INFECTION AND IMMUNITY, Feb. 1989, p. 559–565
0019-9567/89/020559-07$02.00/0

Vol. 57, No. 2

Control of *Escherichia coli* Populations by a Combination of Indigenous Clostridia and Lactobacilli in Gnotobiotic Mice and Continuous-Flow Cultures

KIKUJI ITOH† AND ROLF FRETER*

Department of Microbiology and Immunology, University of Michigan, Ann Arbor, Michigan 48109

Received 5 August 1988/Accepted 19 October 1988

The function of indigenous lactobacilli in the control of other intestinal microbial species is not clear. Still more controversial is the effect of dietary bacterial supplements containing lactobacilli or other species. This situation is unlikely to change unless the mechanisms that control the colonization of ingested bacteria are better understood, and until more detailed information becomes available on the mechanisms by which certain populations of indigenous bacteria can affect the population sizes of other species. We used gnotobiotic mice and a continuous-flow culture system to study the interactions between *Escherichia coli* and (i) clostridia (in chloroform-treated cecal suspensions from conventional mice) and (ii) three strains of lactobacilli isolated from conventional mice. In gnotobiotic mice, the lactobacilli suppressed *E. coli* multiplication in the stomach and the small intestine, but had no demonstrable effect on *E. coli* multiplication in the large intestine. In contrast, clostridia were most effective in controlling *E. coli* multiplication in the large intestine. In the presence of both lactobacilli and clostridia, *E. coli* populations in the various regions of the gastrointestinal tract resembled those found in conventionalized control animals. The control of *E. coli* populations was not related to changes in pH or intestinal motility. In vitro stimulation of the above-described in vivo interactions required a two-stage continuous-flow culture in which the effluent from the first stage represented the influx to the second. The first stage was inoculated with lactobacilli, and the second stage was inoculated with either a pure culture of *E. coli* or *E. coli* and clostridia. In these instances, the *E. coli* populations in the second stage of the culture resembled in size those found in the large intestine of gnotobiotic mice harboring a similar flora. Although there are some current shortcomings of this in vitro model, we expect that a multistage continuous-flow culture can be developed to satisfactorily model the interactions among bacterial populations along the entire gastrointestinal tract.

Following the ideas first enunciated by Metchnikoff (14), lactobacilli have been widely used as dietary supplements. Later workers have added other microorganisms, especially *Escherichia coli* and enterococci, to the armamentarium of such additives (15). This practice, often referred to as "probiotics," is said by some to contribute to the establishment of a beneficial microflora in the gastrointestinal tract and to result in improved general health, more rapid growth (in the case of livestock), and increased resistance to infections. Unfortunately, there is much contradictory evidence concerning the effectiveness of such supplements (4, 16). Tannock (17) writes: "For every article in the scientific literature that claims beneficial results from ingestion of fermented milk, another article will provide evidence to the contrary. Most of the reported studies have not been adequately controlled, statistical analysis of the results is rarely made, and the conclusions are largely subjective." Lee (13) points out that lactobacilli and bifidobacteria are still used in dietary supplements for historical reasons and because of their ease of culture. He submits that these microorganisms are not the logical choice for these purposes because they are not the dominant organisms in the intestinal microflora (a fact which is also true for coliforms and enterococci). Clinical data on the effectiveness of dietary supplements are not very strong (15). On a more positive note, Goldin et al. (7) have presented evidence showing that dietary supplements of lactobacilli can alter the metabolism of the intestinal microflora, especially the synthesis of enzymes such as beta-glucuronidase, nitroreductase, and azoreductase that might be involved in the in vivo synthesis of carcinogens.

The current status of the field may be summarized simply by stating that there is a considerable amount of suggestive evidence that microorganisms which form minority populations in the colonic or fecal flora, such as lactobacilli, coliforms, or enterococci, may nevertheless contribute to the stability of the intestinal ecosystem by their ability to reduce the poulation size of at least some species of undesirable bacteria of external or indigenous origin. The above-mentioned uncertainties concerning the use of bacteria as dietary supplements are a direct consequence of our current ignorance of the mechanisms that control populations of intestinal bacteria. Thus, we do not know what characteristics lactobacilli, coliforms, or enterococci must possess to be able to colonize the gastrointestinal tract after ingestion. Moreover, we do not know to what extent and under what conditions these microorganisms, even after successful colonization, could control the populations of other species in the gastrointestinal tract. The present study is designed to address some of these questions.

Indications for the use of bacterial dietary supplements include a large variety of often ill-defined imbalances in the ecology of the intestinal microflora, which result in a flora that, presumably, is impaired in its ability to control indigenous or invading bacteria that have undesirable metabolic activities or are outright pathogens (16). As an example of

* Corresponding author.
† Present address: Animal and Cellular Systems Laboratory, The Institute of Physical and Chemical Research, Hirosawa 2-1, Wako-shi, Saitama 35101, Japan.

such a deficient flora, we used gnotobiotic mice associated with chloroform-treated cecal suspensions (CTCS) from conventional mice. Such suspensions apparently contain only clostridia (8–10). A single *E. coli* strain was used as a representative of the family *Enterobacteriaceae*, which includes many of the classical enteric pathogens. This technique, which had been developed earlier by one of us (K.I.), produced gnotobiotic mice in which the enlarged cecum that is typical of germfree animals was reduced to normal size, but in which the population level of *E. coli* was only partially reduced and remained much higher than in conventional mice (9). We then asked why the clostridia in this deficient flora were unable to completely control *E. coli* populations and whether (and how) the addition of lactobacilli to the gnotobiotic flora could alter the equilibrium. In view of promising data obtained earlier with continuous-flow (CF) cultures as in vitro models of bacterial interactions in the gut (6), we attempted to reproduce the in vivo bacterial interactions in this system, in the hope that a suitable in vitro model might facilitate future studies of the mechanisms of these bacterial interactions.

MATERIALS AND METHODS

Animals. Germfree CD-1 mice were originally obtained from Charles River Breeding Laboratories, Inc. (Wilmington, Mass.) and then maintained and bred in this laboratory in germfree Trexler-type polyvinyl isolators. Conventional BALB/c mice served as a source of conventional intestinal flora. They were obtained from a colony maintained by William Murphy in the Department of Microbiology and Immunology, University of Michigan, Ann Arbor.

Bacterial isolates. *E. coli* C25 is a streptomycin-resistant mutant of an isolate from human feces which has been described in several previous studies (5). *Lactobacillus acidophilus* 129, *Lactobacillus murinus* 91, and *Lactobacillus fermentum* 106 were isolated from the feces of conventional mice (11).

Cecal suspensions from conventional mice were prepared as follows. Conventional mice were sacrificed by cervical dislocation and transferred to an anaerobic glove box (1) maintained at less than 5 ppm (5 μl/liter) of oxygen in an atmosphere consisting of 5% carbon dioxide, 10% hydrogen, and 85% nitrogen. The oxygen level inside the anaerobic glove box was monitored with a trace oxygen analyzer (Lockwood and McLorie, Inc., Horsham, Pa.). The ceca were removed and homogenized in Teflon pestle tissue grinders (Thomas Scientific, Swedesboro, N.J.) containing 9 ml of prereduced tryptic soy broth supplemented with 0.5% L-cysteine hydrochloride (RTSB).

CTCS from conventional mice were prepared as described previously (9). Chloroform was added to a cecal suspension to give a final concentration of 3%. This was shaken vigorously for about 30 s and subsequently incubated for 1 h at 37°C. After incubation, the chloroform was eliminated from the suspensions by percolation with N_2 gas. As described earlier (8–10), this suspension contained bacterial spores as the sole culturable component. For this reason, CTCS is assumed to contain *Clostridium* spp. only and will be referred to as such in this report.

Inoculation of mice. Overnight broth cultures of *E. coli* were administered to germfree CD-1 mice by adding a few drops to the drinking water. Two days later, cecal suspensions, CTCS, and bacterial cultures were enclosed in glass test tubes with butyl rubber stoppers and removed from the anaerobic glove box. The mice were inoculated with 1 ml of inoculum via the stomach and the rectum with a blunt-end feeding needle attached to a 5-ml syringe. The inoculations were repeated twice at 3-day intervals. The distribution of *E. coli* and lactobacilli in the gastrointestinal tract was determined by culture approximately 3 weeks later as described below.

Quantitative cultures. The population sizes of *E. coli* and lactobacilli were examined by sacrificing the mice by cervical dislocation and homogenizing separately (i) the stomach plus the upper part of the small intestine (to 5 cm from the stomach), (ii) the lower part of the small intestine, and (iii) the cecum and colorectum.

The walls and contents of each segment of the gastrointestinal tract were cultured as follows. Each segment was excised, and the contents were removed with a metal spatula, being careful to avoid scraping of the mucosa. The contents adhering to the walls were gently washed off with 4.5 ml of RTSB, and the tissues were homogenized in a total volume of 9 ml of RTSB. The intestinal contents were combined with the RTSB washings of the tissues and homogenized separately from the washed tissues. Serial dilutions of the homogenates were plated on deoxycholate agar containing streptomycin (100 μg/ml) for the enumeration of viable *E. coli* and on LBS agar (BBL Microbiology Systems, Cockeysville, Md.) for the enumeration of viable lactobacilli.

pH of gastrointestinal tract. The pH was measured directly by inserting a glass electrode (Micro-combination glass pH Probe, MI-410; Micro Electrodes, Inc., Londonderry, N.H.) into the lumen of the otherwise undisturbed stomach, small intestine, and cecum of freshly killed animals.

Transit rate of intestinal contents. A 2.5% suspension of green CR_2O_3 (0.1 ml) was inoculated into the stomach with a blunt feeding needle. The animals were returned to their cages for 1.5 h and then killed by cervical dislocation. The transit rate was calculated as the length of the green area, expressed as a percentage of the entire length of the small intestine.

CF culture. The single-stage CF culture system has been described previously (5). Briefly, it consisted of a culture vessel containing 7 ml of broth medium fed with a peristaltic pump at a continuous dilution rate of −0.16/h. The culture vessels and medium were kept in an anerobic glove box heated to 37°C. The culture medium used in the present study was modified veal infusion broth containing (per liter) Difco veal infusion broth (25 g), yeast extract (5 g), hemin (1 mg), menadione (0.5 mg), $NaHCO_3$ (2 g), germfree mouse fecal extract (1:1 in distilled water) (100 ml), liver infusion (100 ml), and BBL tomato juice broth (8.8 g). The mouse fecal extract was used because it had been shown to improve the ability of CF cultures to reproduce the interactions of *Clostridium difficile* with the mouse intestine (19). The pH was adjusted to 7.3, and the medium was autoclaved for 30 min at 120°C. The medium was allowed to equilibrate with the gas mixture inside the anaerobic glove box for 48 h prior to use. Single-stage CF cultures were inoculated first with *E. coli*. Two days later, the cultures were inoculated again with, in different experiments, either a small piece of cecal wall and adherent cecal contents from one of the groups of mice shown in Table 1 or CTCS or lactobacilli.

A diagram of the two-stage CF culture system is shown in Fig. 1. In two-stage CF cultures, lactobacilli were inoculated into the first stage and *E. coli* into the second stage. Two days later, a small piece of cecum with its contents from one of the groups of mice described in Table 1 was inoculated

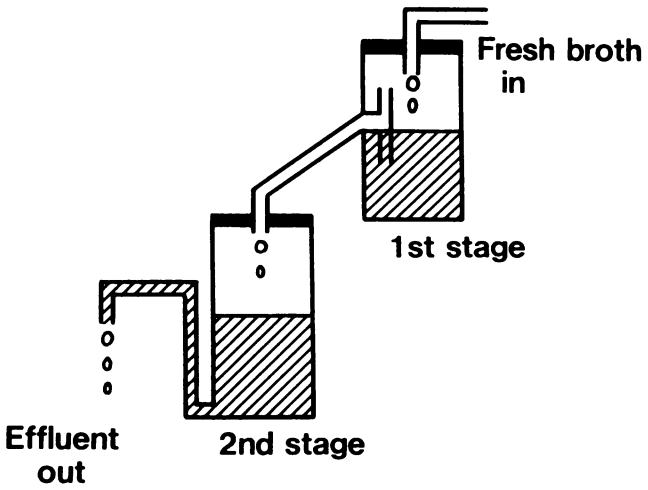

FIG. 1. Schematic representation of the two-stage CF culture system.

into the second stage. All other parameters were as described above for single-stage CF cultures.

Statistical analysis. Statistical analysis was by Student's *t* test.

RESULTS

Gnotobiotic mice. A typical series of experiments to determine the distribution of *E. coli* and lactobacilli in the gastrointestinal tract is shown in Table 1. There were no large differences in the numbers of lactobacilli in various parts of the gastrointestinal tract among the three groups inoculated with this microorganism (Table 1). In contrast, the *E. coli* population levels were very different among the groups. In *E. coli*-monoassociated mice and in the gnotobiotic mice associated with CTCS, the *E. coli* population level was high in the specimens from stomach and small intestine. In contrast, the gnotobiotic control mice associated with cecal suspensions of conventional mice had an *E. coli* population of about 10^3 per organ in these specimens. The average populations of *E. coli* in stomachs and small intestines of mice associated with lactobacilli or CTCS plus lactobacilli were much reduced in size compared with those of the CTCS-associated group, but did not quite reach the low level of those of the control mice associated with a cecal suspension from conventional mice (note, however, the data in Table 2, in which this difference was not apparent).

In the large intestine, in contrast to the findings in the small intestine, association with both CTCS and lactobacilli (but not with either of these components alone) reduced the *E. coli* populations to the level of those of the control mice. The *E. coli* counts throughout the intestinal tract of mice associated with CTCS alone were constant (group 3, Table 1). This suggests that *E. coli* was multiplying mainly in stomach or small intestine or both and that the clostridia in CTCS prevented significant additional multiplication of the transient *E. coli* in the large intestine. Consequently, whereas the multiplication of *E. coli* in the cecum of mice of group 3 was inhibited by the clostridia, there was still a sizable *E. coli* population present in the cecum, which resulted from the influx of *E. coli* into the cecum from proximal sites where, in the absence of lactobacilli, *E. coli* could multiply freely. In other words, while it is semantically correct to state that the presence of lactobacilli affected the *E. coli* populations in the cecum, this statement is not very informative. Ecologically important is the question whether, and where in the gut, the lactobacilli affected the multiplication of *E. coli*, and here our data showed that this occurred only in the stomach and, possibly, in the small intestine.

In the control mice harboring a conventional flora (Table 1), multiplication of *E. coli* appeared to take place mainly in the cecum. This is evidenced by the much increased *E. coli* counts in the cecum compared with the lower *E. coli* counts in the stomach and small intestine. Such a large (over

TABLE 1. Numbers of *E. coli* and lactobacilli in the gastrointestinal tract of various gnotobiotic mice

Group	Composition of flora (no. of mice)	Cecal size (% total body wt ± SD)	Parts of the gastrointestinal tract[a]	Mean $\log_{10}$ CFU/organ ± SD	
				E. coli	Lactobacilli
1	*E. coli* C25 (6)	5.32 ± 0.98	Stomach + USI	8.25 ± 0.91	Not detected
			LSI	8.11 ± 0.67	Not detected
			Cecum	10.01 ± 0.26	Not detected
			Colorectum	9.52 ± 0.19	Not detected
2	*E. coli* C25 and lactobacilli (7)	5.00 ± 0.63	Stomach + USI	5.32 ± 0.74	8.48 ± 0.22
			LSI	5.77 ± 0.41	8.85 ± 0.10
			Cecum	9.04 ± 0.23	9.57 ± 0.07
			Colorectum	8.62 ± 0.59	9.04 ± 0.23
3	*E. coli* C25 and CTCS (6)	1.59 ± 0.09	Stomach + USI	7.26 ± 0.24	Not detected
			LSI	7.03 ± 0.80	Not detected
			Cecum	7.03 ± 1.22	Not detected
			Colorectum	7.13 ± 1.08	Not detected
4	*E. coli* C25, CTCS, and lactobacilli[b] (7)	1.48 ± 0.22	Stomach + USI	4.04 ± 1.44	8.45 ± 0.47
			LSI	4.90 ± 0.88	8.48 ± 0.46
			Cecum	5.14 ± 1.33	8.94 ± 0.33
			Colorectum	5.63 ± 1.13	9.00 ± 0.26
Control	*E. coli* C25 and cecal suspension[c] (7)	1.63 ± 0.13	Stomach + USI	<3.13 ± 1.44	8.92 ± 0.36
			LSI	3.14 ± 1.28	8.53 ± 0.56
			Cecum	5.45 ± 0.63	8.47 ± 0.85
			Colorectum	5.53 ± 0.48	8.51 ± 0.76

[a] USI, Upper part of small intestine (5 cm from the stomach); LSI, lower part of small intestine (all of the small intestine except USI).
[b] Mixture of *L. acidophilus*, *L. murinus*, and *L. fermentum*.
[c] From conventional mice.

TABLE 2. Numbers of *E. coli* and lactobacilli in walls and contents of the gastrointestinal tract of various gnotobiotic mice

Composition of flora (no. of mice)	Parts of the gastrointestinal tract[a]	Mean $\log_{10}$ CFU ± SD of *E. coli*/organ		% of *E. coli* population on walls	Mean $\log_{10}$ CFU ± SD of lactobacilli/organ		% of lactobacillus population on walls
		Walls	Contents		Walls	Contents	
E. coli C25 and cecal suspension[b] (6)	Stomach	<2.82	4.00 ± 1.76		7.44 ± 0.58	7.86 ± 0.67	27.5
	USI	<2.32	2.81 ± 1.20		6.75 ± 1.04	7.29 ± 1.14	22.4
	LSI	<2.98	3.89 ± 0.90		7.01 ± 0.73	7.71 ± 0.73	16.6
	Cecum	5.44 ± 0.25	6.26 ± 0.15	13.1	6.95 ± 0.48	7.86 ± 0.66	11.0
	Colorectum	5.04 ± 0.28	6.80 ± 0.64	1.7	6.84 ± 0.92	8.00 ± 0.54	6.5
E. coli C25, and CTCS, and lactobacilli[c] (6)	Stomach	2.99 ± 1.41	3.99 ± 1.96	9.1	7.90 ± 0.28	8.46 ± 0.41	21.6
	USI	<2.68	<2.97		7.25 ± 0.21	7.96 ± 0.27	16.3
	LSI	3.99 ± 1.31	4.66 ± 1.42	17.6	7.67 ± 0.28	8.35 ± 0.31	17.3
	Cecum	5.51 ± 0.58	6.42 ± 0.63	11.0	7.75 ± 0.37	8.62 ± 0.38	11.9
	Colorectum	5.29 ± 0.57	6.80 ± 0.80	3.0	7.44 ± 0.31	9.00 ± 0.59	2.7

[a] USI, Upper part of small intestine (5 cm from the stomach); LSI, lower part of small intestine (all of the small intestine except USI).
[b] From conventional mice.
[c] Mixture of *L. acidophilus*, *L. murinus*, and *L. fermentum*.

200-fold) increase cannot be explained by a mere accumulation in the cecum of bacteria from the small intestine. If such an accumulation were a significant factor, it should occur in all groups (especially in group 3, Table 1), and this was not observed in these experiments.

Table 2 shows the populations of *E. coli* and lactobacilli in the washed tissue and contents of each segment of the gastrointestinal tract of gnotobiotic mice associated with either conventional mouse cecal flora or CTCS plus lactobacilli. In this experiment, there were no significant differences in *E. coli* and *Lactobacillus* populations between the mice associated with conventional mouse cecal flora and those with CTCS plus lactobacilli, i.e., the slightly elevated *E. coli* populations in the stomach and small intestine of group 4 (Table 1) were not apparent here. The adhesive capacity of lactobacilli (percentage of *Lactobacillus* population on walls, Table 2) was highest in the stomach and small intestine, whereas the adhesion of *E. coli* appeared to be optimal in the lower small intestine and cecum.

It is often suspected that lactobacilli reduce the multiplication rates of enteric bacteria in the gut by lowering the pH. However, there were no large differences in pH in the different regions of the gastrointestinal tract among the various groups of mice studied (Table 3), despite large differences in the *E. coli* populations (Table 1).

Table 4 shows the transit rate of Cr_2O_3 through the small intestine. In conventional mice and in gnotobiotic mice which harbored clostridia, transit was faster than in the other groups. Transit rate did not correlate with the size of *E. coli* poulations in the stomach and small intestine. For example, groups 3 and 4 and the control group had similar transit rates (Table 4), but very different *E. coli* populations (Table 1). This does not rule out the possibility that the high transit rates of the small intestine in animals associated with clostridia were a contributory factor. For example, the *E. coli* populations in the stomach and small intestine of group 4 (Table 1) were lower than those of group 2. These differences are small and probably not significant. On the other hand, one may speculate that the higher rate of peristalsis brought about by the clostridia in group 4 caused a more rapid removal of the *E. coli* from the stomach and small intestine, thus resulting in lower *E. coli* populations (but not necessarily lower *E. coli* multiplication). Be this as it may, intestinal motility had, at best, a minor role in determining the size of the *E. coli* populations and, almost certainly, had no role in determining the rates of *E. coli* multiplication in the various regions of the gastrointestinal tract.

CF cultures. Cecal suspensions from conventional mice and from gnotobiotic mice associated with CTCS plus lactobacilli or with lactobacilli only were inoculated into single-stage CF cultures previously inoculated with *E. coli* C25. The mice were from the same groups described in Table 1. The lactobacilli were eliminated from all CF cultures (Fig. 2). Fifteen days after the first inoculation, lactobacilli were inoculated a second time into two of the CF cultures, and the lactobacilli were again eliminated (Fig. 2). Consequently, it appears that lactobacilli were unable to colonize in the presence of *E. coli* or other cecal flora. This finding is consistent with the data obtained in mice discussed above, which indicate that there was no multiplication of lactobacilli in the large intestine.

It became clear at that point that the in vivo interactions between lactobacilli and *E. coli* could not be simulated by a

TABLE 3. pH in the gastrointestinal tract of germfree, conventional, and various gnotobiotic mice

Type of mice	Composition of flora (no. of mice)	pH (mean ± SD)			
		Stomach	USI[a]	LSI[a]	Cecum
Gnotobiotic	*E. coli* C25 and CTCS (6)	1.95 ± 0.37	6.54 ± 0.21	7.39 ± 0.18	7.00 ± 0.29
Gnotobiotic	*E. coli* C25, CTCS, and lactobacilli[b] (6)	3.02 ± 0.92	6.65 ± 0.21	7.21 ± 0.32	6.73 ± 0.12
Gnotobiotic	*E. coli* C25 and CTCS (6)	2.57 ± 1.03	6.62 ± 0.20	7.63 ± 0.15	6.57 ± 0.19
Gnotobiotic	*E. coli* C25 and lactobacilli (6)	3.53 ± 0.74	6.58 ± 0.05	7.54 ± 0.03	6.75 ± 0.06
Germfree	None (6)	2.07 ± 0.28	6.63 ± 0.24	7.49 ± 0.31	6.62 ± 0.36
Conventional	Conventional mouse flora (6)	3.40 ± 1.46	6.61 ± 0.16	7.29 ± 0.06	6.62 ± 0.36

[a] USI, Upper part of small intestine (5 cm from the stomach); LSI, lower part of small intestine (all of the small intestine except USI).
[b] Mixture of *L. acidophilus*, *L. murinus*, and *L. fermentum*.

TABLE 4. Transit rate of small intestinal contents in germfree, conventional, and various gnotobiotic mice

Group	Type of mice	Composition of flora (no. of mice)	Transit rate (%) (mean ± SD)
1	Germfree	None (6)	37.6 ± 10.0
2	Gnotobiotic	*E. coli* C25 and lactobacilli[a] (6)	50 ± 3.52
3	Gnotobiotic	*E. coli* C25 and CTCS (6)	81.9 ± 11.54
4	Gnotobiotic	*E. coli* C25, CTCS, and lactobacilli (6)	81.5 ± 15.33
Control	Gnotobiotic	*E. coli* C25 and cecal suspension[b] (6)	75.9 ± 6.86
Control	Conventional	Conventional mouse flora[c] (6)	89.1 ± 3.7

[a] Mixture of *L. acidophilus*, *L. murinus* and *L. fermentum*.
[b] From conventional mice.
[c] Germfree mice caged for at least 3 weeks with conventional mice.

single-stage CF culture, for the simple reason that these two populations occupy different habitats in the mouse. Consequently, the two-stage CF culture described above was used. The first stage was to represent the stomach and small intestine, whereas the second stage might be analogous to the cecum. The first stage was inoculated with pure cultures of the three strains of lactobacilli, and the second stage was inoculated with *E. coli*. Two days later, the second stage was inoculated again with, in different experiments, cecal wall and contents from one of the groups of mice described in Table 1. The contents of the second stage were then cultured periodically to determine the populations of *E. coli* and lactobacilli. These cultures revealed similar interactions between lactobacilli, CTCS, and *E. coli* as shown in Tables 1 and 2 for the mouse cecum, i.e. consistently high *Lactobacillus* populations and *E. coli* populations that were significantly reduced only in the presence of CTCS. Figure 3

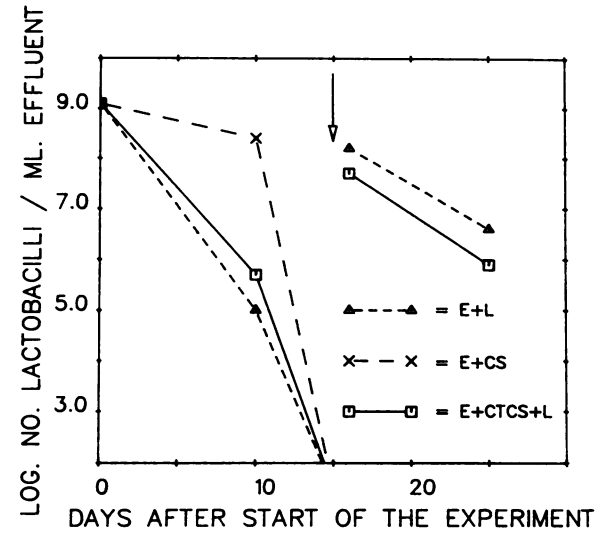

FIG. 2. Number of lactobacilli in a single-stage CF culture. On day 0, the cultures were inoculated with *E. coli* (E) plus one or more of the following: a suspension of cecal contents from conventional mice (CS); clostridial spores in a CTCS from conventional mice (CTCS); or a mixture of *L. acidophilus*, *L. murinus*, and *L. fermentum* (L). On day 15 (arrow), two of the cultures were reinoculated with the mixture of lactobacilli.

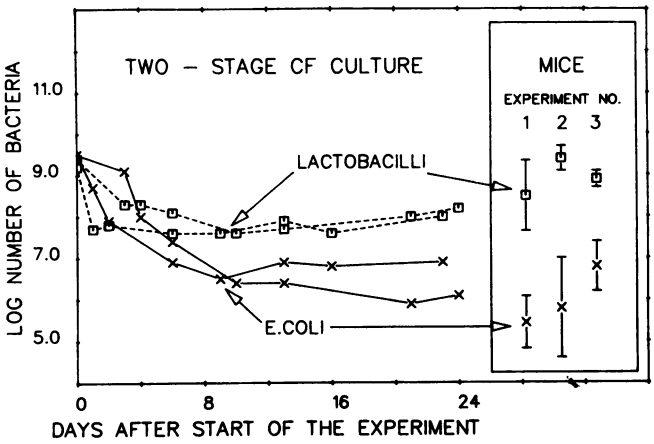

FIG. 3. Number of *E. coli* and lactobacilli in the second stage of two-stage CF cultures. Data from two replicate experiments are shown. On day 0, the first stage was inoculated with a mixture of *L. acidophilus*, *L. murinus*, and *L. fermentum* and the second stage was inoculated with *E. coli*. On day 2, the second stage was inoculated with cecal contents of mice associated with CTCS (group 3, Table 1). The individual data points with error bars shown in the box at the right side of the graph indicate the mean number and standard deviation of bacterial counts in the cecum of mice associated with the same flora as the CF cultures. Each vertical pair of these data points is derived from one replicate experiment, involving six to nine mice each.

illustrates two such experiments in which the second stage was inoculated with *E. coli* and CTCS (clostridia). Because the first stage (containing the lactobacilli) empties into the second stage, the second stage also contained lactobacilli in addition to the two species that had been inoculated directly into the second stage. This arrangement resembles the situation found in mice in which, as described above, the lactobacilli appear to multiply exclusively in the stomach and small intestine and in which the lactobacillus populations found in the cecum represent merely the runoff from those proximal sites of multiplication. For comparison, Fig. 3 also shows data from three replicate experiments in mice which were associated with the same flora as the CF cultures. These animals had been prepared by caging germfree mice for at least 3 weeks with the mice described in group 4 of Table 1. Figure 3 shows that the population levels of lactobacilli and *E. coli* observed in the in vivo experiments were reproduced in the second stage of the CF cultures.

DISCUSSION

The data presented in Table 1 show that the lactobacilli were a major factor in controlling *E. coli* multiplication in stomach and small intestine, but were largely ineffective in affecting *E. coli* multiplication in the large intestine. The suggestion by Lee (13) quoted above, namely, that lactobacilli are unlikely candidates for the control of other intestinal populations because they do not represent a majority population in the intestinal tract, is therefore only partly correct. The gastrointestinal tract consists of a large number of ecological niches, and some microbial species may predominate in one of those, even though they may not be among the predominant populations in the colonic or fecal flora. The inhibitory effect of the lactobacilli in stomach and small intestine was not correlated with changes in pH in the lumen of these organs. A comparison of group 4 with the control group in Table 1 shows, however, that lactobacilli were not

sufficient to reduce the *E. coli* population entirely to the level of that of the conventionalized animals, indicating that additional bacterial species may be involved in the control of *E. coli*.

In contrast to lactobacilli, the clostridia in the CTCS preparation were most effective in controlling *E. coli* in the large intestine and had no effect on its multiplication in the stomach and small intestine. Table 1 shows further that the size of the *E. coli* populations in the stomach and small intestine was not correlated with the size of its populations in the large intestine, a finding which rules out coprophagy as a major source of the former. Recent data from this laboratory (R. Freter, unpublished data) also show that the *E. coli* populations in the stomach and small intestine persist in conventional mice at similar levels as shown in Table 1 (control group), even when coprophagy is prevented. One must conclude, therefore, that the bacterial populations in the proximal regions of the gastrointestinal tract were truly colonizing, i.e., they were maintained by in situ multiplication of the bacteria. *E. coli* populations in the large intestine obviously reflected the accumulated influx from proximal sites which, in all but group 3 of Table 1, was augmented by additional in situ multiplication.

It is apparent, then, that lactobacilli and the clostridia in CTCS occupied two entirely different habitats in the mouse gastrointestinal tract. Both of these habitats were shared by *E. coli*. It is not surprising, therefore, that a single-stage CF culture was unable to reproduce these complex interrelationships. The finding that a two-stage CF culture did duplicate the in vivo findings is encouraging. It must be pointed out, however, that the two-stage in vitro model is not perfect, because it requires a first stage that is inoculated with pure cultures of lactobacilli. When *E. coli* was subsequently introduced into the first stage, it multiplied and eventually replaced the lactobacilli, in clear contrast to what happens in the mouse small intestine. Most likely, the high degree of fitness of the lactobacilli for growth in the stomach and small intestine is related to their high adhesive capacities. Table 2 shows that the percentages of *Lactobacillus* populations that were associated with the walls of the stomach and small intestine were consistently high (16.6 to 27.5%), in contrast to *E. coli*, which did not exceed 17.6% adhering bacteria anywhere in the gastrointestinal tract. Mathematical models of ecological interactions in CF cultures and in the intestine show that even small differences in adhesive capacity can have profound effects on the colonization of bacteria in dynamic systems such as the gut (3, 5). Most likely, then, the most promising approach to the design of an improved two-stage CF culture is to use a first-stage growth tube fabricated of, or coated with, material that allows superior adhesion of lactobacilli. In addition, the first stage may be made smaller, to give it a specific flow rate which is higher than that of the second stage. This may eventually lead to an in vitro model of the small and large intestine that can be used to study the actual mechanisms by which the intestinal populations interact. To our knowledge, the report by Veilleux and Rowland (18) is the only earlier description of a two-stage CF culture that was used to simulate the microecology of the intestine. These workers utilized acidification of the first stage as the device to make it competitive for lactobacilli. This design may serve to make the first stage resemble the stomach, but it will not permit a study of the mechanisms by which lactobacilli interact with coliforms in the small intestine. It is possible, therefore, that the ultimate in vitro model will have to be a three-stage CF culture, with the individual stages representing the stomach, small intestine, and cecum, respectively.

In general, we demonstrated what in retrospect seems to be a very obvious fact, namely, that the gastrointestinal tract consists of a number (most likely considerably more than two) of ecosystems with different characteristics which are controlled by different groups of bacteria. The actual sites and the nature of these ecosystems certainly differ among different animal species. For example, unlike the situation in the mouse, lactobacilli do not colonize the human stomach, but rather the lower ileum. It seems obvious, however, that the gut is not a homogeneous fermentor in any animal species and that all its various composite ecosystems must be considered if one wants to understand the ecology of the intestinal flora. Concerning the practical matter of attempts to change the intestinal microecology by dietary bacterial supplements, recent attempts by others to select strains with superior adhesive properties (2, 12) may be a step in the right direction. It seems unlikely, however, that this characteristic by itself is sufficient to ensure colonization and control of undesirable bacterial species. Most likely, earlier attempts to consistently modify the intestinal flora by feeding dietary supplements containing a single bacterial species have failed because this approach implies an overly simplistic view of the intestine as a single homogeneous fermentor. Rather, control of *E. coli* and other undesirable species in each habitat is likely to involve different mechanisms mediated by different indigenous bacterial populations, and control of intestinal flora by dietary supplements is likely to be successful only if the supplements contain at least the major bacterial species that are important in the ecology of each of the various habitats. Consequently, the interaction of the added microorganisms with other components of the indigenous flora and the mechanisms by which supplemental bacteria can affect the populations of other species must be investigated for each potential habitat. The present study represents a first step in that direction.

ACKNOWLEDGMENT

This investigation was supported by Public Health Service grant AI 20387 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. **Aranki, A., and R. Freter.** 1972. Use of anaerobic glove boxes for the cultivation of strictly anaerobic bacteria. Am. J. Clin. Nutr. **25**:1329–1334.
2. **Conway, P. L., S. L. Gorbach, and B. R. Goldin.** 1987. Survival of lactic acid bacteria in the human stomach and adhesion to intestinal cells. J. Dairy Sci. **70**:1–12.
3. **Freter, R.** 1983. Mechanisms that control the microflora in the large intestine, p. 33–54. *In* D. J. Hentges (ed.), Human intestinal flora in health and disease. Academic Press, Inc., Orlando, Fla.
4. **Freter, R.** 1988. Mechanisms of bacterial colonization of the mucosal surfaces of the gut, p. 45–60. *In* J. A. Roth (ed.), Virulence mechanisms of bacterial pathogens. American Society for Microbiology, Washington, D.C.
5. **Freter, R., H. Brickner, M. Botney, D. Cleven, and A. Aranki.** 1983. Mechanisms that control bacterial populations in continuous-flow culture models of mouse large intestinal flora. Infect. Immun. **39**:676–685.
6. **Freter, R., E. Stauffer, D. Cleven, L. V. Holdeman, and W. E. C. Moore.** 1983. Continuous-flow cultures as in vitro models of the ecology of large intestinal flora. Infect. Immun. **39**:666–675.
7. **Goldin, B. R., L. Swensson, M. Sexton, and S. L. Gorbach.** 1980. Effect of diet and *L. acidophilus* on human fecal bacterial enzymes. J. Natl. Cancer Inst. **64**:255–262.

8. **Hazenberg, M. P., and L. M. C. Custers-van Lieshont.** 1976. Conversion of germ-free mice to the normal state by clostridia. Z. Versuchstierkd. **18**:185–190.
9. **Itoh, K., and T. Mitsuoka.** 1980. Production of gnotobiotic mice with normal physiological functions. I. Selection of useful bacteria from faeces of conventional mice. Z. Versuchstierkd. **22**:173–178.
10. **Itoh, K., and T. Mitsuoka.** 1985. Characterization of clostridia isolated from faeces of limited flora mice and their effect on caecal size when associated with germfree mice. Lab. Anim. **19**:111–118.
11. **Itoh, K., T. Mitsuoka, K. Sudo, and K. Suzuki.** 1983. Comparison of fecal lactobacilli in mice of different strains under different housing conditions. Z. Versuchstierkd. **25**:193–200.
12. **Kleeman, E. G., and T. R. Klaenhammer.** 1982. Adherence of *Lactobacillus* species to human fetal intestinal cells. J. Dairy Sci. **65**:2063–2071.
13. **Lee, A.** 1985. Neglected niches. The microbial ecology of the gastrointestinal tract. Adv. Microb. Ecol. **8**:115–162.
14. **Metchnikoff, E.** 1907. The prolongation of life. P. Putnam & Sons, New York.
15. **Rusch, V.** 1980. Medicine and the microbial world. Microecol. Ther. **10**:163–172.
16. **Shahani, K. M., and A. D. Ayebo.** 1980. Role of dietary lactobacilli in gastrointestinal microecology. Am. J. Clin. Nutr. **33**:2448–2457.
17. **Tannock, G. W.** 1984. Control of gastrointestinal pathogens by normal flora, p. 374–382. *In* M. J. Klug and C. A. Reddy (ed.), Current perspectives in microbial ecology. American Society for Microbiology, Washington, D.C.
18. **Veilleux, B. G., and I. Rowland.** 1981. Simulation of the rat intestinal ecosystem using a two-stage continuous culture system. J. Gen. Microbiol. **123**:103–115.
19. **Wilson, K. H., and R. Freter.** 1986. Interaction of *Clostridium difficile* and *Escherichia coli* with microfloras in continuous-flow cultures and gnotobiotic mice. Infect. Immun. **54**:354–358.