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A Comparison of Two Techniques for Counting Cellulolytic Rumen Bacteria

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SUMMARY

Cellulolytic bacteria were counted by the 'direct' method; estimated from the number of clearings produced in films of cellulose-containing agar medium inoculated with high dilutions of rumen ingesta and by the 'indirect' method; numbers were calculated from 'total culturable' counts on films of a non-specific agar medium and from the percentages of bacteria found to be cellulolytic after isolation in pure culture. The two methods yielded similar results.

Two methods have been mainly used to determine the numbers of cellulolytic bacteria in the rumen. In the 'direct' method as used for instance by Hungate (1947, 1950, 1957), Kistner (1960), Gilchrist & Kistner (1962), Kistner, Gouws & Gilchrist (1962), Gilchrist (1965) and De Wet (1966), colony counts of cellulolytic bacteria are made on a selective medium containing finely divided cellulose. The cellulolytic colonies were identified by the circular clearings formed through the action of cellulase secreted by the bacteria. The number of clearings gives a direct estimate of the numbers of cellulolytic bacteria in the sample. In the 'indirect' method used for instance by Bryant & Burkey (1953*b*), Bryant, Small, Bouma & Robinson (1958) and De Wet (1966) an agar medium designed to support the growth of the widest possible spectrum of rumen bacteria was inoculated with high dilutions of rumen contents. Well-spaced colonies were picked non-selectively and transferred directly to agar slants of non-specific medium. All the isolates obtained from a number of samples, collected at intervals from an animal conditioned to a particular diet, were then examined for cellulolytic activity. From the percentage of isolates capable of hydrolysing cellulose and the mean colony count on the non-selective medium the mean level of cellulolytic bacteria in the rumen was calculated.

When comparing the counts of cellulolytic bacteria by the two methods, counts by the 'indirect' method were almost always higher than those obtained by the 'direct' method. In Table 1 the values for counts by the 'direct' method range from about 1×10^6 to 20×10^6 bacteria (colonies)/ml. whereas those by the 'indirect' method are within the range 1×10^6 to 500×10^6/ml. In no case, however, were counts by the two methods made on the same sample. Although De Wet examined samples from the same animals on the same diets, the samples were taken on different dates. To decide whether these differences are due to inherent differences in counting techniques, or are due to differences between animals or between diets, a comparison of the two methods was made on the same samples from sheep on three high-roughage

diets. This work was done in the course of studies on the cellulolytic flora of sheep on different diets. To get enough isolates in the case of the 'indirect' method in Expts. 1 and 3 it was necessary to obtain them from more than one sheep on a given ration. However, for the purpose of this study this was not considered to be important.

Table 1. *Numbers of cellulolytic rumen bacteria found by different authors using the 'direct' and 'indirect' counting methods*

Reference	Animals used	Ration	Numbers of cellulolytic bacteria/ml. or g. ($\times 10^6$)
Direct method			
Hungate (1957)	Cows	Timothy hay + different supplements	2·87 (12)* 5·57 (12) 3·00 (12) 2·12 (11) 2·27 (12) 3·14 (9) 1·88 (12) 0·83 (13)
Kistner (1960)	Sheep	Lucerne hay	8·2 (2–22)† (11) 18·0 (13·75–25·6) (5) 20·5 (19·25–21·5) (4)
Gilchrist & Kistner (1962)	Sheep	Poor teff hay	3·2 (0·1–10) (17)
Kistner *et al.* (1962)	Sheep	Lucerne hay	17 (1–39) (19)
Gilchrist (1965)	Sheep	Lucerne hay Teff hay Teff hay + urea + molasses	5 (4–6) (3) 2·03 (0·1–5) (3) 1·64 (0·06–3) (4)
De Wet (1966)	Sheep	Wheat straw Wheat straw + urea + molasses	0·87 (0·01–5) (20) 15 (2·5–50) (35)
Indirect method			
Bryant & Burkey (1953*b*)	Cows	Alfalfa hay + concentrates	113, 294
		Wheat straw	502
		Concentrates	338
		Alfalfa hay	258, 114
Bryant *et al.* (1958)	Cow		100
	Calves	Alfalfa hay + grain mixture	1·2 110 320 430 62
De Wet (1966)	Sheep	Wheat straw Wheat straw + urea + molasses	4·1 143

* Number of counts made. † Range.

METHODS

Animals. These were Merino wethers fitted with permanent rumen fistulas.

Diets. The diet for Expt. 1 consisted of 1500 g. of teff hay daily. For Expt. 2 the ration consisted of 1200 g. of teff hay, supplemented with 10 g. urea and 80 g. glucose in 1 l. water which was given *per fistula* at about 09.00 a.m. In Expt. 3 the sheep were fed 1200 g. of treated teff hay daily, the crude protein content of which had been raised

from 3·7 to 10·7% by application of spray-dried egg albumin. Each ration was supplemented with a balanced mineral+trace element lick and vitamin A. In all three experiments the hay was given to the sheep as one feed at about 08.15 a.m. The trace element lick (about 15 g.) was given separately at the same time while vitamin A was given once/week. The sheep were conditioned to the diets for at least 6 weeks before sampling of rumen ingesta began.

Sampling and treatment of rumen ingesta. Samples of rumen ingesta for bacterial counts were taken through the fistula with a sampling tube about 2·5 hr after feeding. On sampling days water was withheld from the sheep from the time of feeding until the sample had been drawn. Ingesta were generally not drawn more often than once/week from a sheep. In Expts. 1 and 2 about 150 ml. ingesta were withdrawn. The sample was blended with an Ultra Turrax, type TP 18/2 homogenizer (Janke & Kunkel, Staufen i. Br., West Germany) operating at 20,000 rev./min. for 30 sec. with the container cooled in ice. A 10 g. subsample was weighed out and then diluted with 90 ml. of an anaerobic diluting solution similar to that used by Bryant & Burkey (1953*a*) except that it contained indigo carmine instead of resazurin. This mixture was again treated with the homogenizer for 60 sec. to ensure maximal release of organisms from solid particles of ingesta and perhaps to decrease the size of aggregates of bacteria. For Expt. 3, in an attempt to obtain a truly representative sample from the rumen, about 600 ml. ingesta were withdrawn. This was mixed by shaking in a stoppered bottle; a 150 ml. sample, treated as described above, was used for making colony counts.

Cellulolytic counts by the 'direct method'. For these counts 1·2% (w/v) ball-milled Whatman no. 1 filter paper was substituted for the carboyhdrates in the medium of Bryant & Robinson (1961) for obtaining 'total culturable' counts. Indigo carmine (0·0005%) was substituted for resazurin. The filter paper cellulose was prepared as follows: 12 g. shredded filter paper and 600 ml. deionized water, were placed in a ball mill of about 1 l. capacity and this was milled with a mixed charge of porcelain balls for about 72 hr at 57 rev./min. Roll bottles of 7 ml. capacity, spun mechanically, were used as culture vessels (Kistner, 1960). Colonies of cellulolytic bacteria were counted after incubation for 4 weeks at 39°.

'Total culturable' and 'indirect' cellulolytic counts. Methods for obtaining colony counts of 'total culturable' bacteria were essentially those of Bryant & Burkey (1953*a*) as modified by Bryant & Robinson (1961). The medium (GCSX medium) contained 0·05% each of glucose, cellobiose, starch and xylan, with indigo carmine (0·0005%) instead of resazurin. Roll bottles were used as for the 'direct' cellulolytic counts. Colonies of 'total culturable' bacteria were counted after incubation for 1 week at 39°.

For the 'indirect' cellulolytic counts generally all the well-isolated colonies were picked from one or more roll bottles containing GCSX medium inoculated with the 10^{-7} or 10^{-8} dilutions of the samples. Inoculum was transferred to slopes of GCSX medium. The cultures were tested for ability to hydrolyse cellulose in a medium containing rumen fluid and 1·2% (w/v) ground α-cellulose prepared from teff hay. Inoculated bottles containing this medium were incubated for at least 4 weeks, after which samples of the well-mixed medium were placed in Wintrobe Hematocrit Tubes (Type A-2456, Clay Adams, Inc., N.Y.) and centrifuged for 30 min. at about 1500 *g*. Cultures showing more than 10% decrease in volume of cellulose when compared

with readings for uninoculated medium were considered to be cellulolytic. Ten % was considered a minimal value consistent with reliable interpretation.

Table 2. *Counts of 'total culturable' and cellulolytic bacteria made by 'direct' counting method on samples of rumen ingesta from sheep fed teff hay diets*

		Number of colony-forming units/1 g. rumen ingesta			
Experiment number*	Sheep	'Total culturable' counts ($\times 10^8$)		Cellulolytic colony counts ($\times 10^6$)	
1	1	9		53	
	1	7		9	
	1	3		8	
	1	2		7	
	1	10	Mean = 5·2	4	Mean = 23
	1	8	$\sigma = \pm 3{\cdot}7$	60	$\sigma = \pm 21$
	1	1	$V\sigma = 71$	42	$V\sigma = 91$
	1	6		28	
	2	0·3		0·7	
	2	0·8		0·8	
	2	5		34	
	3	10		30	
2	4	9		8	
	4	10		7	
	4	4	Mean = 7·7	N.D.	Mean = 38
	4	0·5	$\sigma = \pm 4{\cdot}9$	0·4	$\sigma = \pm 54$
	4	14	$V\sigma = 63$	109	$V\sigma = 142$
	4	11		125	
	4	11		7	
	4	2		11	
3	5	11		37	
	5	37		18	
	5	38		24	
	5	26		24	
	5	32		28	
	5	36		42	
	6	19		43	
	6	13	Mean = 29	17	Mean = 24
	6	35	$\sigma = \pm 10$	19	$\sigma = \pm 11$
	6	28	$V\sigma = 35$	36	$V\sigma = 45$
	6	39		28	
	6	24		30	
	7	28		9	
	7	17		6	
	7	22		23	
	7	48		28	
	7	37		9	
	7	23		16	

* For diets see text. N.D. = not done.

RESULTS

The results obtained for the 'total culturable' and 'direct' cellulolytic counts for the three experiments are given in Table 2. Table 3 gives a comparison of the results for the 'direct' and the 'indirect' methods of counting cellulolytic bacteria. A remarkably close agreement existed in the results obtained with the two methods in

Expt. 3. The differences between the results for the methods in Expts. 1 and 2, although approaching twofold, were not considered to be excessive. The percentages of cellulolytic bacteria calculated from the 'direct' and 'indirect' counts were, respectively: Expt. 1, 4·4 and 7·2; Expt. 2, 4·9 and 3·0; Expt. 3, 0·8 and 0·8.

Table 3. *Derivation of 'indirect' cellulolytic counts and comparison with 'direct' cellulolytic counts made on samples of rumen ingesta from sheep fed teff hay diets*

		Indirect method					Direct method
Experiment number*	Sheep	Number of colonies picked from non-specific medium	Number of colonies found to be cellulolytic	Cellulolytic bacteria (% total number of colonies picked)	Mean 'total culturable' counts†	Mean number of cellulolytic bacteria/1 g. rumen ingesta (calculated)	Mean number of cellulolytic bacteria/1 g. rumen ingesta†
1	1, 2, 3	291	21	7·2	$5{\cdot}2 \times 10^8$	37×10^6	23×10^6
2	4	168	5	3·0	$7{\cdot}7 \times 10^8$	23×10^6	38×10^6
3	5, 6, 7	265	2	0·8	29×10^8	23×10^6	24×10^6

* For diets see text. † See Table 2.

DISCUSSION

In the present study, the 'direct' and 'indirect' methods of colony counts of cellulolytic rumen bacteria yielded essentially the same results when applied to the same samples. This does not exclude the possibility that the differences between the counts reported by various workers using one or other of the methods (Table 1) were partly due to differences in counting techniques. Both methods are subject to particular types of errors. In the case of the 'direct' method, the nature of the cellulose substrate may influence the counts. Certain forms of cellulose are not readily utilized by all strains of cellulolytic rumen bacteria (Halliwell & Bryant, 1963; Hungate, 1966; Shane, 1966). Moreover, if the cellulose substrate is not sufficiently ground the substrate is not homogeneously distributed throughout the medium. This can make detection of small clearings difficult, and it may also prevent the development of colonies from cellulolytic bacteria which are not near to the cellulose substrate. Low cellulolytic counts may also result from competition between cellulolytic bacteria for limiting amounts of accessory nutrients, or from accumulation of metabolic end-products, which prevent some of the bacteria from developing into visible colonies. This is often the case in cultures inoculated with lower sample dilutions.

In the case of 'indirect' counts, the method of determining cellulolysis may influence the results. The method described above showed up weakly cellulolytic strains which were previously missed when the method of Bryant & Burkey (1953*a*) was used for detecting cellulolysis. The extent to which the medium for the 'total culturable' counts supports the growth of all viable bacteria in the sample of rumen contents should not affect the 'indirect' counts of cellulolytic bacteria, provided that the medium meets the environmental requirements of all the cellulolytic species. However, the composition of the medium may affect the apparent percentage of cellulolytic bacteria. Thus the high percentage of cellulolytic bacteria in rumen contents of cows on various

diets reported by Bryant & Burkey (1953*b*) may be related to the fact that these authors included neither starch nor maltose in their medium for 'total culturable' counts and therefore excluded at least one important specifically amylolytic, organism namely *Bacteroides amylophilus*. The 'indirect' method becomes particularly inefficient when the cellulolytic organisms form a small percentage of the 'total culturable' bacterial population. Unless a very large number of isolations is made under these circumstances, considerable errors in cellulolytic counts may occur. However the 'indirect' method does give the proportion of the total population which is cellulolytic, although it is easier to obtain this information by making 'direct cellulolytic counts and total culturable' counts on the same sample. In the present work, percentage values for cellulolytic bacteria were obtained by both methods and the figures for the two methods were in close agreement.

Another factor which can profoundly influence the counts of cellulolytic bacteria obtained by different workers is the method of obtaining the sample. A large proportion of the cellulolytic bacteria in the rumen are attached to the food particles (Hungate, 1966) and may therefore be excluded by straining the ingesta. There is, however, no generally accepted method of collecting and preparing samples of ingesta for making counts. The influence of different procedures on the counts of cellulolytic and other groups of bacteria in the rumen is being investigated in this laboratory.

The time of day at which the samples are taken can influence the counts in two ways: (1) it may affect the proportion of organisms bound to food particles and hence enhance or decrease differences due to the method of sample preparation; (2) it may also reflect diurnal changes in the actual numbers of bacteria in the rumen. Thus the results of 'total culturable' counts on rumen contents of a cow fed a grain ration or a hay ration once a day, as reported by Bryant & Robinson (1961), showed marked diurnal variations with the former and very little change with the latter diet. Assuming that the cellulolytic counts showed a similar trend on each diet the time of sampling could exert a significant influence on the counts in the case of the grain ration, while it would have little effect with the hay diet. It is desirable that differences in counts associated with these different methods should be eliminated as far as possible, or at least be capable of assessment, so that the results of different workers with diets of different composition, in different localities, or with different species of ruminants can be compared with confidence.

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