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Short Communication: Effectiveness of Sample Duplication to Control Error in Ruminant Digestion Studies[1]

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ABSTRACT

Eight ruminally cannulated lactating dairy cows from a study on the effect of dietary rumen-degraded protein on production and digestion of nutrients were used to assess using sample duplication to control day-to-day variation within animals and errors associated with sampling and laboratory analyses. Two consecutive pooled omasal samples, each representing a feeding cycle, were obtained from each cow in each period. The effectiveness of sample duplication in error control was tested by comparing the variance of the difference in treatment means when taking 2 samples from each cow in each period to the variance when taking only one sample. Compared with no duplication, sample duplication improved precision by reducing variance by 50, 40, 31, 23, 23, and 9% for, respectively, rumen-undegraded protein flows, ruminal neutral detergent fiber digestibility, microbial nonammonia N flow, microbial efficiency, organic matter flow, and organic matter truly digested in the rumen. For these same variables, reductions in the standard errors of the difference between treatment means due to sample duplication represented 100, 87, 73, 59, 58, and 27% of the predicted reductions resulting from doubling the number of experimental units without sample duplication. Sample duplication can substantially reduce experimental error originating from day-to-day variation within cows, sample collection, and laboratory analyses, thus improving statistical power in ruminant digestion studies.
Key words: dairy cow, nutrient digestion, experimental unit, error variance

Where researchers are interested in improving both the statistical power to detect differences between treatments and the accuracy of estimates of the true population mean, the most effective allocation of resources is to increase the number of experimental units. Given a fixed number of measurements, it is better to have a single measurement on more experimental units than multiple measurements on fewer experimental units. However, this may not be achievable in ruminant digestion studies because of the high cost of cannulating animals and maintaining those animals under intensive experimental conditions. When day-to-day variation within animals and sampling and analytical errors (e.g., marker infusion, sample collection and processing, laboratory analyses) contribute a substantial proportion of the total error variance, multiple measurements from each animal will improve the statistical power and accuracy of estimates. To assess those sources of variation, more than one sample must be obtained from each cow on each treatment. However, because of the complexity of the digesta sampling process, usually only one composite sample is prepared and one measurement of digesta flow is made; thus, information on variance attributable to sampling and analyses is lacking. The objective of this experiment was to study the effectiveness of sample duplication to control error in digestion studies using ruminants.

Eight lactating dairy cows in early lactation (mean 72 DIM) were fitted with ruminal cannulas and assigned to two 4×4 Latin squares with 28-d periods to study the effect of dietary RDP on nutrient digestion. Experimental diets contained (DM basis) 37% corn silage, 13% alfalfa silage, and 50% concentrate. Proportions of rolled high-moisture shelled corn, solvent soybean meal, lignosulfonate-treated soybean meal (SoyPass; LignoTech USA, Inc., Rothschild, WI), and urea were adjusted to provide similar concentrations of CP from ingredients other than urea to achieve 4 concentrations of RDP across diets: 10.6, 11.7, 12.3, and 13.2% RDP. Cows were fed twice daily at 1000 and 2200 h. The Research Animal Resource Center of the University of Wisconsin-Madison approved all procedures involving animals. Details of the animal management, sampling and analyses of feeds and orts, and diet composition are described elsewhere (Reynal and Broderick, 2005).

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Digesta flow from the rumen was quantified using the omasal sampling technique developed by Huhtanen et al. (1997) and modified by Ahvenjärvi et al. (2000). Indigestible NDF (Huhtanen et al., 1994), CoEDTA (Uden et al., 1980), and $YbCl_3$ (modified from Siddons et al., 1985) were used as digesta flow markers. The external microbial marker ^{15}N was used to measure microbial N flows from the rumen. Except for indigestible NDF, markers were continuously infused for 158 h from d 20 at 0900 h to d 26 at 2300 h using 2 syringe pumps (model 33; Harvard Apparatus, Inc., Holliston, MA). Using the omasal sampling technique, 285-mL spot samples were collected from the omasal canal 6 times daily at 1-h intervals on d 23 to 26. Sampling was at 1000, 1100, 1200, 1300, 1400, 1500 h (d 23 and 25), and at 1600, 1700, 1800, 1900, 2000, and 2100 h (d 24 and 26), such that the samples taken represented two 12-h feeding cycles over 2 d each, the first feeding cycle corresponding to sampling days 23 and 24, and the second feeding cycle corresponding to sampling days 25 and 26. Omasal spot samples were divided into subsamples of 85 and 200 mL. The 85-mL subsamples were held on ice as they were collected and pooled over the 6 sampling times each day. The resulting 510-mL pooled samples were processed to isolate particle-associated bacteria from small and large particles equivalent to small particles (**SP**) and large particles (**LP**), and fluid-associated bacteria from fluids equivalent to fluid phase (**FP**). The 200-mL subsamples were pooled and stored at –20°C as they were collected over the 12 sampling times to yield two 2.4-L omasal composites, one corresponding to sampling d 23 and 24 and another corresponding to d 25 and 26, from each cow in each period. The 2.4-L pooled omasal composites were thawed at room temperature and separated into 3 digesta phases: SP, LP, and FP. Concentrations of Co, Yb, and indigestible NDF, determined in SP and LP, and concentrations of Co and Yb, determined in FP, were used to recombine DM from freeze-dried digesta phases in the correct proportions to reconstitute the omasal true digesta flowing out of the rumen based on the triple-marker method of France and Siddons (1986). Based on the similar background of ^{15}N in microbes and digesta observed by Ahvenjärvi et al. (2002), the ^{15}N background used for computing ^{15}N enrichment in both bacterial and digesta fractions was defined as the ^{15}N content of ruminal contents immediately before infusion. Details about marker preparation, infusion, analyses, and calculations are provided elsewhere (Reynal and Broderick, 2005).

Data were analyzed using Proc Mixed in SAS (SAS Institute, 1999–2000). For variables reported on in this communication, the 2 sets of samples taken from each cow in each period were considered repeated measures and therefore correlated in time, and were analyzed together using the following model:

$$Y_{ijklm} = \mu + S_i + P_j + c_{k(i)} + T_l + ST_{il} + w_{lk(i)} + \varepsilon_{ijklm},$$

where Y_{ijklm} is the dependent variable, μ is the overall mean, S_i is the effect of square i, P_j is the effect of period j, $c_{k(i)}$ is the effect of cow k (within square i), T_l is the effect of treatment l, ST_{il} is the interaction between square i and treatment l, $W_{lk(i)}$ is the interaction between treatment l and cow k (within square i), and ε_{ijklm} is the residual error. All terms were considered fixed except for $c_{k(i)}$, $w_{lk(i)}$, and ε_{ijklm}, which were considered random. The compound symmetry covariance structure was used, with the subject of the repeated measurements defined as period × treatment × cow(square). Random effects $c_{k(i)}$ and $w_{lk(i)}$ were modeled as independent, mean zero, normal random variables with variances σ^2_C and σ^2_W, respectively. The residual error ε_{ijklm} has variance $\sigma^2 + \sigma_1$, where σ_1 is the covariance for repeated measures (SAS Institute, 1996).

For the 6 dependent variables under study, the effectiveness of sample duplication in controlling variation was tested by comparing the variance of the difference in treatment means when taking 2 samples from each cow in each period to the estimated variance of the difference in treatment means when taking one sample from each cow in each period. The 6 variables were selected to represent several laboratory analyses and measurements performed in the companion studies (Reynal and Broderick, 2005; Reynal et al., 2005). In a balanced design in which each of the K cows is measured M times for each treatment, the estimated difference in treatment means is

$$\hat{T}_l - \hat{T}_{l'} = T_l - T_{l'} + \sum_{k(i)} (W_{lk(i)} - W_{l'k(i)})/K + \sum_{k(i)} \sum_{m} (\varepsilon_{ijklm} - \varepsilon_{ijkl'm})/(KM).$$

Under the model assumptions,

$$\begin{aligned} Var\,(\hat{T}_l - \hat{T}_{l'}) &= 2Var\left(\sum_{k(i)} W_{lk(i)}/K\right) + 2Var\left(\sum_{k(i)} \sum_{m} \varepsilon_{ijlkm}/(KM)\right) \\ &= \{2\sigma^2_W/K\} + \{2(KM(\sigma^2 + \sigma_1) + KM(M-1)\sigma_1)/(KM)^2\} \\ &= 2(\sigma^2_W + \sigma^2/M + \sigma_1)/K. \end{aligned}$$

The variances σ^2_W and σ^2 and the covariance σ_1 were computed for the selected variables using PROC MIXED in SAS. The above equation was also used to predict the variance reduction that would result from

Table 1. Estimated and predicted variances and standard errors of difference in treatment means when varying the number of samples from each cow in each period and the number of experimental units

Item	OM flow, kg/d	OMTDR,[1] kg/d	Microbial NAN flow, g/d	Microbial efficiency,[2] g/kg	RUP flow, g/d	Ruminal NDF digestibility, %
Variance components						
Cow × period × treatment(square)[3]	0.446	1.306	491	3.31	0	3.60
Residual[4]	0.743	0.576	1597	5.70	56,954	29.9
Residual, % of total[5]	62.5	30.6	76.5	63.1	100.0	89.2
Variances of difference in treatment means						
8 cows, 1 sample[6]	0.297	0.470	522	2.24	15,557	8.37
8 cows, 2 samples[7]	0.204	0.398	322	1.53	7778	4.63
16 cows, 1 sample[8]	0.149	0.235	261	1.12	7778	4.18
Relative efficiencies[9]						
8 cows, 2 vs. 1 sample	1.45	1.18	1.62	1.47	2.00	1.81
16 vs. 8 cows, 1 sample	2.00	2.00	2.00	2.00	2.00	2.00
Standard errors of difference in treatment means						
8 cows, 1 sample[6]	0.545	0.686	22.85	1.50	124.7	2.89
8 cows, 2 samples[7]	0.452	0.631	17.96	1.24	88.2	2.15
16 cows, 1 sample[8]	0.386	0.485	16.16	1.06	88.2	2.05
Ratios between standard errors of difference in treatment means						
8 cows, 2 vs. 1 sample[10]	0.83	0.92	0.79	0.83	0.71	0.74
16 vs. 8 cows, 1 sample[10]	0.71	0.71	0.71	0.71	0.71	0.71
Median values	12.3	14.4	412	29.4	1386	49.9
Required observations at α = 0.05 (1 sample)[11]						
5% difference	13	14	20	17	45	22
10% difference	3	4	5	4	11	5
Required observations at α = 0.05 (2 samples)[11]						
5% difference	9	12	12	11	19	12
10% difference	2	3	3	3	5	3

[1]OMTDR = OM truly digested in the rumen.

[2]Grams of microbial NAN per kilogram of OMTDR.

[3]Variance of the random effect associated with each cow and treatment-period combination.

[4]Residual variance originated from day-to-day variation and measurement errors within cows.

[5]Proportion of the variance of difference in treatment means associated with day-to-day variation and analytical errors.

[6]Variance or standard error of the difference in treatment means when taking one sample from each of the 8 cows in each period.

[7]Variance or standard error of the difference in treatment means when taking 2 samples from each of the 8 cows in each period.

[8]Predicted variance of the difference in treatment means when taking one sample from each of the 16 cows in each period.

[9]Relative efficiencies of doubling the number of measurements on each cow or doubling the number of cows.

[10]Ratios between standard errors of the difference between treatment means from complex and simple models.

[11]Number of observations required to detect significant differences (α-level of 0.05) between treatment means of 5 and 10% of the median value when taking either 1 or 2 samples per cow per period.

doubling the number of experimental units in a hypothetical trial. The relative efficiencies of doubling the number of measurements on each of the 8 cows or doubling the number of cows and preparing only one sample per cow per period were computed as the ratios between the information (1/variance) from the complex models (2 samples and 8 cows or 1 sample and 16 cows) and the information from the simple model (1 sample and 8 cows; Steel et al., 1996). Ratios between standard errors of the difference between treatment means from complex and simple models were also computed.

In the present study, from 30.6 to 100.0% of the variance of difference in treatment means was associated with day-to-day variation within cows plus sampling and analytical errors (residual variance; Table 1). Because sample duplication can reduce only the residual variance (see equation in the Statistical Analysis section), the greatest improvements in precision were on those variables with highest residual variance. Duplication of sampling resulted in 50, 40, 31, 23, 23, and 9% improvements in precision (lower variance) for, respectively, RUP flows, ruminal NDF digestibility, microbial NAN flow, microbial efficiency, OM flow, and OM truly digested in the rumen. For these same variables, sample duplication reduced the standard errors of the difference between treatment means compared with not duplicating sampling by, respectively, 29, 26, 21, 17, 17, and 8%, representing 100, 87, 73, 59, 58, and 27% of the predicted reductions resulting from doubling the number of cows (29% reduction for all variables). When taking one sample from each cow in each period, between 13 and 45 observations per treatment were re-

quired to detect differences of 5 percentage units between treatments (at $\alpha = 0.05$) for the variables under study. However, sample duplication reduced those required observations to between 9 and 19. Differences of 10 percentage units between treatments (at $\alpha = 0.05$) were detected using between 3 and 11 observations without sample duplication and between 2 and 5 observations with sample duplication (Table 1). Therefore, these results indicate that sample duplication can substantially reduce experimental error originating from day-to-day variation within cows, sampling, or analytical errors, thus improving statistical power in ruminant digestion studies. Moreover, improvements in the precision of sampling and analytical techniques may decrease error rates in digestion studies, allowing for the use of fewer animals while minimizing type II errors. Possible ways to minimize analytical and sampling errors are by 1) collection of several samples over time to account for diurnal variation, 2) use of markers for different digesta phases (Titgemeyer, 1997), 3) use of ^{15}N as a microbial marker instead of total purines (Broderick and Merchen, 1992; Reynal et al., 2005), and 4) separating ruminal isolation and flow measurements for fluid-associated bacteria and particle-associated bacteria (Broderick and Merchen, 1992; Titgemeyer, 1997). A thorough review of the factors that influence digestion studies has been published by Titgemeyer (1997).

To detect biologically important differences among treatments (minimize the probability of a type II error), researchers should carefully select the experimental design and number of replications while minimizing the errors associated with the sampling and analytical techniques used in their laboratories. The use of 4 observations per treatment may not be appropriate for most laboratories, especially when trying to detect biologically important interactions with a 2×2 factorial arrangement of treatments.

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