Processing ruminal ingesta to release bacteria attached to feed particles

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A comparison was made of different methods of processing ingesta to release bacteria attached to solid particles, prior to making viable counts. Initially processing was performed under a stream of anaerobic gas and counts were made using the roll tube technique. Later, processing was done in an anaerobic cabinet and counts were made using the plating technique.

On long hay diets counts of total culturable and lactate-utilizing bacteria were twice as high with the Stomacher and Ultra-Turrax treatment of ingesta when compared with the Minimal treatment. On the chopped hay and high concentrate diets there was little difference between the processing methods in respect of counts of these two groups of bacteria. Counts of cellulolytic bacteria which are attached to ingesta particles were higher with the Minimal treatment than with the other two methods. In contrast, counts of the ciliate protozoa showed a marked difference owing to processing.

Results from the later experiments done in the anaerobic cabinet showed that poor anaerobiosis was responsible for at least some of the lethal action of the Ultra-Turrax and Stomacher.

'n Vergelykende studie is gedoen van die verskillende behandelings van verteringsmateriaal om baterieë wat vassit aan die vaste deeltjies te bevry voordat lewensvatbare tellings gedoen word. Oorspronklik is die behandeling onder 'n stroom anaërobe gas gedoen en tellings is in rolbuise gedoen. Later is die behandelings in 'n anaërobe kabinet gedoen en die tellings is volgens die plaattegniek gedoen.

Op rantsoene van lang hooi was die tellings van die totale telbare en die melksuur-benuttende baterieë twee maal so hoog vir die 'Stomacher' en die 'Ultra-Turrax' behandeling, as vir die Minimum behandeling. Op die gemaalde hooi en hoë konsentraat rantsoene was daar baie min verskil tussen die behandelings ten opsigte van tellings van die twee groepe bakterieë. Tellings van die sellulolitiese bakterieë wat vassit aan die deeltjies was hoër vir die Minimum behandeling as vir die ander twee metodes. In teenstelling hiermee, het die tellings van die siliaat protosoë merkbare verskille getoon ten opsigte van die behandelings.

Resultate van latere eksperimente wat in die anaërobe kabinet gedoen is, het getoon dat swak anaërobiose verantwoordelik was vir ten minste sommige van die nadelige uitwerking van die 'Ultra-Turrax' en die 'Stomacher'.

Keywords: Ruminal bacteria, ciliate protozoa, processing ruminal ingesta, viable counts

Introduction

Since many species of rumen bacteria tend to be preferentially attached to the solid particles of ingesta, it is necessary to free these bacteria prior to making viable counts in any ecological study. Most studies, including those in our laboratories, have used bladed homogenizers to release bacteria from ingesta particles but it appeared that this technique damaged some of the large bacteria and protozoa. Although the Stomacher has not been used for processing samples of ruminal ingesta, it was thought that it offered a more gentle method than the Ultra-Turrax homogenizer so that most of the protozoa and large bacteria would survive. The Stomacher has two paddles, side by side, which alternately pound a polyethylene bag containing the sample and diluent thus compressing the contents against the flat surface of the door. Removal of the micro-organisms is probably brought about by shearing forces as the liquid is swept from side to side, and partly by a series of rapid compressions under the paddles. It has the added advantages that sample and diluent are isolated in a bag from the working surfaces, and that increases in temperature are small compared with those produced by bladed homogenizers (Sharpe & Jackson, 1972).

Thus a comparison was made of the effect of different methods of processing ingesta on viable counts of different metabolic groups of organisms. Initially processing was performed under a stream of anaerobic gas and counts were made using the roll tube technique. In later experiments processing was done in an anaerobic cabinet and counts were made using the plating technique.

Materials and Methods

Animals and diets. The sheep were fed the following diets: 1 000 g chopped lucerne hay once daily at 08h00; 750 g high concentrate diet (71% maize grain + molasses, 18% maize stalks) fed twice daily at 08h00 and 16h00; 1 200 – 1 400 g long teff hay once daily at 08h00. Water was freely available at all times except on sampling days when it was withheld until sampling was completed. Representative samples of ruminal ingesta were obtained by mixing the contents of the rumen and reticulum *in situ* by passing the hand through the rumen cannula (83 mm ID) and then using a beaker to scoop out a sample. The beaker, containing *ca.* 250 g of ingesta, was sealed with a sheet of parafilm and rubber band and rapidly transported to the laboratory.

Processing methods. In the laboratory the sample was mixed and subsamples (*ca.* 10 g) were removed using a ladle. The weight of each subsample was determined and the sample diluted exactly 1/10 with anaerobic diluent. The diluted subsamples were then processed as follows:

MN — Minimal treatment with manual swirling to suspend solid particles;

ST — Stomacher treatment for 1 min in a Colworth Stomacher 400 (Seward & Co, 6 Stamford St, London);

UT — Ultra-Turrax treatment for 1 min in an Ultra-Turrax Homogenizer (20 000 rpm; Janke & Kunkel KG, Staufen i. Br., West Germany).

Microbiological counts. Counts of ciliate protozoa were performed according to the method of Eadie *et al.* (1970). The roll tube techniques and media used for counts of viable bacteria were reported by Mackie *et al.* (1978). Agar plates were prepared, inoculated and incubated in an anaerobic cabinet (Forma Model 1024, Marietta, Ohio; 30% CO_2, 5% H_2, 65% N_2 gas phase). Direct cell counts were made microscopically on Gram-stained slides from the 10^{-3} and 10^{-4} dilutions (20 µl spread evenly over 1 cm^2 and viewed at a magnification × 900).

Results and Discussion

On the long hay (teff) diet counts of total culturable and lactate-utilizing bacteria were twice as high with the Stomacher and Ultra-Turrax treatment of ingesta when compared with the Minimal treatment (Table 1). On the chopped lucerne hay and high concentrate diets there was little difference between the processing methods in respect of counts of these two groups of bacteria. Counts of cellulolytic bacteria which are attached to ingesta particles were higher with the Minimal treatment than with the other two methods of processing (Table 1).

Table 1 Effect of different processing methods on numbers of bacteria in different metabolic groups using the roll tube technique for enumeration

Diet and metabolic group	No. of bacteria (× 10^9 per g ingesta) as determined after processing[a]		
	MN	ST	UT
Teff hay (n = 3)			
Total culturable	2,5	4,4	4,6
Lactate-utilizers	0,06	0,12	0,11
Cellulolytics	0,05	0,01	0,01
Lucerne hay (n = 3)			
Total culturable	1,8	2,0	1,6
Lactate-utilizers	0,12	0,17	0,17
Cellulolytics	0,02	0,01	0,01
High concentrate (n = 3)			
Total culturable	13,9	11,1	10,4
Lactate-utilizers	1,0	1,5	0,9
Cellulolytics	0,05	0,02	0,02

[a]MN — Minimal treatment with manual swirling in anaerobic diluent at 1/10 dilution to suspend ingesta particles

ST — Stomacher treatment for 1 min at 1/10 dilution in Colworth Stomacher 400

UT — Ultra-Turrax treatment for 1 min at 1/10 dilution in Ultra-Turrax Homogenizer

In contrast, the counts of the ciliate protozoa showed a marked difference owing to processing. On all three diets counts of ciliate protozoa were highest with the Minimal treatment and lowest with the Ultra-Turrax treatment although the difference between treatments was least on the teff hay diet (Table 2). On the high concentrate diet there was a four-fold decrease in protozoal counts when the Minimal and Ultra-Turrax treatments were compared. On further analysis of the size distribution of ciliate protozoa in the processed samples (data not presented) it was clear

Table 2 Effects of different processing methods on number of ciliate protozoa ($\times 10^5$ per g ingesta) and the relative decrease when compared with the minimal treatment

Diet (n = 3)	Counts after processing[a]			Relative decrease		
	MN	ST	UT	MN	ST	UT
Teff hay	3,8	3,4	3,1	100	89	82
Lucerne hay	0,9	0,4	0,3	100	44	33
High concentrate	16,1	7,0	4,6	100	43	29

[a]Refer to footnote in Table 1

that all large holotrichs (*Isotricha* sp.) and entodiniomorphs ($100-150 \times 60-90$ mμ) were destroyed when processed with either the Stomacher or Ultra-Turrax homogenizer. Numbers of small entodiniomorphs ($40-60 \times 20-30$ mμ) were also reduced by processing with these two methods but to a lesser extent. Small entodiniomorphs predominated with the teff hay diet and hence it was least affected by the Stomacher and Ultra-Turrax treatments.

Although anaerobic precautions were taken, it was not possible when processing to prevent oxidation of anaerobic diluent, as shown by the indigocarmine redox indicator. When the three methods were ranked according to degree of anaerobiosis i.e. Minimal > Stomacher > Ultra-Turrax, it can be seen that the Ultra-Turrax was the least anaerobic and the Minimal treatment the most anaerobic. However, the effectiveness of the treatments for removing bacteria from solid particles of ingesta was Ultra-Turrax > Stomacher > Minimal, as shown by scanning electron microphotographs (data not presented). Thus the count of the different metabolic groups of organisms is influenced by opposing actions which release and destroy or inhibit to varying extents the different species of which the group is composed. Thus poor anaerobiosis was responsible for at least some of the lethal action of the Ultra-Turrax and Stomacher.

Table 3 Effects of different processing methods on numbers of bacteria in different metabolic groups using the anaerobic cabinet for ingesta processing and plate counts

Diet and metabolic group	No. of bacteria ($\times 10^9$ per g ingesta) as determined after processing[a]		
	MN	ST	UT
Lucerne hay (n = 5)			
Direct microscopic	17,9	31,3	39,4
Total culturable	4,3	8,9	12,9
Lactate-utilizers	2,3	6,1	10,6
Cellulolytics	0,04	0,71	0,87
High concentrate (n = 6)			
Direct microscopic	60,8	99,7	106,0
Total culturable	29,7	61,7	68,2
Lactate-utilizers	21,7	49,5	54,6
Cellulolytics	1,4	2,8	3,3
Pure cultures (n = 3)			
Bacteroides amylophilus	3,1	3,3	3,2
Selenomonas ruminantium	5,0	4,9	4,9

[a]Refer to footnote in Table 1

This was confirmed by a series of experiments in which both processing and plate counts were performed inside an anaerobic cabinet. Counts of total culturable, lactate-utilizers and cellulolytic bacteria all increased 2 – 3 fold when the Minimal and Stomacher or Ultra-Turrax treatments were compared (Table 3) on both the lucerne hay and high concentrate diets. Furthermore, experiments performed on two pure cultures of rumen bacteria *(Bacteroides amylophilus, Selenomonas ruminantium)* processed by the three different methods within the anaerobic cabinet showed no reduction in either direct microscopic or viable counts.

The results of the experiments reported in this paper show that:-

(i) it is essential that samples of ruminal ingesta be processed to remove bacteria attached to ingesta particles and to break chains and clumps of bacteria
(ii) the Ultra-Turrax homogenizer was the most effective treatment for removing bacteria from solid particles of ingesta
(iii) reduced anaerobiosis when processing samples of ruminal ingesta outside the cabinet is an important factor causing a decrease in counts of viable ruminal bacteria
(iv) there is no evidence of mechanical damage to the cells in the pure culture studies although bacteria attached to particles may be damaged by mechanical removal
(v) high counts were obtained when ingesta samples were processed and plated out within the anaerobic cabinet.

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