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THE PHYSIOLOGY AND SOME NUTRITIONAL ASPECTS OF A RUMEN BACTERIUM

By

Norma P. Christopherson

A thesis submitted
in partial fulfillment of the requirements for the
degree of Master of Science at South Dakota
State College of Agriculture
and Mechanic Arts

August 1958

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THE PHYSIOLOGY AND SOME NUTRITIONAL ASPECTS

OF A RUMEN BACTERIUM

This thesis is approved as a creditable, independent investigation by a candidate for the degree, Master of Science, and acceptable as meeting the thesis requirements for this degree; but without implying that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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INTRODUCTION

One of the interesting symbiotic relationships in nature is that existing between the ruminants and their microbial population. This arrangement has solved for them the problem of existing on a herbivorous diet. Most mammals can derive little benefit from the potentially available energy in plant materials, which is locked in the complex, insoluble polysaccharide, cellulose. Cellulase is not a mammalian enzyme, but it is part of the chemical machinery of a number of bacteria, some species of which reside in the rumen.

The rumen has often been referred to as a "fermentation vat". No enzymes are secreted into this incubation chamber which has a microbial population—consisting of protozoa and bacteria—of the order of 10^{10} cells per milliliter. The salivary glands secrete essentially a dilute salt solution consisting principally of sodium bicarbonate and sodium phosphate. The plant materials taken in are mixed with large amounts of this. The result is an excellent medium for the organisms which are present and rapid fermentation takes place.

Many different organisms are present and their biochemical activities have not as yet been completely delineated. All of the organisms have not as yet been identified, nor their relative numbers assessed under different conditions. It is known, however, that the cellulose and other carbohydrates taken in are fermented with the eventual formation of simple fatty acids and gases. These fatty acids are absorbed through the rumen wall, circulate to the various tissues, and are there oxidized. The tissues of ruminants are enzymatically better equipped for the oxidation of fatty acids than are those of non-ruminants.

Another function of the rumen organisms is to provide nitrogenous compounds and vitamins for their host. Their fate is eventually to be destroyed by proteases as they pass out of the rumen as digestion proceeds. For such a reason, ruminants can thrive on rations consisting of hay and simple nitrogenous compounds such as urea or ammonia.

Rumen gases have been reported to consist of 67 per cent carbon dioxide, 26 per cent methane, 6 per cent nitrogen, 0.1 per cent hydrogen sulphide and less than 1 per cent oxygen (5). The main volatile fatty acids present in the rumen are acetic and propionic acids, which account for 80 per cent of the total. Butyric acid is also present. The non-volatile acid, lactic acid, is also known to be present, and it has been suggested that this is one source of acetic acid and carbon dioxide.

Investigations of the biochemical activity of the rumen have been carried out with the use of mixed, washed cell suspensions, which is a valid approach to the study of the over-all physiology of this organ. An extensive study of the individual organisms has not yet been made. This study has as its purpose the investigation of a rumen bacterium, with the hope of learning its physiological characteristics, some of its nutritional requirements, and placing it, if possible, in its taxonomic niche.

REVIEW OF LITERATURE

There has been a great deal of interest in the microflora of ruminants by bacteriologists and nutritionists alike. Most of the publications concerning the bacteriology of both bovine and ovine rumens have appeared since 1940. In their review of the bovine bacteriology, covering a period of approximately ten years, Doetsch and Rovinson (9) list a bibliography of not less than 136 publications on this specific topic. There is, admittedly, much yet to be learned. Five different approaches have been used: (1) microscopic methods, (2) cultural methods, (3) use of washed suspensions of rumen bacteria, (4) artificial rumen techniques, and (5) *in vivo* studies; and each has yielded useful information. The physiological approach, using washed suspensions of resting cells, is probably the most valuable aid in the study of overall function of the rumen.

The earlier cultural studies of this group were carried out with the use of techniques for maintaining strict anaerobiosis at all stages of the operation. Gall, Stark and Loosli (13) describe a method of taking rumen samples and preparing cultures of the material which stresses speed and the need for anaerobic conditions at all times. Their media contained tryptone, peptone, beef extract, glucose, yeast extract, skim milk, cellulose and phosphate buffer; or glucose, tryptone, meat infusion, tomato juice, cellulose and phosphate buffer. Bryant and Burkey (4), in their studies of the bacteria in the bovine rumen, also stressed the importance of maintaining anaerobic conditions. Their culture medium contained rumen fluid, glucose, cellobiose and agar (RGCA medium) plus a complex mineral solution and resazurin indicator. Doetsch,

Robinson and Shaw (10) also carried out similar investigations. They used a medium containing rumen fluid and inorganic salts, glucose, cellobiose, an oxidation-reduction indicator and a carbon dioxide-bicarbonate buffering system. They also used a modified, commercially-available medium (Eugon agar, BBL) which did not contain rumen fluid. From their studies they concluded that: (1) total counts varied little even when held as long as eight hours at room temperature, (2) aeration of samples over periods of up to four hours resulted in little change in total counts, and (3) rumen fluid contains unknown substances for optimal growth of some rumen bacteria. In a later publication, McNeill, Doetsch and Shaw (19) reported further on the requirement for rumen fluid in the nutrition of rumen bacteria. They concluded that rumen fluid served both as a source of nitrogen and growth factors and that the factors found in the rich nitrogenous material ordinarily used for the cultivation of fastidious bacteria did not possess this stimulatory effect. At a still later date, Gilroy and Doetsch (15) reported on a nutritionally rich medium that was able to support growth of all rumen isolates at a rate comparable to that of rumen fluid medium, and one medium that supported growth, but at a slower rate.

In 1951 Gall and Huhtanen (12) published their criteria for judging a true rumen organism. As a conclusion to their study of about 5,000 isolates from the rumens of approximately 350 cattle and sheep from several herds in three states, they set up the following five criteria: (1) anaerobiosis, (2) presence in numbers of one million or more per gram of fresh rumen contents, (3) isolation of a <u>similar</u> type bacterium at least ten times from at least two animals, (4) isolation

from animals in at least two geographical locations, and (5) production by the organisms of end-products found in the rumen from substrates found in the rumen. They also described some of the physiological characteristics of five rumen bacteria.

In none of these studies, however, were the organisms cultured, studied and identified. Morphology, gram reaction, a few photographs and a suggested resemblance to some group was the extent of their descriptions.

The greatest preoccupation has been with the organisms producing cellulose-digesting enzymes. This group has been studied almost to the exclusion of other flora which may be present. Not until quite recently have there been reports of studies of other groups believed to play important roles in rumen fermentations.

Hungate, Dougherty, Bryant and Cello (16) in a series of ten experiments using fistulated and non-fistulated sheep, concluded that an excess of grain or glucose introduced into the rumen caused a marked change in the rumen microflora and the composition of the fluids. They reported a relative increase in the numbers of gram positive bacteria, an accumulation of non-volatile acids and a reduction in volatile acids. They believed Streptococcus bovis to be the organism usually responsible for the high acidity (pH 4.1-4.7) of the rumen contents, especially that produced shortly after the feedings. One case, however, showed the predominant organism to be a gram-positive rod which was facultatively anaerobic and catalase negative. The organism was placed in the genus Lactobacillus and its characteristics suggested a relationship to L. brevis.

An increasing number of reports have appeared in the literature relative to rumen lactobacilli. Mann and Oxford (18) in England isolated 13 gram-positive rods in the course of antibiotic feeding trials with young calves. Ten of the isolates were found to be catalase negative and were studied further by methods suitable for lactobacilli. Four isolates proved to be *L. brevis*, three were provisionally identified as anaerobic variants of *L. lactis*. The remaining three were motile, homofermentative and mannitol-fermenting, and could not be identified with any known species of lactobacilli. Their identifications were made on the basis of morphology and biochemical reactions.

The authenticity of the lactobacilli as "true" rumen inhabitants of the calf rumen might be questioned by some. However, Wasserman, Seeley and Loosli (23) reported on isolations they made from rumen liquor at dilutions of 10^7 or greater from mature ruminants. These animals had been on a urea-molasses supplemented ration. Their medium was a relatively simple one, containing yeast extract, glucose, ammonium chloride, cysteine, phosphate buffer and salts. From the results of their study of the physiology and nutritional requirements of their isolates, they concluded that they were variants of *L. bifidus*, differing from the type species by certain significant characteristics. They believed the relative simplicity of the nutritional requirements and the occurence of these organisms in animals receiving non-protein nitrogen and soluble carbohydrates to be significant. These organisms may be important as converters of non-protein nitrogen to protein nitrogen in the ruminant.

At Wisconsin, Bauman and Foster (1) studied the characteristics of organisms isolated from the rumens of cows fed high and low roughage

rations. There had been previous reports of changes in the ratio of acetic acid to other rumen acids with a change in the type of ration fed, this change being reflected in the amount of butter fat found in milk from cows on such experimental diets. This prompted the above study. Their cultures were maintained under reducing conditions and carbohydrate fermentations were determined by observing an indicator (brom cresol purple) added to the media. In addition, a fermentation train was used to characterize the products of dextrose dissimilation. These cultures were also maintained under anaerobic conditions. The rod cultures isolated from cows on high roughage diets were found to ferment dextrose almost entirely to acetic acid and lactic acid in a molar ratio of 2:1, with only traces of acetoin and carbon dioxide. This suggested to them the possibility of carbon dioxide fixation, which has been previously reported to occur with rumen organisms.

In contrast to the above report on rumen lactobacilli associated with animals on a high roughage diet, Briggs (3) reported an increase of lactobacilli when the ration contained a high amount of concentrates.

Perhaps the most extensive investigation of rumen lactobacilli was that of Jensen, Smith, Edmondson and Merilan (17). They studied 168 cultures and identified 155 of them. Seven species were represented: L. *acidophilus* (12), L. *brevis* (5), L. *buchneri* (30), L. *casei* (4), L. *casei* (non-lactose fermenting) (46), L. *fermenti* (6), and L. *plantarum* (52). L. *acidophilus* and L. *casei* were suspected of being passengers only. Physiology was the basis for classification.

Some of the lactobacilli have been accused of being detrimental to their hosts. Rodwell (21) made investigations of bacteria isolated

from horse stomachs and sheep rumen contents, which possessed active amino acid decarboxylases. Using manometric techniques, he found that several of the strains of lactobacilli were able to decarboxylate histidine at a high rate. Dain, Neal and Dougherty (8) implicated histamine, because of its ability to inhibit rumen motility, as one of the causes of illness and fatalities in over-fed sheep. They reported an increase of histamine formation when the pH of the ingesta fell below 5.0. Below a pH of 4.5 histamine concentrations reached levels high enough to cause death of the animal.

A recent and interesting report on the nutrition of lactic acid bacteria has come from an English group. Ford, Perry and Briggs (11), working at the National Institute for Research in Dairying, at the University of Reading, selected about one quarter of some 350 strains of streptococci and 300 strains of lactobacilli from cow and calf rumens as representative and identified them. Their report concerned the pattern of vitamin requirements of these organisms and their attempt to establish to what extent these patterns are characteristic of the species. They state, also, that they have a longer-term objective——study of the symbiotic interrelationships between the more important rumen species and the nutritional factors which may determine the equilibrium between assorted populations present.

The four lactobacilli species included in the study were: L. plantarum (7 strains), L. fermenti (33 strains), L. acidophilus (19 strains), and L. casei (17 strains). They found only L. plantarum to be homogenous in respect to vitamin requirements. Most of the rumen strains of L. fermenti required vitamin B_6 and riboflavin, in addition to

nicotinic acid, thiamine and pantothenic acid, which have been reported as requirements typical of this species. The vitamin requirements of the other three species seemed to be broadly similar to the requirements reported for a variety of non-rumen strains. Their variance with findings reported in other investigations, they felt, might be attributed to the anaerobic conditions under which their tests were conducted.

The potential value of such comparative nutrition in the study of bacterial taxonomy seems promising. In fact, Professor Carl S. Pederson, in his description of the genus *Lactobacillus* in the Seventh Edition of Bergey's Manual of Determinative Bacteriology (2), has this to say about the confusion which surrounds the taxonomy of this genus: "It is impossible to make an entirely satisfactory differentiation of the species in the genus *Lactobacillus* due to the inadequacy of comparative data. The end-products of fermentation, utilization of carbon compounds and temperature of growth are the criteria relied upon at present. It is quite possible that when more comparative information is made available in regard to nutritional patterns in defined media, serological reactions and variations in sugar fermentations, a more satisfactory arrangement of species may be effected."

PROCEEDURE AND RESULTS

The mixed culture from which the organism under study was isolated, contained both gram-positive and gram-negative forms, and a mold which grew in a dense, heavy mat on the surface of the liquid medium. This culture broth contained the following ingredients per liter: nutrient broth (Difco), 8 grams; yeast extract (Difco), 3 grams; dextrose, 25 grams; 100 milliliters of phosphate buffer, which contained 4.36 grams of monopotassium phosphate and 10.59 grams of disodium phosphate per liter; and distilled water to make one liter. Successive transfers in this same medium gave good growth of all forms present.

The mold was particularly troublesome when attempts were made to isolate the bacterial species present in the culture. In an effort to control its growth, the use of sorbic acid seemed feasible. This unsaturated fatty acid is used to curb the development of a number of such species in dairy and other food products. It has been reported to be ineffective against catalase-negative bacteria and even actually stimulatory to some strains of lactobacilli. Because sorbic acid has a low aqueous solubility, a ten per cent solution of sodium sorbate was prepared, filter sterilized, and added aseptically to the culture medium described above, in concentrations of 0.01 and 0.1 per cent. Mold control was effective at both concentrations and bacterial growth seemed to be stimulated.

Final isolation of the bacterium was made in soft agar shake cultures. To the medium previously described was added 0.5 per cent agar. Tubes of the medium were sterilized and cooled to 45 degrees centigrade, and inoculated by dropwise dilution transfers.

When well-isolated colonies had developed in a tube, the culture was expelled into a sterile Petri dish and individual colonies were picked into tubes of thioglycollate broth (Difco). In this medium all of the colonies developed below the oxidized layer. AC Medium (Difco) was also used for subcultures. Gram stains were made from these cultures and they showed short, gram-positive rods, which did not form spores.

These characteristics suggested the possibility of the organism being a lactobacillus. Since this group has marked acid tolerance, the isolate was tested for its ability to grow in an acid medium. A 50 per cent solution of lactic acid was autoclaved and then added aseptically to thioglycollate broth in concentrations varying from zero to 1.0 per cent. The organisms grew well in the acidified media, and subcultures made after a lapse of three days were successful.

A preliminary carbohydrate fermentation test was run, using the four sugars, dextrose, sucrose, maltose and lactose. The basal medium was a modified thioglycollate broth (BBL No. 01-397). The carbohydrates were added to this medium to give a final concentration of 1.0 per cent. Brom cresol purple indicator, pH range of 5.2-6.8, was added to the medium and Durham tubes were included for detection of gas production. Fermentation of dextrose, sucrose and maltose, but not lactose, was evidenced by a change in color of the pH indicator. No gas was observed in the Durham tubes.

The use of a solid medium seemed desirable for the isolation of individual colonies and the determination of catalase production. Several commercially-available media have been recommended for the cultivation of lactobacilli. Four of these were tried: Tomato Juice Agar

(Difco), Trypsin Digest Agar (Difco), Tryptone Glucose Extract Agar (Difco), and L Agar (BBL). Tomato Juice Agar gave the best surface growth when the cultures were incubated aerobically. Colonies developed more slowly on Trypsin Digest Agar. Growth on both of these media was improved by incubating the cultures in a candle jar; colonies then would appear in 24 to 48 hours. The other two media produced pin-point colonies only, after one week's incubation.

Satisfactory surface growth for detecting catalase production was obtained on Tomato Juice Agar plates. A solution of hydrogen peroxide was dropped on the colonies being tested. The organism of this study was found to be catalase negative, as indicated by the absence of bubbles. Control cultures of catalase-positive organisms were also tested.

On the basis of the evidence accumulated thus far—a gram-positive, non-sporulating, acid-tolerant rod that was catalase negative and anaerobic to microaerophilic—the organism was tentatively placed in the genus *Lactobacillus*.

Because lactobacilli are well-known for their role in the fermentation of silage, and the use of this fodder in the rations of ruminants, a parallel study of an isolate from this source seemed to be of interest.

A sample of good quality corn silage that had been stored in an upright silo for a period of about six months was obtained. The sample was collected in a sterile container from a layer several inches below the surface. A portion of the silage was removed with sterile tweezers to a flask containing sterile, peptone-buffered, distilled water. The contents were thoroughly shaken and samples of the supernatant fluid were plated out on Tomato Juice Agar. The mixture was then allowed to steep

for approximately 12 hours at room temperature. Samples of the liquid portion were then streaked out on Tomato Juice Agar. Colonies of gram-positive, catalase-negative rods were successfully isolated from the mixture that had been steeped for 12 hours. Typical colonies were picked into tubes of sterile nutrient broth, diluted, and again streaked out on Tomato Juice Agar. Gram stains were again made and the colonies retested for catalase production. This isolate proved to be a short, gram-positive, non-sporulating rod which did not produce catalase. It, too, was provisionally placed in the genus Lactobacillus.

For further studies, stock cultures of the rumen strain and the silage strain were made by replating twice on Tomato Juice Agar to secure well-isolated colonies which were transferred to tubes of thioglycollate broth containing 0.1 per cent calcium carbonate. These cultures were incubated at room temperature. Immediately prior to use of the stock cultures, a transfer was made for a new stock culture.

Jensen, Smith, Edmondson and Merilan (17) report the use of V-8 juice agar in their studies of rumen lactobacilli. This medium proved to be superior to Tomato Juice Agar in these studies, and the use of Tomato Juice Agar was discontinued in favor of the V-8 juice medium. A V-8 juice broth, prepared by the same formula, with agar omitted, proved to be very efficient when rapid growth of a large number of cells was desired. Incubation in an atmosphere of increased carbon dioxide tension did not enhance growth on the V-8 juice medium.

On the basis of Costilow and Humphreys' report (7), of nitrate reduction by certain strains of lactobacilli studied by them, the Seventh Edition of Bergey's Manual of Determinative Bacteriology (2) has included

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the following revision in the description of the genus *Lactobacillus*: "Nitrates are not reduced except under certain conditions with *Lactobacillus plantarum*." Indole-Nitrite Medium (BBL) and Nitrate Broth (Difco) with agar and yeast extract added were used to test the nitrate-reducing ability of the rumen and silage organisms. The rumen strain gave positive results repeatedly in both media after an incubation period of three days at 37 degrees centigrade. The silage strain was consistent in not reducing nitrate in either of the two media. All cultures showed good growth. The test reagents used were sulphanilic acid and alpha-napthylamine (6). Zinc dust was added to cultures giving a negative test to confirm the presence of residual nitrate. Uninoculated controls were incubated along with the cultures being tested. These controls were negative for the presence of nitrite.

The nitrate reduction tests were repeated, using in this series Indole-Nitrite Medium containing agar and Indole-Nitrite Medium without agar. Four cultures of each organism were tested for nitrate reduction in each medium, with the following results: all cultures of the silage strain were negative; all cultures of the rumen organism in the agar-containing medium were positive; two of the cultures of the rumen strain in the medium not containing agar were also positive. These results were obtained after an incubation time of three days at a temperature of 37 degrees centigrade. At the end of a two-week period another set of four cultures, as described above, were examined. The results were almost identical with those obtained at three days. In all cases negative controls were included in the tests.

Since Costilow and Humphreys observed nitrate reduction by their

L. plantarum strains only under anaerobic conditions (recently heated medium containing 0.1 per cent agar), the ability of this organism to reduce nitrate under apparently aerobic conditions seemed surprising. Since the foregoing tests had been carried out in 16 by 155 millimeter test tubes half-filled with the medium, it was felt that perhaps sufficiently anaerobic conditions had been attained to satisfy such requirements. Therefore, the following test was set up. Indole-Nitrite Medium without agar was dispensed into 125 milliliter Erlenmeyer flasks in the following amounts: 25 milliliters, 50 milliliters, 75 milliliters, and 100 milliliters. Care was taken to assure easy-fitting cotton stoppers and the medium was thoroughly agitated after autoclaving. Cultures of the rumen organism were tested after three- and six-day incubation periods at 37 degrees centigrade. All tests were negative for nitrate reduction and residual nitrate was present in all cultures.

Cultures in Indole-Nitrite Medium were tested for indole production. All were negative when tested with Kovac's reagent (6).

Thiogel Medium (BBL) was used to test the ability to liquify gelatin. Cultures of both organisms were negative after incubation for a period of one month at room temperature.

A medium containing Casitone (Difco), 2.0 per cent; dextrose, 1.0 per cent; ferrous sulfate, 0.02 per cent; sodium sulfite, 0.04 per cent; sodium thiosulfate, 0.008 per cent and agar, 0.35 per cent; was used to test the rumen organism's ability to produce hydrogen sulfide. The results were negative. The silage organism was not tested.

Both strains showed good growth at 37 degrees centigrade and also at room temperature, 28-30 degrees centigrade. In an effort to assess

the temperature limits of growth, a loopful of stock culture was transferred to thioglycollate broth in screw cap tubes. These cultures were incubated at 17 degrees centigrade and 45 degrees centigrade. In order to assure constant temperatures, the culture tubes were placed in half-pint milk bottles which were filled with water and fitted with collars which accommodated the diameter of the tube. The temperature of the "water jacket" was allowed to equilibrate with that of the incubator before the cultures were placed in them. There was some growth of both strains at 17 degrees centigrade and at 45 degrees centigrade.

The colony characteristics of both strains were somewhat similar. When grown in V-8 juice broth, both organisms produced a heavy, uniform turbidity in 12 to 24 hours. The cells of the rumen organism settled out in a few days, leaving a clear supernatant. In some instances, the cells would gather along the side of the culture tube in a granular floc. The silage strain, on the other hand, produced a silky turbidity which settled out more slowly.

On the surface of V-8 juice agar both strains produced cream-colored colonies which attained a size of one to two millimeters in diameter. The rumen strain formed smooth colonies with raised centers. The colonial type of the silage strain was also smooth, but the surface was flat, with the entire colony being somewhat raised. In subsurface cultures both strains grew into smooth, lens-shaped colonies. All colonies became darker upon aging.

An individual colonial pattern was shown by either organism in thioglycollate broth. The colonies grew downward from the point of inoculation in a streamer or "shooting star" effect. The rumen strain

produced rough, granular "tails", while the silage strain had a smooth, silky "tail".

Preliminary investigation showed that these organisms did not produce any observable change in litmus milk. The organisms were then retested in litmus milk and the following variations of litmus milk: litmus milk + 1.0 per cent dextrose, litmus milk + 1.0 per cent yeast extract, and litmus milk + 1.0 per cent dextrose and 0.5 per cent yeast extract (YGLM). Cultures of both organisms in each of the four media were incubated at room temperature and observed at intervals of one week, two weeks and four weeks. The results are compiled in Table I.

TABLE I. Growth Characteristics in Litmus Milk Media

	Litmus Milk	Litmus Milk + 1.0% dextrose	Litmus Milk + 1.0% yeast extract	YGLM
Silage Strain				
1 week	NC	NC	A,-; R,±	A,±; R,±
2 weeks	NC	NC	A,-; R,±	A,+; R,±
4 weeks	NC	A,±; R,-	A,±; R,±	A,+; R,±
Rumen Strain				
1 week	NC	A,±; R,±	A,-; R,±	A,+; R,±
2 weeks	NC	A,+; R,±	A,±; R,±	A,+; R,+*
4 weeks	NC	A,+; R,+	A,±; R,±	A,+; R,+**

A=acid; R=reduction of litmus; ±=slight change; NC=no change
* lower half of medium was reduced; sufficient acid had been formed to produce a soft curd.
** lower half of medium was reduced; sufficient acid had been formed to produce a solid curd.
No proteolysis was observed in any of the cultures.

An evaluation of the adequacy of some commercially-available nutritive substances was made. Four variations of each substance was prepared as follows: (1) the nutritive substance alone, (2) the nutritive substance + 0.5 per cent dextrose, (3) the nutritive substance + 0.5 per cent dextrose + 0.1 per cent agar, and (4) the nutritive substance + 0.1 per

cent agar. These media were inoculated with 0.1 milliliter of a saline suspension of actively growing cells and incubated at 37 degrees centigrade. Growth was noted in all of the media, but the best growth was observed in the media supplemented with dextrose, or with dextrose and agar. Since acid production is a convenient measure of the metabolism of lactic organisms, the pH of the supplemented cultures was measured with a Beckman glass electrode pH meter after a growth period of ten days. The results of this study are compiled in Table II.

In addition to the above, both organisms were tested for their ability to grow in V-8 juice, diluted one to five, and in Urea Broth (Difco) supplemented with 1.0 per cent dextrose. Both strains were able to grow well in the diluted V-8 juice. The pH of the control was 4.4. The final pH of the culture of the rumen organism was 3.8, and that of the silage organism, 3.7. There was some growth of both strains in the supplemented Urea Broth, but the indicator (phenol red) did not show a change in pH.

Since some investigators have attached significance to sodium chloride and bile salt tolerance in the lactic acid organisms, it seemed of interest to determine the resistance or susceptibility of these two strains. Using V-8 juice broth as the basal medium, reagent grade sodium chloride and Bacto Bile Salts (Difco) were used to prepare culture media containing two per cent and four per cent concentrations of each. The inoculum was a small loopful of a 48-hour culture grown in V-8 juice broth. Incubation was at room temperature. Both strains grew well in either concentration of sodium chloride, the silage organism showing a relatively greater amount of growth. After 24 to 48 hours, a dense

TABLE II. Growth Response to Various Nutritives

Nutritive	Silage Strain (pH)	Rumen Strain (pH)
Bacto Tryptone (Difco), 1%		
Control **	6.9	6.9
+ 0.5% dextrose	5.5	6.6
+ 0.5% dextrose + 0.1% agar	6.4	4.7
Bacto Tryptose (Difco), 1%		
Control	6.7	6.7
+ 0.5% dextrose	4.9	4.7
+ 0.5% dextrose + 0.1% agar	6.0	4.1
Bacto Casitone (Difco), 1%		
Control	7.0	7.0
+ 0.5% dextrose	7.1	6.5
+ 0.5% dextrose + 0.1% agar	6.6	4.8
Neopeptone (Difco), 1%		
Control	6.6	6.6
+ 0.5% dextrose	6.7	4.5
+ 0.5% dextrose + 0.1% agar	5.2	4.2
Bacto Liver (Difco), 0.5%		
Control	5.0	5.0
+ 0.5% dextrose	3.6	4.0
+ 0.5% dextrose + 0.1% agar	4.8	4.0
Yeast Extract (Difco), 0.5%		
Control	6.4	6.4
+ 0.5% dextrose	5.0	4.0
+ 0.5% dextrose + 0.1% agar	5.7	4.0
Lactalysate (BBL), 1%		
Control	6.4	6.4
+ 0.5% dextrose	4.7	5.5
+ 0.5% dextrose + 0.1% agar	5.3	5.3
Phytone (BBL), 1%		
Control	6.8	6.8
+ 0.5% dextrose	4.4	5.0
+ 0.5% dextrose + 0.1% agar	4.9	4.1

** Control=uninoculated media containing 0.5% dextrose

precipitate appeared in the cultures containing bile salts, so that it was impossible to detect turbidity due to bacterial growth. This

precipitate also interfered with a search for organisms in a wet mount. Consequently, a test of survival of the organism was resorted to. After the precipitate had been thoroughly resuspended, a small loopful of a 72-hour culture was transferred into V-8 juice broth. The silage strain grew well upon transfer from the media containing both two and four per cent bile salts. The rumen strain failed to grow upon subculture.

Since these organisms did not produce acid in litmus milk, this indicated that skim milk would not be a suitable medium for determining the ratio of fixed to volatile acids produced by them. Instead, a fermentation medium of the following composition was used: V-8 juice (filtered through gauze), 200 milliliters; tryptose (Difco), 5 grams; tryptone (Difco), 5 grams; yeast extract (Difco), 5 grams; dextrose (Difco), 50 grams; and distilled water, 800 milliliters. The pH was adjusted to 6.8-6.9 with sodium hydroxide and the medium autoclaved at 15 pounds pressure for 15 minutes. Five hundred milliliter flasks containing 400 milliliters of the medium were inoculated with 1.5 milliliters of a heavy growth of the organisms grown in V-8 juice broth and the cultures were incubated at room temperature for two weeks. At the end of this time total and volatile acids were determined. The same determinations were made on a portion of the medium reserved as a control. Total acidity was arrived at by titrating a 10 milliliter portion of the culture with 0.1 normal sodium hydroxide, using phenolphthalein as the indicator. A 250 milliliter portion of the culture was steam distilled, and 100 milliliters of the liter of distillate collected was titrated for volatile acidity. Using the data obtained from this experiment, it was calculated that of the total acid produced by the rumen strain, 18.3 per cent was volatile

acid; 24.5 per cent of the total acid produced by the silage organism was volatile with steam.

The V-8 juice medium described above was also used to determine the type of lactic acid produced. Five hundred milliliter portions of the medium were dispensed into one liter flasks, five grams of calcium carbonate added, and the mixture autoclaved at 15 pounds pressure for 15 minutes. Heavy inoculations were made from actively growing cultures of organisms in V-8 juice medium. After two week's incubation at room temperature, lactic acid was recovered from the fermented mixtures and the optical activity determined.

A 250 milliliter portion of the culture was acidified with concentrated sulfuric acid to a pH of below 2.0, using thymol blue indicator. The calcium sulfate precipitate was removed by filtration with the aid of suction. The filtrate was steam distilled until at least four times the original volume was collected as distillate. The residue was then extracted with diethyl ether for 24 hours in a continuous extraction apparatus to recover the lactic acid present. Water was then added to the ether extract and the ether evaporated off on a steam bath. Chemically pure zinc carbonate was added in excess, the solution boiled for 10 minutes, filtered, and the filtrate treated with a small amount of activated charcoal and again filtered. The filtered solution was evaporated over boiling water until crystals of zinc lactate began to form. Ethyl alcohol was added to give a concentration of 50 per cent and the mixture was placed in the refrigerator over night to complete crystallization. The zinc lactates were then filtered with suction, washed—first, with 95 per cent alcohol and then with ether—and air dried. Samples of the

crystals were weighed, dried over calcium chloride for 24 hours, reweighed, and then dried in an oven at 110 degrees centigrade for eight hours. The samples were then weighed to determine the water of crystallization. The per cent of water lost, as well as the crystalline form of the salt and its solubility, indicated inactive lactic acid. Solutions of the salts were inactive in the polarimeter. Both organisms had produced inactive lactic acid.

A previous attempt to extract sufficient lactic acid from the steam distillation residues by shaking them with three changes of ether had proved unsuccessful. Therefore, the longer, continuous ether extraction was necessary to obtain the quantity of zinc lactates needed for characterization of the acid.

Carbon dioxide is one of the metabolic end-products of heterofermentative lactobacilli. It is usually necessary, however, to provide special conditions for the detection of this product. The first study of gas production used the glucose-nutrient gelatin medium of Gibson and Abdel-Malek (14) supplemented with V-8 juice instead of tomato or cabbage juice as they recommended. The complete medium was dispensed in test tubes, autoclaved for 15 minutes at 15 pounds pressure and then cooled to 45 degrees centigrade. Five cultures of each organism were prepared. After a heavy seeding from a young culture which had been grown in V-8 juice broth, the medium was overlayed with about two milliliters of two per cent melted agar. The cultures were incubated at 37 degrees centigrade. Gas production was evident in some of the cultures within 24 hours. After 48 hours all of the cultures had produced sufficient gas to raise the agar seals above the surface of the medium.

Both organisms were also tested for their ability to produce visible gas in the V-8 juice fermentation medium described for the volatile-fixed acid study. In one trial the medium was dispensed in 18 by 150 millimeter test tubes with 10 by 75 millimeter Durham tubes included for collecting gas. In the other trial the medium contained 1.5 per cent agar and was used as a deep sealed with two per cent plain agar. Both media were heavily inoculated with a young culture that had been grown in V-8 juice broth. Within 24 hours both strains of the organism were positive for gas production in both media. The solid medium showed cracks and disturbances in the agar and in some cases gas bubbles could be seen escaping past the agar seal. In the liquid medium the Durham tubes were half-filled with gas. This gas was completely absorbed upon the addition of a solution of sodium hydroxide to the cultures. Gas production tests were also positive in a V-8 juice agar medium to which the dextrose was added after the otherwise complete medium had been autoclaved.

Carbohydrate fermentations are used extensively in the classification of the lactobacilli, although there reportedly exists great variations in these traits. Both strains were tested for their ability to ferment 18 different carbohydrates. The basal medium contained the following ingredients per liter: Bacto Liver (Difco), 5 grams; tryptone (Difco), 5 grams; tryptose (Difco), 5 grams; yeast extract (Difco), 5 grams; agar, 1.5 grams; distilled water to make one liter. the pH was adjusted to 6.8-6.9 with sodium hydroxide and the medium dispensed into test tubes and autoclaved at 15 pounds pressure for 15 minutes. The following carbohydrates were autoclaved in 20 per cent solutions and added aseptically to the basal medium: dextrose, sucrose, lactose,

mannitol, salicin, trehalose, cellobiose, rhamnose, melibiose, raffinose and melezitose. Ten per cent solutions of maltose, fructose, mannose, arabinose and xylose were sterilized by Seitz filter and added aseptically to the basal medium. Starch and glycerol were added directly to the medium before autoclaving. All carbohydrates were added in a quantity to give a final concentration of one per cent and the finished media were incubated at 37 degrees centigrade for a minimum of 48 hours to assure their sterility. To detect pH changes, brom cresol purple indicator was added to half of the media at the time of preparation. To offset the possibility that the indicator might have an inhibitory effect upon the growth of the organisms, the other half of the media contained no indicator. The inoculum was 0.2 milliliter of a saline suspension of young, actively-growing cells from V-8 juice broth cultures. Each organism was tested with each carbohydrate in quadruplicate, two tests being carried out in the media with added indicator, and two in media without indicator. The cultures were incubated for a total time of two weeks, the first week at 37 degrees centigrade and the second week at 32 degrees centigrade. The cultures were observed daily for the first week and the day on which both tubes containing the indicator showed a definite color change was noted. At the end of the second week the pH of all the cultures not containing the indicator was determined, using a Beckman glass electrode pH meter. All of the cultures showed good growth and the pH determinations of the duplicate cultures were in good agreement. Growth was consistently more luxurious in media containing readily fermentable carbohydrates. The results of the carbohydrate fermentation study are summarized in Table III.

TABLE III. Fermentation of Carbohydrates

Carbohydrate Tested	Rumen Strain (final pH)	Silage Strain (final pH)
Basal	- (6.9)	- (6.9)
Arabinose	- (6.7)	- (6.8)
Cellobiose	- (6.7)	- (6.8)
Dextrose	+ (3.8)[3]	+ (4.1)[2]
Fructose	+ (4.3)[1]	+ (4.4)[1]
Glycerol	- (6.9)	± (6.3)
Lactose	+ (5.3)	- (6.8)
Maltose	+ (4.9)[4]	+ (4.2)[1]
Mannitol	- (6.8)	+ (5.5)
Mannose	+ (5.4)	- (6.9)
Melezitose	- (6.9)	- (6.8)
Melibiose	+ (4.0)[3]	+ (4.5)[2]
Raffinose	+ (4.2)[2]	- (6.8)
Rhamnose	- (6.7)	- (6.8)
Salicin	- (6.6)	- (6.9)
Starch	- (6.8)	- (6.9)
Sucrose	+ (3.8)[1]	- (6.7)
Trehalose	+ (4.0)[3]	- (6.8)
Xylose	- (6.6)	+ (3.8)[1]

Superscript indicates day on which indicator showed definite change.

Because of the growing use of antibiotic-supplemented feeds, it seemed desirable to screen these organisms with several of the common antibiotics. Bacto-Sensitivity Disks (Difco) containing dihydrostreptomycin, penicillin, terramycin, chloromycetin and aureomycin in high concentration were placed on plates of V-8 juice agar which had been seeded with the organisms just prior to pouring. The silage strain was resistant to dihydrostreptomycin and terramycin, while the rumen organism was resistant to dihydrostreptomycin only. Both organisms showed zones of inhibition about the other antibiotic-containing disks. Conclusions can not be drawn as to the relative sensitivity of the organisms to the antibiotics tested, since they were not subjected to the low and medium concentrations.

DISCUSSION AND CONCLUSIONS

The classification of these organisms with the aid of existing keys poses some problems. The silage organism fits easily into the genus *Lactobacillus*. The organism from the rumen passes all the tests for admission to the genus also, with one exception--it reduces nitrate to nitrite. The Seventh Edition of Bergey's Manual of Determinative Bacteriology (2) makes this exception for one member of the genus, *L. plantarum*, which reduces nitrates "under certain conditions". The particular strain being studied here is obviously not *L. plantarum*, since it is definitely a heterofermentative organism. Aside from its nitrate-reducing ability, this strain probably could be classified as *L. fermenti*. Rogosa, Wiseman, Mitchell, Disraely and Beaman (22) made some rather extensive studies in species differentiation of oral lactobacilli, the results of which were published in 1953. On the basis of their investigations they recognize 12 variants of *L. fermenti*. Comparing the carbohydrate fermenting activities of this rumen organism with that of the 12 varieties reported by them, it appears that it most closely resembles their Group Four variant. If growth at a temperature of 45 degrees centigrade is also taken into account, there is more similarity to Group Five characteristics.

The silage organism is not so easily classified on the basis of its fermentative abilities. It, too, is a heterofermentative organism. This strain does not ferment arabinose, which would seem to place it in the *L. fermenti* group also. However, it does not ferment sucrose, lactose or raffinose, which is characteristic of *L. brevis*, according to Bergey's Manual of Determinative Bacteriology (2). They report that this species

usually shows a particularly vigorous fermentation of arabinose. Rogosa, Wiseman, Mitchell, Disraely and Beaman (22) included *L. brevis* in their study also. The organism being studied here does not resemble any of their four variants in all of its fermentation characteristics. It frankly does not have all the fermentation characteristics to place it in any of the other three heterofermentative species. Disregarding its inability to ferment arabinose, it would seem that the silage organism most closely resembles *L. brevis*, as described in Bergey's Manual of Determinative Bacteriology (2).

It is possible, perhaps, to place too much emphasis upon finding a precise location for an isolate within the pre-existing taxanomic structure. If organisms must be classified, the framework of classification must be flexible enough to accommodate the new members, especially when the group is as widely distributed in nature as are the lactobacilli. The present accepted division of the genus recognizes 15 species, 11 of which are homofermentative and four that are heterofermentative. This is considerably more narrow than that found in other genera whose members are of wide natural distribution.

The admission of a number of new species to the group is probably not the answer. Perhaps it is necessary to find some clear-cut, stable characteristics which are typical of a group and define the conditions under which tests for these are carried out. The vitamin and amino acid requirements might be useful in conjunction with the present scheme of carbohydrate fermentations. It is to be hoped that the efforts of the various groups working on these problems may be correlated to bring about some satisfactory scheme for those who must attempt, at least, to classify

these organisms.

The lactobacilli have been given but little attention as rumen organisms. When Gall and Huhtanen (12) set up their five criteria for judging a true rumen organism, they listed anaerobiosis as the first essential. The second of the requirements was the presence of the organism in numbers of at least one million per gram of fresh rumen contents. Rumen organisms have, of course, all been introduced into their present location from some outside source. The ability to survive and multiply in their new location depends upon their ability to meet or adapt to the challenge of their new environment. The lactobacilli are intimately associated with ruminants, being well-known as inhabitants of soil, grain, plants and plant products, such as silages.

It was not possible to determine the actual numbers in which the rumen organism of this study was present in the original material. Jensen, Smith, Edmondson and Merilan (17), however, in their studies of rumen lactobacilli, reported various species present in numbers which varied from between ten to more than 10,000,000 per gram of rumen contents. The variation in numbers appeared to be dependent upon the medium used in the isolations. Their report summarized the investigation of 168 different isolates from rumen fluid.

A possible explanation for the lactobacilli being disregarded as true rumen inhabitants, is the emphasis that has been placed on the maintenance of strict anaerobic conditions when culturing rumen material. Though there have been anaerobic species of lactobacilli reported, the majority of the group are considered to be microaerophilic.

Laying aside the argument as to whether or not the lactobacilli

can survive and grow in the rumen, what might be the significance of such organisms to their host? There is the danger of assuming that *in vitro* findings can be transferred directly to an *in vivo* situation. There is especial danger when the entire environment is in a dynamic state, such as that of the rumen. The reactions which take place in the convenient laboratory test tube may be modified or completely changed by factors entirely unsuspected as yet.

Some general conclusions may be made as to the possible significance of the two organisms that have been studied here. Both are capable of producing considerable amounts of carbon dioxide and volatile acids, as well as lactic acid, from dextrose. Several other carbohydrates are also readily fermented by both organisms (Table III). The rumen strain appeared to function more effectively when anaerobic conditions were provided in the fermentation medium (Table II). The silage organism, on the other hand, seemed to prefer a less anaerobic environment. Starch would not be a suitable carbohydrate source for these bacteria; they would have to depend upon associated organisms for the amylase to release for them fermentable substrates. Urea does not seem to be an adequate nitrogen source. The influence of the B vitamins on these organisms is reflected in their acid-producing response to media containing yeast extract and dextrose and/or liver extract and dextrose (Table II). Plant products seem to supply their nutritional requirements quite adequately, judging by the growth obtained in a medium consisting of only diluted V-8 juice. Phytone, a commercial soy bean preparation, also promoted luxuriant growth.

Under the conditions of the *in vitro* tests, these organisms are

inhibited by certain of the antibiotics which are used in animal feeding, and are not inhibited by others. To what extent this is true *in vivo* can not be ascertained without more extensive tests.

Lewis (20) has reported the conversion of nitrate to ammonia in the rumen, with nitrite being one of the probable intermediates. Large amounts of nitrate in a ration could lead to an accumulation of nitrite in the rumen, its absorption, and methemoglobinemia as a consequence. A nitrate-reducing strain of lactobacilli, such as the rumen strain studied here, might be a contributing factor in such a chain of events.

Such lactobacilli, found in sufficient numbers in the rumen, may be effective in converting soluble carbohydrates into fatty acids and carbon dioxide. They are probably dependent upon associated organisms for their vitamin requirements and a supply of fermentable carbohydrate, but in turn, they provide for them, and their host, necessary fatty acids.

LITERATURE CITED

1. Bauman, H. E., and Foster, E. M. Characteristics of organisms isolated from the rumen of cows fed high and low roughage rations. Jour. Bact. 71:333-338. 1956.

2. Breed, R. S., Murray, E. G. D., and Smith, N. R. Bergey's Manual of Determinative Bacteriology, Seventh Edition. The Williams & Wilkins Co., Baltimore, 1957.

3. Briggs, C. A. E. Viable bacteria in the rumen. Dairy Sci. Abst. 17:714-722. 1955.

4. Bryant, M. P., and Burkey, L. A. Cultural methods and some characteristics of the more numerous groups of bacteria in the bovine rumen. Jour Dairy Sci. 36:205-217. 1953.

5. Cole, H. H., Huffman, C. F., Kleiber, M., Olson, T. M., and Schalk, A. F. A review of bloat in ruminants. Jour. Animal Sci. 4:183-236. 1945.

6. Conn, H. J., and Pelczar, M. J., Jr. Manual of Microbiological Methods. McGraw-Hill Book Company, Inc., New York, 1957.

7. Costilow, R. N., and Humphreys, T. W. Nitrate reduction by certain strains of Lactobacillus plantarum. Sci. 121:168. 1955.

8. Dain, J. A., Neal, A. L., and Dougherty, R. W. The occurrence of histamine and tyramine in rumen ingesta of experimentally overfed sheep. Jour. Animal Sci. 14:930-935. 1955.

9. Doetsch, R. N., and Robinson, R. Q. The bacteriology of the bovine rumen: a review. Jour. Dairy Sci. 36:115-142. 1953.

10. Doetsch, R. N., Robinson, R. Q., and Shaw, J. C. Techniques employed in cultural investigations of the bacteriology of bovine rumen contents. Jour. Animal Sci. 11:536-544. 1952.

11. Ford, J. E., Perry, K. D., and Briggs, C. A. E. Nutrition of lactic acid bacteria isolated from the rumen. Jour. Gen. Microbiol. 18:273-284. 1958.

12. Gall, L. S., and Huhtanen, C. N. Criteria for judging a true rumen organisms and a description of five rumen bacteria. Jour. Dairy Sci. 34:353-362. 1951.

13. Gall, L. S., Stark, C. N., and Loosli, J. K. The isolation and preliminary study of some physiological characteristics of the predominating flora from the rumen of cattle and sheep. Jour. Dairy Sci. 30:891-899. 1947.

14. Gibson, T., and Abdel-Malek, Y. The formation of carbon dioxide by lactic acid bacteria and *Bacillus licheniformis* and a cultural method of detecting the process. Jour. Dairy Sci. Res. 14:35-44. 1945.

15. Gilroy, J. J., and Doetsch, R. N. Nutritional requirements of rumen bacteria. Jour. Dairy Sci. 39:941. 1956.

16. Hungate, R. E., Dougherty, R. W., Bryant, M. P., and Cello, R. M. Microbiological and physiological changes associated with acute indigestion in sheep. Cornell Vet. 42:423-449. 1952.

17. Jensen, R. G., Smith, K. L., Edmondson, J. E., and Merilan, C. F. The characteristics of some rumen lactobacilli. Jour. Bact. 72:253-258. 1956.

18. Mann, S. O., and Oxford, A. E. Studies of some presumptive lactobacilli isolated from the rumens of young calves. Jour. Gen. Microbiol. 11:83-90. 1954.

19. McNeill, J. J., Doetsch, R. N., and Shaw, J. C. Some nutritional requirements of bovine rumen bacteria. Jour. Dairy Sci. 37:81. 1954.

20. Lewis, D. The metabolism of nitrate and nitrite in the sheep. I. The reduction of nitrate in the rumen of the sheep. Biochem. Jour. 48:175-180. 1951.

21. Rodwell, A. W. The occurrence and distribution of amino-acid decarboxylases within the genus *Lactobacillus*. Jour. Gen. Microbiol. 8:224-232. 1953.

22. Rogosa, M., Wiseman, R. F., Mitchell, J. A., Disraely, M. N., and Beaman, A. J. Species differentiation of oral lactobacilli from man including descriptions of *Lactobacillus salivarius* nov spec and *Lactobacillus cellobiosus* nov spec. Jour. Bact. 65:681-699. 1953.

23. Wasserman, R. H., Seeley, H. W., and Loosli, J. K. The physiology and nutrition of a rumen lactobacilli. Jour. Animal Sci. 12:935-936. 1953.