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Distribution of Indigenous Bacteria in the Digestive Tract of Conventional and Gnotobiotic Rats

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The localization and population levels of the indigenous bacterial flora of conventional rats were investigated by cultural and histological techniques. Lactobacilli predominate in the stomach and upper part of the small intestine and associate with keratinized cells of the nonglandular portion of stomach. Mixtures of varying complexity of pure cultures of indigenous bacteria were inoculated into germfree rats. The distribution of these bacteria was examined to investigate the effect of lactobacilli in controlling the composition of other bacterial species in each portion of the digestive tract. In the stomach and the upper part of the small intestine, lactobacilli controlled the population levels of other bacterial species. In the lower part of the small intestine, not only lactobacilli but also the anaerobes which colonized the large bowel influenced the population levels of other bacterial types. Staphylococci isolated from a conventional rat colonized specifically the keratinized cells of the nonsecreting epithelium of the stomach when the rats were free from lactobacilli. This colonization was not observed after inoculation of lactobacilli into the rats.

It is well known that normal enteric flora of the mammalian body contributes significantly to the host's resistance to infectious diseases. For the elucidation of such mechanisms, a number of hypotheses can be made, such as the toxicity of bile acids (S. Sasaki et al., Abstr. Annu. Meet. Jpn. Soc. Bacteriol. 1972 **27**:292 [in Japanese]) and volatile fatty acids (9), a fall in oxidation-reduction potential (9), a competition for nutrients (4), some immunological mechanisms (5), and combinations of the above mentioned.

Furthermore, changes in the composition of intestinal flora are often associated with diseases and possibly may be their cause. Savage and Tannock (13, 16) postulate that particular interference between indigenous microbes and pathogens operates primarily by mechanisms regulating the population levels and localization of indigenous microbes.

We have investigated the localization and population levels of indigenous bacteria in the digestive tract of conventional rats. In the stomach, lactobacilli, streptococci, and coliform bacteria are always detected. Bacteroides and bacilli are sometimes detected but not when the animals are prevented from coprophagy. Staphylococci are sometimes present despite prevention of coprophagy. The population of lactobacilli in the stomach is extremely high, and these bacteria form layers on the stratified squamous epithelium of the nonglandular mucosa.

In the upper part of the small intestine, lactobacilli are detected at about 10^7 per g of contents. Other bacterial species are rarely detected.

In the lower part of the small intestine, long chains of short rods or cocci colonize the villous epithelium, as Savage (11) reported. Lactobacilli, streptococci, and coliform bacteria are also detected, but no strict anaerobes can be cultured.

The large bowels contain bacteria intimately associated with mucosal epithelia. These microorganisms are anaerobic fusiform bacteria and spiral-shaped organisms that colonize the mucus, investing the mucosal epithelium of the cecum and colon (2, 14). Other bacterial species, such as lactobacilli, bacteroides, gram-positive anaerobic cocci, veillonella, streptococci, staphylococci, and coliform bacteria are detectable, but the exact microbial composition in this area is yet unknown.

Of the microorganisms which compose the intestinal flora, lactobacilli (1, 3) in the stomach, coccobacilli (11) on the villi of the ileum, and fusiform bacteria and spiral-shaped organisms (2, 14) in the large bowel are considered

to be autochthonous biota, judging from their mode of colonization, that is, high population levels and association with mucosal epithelia.

In these autochthonous biotas, we examined the lactobacilli which could be cultivated selectively and quantitatively. How these microorganisms affected the population levels of other bacterial species was investigated.

MATERIALS AND METHODS

Germfree rats. Germfree rats, CDF strain, 6 to 12 weeks old, were used. The rats were originally imported from the Charles River Breeding Laboratory and since then have been bred and maintained in our laboratory as a germfree colony. These animals were maintained in Trexler-type flexible vinyl isolators sterilized with peracetic acid. They were fed radiated diets (Oriental Yeast Co., Tokyo, Japan; type NMF). The diets were autoclaved at 126 C for 30 min before introduction into the isolator. The germfree state was tested by collecting and culturing a fresh fecal pellet in accordance with methods recommended by the Japan Experimental Animal Research Association (7).

Microorganisms. Bacteria were isolated from normal conventional rats, of the same strain as that of the germfree rats. Strictly anaerobic bacteria were isolated in an anaerobic glove box on enriched Trypticase soy agar supplemented with fatty acids described by Freter and Abrams (6) and Gifu anaerobic medium (GAM agar; Nissui Co., Tokyo, Japan) with 0.1% Tween 80. These media are enriched with nutrients and fit for isolation and growth of anaerobic bacteria. The media and the diluent (KH_2PO_4, 0.45%; Na_2HPO_4, 0.6%; L-cysteine-hydrochloride, 0.05%; Tween 80, 0.05%; resazurin, 0.0001%; and agar, 0.1%) were prereduced in the glove box before use. About 200 colonies were isolated on the basis of their colony forms and subcultured. Then 40 strains of anaerobes were selected from their cellular morphology and Gram reaction. Growth medium for these anaerobes was prereduced GAM broth with 0.1% Tween 80. These strains were grown in this broth, harvested by centrifugation, resuspended in prereduced dispersion medium (skim milk, 1%; sodium glutamate, 2%; resazurin, 0.0001%; and L-cysteine-hydrochloride, 0.05%) and stored at −70 C. This storing method enables anaerobes to survive for at least 6 months. Other strains used, their origins, and the media for isolation and growth are shown in Table 1.

Inoculation of animals. Five strains of organisms (*Escherichia coli* N-1, *Staphylococcus epidermidis* N-1, *Streptococcus faecalis* var. *liquefaciens* Y-5, *Bacteroides fragilis* GAM-8, and fusiform bacteria EG-1 [Table 1]) were harvested by centrifugation and resuspended in prereduced semiliquid GAM medium (0.1% agar). These suspensions were mixed and sealed with rubber stoppers under a N_2 gas stream without contamination by atmospheric oxygen.

As soon as they were introduced into the isolators, about 0.5 ml of each of the inocula was administered orally with a stomach tube to each rat. Such rats were designated as GB5. Two weeks after inoculation the animals were sacrificed and examined. A week after the first inoculation, a group of the GB5 rats was further associated with 18 strains of *Lactobacillus* (*L. acidophilus*, *L. fermenti*, and *L. salivarius*). These were designated as GB6 rats. The other groups were as follows. After a week of first inoculation of five strains, a mixture of 40 strains of anaerobes (GB5 plus anaerobes group), a mixture of *Lactobacillus* spp. plus anaerobes (GB6 plus anaerobes group), or cecal and ileal contents from normal conventional rats (conventionalized [cvd] group) were administered to rats, and 2 weeks later the animals were sacrificed and examined. Conventional (conv) animals were raised under normal conditions.

Preparation of gastrointestinal specimens. The animals were killed with CO_2 gas. Sections of the alimentary tract used were as follows: stomach, a 5-cm segment of the upper part of the small intestine 5 cm distant from the pylorus; and a 5-cm segment of the lower part of the small intestine 5 cm distant from the ileocecal junction and cecum.

Culture techniques. Samples of gut contents were

TABLE 1. *Strains of bacteria used in contamination: their orgin, media used, and cultural conditions*

Strain	Origin	Isolation media and cultural conditions	Growth media and cultural conditions
E. coli N-1	Conv CDF rat	MacConkey agar, aerobic, 37 C, 20 h.	GAM broth, aerobic, 37 C, 16 h
Staphylococcus epidermidis N-1	Conv CDF rat	Mannit salt agar, aerobic, 37 C, 24 h	GAM broth, aerobic, 37 C, 16 h
Streptococcus faecalis var. *liquefaciens* Y-5	Conv CDF rat	SF agar, aerobic, 37 C, 48 h	Rogosa broth, aerobic, 37 C, 16 h
Bacteroides fragilis GAM-8	Conv CDF rat	Modified NBGT agar, glove box, 37 C, 72 h	Prereduced GAM broth, aerobic, 37 C, 16 h
Fusiform bacteria EG-1	Conv CDF rat	See Methods and Materials	Prereduced GAM broth anaerobic, 37 C, 16 h
Lactobacillus spp. (18 strains)	Conv CDF rat	LBS agar, glove box, 37 C, 72 h	Rogosa broth, aerobic, 37 C, 16 h
Anaerobes (40 strains)	Conv CDF rat	See Methods and Materials	See Materials and Methods

removed and diluted serially with diluent in several 100-fold steps. The remaining gastrointestinal wall was then cut up into small fragments which were washed three times with vigorous agitation in diluent (without agar) and then finally homogenized in a Teflon grinder. The gut contents and the homogenate of washed tissue were then plated in appropriate dilutions of various culture media.

For the isolation of fusiform bacteria and total anaerobic counts, the animals were introduced in the glove box and all cultural procedures were done under oxygen-free conditions. Selective medium for fusiform bacteria was prereduced GAM agar with oleandomycin, 25 μg/ml; colimycin, 100 U/ml; and 0.1% Tween 80.

Expression of data in figures. The data in the figures indicate the range (mean ± standard deviation) and "range of mean." Because of inability to determine tissue concentration of organism by the method employed in our experiments if it is less than 10^3 per gram of specimen, "range of mean" was set up. In taking the mean among specimens when some of them do not show growth of organisms, the mean is determined either by taking that tissue concentration as 10^3/g or as zero along with other measured values. "Range of mean" is the range between the above two values.

RESULTS

Bacterial flora in the stomach contents. In the groups of animals free from lactobacilli (GB5 and GB5 plus anaerobes), *E. coli* and streptococci were dominant; the number of these bacteria was higher than that detected in the other groups of animals containing lactobacilli. The change in the number of staphylococci was similar to that of *E. coli* and streptococci (Fig. 1).

Bacterial flora on the walls of nonsecreting part of the stomachs. In the groups of animals containing lactobacilli, specific colonization of lactobacilli to the walls of nonsecreting stomach was found by culture methods. The adhesion of lactobacilli to the keratinized cells in vivo was also observed with histological techniques (Fig. 2).

In the groups of animals free from lactobacilli (GB5 and GB5 plus anaerobes), *S. epidermidis* N-1 was observed on the keratinized epithelial cells by histological techniques. This colonization was markedly reduced after inoculation of lactobacilli into rats (GB6, GB6 plus anaerobes, cvd, and conv).

To understand more exactly this competition for colonization on the walls of the nonsecreting part of the stomach between staphylococci and lactobacilli, a set of experiments was performed. The first group of germfree rats was monocontaminated with *S. epidermidis* N-1, and 2 weeks later the rats were sacrificed and examined. The other group of germfree rats was first monocontaminated with *S. epidermidis* N-1, and after 1 week the mixture of lactobacilli was inoculated. Two weeks after the second inoculation the animals were sacrificed and examined (Table 2). The population of staphylococci on the walls of the nonsecreting part of the stomach was markedly reduced by the inoculation of lactobacilli. Little reduction of the number of staphylococci was observed in the other parts of the digestive tract.

Bacterial flora in the contents of the upper part of the small intestine. Lactobacilli were

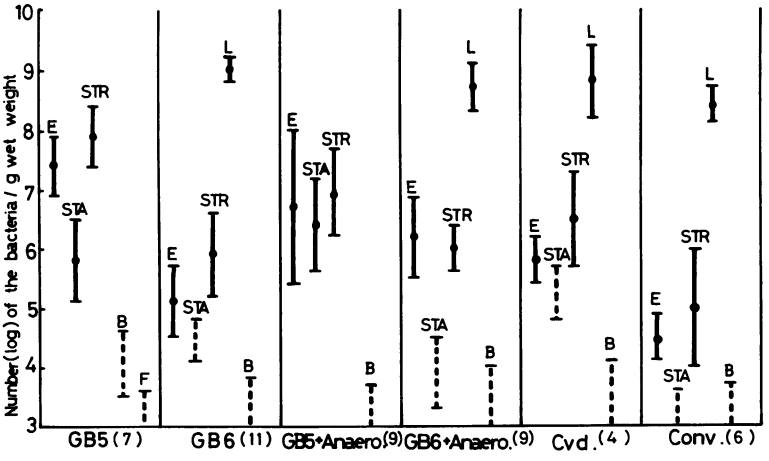

FIG. 1. *Population levels of indigenous bacteria in the contents of the stomachs in GB, cvd, and conv rats. E, E. coli; STA, staphylococci; STR, streptococci; L, lactobacilli; B, bacteroides; F, fusiform; T, total anaerobic counts. Solid lines, mean and standard deviation; dotted lines, range of the mean. Number in parentheses are the number of animals.*

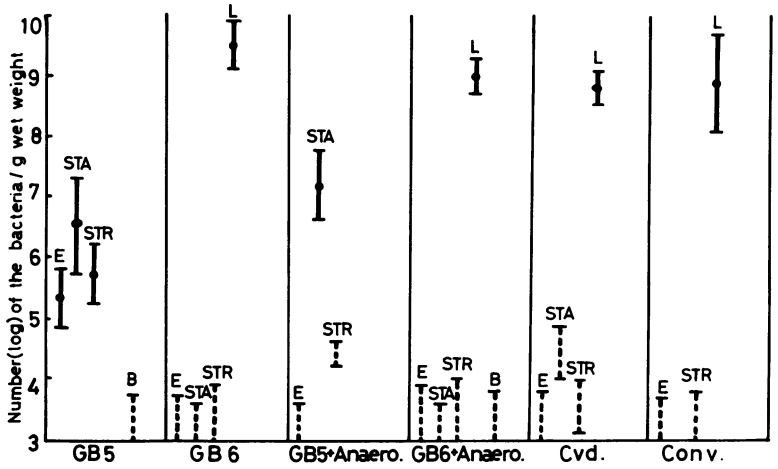

FIG. 2. *Population levels of indigenous bacteria on the walls of nonsecreting part of the stomach in GB, cvd, and conv rats. See the legend to Fig. 1.*

TABLE 2. *Number of Staphylococcus epidermidis N-1 in the digestive tract of gnotobiotic rats*

Contamination	Stomach			Upper part of the small intestine		Lower part of the small intestine		Cecum	
	Contents	Non-secreting wall	Secreting wall	Contents	Wall	Contents	Wall	Contents	Wall
Mono	7.7 ± 0.6[a] (4/4)[b]	7.6 ± 0.4 (4/4)	5.8 ± 0.6 (4/4)	8.0 ± 0.2 (4/4)	5.1 ± 0.5 (4/4)	8.1 ± 0.5 (4/4)	5.6 ± 0.3 (4/4)	8.4 ± 0.4 (4/4)	6.0 ± 0.4 (4/4)
Staphylococci and lactobacilli	6.7 ± 1.3 (5/5)	4.8 ± 1.0 (5/5)	4.5 ± 0.7 (5/5)	7.1 ± 0.5 (4/4)	4.0 ± 0.1 (4/4)	7.8 ± 0.8 (5/5)	5.3 ± 0.9 (5/5)	7.9 ± 0.2 (5/5)	5.0 ± 0.4 (5/5)

[a] Number (log) of the bacteria per gram (wet weight) of sample. Mean ± standard deviation.
[b] Number of animals with bacteria/number of animals examined.

the predominant species in the upper part of the small intestine of animals which contained these bacteria. The number of lactobacilli in the conv rats was somewhat lower than that in the other groups of animals, although there was no significant difference between them statistically (Fig. 3).

Bacterial flora in the contents of the lower part of the small intestine. (i) E. coli. The population level of *E. coli* in the contents of the lower part of the small intestine was the highest in GB5 ($P < 0.01$). In the animals further associated with lactobacilli or anaerobes (GB6 and GB5 plus anaerobes), the number of *E. coli* decreased but was higher than that in GB6 plus anaerobes, cvd, and conv ($P < 0.05$) (Fig. 4).

(ii) Streptococci. The change in the number of streptococci in this part of the digestive tract was similar to that of *E. coli*. That is, the number of these bacteria was the highest in GB5 ($P < 0.01$), decreased in GB6, and decreased even more in GB5 plus anaerobes, GB6 plus anaerobes, cvd, and conv ($P < 0.05$). There was no significant difference statistically between the latter four groups.

(iii) Staphylococci. There was no difference in the population level of staphylococci in the GB and cvd rats. In conv rats, the percentage of detection and the number of these bacteria were lower.

(iv) Lactobacilli. The number of lactobacilli in GB6 plus anaerobes was lower than that in GB6 ($P < 0.05$), and higher than that in cvd and conv ($P < 0.01$).

(V) Bacteroides. The number of bacteroides in GB5 was much higher than that in the other groups. In conventional rats, bacteroides are rarely detected in this portion.

In the lower part of the small intestine, it was observed by histological techniques in cvd and conv rats but not in the other groups of animals that long chains of either rod- or coccal-shaped bacteria (11) colonized the villous epithelium.

Bacterial flora in the cecal contents. (i) E. coli. The population levels of *E. coli* in the cecal contents in GB5 plus anaerobes rats and GB6

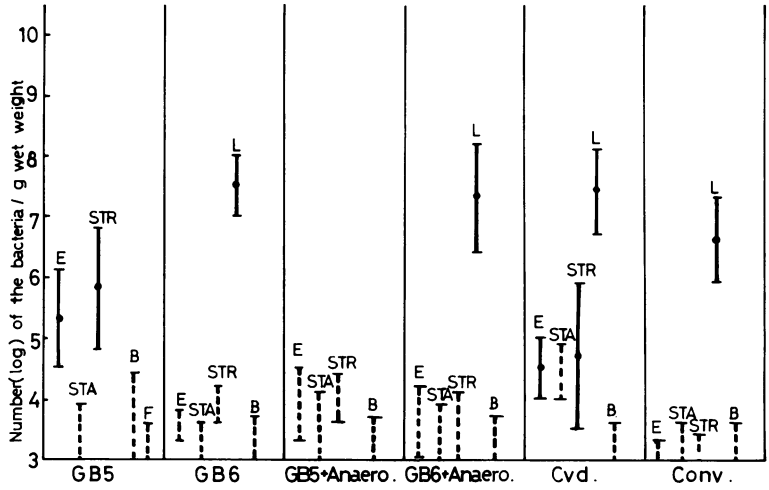

FIG. 3. *Population levels of indigenous bacteria in the contents of the upper part of the small intestine in GB, cvd, and conv rats. See the legend to Fig. 1.*

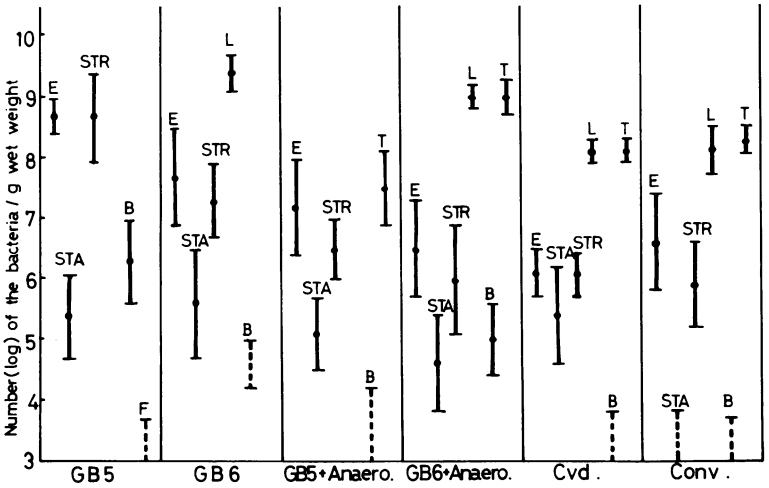

FIG. 4. *Population levels of indigenous bacteria in the contents of the lower part of the small intestine in GB, cvd, and conv rats. See the legend to Fig. 1.*

plus anaerobes rats were lower than that in GB5 and GB6 ($P < 0.01$) but higher than that in the cvd and conv animals ($P < 0.01$) (Fig. 5).

(ii) Staphylococci. There was no difference between GB and cvd rats in the number of staphylococci.

(iii) Streptococci. The number of the streptococci in the GB5 rats was somewhat higher than that in the GB6 ($P < 0.05$), and in the GB5 plus anaerobes and GB6 plus anaerobes it was lower than in GB5 and GB6 ($P < 0.01$).

(iv) Lactobacilli. The population level of lactobacilli in the GB6 rats was higher than that in the cvd and conv animals ($P < 0.01$), and in GB6 plus anaerobes rats it was somewhat higher than in the cvd and conv animals.

(v) Bacteroides. Changes in the population levels of bacteroides were different from those of other bacterial species. That is, the number of these bacteria in the GB5 and GB5 plus anaerobes animals were lower than that in the GB6 and GB6 plus anaerobes rats ($P < 0.05$), but higher than that in cvd and conv animals ($P < 0.01$).

DISCUSSION

In this paper we examined the lactobacilli, one of the autochthonous bacteria of rats (1, 3), and investigated how this organism controlled

the population levels of other bacterial species in each part of the digestive tract.

Stomach. The number of bacteria in the stomach is largely affected by coprophagy (6, 15). As the animals were not prevented from coprophagy in our experiments, the population levels of bacteria in the stomach might be affected by fecal populations. However, the population levels of *E. coli* and streptococci in the stomach in GB5 were much higher than those in GB6, whereas in the cecum there was little difference in them between the two groups. Therefore one may conclude that in the stomach there is an effect of lactobacilli in suppression of other bacterial species, although the mechanism of this suppression is not yet known.

Savage (12) reported microbial interference between indigenous yeast and lactobacilli in the rodent stomach. He observed that when the animals were given penicillin solution in the place of drinking water, the lactobacilli disappeared, and the yeast from the secreting epithelium colonized the nonsecreting epithelium within 24 h. When the penicillin treatment was discontinued, within 5 to 8 days the indigenous lactobacilli again colonized the nonsecreting epithelium. In the present experiments, we observed microbial interference between indigenous staphylococci and lactobacilli on the nonsecreting epithelium of the stomachs of rats. We presume lactobacilli displace staphylococci from the nonsecreting epithelium by interfering with attachment of the staphylococci to the keratinized cells, although desquamation of the epithelial surfaces or secretion of inhibitory substances in the stomach is also a distinct possibility.

Small intestine. In this report, bacterial overgrowth in the small intestine was observed in gnotobiotic animals, especially in GB5 and GB6 (Fig. 3 and 4). This overgrowth in the small bowel may be in part contributed by the bacterial population levels in the stomach. In such animals, further studies are required from the viewpoints of absorption or resistance to infection.

Anaerobes which colonize only the large bowels must affect the composition of the indigenous flora in the small intestine. For example, in the lower part of the small intestine, population levels of *E. coli* and streptococci were reduced after administration of anaerobes which were not detected in this portion (GB5 plus anaerobes and GB6 plus anaerobes; Fig. 4).

Anaerobes in the large intestine probably influence bowel motility and at the same time reduce cecal size. Consequently, they control the population levels of bacteria in the small intestine.

Cecum. It has been reported that in gnotobiotic animals staphylococci were reduced in number after administration of *Escherichia* (8, 10). In our experiments, the number of staphylococci was lowest in GB animals (Fig. 5). This suppression may be caused by *E. coli*. But in cvd animals, no suppression of staphylococci was seen. *E. coli* may lose its ability to control another bacterium when its population is suppressed by other microbes, as Freter and Abrams (6) reported. They also considered that intestinal anaerobes might under certain cir-

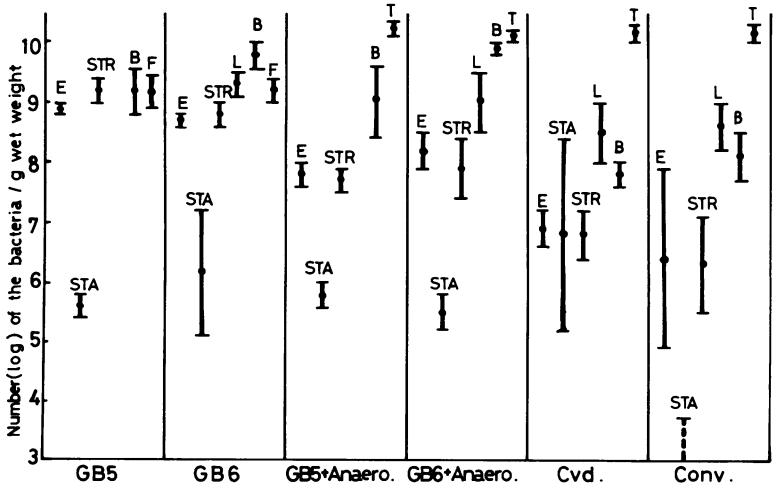

FIG. 5. *Population levels of indigenous bacteria in the cecal contents in GB, cvd, and conv rats. See the legend to Fig. 1.*

cumstances be sufficient to control the populations of the other intestinal bacteria such as *E. coli*. In our experiments, the population levels of *E. coli* in the cecum in GB5 plus anaerobes and GB6 plus anaerobes was between those found in GB5, GB6, and conv. This finding indicates that our collection of anaerobes is inadequate compared with that of Freter and Abrams (6).

Finally, it should be pointed out that in our experiments the population levels of lactobacilli were not affected by other bacteria in the stomach and in the upper part of the small intestine where the lactobacilli are predominant in both conventional and gnotobiotic animals. It is also important to point out that in the cecum, where the lactobacilli are not predominant, there is no effect of lactobacilli on the control of other bacterial populations.

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