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Gastrointestinal Bacteria Of A Healthy Sheep

Paul Berger

THE UNIVERSITY OF NEW MEXICO
ALBUQUERQUE, NEW MEXICO 87106

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GASTROINTESTINAL BACTERIA

OF A HEALTHY SHEEP

Title

Paul Berger

Candidate

Biology

Department

David T. Burdette

Dean

February 16, 1970

Date

Committee

John Adam Bailey

Chairman

Richard D. Hembree

Jack D. Teeter

C. Clayton Hoyt

GASTROINTESTINAL BACTERIA OF A HEALTHY SHEEP

By

Paul Berger
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THESIS

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ABSTRACT OF THESIS

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ABSTRACT

Various portions of the gastrointestinal tract of a healthy sheep were sampled to determine the types of bacteria indigenous to the different areas. These portions included the abomasum, duodenum, jejunum, ileum, cecum, ascending colon, spiral colon, and descending colon. A minimum test set technique was developed and used to identify the bacteria. No definite pattern emerged when one region was compared with another region, although the total bacterial count for both the abomasum and the duodenum was less than that of the other locations sampled. *Escherichia coli* and an infrequent *E. freundii* were the only enteric bacteria found in the gastrointestinal tract. *Streptococcus* species were plentiful, but no one type predominated. Both aerobic and anaerobic sporeformers were common, as were *Corynebacterium* species. Pleomorphic and filamentous anaerobes, identified as *Bacteroides* species, *Sphaerophorus* species, fusiforms, and *Lactobacillus bifidus*, were also common.

TABLE OF CONTENTS

LIST OF TABLES

INTRODUCTION

While a great deal of work has been performed investigating the role of bacteria in radiation injury in such small animals as mice, rats, and dogs, similar studies in larger animals are relatively rare. It is well established, however, that infection plays a prominent role in producing morbidity and mortality after exposure to radiation, except when the radiation doses are large enough to cause death within 2 or 3 days (Rubin and Casarett, 1968; Benacerraf, 1960; Donaldson, 1962; Bond, Fliedner, and Archambeau, 1965). Those organs and tissues which possess phagocytic cells of the reticulo-endothelial system such as the liver, spleen, and lymph nodes are especially prone to bacterial infection--both in large and small animals--after a substantial radiation dose (Gordon et al., 1955; Mobley and DeFeo, 1968).

Various studies using mice and rats have indicated that microbial flora indigenous to the lower gastrointestinal tract is responsible for postirradiation infections (Bradner, Bernstein, and McCarthy, 1955; Bond et al., 1965). Gordon et al. (1955) and others indicate that enteric bacteria are especially prominent in such infections. In order to determine if this is also true for larger animals, one would have to know, among other things, which organisms were causing postirradiation infections in these animals and which organisms were indigenous to the intestines. This investigation concentrates upon the latter point. More specifically, an attempt was made to determine the normal bacterial flora in various portions of the large and small intestines of sheep. Sheep were selected because they are mammals which have a body mass and dimensions which approach those of man. The minimum test set technique,

developed recently by several investigators (Gyllenberg, 1964; Rypka et al., 1967), was used to identify the bacteria isolated in this study.

REVIEW OF THE LITERATURE

Bacteriological Studies

Ruminants such as sheep have many physical characteristics in common with man. Of significance for radiation studies, they are mammals that have a body mass and dimensions approaching those of man. Therefore, in many respects, they should react to radiation in a manner similar to that of humans. There are some fundamental differences, however. Unlike man and most other mammals, the ruminant has four compartments making up the "stomach." The ruminant swallows the food quickly without mastication, and the food passes into the first and largest compartment, the rumen, which is a large fermentation tank. Later, boluses of food are quickly forced back into the mouth where they are ground into smaller portions and reswallowed. The food then returns to the rumen, and being lighter and more fluid that the unmasticated food, passes through the ruminoreticular fold into the second compartment, the reticulum. From there, the food flows directly into the third compartment, the omasum. Chemical digestion occurs in the fourth compartment, the abomasum, which is similar to the stomach of man. Only in this compartment does secretion of digestive juices occur (Dukes, 1955).

The food then passes into the intestines, which are roughly similar to man's. Unlike man, only half of the organic matter consumed enters the small intestines, the rest being absorbed from the stomach (Sineshchekov, 1965).

Bacteriological studies in sheep and other ruminants have been primarily confined to studies of the rumen. It has been studied because it is a natural microbial habitat with a relatively constant

environment and is more susceptible to analysis. In a practical sense, many investigators (e.g., Hungate, 1965) study the rumen in order to learn the best feeds and feeding practices for the domestic ruminant.

The rumen itself is well adapted for the growth and maintenance of certain types of microorganisms. There is a relatively constant supply of food and water and the temperature is nearly constant at 39 C (Bryant, 1959). The pH is usually slightly acidic, about 6-7 (Bryant, 1959; Garton, 1965; Slyter, Bryant, and Wolin, 1966), but sometimes has been found to be between 5.5 and 6.5, especially when the animal has been fed a concentrated diet (Warner, 1965). Anaerobic conditions prevail in the rumen, the redox potential being approximately -0.35 volts (Hungate, 1960).

Different methods have been developed to isolate rumen bacteria, which are fastidious in nutritional requirements and difficult to grow (Ifkovits and Ragheb, 1968; Allison, 1965). Very often rumen fluid is used to supply nutrients characteristic of the habitat, as many of the bacteria isolated from the rumen usually fail to grow in common laboratory media (Bryant, 1959; Hungate, 1960; Allison, 1965). Recently, however, media without rumen fluid has been developed (Caldwell and Bryant, 1966).

Species of microorganisms themselves are apparently unique to the rumen. According to Warner (1965), very few have been found elsewhere, although a thorough search for them has not been made. Most of them have only been found in the last 15 years (Allison, 1965). The total amount of microbial protoplasm is usually about 10% of the volume of the rumen fluid or about 10^{10}-10^{11} cells/g (Hungate, 1960; Warner, 1965), and the total concentration of these organisms is at a maximum

just after feeding and at a minimum just before (Warner, 1965). According to Warner (1965), nearly all organisms found in the rumen are natural to the rumen in a healthy animal eating usual rations. The type of ruminant apparently has little influence on the occurrence of different species of cellulolytic bacteria, which form the most important group of organisms in the rumen (Kistner, 1965).

Sheep, cattle, buffalo, and deer all have nearly the same species of rumen microbes (Kistner, 1965; Pearson, 1967). Kistner (1965) also reports that the nature and concentration of available nutrients are the most important factors influencing both the total numbers of bacteria in the rumen and the proportion of different species. For example, animals fed concentrated diets have lower numbers of bacteria in their rumens than do animals on roughage diets, possibly because the former produces more acidic conditions in the rumen. Other factors are also significant, however. According to Kistner (1965), Hungate found that there existed large differences in the relative proportions of these organisms in samples obtained on the same date from groups of animals receiving the same rations.

Strict anaerobes are by far the most abundant bacteria in the rumen. Very few facultative organisms have been found and even fewer aerobes (Hungate, 1960). Hungate (1950) stated that facultative organisms require a greater variety of enzymes than either strict aerobes or strict anaerobes; types which are encumbered by having genes necessary for the synthesis of both the anaerobic and unused aerobic enzymes may be at a disadvantage.

The rumen organisms, which include protozoa as well as bacteria (Willard and Kodras, 1967; Akkada et al., 1968; Hungate, 1960; Bryant, 1959), live in symbiosis with the ruminant. Several groups of organisms, for example, are able to synthesize the enzyme cellulase (Moir, 1965). Thus the ruminant, unlike other animals, can profitably consume in their diet cellulose, which is one of the most abundant organic substances known. Kistner (1965) maintains that a group of anaerobic cocci, the ruminococci, represents the most abundant group of cellulose digesters in the rumen. The genus *Ruminococcus*, which does not appear in Bergey's Manual (Breed, Murray, and Smith, 1957), includes anaerobic, nonmotile, nonsporeforming, gram-positive, gram-variable, or gram-negative cocci which ferment carbohydrates with the production of certain specific acids (Kistner, 1965; Bryant, 1959). Two species are currently recognized: *R. flavefaciens*, which is usually pigmented yellow, occurs mostly in chains, and produces succinic acid as a major end product; and *R. albus*, which occurs mostly singly or in pairs and produces little or no succinic acid.

Another important cellulolytic microorganism is *Bacteroides succinogenes*. This organism, which Hungate (1960) described initially, is a gram-negative, nonsporeforming, anaerobic rod which actively ferments cellulose with the formation of acetic and succinic acids. No growth occurs on a number of common culture media (Hungate, 1960).

The genus *Butyriovibrio*, also not found in Bergey's Manual (Breed et al., 1957), is also significant in the rumen and may also be an important cellulolytic organism. This genus includes small, anaerobic, nonsporeforming, monotrichous, gram-negative, curved rods which ferment

glucose with the production of butyric acid (Bryant, 1959; Kistner, 1965).

Besides cellulolytic activity, the symbiotic organisms are capable of synthesizing urea and various vitamins, thus making them available to the animal (Moir, 1965).

Besides the cellulolytic microbes, peptostreptococci, various *Bacteroides* species, and *Streptococcus bovis* are also important in the rumen. Somewhat less common anaerobes in the rumen include the lactobacilli, *Ramibacterium*, *Eubacterium*, *Lachnospirae*, fusiforms, *Selenomonas ruminantium*, *Methanobacter*, and *Clostridium*. Coliforms and *Bacillus* species were only found in small numbers (Bryant, 1959). The concentration of rumen protozoa ranged from 10^3-10^6/g, depending on the diet of the host (Hungate, 1960).

The metabolism of rumen organisms has been intensely studied. Blackburn (1965) found that less than 10% of the sheep rumen isolates were proteolytic. Besides cellulose digestion, the degradation of glycine has been studied (Wright and Hungate, 1967), as has been hemicellulose degradation (Dehority, 1967), xylan fermentation (Dehority, 1966), enzyme synthesis and distribution (Joyner and Baldwin, 1966), and many other chemical processes in the rumen.

Many types of bacteria found in soil, water, or food do not grow in the rumen, even though they might be expected to do so. One reason for this is that the rumen organisms produce antibiotics inhibitory to a number of saprophytic bacteria (Warner, 1965).

Once the food leaves the rumen and is reswallowed, it enters the reticulum and then the omasum. I am not aware of a single investigation concerning the bacteria in these two compartments. The last compartment through which the food must pass before entering the intestines is the abomasum or true stomach. When food enters, the abomasum secretes large amounts of gastric juice and maintains secretion until the acidity of the abomasal content reaches pH 1.9-2.8, when inhibition of secretion occurs (Hill, 1965). Thus, the digesta is very rapidly acidified.

The effect of this acidity on rumen and other organisms which may pass into the abomasum should be considered when anticipating the types of organisms in the intestines. Slyter et al. (1966) studied the effect of decreasing pH on the microbial population in the rumen. They found that a mixed culture maintained at pH 6.7 contained the types of bacteria often found in high concentrations in the rumen (e.g., *Butyrivibrio*, *Ruminococcus*, *Bacteroides* species), whereas the culture maintained at pH 5.0 did not. All organisms in the latter culture were nonmotile rods, mostly gram negative; many were unidentifiable although some *Bacteroides* species were found. At pH 5 the number of coliforms nearly doubled and no sporeformers were found. This study suggests that many types of common rumen microbes (e.g., *Butyrivibrio* and *Ruminococcus*) are sensitive to acid and few would survive passage from the abomasum into the intestines.

Published reports of attempts to isolate the normal intestinal flora of ruminants are meager, and these are usually confined to an examination of the bacterial flora of fecal specimens of these animals.

Kenner, Clark, and Kabler (1960) reported that streptococci were present in the feces of sheep in the amount of 38 million/g (wet weight). Enterococci accounted for 24.8% of the streptococci, *Streptococcus bovis* accounted for 40.0%, the *S. equinis* group 6.4%, and enterococcus biotypes 28.8%. No *S. salivarius* was found. While the results represented an average from five sheep, considerable variation in streptococcal densities among sheep did occur, an observation also made by Kjellander (1960). Deibel (1964) agreed that in general *Streptococcus bovis* constituted the predominant group of streptococcus in the feces of sheep. He stated, however, that *Streptococcus faecalis* and atypical *S. faecalis (S. faecium)* together constituted only 10% of the total streptococcal flora. Although Kjellander (1960) concluded that sheep have total counts of about one-half as many streptococci as man, Smith and Crabb (1961) found that these organisms were usually one of the most common groups found in sheep feces. The latter investigators also found that the total streptococcal count decreased as the sheep grew older.

While streptococci and coliforms were usually the most common bacteria in the feces of sheep, lactobacilli and *Bacteroides* were also found in much smaller numbers. Although not writing specifically about sheep or other ruminants, Dubos, Schaedler, and Costello (1963) stated that the enterics and enterococci represent only a very small part of the flora and that lactobacilli, *Bacteroides*, and fusiforms are more typical but have not been satisfactorily enumerated for lack of cultural techniques. Smith and Crabb (1961) found *Bacteroides* in 3 out of 10, and lactobacilli in 5 out of 10 healthy adult sheep,

although they discovered that these bacteria were far more common in the human and the dog. From ovine fecal samples, Firehammer (1965) isolated vibrios which were related to *Vibrio fetus* var. *intestinalis;* he suggested the name *Vibrio fecalis* for them. Anaerobic gram-negative cocci were isolated occasionally from the feces of young lambs by Smith and Crabb (1961), but they never found them in adult sheep. In a survey of 57 healthy sheep by Hoadley and McCoy (1968), no fecal sample contained *Pseudomonas aeruginosa*. *Staphylococcus aureus* was never isolated from any of the 10 healthy sheep examined by Smith and Crabb (1961).

In addition to studies on the fecal flora of sheep, a few studies have been undertaken to investigate specific groups of microorganisms in the intestinal contents of healthy sheep. English (1966) stated that *Clostridium perfringens* lives as a saprophyte under normal conditions in the lower intestine of all domestic animals, as Taylor and Gordon (1940) had observed for sheep. The latter investigators examined 23 intestinal samples consisting of a mixture of the contents of the large and small intestine and found that all of them contained *C. perfringens*, mostly of nonpathogenic types. Similar results were obtained for cattle and pigs. Smibert (1965) isolated *Vibrio fetus* var. *intestinalis* from the feces and intestines of normal sheep, and Roberts, Graham, and Egerton (1968), in their investigation of bulbar necrosis in sheep, presented evidence that *Fusiformis necrophorus* and *Corynebacterium pyogenes* are normal residents in the alimentary tract of sheep.

Numerical Analysis

In order to aid in the identification procedures, a modification of the numerical analysis technique generally used was employed. A number of investigators are attempting, with success, to put this recently developed technique on a firm, statistical basis (Ledley and Lusted, 1959; Lipkin, 1964; Warner et al., 1961; Beers and Lockhart, 1962). This technique involves the listing of some item such as a bacterial species and characterizing this item according to a number of other independent factors, such as morphological and biochemical characteristics. A computer is often employed with the resulting matrix to aid in the identification of an unknown.

Early work using this mathematical technique involved the diagnosis of disease (Lipkin, 1964). Often computers were used to produce a list of possible diagnoses for a patient by comparing his symptoms to the symptoms characteristic of certain diseases in the matrix. The computer could also be programmed to indicate further diagnostic tests which best distinguish among the most probable probabilities.

Although Dybowski and Franklin (1968) found that a recent Medical Literature Analysis and Retrieval System (MEDLARS) search produced 265 references since 1963 associated with numerical analysis, little work has been done in bacteriology. In 1964, Gyllenberg suggested an approach to numerical descriptions of microbial populations. In his report, he described a general method for determining the shortest route to the identification of an unknown bacterium by reducing the number of tests to be performed to a minimum. This method, known as the minimum test set, was further developed and refined by Rypka et al (1967).

Some criticism, however, has been leveled at the use of numerical analysis methods currently in use. Leifson (1966) claims that they are inadequate both in the definition of taxonomic categories, for which they are often used, and for identifying unknown cultures. Dybowski and Franklin (1968) conclude that the minimum test set procedure is incapable of recognizing a strain from outside its basic reference set; any such strain would be identified as the member of the reference set which most resembles it. Beers and Lockhart (1962) claim that not enough quantitative data are known to justify a diagnostic scheme at present, but ultimately numerical analysis will become an important diagnostic tool.

Because of its simplicity, speed, and convenience for studying large numbers of bacteria at one time, the minimum test set technique was used to identify bacteria. The approach has been modified, however, so as to reduce some of the drawbacks that are inherent in this system.

MATERIALS AND METHODS

Experimental Subjects

A female domestic sheep, a 3-year-old Columbia-Rambouillet cross, was used in this study. The sheep was purchased from a local breeder, and the animal was maintained in an outdoor pen with other sheep until several weeks before the experiment began, at which time it was transferred to an indoor pen. Once a day during the morning the animal was fed pellets consisting of alfalfa (70%), milo (20%), and molasses (10%). The experimental subject was clinically healthy, was free of demonstrable disease, and had been vaccinated against *Clostridium perfringens*.

Sampling Methods

The sheep was euthanized with an intravenous injection of heparin. Intestinal samples were quickly obtained by use of sterile cotton swabs and were collected in sterile tubes containing fresh Thioglycollate medium (Difco). Plating procedures were completed within 2 hours after euthanization. Samples were obtained from the abomasum, duodenum, jejunum, ileum, cecum, ascending colon, spiral colon, and descending colon.

Isolation Procedures

Each intestinal sample was streaked from the Thioglycollate medium onto four types of media:

a. Blood Agar plates consisting of Trypticase Soy Agar (BBL) and 5% fresh, citrated sheep blood

b. E.M.B. Agar plates (Difco)

c. Chocolate Agar plates consisting of GC Medium Base (BBL), Hemoglobin (BBL), and Isovitalex enrichment (BBL)

d. Blood Agar plates as above with 7.5 mcg/ml vancomycin added

All plates were streaked in duplicate to increase the opportunity of isolated colonies. One set of both Chocolate Agar plates and Blood Agar plates were incubated under aerobic conditions (increased CO_2 tension) and the other set under anaerobic conditions (using a BBL Gas-Pak). EMB Agar plates were only incubated aerobically and Vancomycin-Blood Agar plates only anaerobically. All plates were incubated at 35 C for approximately 24 hours.

Once this was completed, each different type of colony on each plate was picked into a tube of fresh Thioglycollate medium and incubated at 35 C under the appropriate oxygen conditions. At approximately 24 hours afterward, tubes were examined for growth. If no growth occurred, other methods were used in an attempt to initiate growth. If growth had occurred, the culture was gram stained. If pure, then identification procedures were initiated, using minimum test set techniques and the aid of a computer program. Those cultures which were not pure were streaked onto Trypticase Soy Agar or other appropriate medium for isolation; resulting colonies were transferred to Thioglycollate medium as before, and the process repeated.

Identification Techniques

A computer program was used as an aid in the identification procedure. This program was designed to list the bacteria which might normally be found in sheep and characterize each organism on the basis of about 45 biochemical and morphological tests. Each test was a "yes-no" type of test, e.g., either sucrose was fermented with the production of acid or it wasn't. A positive test and a negative test

were indicated by the numerals 1 and 0, respectively. A "2" either signified that the culture was capable of giving either result, depending on the strain or conditions at which growth occurred, or that there was confusion in the literature. A blank indicated that no information was found for that culture-test combination. Two similar matrices were formed--one for gram-positive, the other for gram-negative bacteria. Information about the characteristics of each microorganism was obtained chiefly from Breed et al. (1957), Cowan and Steel (1965), Bailey and Scott (1962), Wilson and Miles (1955), and Edwards and Ewing (1962). The taxonomic scheme used was derived mainly from Breed et al. (1957), although the Enterobacteriaceae were classified according to the scheme proposed by Edwards and Ewing (1962).

The computer, using these matrices, selected that combination of eight tests of the 45 which offered the greatest number of bacterial separations, i.e., the greatest number of bacteria which were distinguished from other bacteria in the key. In practice, there were two sets of eight tests--one for gram-positive bacteria and the other for gram-negative bacteria; this allowed a greater number of separations. For convenience, accuracy, and rapidity, each combination of eight tests selected by the computer was examined to ascertain whether substitutions of unselected tests could be made which would not significantly decrease the number of separations as originally determined by the computer. Such substitutions were made so that certain tests were common to the test sets of both the gram-positive and gram-negative listings. In addition, certain tests were arbitrarily selected because of their importance or significance. Such a test, for example,

would be bacterial shape. The resulting minimum test sets were as follows:

For Gram-Positive Organisms

Motility
Acid from glucose
Gas from glucose
Acid from lactose
Shape of organism (rod or coccus)
Growth under aerobic conditions
Coagulation of litmus milk
Beta-hemolysis of blood

For Gram-Negative Organisms

Motility
Acid from glucose
Gas from glucose
Shape of organism
Growth under aerobic conditions
Acid from sucrose
Acid from mannitol
Production of indole

The motility and indole tests were performed in semisolid Cystine Trypticase Agar (CTA) (BBL). Tests for acid and gas from glucose were performed with a Durham tube using Tryptic Soy Broth (Difco) which contained 1% glucose. Other carbohydrate reactions were done in CTA to which was added 1% carbohydrate. Bacteria isolated anaerobically were inoculated onto Blood Agar plates and incubated to determine if they could grow under aerobic conditions. If growth occurred, the colonies were inspected for beta-hemolysis. Strict anaerobes were tested for beta-hemolysis by incubating Blood Agar plates anaerobically. For those organisms which grew originally in Chocolate Agar, this medium was used for the aerobic growth test.

Once these test sets were selected, the computer listed all possible combinations of those eight tests and, for each matrix,

grouped the listed bacteria according to each category or combination. Every bacterial species in the program fell into at least one category, and some bacteria fell into several categories. For any category on the computer print-out, there might be no bacteria, one bacterium, or many bacteria. In the latter case, each bacterium was separated from other bacteria in that category by the use of further preselected biochemical tests, morphological tests, or both. Readily available information not considered in the protocol was used in the identification procedures. This, for example, included endospore formation, reactions other than coagulation in litmus milk, zone of growth in Thioglycollate medium, pigmentation, cell arrangement, pleomorphism, and colonial morphology.

RESULTS

Species of bacteria isolated in the sites sampled are shown in Table 1. Table 2 indicates the relative number of bacteria at each site. While the number of different types of bacteria was generally the same for all of the regions sampled (except possibly from the descending colon), the abomasal contents consistently contained fewer total organisms. The duodenum contained only a slightly larger number than did the abomasum.

TABLE 1. Bacteria isolated from various parts of the gastrointestinal tract in a female sheep. Presence of organism indicated by ×.

Species of bacteria	Abomasum	Duodenum	Jejunum	Ileum	Cecum	Ascending colon	Spiral colon	Descending colon
Escherichia coli	×	×	×	×	×	×	×	×
Escherichia freundii					×	×	×	×
Streptococcus faecalis				×				
Streptococcus faecalis var. *zymogenes*	×					×		
Streptococcus faecalis var. *liquafaciens*	×						×	
Streptococcus bovis	×	×					×	
Streptococcus acidominimus	×							
Streptococcus mitis		×	×	×	×		×	
Streptococcus cremoris				×				
Streptococcus dysgalactiae				×				
Streptococcus uberis						×	×	
Streptococcus equinis							×	
Streptococcus salivarius		×						
Corynebacterium enzymicum	×	×		×		×	×	
Corynebacterium acnes					×			
Corynebacterium xerosis							×	
Clostridium septicum				×				
Clostridium tertium					×			
Clostridium cellobioparum	×							

TABLE 1 (cont'd)

Species of bacteria	Abomasum	Duodenum	Jejunum	Ileum	Cecum	Ascending colon	Spiral colon	Descending colon
Bacillus brevis			×					
Lactobacillus bifidus	×				×			
Fusobacterium fusiforme					×			
Eubacterium lentum		×		×				
Brevibacterium vitarumen	×					×		
Sphaerophorus gulosus	×							
Sphaerophorus sp.	×							
Gaffkya sp.		×	×	×				
Bacteroides sp.		×	×	×	×	×		×
Corynebacterium sp.							×	
Bacillus sp.			×					

TABLE 2. Relative number of colonies at each sampling location. The number of +'s indicates the relative numbers of total colonies.

Sampling region	Relative total growth
Abomasum	+
Duodenum	+ +
Jejunum	+ + + +
Ileum	+ + + +
Cecum	+ + + +
Ascending colon	+ + +
Spiral colon	+ + + +
Descending colon	+ + +

DISCUSSION

Numerical Analysis

The minimum test set technique used in this study to identify bacteria is an abbreviated form of one described by Rypka (personal communications). Rypka used over 1,000 bacteria and over 100 tests to develop a scheme for identifying bacteria associated with human diseases. A positive or negative test was indicated when an organism produced a result 95% of the time. My scheme lists only about 200 organisms and 45 morphological and biochemical tests with a positive or negative result occurring when an organism produces a particular result 80% of the time. The great deal of time required to develop a matrix with 95% accuracy is probably justified when the bacterial agents of human disease are being investigated.

Rypka has attempted to produce a system which is completely objective in identifying bacteria. Descriptions of bacteria which include the words "usually," "normally," "customarily," "practically always," and similar subjective terms were not used by Rypka in developing his matrix. When his source material failed to note a definite character or lack of a character, he was extremely reluctant to record his own conclusions. For example, if the references failed to note that streptococci do not form endospores, Rypka declined to indicate a negative result in his matrix.

In contrast, the present matrix includes information which is more subjective than that sanctioned by Rypka. Descriptions of bacteria that are modified by such terms as "practically always," "normally," and "ordinarily" are permitted in this scheme. The computer technique,

unlike Rypka's, is intended to be used in conjunction with other observations to identify the organism.

Taxonomically, both Rypka and I prefer the classification system used in Bergey's Manual (Breed et al., 1967), although, unlike Rypka, I have substituted the scheme proposed by Edwards and Ewing (1964) to classify the enteric bacteria.

Cowan and Steel (1965) also produced an identification scheme, which, like Rypka's method, catalogs the medically important bacteria. Their approach is slightly different from the methods used by Rypka and myself in that they have placed more of their initial emphasis on tests which separate the genera rather than on tests which usually neglected the genera as was true in our cases. In Cowan and Steel's technique, seven tests partially differentiated the genera containing gram-negative bacteria and nine tests partially differentiated gram-positive organisms. A number of tables were then used, each one subdividing a genus and closely related genera into species.

By using this approach, a possible identification error could be made if an isolate failed to react to all of the primary tests in the same manner in which its genus did generally. Recognizing this, the authors contend that any identification using their tables must be made in conjunction with other observations. Thus the technique propounded by Cowan and Steel, as in this investigation, requires some subjective judgment in any bacterial identification. Both approaches record a test positive or negative if the species produces a result 80% of the time.

The minimum test set technique was used in this study because it provided a simple, convenient, rapid, and inexpensive means of identifying many bacteria at one time. This procedure is expedient in research laboratories that cannot accommodate or afford a great deal of equipment, supplies, or time to carry out large-scale bacterial identifications. There are some drawbacks, however, in using such a system. The time required to prepare a usable computer technique is, as I discovered, extensive. It is difficult to produce a tenable matrix. An error may be introduced when information gathered for the matrix is based on a description that does not specify the conditions under which the data were obtained. For example, an investigator may describe an organism as motile, but which in fact is an organism motile only at lower temperatures (15-25 C). Another example is the production of hydrogen sulfide; some tests are more sensitive to this gas than others and, for this reason, contradictory results between the unknown organism and the description of that organism in the matrix may occur.

A potential weakness in using numerical analysis in identifying bacteria became apparent when bacteria not listed in the key were isolated. This may have occurred because the organism was either usually not found in sheep or not described in Bergey's Manual (Breed et al., 1957). When such a strain was isolated and transferred to the minimum test set, the results of the eight primary tests would place the organism in a category which either contained no other organisms, so that it would be suspected of being an unlisted bacteria, or which contained other organisms. In the latter case, the nonlisted bacteria

might be implicated as such by the results of the secondary tests. To further reduce the possibility of an error in identification due to nonlisting, the identification made with the minimum test set was always checked against the description for that organism in Bergey's Manual (Breed et al., 1967), Identification of Enterobacteriaceae (Edwards and Ewing, 1964), Topley and Wilson's Principles of Bacteriology and Immunity (Wilson and Miles, 1955), or other reliable source, mainly comparing the microscopic and colonial macromorphology of the two organisms.

No attempt was made to isolate and identify spirochetes or other organisms which might have been found in the gastrointestinal tract but which were unable to grow on the media used in this study.

Bacteriological Studies

Approximately 150 isolates were taken from the abomasal and intestinal contents of one sheep. From this number, 28 different species were identified. Most of them were gram positive, many were gram variable, and only a few were gram negative. The majority of gram-positive bacteria were either cocci in chains or rods of medium length with rounded ends. The gram-variable strains were always rods which often demonstrated great pleomorphism, branching, and long filaments, and the gram-variable characteristic was observable in the same bacterial cell. Often a repeating gram-positive and gram-negative pattern could be detected.

The gram-negative bacteria were found in great numbers, but few types were represented. All regions sampled contained *Escherichia coli* and this organism was the only one found in all sampling points. Only the cecum and the colon contained *Escherichia freundii*. No other enteric bacterium was found in the gastrointestinal tract of the sheep studied. This result is consistent with the findings of Maki and Picard (1965), who examined the contents of several intestinal areas of cattle. They reported that the only enteric bacteria they found in 15 animals were *E. coli* and *E. freundii*. In their study of the fecal flora of normal cattle, Wilssens and Buttiaux (1958) also found these two organisms in great abundance, but in addition observed a small number of individuals of *Proteus* species. *Salmonella* and *Shigella* were never isolated by either of these two groups of investigators.

Streptococci were evident in great numbers, both in aerobic and anaerobic plates. No one type predominated, although *Streptococcus mitis* was the only streptococcus which was present in the jejunum and the cecum. The typical enterococci were not found in large numbers and were found only in the abomasum, ileum, and colon. Organisms, including *S. bovis* and *S. equinis*, usually associated with other animals (Breed et al., 1957) were often isolated from the sheep. These results are dissimilar to those reported by Maki and Picard (1965), who reported that *S. bovis* greatly predominated in the cow.

One of the more interesting results of this experiment was that no staphylococci were isolated. Maki and Picard (1965) found members of this genus, but only very rarely. I found cocci in tetrads in all sections of the small intestine but never in the large intestine. They were placed in the genus *Gaffkya* because of the tetrads, but they behaved in a manner inconsistent with the two species recognized in Bergey's Manual.

Sporeformers were also present in half the regions sampled. Four species of *Clostridium* and one *Bacillus* strain were discovered randomly. One catalase-positive aerobic sporeformer did not resemble any of the *Bacillus* species described in Bergey's Manual, and thus was termed as "*Bacillus* sp." No sporeformer was identified from the colon. This might have been caused by the difficulty of showing endospore formation with some of the clostridia, this being an important secondary test for sporeformer identification. Both *Clostridium* and *Bacillus* isolates grew well under strictly anaerobic conditions, and a few anaerobic sporeformers were able to adapt to aerobic

conditions after several transfers. It is interesting to note that Clapper and Meade (1963), in their study of bacterial flora in the nose, throat, and lower intestines of healthy dogs, attributed findings of *Bacillus* species to the environment. Maki and Picard (1965) found both aerobic and anaerobic sporeformers in the intestines of cattle as I did in sheep.

Many short anaerobic gram-negative rods, often pleomorphic, were contained in the small intestine. They were often clinically and morphologically related to the genus *Bacteroides*, but did not resemble any of the species described in Bergey's Manual. They were noted as "*Bacteroides* sp." Another similar organism possessed pointed ends and was thus noted as "*Fusobacterium* sp." One organism manifested great pleomorphism with long gram-variable filaments and some branching. This was placed in the genus *Sphaerophorus* because of its similarity with members already in that genus. *Lactobacillus bifidus* was the only member of the genus *Lactobacillus* found in sheep, and this was observed only irregularly. The finding of the preceding genera in the gastrointestinal tract of sheep is supported by Dubos et al. (1963) who, while writing generally and not specifically referring to sheep, stated that there was reason to believe that enterics, enterococci, and clostridia represented only a very small part of the normal intestinal flora and not the most important. Lactobacilli, *Bacteroides*, and fusiforms, they concluded, were far more typical, but for lack of cultural techniques have not been satisfactorily enumerated.

Besides bacteria, molds were sometimes seen on blood and chocolate plates that had been incubated aerobically. No effort was made to

identify these because they are obligate aerobes and probably do not contribute much to the relatively anaerobic environment of the intestines.

The lower total count of bacteria exhibited by the abomasal and duodenal samples was probably due to the acidity of the two regions. By the time the food contents reaches the jejunum, neutralization would have been sufficient to allow more bacterial growth.

Other than an isolate of *Clostridium septicum*, no potential pathogen was noted. *Clostridium perfringens* was never found in any part of the intestine, a result which is supported by Wilssens and Buttieax (1958). In their study of 15 cattle, Maki and Picard (1965) twice isolated this organism--both times from the ileum.

The media used for isolation were selected in an attempt to obtain the majority of organisms. It is recognized that these media might not allow the growth of certain organisms, such as the spirochetes.

The results of this study present a preliminary description of the organisms in the gastrointestinal tract of sheep. The fact that only one sheep was used in this investigation brings the authenticity of the conclusions into question, but this deficiency is not, I believe, of major significance. The sheep used was a representative sample of a large group of sheep which grazed together and were housed in close proximity. Further work will be performed--using both the computer analysis technique, which is described in this study, and conventional methods--in order to substantiate the results of this investigation. Eventually this work will lead to a determination of the

contribution of the normal gastrointestinal flora to radiation injury in larger animals.

SUMMARY

Eight portions of the gastrointestinal tract of a normal, healthy sheep were sampled to determine the types of bacteria indigenous to the different regions. *Escherichia coli* was the only organism present in all regions. Other than this and an occasional *E. freundii*, no other enteric bacteria was found in any region. Streptococci were common in all portions, but no one species predominated above all others. Anaerobic as well as aerobic sporeformers were plentiful, and corynebacteria were especially common. Pleomorphic anaerobes, often long and filamentous, were frequently observed and these were identified as *Bacteroides* species, *Sphaerophorus* species, fusiforms, and *Lactobacillus bifidus*.

A minimum test set developed with the aid of a computer technique was used to identify the bacteria. This method was found to be satisfactory, although the time required to prepare the computer technique was extensive.

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