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Volatile Fatty Acids and the Inhibition of *Escherichia coli* Growth by Rumen Fluid[1]

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Concentrations of volatile fatty acids (VFA) normally found in bovine rumen fluid inhibited growth of *Escherichia coli* in Antibiotic Medium 3. Acetic, propionic, and butyric acids each produced growth inhibition which was markedly *p*H-dependent. Little inhibition was observed at *p*H 7.0, and inhibition increased with decreasing *p*H. A combination of 60 μmoles of acetate, 20 μmoles of propionate, and 15 μmoles of butyrate per ml gave 96, 69, and 2% inhibition at *p*H 6.0, 6.5, and 7.0, respectively. Rumen fluid (50%) gave 89 and 48% inhibition at *p*H 6.0 and 6.5, respectively, and growth stimulation (22%) at *p*H 7.0. Rumen fluid inhibitory activity was heat-stable, was not precipitated by 63% ethyl alcohol, and was lost by dialysis and by treatment with anion-exchange resins but not with cation-exchange resins. These results are consistent with the idea that VFA are the inhibitory substances in rumen fluid. Previous results which indicated that rumen fluid VFA did not inhibit *E. coli* growth were due to lack of careful control of the final *p*H of the growth medium. The *E. coli* strain used does not grow in rumen fluid alone at *p*H 7.0.

We reported that *Escherichia coli* K-12 cannot grow in an in vitro continuous culture of mixed rumen microorganisms (3). This was true even if the continuous culture was inoculated with high levels of *E. coli* and supplied with nutrients that ordinarily support the growth of *E. coli*. These results suggested that a growth inhibitor is present in the rumen environment. Further experiments showed that clarified rumen contents of the bovine do not support the growth of *E. coli*, and, in addition, inhibit growth when added to Antibiotic Medium 3 (Difco). We immediately suspected that volatile fatty acids, which normally accumulate in high concentrations in rumen contents, could possibly inhibit *E. coli* growth. When we added these acids to Antibiotic Medium 3, however, we found no growth inhibition (3). Further study showed that this result was due to our ignorance of the critical effect of *p*H on the ability of volatile fatty acids to inhibit *E. coli*. The volatile fatty acids do not inhibit growth at *p*H 7. They markedly inhibit *E. coli* if the growth medium is adjusted to *p*H 6. This report is concerned with some characteristics of the volatile fatty acid inhibition of *E. coli* along with evidence which strongly suggests that the inhibitor present in rumen contents is the volatile fatty acid fraction.

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MATERIALS AND METHODS

Organism and culture procedures. *E. coli* K-12 was routinely transferred in Antibiotic Medium 3. Growth inhibition experiments were carried out by adding the test material to double-strength Antibiotic Medium 3 with final adjustment of volumes to give single-strength Antibiotic Medium 3. All culture medium components were sterilized by autoclaving, except for rumen fluid or rumen fluid fractions which were sterilized by filtration through a membrane filter (HA; Millipore Corp., Bedford, Mass.) and aseptically added to the medium. Unless stated otherwise, growth experiments were carried out with a total of 5 ml of medium in a culture tube (18 × 120 mm). Inoculations were made with one drop of a 24-hr culture grown in Antibiotic Medium 3. Incubations were at 40 C in air. Growth was estimated by measuring the turbidity of cultures at 660 nm with a Bausch & Lomb Spectronic-20 colorimeter.

Preparation and treatment of rumen contents. Rumen contents were collected and clarified by centrifugation at 44,330 × *g* and 105,400 × *g* as previously described (1).

Alcohol fractionation was carried out with the 105,400 × *g* supernatant, i.e., filterable rumen fluid (FRF). A 100-ml amount of 95% ethyl alcohol was added to 50 ml of FRF, and the mixture was maintained at 4 C for 24 hr. The precipitate which formed was removed by centrifugation at 14,500 × *g* for 10 min. The supernatant solution was adjusted to *p*H 6.0 with NaOH and evaporated in a rotary evaporator (40 C) to approximately 10 ml to remove ethyl alcohol and to concentrate the solution. The concen-

TABLE 1. *Effect of pH on rumen fluid inhibition of E. coli*

pH	Optical density[a]		Inhibition
	No rumen fluid	Plus 50% FRF	
			%
6.0	1.40	0.16	89
6.5	1.22	0.64	48
7.0	1.10	1.32	(−22)[b]

[a] After 24 hr.
[b] Stimulation.

TABLE 2. *Alcohol-soluble fraction inhibition of E. coli*

Fraction added	Optical density[a]
None	0.86
Alcohol-soluble fraction[b]	0.08
Autoclaved alcohol-soluble fraction[b]	0.09
Dialyzed alcohol-soluble fraction[b]	0.98
Untreated FRF[b]	0.10

[a] After 24 hr.
[b] Equivalent to 50% (v/v) FRF in medium.

trated solution was brought to 25 ml with distilled water (alcohol-soluble fraction).

RESULTS

pH and rumen fluid growth inhibition. *E. coli* was grown in Antibiotic Medium 3 at *p*H 6.0, 6.5, and 7.0 with and without centrifuged rumen fluid (FRF). Growth was good at all pH values tested in the absence of FRF, but severe inhibition of growth was observed at *p*H 6.0 when FRF was added (Table 1). Moderate inhibition by FRF occurred at *p*H 6.5, and no FRF inhibition was observed at *p*H 7.0 (in fact, growth stimulation was observed). In the experiment (Table 1), all FRF samples were adjusted to the *p*H of the medium prior to filter sterilization.

Ethyl alcohol fractionation of FRF (as described in Materials and Methods) gave a soluble fraction which showed the same growth inhibition as FRF itself, but was much more easily filter-sterilized than FRF. The inhibition of *E. coli* by the alcohol-soluble fraction is presented in Table 2. Inhibition is equivalent to that obtained with FRF. The inhibitor in the alcohol-soluble fraction is heat-stable (Table 2). Heating was carried out in an autoclave at 120 C for 10 min. Dialysis of the alcohol-soluble fraction (20 ml dialyzed against 2 liters of distilled water for 24 hr at 4 C) resulted in a complete loss of inhibitory activity (Table 2).

The amount of alcohol-soluble fraction necessary to inhibit growth was determined. Figure 1 shows the growth of *E. coli* in Antibiotic Medium 3 at *p*H 6.0 with increasing amounts of the alcohol-soluble fraction. The equivalent of 5% of FRF gave 50% growth inhibition, but a less than linear increase in inhibition was observed with increasing concentrations of the alcohol-soluble fraction.

Volatile fatty acid inhibition of E. coli. Acetic acid inhibited *E. coli* when added to Antibiotic Medium 3 (Table 3). The inhibition exhibited a *p*H dependence which was similar to that found with FRF. No inhibition was obtained at *p*H 7.0, and 80% growth inhibition was obtained at *p*H 6.0. The concentration of acetic acid used in the experiment (Table 3) was slightly below the concentration usually found in bovine rumen contents (4).

The other principal volatile fatty acids in rumen contents also inhibited *E. coli* growth in Antibiotic Medium 3 when the medium was adjusted to *p*H 6.0. Inhibition by acetic, propionic, butyric, isobutyric, valeric, or isovaleric acid is shown in Table 4. Although careful titrations of inhibition versus acid concentration were not carried out,

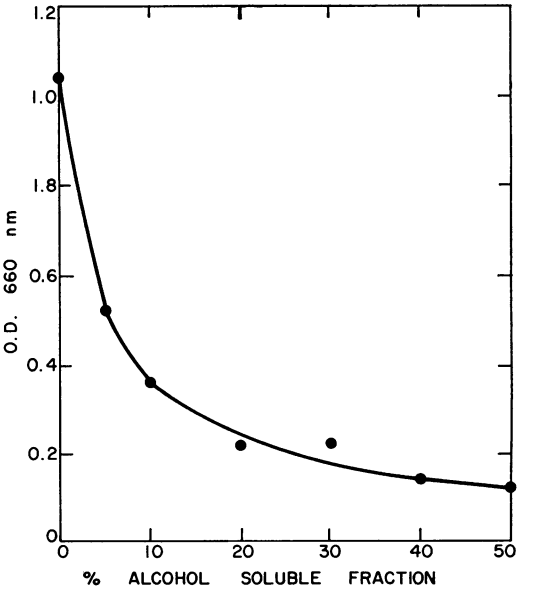

FIG. 1. *Concentration of alcohol-soluble fraction and inhibition of E. coli growth. The alcohol-soluble fraction of rumen fluid was prepared as described in the text and added as indicated to Antibiotic Medium 3. Optical density was measured after 24 hr. Alcohol-soluble fraction concentrations are expressed in terms of the unfractionated rumen fluid equivalent.*

TABLE 3. *Acetic acid inhibition of E. coli*

pH	Optical density[a]		Inhibition
	No acetic acid	Plus 45 mM acetic acid	
			%
6.0	1.10	0.22	80
6.5	0.98	0.58	41
7.0	1.10	1.16	0

[a] After 24 hr.

TABLE 4. *Inhibition of E. coli by volatile fatty acids*

Acid addition[a]	Growth inhibition[b]
	%
Acetic (15 mM)	67
Propionic (10 mM)	57
Butyric (11 mM)	74
Valeric (10 mM)	79
Isobutyric (20 mM)	65
Isovaleric (20 mM)	77

[a] Addition to Antibiotic Medium 3 at *p*H 6.0.

[b] Optical density used to estimate growth after 24 hr; expressed as the percentage of decrease in optical density from the control without acid addition.

preliminary titrations indicated that a nonlinear increase in inhibition was produced by increasing acid concentrations similar to the inhibition response obtained with increasing concentrations of the alcohol-soluble fraction of rumen fluid.

A combination of acetic, propionic, and butyric acids, in concentrations usually found in bovine rumen contents, was tested for inhibition of *E. coli* growth at various *p*H values. The results in Table 5 show that significant inhibition of growth occurred at *p*H 6.5 and 6.0 with little inhibition observed at *p*H 7.0.

Effect of ion-exchange resins on the rumen fluid inhibitor. The *p*H-dependent inhibition of growth by rumen fluid and the alcohol-soluble fraction derived from it, and the *p*H-dependent inhibition by volatile fatty acids known to be present in rumen fluid, strongly suggested that volatile fatty acids were responsible for the inhibition of *E. coli* growth by rumen fluid. It would be expected that treatment of rumen fluid with an anion-exchange resin at *p*H 7.0 would remove the fatty acid anions and result in a loss of the inhibitory activity of rumen fluid. Treatment of rumen fluid with a cation-exchange resin should not remove the inhibitor.

To test the effects of ion-exchange resins, 20 ml of the alcohol-soluble fraction was passed through 10 ml of packed Dowex 50 W-X4 (H^+). A separate 20 ml of the alcohol-soluble fraction was passed through Dowex 1-X8 (Cl^-). The columns were washed with 5 ml of distilled water, and the washes were combined with their respective column eluates. The *p*H of the eluates plus washes was brought to 7.0, and the solutions were evaporated in a rotary evaporator, at 40 C, to approximately 5 ml. The solutions were then adjusted to *p*H 6.0, brought to 10 ml with distilled water, filter-sterilized, and tested for their inhibitory activity. The anion exchanger completely removed the inhibitor from the alcohol-soluble fraction, whereas the cation exchanger did not remove the inhibitor (Table 6). Addition of acetate at *p*H 6.0 to the anion-exchange-treated material restored inhibition.

Will E. coli grow in rumen fluid? *E. coli* is inhibited by rumen fluid and volatile fatty acids only if the *p*H of Antibotic Medium 3 is adjusted to a *p*H below 7.0. If rumen fluid contains all of the ingredients essential for *E. coli* growth, it might be expected that rumen fluid would support growth if it was inoculated at *p*H 7.0. We tested a heated FRF preparation to see if it would support

TABLE 5. *Inhibition of E. coli by a combination of volatile fatty acids*

pH	Optical density[a]		Inhibition
	No fatty acids	Plus fatty acids[b]	
			%
6.0	0.90	0.04	96
6.5	1.18	0.36	69
7.0	1.10	1.08	2

[a] After 24 hr.

[b] Acetate, 60 mM; propionate, 20 mM; and 15 mM of butyrate in Antibiotic Medium 3.

TABLE 6. *Anionic character of rumen fluid inhibitor*

Addition	Optical density[a]	
	Without acetate	With acetate (30 mM)
None	1.20	0.17
Alcohol-soluble fraction[b]	0.17	
Dowex-1 (Cl^-) treated alcohol-soluble fraction[b]	1.12	0.23
Dowex-50 (H^+) treated alcohol-soluble fraction[b]	0.17	0.07
Dowex-1 (Cl^-) + Dowex-50 (H^+) alcohol-soluble fraction[b]	0.14	

[a] After 24 hr.

[b] Final concentration equivalent to 50% (v/v) of FRF.

TABLE 7. *Lack of E. coli growth in a neutralized rumen fluid fraction*

Medium	Initial *p*H	Optical density[a]
AM3[b]	6.0	1.06
AM3[b]	7.0	0.88
AM3 + 50% heated FRF[c]	6.0	0.26
AM3 + 50% heated FRF[c]	7.0	1.16
FRF[c] (100% heated)	6.0	0.04
FRF[c] (100% heated)	7.0	0.04

[a] After 24 hr.
[b] AM3, Antibiotic Medium 3.
[c] FRF, filterable rumen fluid.

TABLE 8. *Growth of E. coli in rumen fluid plus additions*

Additions to heated FRF[a]	Optical density[b]
None	0.06
Glucose	0.13
Phosphate	0.07
Glucose, phosphate	0.24
Glucose, phosphate, yeast extract, tryptone	0.72

[a] All media at *p*H 7.2. Glucose (0.2%), yeast extract (0.1%), tryptone (0.5%), and KH_2PO_4 (0.2%) were added where indicated.
[b] After 24 hr.

growth of *E. coli* with initial *p*H adjustment to 7.0.

FRF was autoclaved at 120 C for 10 min. The small amount of precipitate which formed was removed by centrifugation at 14,500 × *g* for 5 min. The supernatant solution was adjusted to *p*H 6.0 and 7.0 with HCl and filter-sterilized. Portions were used for testing for growth inhibition by adding 5 ml to 5 ml of sterile, double-strength Antibiotic Medium 3. Portions (10 ml) of the sterilized rumen fluid samples, with no other additions, were inoculated with *E. coli*. The results of the experiment (Table 7) clearly show that the *p*H 7.0 rumen fluid sample did not inhibit *E. coli*, but also did not support significant growth.

Addition of glucose, yeast extract (Difco), tryptone (Difco), and phosphate to the heated FRF permitted good growth at *p*H 7.0, but glucose alone or glucose plus phosphate supported only poor growth (Table 8). The final *p*H attained in the glucose, yeast extract, tryptone, and phosphate-supplemented medium was 6.1. Glucose plus phosphate supplementation also yielded a final *p*H of 6.1.

DISCUSSION

The results strongly suggest that the volatile fatty acid fraction of rumen fluid inhibits growth of *E. coli* in Antibiotic Medium 3. The *p*H dependence of inhibition by rumen fluid is the same as that found for volatile fatty acids. Heat stability, loss of inhibition by dialysis, alcohol solubility, and the anionic character of the inhibitor are characteristics that are consistent with volatile fatty acid inhibition of growth.

These results are consistent with the results of Bergeim (1) on the toxicity of butyric acid and acetic acid for yeast and *E. coli* and the more recent experiments of Meynell (5) on volatile fatty acid toxicity for *Salmonella typhimurium*. Bergeim showed that the volatile fatty acid fraction of water extracts of human feces was responsible for the extract inhibition and killing of yeast cells. The fractions contained about 45% butyric acid and 55% acetic acid. He then showed that volatile fatty acid toxicity for both yeast and *E. coli* was a function of the free acid concentration and that toxicity increased with increasing chain length of the acids. Free fatty acids showed both growth-inhibiting and killing actions; the killing action was more pronounced at higher free acid concentrations (lower *p*H values), and the growth-inhibiting action was more pronounced at lower free acid concentrations. Similar results have been found by Meynell (5) for *S. typhimurium*.

There would seem to be little doubt, therefore, that volatile fatty acids could play an important role in excluding *E. coli* and other volatile fatty acid-sensitive organisms from the rumen. Since the *p*H of rumen contents usually is in the range of 6.0 to 7.0, however, it is probable that toxic levels of nonionized volatile fatty acids are present only between *p*H 6.0 to 6.5. Bergeim (1) did not observe any inhibition of *E. coli* growth in an 18-hr period when 0.2 N acetate was used at *p*H 6.5, but 0.05 N acetate inhibited growth at *p*H 6.25.

We have to assume that, in our previous experiments with continuous cultures, the *p*H of the cultures was low enough to permit fatty acid toxicity in order to explain the washout of *E. coli* even when the cultures were supplemented with lactose, yeast extract, and tryptone (3). Unfortunately, we did not continuously measure *p*H, although random checks indicated a *p*H range of 6.0 to 6.5 for the continuous cultures.

Brownlie and Grau (2) showed increases of rumen salmonellae and *E. coli* with decreased and interrupted feeding of cattle. The increases usually correlated with decreased volatile fatty acid concentrations and higher *p*H values in the rumen. In

one case, however, after a single feeding after starvation, rumen salmonellae and *E. coli* decreased at *p*H values and at volatile fatty concentrations in which growth of these bacteria occurred in other experiments. They suggested that other factors may affect the growth of these organisms. The preliminary experiments on growth of *E. coli* in heated FRF at *p*H 7.0 suggest that nutrient limitation could operate to control *E. coli* growth even if *p*H in the rumen is not low enough to cause volatile fatty acid inhibition. These experiments were conducted with modified rumen fluid; the heat treatment does cause precipitation of material and the destruction of bicarbonate. It should, however, be possible to determine more precisely if, in fact, there is a nutritional deficiency in rumen fluid and what the deficiency might be. It is conceivable that proper nutrient supplementation and control of *p*H at 7.0 would permit the establishment of *E. coli* in the in vitro continuous culture rumen ecosystem.

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