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Function of Various Intestinal Bacteria in Converting Germfree Mice to the Normal State

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Earlier work had shown that a collection of anaerobic bacteria, in conjunction with facultative anaerobes, may be implanted into germfree mice, thereby rendering the animals "normal" with respect to a variety of parameters tested. The present experiments indicate that a different collection of anaerobic bacteria, isolated from the cecum of normal mice, was necessary to convert germfree mice to the "normal" state when the animals were fed a crude diet, rather than the refined food which had been used in the earlier work. The nature and level of short-chain fatty acids associated with various natural or synthetic "normal" floras in the ceca of mice did not always correlate with the *Escherichia coli* population present, indicating that fatty acids were not the sole agents inhibiting bacterial populations in the intestine. Experiments are reported which indicate that intestinal anaerobes may under certain circumstances be sufficient to control the populations of other intestinal bacteria such as *E. coli*. In other instances, such as control of *Shigella* populations in the mouse intestine, intestinal anaerobes appeared to act synergistically with an *E. coli* strain, in spite of the fact that the population of the latter was itself suppressed by the anaerobes.

The data presented in this paper are part of a continuing study which has as its ultimate goal the delineation of the mechanisms by which the normal enteric flora maintains its stability and by which it antagonizes the establishment of "invading" microorganisms such as enteric pathogens. Stability of certain elements of intestinal flora has been demonstrated by a number of authors (e.g., 16, 21, 22). There is also an increasing list of published studies concerned with the mechanisms of this stability. Conceptually these may be looked upon as representing two different experimental approaches: (i) the synthetic approach, in which a small number of defined bacterial species is studied in vitro or in vivo by using systems such as antibiotic-treated or germfree animals; and (ii) the deductive approach, in which conclusions are drawn from studies of an unchanged and undefined natural flora. Both approaches have their advantages and shortcomings. The synthetic method allows precise analysis of interactions between relatively simple populations. Such studies (4–6, 8, 11, 14) have yielded a number of mechanisms by which one bacterial species may control the growth of another: inhibitory substances such as fatty acids and deconjugated bile salts, competition for nutrients, and complex biological entities such as colicines (the latter now largely ruled out; see references 6 and 11). The synthetic approach lacks, however, an experimental device for demonstrating that the mechanisms discovered in simplified systems are actually operating in the highly complex natural flora. The duductive approach (2, 13, 15; P. V. Scarpino, R. W. Neimeir, and L. E. Deters, Bacteriol. Proc., p. 103, 1970) does not suffer from the latter shortcoming. However, the inability to analyze and control the complex natural fluora of the intestine does not permit the investigator to proceed much beyond the demonstration of associations between certain phenomena. An example of this is the very elegant demonstration by Meynell (13) that metabolic endproducts (short-chain fatty acids) of anaerobic bacteria are present in the mouse cecum in concentrations which are inhibitory in vitro to a strain of *Salmonella typhimurium*. Although this may indeed be true for the particular strain tested, the available data certainly do not justify the conclusion that "the growth of the normal flora" may be controlled "by their metabolic products, including fatty acids" (13). More decisive studies pertaining to this question were not possible in the past because in vivo fatty acid concentrations could not be varied experimentally without at the same time eliminating all anaerobic bacteria from the intestine.

The overall design of studies in this laboratory

is, hopefully, to combine the virtues of the synthetic and deductive approaches by working with a synthetic "normal" flora which is established in germfree animals by feeding pure cultures of all intestinal bacteria of functional importance. A preceding paper (18) reported the synthesis of a flora in germfree animals which was normal with respect to all criteria tested. This had become possible with the development of an anaerobic chamber culture technique permitting easy cultivation of fastidious intestinal anaerobes (1). The present paper elaborates on this finding by showing that a different population of intestinal bacteria is required for animals fed a different diet.

Further data presented in this paper pertain to the question of which microorganisms in the intestine are actually responsible for the control of other bacterial species or strains. Two points of view on this matter have been held, explicitly or implicitly, by earlier investigators. The first of these supposes that all control functions are exerted by the predominant anaerobes (2, 8, 13). The other view implies that certain "resident strains" of facultative anaerobes, such as *E. coli*, may inhibit the establishment of other "invader" strains of the same species. The various studies on colicines are examples of this line of reasoning. Much can be said against such a supposition. Especially important here is the consideration that the population of a resident *E. coli* strain is itself considerably suppressed by other elements of the normal flora (from a level of about 5×10^9/g as found in animals monocontaminated with *E. coli* (*see* Table 1), to 5×10^6/g or even less, which is the *E. coli* population commonly found in normal animals). One may therefore wonder whether such a resident strain, which barely manages to hold its own, could be expected to suppress other organisms in its environment. As will be shown below, both points of view may be correct under different circumstances. A final set of data indicates that the quality and quantity of intestinal fatty acids do not necessarily correlate with the size of the *E. coli* population in the ceca of gnotobiotic mice.

MATERIALS AND METHODS

Mice. Donors of "whole normal flora" were mice of strain BALB/wm, maintained in this department by William Murphy. The germfree mice were Charles River strain CD-1. These were maintained in Trexler-type flexible vinyl isolators sterilized with peracetic acid. Stainless-steel cages with suspended wire-mesh bottoms were used in the isolators.

The animals were fed diets autoclaved at 16 psi for 25 min, with a 28-inch vacuum cycle 10 min before and after sterilization.

The diets used were: (i) Lobound diet L-356 (in powdered form, General Biochemicals), (ii) Charles River sterilizable rat and mouse diet Formula 7RF (Country Best, Agway Inc.), (iii) Lobound diet L-485 (Teklad Inc.), and (iv) Purina Breeder Chow (Ralston Purina Co.). Prior to sterilization, the diets were stored in sealed plastic bags in a cold room at 4 C.

Microorganisms. Bacteria were isolated from normal mice in an anaerobic glove box. Enriched Trypticase soy agar with palladium black overlay (1) was used for the isolation of strict anaerobes. Facultative anaerobes were isolated on the same medium. The collection of 45 anaerobic bacteria (here designated N-strains) is the same as that described earlier (18) except that five strains were lost from the original number of 50. Facultative anaerobes used in this study included enterobacteria, such as *Aerobacter*, *Proteus*, and *Pseudomonas* species; *Staphylococcus albus*; *Streptococcus faecalis*; *Lactobacillus*; and bacteria resembling the *Actinomyces-Bifidobacterium* group. A second collection of 95 anaerobes (designated F-strains) were isolated as described in the text. It should be emphasized that the number of anaerobes in these collections refers to random isolates. For reasons outlined earlier (18), no detailed attempts have been made at classification. It is therefore likely that a number of these isolates are identical. Judging from differences in morphology and in fatty acid patterns in glucose-broth cultures (19), it seems likely that the total number of different strains in these collections may be in the order of 35 and 60, respectively, but not much fewer.

The N-strain and F-strain collections were originally implanted into germfree mice by inoculation directly into the stomach and rectum (18). These injections were repeated twice at weekly intervals. Mice injected in this manner were maintained in germfree isolators and served as sources of these bacteria for subsequent experiments. To associate experimental germfree animals with either N-strain or F-strain collections, two mice from one of the above groups were simply caged with the experimental animals. Association in this manner was accomplished within approximately 3 weeks, since cecal size and *E. coli* populations (in experiments where *E. coli* was added) reached their lowest values after this period of time (and maintained it thereafter). Germfree mice to be associated with "whole normal flora" were caged with two BALB/wm mice which were introduced into the germfree isolator.

E. coli strains C25 (streptomycin-resistant) and 40T (tetracycline-resistant) and the *Shigella flexneri* strain have been described in earlier publications (5, 14, 18).

Quantitative cultures. The ceca of mice were homogenized in 100 ml of Trypticase soy broth in a Waring Blendor. Serial dilutions of the homogenates were plated on the surface of Desoxycholate Agar (BBL) containing 1 mg of streptomycin per ml. This medium allows the growth of *E. coli* C25 while suppressing all other bacteria used in this study. Tetracycline (100 μg/ml), instead of streptomycin, was added to the medium for the enumeration of *E. coli* 40T. *E. coli* C25 was inhibited by this concentration of tetracycline.

Microscopic bacterial counts. Counts were performed

on organ homogenates prepared as above for quantitative cultures. A Petroff-Hausser chamber was used as described earlier (1).

Determination of fatty acids and pH in cecal contents. Mice were killed by cervical dislocation, and the cecum was removed immediately. A combination glass electrode (A. H. Thomas, no. 4858-L15) was then forced into the cecal lumen, and the *p*H was read on a Beckman model G *p*H meter. Several readings were taken by locating the electrode in different areas of the lumen. Individual readings in different locations of the same organ differed at times by as much as 0.4 *p*H units. In such instances an average value was recorded as "the" *p*H of cecal contents. Cecal contents were then removed. A small portion was weighed immediately, and again after 24 hr at 120 C, for determination of water content. The larger portion of cecal contents was weighed, mixed with 2 ml of 0.01 N NaOH and centrifuged. The supernatant fluid was then acidified with 50% H_2SO_4 to *p*H <2.0. Volatile fatty acids were determined by gas chromatography (19). Nonvolatile fatty acids were determined by chromatography on silica gel columns as described by Kesner and Muntwyler (12). Individual acids were quantitated by estimating the areas under the individual curves by the method of Spackman et al. (17) and by subsequent comparison with standard curves of known fatty acids. Molarities of fatty acids were calculated on the basis of the total amount of water in the contents of the individual ceca.

RESULTS

As reported earlier (18) a collection of 50 anaerobic bacteria plus 80 facultative anaerobes was able to convert germfree mice to a state which was normal with respect to a variety of criteria used. This collection has now been reduced to 45 anaerobes plus 14 facultative anaerobes. Table 1 (expt C) shows that mice associated with these bacteria plus *E. coli* C25 showed the same cecal size and *E. coli* populations as control mice conventionalized with whole normal flora (expt A). However, this was true only when refined diet L-356 was fed. In contrast, mice associated with these same bacteria but fed a crude diet (expt D) had cecal sizes and *E. coli* populations which were much larger than those of control mice on the same diet but associated with whole normal flora (expt B). With respect to these two parameters, the mice of group D were almost exactly halfway between mice monoassociated with *E. coli* C25 (group Co) and the controls in group B. Obviously then, the collection of strains used in experiments C and D lacked those microorganisms which are instrumental in establishing normal functions in mice fed a crude diet.

In view of the above, attempts were made to isolate additional collections of intestinal anaerobes. This was done by making serial dilutions of cecal homogenate and plating these on the surface of anaerobic agar (ETSA) plates with palladium black overlay. All procedures were carried out in the anaerobic glove box, and the media and diluting fluids used were prereduced by storage in the glove box for at least 2 days. After 7 days of incubation, a sufficient number of plates, bearing between 10 and 50 colonies each, were selected, to bring the total number of colonies to between 100 and 200. All colonies from these plates were then picked and subcultured. The pure cultures thus isolated were subsequently mixed again and were fed by stomach tube to mice which had been monoassociated with *E. coli* C25 for at least 2 days. Approximately 4 weeks later, the animals were sacrificed and tested for cecal size and *E. coli* population. A total of nine such collections was established in animals fed the crude L-485 diet. The first eight of these failed to bring the parameters tested into the range shown by animals conventionalized with whole normal flora. A final ninth collection of 95 strains was isolated on ETSA supplemented with fatty acids in a concentration roughly 40% that found in normal mouse cecum (per liter of medium: valeric acid, 0.13 ml; isovaleric acid, 0.13 ml; butyric acid, 1.1 ml; isobutyric acid, 0.35 ml; propionic acid, 0.6 ml; acetic acid, 2.9 ml). (A fatty acid supplement was used only on primary isolation. Subcultures were made on regular ETSA plates.) Use of fatty acids in devising "habitat simulating media" is a procedure advocated by Bryant (10) for rumen bacteria. This collection was designated "F-strains."

The results of a series of experiments comparing mice associated with the F-strains or with whole normal flora, respectively, are shown in Table 2. As may be seen, cecum weights were quite reproducible between experiments and were identical between the two groups. In comparing the mean *E. coli* populations, one must note that these were also quite reproducible between experiments with mice carrying F-strains. In contrast, mean *E. coli* populations in mice associated with whole normal flora differed significantly between experiments. This was in spite of the fact that all donor animals of whole normal flora were obtained from the same closed mouse colony. It is therefore not possible to designate any one closely defined population size of *E. coli* as being representative of "the" normal state.

The population size of *E. coli* C25 in mice carrying F-strains was in the range shown by control animals in experiments 70 and 79 (Table 2), i.e., those control experiments representing the larger mean population sizes. In considering why this is so, one must realize that, in contrast to the F-strains which are all anaerobes, whole

TABLE 1. *The effect of natural and gnotobiotic "normal" enteric flora on size, E. coli population, and fatty acid content of the mouse cecum*

Expt. no.	Flora implanted into germfree mice	Food[a]	Cecum percent body wt[b]	No. of *E.coli* per cecum × 10^{-6}[b]	Molarity of fatty acids in cecum (mean ± standard deviation)										Mean *p*H of cecal contents	No. of animals used for fatty acid and *p*H tests
					Valeric	Isovaleric	Butyric	Isobutyric	Propionic	Acetic	Succinic	Formic	Lactic	Total		
Co	*E. coli* C25 only	L-356	5.0 (3.0–7.0) N[c] = 16	5,700 (2,000–11,000) N = 16												
A	Whole flora from normal mouse + *E. coli* C25	L-356	1.0 (0.5–1.7) N = 18	18 (0.8–72) N = 18	0.0048 ±0.0019	0.0059 ±0.0029	0.0197 ±0.0114	0.0023 ±0.0016	0.0202 ±0.0073	0.1003 ±0.0359	0.0058 ±0.0164[d]	0.0115 ±0.0186	0.0161 ±0.0400	0.1866 ±0.0721	6.47	8
B	Whole flora from normal mouse + *E. coli* C25	CR	2.5 (1.6–2.9) N = 8	4.9 (0.2–63) N = 8	0.0028 ±0.0012	0.0030 ±0.0010	0.0387 ±0.0223	0.0013 ±0.0010	0.0185 ±0.0072	0.1397 ±0.0482	0.0084 ±0.0162	0.0041 ±0.0080	0.0066 ±0.0186	0.2231 ±0.0666	6.48	8
C	45 anaerobes (N-strains) + 14 facultative anaerobes + *E. coli* C25	L-356	1.4 (0.8–1.8) N = 18	4.7 (1.0–11.7) N = 18	None	0.0029 ±0.0012	0.0078 ±0.0081	0.0005 ±0.0006	0.0057 ±0.0012	0.0372 ±0.0113	0.0135 ±0.0119	0.0005 ±0.0020	None	0.0681 ±0.0241	6.95	16
D	45 anaerobes (N-strains) + 14 facultative anaerobes + *E. coli* C25	CR	3.7 (2.9–6.7) N = 18	100 (30–228) N = 18	0.0003 ±0.0009	0.0029 ±0.0012	0.0105 ±0.0056	0.0012 ±0.0007	0.0086 ±0.0032	0.0607 ±0.0183	0.0361 ±0.0374	None	None	0.1203 ±0.0534	Not done	16
E	95 anaerobes (F-strains) + *E. coli* C25	L-485	1.3 (0.9–2.0)[e] N = 75	6.8 (0.4–24)[e] N = 75	None	None	0.069 ±0.0208	None	0.0127 ±0.0026	0.1179 ±0.0311	Trace	None	None	0.1996 ±0.0527	6.53	5

[a] L-356 = Lobund diet no. 356. Refined, containing casein, polished rice, etc.; CR = Charles River diet. Crude, containing ground corn, soybean meal, etc.; L-485 = Lobund diet no. 485. Crude, containing ground corn, soybean meal, etc.

[b] First number indicates mean; numbers in parentheses indicate range.

[c] N = number of animals used for determination of cecal weight and *E. coli* population.

[d] In a few instances where very low concentrations of individual acids were present in some animals of a given group, a number of other mice in the same group had no detectable level at all and were scored as zero concentration. The distribution was therefore not normal, resulting in a standard deviation which was higher than the mean. In these instances, figures for mean and standard deviation are included in the table merely to indicate that the above circumstances obtained, even though the figures do not have strict statistical validity.

[e] Taken from data in Table 2.

TABLE 2. *Effect of a collection of 95 anaerobic bacteria (F-strains) on the population of E. coli C25 and on cecal size of gnotobiotic mice receiving diet L-485*[a]

Determination	Expt. no.	No. of animals in expt.	Cecum, percent body weight[b]	No. of *E. coli* C25 per cecum ($\times 10^{-6}$)[b]
F-strains + *E. coli* C25	81	24	1.51 (0.54–2.18)	5.62 (0.70–15.1)
	84	24	1.13 (0.53–1.66)	9.00 (0.40–23.0)
	82	12	1.39 (1.14–1.82)	8.01 (0.95–24.0)
	79	6	1.43 (1.01–1.92)	6.30 (3.80–10.8)
	87	9	1.29 (0.94–2.12)	2.42 (0.79–3.7)
Controls (whole normal flora + *E. coli* C25)	70	20	1.48 (0.88–1.97)	3.78 (0.30–21.4)
	81	12	1.44 (0.85–2.00)	0.48 (0.007–1.7)[c]
	79	6	1.32 (1.00–1.65)	1.80 (0.40–3.50)
	73	5	1.18 (0.99–1.30)	<0.1 (uniform)

[a] The same batch of food was used throughout these experiments.
[b] Numbers indicate mean; numbers in parentheses indicate range.
[c] Total *E. coli* count in experiment 81 was 9.32 (0.003–73) $\times 10^6$ per cecum.

normal flora contains many other bacteria, including coliforms other than the C25 strain. To demonstrate this, the total number of lactose-fermenting bacteria cultivated on Desoxycholate Agar free from antibiotic are shown for experiment no. 81 in footnote *c* of Table 2. As may be seen, the mean total *E. coli* population in this experiment was 9.32×10^6 per cecum, whereas the population of *E. coli* C25 cultivated in the usual manner on agar containing streptomycin was 0.48×10^6 per cecum in these same animals (Table 2). In contrast, no *E. coli* were detected on antibiotic-free Desoxycholate Agar in mice of experiment no. 73. These data show that a "normal" flora derived from animals of a closed mouse colony is quite variable in its composition, and consequently in its effect on populations of *E. coli* C25. Conceivably, the collection of F-strains represents a flora which, in conventional animals, may at times be the only one controlling the population of other bacteria (as in experiments 70 and 79) while at other times additional bacteria (including coliforms) may add to its effect.

A number of animals associated with F-strains and *E. coli* C25 were examined histologically (18). They resembled control animals associated with whole normal flora with respect to the presence of a mucosa-associated layer of bacteria in the large intestine. However, with respect to the morphological features of the mucosa, mice associated with the F-strains did not show the full degree of cellularity of the lamina propria and the characteristic mucosal architecture of normal control animals (18). Conceivably, this may be due to the lack of facultative aerobes in the F-strain population. As may be recalled, complete restoration of normal histologic features had been achieved in earlier experiments (19) involving the N-strain flora of anaerobes plus 80 facultative aerobes.

In view of the profound effect of diet on the function of the N-strain collection in gnotobiotic mice (Table 1), attempts were made to demonstrate effects of these diets on conventional animals as well. BALB/wm mice (the donors of whole normal flora used in this study) were housed in a germfree isolator, and three groups were fed different diets for 3 weeks. The results are shown in Table 3. The data indicate that both cecal size and total bacterial population were significantly affected by the diet. These findings are consistent with those of Wilkins (T. D. Wilkins and W. R. Long, Bacteriol. Proc., p. 113, 1971) who found that the fusiform bacteria virtually disappeared on a chemically defined liquid diet. Obviously, then, profound effects on diet are observed not only with synthetic collections of "normal" flora, but do occur in conventional animals as well.

In view of the fact that data had been collected on population levels of *E. coli* C25 in animals maintained on different diets and associated with synthetic and whole "normal" floras, the opportunity was taken to compare fatty acid levels in the ceca of these animals. As discussed above, fatty acids have been suspected by many as being the prime regulators of bacterial populations in the intestine. The data are included in Table 1. As may be seen, neither the quality nor molarity of intestinal fatty acids were necessarily related to *E. coli* population size. For example, animals of group C had relatively low concentrations of

fatty acids. In addition, the *p*H of cecal contents was relatively alkaline, a condition which tends to reduce the inhibitory effect of fatty acids (8). In spite of this, *E. coli* population sizes in group C were of the same order of magnitude as those in groups A, B, or E, where high fatty acid concentrations and low *p*H prevailed. Also, the mice in group D had considerably higher *E. coli* populations in spite of higher fatty acid concentrations.

As discussed above, various investigators have held different views as to which groups of bacteria may be involved in the control of intestinal populations. The final experiments in this study were therefore designed to test for possible synergism between intestinal anaerobes, as represented by the F-strains, and strains of *E. coli*. In one experiment, four groups of germfree mice were first associated with *Shigella flexneri*, and two days later with *E. coli* C25 plus F-strains or whole normal flora, as outlined in Table 4. The animals were sacrificed 4 weeks later. The data in Table 4 indicate that the *E. coli* populations in these animals were suppressed to "normal" levels by the F-strains or by the whole normal flora. In spite of this suppression, presence of the *E. coli* in the intestinal flora (group 3) resulted in much greater suppression of the *Shigella* population as compared to that observed in animals carrying only the F-strains. It should be recalled here that earlier studies from this laboratory (5) has shown complete suppression of this same *Shigella* strain by *E. coli* C25 when the two organisms were introduced as the only flora into antibiotic-treated animals. The data in Table 4 show therefore that *E. coli* C25 retains some of this antagonistic activity even when its own population is suppressed by the F-strains.

In contrast to the above findings, the data in Table 5 illustrate an experiment where an *E. coli* strain lost its ability to control another bacterium when its population was suppressed by the F-strains. *E. coli* strains C25 and 40T were implanted into germfree mice, together with the F-strain collection of intestinal anaerobes, as outlined in Table 5. The animals were sacrificed 4 weeks later, and the cecal populations of the two *E. coli* strains were determined by quantitative culture on media containing the appropriate antibiotics. The data (Table 5) show that *E. coli* 40T drastically suppressed the population of strain C25 when these two strains were the only inhabitants of the intestine (group 4). A comparison of groups 1 to 3 indicates, however, that strain 40T no longer had any effect on *E. coli* C25 when the strain 40T population was suppressed to "normal" levels by the F-strains. It appears therefore that relatively small populations of facultative anaerobes may at times affect

TABLE 3. *Effect of diet on cecal size and cecal flora of conventional BALB/wm mice kept in germfree isolators*

Diet	Cecum, % of body wt.	No. of bacteria/cecum[a] ($\times 10^{-10}$)
L-356 food	0.57 (0.38–0.73)[b]	0.94 (0.60–1.30)
Charles River food	1.27 (0.98–1.68)	2.16 (1.40–3.00)
Purina Breeder Chow	0.82 (0.70–0.99)	1.10 (0.80–1.50)

[a] Petroff-Hausser counts.
[b] First number indicates mean; numbers in parentheses indicate range. Ten mice per group.

TABLE 4. *Effect of a collection of 95 anaerobes (F-strains), E. coli C25, and normal flora on the population of Shigella flexneri in the cecum of gnotobiotic mice receiving L-485 food*

Group no.	Bacteria implanted	Cecum, % body wt.	No. of *Shigella*/cecum ($\times 10^{-4}$)	No. of *E. coli* C25/cecum ($\times 10^{-6}$)
1	*Shigella* + F-strains	1.94 (1.08–2.94)[a]	7.57 (1.60–2.10)	
2	*E. coli* + F-strains	1.54 (0.82–2.12)		4.69 (0.70–7.60)
3	*Shigella* + *E. coli* + F-strains	1.49 (0.54–2.18)	<0.1 (<0.1–0.10)	6.56 (2.50–15.1)
4	*Shigella* + *E. coli* + whole normal flora	1.44 (0.85–2.00)	<0.1 (<0.1–0.10)	0.48 (0.033–1.7)[b]

[a] First number indicates mean; numbers in parentheses indicate range. Twelve mice per group.
[b] High numbers of normal *E. coli* in this flora.

TABLE 5. *Interaction between E. coli strains C25 and 40T and F-strains in the cecum of gnotobiotic mice receiving L-485 food*

Group no.	Bacteria implanted	No. of *E. coli* C25/cecum ($\times 10^{-6}$)	No. of *E. coli* 40T/cecum ($\times 10^{-6}$)
1	*E. coli* C25 + F-strains	12.2 (3.80–33.0)[a]	
2	*E. coli* 40T + F-strains		10.2 (4.00–20.0)
3	Both *E. coli* + F-strains	14.3 (4.00–26.0)	15.4 (3.60–44.0)
4	Both *E. coli* strains, no F-strains	83.6 (3.4–2.12)	4,900 (2,400–10,000)

[a] First number indicates mean; numbers in parentheses indicate range. Twelve mice per group.

the populations of other bacteria (Table 4), while under different circumstances effects on other bacteria are observed only when unnaturally high population levels are present in the intestine.

DISCUSSION

It has been known for many years that variations in diet may affect the intestinal flora. Especially well studied is the relation between diet and lactobacillus and coliform populations (3, 9, 20). Very drastic effects of diet on intestinal flora were recently reported by Wilkins (Bacteriol. Proc., p. 113, 1971). The present experiments differ from such studies in that the two diets tested did not cause any significant changes in the *E. coli* populations of mice conventionalized with whole flora from normal mice (expt A and B, Table 1). In contrast, a striking difference between crude and refined diets was noted in mice carrying the N-strain collection of intestinal bacteria (expt C and D, Table 1), in that mice on the crude diet were unable to control the *E. coli* population and cecal size. The finding that these parameters could be returned to normal levels by implanting another collection of intestinal bacteria (F-strains, Table 2) indicates that the control functions of the normal flora may shift from one set of intestinal bacteria to another as the diet is changed.

It seems unlikely, in view of the above finding, that it will be possible to identify one or a few intestinal bacteria as the ones which generally inhibit the population size of all others. It appears rather that a variety of microorganisms may be responsible for such control function, each or each group of these organisms becoming predominant and functional under a given set of circumstances (of which diet is one important determinant). This assumption is also consistent with the data in Table 2 showing that the suppression of *E. coli* C25 in control animals conventionalized with whole normal flora differed considerably from one experiment to another in spite of the fact that the same batch of food was used throughout. One must conclude therefore that significant fluctuations in the performance of a "normal" flora do occur even if the donors of this flora are all taken from the same closed animal colony. Conceivably, such fluctuations may represent shifts in the strains of bacteria exerting control over others. Be this as it may, our initial aim (namely, to synthesize a "normal" flora) appears to have been based on the somewhat naive assumption that there is such a thing as a reproducible and precisely definable "normal enteric flora." It seems obvious now that the best one can do is to synthesize a flora representing a "state which is sometimes found in normal individuals." Within the limits of the data available, the collection of F-strains fulfills this requirement. The most promising direction to take in future experiments appears therefore to study the mechanisms of bacterial interaction in a reproducible and defined synthetic flora such as the F-strains. Only after these mechanisms have been delineated will it be possible to decide whether other states of the "normal enteric flora" differ in minor quantitative respects or whether they represent fundamentally different types of microbial interactions.

The above discussion of various "states" of the normal flora may at first glance seem at variance with the findings of earlier workers that the normal flora is stable. It should be pointed out that there is no conflict. Earlier workers were defining stability in terms of the persistence of certain marked strains and with regard to the general population levels (within wide margins) or certain classes of bacteria (e.g., enterococci, coliforms, anaerobes). This view is entirely valid. The fluctuations discussed above become apparent only when relatively minor (10- to 100-fold) population changes of *E. coli* are considered and when experimental parameters such as the diet are varied.

The fact that the collection of F-strains can

simulate the control functions of a normal enteric flora indicates that the anaerobic glove box method used for the isolation of these strains is capable of growing at least the functionally important intestinal anaerobes. Recent data published by others (7) seem at first to contradict this finding because these workers report that they could cultivate certain intestinal anaerobes only when a glove box procedure was combined with a roll-tube method. It must be pointed out therefore that the glove box used by these workers lacked a system for the continuous removal of oxygen. In our experience, glove boxes operated under such conditions contain oxygen concentrations which cannot be lowered beyond several hundred parts per million. Methylene blue indicator still becomes colorless at such high oxygen concentrations, and reduction of this dye cannot therefore be taken to indicate the presence of a strictly anaerobic atmosphere (5–10 ppm, oxygen) as is used in our glove box for the isolation of intestinal anaerobes. All available data are therefore consistent with the finding that a properly conducted glove box procedure is indeed sufficient for the cultivation of the strictly anaerobic intestinal bacteria.

The data on cecal fatty acid concentrations in Table 1 indicate that these do not always correlate with the population levels of *E. coli*. Most likely, other mechanisms were responsible for controlling the growth of *E. coli* in the animals. It should be emphasized that these data do not rule out the possibility that fatty acids may indeed be very important in the ecology of intestinal flora. They merely indicate that fatty acids may not be the universal controlling agent.

A final set of data shown in the present paper give some guidance to future investigations by pointing out the kind of bacteria which are necessary for the control of populations of enterobacteria such as *E. coli* and *Shigella*. The experiment shown in Table 5 is an example of a situation where the intestinal anaerobes were sufficient. Table 4 illustrates an example of a synergistic action between anaerobes and a facultative anaerobe. As pointed out in the introduction to this paper, the finding of such synergisms is somewhat surprising because the facultative anaerobes are themselves suppressed. Obviously then, the mechanism(s) by which the facultative anaerobes contribute to the control of other intestinal bacteria must be of a type which can be brought into play by very dilute bacterial populations. Examples of such mechanisms are: (i) competition for limiting nutrients and (ii) an indirect effect due to stimulation of certain components of the anaerobic flora.

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