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Effect of Alfalfa Fiber Substrate on Culture Counts of Rumen Bacteria

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A medium has been developed using alfalfa fiber as the sole substrate. It gave high culture counts (3×10^9 to 8×10^9/ml) of rumen bacteria. When this medium was combined with the medium 98-5 of Bryant and Robinson, modified to contain 33% rumen fluid instead of 40% clarified rumen fluid, a higher count was obtained than with either medium alone.

In a complete ecological analysis of a natural microbial habitat, the kinds and numbers of microorganisms should be known (8). The most reliable method to enumerate them is to count them microscopically, but this procedure does not distinguish living from dead organisms, nor is it possible to identify many bacteria by their appearance. Culture counts are widely used to estimate numbers of viable bacteria, with subsequent isolation and identification of desired strains.

The culture count of rumen bacteria is usually 5 to 20% of the direct count (1, 2, 10) in agar media containing soluble sugars and starch added to sterile rumen fluid and mineral solution under anaerobic conditions at 39°C. It seemed possible that a higher culture count might be obtained if the carbohydrate component of the medium more closely simulated the plant materials normally consumed by the ruminant. In particular, some bacteria might be able to use "hemicellulose" (H) polymers of plants but unable to live on starch, cellulose (C), or simple carbohydrates.

Some investigators (4-7) have used Hs from various sources as substrates for rumen bacteria, but few have attempted to use the forage itself as a substrate for culture counts of rumen bacteria.

A medium was developed similar to Jayko's (L. G. Jayko, M.S. thesis, The State College of Washington, Pullman, 1953) H medium, using the alkali-treated, water-insoluble fraction of alfalfa as substrate.

A suspension of alfalfa hay was fractionated in various ways to obtain different substrate preparations. Each of these preparations was included as one-third of a final culture medium. Rumen fluid and deionized water with agar in equal parts comprised the rest of the medium.

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Pebble-milled water-insoluble alfalfa was obtained by extracting 5 g of alfalfa hay, ground to pass a 2-mm sieve, with 1 liter of water for 30 min at 100°C. The residue after filtration contained all the H and C. It was suspended in 250 ml of deionized water and pebble-milled overnight at 4°C. The pebble-milled material was diluted four times before addition to the medium. The filtrate from the water extraction was used as the soluble (S fraction) substrate. In media containing both soluble and insoluble components, 3 volumes of S fraction and 1 volume of the undiluted H+C fraction comprised the substrate.

An H fraction free of C was prepared according to Jayko (M.S. thesis, 1953) by resuspending the residue of the 5 g of hay after water extraction in 250 ml of deionized water containing 9.2 g of NaOH and 1.6 g of KOH, and grinding in the pebble mill overnight at 4°C. The solids were removed by filtration through Whatman no. 1 filter paper. Twenty-five milliliters of the filtrate was diluted to 100 ml; then 1.8 ml of concentrated HCl, 0.06 ml of 85% H_3PO_4, 0.045 g of $CaCl_2$, 0.045 g of $MgSO_4$ and 0.45 g of $(NH_4)_2SO_4$ were added to provide the salts in the proportions desired in the final medium. These brought the pH to 7.2. After equilibration with CO_2, the pH dropped to 6.7. When alkaline treatment was not included in the preparation, equal amounts of alkali (0.92 g of NaOH and 0.16 g of KOH) were added to the medium after the substrate fraction had been diluted to 100 ml.

Alkali-treated whole alfalfa (H+C+S) was obtained by grinding 5 g of unextracted alfalfa in the pebble mill in 250 ml of water plus the alkali and then neutralizing as above (diluted four times with water). Alkali-treated H+C was similarly obtained by grinding the water-extracted material with the alkali.

Dried green wheat (*Triticum vulgare*) foli-

age was treated to give preparations comparable to those from the alfalfa.

One hundred milliliters of the substrate preparation was added to a 500-ml round-bottom Erlenmeyer flask. The flask was held at 47°C in a water bath and air was displaced from its interior with a stream of CO_2 freed of O_2 by passage over hot copper (9). Agar (4.4 g; Difco) was dissolved in 100 ml of boiling deionized water containing 0.3 ml of 0.1% resazurin and added to the flask. Carbon dioxide was bubbled through the medium until the color of resazurin disappeared. Rumen fluid (100 ml) was added. The rumen fluid used in agar media was prepared as follows: fresh rumen contents were incubated at 39°C for 30 min. The large particles either rose to the top or settled on the bottom of the flask. The middle layer of rumen fluid was pipetted anaerobically into plastic centrifuge tubes and centrifuged under CO_2 at $900 \times g$ for 30 min at 4°C to sediment the protozoa and large particles. The final pH in all media was 6.6 to 6.8.

The medium was tubed anaerobically in 4.45-ml quantities in culture tubes (16 by 150 mm) provided with a tooled narrow neck closed with a no. 00 butyl rubber stopper. Sterilization was at 15 lb/in^2 for 15 min.

Before inoculation, 0.05 ml of 2.5% cysteine-hydrochloride in 2.5% $Na_2S \cdot 9H_2O$ was injected via hypodermic syringe (9) into each tube. The cysteine-Na_2S was prepared by dissolving 2.5 g of $Na_2S \cdot 9H_2O$ in 100 ml of oxygen-free deionized water, adjusting to pH 11 with 10% NaOH, adding 2.5 g of cysteine-hydrochloride, tubing anaerobically under N_2 in place of CO_2, and sterilizing at 15 lb/in^2 for 15 min.

The medium 98-5 of Bryant and Robinson (2) and medium 10 of Caldwell and Bryant (3) were used as reference media. Thirty-three percent rumen fluid was used instead of 40% clarified rumen fluid.

Anaerobic procedures have been described (9). The rumen contents for inoculation or for media were usually obtained from a fistulated Jersey heifer fed on alfalfa hay. For inoculum, 10 g of contents was added from the weighing beaker with 90 ml of the 33% rumen fluid broth into a Waring blender which had been gassed out with O_2-free CO_2 and into which CO_2 was introduced continuously during blending. The mixture was blended for 1 min (2). The contents were serially diluted through anaerobic tubes of 33% rumen fluid broth.

Inocula, which consisted of 0.5 ml of the broth dilutions (10^{-5} to 10^{-8}), were injected from each dilution tube into replicate tubes of melted agar containing each medium to be tested. This avoided cumulative errors in serial dilution, since experimental and control tubes to be compared had been inoculated from the same serial dilution tube. After incubation at 39°C for 7 to 10 days, the colonies developing were detected by examination under a binocular microscope at a magnification of 10× or 40×. Colony counts were based on the average of two to four replicate tubes at the dilution of 5×10^{-8} per g of rumen content.

Preliminary experiments compared the colony counts from media containing various treated alfalfa hay. The insoluble materials (H+C) gave slightly higher counts than either the H or alkali-treated whole alfalfa hay (H+C+S). Alfalfa media containing H and C yielded higher numbers (5.9×10^9/g) than media containing H (1.7×10^9/g) or H, C, and S (4.7×10^9/g).

Further experiments indicated that alkaline treatment slightly increased the count on the water-insoluble fraction (Table 1, experiment 1). Addition of the water-soluble fraction decreased the counts in experiment 1. Alkaline treatment of the S fraction did not affect the count on this substrate alone, but neither the treated nor the untreated S fraction gave as high a colony count as the insoluble fraction (H+C) (experiment 2). The alkali-treated water-insoluble fraction (H+C) of wheat hay also gave a slightly higher count than H alone (experiment 3).

In all comparisons, the highest counts were obtained with the alkali-treated H+C fraction. This medium supported about the same colony count as rumen fluid containing glucose, cellobiose, and starch (experiment 4). A combination of the H+C with the medium 98-5 significantly increased the count (experiment 5). A similar increase was obtained when glucose, cellobiose, and starch were added to the H+C medium (experiment 6).

These data indicate that the culture counts can be increased by including the fiber components of the forage in the medium. The favorable effect of the alkali treatment is presumably due to partial solubilization of the fiber components. Removal of the lignin may expose other substances to attack, and partial solubilization of carbohydrate polymers may provide some nutrients in the cultures.

The differences found in these experiments are not impressive from the standpoint of the percentage increase in viable count, but they become quite significant when the absolute numbers of bacteria are considered; the increased counts in Table 1, experiment 5, represent between 3.1×10^9 and 8.1×10^9 bacteria/g.

TABLE 1. *Colony counts in various media for rumen contents from an alfalfa-fed cow*[a]

Expt	Substrates	Alkaline treatment	Colony count[b]			
			Trial 1 (10^9/ml)	Trial 2 (10^9/ml)	Trial 3 (10^9/ml)	Avg ± SE[c]
1[d]	Alfalfa (H + C)	+	5.5	6.9	5.4	5.93 ± 0.48[e]
	Alfalfa (H + C)	−	2.9	4.4	5.2	4.17 ± 0.67
	Alfalfa (H + C)	+	4.4	4.1	3.6	4.03 ± 0.23
	Alfalfa (S)	−				
2[d]	Alfalfa (H + C)	+	6.5	5.1	5.5	5.70 ± 0.42[f]
	Alfalfa (S)	−	3.5	3.0	2.9	3.13 ± 0.19
	Alfalfa (S)	+	3.4	3.0	3.2	3.20 ± 0.12
3[d]	Wheat (H + C + S)	+	2.0	1.1		1.55 ± 0.45
	Wheat (H + C)	+	5.8	2.9		4.35 ± 1.45[g]
	Wheat (H)	+	4.0	2.6		3.30 ± 0.70
4[h]	Alfalfa (H + C)	+	5.1	4.8		4.95 ± 0.15
	Glucose, cellobiose, and starch (98-5)	−	5.1	4.3		4.70 ± 0.40
	Glucose, cellobiose, and starch (medium 10)	−	6.4	3.4		4.90 ± 1.50
5[h,i]	Alfalfa (H + C)	+	6.7	7.2	7.9	7.27 ± 0.35
	Glucose, cellobiose, and starch	−	6.2	7.4	2.3	5.30 ± 1.54
	1/2 alfalfa (H + C)	+	9.3	13.4	10.4	11.03 ± 1.23[e]
	1/2 98-5	−				
6[j]	Alfalfa (H + C)	+	5.3	8.1	6.9	6.77 ± 0.81
	Alfalfa (H + C)	+				
	Glucose, cellobiose, and starch	−	11.0	13.8	8.7	11.17 ± 1.47[g]

[a] Rumen contents were taken between 1,030 and 1,400 h on a different day for each trial.

[b] Colony counts per milliliter were calculated from the dilution tube inoculated with 5×10^{-8} g of rumen contents.

[c] SE, Standard error. For each experiment, the analysis of variance was used to test for both a trial effect and a treatment effect. The trial effect was found to be not significant, although in a few experiments it did approach significance ($0.05 < P < 0.01$). When only two treatments were in an experiment, the overall treatment effect indicated whether or not they differed. Where there were three treatments in an experiment, the treatment effect was broken down into individual degrees of freedom, which compared one designated treatment group against the other two treatment groups.

[d] Each value is the average of duplicate culture tubes.

[e] This treatment is significantly higher than the other treatments, with probability $P < 0.05$.

[f] This treatment is significantly higher than the other treatments, with probability $P < 0.01$.

[g] This treatment, although higher than the other treatments, only approaches significance, with probability $P < 0.10$.

[h] Each value is the average of four replicate culture tubes.

[i] The direct microscope count was 2.9×10^{10}/g of rumen contents.

[j] Each value is average of three replicate culture tubes.

Whether this indicates increased viability of known species or growth of some bacteria not cultivated by previous media is not yet known.

The discrepancy between direct and culture counts of rumen bacteria has been much greater for cattle fed dried forage rations than for those receiving a high proportion of concentrate. A possible explanation has been that culture media omitted an important component of the substrate, the water-insoluble polymers. Particularly the H fraction of alfalfa seemed of possible importance, and the present experiments support this view. The results also indicate that the inclusion of the C fraction, containing chiefly cellulose and lignin, in the medium slightly increases the culture count.

Even with the increased numbers obtained in these experiments, the culture count for rumen contents of cattle fed dry forage is still only 20 to 40% of the direct count, a discrepancy greater than in cattle fed concentrate rations. Important factors may still be missing from the culture medium, but part of the difference between culture/direct count ratios may be due to a greater proportion of dead or injured bacteria in the forage-fed animals. On dry forage, the feed is retained longer in the rumen, and the rate of liberation of soluble carbohydrates is slow, thus tending to increase the proportion of dead bacteria.

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LITERATURE CITED

1. **Bryant, M. P., and L. A. Burkey.** 1953. Culture methods and some of the more numerous groups of bacteria in the bovine rumen. J. Dairy Sci. 36:205–217.
2. **Bryant, M. P., and I. M. Robinson.** 1961. An improved nonselective cultural medium for ruminal bacteria and its use in determining diuranal variation in numbers of bacteria in the rumen. J. Dairy Sci. 44:1446–1456.
3. **Caldwell, D. R., and M. P. Bryant.** 1966. Medium without rumen fluid for nonselective enumeration and isolation of rumen bacteria. Appl. Microbiol. 14:794–806.
4. **Dehority, B. A.** 1965. Degradation and utilization of isolated hemicellulose by pure cultures of cellulolytic rumen bacteria. J. Bacteriol. 89:1515–1520.
5. **Dehority, B. A.** 1966. Characterization of several bovine rumen bacteria isolated with a xylan medium. J. Bacteriol. 91:1724–1729.
6. **Dehority, B. A.** 1967. Rate of isolated hemicellulose degradation and utilization by pure cultures of rumen bacteria. Appl. Microbiol. 15:987–993.
7. **Dehority, B. A.** 1968. Mechanism of isolated hemicellulose and xylan degradation by cellulolytic rumen bacteria. Appl. Microbiol. 16:781–786.
8. **Hungate, R. E.** 1960. Symposium: selected topics in microbial ecology. I. Microbial ecology of the rumen. Bacteriol. Rev. 24:353–364.
9. **Hungate, R. E.** 1969. A roll tube method for cultivation of strict anaerobes, p. 117–132. *In* J. R. Norris and D. W. Ribbons (ed.), Methods in microbiology, vol. 3B. Academic Press Inc., New York.
10. **Maki, L. R., and E. M. Foster.** 1957. Effect of roughage in the bovine ration on types of bacteria in the rumen. J. Dairy Sci. 40:905–913.