Normal Intestinal Flora of Cattle Fed High-Roughage Rations

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ABSTRACT
Maki, LEROY R. (University of Wyoming, Laramie), and KAY PICARD. Normal intestinal flora of cattle fed high-roughage rations. J. Bacteriol. 89:1244-1249. 1965.—Intestinal bacteria were isolated from 15 cattle. Approximately 600 isolates were studied, and their presence and numbers in the duodenum, ileum, cecum, and colon were noted. Bacteria most frequently isolated from all parts of the intestinal tract included Escherichia coli, Streptococcus bovis, and species of Bacillus. Several groups of gram-positive rods could not be classified according to Bergey's Manual. Direct counts of intestinal contents indicated that 1 to 10% of the bacteria present were recovered.

Bacteriological studies of the enteric tract of ruminants have been confined primarily to studies of the rumen. Published reports of attempts to isolate the normal intestinal flora of ruminants are meager. Studies reported have been confined to an examination of the bacterial flora of fecal specimens of these animals. Sakazaki, Namioka, and Miura (1956) stated that Escherichia coli could be found in nearly 100% of the fecal samples examined. Other enteric bacteria encountered included E. freundii, Klebsiella, and Proteus. No estimates of the numbers of the bacteria isolated were given. In their study of the feces of healthy cattle, Wilsens and Butiaux (1958) found E. coli, Streptococcus faecium, S. bovis, micrococcus, and Bacillus spp. to be most often present. In addition, they found that Clostridium butyricum was abundant in the feces of cattle during the winter, and they attributed its presence to the feeding of silage. C. perfringens was never isolated from any of the 37 animals examined. Other studies have been undertaken to investigate specific groups of organisms in intestinal contents of healthy cattle. Studies of the streptococci were undertaken by Ayers and Mudge (1923); coliforms, Carpenter and Woods (1924) and Smith and Crabb (1956); C. welchi, Taylor and Gordon (1940); yeasts, van Uden and Sousa (1957) and van Uden (1960). In most instances, these studies were done on the feces of animals; the presence of specific microbial genera was noted but not their numbers. It would be advantageous to know the number of normal intestinal flora of ruminants in the investigations of diseases of unknown etiology where a change in numbers and kinds of intestinal bacteria may be under consideration as a possible cause. Furthermore, a better understanding of ruminant nutrition may be had by studying the effect of ration on the types, numbers, and distribution of bacteria in the intestinal tract.

This study was confined to investigation of the intestinal bacteria cultured aerobically and anaerobically from the intestinal tract of cows and steers fed high-roughage (alfalfa hay and dryland pasture) rations.

MATERIALS AND METHODS
The cattle used for the study were owned by either the Animal Science or the Veterinary Science divisions of the University of Wyoming. Hereford and Shorthorn cows and steers, 2 years or older in age and maintained for at least 2 months on alfalfa and dryland pasture, were slaughtered at all seasons of the year.

Intestinal samples were obtained as soon as the viscera were removed, and all plating procedures were completed within 1.5 hr after slaughter. Samples were collected in sterile containers from the duodenum, ileum, and occasionally from the cecum, and from the colon just below the ileocecal juncture.

Intestinal inocula were prepared for aerobic and anaerobic studies by weighing 2 g of intestinal contents into a sterile stainless-steel Omni-Mixer Chamber (Ivan Sorvall, Inc., Norwalk, Conn.) filled with oxygen-free CO2. A 198-ml amount of anaerobic diluting fluid (Bryant and Burkey, 1953) was added to prepare a 10^-2 dilution, and the diluted sample was then mixed for 60 sec. Tenfold dilutions were prepared in the anaerobic diluting fluid to 10^-7, and 0.1-ml portions were used as inocula.

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An intestinal fluid-cellobiose-glucose-starch agar medium (ICGSA), a modification of the roll-tube method, was used for total anaerobic roll-tube counts. This medium consisted of the following: 45 ml of salt solution A [0.6% (NH₄)₂SO₄ and 0.3% K₂HPO₄], 45 ml of salt solution B (0.6% NaCl, 0.3% KH₂PO₄, 0.06% MgSO₄·7H₂O, and 0.06% CaCl₂), 133 ml of distilled water, 0.3 ml of 0.1% resazurin, 0.3 g of cellobiose, 0.3 g of starch, and 6.0 g of agar. This medium was prepared anaerobically in 5-ml amounts under the gas mixture, according to the procedures described by Bryant and Burkey (1953).

For selections of specific bacterial types, plates of Eugon Agar (BBL), with and without crystal violet (concentration, 1,750,000) to inhibit Bacillus spp., plates of EMB Agar (Difco) were inoculated with 0.1 ml dilutions which were spread over the surface with sterile spreader rods. All plates were inoculated in duplicate; one set was incubated aerobically, and the second set was incubated anaerobically either in Brewer jars or in cold catalyst jars (Torsil, Torsion Balance Co., Clifton, N.J.). For recovery of clostridia, tubes of Brewer's Thioglycollate Medium (BBL) containing 2 mg/100 ml of polymyxin B sulfate were inoculated with 0.1 ml of intestinal dilutions. All media were incubated at 37 C.

Several plating and enrichment media were tried during this study. These included the sorbic acid polymyxin B thioglycollate medium of Wetter, Marshall, and Cardella (1956) for isolation of clostridia, SF Medium (Difco) for isolation of enterococci, Rogosa medium (Rogosa, Mitchell, and Wiseman, 1951) for lactobacilli, Zarett medium (Zarett and Doetsch, 1949) for Proteus, Violet Red Bile Agar (Difco) for coliforms, Bismuth Sulfite Agar (Difco) and S-Agar (Difco) for salmonellae, and Mylecos Agar (BBL) for fungi. For total plate counts, Trypticase Soy Agar (BBL) and blood-agar were used in addition to the media already described.

Isolated cultures were streaked for purity or serially diluted in ICGSA medium and then maintained in ICGSA medium, Thioglycollate Medium [without indicator, Brewer-modified (BBL), with 0.3% agar and added calcium carbonate], or Eugon Agar slants, depending upon the growth requirements of the isolates. Where possible, all cultures were identified according to Bergey's Manual and the Manual of Microbiological Methods (Society of American Bacteriologists, 1957). In addition, we used methods for identification of Micrococcus and Staphylococcus described by Baird-Parker (1963) and of Pseudomonas by Lysenko (1961). Yeasts were identified by the methods and classification of Lodder and Kreger-Van Rij (1962). Carbon assimilation by yeasts was carried out as described by Ajello et al. (1965).

The average cultural count per gram for each species was calculated by determining the average count of the species in a positive sample. When more than one sample was positive, the average count of all positive samples from the same intestinal area was used.

Direct counts were made of intestinal contents by use of smears (1 cm²) of the 10⁻¹ dilution of intestinal contents prepared according to the direct microscopic method (Breed technique) for counting bacteria in milk. The smears were heat-fixed, Gram-stained, and counted with a calibrated oil-immersion light microscope.

### RESULTS

From 15 animals, 6 duodenal, 14 ileal, 7 cecal, and 6 colic samples of intestinal contents supplied approximately 600 isolates for identification. The numbers and prevalence of these microorganisms in various parts of the intestinal tract were determined.

A summary of the average total counts of ICGSA, EM, and Eugon Agar, as contrasted with those made by direct counts, is given in Table 1. In making the cultural counts, 20 different animals were studied, and the data for direct counts were based upon a study of 6 of these animals. Eugon Agar supported the largest number of organisms from all samples. The ICGSA and EM Agar showed approximately the same total count from duodenal and colon contents; the

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<th>Table 1. Total cultural counts using ICGSA, EM and Eugon Agar and average direct counts of bacteria from the intestinal contents of cattle</th>
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<td>Type</td>
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<td>ICGSA</td>
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<td>Cecal</td>
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* Intestinal fluid cellobiose-glucose-starch agar in roll tubes.
ICGSA supported higher counts from ileal contents and the EMB Agar supported higher counts from the cecum. Counts of EMB and Eugon Agar included many *Bacillus* spp.

Direct counts indicated that cultivable bacteria were from 1 to 10% of the total number of bacteria present in the intestinal tract, assuming that all bacteria stained represented viable microorganisms.

When samples from the same animal were com-
pared, the count from the duodenum was about
10-fold lower than from the ileum, the colic count
was approximately equal to that of the ileum, and
the cecal count was consistently higher than any
other count. This indicated an increase in the
numbers of viable bacteria as the food moves
through the intestinal tract. This was also sup-
ported by direct counts of duodenal and ileal
contents, the ileal counts being 10- to 100-fold
greater.

The variety of microbial species of bacteria
found in all cattle is shown in Table 2. In general,
only those species which were isolated more than
once are listed. The species of bacteria most con-
sistently found in all samples were E. coli, S.
bovis, B. subtilis, and B. pumilus. E. coli was iso-
lated from 20 of the 33 samples, and S. bovis from
19. E. coli was isolated from the intestinal tract of
9 of the 15 animals examined, S. bovis from 10.
Thus, even the most frequently isolated species
were isolated only two-thirds of the time.

Many of the B. subtilis isolates were from an-
erobic plates, and, occasionally, this organism
was isolated from the roll-tube medium. Most
colonies of B. subtilis were very mucoid with
a light-pink pigment. Most B. pumilus isolates were
from aerobic plates and were isolated from higher
dilutions than was B. subtilis.

Molds (Mucor spp.) and Streptomyces spp. ap-
ppeared only on aerobic plates. No actual de-
termination of species was made of these two
groups, because they are obligate aerobes and
probably do not contribute to the vegetative
microbial flora of the intestinal tract.

Several groups of gram-positive branching rods
were isolated from the intestinal tract. The cata-
lase-negative rods were Lactobacillus bifidus; the
catalase-positive rods have not yet been identi-
ified. Other identified bacteria include those of
the genera Sphaerophorus, Brevisbacterium, Bac-
teroides, Staphylococcus, Acaligenes, Pseu-
domonas, Corynebacterium, Arthrobacter, Kurthia,
and Sarcina. In addition, species of Candida and
Torulopsis were isolated and identified.

Other than isolates of C. perfringens and one
isolation of Sphaerophorus and a potential patho-
genic bacteria were isolated. Neither Salmonella
nor Shigella species were isolated in a few trials in
which SS Agar was inoculated with 1:10 dilu-
tions. This is in agreement with the work of
Wilssens and Buttiiaux (1958), who were not able
to isolate these species from cattle feces.

**Discussion**

Several of the media initially tried for the iso-
lation of intestinal bacteria proved to be of little
value. Eosin Methylene Blue Agar consistently
gave higher counts than the Violet Red Bile Agar
and, otherwise being comparable with the Violet
Red Bile Agar, was used for enumeration of col-
iforms. Trypticase Soy Agar and blood-agar (5% 
sheep blood in Trypticase Soy Agar) were in-
ferior to Eugon Agar for total plate counts and
were not used throughout the study. Rogosa’s
medium for lactobacilli, Zarett’s medium for
Proteus, Wetzler’s polymyxin B-sorbic acid me-
dium for clostridia, SF Medium for enterococci,
and SS Agar for salmonellae and shigellae con-
sistently showed no growth at 1:10 dilutions
when inoculated with duodenal, ileal, colon, or
cecal contents. Their use was therefore discon-
tinued after a few trials.

Highest counts of obligatory anaerobes were
consistently obtained from the ICOSA medium.
Most of the subcultures from this medium grew
in Brewer’s Thioglycollate Medium, better
growth being obtained if 0.3% agar and a small
amount of calcium carbonate were present. High
counts were also obtained on EM II and Eugon
Agar. The chief difficulty of using aerobically in-
cubated plates was the overgrowth of Bacillus,
Streptomyces, and Mucor species. The Strep-
tomyces and Mucor species never appeared on
plates incubated anaerobically, indicating that
the presence of these organisms was the result of
ingestion and passage of the spores through the
intestinal tract. Though the Streptomyces and
Mucor species could be transferred anaerobically,
they did not germinate under anaerobic condi-
tions. Many of the Bacillus species, however,
grew well anaerobically, indicating the facultative
nature of some species within this genus. Counts
of Bacillus in pasteurized and unpasteurized
samples were approximately the same. This would
indicate that these species were ingested in the
spore state in the feed and did not germinate ex-
tensively in the intestinal tract. In an attempt to
reduce the number of Bacillus colonies, crystal
violet (final concentration, 1:750,000) was added
to Eugon Agar. This decreased the Bacillus count
10-fold and markedly reduced the size of the sur-
viving colonies.

In general, total counts from the duodenum
were lower than from the ileum, colon, or cecum.
When duodenal, upper ileal, and lower ileal con-
tents were compared, there was a progressive in-
crease in numbers of bacteria. No organism was
peculiar to any portion of the intestinal tract, nor
was any species present in all samples from a par-
ticular area of the intestinal tract. The most
commonly recovered organisms were E. coli and
S. bovis, but even these were not recovered from
all samples.

The only correlation between the area from
which the intestinal content was taken and the
bacteria recovered was the observation that the
predominant bacteria isolated from the duodenum were \textit{E. coli} and \textit{S. bovis}. This would suggest that bile-tolerant organisms constitute the major flora of this portion of the intestinal tract.

Wilsens and Buttiaux (1958) observed that \textit{E. coli} was present in all bovine fecal samples they examined. Apparently, the occurrence and concentration of coliform organisms increase in the lower levels of the intestine until they appear regularly in the feces. Since our colic samples were taken just below the ileocecal juncture, this may explain why \textit{E. coli} was not found in all samples. In addition, an increase in the number of one species might mask the presence of another, even if it were present. \textit{E. coli} was cultured from samples from 9 of the 15 cattle examined. Wilsens and Buttiaux found \textit{Proteus} to be present in about 50\% of the fecal samples they examined from cattle on summer pasture. Moreover, \textit{S. faecium} was present in all fecal samples they examined while \textit{S. bovis} was recovered about 30\% of the time. These workers suspected \textit{S. bovis} to be more abundant than they actually found because of the difficulty they had in actually determining \textit{S. bovis} presence in feces. Our intestinal sampling did not disclose \textit{Proteus}; \textit{S. faecium} was rarely recovered; and \textit{S. bovis} was found in 10 of the 15 animals examined.

The large variety of microorganisms isolated indicates the complexity of the intestinal flora. Bacterial genera isolated and identified were \textit{Escherichia}, \textit{Alkalaeons-Dispar} group, \textit{Streplococcus}, \textit{Bacillus}, \textit{Micrococcus}, \textit{Staphylococcus}, \textit{Sarcina}, \textit{Lactobacillus}, \textit{Clostridium}, \textit{Brevibacterium}, \textit{Arthrobacter}, \textit{Kurthia}, \textit{Microbacterium}, \textit{Sphaerothorpus}, \textit{Alcaligenes}, \textit{Pseudomonas}, \textit{Bacteroides}, and \textit{Corynebacterium}. Streptomycetes and the mold \textit{Mucor} were isolated, but no attempt was made to identify the species. Two yeasts were isolated and identified as \textit{Candida krusei} and \textit{Torulopsis famata}. It is interesting to note that the majority of organisms isolated, other than \textit{E. coli}, were gram-positive. In addition, the bacteria we have been unable to identify are gram-positive rods, many with a distinct tendency to branch. Some of these presently unidentified species may eventually be considered as species of \textit{Kurthia} or \textit{Catenabacterium}.

Direct counts indicated the presence of gram-positive cocci and rods in far greater numbers than were isolated. From 1 to 10\% of the organisms stained were recovered on culturing. The portion of bacteria seen in the direct smears that were actually viable cells is not known. Because of the large numbers and variety of organisms in the rumen, one would expect that some of these organisms would pass through the abomasum in an intact state: nonviable, yet readily stained, or possibly viable, but not multiplying in the intestinal content.

It has been shown that rations high in carbohydrate content change the rumen microflora (Hungate et al., 1952). It would be interesting to see whether a corresponding shift in the intestinal bacteria takes place. The feeding of high-concentrate rations increases the number of \textit{S. bovis} in the rumen (Hungate et al., 1952; Gutierrres et al., 1959). This would suggest that one might expect an increase of \textit{S. bovis} in the intestinal tract of animals fed such a diet. The influence of such a diet on intestinal \textit{pH} has not been reported.

The ecological role of the isolated bacteria has not been determined. Their influence on vitamin production, as well as the breakdown of carbohydrates and proteins for eventual use by the ruminant, needs further study.

It is of interest to compare these findings with the results obtained from other animals. For example, Clapper and Meade (1963) have shown that, in dogs, the organisms isolated most frequently from the rectum were \textit{E. coli}, \textit{Streptococcus mitis}, enterococci, \textit{Aerobacter} spp., and other gram-negative rods of the paracolon and \textit{Proteus} groups. Their isolation of \textit{Bacillus} spp. was attributed to the flora of the environment rather than the health of the animal. In addition, they found that the mold \textit{Mucor} was the mold most frequently isolated, an observation we have found true with cattle. Rogers and Sarles (1964) have found that, in the rat, the enterococci consist primarily of \textit{S. faecalis}, with lesser numbers of \textit{S. durans} and \textit{S. faecium}. \textit{S. bovis} was not found in either the dog or the rat by these investigators and may be an organism which is more common in the ruminant.

\textbf{Acknowledgment}

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\textbf{Literature Cited}


\textbf{Bryant, M. P., and L. A. Burkey.} 1965. Cultural methods and some characteristics of some of the various and more numerous groups of bac-


