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Basis for the Exclusion of *Escherichia coli* from the Rumen Ecosystem

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ABSTRACT

HOLLOWELL, C. A. (University of Illinois, Urbana), AND M. J. WOLIN. Basis for the exclusion of *Escherichia coli* from the rumen ecosystem. Appl. Microbiol. **13**:918-924. 1965.—The possible causes for the exclusion of *Escherichia coli* from the rumen ecosystem were investigated. Evidence presented indicates that oxidation-reduction potential, temperature, *p*H, CO_2, volatile fatty acids, traces of heavy metals, and bacteriophage are not environmental factors which select against *E. coli* in the rumen ecosystem. The feeding of nutrients utilizable by *E. coli* to an artificially inoculated continuous-culture rumen ecosystem did not prevent the washout of the *E. coli* from the continuous cultures. The presence of an inhibitor of *E. coli* growth was demonstrated. High concentrations of rumen fluid (50 to 100%) were necessary to inhibit growth in Antibiotic Medium 3. The inhibitor may operate to control the growth of *E. coli* in the rumen ecosystem. Because of the presence of an inhibitor, the possibility that rumen fluid is deficient in nutrients to support the growth of *E. coli* cannot be eliminated.

Food ingested by the ruminant is subjected to fermentation by a unique microbial population. The many genera and species of bacteria found in the rumen are mainly nonsporeforming anaerobes (Hungate, 1957). Many species are not known to be present in other natural environments. Some genera and species found in other environments, whose known physiological characteristics would not apparently eliminate them as possible components of the rumen ecosystem, are not found in the rumen.

Some of the characteristics of the rumen environment which are responsible for the exclusion of certain microorganisms from the rumen are obvious. Obligate aerobes, organisms incapable of growing at 39 C (the temperature of the rumen), and organisms incapable of growing at *p*H 5.8 to 6.8 (the *p*H range of rumen ingesta) do not grow in the rumen. Rumen bacteria generally have simple nutritional requirements (Bryant, 1959), which suggests that bacteria with complex nutrient requirements may be selected against by the rumen environment. However, many organisms which could conceivably grow in the rumen, on the basis of their known physiology, do not; thus, unknown factors must operate to select against these organisms.

This investigation attempted to obtain information about control principles in the rumen environment by studying the factors which control the development of an unimportant member of the rumen population, *Escherichia coli*. Although the approach was restricted to the use of this single organism, it was thought that an analysis of possible control mechanisms affecting *E. coli* might provide useful information pertaining to the general question of population control in the rumen ecosystem.

E. coli, although found in the rumen, is not usually found in high numbers. One thousand to one million viable *E. coli* cells per milliliter were reported by Mann, Masson, and Oxford (1954), out of a total viable population of 10^9 to 10^{10} bacteria per milliliter usually found in the rumen (Hungate, 1960). Admittedly, the characterization of a particular rumen microbe as "important" is somewhat subjective. "Important" usually refers to an organism which is present in high concentrations in rumen ingesta and which plays a role in the overall fermentation of feed materials.

Possible explanations for the lack of development of *E. coli* in the rumen include: (i) lack of introduction of a large enough inoculum for establishment in the rumen; (ii) inability to obtain nutrients for growth from the rumen fluid or from the feed; (iii) inhibitors in the rumen system such as antibiotics, fermentation products, or

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carbon dioxide; (iv) lysis by bacteriophage; and unfavorable (v) temperature, (vi) *p*H, or (vii) oxidation-reduction potential.

It is possible to eliminate temperature, *p*H, and oxidation-reduction potential as likely causes for the absence of large numbers of *E. coli* from the rumen on the basis of known characteristics of *E. coli*. *E. coli* grows well at 39 C and at a *p*H range of 6.0 to 7.0, and is a facultative organism capable of growth under aerobic or anaerobic conditions. The inability of *E. coli* to obtain nutrients for growth is, perhaps, the most likely explanation for low *E. coli* concentrations in the rumen. *E. coli* does not ferment starch or cellulose, and the rumen polysaccharide-fermenting microorganisms may not produce significant levels of free sugars to feed other microorganisms. There may be some free sugars in feed materials, however, which *E. coli* could use. Aside from the carbohydrate energy source, the rumen environment should not be a nutritionally unfavorable environment for *E. coli*. *E. coli* can use NH_3 as a nitrogen source and does not require vitamins.

The experiments described in this report were designed to test various possible mechanisms for control of the *E. coli* population in the rumen. In some experiments, advantage was taken of a method developed for the maintenance of the rumen ecosystem in continuous culture in vitro (Rufener, Nelson, and Wolin, 1963; Slyter, Nelson, and Wolin, 1964). The continuous culture permitted studies of the fate of *E. coli* in the simulated rumen environment under carefully controlled conditions. The results show that, of the possible mechanisms for control of the *E. coli* population in the rumen, the presence in rumen fluid of an inhibitor of *E. coli* growth is the most probable explanation for the inability of *E. coli* to compete in the rumen ecosystem.

MATERIALS AND METHODS

Organism and culture procedure. The organism used throughout the study was *E. coli* K-12. The medium routinely used to grow *E. coli* was Antibiotic Medium 3 (Difco). When *E. coli* was used as a mass inoculum for addition to the continuous cultures, 10 ml of a 24-hr culture were inoculated in 1 liter of medium in a 2-liter Ehrlenmeyer flask. After incubation at 40 C for 24 hr, the cells were harvested by centrifugation, washed with sterile physiological saline, and suspended to a volume of 25 ml with sterile saline. The suspended cells were then added to the fermentation vessels of the continuous culture system. When *E. coli* was used for the inoculation of small amounts (10 ml) of medium, the bacteria were grown in 10 ml of Antibiotic Medium 3 for 24 hr at 40 C. One drop of the 24-hr culture was used to inoculate each tube of medium.

To determine the numbers of viable *E. coli* cells, serial dilutions were made in sterile distilled water, and the dilutions were plated on E M B Agar (Difco). Typical *E. coli* colonies were counted after 24 hr of incubation at 40 C. If the media used for *E. coli* growth were optically clear, cell growth was measured by determining the optical density of the culture in a Bausch & Lomb Spectronic-20 colorimeter at 660 mμ. Matched colorimeter tubes (18 by 150 mm) were used, and an uninoculated blank was used for each type of medium prepared.

T-2 bacteriophage was kindly provided by J. Drake of the University of Illinois.

Preparation of rumen contents. All rumen contents were collected from a fistulated cow fed a normal hay and grain diet. Rumen contents used as an inoculum for the continuous culture apparatus were placed directly into the fermentation vessels.

Rumen contents were clarified by centrifugation for use in culture media. An initial centrifugation to remove bacteria, protozoa, and other particles was performed in a Spinco model L ultracentrifuge (Beckman Instruments Inc., Spinco Division, Belmont, Calif.) at an average force of 44,330 $\times$ *g* for 30 min. The rumen fluid supernatant solution was clear, but could not be easily forced through a bacterial filter (filter size HA, 0.45 μ; Millipore Filter Corp., Bedford, Mass.) for sterilization. Filtration through the bacterial filter could be accomplished after further centrifugation of the 44,330 $\times$ *g* supernatant solution at an average force of 105,400 $\times$ *g* for 30 min. This centrifugation removed a small amount of viscous material and permitted filtration through the Millipore bacterial filter. The 105,400 $\times$ *g* supernatant solution will be referred to as filterable rumen fluid.

Continuous culture apparatus. The continuous culture apparatus used in this study was described by Slyter et al. (1964). The flow rate of the artificial saliva-water solution into the fermentation vessels was 750 ml per day. Each fermentor contained approximately 500 ml of culture fluid. The artificial saliva-water solution was identical to that described by Rufener et al. (1963). The feeding, gassing with helium to remove air, and collection of effluent were as described by Slyter et al. (1964). Inoculation of *E. coli* into the fermentors was accomplished through a side port. After inoculation, each fermentor was gassed with helium to remove air which entered during inoculation. The samples for plate counts were removed through a sampling tube. The level of the ration used to maintain the microbial population was calculated to be 50% of the calculated total digestible nutrient requirement of an equivalent ruminant (Rufener et al., 1963). Each fermentor was fed twice daily with 7.3 g of hay and 2.3 g of concentrate per feeding. The theoretical washout rate, referred to in this paper is the washout rate for a nondividing organism (growth constant = 0), was calculated from the equation: $\log X_2 = \log X_1 + (K - D)T/2.3$. X_2 is the population after time T, X_1 is the initial popu-

lation, K is the growth constant, and D is the dilution rate (Gerhardt and Bartlett, 1959).

RESULTS

Effect of feeding lactose on the E. coli population in a continuous-culture rumen ecosystem. One possible explanation for the low numbers of *E. coli* in the rumen is the nonavailability of carbohydrate substrates, because *E. coli* cannot ferment the major plant polysaccharides, starch and cellulose. The continuous-culture system was used in an experiment designed to test the possibility that feeding a carbohydrate which *E. coli* can utilize would increase the number of *E. coli* cells in the in vitro rumen ecosystem. Lactose was fed in addition to the normal feed ingredients used to maintain the in vitro ecosystem.

A control fermentor was fed the usual 50% maintenance ration, and another fermentor was fed the 50% maintenance ration plus 1 g of lactose per feeding (the initial lactose concentration in the fermentor was 0.2%, w/v). Each fermentor was fed twice daily at 12-hr intervals. Samples (1 ml) were taken directly from each fermentor at various time intervals, serially diluted, and plated on E M B Agar. The results shown in Table 1 indicate that no increase in the number of *E. coli* cells in the continuous-culture rumen ecosystem occurred due to lactose feeding. In fact, the number of *E. coli* cells in the lactose-fed fermentors dropped from 1,000 per milliliter to 100 to 300 per milliliter after lactose was fed, whereas the control showed a range of *E. coli* cells of 900 to 6,000 per milliliter during the 30.5-hr experiment. These results also show the magnitude of the *E. coli* population in the continuous culture system, ranging from a low of 100 cells per milliliter in the lactose-fed fermentor to 6,000 per milliliter in the control fermentor.

TABLE 1. *Effect of lactose feeding on the Escherichia coli population in the continuous cultures*

Hr of continuous culture*	Cells per ml × 10^{-2}	
	50% maintenance ration	50% maintenance ration + lactose
0	9	10
1.5	90	1
3.5	10	3
12.5†	60	1
24.0	60	1
25.5	10	3
30.5	10	1

* Feed was introduced into the fermentors at 0.5, 12.5, and 24.5 hr.

† Sampled immediately before feeding.

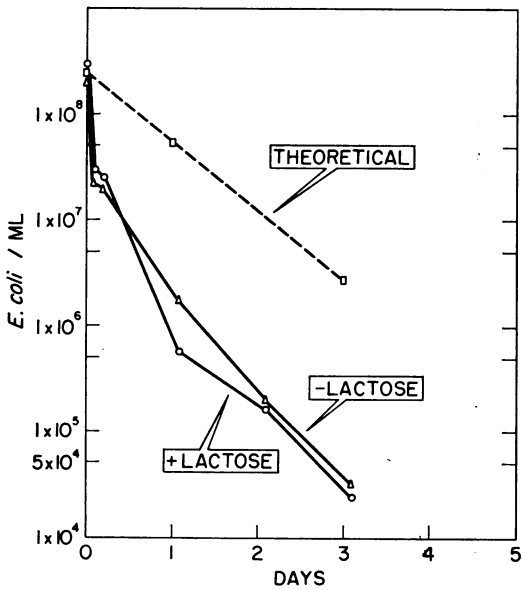

FIG. 1. *Washout of an artificially inoculated population of Escherichia coli from a continuous culture ecosystem fed lactose. The control fermentor was fed a 50% maintenance ration, and the other fermentor was fed the 50% maintenance ration plus 1 g of lactose per feeding. Each fermentor was fed twice daily at 12-hr intervals. The theoretical line refers to the calculated washout rate for a nondividing E. coli population.*

Washout of an artificially inoculated population of E. coli in a continuous-culture rumen ecosystem. Since the concentration of *E. coli* found in the rumen is low, it is possible that *E. coli* cannot compete for an added energy supply with microorganisms which are in high concentration in the rumen. If a large inoculum of *E. coli* was introduced into the in vitro ecosystem at a concentration as high as some of the "important" rumen species, *E. coli* might be able to compete for an added energy supply. An experiment was designed to test this possibility by use of the continuous-culture apparatus. A control fermentor was fed the usual 50% maintenance ration and another fermentor was fed the 50% maintenance ration plus 1 g of lactose per feeding. Each fermentor was fed twice daily at 12-hr intervals. Both fermentors were inoculated with a population of *E. coli* previously grown on Antibiotic Medium 3. The concentration of viable *E. coli* at the start of the experiment was 2×10^8 cells per milliliter for the control fermentor and 3×10^8 cells per milliliter for the fermentor to which lactose was to be added. The cell numbers were followed for 4 days. The results shown in Fig. 1 indicate that the introduction of a large

inoculum of *E. coli* did not permit the establishment of this bacterium in the rumen ecosystem whether or not lactose was available.

Since lactose did not influence the washout rate when *E. coli* was artificially inoculated into the continuous-culture ecosystem, an experiment was conducted to see whether other nutrients would influence the washout rate of an artificially inoculated continuous-culture system. *E. coli*, although it can grow on an energy source, inorganic nitrogen, and salts, might still have to compete for more complex nutrients to become established in the rumen ecosystem. A control fermentor was fed the usual 50% maintenance ration, and another fermentor was fed the maintenance ration plus, at each feeding; yeast extract (Difco), 1.5 g; peptone (Difco), 5.0 g; and glucose, 1 g. Each fermentor was fed twice daily at 12-hr intervals. *E. coli*, previously grown on Antibiotic Medium 3, was used to inoculate both fermentors. The results (Fig. 2) indicate that yeast extract, peptone, and glucose did not permit the establishment of the *E. coli* inoculum in the rumen ecosystem.

Anaerobiosis and temperature. The growth curve of *E. coli* was determined, by optical-density measurements, in Antibiotic Medium 3 at 40 C, in a 95% N_2-5% CO_2 atmosphere. The generation time was calculated to be approximately 20 min, which excluded the possibility that anaerobiosis and temperature were limiting *E. coli* growth in the continuous cultures.

Bacteriophage experiment. Bacteriophage which could infect and destroy *E. coli* could operate to control the level of *E. coli* in the rumen. Bacteriophage infective for *E. coli* K-12 were sought in rumen fluid. Rumen contents from a fistulated bovine were centrifuged at 14,500 × *g* for 15 min to remove bacteria, protozoa, and other particles. A 1-ml amount of the clear rumen fluid was added to 10 ml of Antibiotic Medium 3 which had been inoculated with *E. coli*. Since there was no clearing in the tube after 24 hr of incubation at 40 C, the *E. coli* cells were removed by centrifugation. A 0.5-ml amount of the clear supernatant liquid was spread over the surface of an Antibiotic Medium 3-agar (Antibiotic Medium 3 plus 2% agar) plate which had previously been inoculated with *E. coli* by spreading the inoculum over the entire surface of the plate. Duplicate plates were incubated aerobically for 24 hr at 40 C, at which time the plates were examined for plaque formation. No plaques were observed in any of the plates. A control plating was made with a phage, T2. T2 phage caused plaque formation with the plating procedure described above.

Effect of carbon dioxide on growth in Antibiotic Medium 3. Since the rumen gas contains approximately 65% CO_2, it was desirable to see whether *E. coli* could grow in a CO_2 atmosphere. Filter-sterilized $NaHCO_3$ was added to a 0.1% (w/v) final concentration to Antibiotic Medium 3-agar (Antibiotic Medium 3 plus 2% agar). The serial dilutions of an *E. coli* culture were plated in the agar medium. The plates were incubated at 40 C for 24 hr under an atmosphere of 100% carbon dioxide. As a control, Antibiotic Medium 3 agar plates without $NaHCO_3$ were inoculated with *E. coli* and incubated under 100% N_2 for 24 hr at 40 C. The results were as follows: Antibiotic Medium 3, 2.8 × 10^8 cells per milliliter; Antibiotic Medium 3 plus $NaHCO_3$ plus 100% CO_2 atmosphere, 2.6 × 10^8 cells per milliliter. These results show that single cells of *E. coli* are able to form colonies under 100% carbon dioxide as well as under 100% N_2. The results do not indicate whether the growth rate is lower in the 100% CO_2 atmosphere. Qualitative observations, however, have never indicated a large difference in growth rate of *E. coli* in N_2 or 100% CO_2 atmospheres.

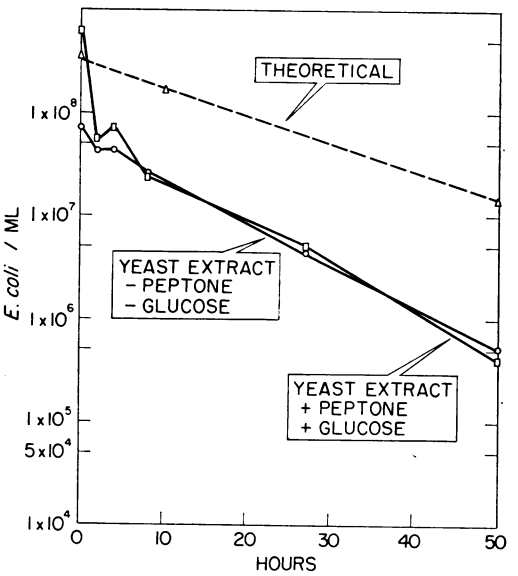

FIG. 2. *Washout of an artificially inoculated population from a continuous-culture ecosystem fed yeast extract, peptone, and glucose. A control fermentor was fed the usual 50% maintenance ration, and the other fermentor was fed the maintenance ration plus (per feeding): yeast extract, 1.5 g; peptone, 5.0 g; and glucose, 1 g. Feeding times and the theoretical line were as indicated in Fig. 1.*

Examination of rumen fluid for inhibitors. Filter-pad assays for inhibitors were performed by saturating sterile filter pads with the supernatant fluid obtained by centrifugation of rumen

contents at 14,500 × *g* for 15 min, and incubating the pads on Antibiotic Medium 3 which had been seeded with *E. coli*. No clear inhibition zones were detected. In another experiment, rumen contents were filtered through cheesecloth, and the filtrate was autoclaved at 121 C at 15 psi for 15 min. The sterile, autoclaved suspension was inoculated with *E. coli* and incubated for 24 hr at 40 C. Plate counts at the end of the incubation period showed that the final cell concentration was almost identical with that obtained in similarly treated Antibiotic Medium 3 (approximately 5.5×10^8 cells per milliliter). Thus, the filter-pad assay and the autoclaved rumen ingesta experiments gave no indication of an inhibitory material in rumen fluid.

An experiment was conducted to determine the ability of *E. coli* to grow in filter-sterilized rumen fluid, and whether the rumen fluid would inhibit growth of *E. coli* in Antibiotic Medium 3. One series of tubes was incubated for 24 hr at 40 C either in a 95% N_2 plus 5% CO_2 atmosphere or in a 100% CO_2 atmosphere. The results presented in Table 2 show that *E. coli* is unable to grow in filter-sterilized rumen fluid. When the components of Antibiotic Medium 3 are added to filter-sterilized rumen fluid, there is only slight growth. These results show the presence of an inhibitor in filter-sterilized rumen fluid. The smaller amount of growth under 100% CO_2 is probably due to the lower *p*H of the media due to the high CO_2 concentration.

Titration of inhibitor in filter-sterilized rumen fluid. The effect of varying the concentration of filter-sterilized rumen fluid in Antibiotic Medium 3 on the growth of *E. coli* was tested. The media containing 10 to 50% filter-sterilized rumen fluid were prepared by adding 5 ml of filter-sterilized, double-strength Antibiotic Medium 3 to matched colorimeter tubes. Various amounts of filter-sterilized rumen fluid ranging from 1 to 5 ml were added, and the volume in each tube was brought to 10 ml with sterile distilled water. To prepare 60 and 80% clarified rumen fluid media, 1.76 g of Antibiotic Medium 3 was added to 60 and 80 ml of clarified rumen fluid, the volume was brought to 100 ml with distilled water, and each solution was filter-sterilized. All tubes were incubated for 24 hr at 40 C under an atmosphere of 95% N_2 plus 5% CO_2. The results of this experiment (Table 3) show that the inhibitory substance in the rumen fluid was in low concentration in rumen fluid, or that a large amount of inhibitor was necessary to inhibit *E. coli* growth.

TABLE 2. *Inhibition by rumen fluid of Escherichia coli growth*

Medium*	Growth after 24 hr†	
	95% N_2-5% CO_2‡	100% CO_2‡
Antibiotic medium 3	0.68	0.43
100% filterable rumen fluid	0.00	0.00
100% filterable rumen fluid plus 100% Antibiotic Medium 3‡	0.16	0.09

* All media were filter-sterilized.
† Expressed as optical density at 660 mμ.
‡ Gas atmosphere for growth.

TABLE 3. *Titration of the Escherichia coli growth inhibitor in rumen fluid*

Amt of rumen fluid added to medium*	Growth after 24 hr†	Growth inhibition
% (v/v)		%
0	0.48	0
10	0.47	2
20	0.34	29
30	0.32	33
40	0.37	23
50	0.30	38
60	0.22	54
80	0.19	60

* Basal medium was Antibiotic Medium 3.
† Expressed as optical density at 660 mμ.

Effect of autoclaving on the inhibitor in filterable rumen fluid. The purpose of this experiment was to investigate the effects of autoclaving on the inhibitory material in filterable rumen fluid. Two 50-ml portions of filterable rumen fluid were autoclaved for 15 min at 121 C at 15 psi. One 50-ml portion contained 0.88 g of Antibiotic Medium 3 and othe other contained only rumen fluid. Another 100-ml portion of filterable rumen fluid was autoclaved, and the small amount of precipitate formed during autoclaving was removed by centrifugation at 15,900 × *g* for 10 min. The clear supernatant solution was separated into two 50-ml portions. To one portion, 0.88 g of Antibiotic Medium 3 was added and the solution was filter-sterilized. The other 50-ml portion was also filter-sterilized. The unautoclaved, filterable rumen fluid used was sterilized by filtration. One portion had 0.88 g of Antibiotic Medium 3 added before filtration. The tubes of media were inoculated and incubated for 24 hr at 40 C in an atmosphere of 95% N_2 plus 5% CO_2. The results in Table 4 indicate that the inhibitor may be partially destroyed by autoclaving filterable rumen fluid for 15 min at 121 C under 15 psi.

Effect of volatile fatty acids on the growth of E. coli in Antibiotic Medium 3. Since short-chain volatile fatty acids are in high concentration in

the rumen (Phillipson, 1942), the possibility of their inhibiting *E. coli* growth was investigated. Salts of the C_2-C_5 acids were added to the Antibiotic Medium 3 to give the following final concentrations: acetate, 60 mM; propionate, 20 mM; butyrate, 15 mM; valerate, 3 mM; and isovalerate, 2 mM. These molar quantities approximate the molar quantities found in the rumen of a ruminant on a hay-grain diet (Annison and Lewis, 1959). After inoculation with *E. coli*, the tubes were incubated for 24 hr at 40 C under a 95% N_2 plus 5% CO_2 atmosphere. No growth inhibition was observed when the fatty acids were added to the medium.

Effect of addition of ethylenediaminetetraacetic acid (EDTA) to filterable rumen fluid on the growth of E. coli. Jones (1964) reported on the inability of *E. coli* to grow in another natural system, seawater, and examined the possibility that iodate and heavy metals cause the inhibition. Iodate was shown to stimulate *E. coli* growth. The addition of EDTA, or other chelating agents, to seawater caused a marked increase in growth. These results indicated that the heavy metals were inhibiting *E. coli* growth in the seawater, and the binding of the heavy metals by the chelating agents reversed the inhibition.

TABLE 4. *Partial destruction of rumen fluid inhibitor by autoclaving*

Medium	Growth after 24 hr*
Antibiotic medium 3	0.62
FRF†	0.00
Antibiotic Medium 3 + FRF	0.18
Autoclaved FRF	0.00
Antibiotic Medium 3 + autoclaved FRF	0.35
Autoclaved, centrifuged FRF	0.00
Antibiotic medium 3 + autoclaved, centrifuged FRF	0.34

* Expressed as optical density at 660 mμ.
† FRF = filterable rumen fluid.

TABLE 5. *Inability of EDTA to reverse rumen fluid inhibition*

Medium	Growth after 24 hr*
AM3†	0.52
AM3 + 100% FRF‡	0.15
AM3 + 10^{-4} M EDTA	0.49
AM3 + 100% FRF + 10^{-4} M EDTA	0.16

* Expressed as optical density at 660 mμ.
† AM3 = Antibiotic Medium 3.
‡ FRF = filterable rumen fluid.

The possibility of the inhibition of *E. coli* growth by heavy metals in rumen fluid was examined. EDTA (10^{-4} M) was added to Antibiotic Medium 3 plus 100% filterable rumen fluid (filter-sterilized); the medium was inoculated and was incubated for 24 hr at 40 C under an atmosphere of 95% N_2 plus 5% CO_2. After 24 hr, the optical density was determined. To make certain that EDTA was not toxic, it was added in the same concentration to Antibiotic Medium 3 without added rumen fluid. The results (Table 5) show that EDTA is not toxic to *E. coli* in Antibiotic Medium 3 and that inhibition of *E. coli* by clarified rumen fluid cannot be reversed by the addition of 10^{-4} M EDTA.

DISCUSSION

The experimental results provide evidence against the possibility that *p*H, oxidation-reduction potential, CO_2, volatile fatty acids, bacteriophage, and temperature are environmental factors which select against the establishment of *E. coli* in the rumen ecosystem. Feeding of nutrients did not affect the rate of washout of *E. coli* from the continuous culture ecosystems even if artificially large inocula of *E. coli* were introduced into the continuous cultures. The results of the washout experiment argue against the possibility that nutrient limitation per se is a primary reason for the inability of *E. coli* to compete in the ecosystem. The major reason for the inability of *E. coli* to become established in the rumen would appear to be the inhibitory action of rumen fluid on the growth of *E. coli* as demonstrated in the reported experiments.

The presence of an inhibitor of *E. coli* in rumen fluid makes it difficult to interpret the results of the continuous-culture experiments on nutrient limitation. Although nutrient limitation is ruled out as a likely primary cause of the inability of *E. coli* to develop in the ecosystem, the nutrient inadequacy of rumen fluid for *E. coli* growth is not ruled out. Addition of nutrients to the continuous cultures would not be expected to stimulate *E. coli* growth if a growth inhibitor is also present.

In the continuous culture experiments, the washout rate of *E. coli* was greater than expected for a nondividing organism. The turnover of the continuous cultures is slow in accordance with the probable in vivo turnover of the rumen system (Rufener et al., 1963). In order for an organism to maintain a steady state in the continuous cultures, only a very slow growth rate is required. A generation time of about 11 hr is sufficient to maintain a steady state. The results, however, indicate that no division of *E. coli* is achieved. The washout pattern actually indicates that the

continuous-culture medium is bactericidal for *E. coli*. It is not known, however, how closely the actual washout rate of a nondividing organism, unaffected by the culture medium, would approach the theoretical values calculated.

The nature of the filterable rumen fluid inhibitor is not known. It is either in low concentration in the rumen fluid or large amounts are required for inhibiting *E. coli* growth. Preliminary experiments have indicated a partial instability to autoclaving at 15 psi at 121 C for 15 min (Table 5). The inhibitory activity was found in a precipitate prepared by mixing 1 volume of clarified rumen fluid with 9 volumes of 95% ethyl alcohol. A precipitate formed by adding $(NH_4)_2SO_4$ to rumen fluid to 90% saturation did not have any inhibitory activity. The short-chain volatile fatty acids of rumen fluid have been excluded as the possible cause of inhibition. A similarity between rumen fluid inhibition of *E. coli* and the inhibition of *E. coli* by seawater has been ruled out. The addition of EDTA to Antibiotic Medium 3 plus 100% rumen fluid did not overcome the inhibitory action of the rumen fluid, whereas EDTA does overcome the inhibition of seawater (Jones, 1964).

It is of interest to note that the autoclaving of rumen fluid containing the microorganisms of the rumen (unclarified) leads to the formation of an uninhibitory medium which supports good growth of *E. coli*. This cannot be caused solely by the destruction of the inhibitory substance, because autoclaved microbe-free rumen fluid does not support *E. coli* growth (Table 3). The autoclaved microorganisms could supply a missing nutrient or an inhibitor antagonist which overcomes the activity of a partially destroyed inhibitor.

ACKNOWLEDGMENT

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