IMPROVED TECHNIQUES FOR ISOLATING AND PURIFYING RUMEN ORGANISMS

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The isolation and purification of anaerobic bacteria present in the rumen is a difficult task. The isolation of rumen organisms from rumen contents diluted as much as 100 billion times was accomplished by a technique described by Gall *et al.* (1947). Hungate (1947) published a method for isolating anaerobic cellulose digesters from the rumen, and Sijpesteijn (1948) also described anaerobic techniques for isolating cellulose-decomposing bacteria from the rumen of cattle. None of these techniques is completely satisfactory for the isolation of many important rumen organisms.

Although the earlier cultural procedure described by Gall *et al.* (1947) is satisfactory for the isolation of many rumen organisms, this technique had several weaknesses which made the isolation of several of the more delicate rumen organisms rather difficult. The studies resulting in the improved technique were designed to eliminate such flaws as low pH and high Eh in the unbuffered dilution blanks, and high Eh and glucose concentration in the broth, and the difficulty of purifying mixed cultures.

MATERIALS AND METHODS

A description of the improved technique includes preparation of the dilution blanks, broth, agar, and samples, the inoculation of the broth cultures, and the purification of mixed cultures.

Preparation of dilution blanks. The diluting fluid is composed of 0.75 per cent $NaHCO_3$, 0.07 per cent K_2HPO_4, and 0.07 per cent KH_2PO_4. For each sample it is necessary to sterilize one wide mouth French square bottle containing 90 ml of diluting fluid and five round screw cap bottles with 99 ml of diluting fluid. After sterilization, CO_2 gas is passed through each bottle for two minutes. Immediately before use, two drops of sterile 10 per cent cysteine solution in 2.3 per cent $NaHCO_3$ are added to each blank. The method of preparing the cysteine-bicarbonate solution is described. Add 20 grams of cysteine hydrochloride to 100 ml of approximately 1 N NaOH. The pH will be about 7.0. More or less concentrated NaOH will be required depending on the particular batch of cysteine hydrochloride. To 4 ml of this solution (15 per cent as cysteine) in a test tube add 2 ml of 7 per cent $NaHCO_3$, seal with melted vaspar, and autoclave at 15 pounds pressure for 10 minutes. These tubes can be stored at room temperature for months without appreciable loss due to oxidation. The final concentration as cysteine will be 10 per cent. The final pH of the diluting fluid is 6.8 and the Eh equals -200 mv as determined by the Beckman meter.

Preparation of cultural broth and agar. Ten ml of broth of the following com-

position are used in each of 6 tubes for the cultural series: 1 per cent of peptone, tryptone, beef extract, and yeast extract; and 0.1 per cent each K_2HPO_4, KH_2PO_4, and glucose. To this broth are added one drop of a 0.5 per cent suspension of finely divided cotton and two drops of the cysteine-bicarbonate solution described for use in the dilution blanks. The broth is sealed with vaspar and autoclaved for ten minutes under 15 pounds pressure with the tubes partly immersed in water to prevent the vaspar seal from blowing. Directly before inoculation with the properly diluted rumen contents, the vaspar seal is displaced. The agar used has a composition similar to the broth, with the cotton and cysteine-bicarbonate omitted and 1.5 per cent agar added.

Preparation of sample. Ten grams of rumen contents are weighed out quickly and placed as soon as possible into the wide mouth French square bottle containing 90 ml of diluting fluid. Carbon dioxide gas is bubbled through this 10^{-1} dilution for two minutes. The sample is then placed in a mechanical shaker and shaken for three minutes, after which it is ready for further dilution before culturing. Dilutions 10^{-3}, 10^{-5}, 10^{-7}, 10^{-9}, and 10^{-11} are made by transferring 1 ml of the former dilution into 99 ml of the diluting fluid in the next higher dilution blank.

Inoculation of the broth. Inoculations of dilutions 10^{-6} through 10^{-11} are made in the usual manner with a 1.1 ml pipette from the dilution blanks into the broth. The broth is then resealed by gently heating the displaced vaspar seal. The cultures are incubated at 38 C until growth occurs or until a 30-day incubation period has elapsed. If the field technique is used, the procedure described in the paper by Gall *et al.* (1949) can be used with the modifications described in this paper to improve the technique.

Purification of mixed cultures. After the culture has grown, it is purified by an anaerobic plating procedure using a Brewer anaerobic dish (Brewer, 1942). Inoculation is conducted by transferring an inoculating needle of a turbid broth culture into a tube of melted agar. This is mixed by rolling between the hands, and another transfer is made from this into a similar tube of melted agar containing 0.1 per cent cysteine. The second tube of agar is poured into the bottom of the Brewer dish containing one drop of sterile 7 per cent $NaHCO_3$. After this has hardened, more agar containing 0.1 per cent cysteine is poured over the hardened layer of agar in the dish to such a depth that it will make contact with the inner ring of the cover when the top is placed carefully on the dish. This procedure leaves an air bubble in the plate and an anaerobic zone completely covered with agar. $NaHCO_3$ is included in all media because a soluble source of CO_2 has been found necessary for the growth of some rumen organisms. If the original culture to be purified does not show a fairly heavy turbidity, it may be possible to employ the first tube of agar for the inoculation in place of making the transfer into the second tube. In this event, of course, the first tube of agar should contain 0.1 per cent cysteine.

Several types of growth may then occur, depending upon the oxygen requirement of the bacteria as shown in figures 1 through 4. Strict aerobes will grow only in the area of the air bubble and the outermost fringe of the plate (figure 1),

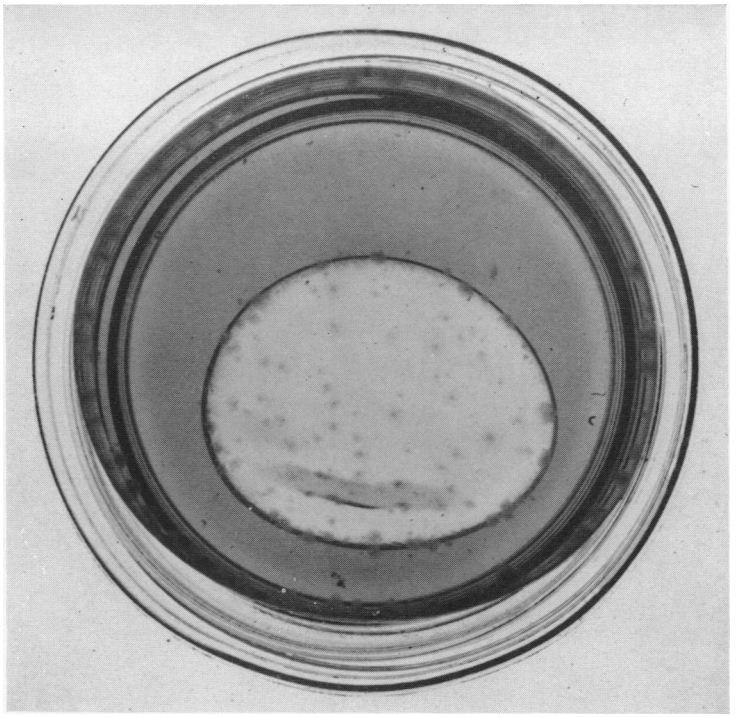

Figure 1. Strict aerobe showing growth only in air bubble and outer, air-exposed fringe—unidentified airborne micrococcus.

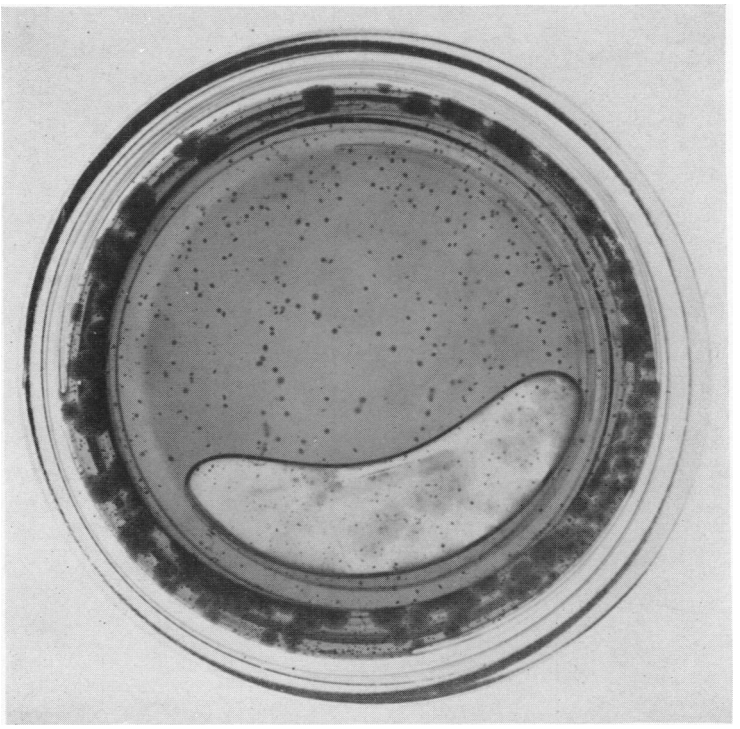

Figure 2. Facultative aerobe showing growth throughout with largest colonies in air bubble and outer fringe—unidentified bacillus.

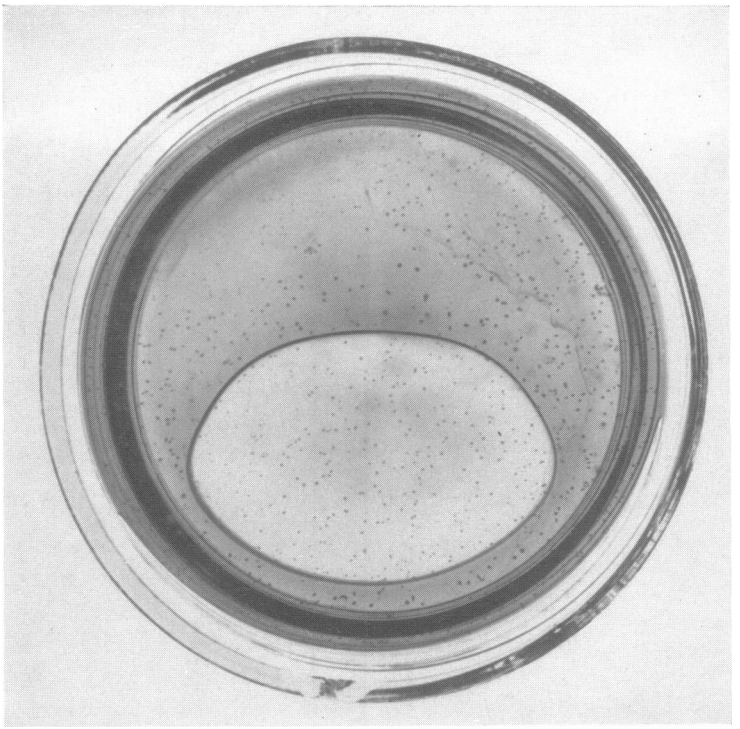

Figure 3. Facultative anaerobe showing growth throughout with somewhat larger colonies in anaerobic zone. Rumen organism—RO-6TBR.

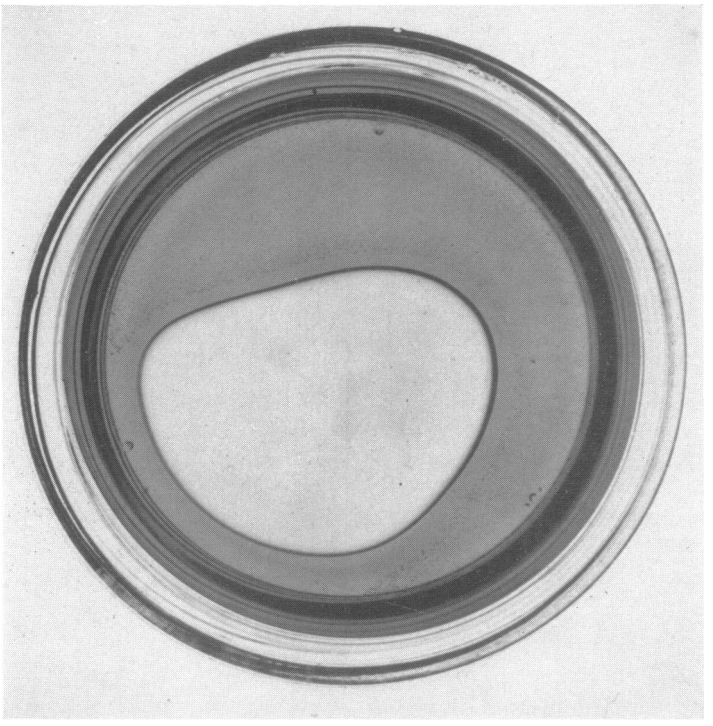

Figure 4. Strict anaerobe showing growth only in anaerobic zone. Rumen organism—RO-L_2.

whereas facultative aerobes will grow heaviest around the air bubble and the outermost fringe of the plate (figure 2), but may extend into the anaerobic zone. Facultative anaerobes will grow in both the anaerobic and aerobic zones, but growth will be heaviest in the anaerobic zone (figure 3). Strict anaerobes will grow only in the anaerobic zone and no closer than 2 to 5 mm to the aerobic zone (figure 4). Individual colonies can then be picked from the desired zone after growth, usually in 24 hours or less.

An oxygen tension gradient is thus established with both a positive and negative oxidation-reduction potential being maintained on the same plate. Each zone permits only the growth of those organisms with a particular oxidation-reduction potential requirement for the initiation of growth. True surface cultivation of anaerobes is not possible by this method, nor is it even desirable. The lifting of the top of the Brewer dish causes a certain maceration of surface colonies with subsequent difficulties in purification. There is no disturbance of the colonies when they are covered with agar. One precaution is necessary when plating bacteria by this method. If no aerobes are present in the culture to use up the oxygen in the air bubble, the cysteine is gradually oxidized to cystine around the bubble. The insoluble crystals of cystine may then slowly grow larger and resemble colonies. These "pseudo-colonies" of cystine crystals may be recognized readily as such under the low power of a microscope and even visually after a certain amount of experience. The "pseudo-colonies" are hard when picked and do not break apart like bacterial colonies.

This technique can also be used for quantitative counts of the bacteria present in a sample by making the desired dilutions in tubes of melted agar containing 0.1 per cent cysteine or in dilution blanks which can then be treated as the foregoing inoculum. Estimates can be made of the relative numbers of aerobes and anaerobes in the samples by observing the density and difference in size of the colonies in the different zones.

DISCUSSION

The old technique for culturing rumen organisms was not completely satisfactory. The Eh, pH, and salt concentration of the diluting fluid were not ideal for the survival of rumen organisms. Studies of the natural buffers of the rumen contents indicated that a combination of $NaHCO_3$, K_2HPO_4, and KH_2PO_4 would give a buffering action similar to the rumen. The addition of cysteine hydrochloride maintained the Eh at -200 mv, and the concentration of salts used is close to physiological saline. This new diluting fluid has lengthened the survival time of the delicate rumen organisms from a few minutes to about three hours. This will greatly facilitate culturing in the field, since it will be possible to obtain samples from several animals in the pasture before returning to the laboratory to culture the rumen contents.

The addition of the cysteine-bicarbonate solution to the sealed broth before autoclaving maintains a suitable Eh and pH for several weeks. The lowered concentration of glucose limits the amount of acid and other possible toxic substances produced by the bacteria by limiting bacterial growth. This has resulted

in the appearance of more of the delicate rumen bacteria in mixed cultures in the lower dilutions.

The new procedure using Brewer anaerobic petri plates allows rapid purification of mixed cultures since it eliminates the use of agar shakes which must be broken in order to pick isolated colonies. Isolated colonies can be picked easily from the Brewer plates, and even the more delicate types of rumen organisms will grow under these conditions.

The new technique for growing rumen bacteria has greatly improved the culturability of rumen organisms, especially the delicate types frequently found in samples of rumen contents from adult animals fed largely on roughage. Table 1 shows that the culturability of the bacteria from rumen samples obtained from

TABLE 1

Comparison of culturability of rumen bacteria and occurrence of curved rod types in rumen contents of 20 4-to-6-month-old calves cultured by the old and new techniques

	NO. OF ANIMALS	NO. OF ANIMALS SHOWING GROWTH IN DILUTION					NO. OF ANIMALS FROM WHICH CURVED RODS WERE ISOLATED
		10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	
Old techniques	10	10	8	1	1	0	3
New techniques	10	10	10	10	8	5	9

TABLE 2

Comparison of the culturability of rumen bacteria from the rumen contents of 23 adult cattle on pasture by the old and new techniques

	NO. OF ANIMALS	NO. OF ANIMALS SHOWING GROWTH IN DILUTION				
		10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}
Old techniques	11	11	11	8	1	0
New techniques	12	12	12	8	6	5

calves of comparable age fed a ration of hay and grain was increased, and that more of the delicate curved rods were isolated when the new technique was used. The results in table 2 show that the culturability of the bacteria in rumen contents from adult cattle was increased by the new technique.

SUMMARY

An improved technique has been devised for the isolation of rumen bacteria from high dilutions of rumen contents. This procedure is designed to create a suitable pH and Eh in both the dilution blanks and the broth, and to control the production of acid by the more rapidly growing organisms. Culturability of rumen bacteria is increased, and the growth of several delicate anaerobes typical of the adult roughage-fed ruminant is encouraged by this method.

An anaerobic plating technique for the purification of mixed cultures is described. Quantitative estimates of the bacteria present in a sample can be obtained using this method.

REFERENCES

BREWER, J. H. 1942 A new petri dish cover and technique for use in the cultivation of anaerobes and microaerophiles. Science, **95**, 587.

GALL, L. S., STARK, C. N., AND LOOSLI, J. K. 1947 The isolation and preliminary study of some physiological characteristics of the predominating flora from the rumen of cattle and sheep. J. Dairy Sci., **30**, 891–899.

GALL, L. S., BURROUGHS, W., GERLAUGH, P., AND EDGINGTON, B. H. 1949 Special methods for rumen bacterial studies in the field. J. Animal Sci., **8**, 433–440.

HUNGATE, R. E. 1947 Studies on cellulose fermentation. III. The culture and isolation of cellulose-decomposing bacteria from the rumen of cattle. J. Bact., **53**, 631–645.

SIJPESTEIJN, A. K. 1948 Cellulose-decomposing bacteria from the rumen of cattle. Thesis, University of Leiden, Holland.