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Evaluation of Subsampling and Fixation Procedures Used for Counting Rumen Protozoa†

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Total numbers of protozoa can be significantly lower in rumen fluid than in whole rumen contents, depending on the time of sampling and the procedure used to separate the fluid and solid fractions. Moreover, generic distribution in rumen fluid was significantly affected in all cases tested. The percentage of *Entodinium* spp. increased, whereas percentages of *Diplodinium* spp. and *Ophryoscolex* spp. decreased. Microscopic observation of fresh and fixed rumen contents did not indicate any marked attachment of protozoa to particulate matter. In addition, dilution of whole rumen contents with water, 5 mM sucrose, or 0.1% Tween 80 before fixation did not affect total numbers or generic composition of protozoa. It was thus concluded that attachment to feed particles is probably not a problem in counting procedures. Blending of whole rumen contents to facilitate subsampling caused a decrease in numbers of protozoa. The concentration of formaldehyde used for preservation of rumen contents, 4, 10, or 18.5%, did not affect the total count.

Methods for counting rumen protozoa have been studied in detail by Purser and Moir (17) and Boyne et al. (7). In general, these authors concluded that three factors are important: (i) the counting chamber must be deep enough to accommodate the large protozoa; (ii) filling procedures must provide an even distribution of cells throughout the chamber; and (iii) the viscosity of the diluent must be such that cells will remain in suspension during pipetting but settle to the bottom of the counting chamber within 5 to 10 min. In contrast, little if any attention has been focused on subsampling of rumen contents for counting protozoa. Most numbers of rumen protozoa reported in the literature are based on counts from strained rumen fluid (2, 9, 10, 12–14, 16, 18–20). A variety of materials, including gauze, cheesecloth, metal sieves, bolting cloth, etc., have been used for straining rumen ingesta. Since many bacteria are attached to feed particles, counts are routinely determined in whole rumen contents (8, 11). Counting protozoa in just the fluid fraction appears inconsistent and, because attachment of several genera of protozoa to plant materials has been observed (1, 5, 6, 15), could potentially give different results. The present study compares numbers of protozoa determined in whole rumen contents with those determined in rumen fluid separated from whole contents by several different procedures.

MATERIALS AND METHODS

Rumen contents were obtained from four sheep and a steer, all with rumen fistulas. Two of the sheep were fed chopped orchard-grass hay, one was fed 60% corn and 40% hay, and the last was fed a complete pelleted ration (45% corn cobs, 35% alfalfa meal, 13.1% oats, 5% molasses, 0.4% urea, 1.5% mineral-vitamin supplement). The steer was fed long orchard-grass hay. All animals were fed once daily in the morning. Composite samples of contents from various locations within the rumen were collected through the fistula with the aid of a rigid plastic tube (inside diameter, 1.5 cm) for sheep and by hand for the steer.

Rumen contents were subsampled in several ways. When sheep rumen contents did not contain large pieces of particulate matter, a cut-off 10-ml measuring pipette (inside diameter, 8 mm) was used. Otherwise, a small plastic 10- to 15-ml cup was filled to the rim with a thoroughly mixed sample of whole contents. The subsample was placed in a small beaker and preserved by adding an equal volume of 50% Formalin (18.5% concentration of formaldehyde). After being mixed, the sample was stored in a culture tube (20 by 150 mm). For steer rumen contents, which generally contained fairly large hay particles, a larger cup or 50-ml beaker was used as the sampling container and was then filled again with 50% Formalin.

The counting technique was an adaptation of the procedure described by Purser and Moir (17). A 1.0-ml aliquot of the formalinized sample was pipetted with a 1.0-ml wide-orifice (3 mm) pipette (Bellco Glass Inc., no. 1231-01001) into a culture tube (16 by 150 mm). Two drops of brilliant green dye (2 g of brilliant green dye and 2 ml of glacial acetic acid diluted to 100 ml with distilled water) were added, and the contents were mixed and allowed to stand for at least 4 h. Allowing the mixture to stand overnight generally resulted in better staining. After staining, 9 ml of 30% glycerol solution was added, resulting in a 1:20 dilution of the original rumen contents. The diluted sample was pipetted into a Sedgewick-Rafter counting chamber by a wide-orifice pipette. Further dilutions, if required, were made with 30% glycerol and wide-orifice pipettes. If the original 1:2 dilution of rumen contents could not be satisfactorily pipetted with the wide-orifice pipette, a further dilution was made with 25% Formalin (9.25% formaldehyde).

Protozoa were counted at a magnification of ×100 with a counting grid 0.5 mm square in the eyepiece. By using a calibrated microscope stage, 50 grids, evenly spaced over the entire chamber surface, were counted. The chamber was then rotated 180°, a second 50-grid count was made, and these two counts were averaged. Dilutions giving 100 to 150 protozoa per 50 grids were found to be the most satisfactory since higher numbers markedly increased counting time. For routine counting, two 1.0-ml subsamples were pipetted from the original formalinized sample, stained, diluted, and counted. The coefficient of variation between 50 grid counts on subsamples from the same sample of formalinized rumen contents was generally between 5 and 6%. Protozoan num-

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TABLE 1. Numbers of protozoa in fluid and solids fractions of whole rumen contents squeezed through two layers of cheesecloth

Sample no.[a]	Vol (%)		Protozoa per ml ($\times 10^5$)				Recovery (%)[b]
	Fluid	Solids	Fluid	Solids	Fluid + solids[c]	Whole rumen contents	
1	80.3	19.7	1.48	3.02	1.78	1.82	97.8
2	75.3	24.7	1.30	1.46	1.34	1.40	95.7
3	69.3	30.7	1.68	2.04	1.79	2.43	73.7
4	68.0	32.0	1.67	1.18	1.51	2.56	59.0
5	73.0	27.0	1.54	1.69	1.58	2.09	75.6
6	75.7	24.3	2.51	2.20	2.43	2.78	87.4
Mean ± SEM	73.6 ± 1.8	26.4 ± 1.8	1.70 ± 0.17	1.93 ± 0.26	1.74 ± 0.15	2.18 ± 0.21	79.8

[a] All samples were taken just before feeding from sheep fed chopped orchard-grass hay.
[b] (Fluid plus solid)/(whole rumen contents) × 100.
[c] Corrected for percentage volume.

bers per milliliter of diluted rumen contents were calculated from the average of the two subsample counts.

Data were analyzed statistically by analysis of variance. The Duncan new multiple range test and least significant difference procedures were used for mean separation and probability levels.

RESULTS

Rumen contents were squeezed through a double layer of fine cheesecloth, fluid and solids volumes were measured, and protozoa were counted in each fraction (Table 1). On a per unit of volume basis, numbers of protozoa were not significantly different between the fluid and solid fractions. By using the volume percentages for each fraction, the numbers of protozoa per milliliter of rumen contents was calculated and compared with the standard count determined for whole rumen contents. The standard count was not significantly different from the values for fluid plus solids or for either fraction alone. Recoveries based on the standard count ranged from 59.0 to 97.8%.

Results of generic distribution determinations revealed that the percentage of *Entodinium* spp. was significantly higher ($P < 0.005$) in the fluid fraction and lower in the solids fraction (Table 2). The reverse was observed for *Diplodinium* spp., counts of which were significantly lower in the fluid and higher in solids. Changes in the percentage *Ophryoscolex* spp. were similar to those for *Diplodinium* spp. No significant differences were observed for *Isotricha* spp. or *Dasytricha* spp.; however, percentages of *Isotricha* spp. tended to be lower in the fluid fraction, and those of *Dasytricha* spp. tended to be lower in the solid fraction. Calculated generic composition of the fluid-plus-solid fractions did not differ from that of whole rumen contents.

Since squeezing through two layers of cheesecloth clearly affected numbers and generic composition, an experiment was conducted to compare the effects of squeezing versus straining, one or two layers of cheesecloth, and time of sampling (Table 3). For samples of rumen contents taken just before feeding, the only significant difference in numbers of protozoa occurred in the fluid obtained by straining through two layers of cheesecloth. In contrast, for rumen samples taken 3 h after feeding, numbers of protozoa determined in whole rumen contents were significantly higher ($P < 0.05$) than those in all fluid fractions except the one squeezed through a single layer of cheesecloth. The percentage of solids would be higher in the samples taken after feeding, suggesting that the solids can serve as a filter mat.

Differences in generic composition were essentially similar for samples taken just before feeding and 3 h after feeding, so these data were combined (Table 4). The percentage of *Entodinium* spp. was significantly lower, and percentages of *Diplodinium* spp. and *Ophryoscolex* spp. were significantly higher ($P < 0.001$), in whole rumen contents than in all of the fluid fractions. The percentage of *Isotricha* spp. was significantly lower in the fluid obtained by squeezing through two layers of cheesecloth. The percentage of *Dasytricha* spp. was not different in whole rumen contents and the different fluid fractions. The significance of any differences between the different fluid fractions has been omitted since they are not relevant to the study.

Microscopic observation of whole rumen contents before and after fixation with Formalin did not suggest any marked attachment of protozoa to particulate matter. An experiment to investigate the possibility of protozoan attachment to solids was conducted in which numbers of protozoa in rumen contents squeezed through a single layer of cheesecloth and then fixed with Formalin were compared with numbers in samples of rumen contents diluted with 38°C tap water or 18.5% formaldehyde and then strained through a single layer of cheesecloth. No differences were observed between these treatments. A more complete experiment was then conducted in which the standard count on whole rumen contents was compared with the following sampling procedures: (i) whole rumen contents were diluted with an equal volume of 18.5% formaldehyde, another equal volume of 9.25% formaldehyde was added, and the sample was counted; (ii) whole rumen contents were diluted with an equal volume of 38°C tap water, another equal volume of 27.8% formaldehyde was added after 3 to 5 min, and the sample

TABLE 2. Generic distribution of protozoa in fluid and solids fractions of whole rumen contents squeezed through two layers of cheesecloth

Genus	% of total count				SEM
	Whole rumen contents	Fluid	Solids	Fluid + solids	
Entodinium	73.7[a]	88.2[b]	49.1[c]	77.4[a]	1.1
Diplodinium	17.4[a]	7.7[b]	38.5[c]	16.6[a]	1.4
Ophryoscolex	3.7[a]	0.5[a]	9.6[b]	3.0[a]	1.0
Isotricha	1.6	0.7	1.3	0.9	0.3
Dasytricha	3.0	2.4	0.9	2.0	0.5

[a,b,c] Means in the same row with different superscripts are significantly different at $P < 0.005$.

TABLE 3. Comparison of numbers of protozoa in rumen fluid obtained by squeezing or straining through one or two layers of cheesecloth[a]

Sampling time (h after feeding)	No. of samples	Protozoa per ml ($\times 10^5$)					SEM
		Rumen contents	Rumen fluid passed through cheesecloth				
			Squeezed		Strained		
			1 layer	2 layers	1 layer	2 layers	
0	11	6.03[b]	6.32[b]	6.12[b]	5.28[b,c]	4.95[c]	0.34
3	10	4.61[b]	4.25[b,c]	4.19[c,d]	3.78[d]	3.83[c,d]	0.14

[a] At both sampling times, six samples were from sheep fed orchard-grass hay, and three samples were from sheep fed a complete pelleted ration. Two samples at 0 h and one sample at 3 h were from sheep fed 60% corn and 40% hay.
[b,c,d] Means in the same row followed by different superscripts are significantly different at $P < 0.05$.

was counted; (iii) procedure ii was followed with 5 mM sucrose solution used in place of tap water; and (iv) procedure ii was followed with 0.1% Tween 80 solution used in place of tap water. All four procedures gave similar total counts and generic compositions, and these values were not different from those obtained by the standard procedure. Fixation of rumen contents with 18.5% formaldehyde solution was compared with 10 and 4% formaldehyde solutions. Based on 12 experiments using rumen contents from three animals each fed different diets, no differences in numbers of protozoa were observed with the different formaldehyde concentrations.

One of the principal reasons that many investigators have separated the fluid portion from rumen contents has probably been for ease of subsampling. Blending whole rumen contents (in a Waring blender) was investigated as a means of reducing particle size to facilitate the sampling procedure. Numbers of protozoa were reduced by 93% when rumen contents were blended for 5 min at a rheostat setting of 100. With a rheostat setting of 50, numbers of protozoa in samples blended for 1, 2, and 3 min were reduced by approximately 21, 31, and 31%, respectively. The holotrichs appeared to be affected most, disappearing almost completely with blending.

Two experiments were conducted in which four separate samples were taken for counting from one large sample of rumen contents. Replicate samples from the same formalinized subsample were counted as previously described. The coefficients of variation for numbers of protozoa between subsamples of whole rumen contents were 3.64 and 5.54%, respectively, for sheep fed a pelleted ration and chopped hay.

DISCUSSION

The number of protozoa per unit volume of fluid and solids was not significantly different in samples taken before feeding; therefore, total numbers of protozoa in whole rumen contents were not different from the count in rumen fluid obtained by squeezing or straining through cheesecloth. On the other hand, numbers of protozoa in rumen contents taken 3 h after feeding were significantly higher than numbers in all fluid fractions, except the fluid obtained by squeezing through a single layer of cheesecloth. This difference probably results from an increased percentage of solids after feeding, which simply acts as a filter mat and retains more of the protozoa. Lower counts in the strained fluid at both sampling times further support this suggestion. Attachment of protozoa to fresh particulate matter might also be a contributing factor, although results of the present experiments do not support this conclusion.

Generic composition of protozoan populations was altered by all procedures used to separate fluid from solids. The percentage of *Entodinium* spp. increased, whereas that of *Diplodinium* spp. and *Ophryoscolex* spp. decreased. Except in the fluid obtained by squeezing through two layers of cheesecloth, holotrich percentages were not affected.

These data clearly indicate that in experiments involving counts of protozoa, separation of the fluid from the solids could be misleading with regard to total numbers and definitely in error for generic distribution. This change in generic distribution would also be of importance in any type of experiment in which protozoa are separated and analyzed for specific markers, e.g., 2-aminoethylphosphonic acid, ATP, DNA.

Although several investigators have reported definite attachment of *Isotricha* spp. and *Epidinium* spp. to particulate matter, based on observations by light and electron microscopy (1, 3–6, 15), no evidence of attachment as a factor in counting total protozoa was obtained in the present study. Unfortunately, the genus *Epidinium* was absent from all but one sample, and numbers of *Isotricha* spp. were very low. Both *Isotricha* spp. and *Epidinium* spp. exhibit a chemotac-

TABLE 4. Comparison of generic distribution of protozoa in rumen fluid obtained by squeezing and by straining through one and two layers of cheesecloth[a]

Genus	% of total					SEM
	Whole rumen contents	Rumen fluid passed through cheesecloth				
		Squeezed		Strained		
		1 layer	2 layers	1 layer	2 layers	
Entodinium	82.6[b]	89.7	91.7	88.7	89.4	0.7
Diplodinium	10.4[b]	5.4	4.4	5.2	4.7	0.5
Ophryoscolex	2.4*b*	1.0	0.6	0.8	0.6	0.2
Isotricha	1.4	1.0	0.7[c]	1.5	1.3	0.1
Dasytricha	3.5	3.1	2.8	3.9	3.9	0.3

[a] Combined data from samples listed in Table 3.
[b] Significantly different from all other means in the same row at $P < 0.001$.
[c] Significantly different from value for whole rumen contents at $P < 0.01$.

tic response to sucrose and other soluble carbohydrates in feed particles, generally followed by attachment of the cell to the particle (1, 5, 15). Orpin and Letcher (15) found that the number of attached *Isotricha* cells decreased when cellulose was impregnated with sucrose concentrations above 1 mM; however, dilution with a 5 mM sucrose solution before fixation had no measurable effect on numbers of *Isotricha* spp. One sample of rumen contents used in the sucrose dilution experiment did contain about 9% *Epidinium* spp., but no difference was observed between treatments. A nonspecific surfactant, Tween 80, was also without effect on total numbers or generic distribution. Since Bauchop (5) specifically states that *Epidinium* spp. were the only protozoa observed to be attached to plant tissues, even though a complex protozoan population was present, it would be useful to repeat these particular experiments with rumen contents containing the genus *Epidinium* and higher numbers of *Isotricha* spp.

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