Estimation of rumen outflow in dairy cows fed grass silage-based diets by use of reticular sampling as an alternative to sampling from the omasal canal¹

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ABSTRACT

A study was conducted to compare nutrient flows determined by a reticular sampling technique with those made by sampling digesta from the omasal canal. Six lactating dairy cows fitted with ruminal cannulas were used in a design with a 3 × 2 factorial arrangement of treatments and 4 periods. Treatments were 3 grass silages differing mainly in neutral detergent fiber (NDF) concentrations: 412, 530, or 639 g/kg of dry matter, each combined with 1 of 2 levels of concentrate feed. Digesta was collected from the reticulum and the omasal canal to represent a 24-h feeding cycle. Nutrient flow was calculated using the reconstitution system based on 3 markers (Co, Yb, and indigestible NDF) and using ¹⁵N as a microbial marker. Large and small particles and the fluid phase were recovered from digesta collected at both sampling sites. Bacterial samples from the reticulum and the omasum were separated into liquid- and particle-associated bacteria. Reticular samples were sieved through a 1-mm sieve before isolation of digesta phases and bacteria. Composition of the large particle phase differed mainly in fiber content of the digesta obtained from the 2 sampling sites. Sampling site did not affect marker concentration in any of the phases with which the markers were primarily associated. The ¹⁵N enrichment of bacterial samples did not differ between sampling sites. The reticular and omasal canal sampling techniques gave similar estimates of marker concentrations in reconstituted digesta, estimates of ruminal flow, and ruminal digestibility values for dry matter, organic matter, starch, and N. Sampling site × diet interactions were also not significant. Concentration of NDF was 2.2% higher in reconstituted omasal

digesta than in reconstituted reticular digesta. Ruminal NDF digestibility was 2.7% higher when estimated by sampling the reticulum than by sampling the omasal canal. The higher estimate of ruminal NDF digestibility with the reticular sampling technique was due to differences in NDF concentration of reconstituted digesta. This study shows that nutrient and microbial protein outflow from the rumen can be measured using a reticular sampling technique. The reticular sampling technique provides a promising alternative to sampling from the omasal canal because there is less interference with the animal and it does not require advanced sampling equipment.

Key words: dairy cow, omasal canal, reticulum, ruminal flow

INTRODUCTION

Different marker techniques and sampling methods have been used with ruminally or duodenally cannulated animals to determine nutrient outflow and microbial protein synthesis in the rumen. Improved accuracy of digesta flow measurements when using a triple marker system (Ahvenjärvi et al., 2003), and reduced interference from endogenous N flow and abomasal digestive processes are the 2 advantages of the omasal canal sampling technique (Ahvenjärvi et al., 2000). However, compared with samples collected from the duodenum, composition of omasal samples deviated more from calculated true digesta and the omasal large particle (LP) phase consists of more heterogeneous particulate matter (Ahvenjärvi et al., 2000). This could increase errors in cases of unrepresentative digesta sampling. In experiments in which the omasal canal sampling technique was used, Ahvenjärvi (2002) also observed reductions of 5 to 10% in total DMI. Huhtanen et al. (1997) suggested that the sampling procedure, rather than the sampling device, was responsible for the reductions in intake caused by either frequent sampling or a temporary imbalance in sodium supply. However, there is not enough information to differentiate clearly between

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these potential causes for the observed reductions in intake. Flow of particulate matter from the reticulum through the omasal canal is associated with reductions in particle size and proportions of digestible material remaining in the particles. However, reticular digesta selected for a particle size below 1 mm was suggested to be representative of digesta leaving the reticulo-rumen through the omasal canal (Krizsan et al., 2009). The objective of this study was to compare the outflow of nutrients and N fractions from the rumen determined by using a reticular sampling technique as an alternative to sampling from the omasal canal of lactating dairy cows fed 3 sources of grass silage each at 2 levels of concentrate supplementation.

MATERIALS AND METHODS

Animal Management, Experimental Design, and Diets

Six multiparous early-lactation Norwegian Red cows averaging 594 ± 62 kg of BW and 34 ± 5 kg of milk/d of milk yield at the beginning of this study, and fitted with 10-cm ruminal cannulas (Bar Diamond Inc., Parma, ID) were used in this experiment. All animals were registered and cared for according to guidelines approved by the Norwegian University of Life Sciences Animal Care and Use Committee and the National Animal Research Authority, and the experiment was carried out in accordance with the laws and regulations controlling experiments performed with live animals in Norway. The cows were part of a larger experiment with the aim of studying forage particle passage rates and microbial protein synthesis on the same diets. Some of these results have been reported earlier (Krizsan et al., 2009). The experiment was performed with a 3×2 factorial arrangement of treatments in 4 periods. Each cow was randomly assigned to 1 of 6 treatment sequences. The sequences were balanced to handle carryover effects from previous periods. Treatments consisted of 3 grass silages, which differed mainly in NDF concentration: 412 (**G1**), 530 (**G2**), and 639 (**G3**) g/kg of DM, each supplemented with 5 or 9 kg of concentrate as fed. Additional silage-specific chemical composition was (mean $\pm$ SD): G1 (310 ± 14.3 g of DM/kg, 922 ± 1.7 g of OM/kg of DM, and 215 ± 6.1 g of CP/kg of DM), G2 (349 ± 10.0 g of DM/kg, 923 ± 1.6 g of OM/kg of DM, and 127 ± 4.7 g of CP/kg of DM), and G3 (242 ± 9.0 g of DM/kg, 934 ± 1.7 g of OM/kg of DM, and 125 ± 5.2 g of CP/kg of DM). Silages were prepared from a first and third growth of a second-year sward consisting of timothy (66%; *Phleum pratense*) and meadow fescue (20%; *Festuca pratensis*) grass, and red clover (5%; *Trifolium pratense*) from a second-year sward. Smooth meadow grass (*Poa pratensis*) and weeds made

up the remaining 4 and 5%, respectively. The field was applied with 71 kg of N/ha on April 4 and 63 kg of N/ha on June 25 of 2007. The grass was harvested on May 15 (G1), June 11 (G3), and August 6 (G2) of 2007, using a mower conditioner (FC302G, Kuhn, Saverne, France), and an acid-based additive (740 g/kg of formic acid plus sodium formate) containing 20 g of lactose/kg (GrasAAT N Lacto, Addcon, Bonn, Germany) was applied at a rate of 4.5 L/t at the pickup on a round baler (GP 1260, Orkel, Fannrem, Norway). The round bales were processed through a feed mixer (Euromix I, Kuhn) continuously during the trial to equalize chop length between the silages, and silages were then stored at -20°C until used in the experiment. Chemical composition of the pelleted concentrate was (mean $\pm$ SD): 871 ± 0.28 g of DM/kg, 947 ± 3.9 g of OM/kg of DM, 3.9 ± 1.29 g of NDF/kg of DM, and 158 ± 10.7 g of CP/kg of DM. The concentrate was composed of the following ingredients: wheat starch 69%, fishmeal 8%, CN 8%, molasses 5%, urea 1%, rumen-protected fat (Energizer-RP10, Chance & Hunt, Cheshire, UK) 3%, soybean oil 2%, and a vitamin and mineral premix (Pluss Multitilskudd Stofe og geit, Felleskjøpet Agri BA, Gardermoen, Norway) 4%. Cows were provided feed on 7 occasions daily as follows: at 0600 h manually and from automatic belt feeders, placed at the loft and in connection with the individual cribs by slides, set at times 0900, 1200, 1500, 1800, 2100, and 2400 h. Silages and concentrate feed were evenly distributed over all meals. During the first 13 d of each period, cows had free access to silage; thereafter, intake was restricted to 95% of ad libitum intake to minimize between- and within-day variations in intake caused by intensive sampling. The cows were housed in tie stalls bedded with sawdust except during sampling days, and they had free access to water throughout the experiment.

Sampling Procedures and Preparation of Digesta Samples

Each experimental period lasted for 24 d, with the last 11 d used for sample collection. Silage and total DMI were measured between d 14 and 24, and total tract digestibility of OM of the diets was determined from total fecal collection from d 14 to 17. Milk yield was recorded daily, but only data from d 14 to 24, along with data on intake and digestibility, are reported.

Continuous infusion of the flow markers Yb-acetate (2.9 g/d of Yb) and CoEDTA (3.0 g/d of Co) was begun on d 4 in each period, following priming doses of Yb-acetate (1.3 g of Yb) and CoEDTA (1.3 g of Co) given via the rumen cannula. Marker infusions were continued until the last digesta sample was collected on d 17. From d 11, infusion of an aqueous solution of

10% atom excess ($^{15}\text{NH}_4$) $_2\text{SO}_4$ [Sigma Aldrich (Isotec), Miamisburg, OH], providing 200 mg/d of ^{15}N , was conducted using a peristaltic pump (Cenco Instruments MIJ N.V., Breda, the Netherlands), and thereafter, pumps were assembled with the individual lines providing the flow marker infusate directly into the rumen of each cow. The infusion began at least 60 h in advance of the first digesta sample collection in all periods to allow uniform distribution of ^{15}N from incorporation of $^{15}\text{NH}_3$ in the ruminal microbial pools (Broderick and Merchen, 1992). Before beginning the infusion of ^{15}N , a sample of whole ruminal contents was taken from each cow in every period and stored at -20°C for later determination of ^{15}N background.

Digesta flow entering the omasal canal was estimated using the triple marker method described by France and Siddons (1986). Indigestible NDF (**iNDF**; Huhtanen et al., 1994; Ahvenjärvi et al., 2003), which was mainly associated with the LP fraction, Yb concentrated in the small particle (**SP**) digesta phase, and Co following the fluid phase (**FP**; Udén et al., 1980) were used in combination to calculate digesta flow. The omasal sampling technique developed by Huhtanen et al. (1997), as modified by Ahvenjärvi et al. (2000), was used to collect 12 digesta samples from the omasum on d 14 to 17, with a 6-h interval between sampling occasions each day. On the last 2 d, sampling occasions were moved 2 h later than on the previous day. At every sampling, an 800-mL digesta sample was collected and divided into 3 subsamples as follows: 400-mL subsamples from every time point were pooled and held at -20°C as they were collected to yield a 4.8-L omasal composite sample, 250-mL subsamples at 4 collections were pooled and kept in a refrigerator to yield a 1.0-L digesta sample, 1 subsample was collected from each of the 3 digesta collection days, and finally, the balance of 150-mL subsamples were pooled over all 12 sampling times and held at -20°C during the collection for use as a spare sample. Reticular samples were collected on the same 3 d as the omasal digesta samples, applying a similar time schedule. The sampling occasions from the reticulum were always 3 h later than the collection of omasal digesta. Reticular samples were collected using a 250-mL wide-necked plastic bottle with a rubber stopper. The stoppered bottle was placed in the reticulum; the stopper was removed in the reticulum, and after the bottle was filled with digesta, the stopper was replaced before removal. The procedure was repeated 4 times to yield 1.0 L of reticular digesta. The 1.0-L reticular sample of digesta was manually filtered through a 1,000- μm sieve, and particles retained on the sieve were discarded; the sample was then divided into subsamples by using the procedure described for the 800-mL omasal digesta sample.

The 4.8-L pooled omasal and reticular composites were thawed at room temperature and separated into the 3 digesta phases: LP, SP, and an FP. Samples were initially filtered through a 100- μm filter made of polyamide fabric with a pore area equal to 44% of the total surface (Sefar Nitex 100/44, Sefar, Thal, Switzerland). Omasal and reticular particulate matter retained on the filter was rinsed with 200 mL of 0.9% (wt/vol) NaCl solution and defined as the LP phase. The filtrate was centrifuged at $1,000 \times g$ for 10 min at 5°C ; the supernatant was decanted and defined as the FP, whereas the remaining pellet represented the SP phase. The separated digesta phases were stored frozen until freeze-dried and ground. The LP phases were ground through 1.5-, 1.0-, and 0.5-mm screens to give samples of 3 different sizes, and SP phases were ground to pass 1.0- and 0.5-mm screens to give samples of 2 different sizes, using the same cutting mill (Retsch SM 100, Retsch GmbH, Haan, Germany). To obtain SP representative of a screen size of 1.5 mm, samples were ground with a mortar and pestle and manually sieved through a 1.5-mm sieve. All FP samples were ground with a mortar and pestle.

At the end of each of the three 24-h sampling days, bacteria were isolated from the three 1.0-L composites made from digesta collected from both the omasal canal and the reticulum of each cow. The following procedure for bacterial isolation was modified from that described by Reynal et al. (2005). Samples were filtered through the 100- μm polyamide fabric described above and the particulate matter remaining on the filter was washed with 800 mL of 0.9% (wt/vol) NaCl solution. The filtrates from each of the 6 cows were further processed for isolation of liquid-associated bacteria (**LAB**) by centrifugation at $1,000 \times g$ for 10 min at 5°C . Particulate matter retained on the 100- μm filter and the pellets generated from the first centrifugation step of LAB isolation from each cow were transferred to a 1,000-mL bottle. To detach particle-associated bacteria (**PAB**), 700 mL of 0.9% (wt/vol) NaCl solution containing 0.1% (vol/vol) Tween 80 was added to the 1,000-mL bottles, the contents were homogenized for 20 s using a household blender, and bottles were held overnight at 4°C . Supernatants from the initial $1,000 \times g$ centrifugation were transferred to clean centrifuge bottles and recentrifuged at $11,250 \times g$ for 30 min at 5°C ; the resulting supernatants were decanted and discarded. The remaining pellet was resuspended in 200 mL of McDougall's buffer (McDougall, 1948) and recentrifuged at $16,250 \times g$ for 20 min at 5°C to yield LAB. The next day, contents of the 1,000-mL PAB bottles were filtered through the 100- μm filter. To harvest PAB, the filtrate was subjected to the same differential centrifugation procedure described for LAB. The resulting LAB and PAB pellets

were stored at -20°C until freeze-dried. Bacterial composites were made by pooling equal amounts of sample DM from the 3 sampling days.

Composite fecal samples for marker analysis were made from a 3-d total fecal collection. Feces were quantitatively measured 3 times a day and 10% of the total weight on each occasion was stored and pooled at the end of the collection.

Indigestible NDF and Chemical Analyses

Indigestible NDF was determined in duplicate LP samples and in triplicate SP and fecal samples ground to pass a 1.5-mm screen. Sample amounts of 2 g were added to preweighed polyester bags with a pore size of $12\text{ }\mu\text{m}$ and a pore area equal to 6% of the total surface (Saatifil PES 12/6, Saatitech S.p.A., Veniano, Como, Italy). The bags were incubated for 288 h in the rumen of 2 dry cows fed a diet consisting of two-thirds hay and one-third concentrate (DM basis) at maintenance level. After removal from the rumen, the bags were rinsed in a household washing machine, dried at 45°C for 48 h, and weighed and residues were analyzed for NDF, including residual ash determination. The concentration of NDF was determined in an Ankom 200/220 Fiber Analyzer (Ankom Technology Corp., Fairport, NY). Heat-stable α -amylase and sodium sulfite (Mertens et al., 2002) were used in the determination of NDF.

The freeze-dried individual digesta phases were further analyzed for DM, OM, NDF, N, starch, NAN, ^{15}N atom percent excess, Yb, and Co. Dry matter content was determined by oven-drying at 105°C for 4 h, and ash concentrations were determined by ignition of the dried sample at 550°C for 4 h according to the method of Malkomesius and Nehring (1951). Total N concentration was determined by the Kjeldahl method on a Kjeltech Auto 1035/1038 instrument (Tecator AB, Höganäs, Sweden) using a Cu catalyst. Starch concentration was analyzed according to method 7611 of the American Association of Cereal Chemists (2003) on a Roche Cobas Mira S instrument (Roche Diagnostics, Basel, Switzerland). The abundance of ^{15}N and concentrations of NAN were analyzed with an ECS 4010 elemental combustion system (Costech Analytical Technologies Inc., Valencia, CA) interfaced with a Delta Plus XP mass spectrometer (Thermo Finnigan, San Jose, CA). Samples containing approximately $100\text{ }\mu\text{g}$ of N were weighed to the nearest microgram into $5 \times 8\text{ mm}$ tin capsules (Elemental Microanalysis Ltd., Okehampton, UK). Open capsules were placed into 96-well microtiter plates and $50\text{ }\mu\text{L}$ of 72 mM K_2CO_3 solution was added to each capsule. The microtiter plates were placed in a 60°C oven overnight to volatilize ammonia. Samples

were analyzed in duplicate, and samples with similar ^{15}N enrichment were analyzed as groups, separated by blanks, to avoid ^{15}N carryover between samples. L-Glutamic acid standards were included in every run. Individual digesta phases, fecal samples, and marker fluids were analyzed for Yb and Co, using inductively coupled plasma optical emission spectroscopy on a Perkin-Elmer Optima 5300 DV instrument (Perkin-Elmer, Shelton, CT). Samples of approximately 300 mg were added to 2.0 mL of distilled water plus 3.5 mL of a solution containing 69.2% (wt/vol) of purified HNO_3 and diluted to a final volume of 50 mL with distilled water. Samples were digested in a high-pressure microwave reactor reaching a final temperature/pressure of $250^{\circ}\text{C}/160\text{ bar}$ [UltraClave, Milestone srl, Sorisole, Italy]. Composite bacterial samples and background samples from each cow in every period were analyzed for NAN and ^{15}N as described above. Additionally, DM and ash content was determined as described in the composite bacterial samples.

Calculations

Digesta composition and nutrient flows for both sampling sites were calculated using the triple marker method (France and Siddons, 1986). Marker concentrations in the different digesta phases, corrected for fecal recovery, were used as the basis for calculation of reconstitution factors (**RF**). The RF for SP and LP phases were used to mathematically reconstitute reticular and omasal digesta composition. Reconstitution factors indicate the units of digesta phase that must be removed from (if negative RF), or added to (if positive RF), a unit of unrepresentative digesta to yield reconstituted (or true) digesta. Flow of OM was not corrected for VFA flow (Ahvenjärvi et al., 2000), but flows of DM, OM, and NAN were corrected for microbial DM, OM, and NAN flows, respectively. Total NAN flow was assumed to be composed of LAB and PAB NAN and nonammonia nonmicrobial N. The LAB NAN flows were calculated from ^{15}N enrichment above the background of FP, divided by ^{15}N enrichment of LAB above the background, and multiplied by the NAN FP flow from the respective sampling site. The same calculation was performed for NAN flows for PAB, except that ^{15}N enrichment above the background in SP + LP and NAN flows of SP + LP were used, assuming that PAB was representative of bacteria flowing with the SP + LP phase. Proportions of SP + LP were previously adjusted using the RF. The nonammonia nonmicrobial N flow was calculated as the difference between total NAN flow and total microbial NAN flow.

Statistical Analysis

All data were analyzed using the MIXED procedures of SAS (SAS Institute, 2003) by the following model:

$$Y_{ijklm} = \mu + S(C)_{il} + P_j + D_k + S_l + T_m + (DS)_{kl} + (ST)_{lm} + e_{ijklm},$$

where Y_{ijklm} is the dependent variable, μ is the overall mean, $S(C)_{il} \sim N(0, \sigma_{s(c)}^2)$ is the random effect of site l within cow i , P_j is the effect of period j , D_k is the effect of diet k , S_l is the effect of digesta sampling site l , T_m is the carryover effect of the preceding diet m , $(DS)_{kl}$ is the interaction between diet k and digesta sampling site l , $(ST)_{lm}$ is the interaction between digesta sampling site l and the carryover effect of the preceding diet m , and $e_{ijklm} \sim N(0, \sigma_e^2)$ is the random residual error. Carryover effects of the preceding diet and the interaction terms including the carryover effect were removed from the model when not significant ($P > 0.05$). Least squares means are reported and means separation was done by least significant difference to test the differences between sampling sites. The standard errors of the mean of OM and NDF in reticular LP phase DM were 1.03 times higher because of 1 missing observation. This also influenced the standard errors of the mean for OM, NDF, and the iNDF:NDF ratio in the reconstituted reticular sample, in true ruminal digesta of OM and NDF, and for microbial efficiency. Sums of squares for diet effects on production parameters were separated using orthogonal contrasts into single degree of freedom comparisons of a linear effect for concentrate, and linear and quadratic effects for silage NDF concentration.

RESULTS AND DISCUSSION

Intake, Milk Production, and Digestibility

Total DMI decreased linearly ($P = 0.01$) from 17.0 to 15.4 kg of DM/d with increased silage NDF concentration. The decrease in total DMI reflected a linear decrease ($P < 0.01$) in silage DMI, decreasing from 12.2 to 9.4 kg/d with increased silage NDF concentration. Apparent total tract digestibility of OM decreased ($P < 0.01$) linearly from 84.4 to 77.2% with increased silage NDF concentration. The reduced silage DMI and total tract digestibility of OM with increasing concentration of NDF in silage DM are in agreement with the results presented by Rinne et al. (2002). Apparent total tract digestibility values of dietary OM were higher ($P =$

0.02) for cows fed a high level of concentrate supplementation compared with those fed a low amount (82.2 vs. 79.0%, respectively). Increased OM apparent digestibility with increased concentrate supplementation has been reported for forage-based diets (Tyrrell and Moe, 1975). A higher digestibility of concentrate ingredients compared with grass silage would be expected to increase total diet digestibility. Milk production was highest for cows fed the G1 diets (29.4 kg/d) and lowest for cows fed the G2 and G3 diets (24.5 and 24.0 kg/d, respectively; $P = 0.03$).

DM Recovery and Compositional Characterization of Digesta Phases

Composition of the individual recovered digesta phases from the reticulum and the omasum are given in Table 1. Reticular digesta was characterized by 0.10 lower recovery of LP DM ($P < 0.01$) and 0.07 and 0.03 greater recovery of SP DM ($P < 0.01$) and FP DM ($P = 0.05$) than the corresponding phases in omasal digesta, respectively. The median size of reticular particles was 0.88 versus 0.34 mm for omasal particles across all diets (S. J. Krizsan; unpublished data). Ahvenjärvi et al. (2001) also found more LP in reticular digesta than in omasal digesta and reported mean particle sizes of 1.13 and 0.44 mm, respectively. Further, the mean particle size of duodenal digesta was 0.42 mm and the distribution of duodenal particulate OM differed only slightly from that of samples collected at the omasum (Ahvenjärvi et al., 2001). Hristov (2007) reported a larger ($P = 0.01$) proportion of DM recovered in the LP phase in reticular compared with duodenal digesta (0.56 vs. 0.42), comparable proportions of DM recovered in the FP (0.28), and the lowest recovery of DM in the SP (0.16 in reticular digesta vs. 0.29 in duodenal digesta). Hristov (2007) also separated the particulate matter by using a 100- μ m filter, but substantially less DM was recovered as LP in his study. The mean RF of 0.083 and -0.046 for LP in reticular and omasal samples (Table 1), respectively, indicated a relative over-recovery of LP DM during omasal sampling. Reconstitution factors of -0.020 and 0.112 for the SP phase indicated that the reverse was true for SP DM and, relative to reticular samples, DM from SP had to be added to omasal samples during reconstitution. The difference between sampling sites ($P < 0.01$; Table 1) in absolute values of the RF for the LP indicated that the correction was larger for the DM proportion of reticular LP than omasal LP. The opposite was true for the RF for the SP (i.e., the correction was smaller for the DM proportion of reticular SP than omasal SP; $P < 0.01$; Table 1). This indicated that the LP phase in reticular samples and the SP phase in omasal samples would be

Table 1. Composition of digesta phases (% of DM unless otherwise specified) and reconstitution factors for digesta sampled from the reticulum and the omasum in dairy cows

Item	Sampling site		SEM	<i>P</i> -value ¹	
	Reticulum	Omasum		Site	S × D
Proportion of DM recovered					
Large particles	0.17	0.27	0.009	<0.01	0.22
Small particles	0.34	0.27	0.008	<0.01	0.46
Fluid phase	0.49	0.46	0.009	0.05	0.60
DM, %					
Large particles	19.3	18.3	0.50	0.21	0.64
Small particles	7.99	8.04	0.249	0.86	0.66
Fluid phase	2.04	1.98	0.043	0.37	1.00
OM					
Large particles	90.6	92.1	0.35	0.01	0.16
Small particles	85.6	85.6	0.26	0.85	0.74
Fluid phase	53.1	52.9	0.51	0.75	0.94
NDF ²					
Large particles	51.0	58.0	1.02	<0.01	0.78
Small particles	22.7	24.7	0.86	0.13	0.81
Fluid phase	NA ³	NA			
Indigestible NDF					
Large particles	24.4	24.7	0.43	0.56	0.01
Small particles	7.7	8.6	0.36	0.11	0.24
Fluid phase	NA	NA			
Starch					
Large particles	3.4	2.9	0.22	0.13	0.25
Small particles	5.4	5.6	0.33	0.60	0.33
Fluid phase	1.9	1.5	0.39	0.39	0.01
NAN					
Large particles	4.40	3.46	0.136	<0.01	0.56
Small particles	6.22	6.12	0.893	0.42	0.73
Fluid phase	2.68	2.94	0.083	0.05	0.19
¹⁵ N enrichment, atom percent excess					
Large particles	0.033	0.031	0.0017	0.57	1.00
Small particles	0.040	0.037	0.0019	0.37	1.00
Fluid phase	0.053	0.053	0.0026	0.81	0.90
Proportion of microbial NAN in sample NAN ⁴					
Large + small particles	0.64	0.62	0.040	0.69	0.98
Fluid phase	0.90	0.94	0.057	0.68	0.86
Yb, mg/kg of DM					
Large particles	204	157	7.5	<0.01	0.58
Small particles	443	437	17.4	0.82	1.00
Fluid phase	120	130	3.7	0.04	0.97
Co, mg/kg of DM					
Large particles	92.5	90.2	3.49	0.66	0.43
Small particles	177	174	5.8	0.73	0.83
Fluid phase	488	505	21.9	0.55	0.99
Reconstitution factors ⁵					
Large particles	0.083	−0.046	0.0170	<0.01	0.27
Small particles	−0.020	0.112	0.0142	<0.01	0.03

¹Probability of a significant effect of sampling site and the interaction between sampling site and diet (S × D).

²Values for NDF are exclusive of residual ash.

³NA = not analyzed.

⁴Estimated as ¹⁵N enrichment in the particulate or fluid phase/¹⁵N enrichment of particle- or liquid-associated bacteria.

⁵The reconstitution factor for the fluid phase is set to 1.

most influenced by unrepresentative sampling in this study.

The individual digesta phases between the 2 sampling sites differed mostly in the composition of the LP phase. Large particles from reticular samples had 1.5 and 7 percentage units lower concentrations of OM

($P = 0.01$) and NDF ($P < 0.01$), respectively, compared with LP recovered from omasal digesta samples. Some composition differences between the individual digesta phases recovered from the reticulum versus the omasum could be expected because of digestive processes that continue in the omasum. It could not be

determined whether digesta samples collected from the omasal canal contained any of the digesta that may be expelled back into the reticulum or if they consisted mainly or exclusively of digesta flowing directly from the reticulum (McBride et al., 1984). However, because concentrations of OM and NDF were lower in the reticular LP phase, there was no evidence that mineral absorption and digestion of fiber in the omasum (Punia et al., 1988; Ahvenjärvi et al., 2000, 2001) accounted for the observed differences in digesta composition. Sand and other dense material also present in the bottom of the reticulum could have been collected with the digesta samples. This could have increased the ash concentration of the samples and thereby diluted the relative DM proportions of other components. Nonammonia N concentration was 0.94 percentage unit higher ($P < 0.01$) in the LP phase and 0.26 percentage unit lower ($P = 0.05$) in the FP from digesta collected in the reticulum than in the respective phases recovered from omasal digesta. It is possible that the selection of reticular particles below 1 mm was not completely representative of particulate matter belonging to the LP phase that are likely to leave the reticulum. On average, 19% of omasal particulate matter was retained on the 2,500- μm sieve in other samples collected during this experiment (S. J. Krizsan, unpublished data). These results suggest that the 1-mm sieve may have been too small and may have distorted the particulate matter composition somewhat. A larger sieve could be tested for preparing reticular samples, but the error was apparently not large because NDF flows were not different ($P = 0.54$) between sampling sites. Differences in the LP phases could have been due to inclusion of particles associated with microbial and protozoal matter, which would have been selectively retained in the reticulum. The ^{15}N enrichment of the LP phase and estimated proportion of microbial NAN in SP + LP NAN did not differ ($P \geq 0.57$; Table 1) between sampling sites and gave no support to the above-mentioned theory. However, omasal sampling cannot be considered an absolute reference and errors may also be associated with the composition of samples collected from the omasal canal. The slightly higher concentration of NAN in FP from omasal digesta also seems difficult to explain because neither the ^{15}N enrichment of the FP phase nor the proportion of microbial NAN in FP were different ($P \geq 0.68$) between sampling sites. The site $\times$ diet interactions for iNDF concentrations in the LP phase and for starch concentration in the FP were significant ($P = 0.01$; Table 1). The iNDF content in the LP sampled from cows fed G1 plus the high level of concentrate was 1.1 percentage units higher in iNDF, and G3 fed with the high level of concentrate was 4.6 percentage units lower in iNDF in reticular digesta ver-

sus omasal digesta (data not presented). Starch concentration in the FP from reticular digesta was different ($P < 0.01$) between diets, whereas no dietary effect was detected ($P = 0.33$) in FP recovered from omasal digesta (data not presented). The FP recovered from reticular digesta versus omasal digesta in cows fed G3 silage plus the high level of concentrate displayed the largest difference in starch content (4.0 vs. 2.2% of DM; data not presented). No conclusions were drawn from these interactions.

The concentrations of the external markers in the reticular and omasal digesta phases are given in Table 1. None of the markers in the triple marker system are exclusively associated with one digesta phase or are uniformly distributed across all phases. However, markers in the triple marker system should be in the highest concentration in their principal phase relative to the others. The reconstitution method assumes that although the sampled digesta may be unrepresentative with regard to the proportions of phases recovered, the compositions of the different phases are reliable (France and Siddons, 1986). Cobalt was primarily associated with the FP, Yb with the SP phase, and iNDF with the LP phase (Table 1). Sampling site did not affect ($P \geq 0.55$; Table 1) marker concentration in any of the phases with which the markers were primarily associated. However, Yb concentration was affected by sampling site ($P \leq 0.04$) in the LP phase and in the FP, but it is less important for the functionality of the marker system in the reticular sampling technique.

Bacterial Reference Samples

Composition and isotopic enrichment of LAB and PAB isolated from digesta taken from the reticulum or omasum are given in Table 2. No sampling site $\times$ diet interactions were detected for composition of bacterial samples ($P \geq 0.11$). However, concentration of OM in DM of LAB was 2.7 percentage units higher when isolated from omasal digesta versus reticular digesta. Faichney et al. (1997) suggested that synthesis of LAB-N, but not PAB-N, continued in the omasum, based on calculation of transfer quotients for rumen and duodenal bacterial pools, but no difference between sampling sites ($P = 0.96$; Table 2) in NAN concentrations in OM of LAB was observed in this material. Alternatively, differences in transfer quotients could be due to a release of PAB-N into the LAB-N fraction when exposed to the low pH of the abomasum, thereby reducing its enrichment (Faichney et al., 1997). The ^{15}N enrichment for LAB and PAB did not differ between sampling sites ($P \geq 0.71$), which implies that the differences in OM and NAN concentration for LAB were relatively small and could be due to feed OM and NAN

Table 2. Composition and ^{15}N enrichment of liquid- and particle-associated bacteria isolated from the reticulum or omasum of dairy cows

Item	Sampling site		SEM	<i>P</i> -value, ¹ site
	Reticulum	Omasum		
OM, % of DM				
Liquid-associated bacteria	76.7	79.4	0.39	<0.01
Particle-associated bacteria	75.6	75.5	0.74	0.91
NAN, % of OM				
Liquid-associated bacteria	10.0	9.99	0.168	0.96
Particle-associated bacteria	8.99	9.32	0.138	0.11
^{15}N enrichment, atom percent excess				
Liquid-associated bacteria	0.059	0.058	0.0033	0.71
Particle-associated bacteria	0.057	0.056	0.0032	0.81

¹Probability of a significant effect of sampling site. Sampling site $\times$ diet interactions were not significant ($P \geq 0.11$).

contamination during the isolation. Furthermore, ^{15}N enrichment above the background of LAB and PAB were very similar in this study. Different ^{15}N enrichments of LAB and PAB caused by differential utilization by LAB and PAB of N from the free ammonia pool have been reported (Martin et al., 1994; Faichney et al., 1997; Ahvenjärvi et al., 2002; Reynal et al., 2005; Brito et al., 2006). However, variable enrichment of the ruminal ammonia pool resulting from different doses of ^{15}N will only explain differences among studies, but differences in enrichment can also result from factors such as variation in dietary NPN.

Composition of Reconstituted Digesta, Nutrient Flow, and Ruminal Digestibility

The reticular and omasal canal sampling techniques gave similar estimates of marker concentrations in reconstituted digesta, of nutrient and microbial protein outflow from the rumen, and of ruminal digestibility values of DM, OM, starch, and N (Table 3). No sampling site $\times$ diet interactions were significant ($P \geq 0.24$). Concentration of NDF in reconstituted omasal digesta was 2.2 percentage units higher ($P = 0.01$) than in reconstituted reticular digesta. The iNDF:NDF ratio of 0.444 and 0.405 tended ($P = 0.10$; Table 3) to be different in reconstituted digesta from the reticulum versus the omasum. This was probably due to the higher concentration of NDF in the omasal LP phase. Ruminal digestibility of NDF also was 2.7 percentage units higher ($P = 0.04$) when estimated by sampling from the reticulum compared with sampling from the omasal canal. The ruminal digestibility of potentially digested NDF also differed ($P = 0.02$; data not presented) and was 82.9 and 79.6% when estimated by sampling from the reticulum compared with sampling from the omasal canal. Flow estimates did not differ ($P > 0.54$; Table 3) between sampling sites. The explanation for higher

estimates of ruminal fiber digestibility with reticular sampling was the difference in NDF concentration of reconstituted reticular and omasal digesta. The selection or sieving technique applied to the reticular samples could be responsible for the differences. A numerically higher iNDF:NDF ratio for digesta obtained from the reticulum indicates that reticular particles were slightly less digestible than those obtained from the omasal canal.

Measurements of starch digestibility in the rumen were not different ($P = 0.91$; Table 3) between sampling sites, and both estimates were high (on average 93.4%). These high estimates may be reliable because starch in the concentrate was provided as pure wheat starch, which made up 69% of concentrate DM.

In general, few differences were detected between the sampling sites; of all the traits presented in this study, the interactions between sampling site and diet were significant ($P \leq 0.03$) for only 3 traits. However, as already mentioned, sampling from the omasal canal might not be considered an absolute reference method. A measure of variation for the 2 methods separately was summarized as the coefficient of variation for the estimation of the most central traits. The coefficient of variation for the true ruminal digestibility values of OM and NDF, and flows of NAN and microbial NAN when estimates were based on sampling from the reticulum and omasal canal, were 3.8, 2.1, 10.9, and 8.2%, and 3.8, 3.9, 12.7, and 8.9%, respectively. There was no indication of any substantial variance inhomogeneity in estimates of principal traits between the 2 methods.

CONCLUSIONS

The results from this study show that nutrient and microbial protein outflows from the rumen can be measured from reticular sampling combined with a triple marker system. As with the omasal canal sampling

Table 3. Composition of reconstituted digesta, nutrient flow, and ruminal digestibility estimated based on reticular and omasal sampling techniques in dairy cows

Item	Sampling site		SEM	<i>P</i> -value, ¹ site
	Reticulum	Omasum		
Digesta composition, % of DM				
OM	77.4	77.7	0.39	0.64
NDF	19.8	22.0	0.52	0.01
Indigestible NDF:NDF	0.444	0.405	0.0151	0.10
Starch	3.5	3.4	0.33	0.75
NAN	4.43	4.47	0.089	0.77
Microbial NAN	2.9	2.9	0.14	0.82
Marker concentration				
Co, mg/kg of DM	330	339	12.6	0.58
Yb, mg/kg of DM	267	274	9.3	0.56
Indigestible NDF, g/kg of DM	94.1	96.3	6.45	0.81
Nutrient flow, kg/d				
DM	4.4	4.4	0.20	0.94
OM	3.5	3.5	0.16	0.87
NDF ²	1.5	1.6	0.11	0.54
Starch ³	0.25	0.24	0.025	0.73
NAN	0.322	0.318	0.0135	0.84
NANMN ⁴	0.126	0.128	0.0091	0.89
Microbial NAN	0.208	0.201	0.0128	0.71
True ruminal digestibility, %				
DM	71.8	71.6	1.24	0.91
OM	76.2	76.2	1.02	0.98
NDF ²	73.8	71.1	0.81	0.04
Starch ³	93.4	93.3	0.61	0.91
NAN	67.3	66.9	2.73	0.91
Microbial efficiency, g of NAN/kg of OMTDR ⁵	18.3	17.8	0.56	0.61

¹Probability of a significant effect of sampling site. Sampling site $\times$ diet interactions were not significant ($P \geq 0.24$).

²Not corrected for microbial NDF flow because bacterial samples were not analyzed for NDF.

³Not corrected for microbial starch flow because bacterial samples were not analyzed for starch.

⁴NANMN = nonammonia nonmicrobial N.

⁵OMTDR = OM truly digested in the rumen.

technique, sampling from the reticulum would also avoid interference from abomasal degradation of microbial N and endogenous N secretions. The reticular sampling technique provides a promising alternative to sampling from the omasal canal because there is less interference with the animal and no elaborate sampling equipment is required.

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