S.-Afr. Tydskr. Veek. 1983, 13(1)

Media lacking rumen fluid for enumeration of rumen bacteria

S.K. Baker and R.J. Moir

Department of Animal Science and Production, University of Western Australia, Nedlands, Western Australia 6009

A medium without rumen fluid but containing trypticase, hemin and volatile fatty acids was developed for the enumeration of rumen bacteria. Counts of total culturable and amylolytic bacteria were as high or higher than those obtained using medium CC (Zeigler-Leedle & Hespell, 1980) or medium 10 (Caldwell & Bryant, 1966). However, counts of cellulolytic bacteria were low probably owing to the absence of blending to remove bacteria from ingesta particles.

'n Medium sonder rumenvloeistof, maar wat triptikase, hemien en vlugtige vetsure bevat, is ontwikkel vir die vermeerdering van die bakterieë van die grootpens. Tellings van totale kweekbare en amilolitiese bakterieë was net so hoog of hoër as dié verkry met medium CC (Zeigler-Leedle & Hespell, 1980) of medium 10 (Caldwell & Bryant, 1966). Die tellings van sellulolitiese bakterieë was egter laag, waarskynlik as gevolg van die afwesigheid van menging om die bakterieë van die verteringsmateriaal te verwyder.

Keywords: Rumen ecology, rumen bacteria, enumeration of rumen bacteria, selective culture media, culture techniques, rumen population, cellulolytic bacteria, amylolytic bacteria

Introduction

Media containing rumen fluid are unsuitable as niche-simulating media because their composition cannot be adequately and repeatably defined. A semi-defined culture medium for enumerating rumen bacteria is described. This medium lacks rumen fluid and the composition of the inorganic salt solutions (Table 1) is based on work by Matturi (1978), and differs from those of commonly used media. The composition is based on the concentrations of each cation, and of phosphate and sulphide required to give maximum degradation of cellulose with washed suspensions of rumen bacteria. Techniques were developed to allow one person to prepare and rapidly dispense agar media for experiments requiring large numbers (up to 7 000) of tubes of culture media.

Methods

Media were prepared in batches of several litres in round-bottom flasks. After autoclaving the flasks remained sealed and mineral solution B and sodium bicarbonate (Table 1) were added aseptically to the sterile medium using a syringe. The dilution medium had a similar composition, except that carbohydrates and vitamins were omitted and 13% (v/v) of each of Minerals A and B and 0,18% agar were used. A pump incorporating a glass syringe and operating under a slight positive CO_2 pressure from inside the flask was used to dispense the sterile agar medium into sterile CO_2-filled culture tubes; strictly anaerobic techniques were used. Prior to inoculation the agar media were remelted and reducing agents, trypticase, hemin, vitamins and carbohydrates were added to each culture tube.

The growth of rumen bacteria on medium 5-4, medium 10 (Caldwell and Bryant, 1966) and medium CC (Zeigler-Leedle & Hespell, 1980) were compared. The composition of the media was as reported by the authors, but preparation and inoculation procedures were the same as those used in our laboratory. The carbohydrates in the three media were replaced by ball-milled Whatman No. 1 filter paper (0,4% w/v) (added to the medium before autoclaving), or by soluble starch (0,05% w/v), for enumerating cellulolytic or amylolytic bacteria in primary culture.

Digesta samples were taken through a rumen cannula from sheep four hours after feeding 800 g (dry weight) chaffed oaten hay. Inocula were transferred without blending using a syringe with a 19-gauge needle. Inoculation of the roll tubes was completed within two hours of taking rumen samples; during this time samples were kept at 4°C.

Table 1 Composition of medium 5-4

Ingredient	% of medium 5-4
Minerals A[a]	15,0
Minerals B[a]	15,0
Resazurin	0,0001
Agar (Difco Bacto-agar)[b]	1,2
Volatile fatty acids[c]	0,31
$NaHCO_3$	0,4
Trypticase[d]	0,2
Hemin[e]	0,0001
Cysteine HCl/Na_2S[e]	0,05
Vitamins[f]	0,1
Carbohydrates[g]	see below
Final pH	6,7 – 6,8

Prepared with 100% CO_2 gas phase.

[a]Minerals A: K_2HPO_4 0,24%, NaCl 0,6%, KCl 0,6%, $MgSO_4$ 0,24%, $FeCl_3$ 0,19 mg%, $CoCl_2 \cdot 6H_2O$ 0,53 mg%, $ZnCl_2$ 0,20 mg%, $CuCl_2 \cdot 2H_2O$ 0,18 mg%

Minerals B: $CaCl_2 \cdot 6H_2O$ 0,3%, $(NH_4)_6\ Mo_7O_{24} \cdot 4H_2O$ 0,09 mg%.

[b]Medium 10 and medium CC were also prepared with 1,2% agar.

[c]VFA: final concentrations in medium: *n*-valeric, *iso*-valeric, D,L-α-methylbutyric each $9{,}0 \times 10^{-4}$ M, *iso*-butyric $1{,}1 \times 10^{-4}$ M.

[d]N:S ratio in medium 5-4 of 8:1 (Matturi, 1978).

[e]Caldwell & Bryant, 1966.

[f]Bryant & Robinson, 1962.

[g]Non-selective medium (medium 5-4): 0,04% each succinic acid, maltose, glucose, cellobiose, xylose, Na-lactate.

Cellulose agar (C5-4): 0,4% ball-milled Whatman No. 1 filter paper.

Starch agar (S5-4): 0,05% soluble starch.

Results and Discussion

The concentration of viable rumen bacteria estimated from growth in medium 5-4 was of the order 10^9 to 10^{10} per ml digesta and was as high or higher than that from medium CC or from medium 10 (Table 2). In experiment 6, the dilution media were poorly poised and this would have contributed to the low counts obtained, especially on medium CC.

The methods used in our laboratory for preparing and rapidly dispensing agar media had no adverse effect on the number of rumen bacteria on those media (Table 2). Similarly the concentrations of amylolytic and cellulolytic rumen bacteria estimated from the number of colonies in the starch medium (S5-4) and in the cellulose medium (C5-4) were as high or higher than those estimated from numbers on the corresponding starch and cellulose media based on medium CC and medium 10. In these experiments the starch media

Table 2 Concentrations of total culturable rumen bacteria estimated from colony counts on three non-selective media (× 10^9 per ml of digesta)[a]

	Method of preparing and dispensing agar media[b]					
	A			B		
Medium	Exp. 2	Exp. 3	x[c]	Exp. 6[e]	Exp. 7	x[c]
5 – 4	5,28 (a)[d]	10,04	7,66 ± 0,31	4,04	8,63 (b)	6,34 ± 0,58
CC	4,81 (a)	16,29	10,55 ± 0,48	0,82	10,50 (b)	5,68 ± 0,70
10	3,58 (a)	5,93	4,76 ± 0,23			

[a]Five replicate dilution series were used in each experiment

[b]Methods of preparing and dispensing media: A developed in our laboratory; B used by Hungate in our laboratory but essentially according to Caldwell & Bryant (1966)

[c]Mean ± pooled estimate of standard deviation

[d]Estimates followed by the same letter do not differ significantly ($P > 0,05$)

[e]Dilution media poorly poised which may have contributed to the low colony counts

were incubated for two days before colonies were counted, but in later experiments the incubation time was reduced.

On medium 5-4 the delay before colonies were observed was shorter, and the colonies larger at the completion of incubation. Clearings in the cellulose agar (C5-4) were visible after 3 to 7 days incubation. Growth of cellulolytic bacteria in cellulose agar media based on medium CC and medium 10 was poor, although good growth was achieved in broth media containing strips of filter paper (Table 3). Cellulolytic colonies in dilutions of 10^{-7} ml of rumen digesta from sheep fed a diet of chaffed lucerne and oaten hays have been observed in medium C5 – 4.

Table 3 Concentrations of cellulolytic and amylolytic rumen bacteria estimated from colony counts on starch and cellulose media

	Medium		
Carbohydrate	5-4	CC	10
Soluble starch[a] (in agar)	6,85 ± 0,22	7,36 ± 0,24	6,51 ± 0,21
Cellulose agar[b]	1,32 ± 0,47	NG[e]	NG
Filter paper[c] (in broth)	5,12 ± 1,5	6,18 ± 6,5	4,11 ± 0,95
Cellulose agar[d] + 0,05% cellobiose	325 ± 96	276 ± 87	278 ± 87
Cellulose agar[d] (no cellobiose)	1,12 ± 0,48	1,28 ± 0,48	NG

[a] Mean ± standard deviation of concentration of amylolytic bacteria (× 10^9 per ml digesta) from 5 dilution series in each of 2 experiments.

[b] Mean ± standard deviation of concentration of cellulolytic bacteria (× 10^6 per ml digesta) from 3 dilution series in each of 2 experiments.

[c] MPN estimate (± estimate of standard deviation) of cellulolytic bacteria (× 10^6 per ml digesta) from 2 experiments each with 5 replicates dilution series.

[d] Mean ± standard deviation of concentration of bacteria (× 10^4 per ml digesta) using Method B (Table 2) of media preparation.

[e] NG: no visible clearing of the cellulose.

On cellulose media, only those colonies surrounded by a clearing of the cellulose were counted. When 0,05% cellobiose was added to the cellulose media the estimates of concentrations of cellulolytic rumen bacteria were 100-fold higher (Table 3). In medium C5 – 4 without added cellobiose but containing phytone in place of trypticase the number of bacteria (× 10^7) per ml digesta was 5,70 ± 0,37, estimated from all the colonies, whether or not they were surrounded by a clearing of the cellulose.

Results from experiments where the N:S ratio of the medium, the source of organic nitrogen and the composition of the gas phase were varied, are being analysed at present. Lower volatile fatty acids were not included in medium 5 – 4.

Three estimates of the concentration of rumen bacteria were made from a number of colonies on non-selective, cellulose and starch media. From the colony counts in successive tubes in each dilution series a mean colony count and a maximum likelihood estimate using the method of generalized linear models (Nelder & Wedderburn, 1972) were identical. Because the third estimate, using the method of most probable numbers (MPN) had a higher standard error (broth media, Table 3), enumeration of cellulolytic bacteria on agar media was favoured. As an example, three estimates can be made of the number of cellulolytic bacteria (× 10^6) per ml of digesta from colony counts on medium C5 – 4: 36,3 ± 9,0 (mean colony count at dilutions of 10^{-6} ml), 1,32 ± 0,47 (generalized linear model), and 0,70 ± 0,25 (MPN).

Medium 5-4 has the advantage of allowing the growth of numbers of rumen bacteria on selective or non-selective media in roll tubes which is comparable with that on media based on medium CC which contains rumen fluid. Colony counts from rumen digesta of animals fed poor quality diets are often low. In our studies a low protein oaten hay diet was fed to the sheep, but bacterial numbers on medium 5-4 were as high or higher than previously reported from medium CC (16,9 × 10^8 per ml rumen contents (Zeigler-Leedle & Hespell, 1980)) and medium 10 (2,84 × 10^9 per g rumen contents (Caldwell & Bryant, 1966)) inoculated with

rumen fluid from animals fed high quality forage diets. The number of cellulolytic bacteria was low when compared with values reported in the literature and the best count on cellulose roll tubes containing 0,05% cellobiose formed only 0,05% of the toal count. However, these samples were not blended or homogenized to remove bacteria attached to ingesta particles and which would increase cellulolytic counts.

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