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Enumeration of Anaerobic Bacterial Microflora of the Equine Gastrointestinal Tract

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Samples from the duodenum, jejunum, and ileum, as well as from the cecum and colon, were obtained from 11 mature grass-fed horses. Viable counts of total culturable and proteolytic bacteria were made on habitat-simulating media containing 40% clarified ruminal fluid. The mean pHs in the duodenum, jejunum, and ileum were 6.32, 7.10, and 7.47, respectively; the mean pH decreased to 6.7 in the hindgut. The acetate concentration increased along the length of the small intestine and was the only volatile fatty acid present in this gut segment. Molar proportions of acetate, propionate, and butyrate in the hindgut were 85:10:3. Differences in bacterial counts on habitat-simulating media containing equine cecal fluid or clarified ruminal fluid were negligible. Bacterial counts showed a substantial population in the duodenum (ca. 2.9×10^6 per g [wet weight] of sample), and this increased to 29.0×10^6 in the jejunum and 38.4×10^6 in the ileum. Proteolytic bacteria formed a high proportion of the total culturable bacteria, especially in duodenal samples. Counts of proteolytic bacteria per gram (wet weight) of sample were 3.0×10^6, 15.6×10^6, and 22.0×10^6 in the duodenum, jejunum, and ileum, respectively. There was a close relationship between lumenal and mucosal bacterial counts, although actual values were lower in mucosal samples. The mucosal bacterial population in the duodenum was high relative to the lumenal population. Although the comparison of bacterial populations in the hindgut of the horse and white rhino was limited to a single animal, the results were of interest. Counts were higher in the cecum than in the colon for both the horse and the white rhino. Counts of cellulolytic and hemicellulolytic bacteria in the horse were 10- to 100-fold higher than those in the white rhino, despite higher total culturable counts in the white rhino. The results of the study with the horse are discussed in relation to the possible role of the intestinal bacterial flora, especially the mucosal bacterial population, in the etiology of colic.

The most abundant nonruminant, large terrestrial mammals belong to the family *Equidae*, which is represented by the horse, the ass, and the zebra (21). The horse has a combination of a large cecum and an even larger colon where fermentation and absorption occur, although they are not considered to be as digestively efficient as those in ruminants under grazing conditions (21, 25, 33). Domestication of the horse has made a tremendous impact on the history of mankind, and today, some 40.5 million horses of many different breeds are used for draft power, transport, recreation, sport, and in some cases, food production (7).

Despite their importance, formal research on horses has lagged far behind that on food animals, particularly in the basic physiological studies of the gastrointestinal system. The ruminal microbial ecosystem has been the most thoroughly studied gut system, particularly the quantitative aspects and the contribution of the rumen to the host's nutrition (3, 17, 24, 25). The significance of hindgut fermentation has not been studied in the same detail, although there are remarkable similarities with respect to microbial digestion, secretion, and absorption (2, 31). In contrast, there is a paucity of information on the microbial population inhabiting the gastrointestinal tract of the horse. The few reports published thus far have focused on the contribution of the microbial population in the hindgut to the nitrogen and energy requirements of the host animal (14, 15, 20). We are not aware of any report which has considered the normal bacterial population in the small intestine of the horse.

Colic is an important medical problem in horses that frequently results in death. The initiating cause is often gastrointestinal disease which causes distension or spasm of the gut, with the resultant exaggerated response of the horse to abdominal pain (16, 30). Although the enigma of colic in its various forms has long perplexed and challenged clinicians, we are no closer to understanding the cause of the problem than we were decades ago (34; E. E. L. Gerring, Editorial, Equine Vet. J. **18**:243, 1986). Since the equine gut is peculiar in that it has so much functional disturbance and the onset of colic is so acute, the anterior portion of the gut was chosen as the focus for this investigation. Since there was no information available on the normal bacterial flora, the initial aim of the study was to describe the organisms that exist in the small intestines of grazing horses. Attention was also paid to defining environmental conditions and to the relationship between lumenal and mucosal populations. Further studies on the characterization and identification of the bacterial isolates will be included in a separate report (C. A. Wilkins and R. I. Mackie, manuscript in preparation).

The white or square-lipped rhino (*Ceratotherium simum*) is a large grazing herbivore and is similar to the horse, at least with respect to the anatomy of its gastrointestinal tract (5). The intestinal ciliate protozoa that inhabit the hindgut of the white rhino have been described recently (32). The opportunity to enumerate the different functional groups of bacteria that are present in the hindgut of the white rhino was used since material from this animal is rarely obtainable.

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MATERIALS AND METHODS

Animals and diet. Mature (age 3 to 5 years) Anglo-Arab horses, six gelded males and five females, were sacrificed in two groups; the first group of three horses was sacrificed between July and August 1985 and the remainder were sacrificed between May and September 1986. The animals were maintained on natural, unimproved mixed grass pasture with no supplementation and are routinely slaughtered for vaccine production purposes at the Veterinary Research Institute, Onderstepoort, Republic of South Africa. The horses were brought on foot to holding pens at the abattoir at the Veterinary Research Institute and allowed access to hay and water for 20 to 24 h before they were sacrificed at 9 a.m. The horses were stunned with a captive bolt and then were exsanguinated.

Sampling procedure. The intact gastrointestinal tract was removed from the abdominal cavity within 10 min of death. The various gut segments were ligated, the ingesta from each segment were squeezed into a beaker, the pH was measured, and a portion (100 g) was transported to an adjacent laboratory in screw-cap, glass bottles filled to capacity for microbiological counts and chemical analyses. Two subsamples (10 g) were weighed into wide-mouth McCartney bottles, and 10 ml of 1.0 M $HClO_4$ was added to one subsample, while 10 ml of 0.5 N NaOH was added to the other. All samples were centrifuged and stored in a refrigerator for analysis.

Sampling sites. Samples were taken from five sites in the gastrointestinal tract: three sites in the small intestine and two sites in the hindgut. Samples from the small intestine and two sites in the hindgut. Samples from the small intestine were obtained (i) 1 m posterior to the pylorus (duodenum), (ii) 1 m anterior to the ileo-cecal junction (ileum), and (iii) midway between these two sites (jejunum). After collection of the lumenal or gut contents from these ligated segments, the wall was washed under running water and the gut mucosa was removed from the remainder of the gut wall by scraping it with a blunt knife. Samples of digesta from the hindgut were obtained (i) from the body of the cecum and (ii) at the pelvic flexure of the ventral colon.

Bacterial counts. Subsamples (5 to 10 g [wet weight]) were weighed into 250-ml conical flasks and taken into an anaerobic cabinet (model 1024; Forma, Marietta, Ohio) with a 30% CO_2–65% N_2–5% H_2 gas phase. The weighed samples were diluted 1:10 with cold anaerobic diluent and processed for 1 min with an homogenizer (20,000 rpm; Ultra-Turrax; Janke & Kunkel, Stauffen im Briesgau, Federal Republic of Germany). Each sample was serially diluted in anaerobic diluent (19), and appropriate dilutions were inoculated onto agar media. Counting precision was maximized by inoculating three dilutions onto different plates, typically 10^{-4} to 10^{-6} for intestinal samples and 10^{-6} to 10^{-8} for hindgut samples. A repeating dispenser (Eppendorf Multipette) was used to dispense 10 droplets (20 μl each) onto the agar plates through a template. After absorption of the droplets, the plates were inverted, placed in screw-cap containers, and incubated at 38°C inside a cabinet. Colony counts of total culturable and proteolytic bacteria were made after 5 days of incubation. The variation and precision of this counting technique have been reported previously (22).

In general, the media utilized for the counts were based on those commonly used for the ruminal ecosystem in our laboratory (18, 22). A habitat-simulating medium for enumerating total culturable bacteria from the small intestine contained 0.05% glucose and starch, 0.02% lactate, and 0.05% Trypticase (BBL Microbiology Systems, Cockeysville, Md.) (GSLT medium). This medium was modified to include either 40% clarified ruminal fluid or clarified equine cecal fluid. Clarified equine cecal fluid was prepared in the same way as clarified ruminal fluid, which was prepared as described previously (19). Proteolytic bacteria in the small intestine were enumerated on a medium that was modified to contain 1.0% casein but no Trypticase, 0.025% glucose and starch, and 0.01% lactate. Lactobacilli were enumerated on agar described by Rogosa et al. (26). A general nonselective medium (anaerobe blood agar according to the Centers for Disease Control, Atlanta, Ga.; obtained from Merck Diagnostica, Darmstadt, Federal Republic of Germany) containing 5% defibrinated horse blood was also evaluated. The medium (GCSX) for enumerating total culturable bacteria in the hindgut has been described previously (16, 20).

White rhino experiment. The white rhino was shot in the Pilanesburg Game Reserve, which is ca. 160 km west of Onderstepoort, Republic of South Africa. Samples of whole digesta were obtained from the cecum and colon and transported in sealed, screw-cap glass bottles on ice. The samples were taken within 2 h after the animal was shot and while the carcass was still warm. An additional 3 h was required before the samples arrived at the laboratory. The same batch of media for enumerating the different functional groups of bacteria in the horse and white rhino was used; the composition has been described previously (11, 18, 19, 22).

Analytical procedures. The pH of ingesta samples was measured immediately after collection by using a digital, portable pH meter (CG 817; Schott Geräte, Hofheim, Federal Republic of Germany) that was calibrated just before use. The concentration of NH_3 N was determined by the phenol-hypochlorite method (4). D-Lactate and L-lactate were measured by using specific enzymatic methods (8) with biochemicals obtained from Boehringer GmbH (Mannheim, Federal Republic of Germany). The values were added and reported as total lactate. Volatile fatty acid (VFA) concentrations were determined by gas chromatography by using a 2-m glass column packed with 60/80 Carbopak C–0.3% Carbowax 20 M–0.1% H_3PO_4 and a flame ionization detector. Pivalic acid was used as an internal standard (6).

RESULTS

Biochemical measurements in the gastrointestinal tract. As expected, the pH increased along the length of the small intestine and then decreased slightly in the hindgut because of VFA production. The mean pHs in the duodenum, jejunum, and ileum were 6.32, 7.10, and 7.47, respectively (Fig. 1a). The pHs in the cecum and colon (6.70 and 6.67, respectively) were similar to those reported in the rumens of animals fed roughage and in the ceca of sheep (3, 20, 34). Values for NH_3 N (Fig. 1b) were considerably higher in the duodenum (9.82 mM) than in the jejunum (3.99 mM) and ileum (5.64 mM). The value in the colon (12.71 mM) was higher than that in the cecum (6.08 mM). This was probably due to differences in dry matter content between the sites rather than to differences in microbial activity.

The acetate concentration also increased along the length of the small intestine and was the only VFA present in this gut segment (Fig. 1c). The concentration of acetate in the cecum and colon was high (ca. 99 mM). Propionate and butyrate were also present in the hindgut, together with lesser amounts (0.5 to 1.2 mM) of isobutyrate, isovalerate, and valerate (Fig. 1c). The molar proportion of acetate, propionate, and butyrate in the hindgut was 85:10:3 (Table

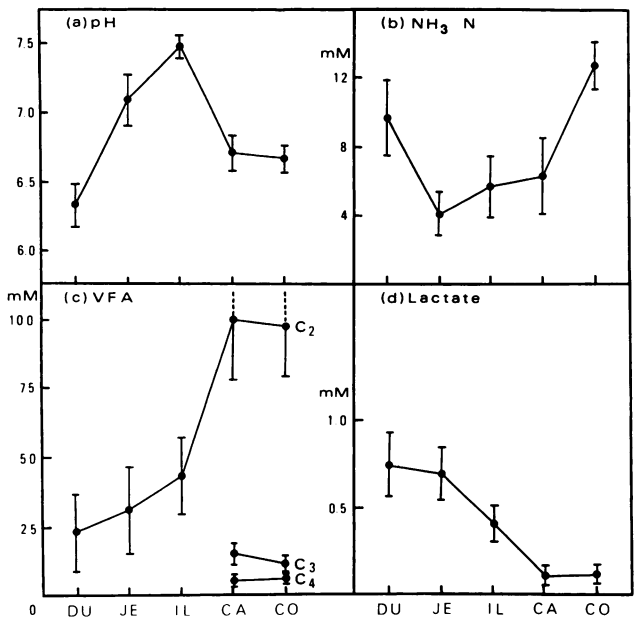

FIG. 1. Biochemical measurements along the length of the gastrointestinal tract of the horse (mean ± standard deviation; n = 11). Gut segment abbreviations: DU, duodenum; JE, jejunum; IL, ileum; CA, cecum; CO, colon. VFAs C_2, acetate; C_3, propionate; C_4, butyrate.

1), which is indicative of fiber fermentation. The lactate concentration (Fig. 1d) was low and decreased along the length of the tract, being highest in the duodenum (0.75 mM) and lowest in the hindgut (ca. 0.1 mM).

Influence of medium formulation on bacterial counts. Preliminary studies were performed on the gut samples obtained from the initial group of three horses in order to determine the most suitable medium for the enumeration of bacteria along the gastrointestinal tract (Table 2). Attention was directed toward enumerating total culturable and proteolytic bacteria. In all cases the highest counts were obtained on the habitat-simulating type of medium for total culturable bacteria (GSLT in the small intestine; GCSX in the hindgut). When the effect of adding either equine cecal fluid or ruminal fluid on bacterial counts was compared, the differences were negligible or slightly higher for ruminal fluid-containing media, especially when proteolytic bacteria were counted on the casein-containing media (Table 2). The proteolytic bacteria appeared to make up a substantial proportion of the bacterial flora not only in the small intestine (ca. 60%) but also in the hindgut, where they were even greater in number (ca. 100%). Since ruminal fluid was readily available in large uniform batches, and based on the results from the hindgut, we decided to use only ruminal fluid-based medium to study the bacterial flora in the second group of eight horses.

The lactobacilli that were present in the gut were enumerated on a selective medium described by Rogosa et al. (26). Interestingly, a high proportion (95%) of the total culturable bacteria could be accounted for by this group of organisms in duodenal samples (Table 2). This proportion dropped markedly in the jejunum (17.6%) and ileum (5.2%). There appeared to be little advantage in utilizing the nonspecific anaerobe blood agar medium containing 5% defibrinated horse blood when compared with the habitat-simulating total culturable medium (Table 2).

Bacterial population in the gastrointestinal tract. The viable counts of total culturable and proteolytic bacteria along the length of the gut are presented in Table 3. The results indicate that even in the proximal duodenum a substantial bacterial population (ca. 3×10^6/g of sample) was present. This value increased 10-fold when samples of jejunal material were examined. The relative increase between jejunal and ileal samples was considerably lower than the increase between the duodenal and jejunal samples. This trend was similar for both total culturable and proteolytic bacteria. The counts of these two groups of bacteria were ca. 100-fold higher in the cecum and colon than in the intestinal samples, confirming that these are more favorable sites for microbial colonization and fermentation.

Comparison of the bacterial population in the hindgut of the horse and white rhino. Bacterial counts were higher in the cecum than in the colon for both the horse and the white rhino (Table 4). In the case of the horse, the counts were 1.5 to 2.7 times higher in the cecum than in the colon. The difference was much greater in the white rhino, with cecal counts that were 9 to 11.3 times higher than the colon counts, with the exception of the lactate utilizers (2.8 times higher) and the hemicellulolytic organisms. When the cecal contents of the horse were compared with those of the white rhino, counts of total culturable and glucolytic bacteria were more than twice as high in the white rhino but were considerably less for all the other functional groups. In the colon, bacterial counts in all functional groups were higher in the horse than in the white rhino. It is of interest that the counts of cellulolytic and hemicellulolytic bacteria in the cecum of the horse were 10- to 18-fold higher than they were in the white rhino.

DISCUSSION

Although the nutritional contribution of the microbial population in the hindgut of the horse has been considered in several reports (14, 15, 20), the small intestine has been neglected, and this region is of prime importance to the normal gastrointestinal function of these herbivorous mammals. The bacterial population in the small intestine of sheep has been described recently (23). The biochemical activities and microbial composition of the intestinal bacterial flora reflect the anaerobic environment of the intestinal tract. The relatively high pH in the small intestine, especially in the jejunum and ileum, may result from bicarbonate in exocrine pancreatic and intestinal secretions. Diffusion of urea into the intestine from blood is not supported by the data (Fig. 1) since a decrease in the NH_3 N concentration in the jejunum

TABLE 1. Mean concentration of individual VFAs in the cecum and colon of the horse[a]

Individual VFA	Mean concn (mM) in[b]:	
	Cecum	Colon
Acetate	99.9 ± 30.8	98.5 ± 25.9
Propionate	12.5 ± 2.1	11.0 ± 2.1
Isobutyrate	0.8 ± 0.4	1.2 ± 0.4
Butyrate	3.8 ± 1.2	3.3 ± 0.6
Isovalerate	0.5 ± 0.3	0.8 ± 0.6
Valerate	0.5 ± 0.2	0.5 ± 0.2

[a] Eleven horses were sampled.
[b] Values are means ± standard deviations.

TABLE 2. Bacterial colony counts on different media inoculated with gut contents from the gastrointestinal tract[a] of the horse

Basal medium	Addition to medium	No. of bacteria/g of gut contents, 10^6[b]			No. of bacteria/g of gut contents, 10^8[b]	
		DU	JE	IL	CA	CO
GSLT or GCSX	Equine cecal fluid	1.96	8.07	25.50	21.20	12.70
	Ruminal fluid				19.70	12.50
Casein	Equine cecal fluid	1.07	6.50	15.92	16.50	7.93
	Ruminal fluid				19.40	13.70
Agar of Rogosa et al. (26)	None	1.87	1.42	1.32	3.50	2.10
Anaerobe blood agar	Defibrinated horse blood	1.75	3.25	1.50	5.25	3.50

[a] Gut segment abbreviations: DU, duodenum; JE, jejunum; IL, ileum; CA, cecum; CO, colon.
[b] Mean values for the first group of three horses.

and ileum was observed. The decrease in pH in the cecum and colon can be ascribed to extensive fermentation and production of VFAs in this region of the gastrointestinal tract. Lactic acid is also produced as an end product of anaerobic fermentation by bacteria and yeasts. However, in the absence of any readily fermentable soluble sugars in the diet of the horses sampled, it is not surprising that lactate levels were negligible (Fig. 1d). Lactic acid concentrations of 14.0, 4.6, 0.4, and 0.6 mM have been reported in the stomach, cranial ileum, cecum, and dorsal colon, respectively, of the horse (1). Bacteria that are capable of lactate production and utilization were isolated from the stomach and intestine of the horse. The concentration of acetate increased between the duodenum and ileum from 23 to 44 mM and was closely related to the increase in numbers of viable bacteria. Comparable acetate concentrations in the stomach (8.5 mM), cranial ileum (9.8 mM), cecum (64 mM), and dorsal colon (75 mM) of grass-fed horses have been reported (1). Acetate is a common fermentation end product in intestinal anaerobes of the genera *Bacteroides*, *Bifidobacterium*, *Eubacterium*, *Propionibacterium*, *Selenomonas*, and *Streptococcus* (12) and is indicative of a diet that is low in rapidly fermentable sugars or concentrates. The VFAs, mainly acetate, are known to be an important source of carbon and energy for the host animal tissues in ruminants as well as in monogastric animals with extensive hindgut fermentation (3, 21, 24). Although acetate can be absorbed and utilized by the intestinal mucosa, it is unlikely that it makes a major nutritional contribution (10).

TABLE 3. Colony counts of total culturable proteolytic bacteria in the gastrointestinal tract of the horse[a]

Gut segment	Location	No. of bacteria/g of sample[b]	
		Total culturable	Proteolytic
Duodenum	Lumen	2.94 ± 1.75	3.04 ± 1.89
	Mucosa	2.14 ± 1.26	1.77 ± 0.96
Jejunum	Lumen	29.02 ± 16.24	15.55 ± 8.72
	Mucosa	6.33 ± 4.41	2.88 ± 1.00
Ileum	Lumen	38.36 ± 13.24	22.03 ± 10.91
	Mucosa	9.12 ± 5.36	9.15 ± 3.11
Cecum		25.85 ± 11.26	15.75 ± 7.12
Colon		6.07 ± 2.75	3.02 ± 1.44

[a] Eleven horses were sampled.
[b] Values are means ± standard deviations. Values for the cecum and colon are 10^8; all other values are 10^6.

The cultural conditions employed in these experiments were adequate for growth of both intestinal and hindgut bacteria. The recoveries were ca. 10% of the total direct microscopic counts in the small intestine and ca. 30% of the total direct microscopic counts in the cecum and colon (Wilkins and Mackie, in preparation). The lower viable counts in the small intestine could be caused by factors such as bactericidal effects of gastric activity, activity of hydrolytic enzymes, bile salt secretion, and the combined effect of propulsive peristaltic movements down the tract which move digesta down the lumen at a rate that is more rapid than the bacterial generation time (28). The holdup of digesta in a large compartment such as the hindgut, together with favorable environmental and nutritional conditions, allows the proliferation of a large, diverse population of anaerobic, fermentative bacteria (17, 25). For the detection of intestinal bacteria, use of a habitat-simulating type of medium containing 40% either ruminal or equine cecal fluid for the provision of unidentified growth factors is important when isolating organisms with unknown nutritional requirements. The presence of casein supported the growth of a large number of bacteria, especially in the duodenal samples. Counts on the medium described by Rogosa et al. (26) for lactobacilli were also high in the duodenal samples.

The proteolytic bacteria formed a high proportion of the total culturable bacteria, especially in the duodenal samples but also in the other gut segments. This indicates that the small intestine is a good site for the proliferation of bacteria that are capable of protein degradation and that adequate levels of this substrate are present. Kern et al. (14) have

TABLE 4. Colony counts of different functional groups of bacteria in the cecum and colon of the horse and white rhino[a]

Functional group of bacteria	No. of bacteria/g of ingesta, 10^8			
	Horse		White rhino	
	Cecum	Colon	Cecum	Colon
Total culturable	6.0	3.5	15.8	1.4
Glucolytic	3.5	2.0	8.8	0.9
Amylolytic	4.5	2.2	1.0	0.1
Lactate utilizers	3.0	1.1	1.4	0.5
Proteolytic	2.3	1.1	0.9	0.1
Hemicellulolytic	3.6	2.4	0.2	0.2
Cellulolytic	10^6	10^6	10^5	10^4

[a] A single animal was studied in each case.

found that proteolytic activity per gram of ingesta is 30-fold or more higher in the ileum than in the cecum or colon of ponies. These results suggest that proteolytic activity may be widely distributed among a primarily saccharoclastic bacterial flora, as has been described in the rumen (27). This would be a likely explanation for the findings of this study in gut samples from the cecum and colon. The data presented here provide evidence of the close relationship that is found between the total culturable and proteolytic bacteria in gut segments of the small intestine. The relationship between counts in lumenal and mucosal samples was also evident (Table 3). The mucosal bacterial counts were proportionately higher in duodenal samples than in the jejunal or ileal samples. The mucosal counts were 73, 22, and 24% of the lumenal counts in the duodenum, jejunum, and ileum, respectively, for total culturable bacteria. The corresponding values for proteolytic bacteria were 58, 19, and 42%.

The bacterial community inhabiting the small intestine can be divided, at least for sampling purposes, into two distinct populations. The lumenal population is often associated with particles of digesta and exist in areas where the rate of passage of digesta does not exceed the rate at which the bacteria can multiply (29). The mucosal flora is formed by adhesion of bacteria to epithelial surfaces and by colonization of the mucous layer overlying the mucosa and can exist in any gut segment (28). Presumably, it is the mucosal flora which has a major influence on the morphological structure of the small intestine and the turnover and enzymatic activity of enterocytes (9), as well as the stimulation of host immunological mechanisms and resistance to colonization by pathogenic bacteria (9, 28). Although there is little direct evidence for the passage of lumenal bacteria into the mucosal flora or vice versa, it is likely that stable communities of bacteria that colonize epithelial surfaces are essential to generate any functional lumenal flora (29). The method of obtaining mucosal scrapings used in this study would underestimate this population, since not only the epithelial surface would be included, resulting in some dilution of colonized material by the uncolonized mucosa below the surface. However, the results of this study do provide confirmation of the high positive correlation between the lumenal and mucosal bacterial populations in the small intestine of the horse. Further evidence of this relationship describing the composition of these two populations will be included in a subsequent paper (Wilkins and Mackie, in preparation).

Total culturable bacterial counts in the cecum of the horses in the present experiment (25.9×10^8/g of content) were considerably higher than those obtained from cecally fistulated horses maintained on bluegrass pasture (2.5×10^8 to 3.7×10^8/g of content) (20). Counts from cecally fistulated ponies maintained on timothy hay were 3.6×10^8/g of content (13). Kern et al. (14) have compared the whole length of the pony gastrointestinal tract with that of steers fed the same diet, mainly grass hay. The VFA concentration in the pony cecum and colon were 97.4 and 25.6 mM, respectively, while the values in the steer rumen and cecum were 98.6 and 34.3 mM, respectively. The viable bacterial count in the steer rumen was ca. threefold higher than that in the pony cecum (16.6×10^8 versus 4.9×10^8/g of ingesta, respectively). Viable counts in the steer cecum (2.3×10^8/g of ingesta) were half those in the pony cecum.

This comparative study on the different functional groups of bacteria in the hindgut of the horse and the white rhino yielded valuable information and merits further investigation when material is obtainable. There is a need to compare counts in the cecum and colon on a dry matter basis to assess whether higher cecal counts are related to a higher dry matter content. The results indicate that the cecum is a more favorable site for microbial growth and fermentation than the colon. Although the white rhino surpasses the black rhino in its ability to digest organic matter and plant cell walls, little is known about the anatomy of the digestive tract of the white rhino (13). Both species of rhino have mean retention times similar to that of the zebra (13) and, presumably, that of the horse. It would be interesting to compare gastrointestinal flora in the white rhino and the zebra after they consumed identical diets. It is worth noting that, under zoo conditions, both the zebra and white rhino are not reported to suffer colic.

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