Postnatal changes in bacterial populations in the gastrointestinal tract of dogs

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Objective—To describe postnatal changes in the populations of bacteria in the gastrointestinal tract (GIT) of dogs.

Animals—110 Beagles ranging from neonatal to adult dogs.

Procedure—Contents of the stomach and proximal and distal portions of the colon and contents of the mid region of the small intestine were collected from puppies at 1 day after birth and subsequent suckling, puppies at 21, 42, and 63 days after birth, and adult female dogs (ie, dams of the puppies) for enumeration of bacterial populations.

Results—The entire GIT was colonized at day 1 by all groups of bacteria studied, aerotolerant forms were dominant. During subsequent postnatal development there were changes in the relative proportions of the various groups of bacteria with anaerobic groups increasing in absolute and relative numbers.

Conclusions and Clinical Relevance—Establishment of bacterial populations in the GIT of dogs is a gradual process that begins immediately after birth. Age-related changes in the relative proportions of bacterial groups coincided with changes in diet and physiologic processes of the host and can influence nutritional state and disease resistance of developing animals. Differences among regions of the GIT suggest that fecal samples may have limited use for understanding the populations of bacteria and the age and diet-related changes in various regions of the GIT (Am J Vet Res 2003, 64:646-651).

Most of the information known about bacteria of the gastrointestinal tract (GIT) of dogs is from mature dogs used for biomedical research (eg, intestinal resection), studies on responses to diet composition, or investigations of small intestinal bowel overgrowth (SIBO). Only a few studies have focused on age-related changes in bacteria of the GIT of dogs. Those studies revealed that there are dramatic changes in quantitative and qualitative characteristics of the fecal bacteria of dogs during postnatal development, with the influence of age being most pronounced in distal regions of the GIT. However, only 1 study has evaluated the distribution of bacteria along the length of the alimentary canal, and that study was restricted to only a few broad age groups.

In adult dogs, a complex set of interactions exist among bacteria residing in the GIT, diet, and structure and functions of the GIT, and these play an important role in health and nutrition. The objective of the study reported here was to determine changes in the distribution and abundance of key members of the population of bacteria residing in the stomach, small intestine, and proximal and distal portions of the colon by using dogs ranging from puppies at 24 hours after birth to adult dogs. The same dogs were used to study postnatal development of anatomic and histologic characteristics of the small intestine, digestive secretions, and nutrient absorption. Populations of T lymphocytes in the mucosa of the small intestine of these dogs are currently being evaluated. Thus, in combination with those other studies, the study reported here provided a unique opportunity to determine relationships among age-related changes in the population of bacteria, diet, and changes in GIT characteristics.

Materials and Methods

Animals—Ninety-five puppies representing 15 litters and the 15 dams of those litters were used to conduct the study. Dogs were procured, housed, and fed as described elsewhere. Briefly, populations of bacteria in the stomach, small intestine, and colon were evaluated in samples obtained from 6 groups of dogs (unsuckled neonates within 1 hour after birth [n=14], 1 day-old neonates after initial suckling [20], puppies at 21 days of age during suckling [19], puppies at 42 days of age during feeding of a weaning diet in combination with suckling [21], puppies at 63 days of age that were 1 week after weaning [21], and adult females [ie, dams of the puppies, 15]).

Collection of samples—Dogs were euthanatized, and samples were collected. For dogs 42 days old, samples were collected 3 to 7 hours after caretakers provided the morning feeding. Gastric contents of each dog were placed in a sterile capped vial. Hemostats were used to isolate sections (approx 5 cm long) from the mid region of the small intestine, proximal portion of the colon immediately distal to the cecum, and distal portion of the colon approximately 5 to 7 cm proximal to the anus. The 3 isolated sections were removed by incising the intestine outside of the hemostats, thereby minimizing exposure of the intestinal contents in each section to atmospheric conditions and contaminants. The 3 sections and vial of gastric contents were immediately transferred to an anaerobic chamber filled with a gas mixture of nitrogen (80%), carbon dioxide (10%), and hydrogen (10%), a palladium catalyst was used to maintain anaerobic conditions.

Gastric contents and intestinal segments from the mid region of the small intestine and the proximal and distal portions of the colon were weighed inside the anaerobic chamber. The populations of bacteria that were adherent to mucosa in the mid region of the small intestine were examined by gently scraping the mucosa from the segment with a sterile glass microscope slide. Five samples (1 of gastric contents, 3 of luminal contents, and the small intestine mucosa) were obtained from each dog, homogenized, and serially diluted in reduced yeast broth.
Microbial culture—Samples were inoculated onto culture media by use of an autoplater. Anaerobic and aerobic bacteria were enumerated in all 5 samples from each dog by use of an anaerobic blood agar and tryptic soy agar with 5% sheep blood, respectively. Luminal contents and mucosa of the small intestine and distal portion of the colon were also used to enumerate enteric bacteria, bifidobacteria, lactobacilli, and bacteroides. Aerobic bacteria were incubated in atmospheric conditions for 2 to 3 days, and anaerobic plates were incubated in the anaerobic chamber for 4 or 5 days. All plates were incubated at 37°C.

Colonies were identified on the basis of results of gram staining, colony morphology, aerotolerance, and biochemical characteristics. Any colonies suspected of being bifidobacteria were screened by use of the fructose-6-phosphate phosphoketolase assay. Gas chromatographic analysis of membrane fatty acids was used to confirm identities of suspect colonies. The number of colony-forming units (CFUs) for each bacterial group was expressed as a function of sample wet weight.

Statistical analysis—Influences of age and region of the GIT and their interactions were examined by use of ANOVA in a statistical program. Logarithmically transformed bacterial counts were used for analysis. When a significant effect of age or region of the GIT was detected, specific differences were identified by use of the Duncan test. For all comparisons, a value of P < 0.05 was accepted as significant.

Results

Results for the GIT—The various regions of the GIT contained milk (puppies at days 1 and 21), solid food (puppies at day 63 and adult dogs), or a combination of milk and solid food (puppies at day 42). All regions of the GIT were colonized at day 1. There was a significant effect of age for all groups of bacteria but not all regions of the GIT. Bifidobacteria were not detected in any samples obtained from the puppies or the adult dogs.

Gastric contents—Numbers of anaerobic bacteria were significantly higher at day 1, compared with values at day 21 (Fig 1). Intermediate values were detected in older puppies and adult dogs. The pattern for aerotolerant forms was similar to that for the anaerobic bacteria, but a significant effect of age was not detected.

Luminal contents of the small intestine—The highest numbers of aerobic and anaerobic bacteria in luminal contents of the small intestine were detected at day 1 (Fig 2). Thereafter, values were significantly lower and did not vary among older puppies and adult dogs. Among the groups of bacteria that were evaluated, enteric bacteria were dominant at day 1. However, the number of enteric bacteria decreased until day 42 and then remained stable in 63-day-old puppies and adult dogs. Number of clostridia in luminal contents of the small intestine contents was highest at days 1.
and 21, with the lowest values at day 42. A significant effect of age was not detected for the number of lactobacilli. The number of bacteroides increased significantly between days 1 and 63, but values for adult dogs were lower and not different from those of puppies at day 1.

Mucosa of the small intestine—We did not distinguish between bacteria that were adherent to the epithelial cells and those associated with the mucus layer underlying the epithelial cells. The numbers of bacteria in the mucosal samples were generally lower than those in the luminal contents (Fig 3); however, the age-related patterns of change for the mucosal samples were similar to those for the luminal contents of the small intestine.

Luminal contents of the proximal portion of the colon—Numbers of anaerobic and aerobic bacteria in luminal contents obtained from the proximal portion of the colon did not change between day 1 and 21 (Fig 4). Although numbers of anaerobic and aerobic bacteria decreased after day 21, the rate and magnitude of the decrease were greater for aerobic bacteria. Consequently, aerotolerant bacteria represented a smaller proportion of the bacteria that grew anaerobically after weaning (puppies at day 63 and adult dogs), compared with the proportion for younger puppies.

Luminal contents of the distal portion of the colon—Significant effects of age were not detected for the total numbers of anaerobic and aerobic bacteria in luminal contents of the distal portion of the colon. However, numbers of anaerobic bacteria in luminal contents of the distal portion of the colon did not decrease with age, whereas the numbers of aerotolerant forms decreased, but not significantly (P = 0.10), in luminal contents of the distal portion of the colon between puppies at day 1 and adult dogs (Fig 5). Significant effects of age were detected for each of the 4 specific groups of bacteria. Numbers of enteric bacteria and clostridia decreased but not until after day 21, whereas the numbers of lactobacilli increased > 100-fold between days 1 and 21, with the highest number at day 63. Numbers of bacteroides increased approximately 1,000-fold between days 1 and 21 with no additional changes thereafter.

Initial populations of bacteria (day 1) had a higher proportion of species that were aerotolerant (e.g., enteric bacteria). When data for all 5 samples were pooled, these forms represented only 50 to 60% of anaerobic bacteria (Fig 6). The proportion of aerotolerant bacteria decreased with increasing age, and by day 42 and into adult dogs, mean number of aerotolerant bacteria was < 40% of that of anaerobic bacteria and significantly (P = 0.01) less than the proportion of aerobic bacteria on day 1.

**Discussion**

In the study reported here, we enumerated a small percentage of the known bacterial species in the GIT o
dogs, although additional species are likely to be identified as indicated by a study\textsuperscript{18} in which investigators reported culturing \textit{Serpulis cama}s. However, the groups of bacteria selected for our study were considered to be important constituents of the bacterial populations in the GIT of dogs.\textsuperscript{19,21} At most ages and sample collection sites, the combined number of bacteria for the 4 groups that were enumerated represented < 10\% of the bacteria that grew during anaerobic culture. In addition to bacteria, the GIT harbors various species of yeasts, fungi, and other microorganisms.

The increase in total number of bacteria and the proportions of anaerobic forms from stomach to colon correspond with other findings for dogs,\textsuperscript{19,21} and other mammals.\textsuperscript{22} Typically, the duodenum and proximal portion of the jejunum harbor lower numbers of bacteria (10\textsuperscript{7} CFU/g). An exception is the increased number of bacteria in the duodenum of dogs with SIBO.\textsuperscript{6,23} Much less is known about the populations of bacteria in the less accessible mid regions of the GIT (ie, jejunum, ileum, and proximal portion of the colon). However, the higher number of bacteria in the mid region of the small intestine reported here and in another study,\textsuperscript{39} compared with values reported\textsuperscript{39} for the proximal portion of the small intestine, indicate that bacterial numbers increase rapidly in the GIT distal to the duodenum.

Despite a paucity of data, the pattern of development for bacteria in the GIT of dogs is similar to that known for other mammals. Specifically, the sterile GIT of neonatal dogs is rapidly colonized, apparently by bacteria in the birth canal and the surrounding environment. By 24 hours after birth, numbers of bacteria in the various regions of the GIT, including the distal portion of the colon (ie, feces), were comparable to those of the adult female Beagles (ie, dams) and to values reported elsewhere for developing,\textsuperscript{8} adult,\textsuperscript{19,21} and geriatric dogs.\textsuperscript{7} After the numbers of bacteria have stabilized, further age-related changes involve shifts in the relative proportions of the various groups that constitute the bacterial populations in the GIT of dogs and other mammals, with the changes more pronounced in the colon.

The age-related increase in the relative proportion of anaerobic bacteria is consistent with the higher number of bacteroides in dogs > 21 days old, particularly in the distal portion of the colon in which anaerobic conditions predominate. Similar age-related decreases in the relative number of aerobic bacteria have been reported in pigs,\textsuperscript{4} humans, and other species.\textsuperscript{24} However, the percentage of aerobic bacteria in the GIT of dogs was higher, compared with the percentage for bacterial populations in rodents,\textsuperscript{26} pigs,\textsuperscript{4} and humans,\textsuperscript{25} but was similar to that of mink.\textsuperscript{27} Because facultative anaerobic bacteria can exceed the number of obligate anaerobic bacteria in the colonic contents of dogs,\textsuperscript{19,26} it is likely that a substantial percentage of the bacteria that grew in the anaerobic cultures were aerotolerant. Interestingly, and for as yet unknown reasons, maintaining dogs in open environments, such as in the study reported here, results in a greater proportion of facultative anaerobic bacteria, compared with results when dogs are conventionally housed in enclosed facilities.\textsuperscript{20,21}

Postnatal changes in bacterial populations of the GIT conform to the principles of successional ecology and extend into senescence.\textsuperscript{28} In other ecosystems, successional changes in the populations of organisms are partly dependent on interactions among the constituent species. Apparently, the metabolic processes of the bacteria that are initially dominant in the colon reduce the oxygen tension and pH and increase the reduction-oxidation potential of the luminal contents. Moreover, competition (for binding sites and nutrients) and interactions (eg, production of bacteriocinids) among the various species of bacteria in the GIT can influence the relative numbers of the various groups.\textsuperscript{39} These and other changes facilitate the proliferation of groups of anaerobic bacteria, allowing them to eventually supplant the aerotolerant forms and dominate the populations of bacteria. Initial detection of segmented filamentous bacteria in the GIT of dogs at 54 days of age\textsuperscript{31} is consistent with such successional changes.

The populations and metabolic characteristics of bacteria in the GIT of adult dogs are responsive to diets prepared with various sources of protein,\textsuperscript{23} supplemented with nondigestible oligosaccharides,\textsuperscript{32,33} regardless of whether those diets are dry or moist.\textsuperscript{35} Similar responses to diet are known for infants,\textsuperscript{36} and adult humans,\textsuperscript{37} pigs,\textsuperscript{38} rodents,\textsuperscript{26} and cats.\textsuperscript{39,40} Therefore, it is not surprising for the study reported here that weaning was associated with large-scale changes in bacteria of the GIT of dogs, which is similar to other reports for diets,\textsuperscript{8} humans, and pigs.\textsuperscript{24}

Bacteria of the GIT are also responsive to changes in physiologic processes of the host. Examples include SIBO with pancreatic insufficiency,\textsuperscript{41} and surgical manipulations of the intestine that alter populations and metabolic characteristics of the resident bacteria.\textsuperscript{42} Corresponding to this, the most noticeable changes in the bacterial populations in the stomach and small intestine were detected between days 1 and 21, a period when there are increases in secretion of pepsin from the stomach and digestive enzymes from the pancreas of dogs.\textsuperscript{13} Postnatal changes in the ability of the intestine to absorb nutrients,\textsuperscript{44} recycle bile acids,\textsuperscript{42} and develop enteric immune functions\textsuperscript{45} are also likely to influence the populations and metabolic characteristics of resident bacteria.

Conversely, changes in resident bacteria can alter structure and function of the GIT by altering patterns of gene expression by enterocytes,\textsuperscript{44} thereby influencing the health and nutritional status of the host. This has been established for SIBO, which is associated with altered mucosal architecture and increased permeability.\textsuperscript{47} Similarly, enteropathogenic bacteria damage the mucosa and reduce functional capacities of the GIT.\textsuperscript{48} In contrast, higher numbers of lactobacillus and other lactic acid-producing bacteria increase the size and nutrient absorptive capacities of the small intestine of dogs.\textsuperscript{49,50} In another study,\textsuperscript{48} conducted by use of samples obtained from the dogs reported here, saturable transport of octanoic acid in the proximal portion of the colon was detected at day 42 and thereafter, coinciding with the onset of consumption of solid food and changes in bacterial populations. Although concentrations of short-chain fatty acids were not measured in
these dogs, changes in dietary inputs and bacterial populations at weaning are associated with increased fermentation and production of short-chain fatty acids in other species. In adult dogs, short-chain fatty acids are rapidly absorbed by the colon and provide an estimated 5 to 7% of metabolic energy.

Resident bacteria are a critical determinant of health of the host. This is evident from the detrimental influences of pathogenic strains of clostndia, salmonelae, and Escherichia coli when they become established in the GIT. Of particular importance to developing and mature animals is the ability of the populations of bacteria in the GIT to provide resistance against invading pathogens. In other ecosystems, the ability of invading organisms to colonize and become established is enhanced at early stages of succession. Similarly, disturbing the populations of bacteria in the mature GIT facilitates invasion and establishment of pathogens in the GIT, with antibiotic-induced diarrhea as an example. Interestingly, the numbers of enteric bacteria and clostndia in the study reported here were higher during early development of Beagles before the numbers of bacteria in the GIT had stabilized, which is similar to results in dogs in another study. Corresponding to this, infective doses for GIT pathogens are lower during early development, and there is increased risk for necrotizing enterocolitis and other bacteria-related pathologic conditions of the GIT during that period. Instability of the resident bacteria during early development may explain the reason that administration of probiotic Lactobacillus organisms is more effective in young dogs, compared with results in older dogs.

Evidence exists to suggest that several groups of bacteria provide health benefits to the host. For example, lactic acid-producing bacteria, which include lactobacil, bifidobacteria, and other groups, reportedly stimulate immune functions, increase resistance to health challenges and reduce accumulation of environmental contaminants. Lack of bifidobacteria in the Beagles reported here contrasts with reports for other dogs. However, application of an assay to confirm the presence or absence of Bifidobacterium spp verified that this group of bacteria was not in the dogs or was at densities below the limit of detection (<10 CFUs/g). Lactobacillus, a member of the lactic acid-producing bacteria, was detected at all ages and is consistently reported in dogs, therefore, this group of bacteria may be more important contributors than bifidobacteria to the health and nutritional status of dogs.

To gain insights into the possible relevance of the changes in the bacterial populations to health of the host, ratios were calculated for the numbers of entenc bacteria and clostndia, which included many potential pathogens, relative to lactobacilli. When data for all sample collection sites were pooled, the highest ratios were at day 1 (Fig 6), corresponding with the rapid colonization of the canine GIT by entenc bacteria and clostndia, which is similar to other reports in dogs and pigs. The ratios decreased significantly (P = 0.01) and remained lower as a result of the increase in lactobacilli in conjunction with a decrease in entenc bacteria and clostndia.

Diets for specific stages of development must provide adequate amounts of energy and nutrients. They should also enhance development of a healthy GIT ecosystem, including the resident bacteria, and thereby improve health and disease resistance. Attempts to define the normal populations of bacteria in the GIT of dogs at various stages of development will be complicated by effects attributable to the influences of breed, environment, health, age at weaning, diets, and other factors. Despite this, general patterns of age-related changes detected in the study reported here should be similar among all dogs and will be useful for additional studies directed at defining the complex but poorly understood interactions among resident bacteria, structural and functional characteristics of the GIT, and diet at various stages of development.

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