A study of a few of the bacteria in the rumen of sheep
by Harvey C Carlson

A THESIS Submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of Master of Science in Bacteriology at Montana State College
Montana State University

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A procedure whereby samples of rumen contents can be cultured in an agar medium under a carbon dioxide environment was employed for isolating organisms in high dilutions from sheep with artificial fistula. Various morphological and physiological tests were performed on two micrococcus-like bacteria and two gram positive obligately anaerobic filamentous Organisms that divided into short rods after prolonged incubation; A representative from each of these two morphological groups was selected and total acid, total volatile acid, and gas production were determined. Bine months later, attempts were made to isolate similar organisms from' another sheep. The gram positive micrococcus-like organisms were present in both animals but the rod shaped bacteria wore not found in the second sheep. Possible reasons for this are discussed.

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ABSTRACT

The importance of the rumen or fore-stomach in the digestion of food material by the cow and sheep is discussed with special emphasis on the bacteria indigenous to this organ. Mention is made of the breakdown as well as the synthesis of compounds in the rumen by these organisms. A procedure whereby samples of rumen contents can be cultured in an agar medium under a carbon dioxide environment was employed for isolating organisms in high dilutions from sheep with artificial fistula. Various morphological and physiological tests were performed on two micrococcus-like bacteria and two gram positive obligately anaerobic filamentous organisms that divided into short rods after prolonged incubation. A representative from each of these two morphological groups was selected and total acid, total volatile acid, and gas production were determined. Nine months later, attempts were made to isolate similar organisms from another sheep. The gram positive micrococcus-like organisms were present in both animals but the rod shaped bacteria were not found in the second sheep. Possible reasons for this are discussed.

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INTRODUCTION

One of the most interesting relationships in biology is the ability of two forms to live together in such intimacy that a separate existence of the two is not found in nature. A prominent example of this association is the symbiotic relationship of the bacterial flora present in the fore-stomach or rumen of the Ruminantia. It was the purpose of this study to investigate the microflora present in the rumen of sheep at Montana State College and learn something of the types of microorganisms present and the role which they play in the rumen.

REVIEW OF LITERATURE

Shalk and Amadon (1928) in an early work on the physiology of the bovine stomach have shown that this organ is quite voluminous, having a capacity of 20 to 40 gallons depending upon the size of the animal. This stomach is compound with four cavities or chambers present. These include the reticulum, the rumen, the omasum and the abomasum. They point out that of the four compartments, the abomasum is the true stomach and the only one containing digestive glands. The reticulum and the rumen are really not separate compartments but one large fermentation vat whose contents represent about one-fifth to one-fourth of the total weight of the animal. Food arriving from the mouth through the esophagus reaches the rumen where it is thoroughly mixed by the churning action of the powerful rumen walls. This food material is held in the rumen for varying periods of time until it is regurgitated to the mouth for remastication. The storage of the

food material in the rumen and the subsequent second mastication by the molars results in a finely divided mixture which is easily digested by the enzymes in the lower levels of the digestive tract. From the rumen this finely divided ingesta passes into a smaller compartment, the omasum or third stomach. In this compartment the coarse particles remaining from the preceding rumination are triturated or rubbed to pieces by the moving action of the numerous folds of laminae lining the walls. The liquid and more finely divided portion of the food material passes into the abomasum where digestion by the stomach enzymes takes place.

Early students of the nutrition of the ruminant animals felt that the rumen served only as a storage chamber for food awaiting further mastication before passage to the glandular stomach. Ritzman and Benedict (1938) stated that, "The function of the organ (the rumen) is of a purely mechanical character," and "the greater efficiency of the ruminant in digesting coarse fodder is due to provision of a mechanical rather than a chemical nature."

Hastings (1944) disagreed with Ritzman and Benedict and suggested that in addition to a mechanical action, a chemical action was also being carried out within this organ by various microorganisms present. He advised one to observe a drop of liquid from the rumen under high magnification and notice the vast variety of microorganisms, both protozoa and bacteria, that are present. In support of his suggestion he also listed several factors that would enable a population of microorganisms to be maintained in the rumen. First of all, the food intake is largely

carbohydrate, an excellent medium for microorganisms, and this intake is at regular intervals. Secondly, the large volume of saliva entering the rumen from the mouth keeps the hydrogen ion concentration at a point favorable for the growth of bacteria and protozoa. The saliva of the ruminants contains a high percentage of $NaHCO_3$, which neutralizes the acids formed by microbial action on the carbohydrates present. Thirdly, the walls of the rumen are in constant motion which prevents the stagnation of any part of the contents and allows the environment to become controlled throughout. Hastings points out that the rumen is serving a much more important function than merely a mechanical nature by harboring and aiding in the multiplication of vast numbers of microorganisms. These rumen microorganisms attack the food material which is in various stages of mechanical disintegration and through the action of their cellular enzymes, act by beginning to digest portions of this material before it reaches the glandular stomach. A symbiotic role thus exists between the ruminant animals and the microflora and microfauna of their digestive tract. The ruminant provides the organisms with their substrate and in return part of the carbohydrate of the ruminant feed is digested by the organisms. It would appear that the cow and the sheep therefore possess a more efficient digestive system, because of the several levels of food breakdown and digestion, than other animals which depend upon the digestion of food in the stomach or intestines alone.

Since chemical changes have been found to occur in the rumen, the emphasis in ruminant digestion has shifted to a more thorough study of the

microorganisms present and the part these organisms play in these changes. The interest has centered mainly in the types of organisms present and in their function within the rumen. Various procedures have been developed to obtain samples of rumen contents but the most successful has been the use of the rumen fistula (Shalk and Amadon, 1928, and Quin et al, 1938). The use of fistulated animals enables the investigator to remove samples from the animal while keeping the rumen and its contents intact for future study.

The types and numbers of microorganisms present in the rumen of cattle and sheep have been described by several investigators. Hastings (1944) has estimated that 10 per cent of the insoluble matter in the rumen consists of bacteria. Gall (1946), Gall et al (1947, 1948) placed the number of microorganisms present in the ruminants that she examined at about 100 billion per gram of fresh rumen contents. Van der Wath (1948) developed a direct microscopic method for counting microorganisms of the rumen, but adds that these counts (1-2 billion per ml.) are not necessarily true total counts, since an unknown percentage of organisms penetrate into or become attached to food particles. Gall observes the wide disagreement in the results of the two observations and feels that, since in her experience rather drastic shaking was necessary to free the bacteria from the food particles, there is a serious limitation in van der Wath's technic. Using an agar dilution series, Hungate (1947), calculated that the original inoculum he used from the rumen of a cow contained 40-60 million cellulose digesting bacteria per ml. Hungate's work was concerned chiefly with

cellulose digestion so the number reported does not represent a total bacterial count of the rumen examined. According to Bortree et al (1946) there is a rapid increase in the numbers of organisms present in the rumen within 2 hours after the animals have been fed and these high counts are maintained or increased for several hours, after which the numbers gradually return to the range observed prior to feeding.

Many investigators have felt that before a true picture of the assimilation of carbohydrates and protein substitutes in the rumen can be obtained, a knowledge must be had of the functional activities of the rumen microorganisms. Several attempts to obtain part of this knowledge by direct microscopic observation of the characteristics of the normal rumen microflora and microfauna have been made.

Baker (1942) found that rumen contents obtained from slaughterhouse material and from fistulated animals always contained protozoa of the families Ophryoscolecidae and Isotrichidae and a variety of bacteria which stained blue when treated with iodine. These were (1) Oscillospira guilliermondi Chatton and Perard, a colorless sporeforming oscillarian; (2) a giant spirillum divided internally by transverse septa into spherical or ovoid compartments; (3) large Sarcina; (4) a navicular bacterium forming rosettes of 5-30 organisms; and (5) chains of cocci.

Hungate (1943, 1946) obtained from the rumen, species of protozoa belonging to the genera Diplodinium, Isotricha and Entodinium. He showed by culturing these organisms in vitro that they are capable of digesting cellulose.

Phillipson (1946), studying the microflora and microfauna of the rumen of calves, found (1) ciliated protozoa which are indigenous to the rumen; (2) pseudo yeasts which he believed to be Schizosaccharomyces ovis; (3) iodophillic bacteria; and (4) propionic acid bacteria. The finding of a yeast is of interest because Quin (1943) has indicated that S. ovis plays a dominant role in the rapid evolution of gas in the fore-stomachs of ruminants after ingestion of lucerne and hence may be an important factor in the pathogenesis of bloat when on this feed.

Pounden and Hibbs (1948a, 1948b) studied the influence of ration on the morphological type of microorganisms present in the rumen of cattle, and observed a change in microflora when the animals were taken off a predominantly grain ration and put on one predominantly hay. Their "hay" and "grain" flora were:

Hay flora:	Group I	Quite large gram positive cocci in closely knit pairs.
	Group II	Large gram positive, thick, fairly square-ended rods.
		Very large gram negative cigar-shaped rods.
		Smaller gram negative short rods in fours or multiples of four.
Grain flora:	Group I	Medium sized, comparatively thin, gram positive rods (somewhat granular staining and of variable length).
	Group II	Gram negative rods resembling coliform bacteria.

From the evidence presented, the following picture takes form as we assemble the facts about the population of the rumen. Two types of microorganisms are always present, protozoa and bacteria of various kinds. The

protozoa were at one time assigned an important role in ruminant digestion until defaunating experiments with $CuSO_4$ showed that digestion of the protein and carbohydrate constituents was not seriously impaired when all protozoa were removed (Becker, 1930, 1932). The most predominating characteristic of the bacteria as observed microscopically is their ability to give a blue color when stained with iodine. Baker (1942) has explained this as being due to the presence within the bacteria of "bacterial starch" or granulose. All of the major morphological groups of bacteria are found in the rumen. The most predominating shapes are rods and cocci which may be either gram positive or gram negative. Comparing the work of Pounden and Hibbs, Phillipson, and Baker, the possibility exists that there is never a "normal" rumen flora and fauna but that these vary with diet, different animals, and geographical location.

In addition to the above work emphasizing the flora of the entire rumen, certain investigators have isolated single organisms from the rumen and studied these organisms *in vitro*. Hopffe (1919) isolated from the rumen, a cellulose digesting *Aspergillus*, which she named *Aspergillus cellulosae* Hopffe. Pochon (1934, 1935 and 1941) cultured from the ox, sheep, antelope, and water buffalo a cellulolytic bacterium which he named *Plectridium cellulolyticum* Pochon. At first, it was very exacting in its growth requirements but by a process of gradual acclimation, it acquired the ability to grow in a semi-synthetic medium. Hungate (1944) isolated *Clostridium cellobioparus* Hungate from the rumen of cattle. This organism was easily isolated and grew well in both glucose and cellulose media. The

growth requirements appeared to be satisfied by an inorganic medium with the addition of biotin and a carbohydrate. Even after four years of pure culture, no loss of cellulose digesting capacity was observed.

Hungate (1947) also isolated ten strains of cellulolytic rumen bacteria from cattle. Of these, four strains were gram negative cocci and six strains were gram negative rod-shaped bacteria. They stained readily with carbol fuchsin but not with methylene blue. He believed that for the present it was preferable to delay naming the organisms until their characteristics could be more completely studied. With later studies on one of the rod-shaped strains, Hungate (1950) felt that this bacterium could be named Bacteroides succinogenes because it possessed the characteristics of the genus Bacteroides and the ability to produce large amounts of succinic acid.

Sijpesteijn (1948) obtained from the rumen of cattle two strains of gram negative, anaerobic oval bacteria to which she assigned the name Ruminobacterium parvums. This bacterium grew well on an inorganic medium with a pH range of from 6.0 to 6.9. At the same time, a cellulose-digesting streptococcus was isolated from the rumen and named Ruminococcus flavefaciens Sijpesteijn. In her experimentation, she observed that R. flavefaciens would develop better if grown in the presence of a synergistic bacterial species, Clostridium sporogenes. All her tests with this organism were subsequently carried out with a mixed culture. It would seem that this author has been careless concerning the rules of taxonomy by creating a new genus and species based solely on the results from impure cultures.

The reactions obtained from a mixed culture could never be as valid for naming an organism as those carried out on a single pure culture.

A review of the literature indicates that the greatest amount of investigation has been concerned with the rumen bacteria that attack cellulose. Since, as Norman and Fuller (1942) show, cellulose is the major structural constituent of all plant material and probably the most abundant single organic compound known, this emphasis on the digestion of cellulose by microorganisms present in the rumen is understandable. However, as Hungate (1947) points out, there are at least 1000 non-cellulose fermenting bacteria to every cellulose fermenter. Very important reactions other than cellulose digestion are taking place in the rumen and these are also being carried out by the numerous microorganisms present in this symbiotic relationship. The various substrates are broken down by microorganisms and the products from this decomposition (volatile and non-volatile acids) are utilized not only by the ruminants but also by other bacteria present to synthesize numerous compounds. Some of these, carbon dioxide and methane, are lost from the digestive system, but many others are utilized by the host after further digestion. Wegner et al (1941) showed that six vitamins of the B complex, namely, thiamine, riboflavin, nicotinic acid, pantothenic acid, pyridoxine, and biotin, are synthesized in the rumen of cattle fed normal diets. Phillipson (1946) demonstrated the synthesis of various members of the B complex vitamins as well as starch and glycogen. Owen (1947) points out that the rumen bacteria synthesize protein from ammonium nitrogen in the rumen and that they also store carbohydrate in the form of

bacterial polysaccharide.

The nutrition of the ruminant closely parallels rumen bacterial nutrition and continued investigation into the bacteria present as well as their metabolism is required before a true picture can be obtained concerning the more efficient feeding of the common domestic ruminant animals.

MATERIALS AND METHODS

The ruminant used in this study was a Rambouillet ram generously furnished by the Department of Animal Industry at Montana State College. Sheep were chosen for this study because of their convenient size and availability and because of their importance in the economy of Montana. During the entire period of experimentation the ram was on a diet of molasses treated grain, some hay, and free access to green pasture and running water.

In order to study effectively the microflora of the rumen it is desirable to have an opening in the side of the animal through which experimental material can be obtained. A rumen fistula was established in the ram using the procedure recommended by Quin et al (1938). The required surgery was performed by Dr. Hadleigh Marsh and Dr. Lee Seghetti of the Montana State College Veterinary Research Laboratories. In place of Quin's ebonite fistula tube, a lucite tube with a threaded cap was substituted. The cap allowed the environment of the rumen to be maintained similar to that of a normal intact rumen by preventing the loss of rumen contents and gases. Rumen contents were obtained by removing the cap from the plug and inserting a length of plastic tubing fitted with a suction bulb

through the hole and into the rumen. The bulb and tubing were flushed with rumen fluid 2 or 3 times to insure anaerobic collections. The more fluid portion of the rumen contents was obtained by drawing the liquid into the bulb and discharging it into a test tube which was filled to the brim with the fluid and closed with a rubber stopper. All of these procedures were carried out with a minimum of exposure to air since it has been found that many rumen microorganisms are rapidly killed in the presence of oxygen.

To duplicate the anaerobic environment of the organisms in the rumen, it was advisable to maintain strict anaerobic conditions while culturing the microorganisms in vitro. Anaerobic conditions were maintained throughout all of the dilution and culture procedures. The sterile water blanks used for preparing the dilution and the tubes of media used for culturing were flushed with oxygen-free carbon dioxide both before and after inoculation. The carbon dioxide was made oxygen free by passing it through a chromous acid solution (Stone and Beeson, 1936; Hungate, 1950). It was found advisable to have the carbon dioxide pass through two bottles of the oxygen absorbent and to equip the gas intake in the first bottle with a sintered glass gas diffuser in order to produce a greater gas surface to the chromous acid solution. The absorbent required replacing approximately once a month.

The first dilution of the rumen fluid was made by adding 1 ml to a sterile 99 ml water blank which had been flushed with carbon dioxide. The water blank was shaken vigorously until the contents were thoroughly

mixed. One milliliter of this 1:100 dilution was transferred to a similar 99 ml water blank to give a 1:10,000 or 10^{-4} dilution. This procedure was repeated until 10^{-6} and 10^{-8} dilutions were obtained. The inoculations into the culture media were made from the 10^{-2}, the 10^{-4}, the 10^{-6}, and the 10^{-8} dilution bottles.

The medium used for the initial culture of the organisms was developed by Gall (1946) and was composed of the following ingredients:

Peptone	1.0%
Yeast extract	1.0%
Beef extract	1.0%
Tryptone	1.0%
Glucose	1.0%
K_2HPO_4	0.5%
Agar	1.5%
Distilled water	

The medium was prepared and autoclaved at 120°C for 15 minutes in an Erlenmeyer flask. At the same time, a can containing 6 x 5/8" pyrex test tubes and the required number of rubber stoppers (No. 0) placed in a deep petri dish was sterilized. Just after the autoclave door was opened, the test tubes were sealed with the rubber stoppers. Using care, the tubes could be closed without incurring contamination.

The medium in the flask was kept anaerobic while cooling by introducing carbon dioxide through a straight pasteur pipette. When the agar had reached a temperature below 60°C, enough sodium bicarbonate, which had been sterilized by filtration to prevent thermal decomposition, was added to make a concentration of 0.0375 per cent. This combination of carbon dioxide and sodium bicarbonate buffered the medium at a pH of about 6.0.

The pasteur pipettes were prepared by plugging each end of a 10 cm glass tube with cotton and autoclaving in a metal container. At the time of use, the required number and size of pipettes were drawn out by heating in a flame.

The next step was to transfer the medium from the Erlenmeyer flask into the test tube. The stopper and top of the test tube to be filled were flamed. With the test tube placed between the palm and the third and fourth fingers of the left hand, the rubber stopper was removed with the thumb and first finger of the same hand. Just prior to and during the addition of the medium, the tube was flushed with carbon dioxide to replace the oxygen and other gases present. A pasteur pipette employed for this operation was long enough to extend at least half way into the tube. The pipette had been bent to an angle of about 30° and was sterilized by passing through a bunsen flame.

About 10 ml of medium was added to the tube. This quantity covered the tip of the pasteur pipette resulting in the formation of large carbon dioxide-filled bubbles. As the agar began to bubble out, the rubber stopper was replaced securely. This precaution insured the complete exclusion of oxygen and an atmosphere of carbon dioxide. This procedure was repeated until all tubes were filled. The tubes of medium were placed in a 50°C water bath to keep the agar melted until time to inoculate.

Tubes of media were inoculated with 0.1 or 1.0 ml aliquots of the 10^{-2} through 10^{-8} dilutions of rumen fluid, giving the following dilution series in the agar test tubes: 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8},

and 10^{-9}.

The inoculated tubes were rapidly cooled under running cold water. By repeatedly inverting them during this process the medium was kept flowing over all inner surfaces of the tube and in constant motion until just before the agar was ready to set. The tubes were then momentarily inverted for the last time, forming a tight seal of medium around the stopper and leaving on the upper portion of the tube a thin film of agar medium which almost instantly solidified. The tubes were held stationary in a slanting position under the cold water tap until the entire tube of medium had set. This gave all degrees of thickness of the agar layer, varying from the solid butt to layers only a fraction of a millimeter thick on the upper portions. Considerable experience with this technique was necessary before uniform results could be obtained. The cultures were then incubated at 37°C until growth appeared.

Colonies appeared throughout the medium. Representative colonies were picked to fresh tubes of sterile media. It was convenient to choose those colonies that developed in the thin upper portions of the tube. These could be picked with less chance of including contaminants. Picking from this thin agar was comparable to picking from a plate. The rubber stopper was carefully loosened and then removed by the palm and fourth finger of the right hand. The selected colony was picked using a sterile pasteur pipette, the tip of which had been bent at a 90° angle and broken off squarely about 5 mm below the bend. Gentle suction was applied to the pipette through a rubber mouth tube as the growth was picked. The contents

of the pipette were discharged into another agar series or into a broth medium.

EXPERIMENTAL RESULTS

As has been indicated by many investigators, the population of the microflora of the rumen is very large and quite varied. How much of this population is indigenous and how many are merely "transients" which were ingested with the feed is not known. One must be aware that any single organism obtained from the rumen would therefore not necessarily be performing an important function in the rumen but instead might be a transient which had found a temporarily favorable environment for growth.

To make sure that any organisms obtained from the ram were not just daily transients, it was advisable to obtain cultures of these organisms on various days extending over a period of a week. The inoculation and culture of the organisms on Gall's medium were made according to the previously described procedure. Representative colonies of the organisms present were transferred to Gall's broth and Gall's agar and retransferred until pure cultures were assured. When isolated colonies were selected for picking, a pure culture was usually obtained within a relatively few transfers. Of the cultures isolated, representative types were selected for further study.

The colonies which developed in the agar series were generally lenticular in shape. The organisms forming these colonies were gram positive cocci. Other distinctive colonies found in smaller numbers exhibited a very fuzzy or filamentous appearance. The organisms were gram positive