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Effect of *p*H on Population and Fermentation in a Continuously Cultured Rumen Ecosystem[1]

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ABSTRACT

SLYTER, L. L. (University of Illinois, Urbana), M. P. BRYANT, AND M. J. WOLIN. Effect of *p*H on population and fermentation in a continuously cultured rumen ecosystem. Appl. Microbiol. **14**:573–578. 1966.—The effect of *p*H on rumen fermentation and microbial population was studied in a continuously cultured rumen ecosystem. A marked decrease in the production of volatile fatty acids and methane from alfalfa hay occurred when the cultures were maintained at *p*H values below 6.0. The decrease in acetate and methane production was greater than that of propionate production. The culture maintained at *p*H 6.7 contained the types of bacteria often found in high concentration in the rumen, whereas the culture maintained at *p*H 5.0 had a high percentage of bacteria which could not be identified with the major rumen bacteria found in rumens of animals fed alfalfa hay. Replacement of the bicarbonate-phosphate buffer used to maintain fermentor *p*H at 6.7 with phosphate alone did not greatly alter the fermentation products produced from a hay-concentrate substrate.

The relative proportions of individual volatile fatty acids (VFA) produced in the rumen varies with the type of diet fed. Propionic acid, for example, generally represents a greater proportion of the total VFA fraction in ruminants fed concentrates than in animals fed hay (4). The amounts and proportions of the individual VFA are important, because they affect milk fat levels (3), heat increment (1, 2), ketone body production (10, 13), and efficiency of weight gains in cattle (11).

The effect of *p*H upon fermentation by mixed rumen microorganisms in vitro during short-term experiments has been studied (Brown and Tucker, J. Dairy Sci. **45**:690, 1962). However, the effect of *p*H changes upon VFA and CH_4 production and upon the rumen microflora is not well established. The present report describes fermentation changes induced by changing the *p*H of a continuously cultured rumen microbial ecosystem. The *p*H effect upon the microbial population in terms of deoxyribonucleic acid (DNA), viable counts, and types of organisms found was also determined.

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MATERIALS AND METHODS

Experimental procedure. Rumen fluid inocula for experiments 1 and 3 were obtained from a rumen fistulated steer fed alfalfa *ad libitum* plus 5 lb of concentrate mix daily, or from a rumen fistulated lactating cow receiving a relatively high concentrate diet (24 lb of concentrate and 18 lb of alfalfa hay daily). The rumen fluid inocula, kept at about 38 C, were placed into the fermentors within 20 min after collection. The artificial rumen apparatus used in these studies was described previously (17), except an overhead mixing drive assembly was used instead of a magnetic drive mixer, and the fermentors were housed in a temperature-controlled, stainless-steel, water bath instead of a Plexiglas bath. The operational procedures concerning rate of saliva inflow, removal of effluent, exchange of gas-collection bladders, the preparation of the feedstuffs, and the addition of substrate at approximately 12-hr intervals, unless otherwise stated in the Results section, were performed as previously described (17). Cation-exchange resins were not used in these experiments. Helium or N_2 was flushed through the system to prevent air contamination during substrate addition. Helium was flushed through the fermentors when the experiments were started. Substrate was added to the continuous cultures in all cases in amounts calculated to represent 50% of the total digestible nutrient (TDN) maintenance requirement of a ruminant scaled down to the in vitro fermentor volumes (for calculation of TDN levels, see 16). The concentrate used in these experiments was the University of Illinois Herd Mix No. 50 containing 14.5% crude protein. Herd Mix

No. 50 consisted of the following (in pounds): ground, shelled, yellow corn, 1,500; ground oats, 200; soybean oil meal, 50% protein, 250; $Ca_2(PO_4)_2$, 25; and trace mineralized salt (NaCl), 25.

In the experiment concerning the effect of *p*H on the continuous culture system, a complete roughage diet (9.9 g of alfalfa hay) was fed to each of four fermentors at approximately 12-hr intervals for 19 days. The inoculum for the fermentors was obtained from the high concentrate-fed lactating cow. The fermentors were treated similarily for the first 10 days. After 10 days of continuous culture, the *p*H of the artificial saliva solution flowing into three of the fermentors was changed from 8.3 to 6.85, 6.2, and 5.7, respectively. Solutions at these *p*H values were shown by preliminary experimentation to give a final *p*H of about 6.0, 5.5, and 5.0 in the fermentors under the conditions of the experiment. A bicarbonate-phosphate saliva solution (14) and tap water in a 3:2 ratio was infused for the first 10 days to all the fermentors. Thereafter, only the control fermentor received this solution. The concentration of NaCl, KCl, $MgCl_2$, and $CaCl_2$, as well as the total molarity of the saliva solutions supplied to each fermentor, was the same throughout the experiment. After the 10th day, the solutions differed in the proportion of sodium bicarbonate to sodium phosphate infused into the fermentors. The proportion of bicarbonate decreased and the proportion of sodium phosphate increased in the artificial saliva as the *p*H of the artificial saliva infused was decreased. Fermentors receiving the lower *p*H buffers received a mixture of solutions A and B. Solutions A and B each contained 3 volumes of artificial saliva "A" and "B," respectively, and 2 volumes of tap water. Artificial saliva A differed from the control artificial saliva (14) in that 26 mM Na_2HPO_4 was replaced by 26 mM NaH_2PO_4. Artificial saliva B differed from the control artificial saliva in that 117 mM $NaHCO_3$ and 26 mM Na_2HPO_4 were replaced by 143 mM NaH_2PO_4. Artificial saliva solutions were prepared by adding solution B to A until the appropriate *p*H was obtained. Times of sample collection in experiment 2 for chemical and microbiological determinations are described in the Results section.

To study the effect of the omission of bicarbonate from the infusion solution, three fermentors, inoculated with rumen fluid, were continuously cultured for a 10-day period. The rumen fluid inoculum was from the fistulated steer. A bicarbonate-phosphate saliva solution (14), and tap water in a 3:2 ratio was infused at a dilution rate of 1.5 times the fermentor liquid volume per day for 7 days, followed by the infusion at a similar flow rate for 3 days of an all-phosphate artificial saliva solution of similar *p*H and molarity. A mixture of 7.3 g of alfalfa hay and 2.3 g of concentrate was fed every 12 hr to each fermentor.

Chemical and microbiological determinations. The daily production and concentration of VFA, ethyl alcohol, and gases and the concentration of DNA were determined as previously described (17). Lactic, succinic, and formic acid were determined by the method of Ramsey (15).

Coliform counts were determined by use of Violet Red Bile Agar (Difco). Viable-bacteria counts were determined by the anaerobic roll tube procedure of Hungate (12), as modified by Bryant and Robinson (8), by use of medium 98-5 with a 100% CO_2 gas phase; inoculation was via pipette rather than via syringe and needle. The total count was determined by averaging the counts from four roll tubes prepared at each of two concentrations (0.2 and 1 ml of 10^{-7} dilution) from each fermentor. The method of Bryant et al. (7) and Bryant, Robinson, and Lindahl (9) was used in the presumptive identification of microorganisms picked from roll tubes after 72 hr of incubation.

RESULTS

Effect of pH on DNA concentrations in the continuous cultures. Quadruplicate fermentors were run continuously for 10 days. The initial rumen fluid inoculum was from an animal fed a high-concentrate diet, but the in vitro feed source consisted entirely of alfalfa hay. The DNA concentrations in the fermentors were followed with time. On the 10th day, the buffers of the saliva-water infusion were changed in three of the fermentors to produce a total of four different *p*H environments. The *p*H patterns in the four fermentors for the 19-day period of the experiment are shown in Table 1. The fermentor DNA concentrations were followed after the buffer changes, and the DNA concentration data for the entire period of the experiment are shown in Fig. 1.

Prior to the buffer changes, the trend in the DNA concentration was toward a decrease for the first 4 days of the experiment, followed by a gradual concentration increase up to the 10th day. This pattern contrasts markedly with results obtained previously (17), in which DNA concentrations decreased for only 1 day and then increased to approximately a steady-state value by the 5th day of continuous culture. In these earlier experiments, rumen fluid inocula were obtained from animals fed a hay-concentrate diet similar to the diet used to maintain the continuous cultures in vitro.

When the buffers were changed in the present experiment, the DNA concentrations decreased with decreasing *p*H. The decrease was especially noticeable in the fermentor which received the lowest *p*H infusion solution and was maintained at a *p*H of 5.0 from the 13th to the 19th day of the experiment.

Effect of pH on the microbial population. On the 17th day of continuous culture, when *p*H changes in the fermentors had been established, samples were taken from each of the fermentors for bacteriological analysis. Table 2 shows the total viable counts and DNA values for the sampling period for all of the fermentors, and the coliform counts in the fermentors maintained at

TABLE 1. *The pH of fermentor contents before and after changing the pH of infused saliva solutions*

Days	Fermentors			
	1	2	3	4
0–10[a]	6.9 ± 0.1[b]	6.85 ± 0.1	6.9 ± 0.1	6.9 ± 0.3
11–12[c]	6.8 ± 0.15	6.4 ± 0.1	5.95 ± 0.45	5.7 ± 0.3
13–19[c]	6.7 ± 0.15	6.0 ± 0.1	5.5 ± 0.2	5.0 ± 0.15

[a] The *p*H of the artificial saliva infused into each fermentor was 8.3.
[b] The *p*H was determined on fermentor samples collected daily 12 hr postfeeding (or just prior to feeding). The value represents the average with the deviation from the average.
[c] The *p*H values of the artificial saliva solutions infused into fermentors 1, 2, 3, and 4 were 8.3, 6.9, 6.1, and 5.7, respectively.

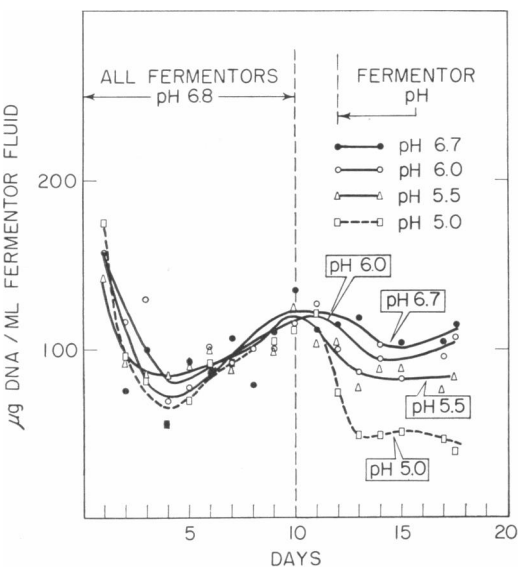

FIG. 1. *Effect of pH on DNA concentration in fermentors sampled 12 hr after feeding. DNA values are the mean of two samples. The average flow rate was 777, 745, 766, and 746 ml per day in fermentors at pH 6.7, 6.0, 5.5, and 5.0, respectively.*

*p*H 6.7 and 5.0. It was surprising to find that, although the DNA of the fermentors decreased with decreasing *p*H, the total viable count tended to increase with decreasing *p*H. The coliform counts were similar in the fermentors at *p*H 6.7 and 5.0. Qualitative examination of the fermentors showed that oligotrich protozoa (*Entodinium*) were in low concentration in the *p*H 6.7 and 6.0 fermentors throughout the 19 days of continuous culture, but were only present in the *p*H 5.5 and 5.0 fermentors up to 2 days after the artificial saliva *p*H was changed. Holotrich protozoa, which were present in the original rumen fluid inoculum, disappeared from all fermentors after 4 to 5 days of continuous culture.

TABLE 2. *Counts[a] of bacteria and concentration of DNA in fermentors incubated at different hydrogen ion concentrations*

Fermentor	*p*H	Coliforms (× 10^5)	Total viable cells (× 10^8)	DNA	Viable bacteria/DNA
				μg/ml	
1	6.7	1.3	7.6	115	6.7
2	6.0	—	9.4	108	10.5
3	5.5	—	36.7	83	44.4
4	5.0	2.4	16.4	40	41.0

[a] Colonies were counted after 72 hr of incubation. Results expressed as counts per gram (10.4 ml = 10 g) of fermentor contents.

Thirty-two strains were isolated from roll tubes inoculated with the *p*H 6.7 fermentor contents, and studied for possible presumptive identification. Among these strains, 13% were cocci and the rest were rod-shaped; 97% were gram-negative; 47% were motile; at least 94% were strict anaerobes; none produced a final *p*H of less than 5.0 in glucose medium; and spores were not seen in any of the cultures. Presumptively identified strains included *Butyrivibrio* species (21%), atypical non-gas-producing strains of *Butyrivibrio* species (12%), B385-like strains (3%), *Bacteroides amylophilus* (9%), *Bacteroides ruminicola* (6%), *Lachnopira multiparus* (3%), and *Ruminococcus* species (3%). As has been the case in previous studies (5) of cattle fed mainly hay diets, many of the strains (42%) could not be presumptively identified by use of the few characteristics which were determined.

Twenty strains were isolated from the *p*H 5.0 fermentor, and were examined microscopically by use of Gram stains and wet mounts. All of these strains were nonmotile and rod-shaped, and only 65% were gram-negative. Again, no spores were observed. Four of the gram-negative strains were selected and studied further for

TABLE 3. *Fermentation changes caused by pH changes in continuous cultures*

Fermentor	pH	Amt (mmoles) produced per day							
		H_2	CH_4	Ethyl alcohol	Acetic acid	Propionic acid	Butyric acid	Valeric acid	Total VFA
1	6.9[a]	NA[c]	15.2 (2.3)[d]	2.2 (0.4)	41.9 (3.1)	11.0 (1.5)	4.3 (1.2)	3.6 (0.8)	150.3
	6.7[b]	0.1	13.6 (0.7)	2.2 (0.7)	41.4 (3.0)	11.3 (1.0)	4.4 (0.7)	2.9 (0.3)	147.9
2	6.9[a]	NA	16.1 (1.4)	1.7 (0.6)	43.0 (5.4)	9.0 (1.7)	4.9 (0.7)	4.1 (0.4)	156.7
	6.0[b]	0.1	13.5 (1.0)	1.7 (0.6)	41.2 (4.2)	12.6 (2.6)	6.0 (0.4)	3.7 (0.4)	164.4
3	6.9[a]	NA	14.0 (1.0)	NA	43.6 (5.8)	13.8 (1.3)	8.1 (1.0)	2.3 (0.5)	185.7
	5.5[b]	0.3	5.8 (0.8)	6.3 (1.8)	25.4 (5.8)	10.3 (1.3)	4.8 (0.9)	1.1 (0.4)	97.7
4	6.9[a]	NA	12.5 (1.8)	1.8 (0.7)	36.0 (1.7)	11.4 (1.5)	3.6 (0.8)	2.2 (0.2)	133.0
	5.0[b]	1.2	1.8 (0.8)	4.0 (0.0)	14.2 (2.3)	8.6 (1.0)	2.3 (0.4)	1.7 (0.3)	74.6

[a] Average value for 5 to 10 days of continuous culture.
[b] Average value for 14 to 19 days of continuous culture.
[c] NA = not analyzed.
[d] Figures in parentheses are standard deviations.

TABLE 4. *Effect of artificial saliva infused upon the molar percentage of acids produced in a continuously cultured artificial rumen*

Fatty acid	Saliva infused	
	Bicarbonate-phosphate[a]	Phosphate[b]
Acetic	66.5[c]	65.9
Propionic	20.8	21.0
Butyric	12.0	11.6
Valeric	1.7	1.5

[a] Mean of 4th to 7th day of saliva infusion.
[b] Mean of 8th to 10th day of saliva infusion (subsequent to bicarbonate-phosphate infusion).
[c] Results expressed as molar percentage of acids produced.

possible presumptive identification. All were anaerobic, noncellulolytic, glucose-fermenting, non-gas-forming members of the genus *Bacteroides*, and were similar to *B. ruminicola* except that all produced a lower *p*H in glucose medium (*p*H 4.4 to 4.7) and one of these failed to hydrolyze starch. Three of the gram-positive rods were studied further. One of these was similar to previously described *Lactobacillus* species (7), except that it hydrolyzed starch. The other two strains were anaerobic rods which could not be identified.

Effect of pH on fermentation products. The average daily production of all measured products for all of the fermentors for the 5- to 10-day period of operation (before changing the artificial saliva *p*H) and for the 14- to 19-day period (after changing the artificial saliva *p*H) are given in Table 3. The total products measured decreased markedly as the *p*H dropped from 6.0 to 5.5 and 5.0. The most significant changes in the production of individual fermentation products were in the decrease of acetate and methane production which accompanied the decrease in *p*H of the fermentors (Table 3). Small increases in the amounts of hydrogen and ethyl alcohol were also detected in the low *p*H fermentors after the *p*H change took place. Propionic acid production was decreased by the decrease in pH of the fermentors. The molar percentage of propionate in the VFA total increased with decreasing *p*H owing to a greater decrease in production of some of the other volatile fatty acids. Butyrate and valerate production decreased markedly in the *p*H 5.5 fermentor after the *p*H changed, but butyrate production was much higher in this fermentor than in the other three fermentors before the artificial saliva *p*H was changed. The reason for this difference in the *p*H 5.5 fermentor is not known. Effluents from all fermentors, collected on days 15, 17, and 19, were analyzed for succinic, lactic, and formic acids, but none of these acids was detected.

Omission of bicarbonate from the artificial saliva. An experiment was performed to determine whether the omission of bicarbonate from the usual *p*H 8.3 artificial saliva solution produced any significant change in the fermentation pattern in the continuous cultures. In this experiment, three fermentors were continuously cultured for 10 days. The initial rumen fluid inocula were from an animal fed a diet of alfalfa hay and concentrate, and a similar feed mixture was used as a source of nutrients for the maintenance of the continuous cultures. A bicarbonate-phosphate solution was infused into the cultures for the first 7 days of operation, and a bicarbonate-free artificial saliva solution was infused during the last 3 days of operation.

During the first 7 days, the *p*H of the fermentors

remained at 6.7. The fermentor pH values dropped to 6.3 in 2 days when the bicarbonate was omitted from the infusion. The proportions of the various VFA produced during the different infusion periods are shown in Table 4. The VFA production per day fell about 11% in the fermentors receiving the bicarbonate-free infusion, but there was less than a 1% variation in the molar percentage of any of the individual VFA in the total VFA mixture between the fermentors which received and did not receive bicarbonate. Methane production decreased only slightly when bicarbonate was omitted from the infusion.

DISCUSSION

The in vitro continuous cultures maintained a fermentation pattern similar to that of the in vivo rumen ecosystem between pH 6.0 and 6.7, but there was a marked change in the fermentation pattern when the pH was dropped below 6.0. The total production of VFA decreased, which could mainly be accounted for as a decrease in the production of acetic acid and butyric acid. Production of methane also diminished with decreasing pH, and was almost completely abolished at pH 5.0. The change in fermentation patterns was accompanied by changes in the microbial population. DNA concentration of the fermentors decreased with decreasing pH. The fermentor maintained at pH 6.7 contained the types of bacteria often found in high concentration in the rumen (6). Bacteriological analysis of the pH 5.0 fermentor showed a high percentage of bacteria which could not be identified with the major species found in rumens of animals fed alfalfa hay. Although the pH 5.5 and 6.0 fermentors were not examined, it seems reasonable to expect that, in the pH 5.5 fermentor, the bacterial population would vary from the major rumen types as the fermentation pattern varied from the normal rumen pattern.

The fact that the total viable bacterial population increased with decreasing pH was surprising, especially when it was found that the DNA concentrations of the fermentors appeared to decrease with decreasing pH. Several possible explanations can be thought of to explain this result. It is conceivable that large microorganisms, which do not contribute significantly to the total viable count on the nonselective medium used, do contribute significantly to the DNA of the higher pH cultures. These large microorganisms may be selected against by the lower pH values and replaced by smaller bacteria. The DNA content of a cell the size of *Escherichia coli* is about 8.3×10^{-15} g. If all of the viable cells measured in the pH 6.7 fermentors were of the same size and had the same DNA content as *E. coli*, these cells would contribute about 6.7 μg of the total of 114 μg/ml of DNA actually found in the fermentors. The numbers of protozoa in the fermentor would appear to be too small to entirely account for the difference in the DNA (about 2,000 per milliliter; mostly entodinia). Another possible explanation of the change in the bacteria-DNA ratio with changing pH is that the viability of bacteria in the pH 6.7 fermentor is a much lower proportion of the total bacteria active in the fermentor than is the case at pH 5.0; i.e., more of the total bacteria from the pH 5.0 fermentor grow in the culture medium used for isolation. In this respect, it would be of interest to compare DNA analyses, culture counts, and direct microscopic counts of the population in fermentors held at the different pH values.

The substitution of a bicarbonate-free infusion solution for the usual bicarbonate-containing infusion did not greatly alter the fermentation pattern of the continuous cultures. Although the feeding regimen used was different from that used for the experiment in which fermentor pH was allowed to drop to low values, the suggestion is that the alteration in fermentation patterns at low pH was not due to a lack of bicarbonate per se but was due to the change itself.

It is of interest that the greatest change in fermentation product output occurred with compounds that arise from fermentation intermediates containing 2 carbon units (acetate and butyrate) and 1 carbon unit (methane) when the pH was lowered in the fermentors. Since both of these units normally arise from the cleavage of pyruvate into a 2-carbon and 1-carbon unit, it is possible that the inhibition of the cleavage reaction could be the basis of the inhibition of acetate, butyrate, and methane production at low pH. Of course, the organisms which produce the inhibited products could be sensitive to low pH for reasons which have nothing to do with the pyruvate cleavage reaction, but the possibility of a specific target site is an attractive one.

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