Enumeration and Recovery of Bacterial Isolates from Ruminants Fed with Different Dietary Regimes and Their Antibacterial Activity

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ABSTRACT : The study evaluated different synthetic and semisynthetic media for maximal recovery of rumen bacteria and expression of their antibacterial activity. Rumen Glucose Cellobiose Agar (RGCA) medium was found to be the best for recovery of rumen bacteria. However, L-10 medium was the best for expression of antibacterial activity of ruminal isolates followed by Easy, M-10, RGCA and M-98-5 medium. The present study recommends the use of L-10 medium as the medium of choice for screening of antibacterial activity of ruminal isolates. Comparative evaluation of bacterial counts on different dietary regimes indicated significant difference between different growth media on a specific diet and between diets on specific growth media within a species. However, there is no overall significant difference between total bacterial counts obtained from rumen liquor of cattle and buffalo with respect to either the feeding regime or growth media. Feeding straw based diet to the animal is the best for high recovery of rumen bacteria. *(Asian-Aust. J. Anim. Sci. 2001. Vol. 14, No. 6 : 811-815)*

Key Words : Rumen, Feeding Regime, Recovery, Buffalo, Cattle, Antibacterial Activity

INTRODUCTION

Rumen represents a very complex ecosystem and an efficient biological fermentation vessel where a variety of fermentation reactions essential for growth and productivity of the ruminant are brought about with the help of microorganisms (Hobson and Stewart, 1997). The eubacterial ruminal population consists predominantly of an array of highly diversed anaerobes armed with fibrolytic activities (Stewart and Byant, 1988) and few of them also possess antibacterial potential (Kalmokoff and Teather, 1997). However, many of them remain under represented due to their poor growth rate and slow generation time and because of their obligate nature, majority of ruminal bacteria fail to grow in formulated synthetic media. The present study aims at evaluation of different synthetic and semisynthetic growth media for maximal recovery of rumen bacteria and expression of their antibacterial activity.

MATERIALS AND METHODS

Bacterial cultures

All the bacterial cultures used as indicator strains for assay of antibacterial activity of isolates were obtained from different sources as indicated in table 1.

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Collection of rumen liquor samples

The samples of rumen liquor were collected randomly from different fistulated cattle and buffaloes of 12-14 and 34-36 months old age group preadapted on different feed regimes (roughage; silage; green fodder; straw concentrate; feed concentrate) over a period of two months. Rumen liquor samples were collected using a polyvinyl pipe through the fistula into pregassed flasks and immediately transported to the laboratory. During transportation, adequate precautions were taken to maintain air tight condition and a temperature of 39℃ by keeping the flask inside a thermally insulated bucket containing warm water at 40℃. The samples were gassed with CO_2 for 5 minutes and transferred immediately inside the microprocessor controlled anaerobic workstation (Don Whitley Scientific, UK) saturated with ultra pure oxygen free nitrogen, carbon dioxide and hydrogen gas at a ratio of 80:10:10 and maintained at 39℃ and 70 percent humidity. The samples were homogenized to dislodge the bacteria associated with feed particles by stirring for 10 minutes over a magnetic stirrer and processed for further analysis.

Preparation of media

The growth media namely Rumen Fluid Glucose Cellobiose Agar (RGCA) (Bryant & Robinson, 1962), L-10 Medium (Kalmokoff & Teather, 1996), Easy Medium (Champion et al., 1988), Medium 10 (Caldwell and Bryant, 1966) and Medium-98-5 (Bryant & Robinson, 1962) used in this study for enumeration and isolation of rumen bacteria were prepared under strict anaerobic conditions. Strictly anaerobic techniques of Hungate (1969) and Macy et al. (1972) were followed during the preparation of pre-reduced media.

Table 1. Bacterial cultures and their sources

Name of bacteria (indicator strain)	Source
Ruminococcus albus B119	Bryan A. White, Department of Animal Sciences, University of Illinois, Urbana, Illinois, USA
Ruminococcus flavefaciens C94	- do -
Prevetolla ruminicola S-23	- do -
Selenomonas ruminantium D	- do -
Butyrivibrio fibrisolvens D1	Ron M. Teather, Centre for Food and Animal Research, Ottawa, Ontario, Canada
Butyrivibrio fibrisolvens OR 3	- do -
Butyrivibrio fibrisolvens OR 12	- do-
Butyrivibrio fibrisolvens OR 515	- do -
Streptococcus bovis SB3	- do -
Streptococcus bovis 26	- do -
Eubacterium ruminantium GA-195	C.W. Forsberg, Dept. of Microbiology, University of Guelph, Canada
Fibrobacter succinogenes BL-2	- do -
Bacteroides amylophilus 70	- do -
Ruminococcus flavefaciens OF-2	Mol. Biol. Unit, NDRI, Karnal, India
Ruminococcus albus A-6	- do -
Bacillus cereus NCDC-66	- do -
Salmonella typhi NCDC-133	- do -
Pseudomonas aeruginosa NCDC-110	- do -
Staphylococcus aureus NCDC-105	- do -
Lactobacillus casei ED-108	Multiple drug resistant milk isolate, Mol. Biol. Unit, NDRI, Karnal, India

Enumeration of rumen bacteria

Appropriate ten-fold dilutions (10^{-1} to 10^{-8}) of the rumen liquor samples were prepared inside the anaerobic glove box in screw capped tubes predispensed with anaerobic diluent (Dehority, 1963). The screw capped tubes, petri-dishes, tips and tooth picks (all sterile) were placed inside the anaerobic chamber one day before use for equilibration under anaerobic conditions. Premolten media namely L-10, M-10, Easy, RGCA, M-98-5 in molten state (tempered to 50~60℃) were transferred into the chamber and poured in the petri plates one day before the experiment. An aliquot of 100 μl of appropriate dilutions (10^{-1} to 10^{-7}) of each sample was spread onto the pre-dried media plates with the help of sterile glass spreader (hockey sticks) and incubated inside the anaerobic glove box for 24 to 48 hrs. A number of typical but morphologically different colonies developed after incubation on each media plates were randomly picked with the help of sterile tooth picks into the respective broth tubes and incubated for 24 hrs inside the glove box. The purity of the cultures was checked by Gram's staining and confirmed by reisolation on solid medium until colonies of only one type were obtained. The cultures were also checked for growth under aerobic conditions using respective growth media.

Assay for Antibacterial activity of rumen bacterial isolates

Well isolated colonies developed on different growth media plates during the primary isolation were randomly picked with sterile tooth picks, replica plated on the respective agar media and incubated at 39℃ for 24 hrs. As many as 50 colonies were transferred on each plate maintaining equidistance between each spot. After incubation, the plates were overlaid with 8 ml of Easy Medium (soft agar) seeded with indicator organisms (table 1) at the rate of 10^{6} cfu/ml after standardization of the cell suspension and allowed to solidify. While pouring soft agar, adequate care was taken not to disturb the bacterial colonies developed on the media plates. The plates were then incubated overnight at 39℃ and examined for clear zones of inhibition of the indicator strain around the respective colonies. The colonies showing a minimum of 1 mm zone of inhibition were marked. The selected colonies exhibiting antibacterial activity were picked from the master plate with the help of tooth pick and transferred to broth tubes and incubated for 24 hrs at 39℃ in the anaerobic glove box. The broth tubes inoculated with the selected test cultures were checked for purity by Gram staining. The cultures were streaked on corresponding media plates repeatedly till pure culture of the respective isolate was obtained. An aliquot of 3 μl of the pure broth culture was

spotted on different media plates (L-10, M-10, RGCA, Easy, M-98-5) and the plates were incubated for 24 hrs followed by overlaying with 8 ml of easy medium (soft agar) seeded with 10^6 cfu/ml of the indicator organisms namely *S. bovis* SB3 and *R. albus* B-199. The plates were again incubated for overnight and checked for clear zones of inhibition around the spots.

Statistical analysis

Two-way ANOVA was applied to rumen bacterial counts obtained from different rumen samples under the influence of different variables using GraphPad Prism version 3.02 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. Critical difference (CD) was calculated for $p<0.05$ for each ANOVA perfomed to delineate the significant effect produced by a particular variable individually or in combination. Difference between means of each treatment were determined at $p<0.05$ using Duncans new multiple range test (Duncan, 1955).

RESULTS AND DISCUSSION

A total of 60 rumen liquor samples comprising 33 from buffalo and 27 from cattle were analysed anaerobically for rumen microbial population in terms of total bacterial counts ($\log_{10}$ cfu/ml). The comprehensive data pertaining to the same has been presented in table 2. As can be evidenced from the data presented therein, the total ruminal bacterial counts ranged from $\log_{10}3.93\sim9.38$ and $4.34\sim9.66$ cfu/ml in rumen liquor samples collected from buffalo and cattle respectively. On critical appraisal of the counts from different animals, it was observed that there was no significant difference between the average counts obtained from cattle and buffalo rumen. On comparative evaluation of different growth media for the recovery of ruminal bacteria indicated that RGCA medium was the best (table 2) as it significanty differed from rest of the growth media on the basis of the calculated critical difference (CD=0.42) and Duncans multiple range test. The recovery on L-10, M-10 and Easy medium was mediocre and was almost comparable in case of all the rumen liquor samples collected from different sources as they did not show any significant difference among each other. However, the recovery on M-98-5 was minimal as indicated by lowest corresponding average counts. It was also observed that the type of feed given to the ruminant could also influence the rumen microbial population in the ruminants considerably as has been indicated in table 3. Separate ANOVA was performed for the data presented in table 3 for buffalo and cattle. The results of ANOVA indicated that the feeding regime and growth media had significant effect on recovery of rumen bacteria for each species. The maximal average log counts were obtained from rumen liquor samples collected from buffaloes fed with straw based diet. However, based on the calculated critical difference and Duncans multiple range test, the total micorobial count obtained from buffalo fed with straw based diet or feed concentrate or green fodder did not differ significantly but the samples obtained from silage fed buffalo differed significantly from the rest. Almost, a similar trend was observed when rumen liquor samples collected from cattle fed with different diets were analysed. Based on Duncans multiple range test, it was deciphered that the recovery of bacteria from cattle fed with straw based diet and feed concentrate exhibited a similar pattern. However, cattle fed with green fodder or silage resulted into poor recovery of rumen bacteria on different growth media that exhibited significant difference in comparison to animals fed with straw based diet or feed concentrate. However, no significant difference was observed between the species (buffalo and cattle), when a separate ANOVA was performed for different feeding regimes ignoring growth media. The critical difference obtained from ANOVA of mean counts of different feeding regime irrespective of growth media indicated that feeding straw based diet to animals was the best for getting highest recovery of rumen bacteria. On the other hand, animals fed with feed concentrate or green fodder resulted mediocre bacterial recovery followed by silage fed animals resulting into poor recovery of rumen bacteria. However, it is obvious that the over all population structure of rumen bacteria could be

Table 2. Enumeration of bacterial population on different growth media from rumen liquor samples from buffalo and cattle

Source	Total no. of Samples	Total counts (log cfu/ml)*					P value
		L-10	M-10	Easy	RGCA	M-98-5	
Buffalo	33	4.17-8.69 (6.70)^b	4.82-8.68 (6.75)^b	4.29-8.60 (6.45)^{bc}	5.98-9.38 (7.68)^a	3.93-6.81 (5.37)^c	0.012
Cattle	27	4.34-7.48 (5.91)^b	4.38-7.34 (5.86)^{bc}	4.49-8.17 (6.33)^b	5.67-9.66 (7.67)^a	4.41-6.32 (5.37)^c	0.012

* Values in parentheses indicate the average total plate count. a, b, c: Means with different superscripts in the row differ ($p<0.05$).

Table 3. Enumeration of bacterial population on different growth media from rumen liquor samples collected from cattle and buffalo under different feeding regimes

Source*	No. of samples	Feed regimes	Total counts (log cfu/ml)**					P value	Row mean
			L-10	M-10	Easy	RGCA	M-98-5		
Buffalo (33)	11	Straw based	5.76-8.69 $(7.22)^{ab}$	5.41-8.68 $(7.05)^{b}$	4.44-7.42 $(5.93)^{c}$	6.22-9.38 $(7.80)^{a}$	5.13-6.22 $(5.68)^{c}$	0.027	6.73 ± 0.40^{A}
	7	Silage	5.52-6.49 $(6.00)^{b}$	4.82-6.30 $(5.56)^{c}$	4.32-6.82 $(5.57)^{c}$	5.98-7.56 $(6.77)^{a}$	3.93-6.11 $(5.02)^{d}$	0.033	5.78 ± 0.29^{B}
	6	Green fodder	5.14-6.46 $(5.80)^{d}$	6.56-6.92 $(6.74)^{b}$	4.29-8.60 $(6.45)^{bc}$	6.26-8.45 $(7.36)^{a}$	5.36-6.81 $(6.08)^{c}$	0.026	6.48 ± 0.27^{A}
	9	Feed concentrate	4.17-8.50 $(6.36)^{b}$	5.30-7.70 $(6.50)^{b}$	5.93-6.86 $(6.39)^{b}$	6.36-8.34 $(7.35)^{a}$	5.40-6.71 $(6.05)^{b}$	0.028	6.53 ± 0.21^{A}
Cattle (27)	10	Straw based	5.84-8.23 $(7.04)^{b}$	5.23-7.75 $(6.49)^{bc}$	5.30-8.17 $(6.74)^{b}$	7.73-9.66 $(8.69)^{a}$	4.61-6.86 $(5.73)^{c}$	0.027	6.93 ± 0.48^{A}
	5	Silage	4.34-7.12 $(5.73)^{c}$	4.45-6.90 $(5.68)^{c}$	5.85-6.72 $(6.29)^{b}$	5.67-8.24 $(6.96)^{a}$	4.98-6.32 $(5.65)^{c}$	0.033	6.06 ± 0.25^{C}
	6	Green fodder	4.68-6.44 $(5.66)^{c}$	4.38-7.34 $(5.86)^{bc}$	4.49-7.75 $(6.12)^{b}$	6.72-8.78 $(7.75)^{a}$	5.22-6.71 $(5.97)^{bc}$	0.026	6.27 ± 0.37^{BC}
	6	Feed concentrate	5.64-7.48 $(6.56)^{b}$	6.21-6.75 $(6.48)^{b}$	4.63-8.11 $(6.37)^{b}$	6.94-9.47 $(8.20)^{a}$	4.41-6.72 $(5.57)^{c}$	0.028	6.63 ± 0.42^{AB}

* Values in parentheses indicate total number of samples collected. ** Values in parentheses indicate the average total plate count. a, b, c, d: Means with different superscripts in the row differ ($p<0.05$). A, B, C: Means with different superscripts in the column differ ($p<0.05$).

different for each feeding regime, hence our observation reflects only the total bacterial counts and not the bacterial types. In this study, a fairly high recovery of bacteria from rumen liquor samples collected from different sources was recorded on RGCA as compared to those on other media such as L-10, M-10, Easy and M-98-5. This could possibly be attributed to the compositional richness of RGCA medium as it contains undefined nutritional factors present in the clarified rumen fluid used as supplement along with complex sugars such as cellobiose and xylan, and growth stimulating factors like hemin and volatile fatty acids. Our results in this regard are in agreement with those of Bryant and Burkey (1953) and Bryant and Robinson (1962) who also recorded high recovery of rumen bacteria from rumen liquor samples on media supplemented with growth stimulating factors. In this context, the studies conducted by Bryant (1972) and Holdeman et al. (1977) also support that RGCA was the medium of choice for general purpose isolation of rumen bactera. In the second part of this study, a large number of rumen bacterial isolates recovered from the two animal species were randomly picked and screened for antibacterial activity against some indicator strains as indicated in table 1. A total of 272 and 209 Colonies isolated from buffalo and cattle respectively exhibited antibacterial activity. All these isolates were checked for exhibition of antibacterial activity on different growth media and the observations are recorded in table 4. It is evident from the table that L-10 medium was the best in terms of expression of antibacterial activity since maximum number of isolates (169 isolates from buffalo rumen and 122 isolates from cattle rumen) exhibited antibacterial activity against *Streptococcus bovis* SB3 when tested on L-10 medium. On comparative basis, L-10 medium was the best followed by Easy, M-10, RGCA and M-98-5 medium. Though RGCA medium yielded highest recovery of ruminal bacteria, most of the isolates preferred L-10 medium to exhibit antibacterial activity against *S. bovis* SB3, *Ruminococcus albus* B-199 and *Eubacterium ruminantium* GA-195. These results suggest that the cultural requirements for growth and production of antibacterial substances are different. However, our results can not be corroborated due to nonavailability of published literature pertaining to this aspect. Analysis of variance between different media on a specific diet suggested significant differences within a species. However, there was no significant difference between the species. Similarly, analysis of variance between diets on a specific media indicated significant difference within species but we did not observe any overall difference between buffalo and cattle for either of the cases. From this study, it can be concluded that animals fed with straw based diet yield better recovery of rumen bacteria and RGCA medium is the best among the various media tested in this study. On the

Table 4. Rumen bacterial isolates exhibiting antibacterial activity against *Streptococcus bovis* SB3 on different growth media

Source	Total no. of isolates	No. of isolates exhibiting antibacterial activity*				
		L-10	RGCA	Easy	M-10	M-98-5
Buffalo	272	203 (74.63)	103 (37.87)	169 (62.13)	116 (42.65)	89 (32.72)
Cattle	209	181 (86.60)	93 (44.49)	122 (58.37)	110 (52.63)	76 (36.36)

* Values in parentheses indicate the percent value.

other hand, L-10 medium may be used as the medium of choice for screening of ruminal isolates for antibacterial activity.

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