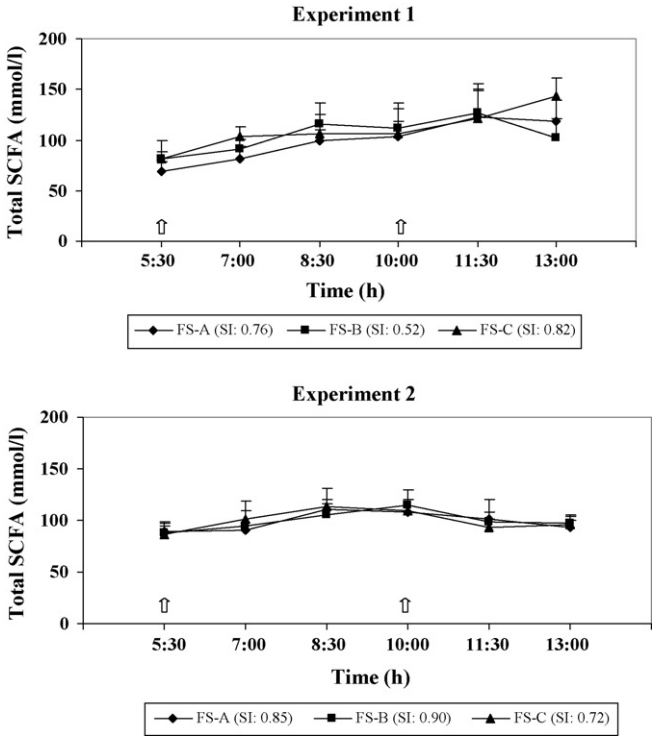

Fig. 4. Mean course of total SCFA concentration (mmol/l) in the rumen of cows fed different feeding sequences (FS) ($n = 4$). Arrows on the axis indicate feeding times (SI, synchronization index).

not be proved statistically. In the first experiment, however, there was an indication that the daily means total SCFA concentrations were lower in FS-A than FS-C at P=0.069. Concentration of SCFA in the first experiment increased just after morning feeding and continued increasing after the second feeding time whereas in the second experiment SCFA concentration reached a peak at 10:00 h for all three feeding sequences (Fig. 4). The daily mean SCFA concentrations for the first and second experiment were on average 104.0 and 101.4 mmol/l, respectively, and were in the range of normal rumen SCFA concentration of 70–130 mmol/l suggested by France and Siddons (1993).

Although the cows were fed the same diet within each experiment, the mean proportions of acetic and propionic acids were different between sequences in the first experiment, but not in the second experiment (Table 5). Cows fed FS-B and FS-C had lower molar proportions of acetic acid and higher molar proportions of propionic acid than those fed FS-A, resulting in a lower ratio of acetic to propionic acid. It is unclear why feeding a protein source (peas + urea) first in the morning (FS-C) produced more propionic acid in the rumen than when the cows were fed energy source (wheat) first (FS-B), and at the same time they produced the same proportion of acetate. Generally, feeding starch-rich concentrates, such as in pea and wheat, support the developments of propionate-producing bacteria in the rumen and is associated with an increase of propionic acid in the rumen (France and

Table 5
Mean molar proportions of individual SCFA and ratio between acetic and propionic acid in the rumen of the cows fed the different sequences (LS-means ± S.E.)

Fatty acids	First experiment			Second experiment		
	FS-A (SI: 0.76) ($n=4$)	FS-B (SI: 0.52) ($n=5$)	FS-C (SI: 0.82) ($n=4$)	FS-A (SI: 0.85) ($n=4$)	FS-B (SI: 0.90) ($n=4$)	FS-C (SI: 0.72) ($n=4$)
Acetic acid	$0.63^{a} \pm 0.00$	$0.59^{b} \pm 0.01$	$0.58^{b} \pm 0.01$	0.66 ± 0.00	0.66 ± 0.00	0.65 ± 0.00
Propionic acid	$0.19^{b} \pm 0.01$	$0.20^{b} \pm 0.01$	$0.22^{a} \pm 0.01$	0.18 ± 0.00	0.19 ± 0.00	0.18 ± 0.00
Butyric acid	$0.14^{b} \pm 0.00$	$0.16^{a} \pm 0.00$	$0.15^{b} \pm 0.00$	$0.13^{a} \pm 0.00$	$0.12^{b} \pm 0.00$	$0.13^{ab} \pm 0.00$
Valeric acid	$0.02^{b} \pm 0.00$	$0.03^{a} \pm 0.00$	$0.03^{a} \pm 0.00$	$0.01^{b} \pm 0.00$	$0.01^{b} \pm 0.00$	$0.02^{a} \pm 0.00$
Branched chain fatty acids*	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	$0.02^{b} \pm 0.00$	$0.03^{a} \pm 0.00$	$0.03^{a} \pm 0.00$
C2:C3	$3.3^{a} \pm 0.1$	$2.9^{b} \pm 0.1$	$2.8^{b} \pm 0.1$	3.7 ± 0.0	3.6 ± 0.0	3.7 ± 0.0

Values with different superscript letters (a and b) within experiment differ significantly.
* Branched chain fatty acids: isobutyrate + isovalerate.

Siddons, 1993; Van Soest, 1994). Only few experiments have been conducted to compare the effects of feeding sequence of concentrates on the relative concentrations of individual fatty acids in the rumen. Robinson et al. (1997) observed a significant effect of time of feeding protein supplements on the patterns of ruminal fermentation.

Feeding sequences also altered the ratio of rumen ammonia and fatty acid concentrations in the rumen during the day. This ratio gives an indication of the relationship between degradation of carbohydrates and availability of N in the rumen. Table 7 shows the ratios of ammonia-N to total SCFA concentration in the rumen for each feeding sequence. In both experiments, FS-B had lower values and less variation for this ratio than FS-A and FS-C.

3.2.3. Organic matter fermentation

Means of flows and apparent ruminal digestibilities of OM, NDFom, and ADFom as well as OM fermentation are shown in Table 6. Significant differences within experiment were only seen in Experiment 2 for the flow and the apparent ruminal digestibility of organic matter. The flow of OM was lower for FS-A than FS-B. The mean apparent ruminal digestibility of OM and fermentability of OM tended to be lower, whereas digestibility for ADFom and NDFom were numerically higher in Experiment 2 than in Experiment 1. The higher OM degradation can be explained by the higher proportion of easily degradable feedstuffs in Experiment 1 and the lower NDFom and ADFom digestibilities by the lower pH value.

3.2.4. Flow of utilizable crude protein (uCP) at the duodenum and microbial protein synthesis

Flow of uCP (NAN $\times$ 6.25 $-$ endogenes protein) at the duodenum were numerically higher for FS-B than for FS-A and FS-C in both experiments (Table 7). Relative to the uCP values calculated according to GfE (2001) in Table 4, the measured flows amounted to 0.94, 0.98, 0.94, and 0.98, 1.09, 1.01 for FS-A, FS-B, FS-C in the first and second experiment, respectively, showing the good agreement between the calculated and the measured values.

Synthesis of microbial protein (MP) showed the same trend in both experiments where FS-B had the highest and FS-A the lowest values (Table 7). In accordance with this urea concentration in the milk (MU) was lowest in FS-B and highest in FS-A (Table 7). In the first experiment, microbial synthesis in FS-B was 1672 g MP/day, being 10% and 6% higher than that for FS-A and FS-C, respectively. In the second experiment, 2037 g MP was produced per day in FS-B, an increase of 18% and 14 % over that in FS-A and in FS-C, respectively. Efficiency of MP synthesis followed the same trend as MP synthesis, whereas in Experiment 2 the higher efficiency of MP synthesis for FS-B, either expressed by g MP/kg FOM or g MP/MJ ME, was significantly higher than for FS-A and FS-C (Table 7). Efficiency of MP synthesis in the present study averaged 195 g MP/kg FOM (31 g microbial N/kg FOM). These values were in the normal range of efficiency of MP synthesis reported in the literature (Lebzien and Voigt, 1999) (Table 8).

The higher efficiency of MP synthesis in FS-B was caused by higher MP flow at the duodenum and relatively lower OM digestion in the rumen (Table 6). The high mean value of microbial N synthesis (245 g microbial CP/kg FOM) in FS-B in the second experiment was mainly caused by very low FOM values of two cows. It might be however that feeding an energy-rich concentrate at the first feeding time in the morning stimulates microbial activity.

Table 6
Mean flows of organic matter (OM), NDFom, and ADFom at the duodenum, their apparent ruminal digestibilities as well as amount of fermented organic matter (FOM)

	First experiment			Second experiment		
	FS-A (SI: 0.76) ($n=6$)	FS-B (SI: 0.52) ($n=7$)	FS-C (SI:0.82) ($n=5$)	FS-A (SI: 0.85) ($n=4$)	FS-B (SI: 0.90) ($n=5$)	FS-C (SI: 0.72) ($n=4$)
Organic matter						
kg/day	7.42 ± 0.99	8.03 ± 0.99	8.16 ± 0.87	9.90^{b} ± 0.53	11.29^{a} ± 1.68	10.25^{ab} ± 1.19
Apparent digested in the rumen	0.46 ± 0.08	0.41 ± 0.06	0.40 ± 0.06	0.38^{a} ± 0.03	0.28^{b} ± 0.01	0.36^{ab} ± 0.08
Fermented organic matter						
kg/day	9.3 ± 0.9	8.6 ± 0.5	8.4 ± 0.7	9.2 ± 0.5	8.3 ± 1.9	9.0 ± 1.1
g/100 g OM intake	670 ± 57	638 ± 31	618 ± 53	579 ± 33	528 ± 22	566 ± 67
NDFom						
g/day	2537 ± 446	2784 ± 321	2740 ± 305	2745 ± 281	2634 ± 669	2925 ± 339
Apparent digested in the rumen	0.43 ± 0.10	0.36 ± 0.07	0.37 ± 0.07	0.53 ± 0.05	0.54 ± 0.12	0.50 ± 0.06
ADFom						
g/day	1183 ± 210	1325 ± 114	1356 ± 110	1365 ± 213	1320 ± 38	1380 ± 0.2
Apparent digested in the rumen	0.46 ± 0.10	0.39 ± 0.0 5	0.37 ± 0.05	0.59 ± 0.06	0.60 ± 0.12	0.58 ± 0.05

$a > b$ (P<0.05) within experiment. FOM: OM intake − (OM at the duodenum − microbial OM); microbial OM: microbial-N × 11.8 (Schafft, 1983).

Table 7
Flow of utilizable CP (uCP) at the duodenum, microbial protein (MP) synthesis and milk urea of cows fed different feeding sequences in two experiments in relation to synchronization index and ratio NH_3-N to SCFA

	First experiment			Second experiment		
	FS-A ($n=6$)	FS-B ($n=7$)	FS-C ($n=5$)	FS-A ($n=4$)	FS-B ($n=5$)	FS-C ($n=4$)
Synchronization index	0.76	0.52	0.82	0.85	0.90	0.72
NH_3-N:SCFA (mmol/l:mmol/l)	0.12 ± 0.11	0.07 ± 0.05	0.09 ± 0.08	0.14 ± 0.09	0.11 ± 0.06	0.14 ± 0.08
Utilizable crude protein (g/day)[a]	1985 ± 215	2018 ± 352	1983 ± 147	2624 ± 238	2908 ± 96	2714 ± 217
Microbial protein						
g/day	1515 ± 215	1672 ± 398	1576 ± 234	1723 ± 257	2037 ± 183	1777 ± 221
g/kg FOM[b]	163 ± 35	194 ± 41	188 ± 22	187[b] ± 24	245[a] ± 40	197[b] ± 37
g/MJ ME	9.0 ± 1.3	10.2 ± 2.5	9.5 ± 1.3	9.0[b] ± 1.3	10.8[a] ± 1.3	9.3[b] ± 1.3
Milk urea (mg/100 ml)	27.4[a] ± 4.7	21.6[b] ± 2.1	23.4[ab] ± 7.0	28.0 ± 4.4	27.3 ± 4.1	28.0 ± 3.9

$a > b$; $P<0.05$ within experiment.
[a] Utilizable CP: (non-ammonia-N − endogenes N) × 6.25 = MP + UDP, endogenes N = 3.6 × kg DM at the duodenum (Brandt et al., 1980).
[b] FOM (fermented organic matter): OM intake − (OM at the duodenum − microbial OM), microbial OM: microbial N × 11.8 (Schafft, 1983).

Table 8
Synchronization index and microbial N synthesis according to different authors

Source	Methods/animal	Synchronization index	Efficiency of microbial N synthesis
Blümmel et al., 2001	*In vitro*	0.78	26.3[a]
		0.78	28.0
		0.79	28.0
		0.82	28.8
		0.83	30.5
Sinclair et al., 1993	*In vivo*, sheep	0.58	27.0[b]
		0.93	30.8
Sinclair et al., 1995	*In vivo*, sheep	0.63	27.5[a]
		0.93	30.7
Own investigations	*In vivo*, dairy cows	0.52 (FS-B)	31.1[c]
		0.76 (FS-A) first experiment	26.1
		0.82 (FS-C)	30.1
	In vivo, dairy cows	0.72 (FS-C)	31.5[c]
		0.85 (FS-A) second experiment	29.9
		0.90 (FS-B)	39.2

[a] g microbial N/kg FOM. Calculated on the assumption that 1 kg microbial biomass contains 84.7 g N (Schafft, 1983).
[b] g microbial N/kg $OM_{truly\ digested}$ in the rumen based on [^{3}H] leucine.
[c] g microbial N/kg FOM.

With regard to the question of whether the SI is related to the efficiency of microbial N synthesis, Blümmel et al. (2001) and Sinclair et al. (1995) reported a relationship between index and efficiency of microbial N synthesis, whereas Sinclair et al. (1993) could not find a significant effect (Table 8). In the present experiments, there was an inconsistency between index and efficiency of microbial N synthesis. Table 7 shows that the lowest index in the first experiment resulted in the highest efficiency of microbial protein synthesis, but in the second experiment the highest index had the numerically highest efficiency of microbial protein synthesis. According to a literature review (Chamberlain and Choung, 1995) microbes may not require close synchrony between NH_3-N and energy because NH_3 is usually available.

Inconsistent results reported in the present experiment and in the literature suggest that SI calculated on the basis of *in sacco* results could not really explain the variation on the efficiency of MP synthesis. A number of reasons might be responsible for this:

1. Although the *in sacco* technique has been used extensively to compare degradation characteristics among feedstuffs and to improve our understanding of ruminal digestion processes, this technique has considerable variations among and within laboratories (Huhtanen, 2005; Madsen and Hvelplund, 1994). The sources of variation include preparation of samples, characteristics of bags, procedure and locality of incubation, washing, drying, animals, feeding of animals, and correction or not for small particles lost through the bag pores without being degraded (Madsen and Hvelplund, 1994).
2. The SI also does not consider the feeding behaviour, which is different from animal to animal. In the present experiments feeding was divided into four different feeding

times but the feed offered was not always completely eaten at the allocated time. In fact, some cows postponed eating the offered feed and preferred to have their meal with a low eating rate. This means that although there was no feed refusal left on the following day, consumption of feed failed to distribute nutrients as it was planned. The SI, however, calculates the amount of nutrients offered at each feeding time assuming that feed is consumed by the animal. Sinclair et al. (1993), who studied synchronization in sheep reported that amount of feed was restricted, so that the diets were consumed within 5 min after being offered, suggesting that there was no effect of feeding behaviour.

3. The amount of OM and N available in the rumen *in vivo* might be different from that calculated from *in sacco*, because passage of undegraded nutrients is not accounted for in the calculation of the index.
4. Rumination and N-recycling are not included in the calculations.
5. Rumen microbes are able to cope effectively with fluctuating supplies of energy and N in the rumen (Chamberlain and Choung, 1995). Therefore, the response in microbial synthesis to changes in the degree of synchrony of energy and N is smaller than expected.

Variation in the NH_3-N:SCFA ratio (Table 7) during the day seemed to reflect efficiency of microbial protein synthesis somewhat better than SI. Within experiment, a lower variation of this ratio (given as ±S.E.) was related to a numerically higher synthesis. This may demonstrate, that a more constant supply of ruminal available nutrients leads to higher MP synthesis, but this was not reflected by SI. Between experiments a correlation between variation in the NH_3-N:SCFA ratio and microbial protein synthesis did not exist.

Nevertheless, a method for estimating synchrony cannot be derived from these experiments. In contrast to SI, feeding sequence seemed to be related to the differences in MP synthesis or efficiency of MP synthesis. Table 7 shows that MP synthesis or efficiency of MP synthesis as well as uCP flow at the duodenum were higher in FS-B in both experiments. MP synthesis either expressed as g/day, g/kg FOM, or g/MJ ME was consistent in both experiments with the ranking order of FS-B > FS-C > FS-A. Feeding energy-rich concentrate at the first feeding time followed by feeding protein-rich concentrate at the next feeding, as in FS-B, perhaps increased the rate of fermentation and stimulated ammonia uptake for microbial protein synthesis. Rumen ammonia concentration in FS-B, especially in the first experiment, supported this assumption. Henning et al. (1993) reported that continuous infusion of energy resulted in a higher efficiency of microbial growth than did pulse dosing. The authors suggested that feeding strategies should be directed at first obtaining the most even ruminal energy supply pattern with a particular dietary feeding regimen and then providing the appropriate amount of ruminally available N. Few experiments have been conducted to examine the relationship between feeding sequence and MP synthesis. Some (Kolver et al., 1998; Vaughan et al., 2002) indirectly indicate that feeding sequence could affect MP synthesis. Voigt et al. (1978), who conducted the experiments using the feeding sequence methods could be of interest. They fed barley or maize 90 min before or 90 min after feeding roughage (*Rye-grasses*) either chopped or pelleted. More OM was digested in the rumen when cows fed concentrate 90 min after forage. However, the feeding sequence did not modify passage of duodenal N, bacterial-N, and non-bacterial-N. Numerically, feeding concentrates before forage gave more bacterial N than the opposite sequence. But there was a quite high variation of bacterial-N flow at the duodenum. Compared with

the present experiments, the experiments of Voigt et al. (1978) were not designed to have different sources of protein and energy in a separate feeding.

4. Conclusions

The results obtained from the present study show that the feeding method altered release of N and energy in the rumen. However, synchronization of energy and protein supply in the rumen in terms of the index proposed by Sinclair et al. (1993) which was calculated based on *in sacco* experiments was not associated with higher microbial protein synthesis. In addition, feeding energy-rich concentrate at the first feeding in the morning prior to protein-rich concentrate seemed to increase microbial protein synthesis.

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