A comparison of ruminal or reticular digesta sampling as an alternative to sampling from the omasum of lactating dairy cows

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ABSTRACT

The objective of this study was to develop and compare techniques for determining nutrient flow based on digesta samples collected from the reticulum or rumen of lactating dairy cows with estimates generated by the omasal sampling technique. Pre-experimental method development suggested, after comparing with the particle size distribution of feces, application of primary sieving of ruminal and reticular digesta from lactating cows through an 11.6-mm sieve, implying that digesta particles smaller than this were eligible to flow out of the rumen. For flow measurements at the different sampling sites 4 multiparous, lactating Nordic Red cows fitted with ruminal cannulas were used in a Latin square design with 4 dietary treatments, in which crimped barley was replaced with 3 incremental levels of protein supplementation of canola meal. Digesta was collected from the rumen, reticulum, and omasum to represent a 24-h feeding cycle. Nutrient flow was calculated using the reconstitution system based on Cr, Yb, and indigestible neutral detergent fiber and using ¹⁵N as microbial marker. Large and small particles and the fluid phase were recovered from digesta collected at all sampling sites. Bacterial samples were isolated from the digesta collected from the omasum. Several differences existed for digesta composition, nutrient flows, and estimates of ruminal digestibility among the 3 different sampling sites. Sampling site × diet interactions were not significant. The estimated flows of DM, potentially digestible neutral detergent fiber, nonammonia N, and microbial N were significantly different between all sampling sites. However, the difference between DM flow based on sampling from the reticulum and the omasum was small (0.13 kg/d greater in the omasum). The equality between the reticulum and the omasum as sampling sites was supported by the following regression: omasal DM flow = 0.37 (±0.649) + 0.94 (±0.054) reticular DM flow ($R^2 = 0.96$ and root mean square error = 0.438 kg/d). More deviating nutrient-flow es-

timates when sampling digesta from the rumen than the reticulum compared with the omasum suggested that sampling from the reticulum is the most promising alternative to the omasal sampling technique. To definitively promote sampling from the reticulum as an alternative to the omasal sampling technique, more research is needed to determine selection criteria of reticular digesta for accurate and precise flow estimates across a range of diets.

Key words: dairy cow, digesta flow, microbial protein synthesis, rumen digestibility

INTRODUCTION

Accurate estimation of nutrient outflow from the rumen is a prerequisite for proper ration formulation to meet the nutrient requirements of dairy cattle (NRC, 2001; Fox et al., 2004). The abomasum or duodenum in fistulated sheep and cattle has commonly been used as a digesta sampling site to study digestion in the ruminant fore-stomachs. Abomasal or intestinal surgical procedures are often more difficult and involve longer animal recovery times than ruminal cannulation and may shorten the lifetime of the experimental animals, and cannulas at these locations require extensive maintenance (Faichney, 1993; Harmon and Richards, 1997). Huhtanen et al. (1997) proposed an omasal sampling technique for obtaining spot samples of ruminal digesta through the reticulo-omasal orifice. Improved accuracy of digesta flow measurements using a triple-marker system (Ahvenjärvi et al., 2003) and reduced interference from endogenous N flow and abomasal digestive processes are the advantages of the omasal sampling technique compared with sampling digesta from the duodenum (Ahvenjärvi et al., 2000). However, sampling from the omasum can reduce feed intake (Ahvenjärvi et al., 2002), and compared with samples collected from the duodenum, composition of omasal samples deviated more from calculated true digesta (Ahvenjärvi et al., 2000). This could increase errors in cases of unrepresentative digesta sampling if using less than 3 markers.

Björnhag et al. (1984) introduced a reticular sampling device to replace duodenal sampling in sheep. Hristov

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(2007) compared reticular and duodenal sampling in dairy cows and concluded that the reticular sampling technique can provide reliable estimates for ruminal digestibility. Furthermore, Krizsan et al. (2010) pointed out reticular sampling as a promising alternative to sampling from the omasum, with only a small digesta composition difference in NDF when sampling from the reticulum compared with the omasum. The composition difference resulted in a slightly higher estimated ruminal NDF digestibility when sampling from the reticulum compared with the omasum. Krizsan et al. (2010) applied the triple-marker method (France and Siddons, 1986) and sieving of the digesta through a 1-mm sieve size directly after it was collected from the reticulum. Hristov (2007) and Rotta et al. (2014) did not use any sieving to discard large particles not eligible for escape through the reticulo-omasal orifice. Krizsan et al. (2010) concluded that the selection of reticular particles below 1 mm was not completely representative of particulate matter belonging to the large-particle phase that are likely to leave the reticulum, and it appears that the triple-marker method was not able to completely correct for this unrepresentative sampling. Because flow of particulate matter from the reticulum through the reticulo-omasal orifice is associated with reductions in particle size (Ahvenjärvi et al., 2000), it was hypothesized that sieving of reticular or ruminal digesta through a sieve size yielding a particle size distribution comparable with that of fecal particles would provide a more representative sample. Poppi et al. (1980) and Udén and Van Soest (1982) suggested negligible changes in digesta particle size distribution after the abomasum. Sampling digesta from the rumen is the most reasonable alternative for estimates of ruminal nutrient outflow in small ruminants without the application of more advanced sampling equipment.

The hypothesis of this study was that the omasal sampling technique can be replaced by sampling of digesta from the reticulo-rumen of dairy cows, applying a reconstitution system based on 3 markers, and primary sieving of the collected digesta. Therefore, the objectives of this study were first to determine an appropriate sieve size selection for digesta collected from the reticulum or rumen of lactating dairy cows based on the fecal particle size distribution, and second compare nutrient flows based on sampling from these sites, applying the most appropriate sieving, with estimates based on omasal sampling.

MATERIALS AND METHODS

All animals were registered and cared for according to guidelines approved by the Swedish University of Agricultural 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 Sweden.

Sieve Size Selection for Rumen and Reticular Digesta

Digesta was sampled from the rumen (the cranio-dorsal blind sac), reticulum, omasum, and rectum from 3 mid-lactation cows fed TMR consisting of on average 60% grass silage and 40% concentrate [composed of crimped barley and canola meal; ExPro-00SF (Aarhus Karlshamn AB, Malmö, Sweden) and Solid 220 (Lantmännen Lantbruk AB, Stockholm, Sweden)], averaging (mean $\pm$ SD) 22.6 ± 3.8 kg of DMI/d and 27.6 ± 12.6 kg of milk/d during the sampling. Sampling was conducted at 3 different occasions. Samples of rumen and reticular digesta were collected using a 250-mL wide-necked plastic bottle with a rubber stopper according to Krizsan et al. (2010). Omasal digesta samples were collected by using the omasal sampling technique according to Huhtanen et al. (1997) and as modified by Ahvenjärvi et al. (2000). Ruminal and omasal samples were taken 4 times daily at 4-h intervals: 0600, 1000, 1400, and 1800 h. Spot samples of reticulum and feces were obtained 1 h after the omasal and rumen sampling the same 3 d at 0700, 1100, 1500, and 1900 h. Individual samples from each cow within sampling site and time point and for each applied sieve size selection were wet sieved separately. Three samples of 250 mL of rumen, reticular, and omasal digesta from each cow per sampling time per sampling site were distributed as follows: one was directly poured in a 1-L plastic bottle without primary sieving, the next 2 samples were sieved immediately after collection by custom-made sieves of 5.6 and 11.6 mm, and thereafter the filtrates were poured in 1-L plastic bottles. The sieves were made of 7-cm-high PVC tube (Uponor AB, Virsbo, Sweden) with 14-cm i.d., and the bottom of the tubes was covered by different-pore-size nets of stainless steel (Jowema, Anderstorp, Sweden). Samples were stored at -20°C before wet sieving. Thawed samples of 50 g on a fresh-weight basis from each of the rumen, reticular, and omasal digesta samples ($n = 4$ per cow and sampling site), respectively, were sieved through a series of sieves of 1,250, 630, and 160 μm representing large, medium, and small particle size fractions, respectively, using a Fritsch analysette 3 PRO (Fritsch GmbH, Idar-Oberstein, Germany) sieve shaker. The subsamples were sieved for 10 min with continuous water flow of 3.5 L/min at a rate of 3,000 vibrations/min, and the amplitude was adjusted to 2.0 mm. Fecal samples were suspended in 200 mL of water before sieving by the same method as described for the

digesta samples. After sieving, material from each sieve was quantitatively collected to preweighed 6- μ m polyester bags (07-6/5 Sefar Petex from Sefar AG, Heiden, Switzerland), dried at 60°C for 48 h, and weighed to determine the DM distribution of different particle size fractions.

Animal Management, Experimental Design, and Diets

For flow measurements at the different sampling sites, 4 multiparous, lactating Nordic Red cows were used, averaging (mean $\pm$ SD) 681 $\pm$ 54.8 kg of BW, 111 $\pm$ 16 DIM, and 29.1 $\pm$ 9.1 kg of milk/d at the start of the study. All cows were fitted with 10-cm ruminal cannulas (Bar Diamond Inc., Parma, ID). The cows were housed in tie stalls bedded with sawdust, had free access to water and salt blocks throughout the experiment, and were offered feed twice daily at 0600 and 1800 h. Orts were recorded once daily, and feeding rate was adjusted to yield orts of approximately 10% of intake except during the 4 d of sampling from the rumen, reticulum, and omasum; then intake was restricted to 95% of ad libitum intake to minimize between- and within-day variations in intake because of intensive sampling.

This study included 4 dietary treatments, in which crimped barley was replaced with 3 incremental levels of protein supplementation of canola meal (CM). The diets were provided to the cows according to a Latin square design in 4 experimental periods. In addition, the diets consisted of grass silage. Silages were prepared from the primary and regrowth of second-year sward consisting of timothy (80%; *Phleum pratense*) and red clover (20%; *Trifolium pratense*). The swards were fertilized with 80 kg of N/ha on May 21 and 40 kg of N/ha on July 3 in 2012. The grass was harvested on June 16 and July 31 in 2012 using a mower conditioner (GMT 3605 FlexP; JF-Stoll A/S, Sønderborg, Denmark) and a precision chop forage wagon (ES 5000 MetaQ ProTec; JF-Stoll A/S). The grass was treated with an acid-based additive (ProMyr XR 630; Perstorp Holding AB, Perstorp, Sweden) at a rate of approximately 3.5 L/t and ensiled and stored in bunker silos until use in the experiment. Crimped barley was ensiled using propionic acid (Perstorp Holding AB) applied at a rate of approximately 4 L/t. The CM was a commercial heat-treated protein supplement (ExPro-00SF). Feed rations were prepared each day, and silage and concentrate were fed separately. The DM concentration of the grass silages was determined twice a week and once a week for the concentrate ingredients. Forage and concentrate proportions were adjusted to a ratio of 40:60 on DM basis twice a week.

Sampling Procedures and Preparation of Digesta Samples

Each experimental period lasted for 21 d with the last 10 d used for sample collection. Total DMI was measured between d 12 and 21, and total-tract digestibility of the diets was determined by fecal spot sampling during d 18 to 21. The acid-insoluble ash (Van Keulen and Young, 1977) and indigestible NDF (iNDF; Huhtanen et al., 1994) marker methods were used to estimate total-tract digestibility. Milk yield was recorded daily, but only data from d 12 to 21, along with data on intake and digestibility, are reported.

Continuous infusion of the flow markers Yb-acetate (2.8 g/d of Yb) and CrEDTA (2.8 g/d of Cr) were started on d 15 in each period. The CrEDTA was prepared as described by Binnerts et al. (1968). Marker infusions were continued until the last digesta sample was collected on d 21. Also from d 15, infusion of 10% atom excess ($^{15}\text{NH}_4$) $_2\text{SO}_4$ [Sigma Aldrich (Isotec), Miamisburg, OH], providing 186 mg/d of ^{15}N , was conducted using a peristaltic pump (Ismatec Reglo Digital; IDEX Health and Science SA, Glattbrugg, Switzerland) assembled with the individual lines providing the flow-marker infusate directly into the rumen of each cow. The infusion started at least 60 h before 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 starting 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 was estimated using the triple-marker method described by France and Siddons (1986). Indigestible NDF, which is mainly associated with the large-particle fraction (Ahvenjärvi et al., 2003), Yb concentrated in the small-particle digesta phase, and Cr following the fluid phase (Downes and McDonald, 1964) were used in combination to calculate digesta flow. The omasal sampling technique was used to collect 12 digesta samples from the omasum on d 18 to 21 with 4-h interval between the 3 sampling occasions each day to cover sampling hourly during a complete 12-h feed cycle. On the last 3 d, sampling occasions were moved 1 h later than on previous day. At every sampling, a 650-mL digesta sample was collected and divided in 2 subsamples as follows: 400 mL from every time point was pooled and held at -20°C as they were collected to yield a 4.8-L omasal composite sample, 250 mL at 3 collections were pooled and kept in a refrigerator to yield a 750-mL digesta sample, one from each of the 3 digesta collection days. Rumen and reticular samples were collected the same 3 d as the omasal digesta

samples applying a similar time schedule. The sampling occasions from the omasum were always 1 h later than the collection of reticular and rumen digesta. Rumen and reticular samples were collected as described earlier and according to Krizsan et al. (2010). The sampling was repeated 2 times to yield 400 mL of digesta. The rumen and reticular samples of digesta were manually filtered through the 11.6-mm sieve (selected on the basis of the preliminary study), and particles retained on the sieve were discarded; then the sample was pooled and held at -20°C as they were collected to yield a 4.8-L composite sample. Fecal samples (approximately 250 g) were collected the same 4 d and time points as the omasal digesta samples. Fecal grab samples were directly pooled within cow during collection in each period.

The 4.8-L pooled digesta composites were thawed at room temperature and separated into the 3 digesta phases: large particles, small particles, and a fluid phase according to the same procedures as described by Krizsan et al. (2010). The separated digesta phases were stored frozen until dried and ground. Because of limited freeze-drying capacity, large particles were oven dried, and small particles and fluid phase were freeze-dried. The large-particle phases were ground through 2.0- and 1.0-mm screens to give samples of 2 different sizes, and small-particle phases were ground to pass 2.0-mm screens using the same cyclone mill (Cyclotec 1093 Sample mill; Foss Analytical A/S, Hillerød, Denmark). To obtain a small-particle representative using a screen size of 2.0 mm in a cutter mill, samples were ground with mortar and pestle and manually sieved through a 2.5-mm sieve. All fluid-phase samples were ground with mortar and pestle.

At the end of each of the four 12-h sampling days, bacteria were isolated from the four 750-mL composites made from digesta collected from the omasum of each cow with slight modifications of the method described by Krizsan et al. (2010). The supernatants from the $1,000 \times g$ for 10 min at 5°C centrifugations that aimed to separate liquid- and particle-associated bacteria from feed particles were combined to give one bacterial sample. A pooled bacterial isolate was thereafter separated and cleaned by high-speed centrifugation as described by Krizsan et al. (2010). The resulting bacterial pellets were stored at -20°C until being freeze-dried. Bacterial composites were made by pooling equal amounts of sample DM from the 3 sampling days.

Milk samples were collected from morning and evening milking on d 18 to 20 of each period. The samples were analyzed for the concentrations of fat, protein, and lactose by near infrared reflectance analyzer (Eurofins Steins Laboratory AB, Jönköping, Sweden).

iNDF and Chemical Analyses

Concentration of iNDF in all feed, digesta (particulate fractions of digest samples), and fecal samples was determined following a 288-h in situ incubation (Huhtanen et al., 1994) in the rumen using 2 cows. The 2 Nordic Red cows used for the in situ incubations were fed a diet consisting of 60% grass silage and 40% concentrate (mainly composed of crimped barley and canola meal; ExPro-00SF) on a DM basis. Samples were analyzed according to recommendations by Krizsan et al. (2013) using polyester bags with pore size of $6\text{ }\mu\text{m}$ and a pore area equal to 5% of the total surface (07-6/5 Sefar Petex). Indigestible NDF was expressed exclusive of residual ash.

The dried individual fecal and digesta phases were further analyzed for DM, and Yb and Cr. Feed samples were also analyzed for DM. Furthermore, dried individual feed, fecal, and reconstituted true digesta samples from all 3 sampling sites were analyzed for OM, NDF, N, NAN, and ^{15}N atom percent excess (**APE**). Feed and fecal samples were also analyzed for acid-insoluble ash according to Van Keulen and Young (1977). Residual moisture of all samples was determined by oven drying for 16 h at 105°C . Ash concentration was determined by ignition of the dried sample at 500°C for 4 h. The samples were analyzed for NDF using heat-stable α -amylase (Mertens et al., 2002) in an Ankom²⁰⁰ Fiber Analyzer (Ankom Technology Corp., Macedon, NY). Ash was determined in the bag residues, and NDF is expressed free of residual ash. Concentrations of N were determined by Kjeldahl digestion of a 1.0-g sample in 12 M sulfuric acid using Foss Tecator Kjeltabs Cu (Höganäs, Sweden) in a Block Digestion 28 system (Seal Analytical Ltd., Mequon, WI) with determination of total N by continuous-flow analysis using an Auto Analyzer 3 (SEAL Analytical Ltd.). The abundance of ^{15}N and concentrations of NAN were analyzed with a Flash 2000 Organic Elemental Analyzer interfaced with an Isotope Ratio Mass Spectrometer Delta V Advantage (Thermo Fisher Scientific Inc., Waltham, MA). Samples were prepared and ammonia volatilized as described by Krizsan et al. (2010). Samples were analyzed in duplicate, and samples with similar ^{15}N enrichment were analyzed as groups to avoid ^{15}N carryover between samples. Individual digesta phases and fecal samples were analyzed for Yb and Cr using inductively coupled plasma optical emission spectroscopy on a Perkin-Elmer Optima 7300 DV (Perkin Elmer, Shelton, CT). Samples of approximately 1.5 g of DM were added to 10 mL of a solution containing 65% wt/vol of purified HNO_3 . The samples were boiled in Kjeltac tubes in a block digester system (Tecator TM Digestion Auto; Foss Analytical A/S) with stepwise increasing

temperature: 1 h at 60°C, 1 h at 100°C, and finally 4 h at 125°C. After 2 h at 125°C, an additional 5 mL of the HNO₃ solution was added. The samples were diluted to a final volume of 50 mL with distilled water after cooling. Composite bacterial samples and background samples from each cow in every period were analyzed for NAN and ¹⁵N as described above. Additionally, DM and ash contents were determined as described in the composite bacterial samples.

Calculations

Dry matter flows for all 3 sampling sites were calculated using the triple-marker method. The method requires the same number of markers as physically separated digesta phases. The true digesta composition was solved by matrix calculation based on marker concentrations in the digesta phases and daily marker doses according to Armentano and Russell (1985). Daily marker doses were estimated from fecal output. Nutrient flows were calculated from nutrient composition in reconstituted true digesta samples and DM flows. To calculate true digestibility of OM, the flow of OM was corrected for VFA flow according to the equation by Huhtanen et al. (2010), and the flow of DM, OM, and NAN was corrected for microbial DM, OM, and NAN flows, respectively. Total NAN flow was assumed to be composed of microbial NAN and nonammonia, nonmicrobial N. The microbial NAN flows were calculated from ¹⁵N enrichment above the background:

$$\begin{aligned} \text{Microbial NAN flow (g/d)} &= \text{NAN flow (g/d)} \\ &\times (\text{TD } ^{15}\text{N APE/bacterial } ^{15}\text{N APE}), \quad [1] \end{aligned}$$

where TD ¹⁵N APE = ¹⁵N enrichment of the reconstituted true-digesta sample above background and bacterial ¹⁵N APE = ¹⁵N enrichment of microbial sample above background. The nonammonia, nonmicrobial N flow was calculated as the difference between total NAN flow and total microbial NAN flow.

Statistical Analysis

Data for digesta particle size distribution were analyzed using the GLM procedures of SAS (SAS Inc. 2002–2003, Release 9.2; SAS Institute Inc., Cary, NC) by correcting for time point and treatment. Each sampling site with the applied sieve size selection, if any, was given a treatment number to be able to analyze the data statistically. Least squares means of treatment are reported, and mean separation was done by least significant difference to test differences between digesta and fecal particle size distribution.

Data comparison between sampling sites was analyzed using the MIXED procedures of SAS by the following repeated-measurement model:

$$Y_{ijkl} = \mu + C(P)_{ij} + P_j + D_k + S_l + (DS)_{kl} + e_{ijkl},$$

where Y_{ijkl} = dependent variable, μ = overall mean, $C(P)_{ij} \sim N(0, \sigma_{C(P)}^2)$ is the random effect of cow i within period j , P_j = effect of period j , D_k = effect of diet k , S_l = effect of digesta sampling site l , $(DS)_{kl}$ = interaction between diet k and digesta sampling site l , and $e_{ijkl} \sim N(0, \sigma_e^2)$ is the random residual error. The effect of sampling site was evaluated as a repeated measure using the Ar(1) function with cow within period as subject. Least squares means are reported, and mean separation was done by least significant difference to test differences between treatments. Sums of squares for diet effects on production parameters were separated using orthogonal contrasts into single-degree-of-freedom comparisons of linear and quadratic effects of dietary CP concentration.

RESULTS AND DISCUSSION

Sieve Size Selection for Rumen and Reticular Digesta

The particle size distribution of ruminal, reticular, and omasal digesta subjected to no sieving or primary sieving with 2 different sieve sizes in comparison with fecal particle size distribution from 3 lactating dairy cows are presented in Table 1. Ruminants selectively retain large particles and structures containing a high proportion of digestible material in the rumen (Ahvenjärvi et al., 2001). Particles escape from the rumen at a critical particle size or after depletion of fermentable substrates. The probability of particles to escape from the rumen has been shown to increase with decreasing particle size (Poppi et al., 1980; Rinne et al., 2002; Bayat et al., 2007; Huhtanen et al., 2007) and with increased functional specific gravity (Sutherland, 1988). Ahvenjärvi et al. (2001) suggested that critical particle size was the major factor controlling the passage of feed particles from the reticulum of dairy cows because iNDF:NDF ratio within different particle size fractions was similar throughout the fore-stomachs. We hypothesized that if digesta flow from the reticulo-rumen is primarily determined by particle size or distribution, it could be determined based on sampling from the reticulo-rumen if the digesta is made similar to the particle size distribution in feces. This is further supported by the fact that little reduction in particle size has been suggested to occur between the abomasum and

Table 1. Effect of digesta sampling site and primary sieving (no sieve, a 5.6-mm sieve, or an 11.6-mm sieve) on particle size distribution (% on DM basis) retained on 3 sieves sizes of 1,250, 630, and 160 μm^1 ($n = 4$)

Particles ²	Rumen ³			Reticulum			Omasum			Rectum
	No	5.6 mm	11.6 mm	No	5.6 mm	11.6 mm	No	5.6 mm	11.6 mm	No
Large	40.5	3.87**	35.9	48.1**	3.67**	37.0	30.0	13.7**	34.0	36.3
Medium	12.3	11.7	11.3	9.49	9.22	12.0	10.3	13.6	11.8	12.7
Small	47.2	84.4**	52.9	42.4*	87.1**	50.9	57.6	72.8**	54.2	51.0

¹The SEM for large and small particles was 2.70% except for rumen digesta without primary sieving, where one missing observation caused a slight increase of the SEM to 3.16%. The SEM for medium particles was 1.32% except for rumen digesta without primary sieving, where one missing observation caused a slight increase of the SEM to 1.54%.

²Samples were sieved through a series of sieves of 1,250, 630, and 160 μm , representing large, medium, and small particle size fractions, respectively.

³Rumen samples collected from cranio-dorsal blind sac.

*,**Asterisks indicate the level of the least significant difference to fecal particle size distribution: ** $P \leq 0.01$; * $P \leq 0.05$.

feces (Poppi et al., 1980; Udén and Van Soest, 1982) or between omasum and feces (Ahvenjärvi et al., 2001), which would be likely when large particles mainly consist of fibrous material. Primary sieving of ruminal and reticular digesta through the 5.6-mm sieve decreased the contribution of large particles in the digesta sample, implying that a large proportion of particles eligible for passage were retained on a filter formed by the 5.6-mm sieve and the large digesta particles withheld on it. The reduction of the proportion of large particles was greater for reticular digesta than digesta collected in the cranio-dorsal blind sac, which is in agreement with a small proportion of newly ingested feed present in the reticulum (Sjaastad et al., 2003). By applying a primary sieving through an 11.6-mm sieve after digesta collection, particle size distribution of ruminal, reticular, and omasal digesta did not differ from the particle size distribution of feces from the same lactating dairy cows. Particle size distribution of ruminal and omasal digesta did not differ from fecal particle size distribution when no primary sieving was applied, in agreement with the results of Ahvenjärvi et al. (2001). Huhtanen et al. (1997) observed similar particle size fractions of omasal and fecal DM but criticized collected digesta from the omasum for being unrepresentative. However, this should be more related to fluid versus particle proportion in measurements of nutrient flow, not likely to distribution between different particle size fractions. Because the omasal sampling technique was developed not to include any primary sieving, it was only applied to the digesta collected from the reticulo-rumen in this study.

Diet Composition, Intake, Digestibility, and Milk Production

The experimental diet ingredient and chemical compositions are presented in Table 2. The diets primarily displayed variation in the CP and starch concentrations

as a consequence of the replacement of crimped barley with an incremental amount of CM. In these kind of studies, it is valuable to conduct the measurements on cows fed diets with a potential range in expected DMI and production performance, and apply a Latin square design. It is important that flow measurement techniques are robust over a range of different diets.

Total DMI increased linearly ($P = 0.02$) from 15.9 to 16.8 kg of DM/d with increased dietary CP concentration. Total-tract digestibility of NDF increased ($P = 0.02$) linearly from 595 to 630 g/kg with increased dietary CP concentration. Likewise, feeding more CM linearly increased yield of ECM (from 19.9 to 24.4 kg/d). The production responses were in agreement with the production trial that included comparable dietary CM supplementation by Gidlund et al. (2013). It has been recognized that inclusion of CM in dairy rations increases DMI and yields of milk and milk protein (Huhtanen et al., 2011; Martineau et al., 2013). Total NDF digestibility increased with the level of CM supplementation despite increased dietary iNDF concentration. This indicates that substitution of starch by protein improved cell-wall digestion by alleviating the negative effects of starch or that increased supply of AA had a stimulatory effect on cell-wall digestion, or both. It is also possible that the intrinsic rate of potentially digestible NDF digestion is faster for CM than barley.

Composition of Reconstituted Digesta, Nutrient Flow, and Ruminal Digestibility

Digesta composition, nutrient flows, and estimates of ruminal digestibility had several significant differences between the 3 different sampling sites in this study (Table 3). No sampling site $\times$ diet interactions on any trait were significant ($P \geq 0.06$). The concentrations of Cr and Yb in omasal digesta were not different from the concentrations in digesta from the reticulo-rumen

Table 2. Ingredient and diet composition (n = 4)

Item	Diet ¹				Ingredient		
	C	CL	CM	CH	Silage ²	Crimped barley	Canola meal ³
Ingredient (g/kg of DM)							
Silage	544	549	561	552			
Crimped barley	441	357	271	198			
Canola meal ³	0	78.9	154	235			
NaCl	3.42	3.38	3.30	3.36			
Minerals ⁴	11.4	11.3	11.0	11.2			
Nutrient composition (g/kg of DM)							
DM (g/kg)	411	427	440	465	232	611	848
OM	926	921	917	912	910	976	918
CP	132	148	164	180	142	122	328
Fat	21.3	26.0	30.5	35.5	NA ⁵	23.6	83.7
NDF	340	351	364	368	545	145	279
Indigestible NDF	79.9	84.4	88.6	92.7	103	54.9	106
Starch	247	204	160	125	NA	560	54.3
Water-soluble carbohydrates	19.0	22.5	25.2	29.4	5.3	49.4	80.3

¹Control diet (C) without canola meal, and low (CL), medium (CM), and high (CH) dietary canola-meal inclusions were achieved by replacing crimped barley.

²Average silage composition of the first- and second-cut timothy and red clover silages used in the experiment. Fermentation quality: pH 3.70; lactic acid, 105 g/kg of DM; acetic acid, 23 g/kg of DM; propionic acid, 2.6 g/kg of DM; butyric acid, 0.3 g/kg of DM; and NH₃-N, 45 g/kg of N.

³A commercial heat-treated protein supplement of canola meal (ExPro-00SF; Aarhus Karlshamn AB, Malmö, Sweden).

⁴Effekt Hög Zn; Lantmännen Lantbruk AB, Stockholm, Sweden.

⁵Not analyzed.

($P \geq 0.06$). The concentration of iNDF was different between reticular, and ruminal and omasal digesta ($P = 0.02$) but not between ruminal and omasal digesta ($P = 0.53$). However, the concentrations of all 3 markers in digesta from the reticulum were significantly different from the rumen ($P \leq 0.04$). Concentration of OM in digesta collected from the omasum was different from that in digesta collected from the reticulo-rumen ($P = 0.01$). The estimated flow of DM was significantly different between all sampling sites ($P < 0.01$). Numerically, the difference between flow estimates based on sampling from the reticulum and the omasum was very small (0.13 kg/d greater in the omasum) compared with when sampling from the rumen (more than 1 kg/d difference). The similarity in DM flows between the reticulum and the omasum was further supported by the linear relationship between the 2 sampling sites (Figure 1). Flow of OM was also smallest when sampling from the rumen ($P < 0.01$), but estimates based on digesta collected from the reticulum versus the omasum did not differ ($P = 0.79$). The differences in DM and OM flows were reflected in greater ruminal digestibility of DM and OM when estimated from digesta collected from the rumen compared with the other sampling sites ($P < 0.01$). Sand and other dense material present in the bottom of the reticulum can be collected with the digesta samples. This can alter ash concentration of these samples and, thereby, DM proportions of other components.

The differences in NDF concentration and iNDF:NDF ratio in digesta collected from the rumen compared with the omasum ($P \leq 0.04$) were reflected in a greater estimated NDF flow and digestibility when sampling from the omasum compared with the rumen ($P \leq 0.05$). Furthermore, all sampling sites generated different estimates of ruminal potentially digestible NDF flow and digestibility ($P < 0.01$). Digesta concentration and flow of NAN were also different between all sampling sites ($P < 0.01$) and generated significant different flow of microbial NAN at all 3 sampling sites ($P < 0.01$). The microbial efficiency was different between all sampling sites, being smallest when sampling from the omasum and greatest when estimated from digesta collected from the rumen ($P < 0.01$).

In the study by Ahvenjärvi et al. (2001), sampling sites from the rumen to the omasum did not differ in potentially digestible NDF concentration within sieve sizes between 0.080 and 1.25 mm. In addition, an equal distribution of particulate OM between digesta from reticulum and omasum passing the 1.25-mm sieve further supported the suggestion that a critical particle size close to 1 mm was the major factor controlling the passage of feed particles through the reticulo-omasal orifice in dairy cows. This is in contrast to the approach and results of this study and more in agreement with the results of Krizsan et al. (2010). Furthermore, earlier research by Mertens (1997) has considered feed particles physically effective for dairy cows based on

Figure 1. The linear relationship between omasal DM flow and reticular DM flow in kilograms per day. The fitted regression equation is given in the figure. The coefficient of determination and root mean square error of the regression equation were 0.957 and 0.438 kg/d, respectively.

Table 3. Composition of reconstituted digesta, nutrient flow, and ruminal digestibility estimated based on ruminal, reticular, and omasal sampling techniques in dairy cows (n = 16)

Item ¹	Sampling site			SEM	P-value: site ²
	Rumen	Reticulum	Omasum		
Marker concentration					
Cr (mg/kg of DM)	163 ^a	156 ^b	160 ^{ab}	6.4	0.02
Yb (mg/kg of DM)	169 ^a	163 ^b	167 ^{ab}	7.8	0.02
iNDF (g/kg of DM)	123 ^a	118 ^b	122 ^a	2.2	0.02
Digesta composition (g/kg of DM)					
OM	777 ^a	774 ^a	763 ^b	5.0	0.01
NDF	214 ^b	222 ^{ab}	240 ^a	8.5	0.04
iNDF:NDF	0.584 ^a	0.538 ^a	0.511 ^b	0.0181	0.03
NAN	42.1 ^a	39.7 ^b	37.5 ^c	0.61	<0.01
Nutrient flows ³ (kg/d)					
DM	6.23 ^c	7.33 ^b	7.46 ^a	0.339	<0.01
OM	4.09 ^b	4.87 ^a	4.82 ^a	0.230	<0.01
NDF	2.44 ^b	2.62 ^{ab}	2.78 ^a	0.127	0.05
pdNDF	1.04 ^c	1.21 ^b	1.41 ^a	0.068	<0.01
NAN (g/d)	517 ^a	475 ^b	436 ^c	12.3	<0.01
NANMN (g/d)	174	181	170	8.3	0.17
Microbial NAN (g/d)	342 ^a	294 ^b	267 ^c	18.0	<0.01
True ruminal digestibility (g/kg)					
DM	612 ^a	540 ^b	531 ^b	10.1	<0.01
OM	722 ^a	666 ^b	669 ^b	8.6	<0.01
NDF	565 ^a	533 ^{ab}	507 ^b	15.4	0.04
pdNDF	754 ^a	711 ^b	670 ^c	13.3	<0.01
NAN	433	451	424	12.3	0.27
Microbial efficiency (g of NAN/kg of OMTDR)	32.6 ^a	30.2 ^b	27.4 ^c	1.01	<0.01

^{a-c}Means within a row with different superscripts differ ($P < 0.05$).

¹iNDF = indigestible NDF; pdNDF = potentially digestible NDF; NANMN = nonammonia, nonmicrobial N; and OMTDR = OM truly digested in the rumen.

²Probability of significant effect of sampling site. The 2-way interaction sampling site × diet was not significant ($P \geq 0.06$).

³Flow of OM was corrected for VFA flow (Huhtanen et al., 2010), and flows of DM, OM, and NAN were corrected for microbial DM, OM, and NAN flows, respectively.

1.18 mm as a threshold particle size for particles being likely to leave the rumen. Krizsan et al. (2010) only observed a digesta composition difference in NDF when sampling from the reticulum compared with the omasum that resulted in a 2.7% greater estimated ruminal NDF digestibility when sampling from the reticulum compared with the omasum. In this study ruminal NDF digestibility only tended to differ between the sampling sites ($P = 0.06$). The estimate was clearly greatest when sampling from the rumen, but ruminal NDF digestibility was also numerically 2.6% greater when sampling from the reticulum compared with the omasum. Krizsan et al. (2010) applied the total fecal collection method to determine marker recovery. The present study calculated fecal output based on marker methods (acid-insoluble ash and iNDF) that likely contributed to a greater random variation in flow estimates of this study.

It seems likely that the mechanisms of selective retention of reticular digesta were influenced by fermentable substrates still remaining in the feed particles to a greater extent than when smaller digesta particles were selected in the study by Krizsan et al. (2010). This is also in agreement with the conclusion by Hristov et al. (2003) that found that the functional specific gravity was an important factor determining the rate of outflow and residence time of feed particles within the reticulo-rumen. The particle size distribution of ruminal digesta both with and without applying primary sieving displayed a distribution comparable to that of the fecal particles. It was only the particle size distribution of reticular digesta that had been subjected to the primary sieving that was comparable with the fecal particle size distribution. The digesta in the rumen was collected from the cranio-dorsal blind sac from where the particles next are transferred to the reticulum. Material is shuttled back and forth between the reticulum and the cranio-dorsal blind sac until it is passed on to the omasum (Sjaastad et al., 2003). However, the reticulum contains newly ingested particles as well as well-digested material, and transportation by contraction creates a flow of particles out through the omasal orifice as well as a flow of feed particles back to the central rumen (Sjaastad et al., 2003). Because of a suggested backflow of large particles from the omasum to the reticulum (McBride et al., 1984; Mathison et al., 1995), it is also likely with larger particles present in digesta collected from the omasum. However, less in the omasum compared with the reticulum as suggested by the results of this study. This study does not support that sampling from the rumen could be an alternative to the omasal sampling technique.

Hristov (2007) compared sampling from the rumen and reticulum with duodenal sampling without apply-

ing any primary sieving to the digesta. He concluded that the rumen method overestimated DM, OM, and NDF flows, whereas the reticular underestimated that of DM and OM compared with the duodenal sampling technique. Likewise, Rotta et al. (2014) did not apply any primary sieving in the comparison of reticular versus omasal sampling. They also observed a lower flow of DM and OM when sampling from the reticulum compared with the omasum. Furthermore, contrary to this study and Krizsan et al. (2010), the flow of NDF was greater when sampling digesta from the reticulum compared with the omasum in the study by Rotta et al. (2014). Hristov (2007) did not observe any difference in microbial protein synthesis between the sampling sites and techniques, but their standard errors for the NAN; nonammonia, nonmicrobial N; and the microbial protein were greater than in this study. Flow and ruminal digestibility of NAN estimated based on reticular and omasal sampling did not differ in the study by Krizsan et al. (2010). However, in this study greater concentration and flow of NAN were observed in reticular compared with omasal digesta. A lower flow of NAN may be attributed to protozoa that under normal conditions are present to a much smaller extent in digesta from the omasum compared with from the rumen (Michalowski et al., 1986).

CONCLUSIONS

This study does not support the hypothesis that a critical particle size distribution alone is influencing the mechanism of selective retention in the reticulum of dairy cows. The NAN and potentially digestible NDF fractions displayed the greatest difference in estimated ruminal outflow between the sampling sites. Flow estimates more often differed when digesta was collected from rumen than from the reticulum compared with the omasum. This suggests that sampling from the rumen is a less promising alternative to the omasal sampling technique compared with sampling from the reticulum. Knowledge of which characteristics are most influential on the mechanism of selective retention of feed particles in the reticulo-rumen across a range of diets is limited. More research is needed to investigate whether a universally applicable critical particle size distribution exists across different diets sufficiently close to representative digesta leaving the rumen.

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