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Effect of pH on Growth Rates of Rumen Amylolytic and Lactilytic Bacteria

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The relationship between the pH of the medium and specific growth rates, in well-buffered media at 38.5°C, was determined for three strains of *Butyrivibrio fibrisolvens* and for one strain each of *Streptococcus bovis, Selenomonas ruminantium* subsp. *lactilytica, Megasphaera elsdenii, Veillonella alcalescens*, and *Propionibacterium acnes*. The pH optima for growth were between 6.1 and 6.6 for all six species, and the upper pH limits were between 7.3 and 7.8. The lower limit pH values for growth on glucose were 5.4 for *B. fibrisolvens*, near 5.0 for *V. alcalescens*, and between 4.4 and 4.8 for the other four species. These values fall within the minimum pH ranges found when these species are grown in poorly buffered medium with nonlimiting glucose concentrations. Acid sensitivity per se could cause the washout of *B. fibrisolvens*, but not of the other five species, from the rumens of animals on high-starch diets.

In a recent study in our laboratory, Mackie et al. (9) found a succession of species within the amylolytic and especially lactilytic bacterial groups in the rumens of sheep as the grain content of the diet was progressively increased. They considered this to be a shift from acid-sensitive to more acid-tolerant species as the pH of the rumen decreased. At that time, however, there was little information in the literature about the effect of pH on the growth rates of rumen bacteria, as opposed to the initial medium pH permitting growth. The present study was undertaken to supply this information. Since then, Russell and co-workers have presented data on the effect of pH on the maximum specific growth rates of *Megasphaera elsdenii, Butyrivibrio fibrisolvens, Selenomonas ruminantium, Bacteroides ruminicola*, and *Streptococcus bovis* (16); and Russell and Dombrowski have presented data on the effect of pH on the efficiencies of growth of the above species, as well as of *Lactobacillus vitulinus, Ruminococcus albus, R. flavefaciens*, and *Bacteroides succinogenes* (15). The latter paper gives a good indication of the lower pH limits for growth of these species. The present results confirm and amplify their work.

(A preliminary report of part of this work was presented at the 5th International Symposium on Ruminant Physiology, Clermont-Ferrand, France, 3 to 7 September 1979 [5].)

MATERIALS AND METHODS

Organisms and inoculum preparation. The amylolytic organisms *B. fibrisolvens* Ce51 (17), A32, and A88 and *S. bovis* 21.09.6C came from our own collection. The lactate-utilizing bacteria *S. ruminantium* subsp. *lactilytica* ATCC 19205, *M. elsdenii* ATCC 25940, *Veillonella alcalescens* subsp. *alcalescens* ATCC 17745, and *Propionibacterium acnes* ATCC 6919 were obtained from the American Type Culture Collection, Rockville, Md. For most of this study, the inocula came from chemostat cultures in chemically defined medium similar to PCA medium (see below) containing 1% (wt/vol) glucose or sodium DL-lactate as the energy source. The pH of the chemostat cultures was controlled at 6.5, and dilution rates were set at between 0.3 and 0.4 h^{-1}, unless otherwise stated. However, for subsequent single comparisons of growth rates in PCA and PCA2 media, the cultures were transferred three times in batch culture in PCA medium (pH 6.5), allowing the turbidity to increase not more than two- to three-fold before making the next transfer. Regardless of the source of the inoculum, 0.2 ml was used per 10-ml portion of medium of known pH value.

Media. The composition of defined PCA medium, which was used for most of this work, was based on that of the medium of Roché et al. (11), with the following concentrations and modifications: KH_2PO_4, 20.0 mM; K_2HPO_4, omitted; L-alanine, 0.57 mM; L-glutamic acid, 0.26 mM; acetic acid, 50.0 mM; propionic acid, 1.0 mM; isobutyric acid, 0.4 mM; 2-methylbutyric acid, 0.4 mM; valeric acid, 0.4 mM; isovaleric acid, 0.4 mM; diaminopimelic acid, 0.17 mM; succinic acid, 0.37 mM; citric acid, 0.4 mM; pyridoxal-5-phosphate, 0.0004 mM; calcium pantothenate, 0.00013 mM; hemin, 0.0015 mM; dithiothreitol, omitted; cysteine $HCl{\cdot}H_2O$, 0.71 mM; $Na_2S{\cdot}9H_2O$, 0.52 mM; glucose, 55.5 mM, or sodium DL-lactate, 89.23 mM; cellobiose, omitted; and $NaHCO_3$, added (as solid) as required to adjust the pH to the desired value while equilibrating with 98% CO_2–2% H_2 at 38.5°C.

PCA2 medium differed from PCA medium in that

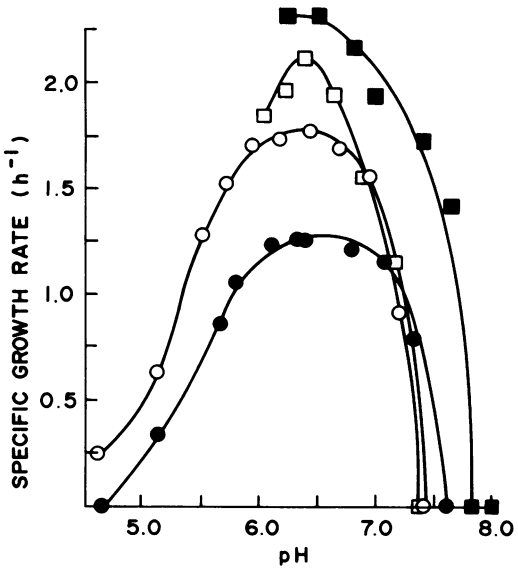

FIG. 1. Effect of pH on the specific growth rate of *S. bovis* 21.09.6C at 38.5°C, with glucose as the substrate. Bacteria were grown in PCA medium, with inocula grown in chemostat culture (○), in a simple defined medium, with inocula grown in chemostat culture (●), in PCA medium, with inocula grown in batch culture (□), and in PCA2 medium, with inocula grown in batch culture (■).

pH values were adjusted with $NaHCO_3$ while equilibrating with 68% N_2–30% CO_2–2% H_2. At similar pH values, PCA2 medium had a considerably lower sodium content and tonicity than PCA medium (at pH 7.4, ca. 4.3 versus 12.25 g of sodium liter^{-1}; 0.4 versus 0.98 osmoles kg^{-1}). Its buffering capacity was also lower but was adequate above pH 5.0.

S. bovis was also grown in a medium that contained glucose, NH_4^+, minerals, trace elements, vitamins, acetate, and sulfide in the same concentrations as in PCA medium but from which amino acids, purines, pyrimidines, and other growth factors were omitted. In the case of *V. alcalescens*, 46.68 mM ammonium DL-lactate was substituted for the higher concentration of sodium lactate in both PCA and PCA2 media since initial growth-rate measurements indicated a sensitivity to either higher sodium concentrations or higher osmolalities. *P. acnes* would not grow well in our defined media, but a modification of PCA2 medium in which 2% (wt/vol) Trypticase (BBL Microbiology Systems)–0.2% yeast extract –0.1% Tween 80 replaced amino acids, B vitamins, purines, and pyrimidines supported good growth.

The pH of the deoxygenated (11) medium without reducing agents was adjusted sequentially from 4.5 to 8.0 in 13 steps while equilibrating with the appropriate gas mixture at 38.5°C and under conditions of oxygen exclusion. After each adjustment, the required number of 9.8-ml portions was sterilized by membrane filtration while dispensing into sterile, rubber-stoppered, 30-ml, optically matched culture bottles which had previously been purged with the same gas mixture.

Cysteine and sulfide from a 50-fold-strength sterile stock solution were injected in 0.2-ml volumes into the individual culture bottles a few hours before inoculation of the media. Three bottles of every subbatch of medium were used to determine any deviation of the initial pH from the design value.

Determination of specific growth rates. Growth was measured, in triplicate, as an increase in turbidity at 578 nm. When growth occurred, readings were continued until the turbidity reached the limit of a satisfactory linear relationship with biomass. Well-documented techniques (6) were used to calculate specific growth rates from the turbidity data. Experience showed that results from inocula obtained from chemostat cultures in steady state were reproducible at different times (5). Results obtained with different inocula grown in batch culture tended to be more variable.

Checks on final pH values and culture purity. The pH values of all media in which growth occurred were checked upon termination of the turbidity measurements. In the media with the least buffering capacity, the changes associated with growth did not exceed 0.15 pH units. The same material was used to prepare Gram-stained films for microscopic examination. The incidence of contamination was very low, and results obtained with contaminated cultures were discarded.

RESULTS

S. bovis. The effect of pH on the maximum specific growth rate of *S. bovis* 21.09.6C is shown in Fig. 1. PCA2 medium was used in all cases to determine the upper limit of pH tolerance, which in this strain was about 7.7. The optimum pH for growth was 6.4, and the lower limit for growth was just below pH 4.5. Whereas the growth rate in the simplified medium was markedly lower, the pH response was very similar, except that the lower limit for growth was about pH 4.6. These values are in agreement with the results of Russell and Dombrowski (15), who found that their strain washed out of a continuous culture run at a dilution rate of 0.158 h^{-1} when the pH was lowered to 4.55.

Of significance for maintaining the numbers of a species in the rumen when readily fermentable carbohydrate is fed to animals is the pH at which the growth rate falls to the washout rate of the rumen contents. The dilution rate of the rumen fluid is about 0.08 h^{-1} in animals fed a high-roughage diet (3). It decreases when concentrates are fed because of the reduction in the flow of saliva. The dilution rate of the solid digesta is 0.04 h^{-1} or less (3). The growth rate of *S. bovis* did not decline to these values, even in the simplified medium, until the pH was very nearly at the lower limit for growth.

B. fibrisolvens. Figure 2 shows the relationship between pH and the specific growth rate of *B. fibrisolvens* Ce51. The curves for strains A32 and A88 showed the same pH range and optimum pH for growth, but the growth rates at the optimum pH were lower, viz., 0.56 and 0.73 h^{-1}, respectively, compared with 0.76 h^{-1} for strain

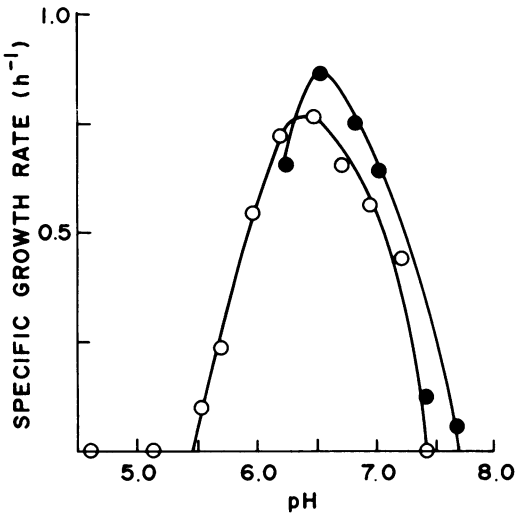

FIG. 2. Effect of pH on the specific growth rate of *B. fibrisolvens* Ce51 at 38.5°C, with glucose as the substrate. Bacteria were grown in PCA medium, with inocula grown in chemostat culture (○), and in PCA2 medium, with inocula grown in batch culture (●).

Ce51. The pH range for growth of this species thus appears to be 5.4 to 7.7, with a fairly pronounced optimum pH for growth between 6.3 and 6.5. The lower limit for growth agrees reasonably well with the results of Russell and Dombrowski (15), who found that the growth rate of strain A38 fell below the dilution rate of 0.162 h^{-1} when the pH of the incoming medium was lowered to 5.70.

***S. ruminantium* subsp. *lactilytica*.** On glucose, *S. ruminantium* subsp. *lactilytica* ATCC 19205 grew at a pH range of about 4.5 to 7.6 (Fig. 3), with maximum growth at pH 6.5. Whether the inoculum was grown in glucose or lactate medium had virtually no effect on the growth rates at different pH values. The growth rates on lactate were considerably lower than those on glucose, which was also reported for *S. ruminantium* HD4 by Russell et al. (14). For growth on lactate, the substrate on which the inoculum was produced had an effect both on the pH range for growth and on the peak specific growth rate. When the inoculum from glucose medium was used, growth occurred over the pH range of 4.6 to 7.0, with a peak growth rate of only 0.22 h^{-1} between pHs 6.1 and 6.3. On the other hand, when the inoculum from lactate medium was used, the pH range for growth shifted slightly upscale (5.0 to 7.1), and a pronounced peak growth rate of 0.56 h^{-1} was observed at pH 6.6.

The lower limit pH values for growth in glucose medium found in the present study are close to the range of lower limit pH values for growth in poorly buffered glucose medium (pHs 4.3 to 4.4) reported previously for the three subspecies of *S. ruminantium* (1, 10). By applying their continuous culture with a pH-shift technique and a dilution rate of 0.155 h^{-1}, Russell and Dombrowski (15) arrived at a somewhat higher value for the lower limit of growth of strain HD4, viz., pH 4.85.

***M. elsdenii*.** *M. elsdenii* ATCC 25940 grew over a pH range of 4.6 to 7.8; the optimum pH

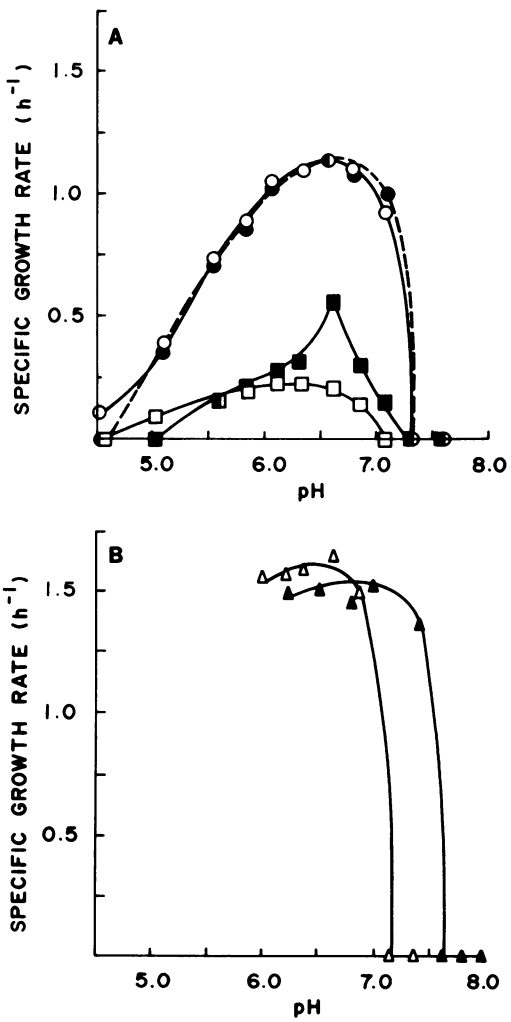

FIG. 3. Effect of pH on the specific growth rate of *S. ruminantium* subsp. *lactilytica* ATCC 19205 at 38.5°C. (A) Bacteria were grown in PCA medium with 1% (wt/vol) glucose, with inocula grown in chemostat culture in glucose (○) or lactate (●) medium, and in PCA medium with 1% (wt/vol) sodium DL-lactate, with inocula grown in chemostat culture in glucose (□) or lactate (■) medium. (B) Bacteria were grown in PCA medium (△) or PCA2 medium (▲), with the same inocula produced in glucose medium in batch culture. This experiment was run 11 months later than those shown in (A).

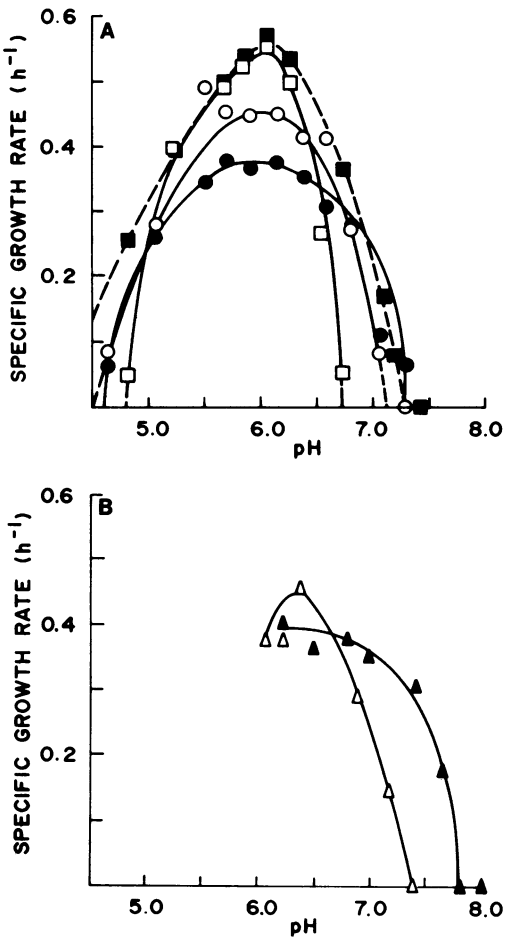

FIG. 4. Effect of pH on the specific growth rate of *M. elsdenii* ATCC 25940 at 38.5°C. (A) Bacteria were grown in PCA medium with 1% (wt/vol) glucose, with inocula grown in chemostat culture in glucose (○) or lactate (●) medium, and in PCA medium with 1% (wt/vol) sodium DL-lactate, with inocula grown in chemostat culture in glucose (□) or lactate (■) medium. (B) Bacteria were grown in PCA medium (△) or PCA2 medium (▲), with the same inocula produced in glucose medium in batch culture. This experiment was run 11 months later than those shown in (A).

for growth was about 6.05 (Fig. 4). Between pHs 5.0 and 6.5, growth rates on lactate medium were higher than in glucose medium, but outside of this range the reverse was true. It is interesting to note that Russell and Baldwin (12), in their study on substrate preferences of different species of rumen bacteria, maintained the pH of their cultures between 6.75 and 6.9 and found a lower growth rate on lactate than on glucose or maltose for *M. elsdenii* B159.

***V. alcalescens* subsp. *alcalescens*.** The relationship between pH in the range of 4.5 to 8.0 and the specific growth rate of *V. alcalescens* subsp. *alcalescens* ATCC 17745 is shown in Fig. 5. In PCA2 medium, the lower limit for growth was between pHs 4.5 and 5.0, the upper limit was at approximately pH 7.7, and the highest growth rate occurred at pH 6.5. The minimum pH at which Douglas (2) obtained growth of one strain of this species was 4.8. At similar pH values between 5.0 and 6.4, the growth rates in PCA medium were considerably lower than in PCA2 medium. It would thus appear that, even with the decreased substrate concentration, the tonicity of PCA medium was still above the optimum.

***P. acnes*.** The specific growth rates of *P. acnes* ATCC 6919 at different pH values are shown in Fig. 6. With glucose as the substrate, the lower and upper pH limits for growth were 4.7 and 7.5, and maximum growth rates occurred between pHs 6.2 and 6.4. In lactate medium, the upper limit for growth was similar, but the lower limit shifted to about pH 5.2, and the optimum pH shifted to 6.6. Below pH 6.7, growth on glucose was markedly faster than on lactate. Above this value, the difference between growth rates decreased. An observation which was made in triplicate, but for which we can offer no explanation, was that cultures grown from lactate-grown inocula grew faster than those grown from glucose-grown inocula. This was particularly the case with cultures in glucose medium.

Table 1 shows a comparison of the specific growth rates of the six bacterial species studied at pH values between 5.0 and 6.75 in glucose

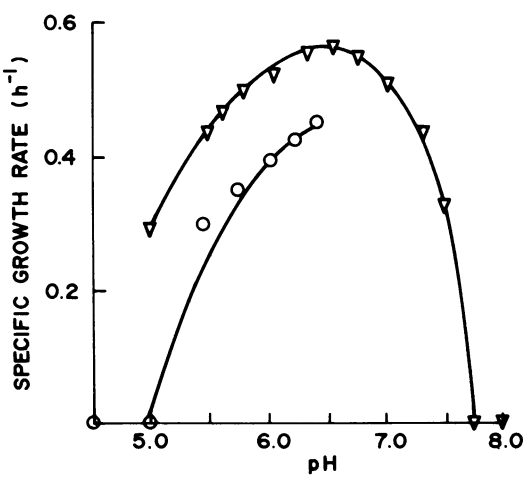

FIG. 5. Effect of pH on the specific growth rate of *V. alcalescens* subsp. *alcalescens* ATCC 17745 at 38.5°C, with 0.5% (wt/vol) ammonium DL-lactate as the substrate. Bacteria were grown in PCA medium (○) or PCA2 medium (▽), both with the same inocula produced in batch culture.

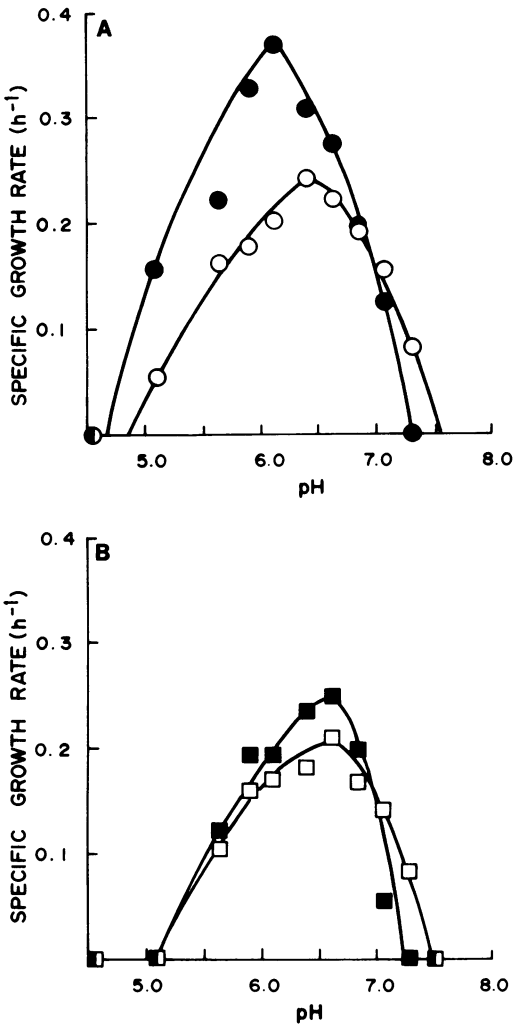

FIG. 6. Effect of pH on the specific growth rate of *P. acnes* ATCC 6919 at 38.5°C. (A) Bacteria were grown in a semidefined medium with 1% (wt/vol) glucose, with inocula grown in batch culture in glucose (○) or lactate (●) medium. (B) Bacteria were grown in a semidefined medium with 1% (wt/vol) ammonium DL-lactate, with inocula grown in batch culture in glucose (□) or lactate (■) medium.

and, when appropriate, lactate media. Since the actual pH values of different batches of media were scattered around the design values, the data for this table were calculated from the curves for PCA or PCA2 medium (whichever gave the highest growth rates) (Fig. 1 through 5) and the curves for the minerals-Trypticase-yeast extract-Tween 80 media (Fig. 6).

DISCUSSION

Of the six species studied, *B. fibrisolvens* was the most acid sensitive, its growth rate falling sharply below pH 6.0. Hence, it would be expected that it would decrease in number in relation to the more acid-tolerant amylolytic species, particularly *S. bovis*, and the lactobacilli when the pH falls below this value for any significant part of the day. On the other hand, it would not be washed out completely until the pH was continuously below 5.6, a value at which its specific growth rate equals 0.08 h^{-1}. This corresponds to a retention time of 12 h, which is fairly typical of the fluid portion of rumen contents. In fact, Mackie et al. (9) found that *B. fibrisolvens* constituted 5 to 30% of the amylolytic bacteria, even when the diet contained 71% grain plus molasses and the pH of the rumen fell below 6.0 for most of the day. In contrast, *S. bovis*, despite its greater acid tolerance, higher maximum specific growth rate, and simple nutritional requirements, was found only sporadically. This may be due to its low affinity for maltose, cellobiose, and glucose (13) which would only permit it to multiply rapidly when the concentration of one of these carbohydrates is unusually high in the rumen. This is the case when ruminants are suddenly overfed with grain or soluble carbohydrate (4, 7).

Concerning the incidence of different species of lactilytic bacteria in the rumens of sheep on high-concentrate diets, Mackie and Gilchrist (8) found that *Veillonella* species were not found among viable counts of high sample dilutions after the sheep had been on a 24% grain-plus-molasses diet for 1 week. Likewise, *Selenomonas* species were not observed after the grain-plus-molasses content of the diet was raised to above 44%. On the other hand, *Propionibacterium* species were found at all levels of concentrates, and this was also true for *Megasphaera* species, although these occurred less regularly than *Propionibacterium* species. *Anaerovibrio*-like organisms increased in number as the proportion of readily fermentable carbohydrate fed was increased, and they became the predominant lactilytic organisms when the grain-plus-molasses content of the diet exceeded 44%.

From the comparison of the growth rates of the six species at different pH values (Table 1), it seems unlikely that the disappearance of *Veillonella* species from among the predominant lactilytic species in the rumens of sheep fed a 24% grain-plus-molasses diet could have been due to its sensitivity to low pH, because the pH of the rumen contents at that stage did not fall below 5.7. At that point, its specific growth rate should have been equal to that of *M. elsdenii* and considerably higher than that of *P. acnes*, both of which were found with this diet. It is more likely that the overgrowth of *V. alcalescens* by the other species was due to the fact that it alone

TABLE 1. Comparison of specific growth rates of bacterial species at different pH values and on two types of media

pH	Specific growth rate (h^{-1}) in:								
	Glucose-containing medium					DL-Lactate-containing medium			
	S. bovis[a]	*B. fibrisolvens*[a]	*S. ruminantium*[a]	*M. elsdenii*[a]	*P. acnes*[b]	*S. ruminantium*[c]	*M. elsdenii*[c]	*P. acnes*[d]	*V. alcalescens*[e]
5.00	0.46	NG[f]	0.28	0.26	0.04	NG	0.32	NG	0.30
5.50	1.26	0.07	0.68	0.39	0.11	0.17	0.46	0.09	0.44
5.75	1.54	0.33	0.85	0.44	0.16	0.21	0.51	0.13	0.49
6.00	1.70	0.56	0.98	0.46	0.20	0.25	0.55	0.20	0.53
6.25	1.76	0.75	1.09	0.44	0.23	0.32	0.54	0.23	0.55
6.50	1.78	0.76	1.13	0.39	0.24	0.45	0.47	0.25	0.56
6.75	1.68	0.68	1.12	0.29	0.21	0.40	0.36	0.23	0.54

[a] Growth was measured in PCA glucose medium, with inocula from chemostat cultures in the same medium.
[b] Growth was measured in minerals-glucose-Trypticase-yeast extract-Tween 80 medium based on PCA2 medium, with inocula from batch cultures on the same medium.
[c] Growth was measured in PCA lactate medium, with inocula from chemostat cultures in the same medium.
[d] Growth was measured in minerals-lactate-Trypticase-yeast extract-Tween 80 medium based on PCA2 medium, with inocula from batch cultures on the same medium.
[e] Growth was measured in PCA2 lactate medium, with inocula from batch cultures on the same medium.
[f] No growth after 36 h of incubation.

could not benefit from the increase in the carbohydrate content of the diet, being unable to utilize sugars. Again, the absence of *Selenomonas* species from the dominant lactilytic bacteria when the sheep were fed a diet containing more than 60% grain-molasses could not be attributed solely to pH, which never fell below 5.5, a value at which its growth rate on glucose was still 0.86 h^{-1}. Only if competition from other amylolytic organisms forced it to utilize lactate as its main substrate, on which its specific growth rate is very low, would its numbers have been expected to fall.

Based on these results it appears that pH alone is of less importance than has been postulated (8) in determining the shifts in the bacterial population in the rumen when concentrates are fed. Shifts in the nature or concentrations of available nutrients in the rumen, in relation to the substrate preferences (12) and affinities (13) of the competing bacterial species, may play a greater role and deserve further investigation.

ACKNOWLEDGMENTS

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