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Changes in the Mouse Intestinal Microflora During Weaning: Role of Volatile Fatty Acids

ADRIAN LEE AND ERICA GEMMELL

Department of Medical Microbiology, University of New South Wales, Sydney, Australia

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The influence of volatile fatty acids on the ecology of the bacterial flora of the mouse intestinal tract has been studied in three situations where large fluctuations in the composition of the microflora have been observed. Young mice were shown to ingest solid food particles when 11 days old; this correlated with the appearance of strictly anaerobic fusiform bacilli in the intestinal lumen and a 10,000-fold decrease in numbers of coliform bacilli. Over the same period, volatile fatty acids were shown by gas-liquid chromatography to appear in the intestinal content. It is suggested that the fusiform bacilli are responsible for the presence of the volatile acids (especially butyric acid) which exert an inhibitory effect on the coliform bacteria, resulting in the decline in numbers. When germ-free mice are placed in a specific pathogen-free mouse colony, changes in the intestinal flora occurred which were similar to those observed in the young mice approaching weaning. Once again, the decline in the coliform population correlated with the appearance of significant levels of butyric acid in the large intestine. In a further series of experiments, mice were fed penicillin and levels of the intestinal fatty acids were measured. The antibiotic eliminated the anaerobic fusiforms from the intestine, resulting in the disappearance of significant levels of butyric acid and a million-fold increase in the numbers of coliform bacilli.

Shortly after birth, the gastrointestinal tracts of rodents are colonized with large numbers of aerobic bacteria belonging to the family *Enterobacteriaceae* (the coliforms). From the 12th day of life, the numbers of these organisms decline and reach minimum levels at about day 21. These levels are maintained for the rest of the animal's life unless it is placed under stress or administered antibiotics. Previous studies have shown a correlation between the fall in coliform numbers and appearance of strictly anaerobic bacteria which rapidly become the dominant members of the intestinal flora of the adult animal; no explanation for the bacterial antagonism was proposed, however (10, 14).

In other contexts, several ways whereby the presence of one bacterial species may influence the behavior of another have been suggested, e.g., changes in redox potential (11) and competition for fermentable carbon sources in a reduced environment (5). An alternative mechanism, supported by some experimental evidence, has implicated short-chain volatile fatty acids, especially butyric acid (3, 11). Thus, Bohnoff and Miller (3) demonstrated that adult mice, fed previously with antibiotics, became highly susceptible to oral *Salmonella* infection; they believed antibiotics eliminated members of the normal flora which were capable of forming volatile fatty acids in vivo.

In the present communication, the influence of the volatile fatty acids on the ecology of the microflora of the mouse intestinal tract is considered in greater detail. To do this, three rather different models have been used, namely, (i) neonatal mice, (ii) germ-free mice, and (iii) adult specific pathogen-free (SPF) mice treated orally with penicillin. In each case, the intestinal microflora is vastly different from that of the adult or normal animal and thus provides a means of correlating the production of the fatty acids with the presence or absence of the strictly anaerobic autochthonous organisms.

MATERIALS AND METHODS

Animals. Mice were obtained from the SPF colony maintained in the Department of Medical Microbiology at the University of New South Wales (UNSW). This colony originated from germ-free animals (Charles River Breeding Laboratories, Inc., North Wilmington, Mass.) and has been maintained under conditions appropriate to retain the SPF state. For one series of experiments, germ-free mice were kindly supplied by Margaret Holmes from the C3H colony

maintained at the Walter and Eliza Hall Research Institute, Melbourne.

Extraction of volatile fatty acids. Approximately 1 g of cecum and large intestine was accurately weighed and homogenized in 5 ml of distilled water with a Teflon grinder. The homogenate was centrifuged to remove debris; the supernatant fluid was made alkaline (ca. *p*H 9.0) with 0.1 N NaOH and stored, until distillation, in a deep freezer. The thawed alkaline mixture was adjusted to *p*H 2.8 to 3.2 with 1 M phosphate buffer (equal volumes of 1 M NaH_2PO_4 and 1 M Na_2HPO_4). This solution was immediately transferred to a Markam still, and 1 g of $MgSO_4$ was added. The volatile fatty acids were steam-distilled into 10 ml of 0.1 N NaOH until 300 ml of distillate had been collected. The distillate was reduced at 100 C in a hot-air oven and finally evaporated to dryness at 58 C. The volatile acids were liberated, when required for analysis, by 0.8 ml of 30% phosphoric acid (9).

Gas chromatographic separation of the volatile fatty acids. The volatile fatty acids were routinely quantitated on a column of 15% Carbowax 20M (Analabs, Inc.) on ChromoSorb W DMCS (60 to 80 mesh obtained from Johns-Manville Corp.) packed in stainless-steel tubing (internal diameter of 3 mm). The column was maintained isothermally at 147 C; the nitrogen carrier gas and hydrogen flow rates were 15 cc/min.

Individual volatile acids were determined on an Aerograph 1520 gas-liquid chromatograph with output from a flame ionization detector coupled to a 1 mv Leeds and Northrup Speedomax Recorder with chart integrater. Peaks were identified and quantitated by reference to chromatograms of 0.1% standards of volatile fatty acids which had been distilled in an identical manner to the intestinal samples.

Enumeration of intestinal bacteria. Coliform bacteria (lactose- and late lactose-fermenting organisms) were counted as follows. Samples of intestinal homogenate were cultured on tergitol 7 agar (Difco) with added tetrazolium by using the loop dilution technique described previously (15). For counting anaerobic fusiform bacteria, smears of intestinal homogenate were prepared and stained by Gram's method. The proportion of tapering fusiform rods was estimated as described in a previous publication (10).

RESULTS

Changes in the intestinal microflora of the UNSW SPF mouse during weaning. Previous studies (6, 10, 13–15) were carried out with American strains of SPF mice. It was important, therefore, to determine whether a similar pattern of bacterial colonization was found in the mice used in this study. Figure 1 shows the development of the flora in a representative litter of UNSW mice. Measurements were carried out exactly as in a previous paper (10). The predicted changes in the flora as the mice approach weaning were seen. Coliforms were present in very large numbers until about day 14 when they began to decline to levels found in the litter mother. This represents a drop in bacterial count of 10,000 organisms per g of large bowel. The strictly anaerobic tapered-fusiform rods first appeared at day 14 and increased in number until day 22 when they became the dominant members of the bacterial flora.

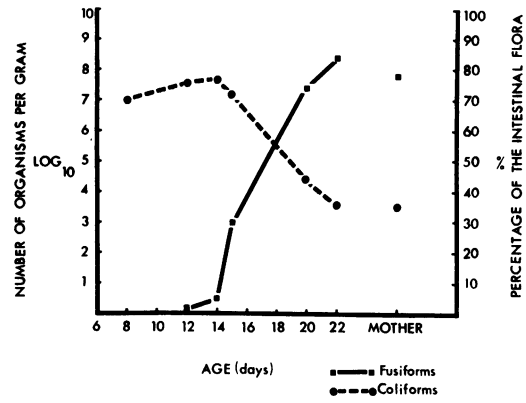

FIG. 1. *Development of the bacterial flora in the large intestines of a litter of UNSW mice. The levels of coliform bacilli, determined by cultural methods, are shown as the numbers of organisms per gram of intestinal content. The fusiform populations are expressed as percentages of the total intestinal flora based on microscopic examinations.*

Time of weaning of the UNSW mouse. In previous studies, the period of weaning had been estimated by casual observation of the baby mice. It seemed important for subsequent studies to determine the weaning time of the UNSW mouse more accurately. To do this, the normal diet provided to the litter mothers was ground, mixed with 10% activated charcoal, and then repelleted. It was assumed that as soon as the baby mouse began to ingest the solid diet the black particles of food would clearly be visible in the previously milk white stomach. Figure 2 shows the stomachs of young mice of four litters which were killed at different days after birth. Black food particles were first seen at day 11, whereas it was obvious that the stomachs became totally black at about day 14. Presumably, about this time, the young mice were taking considerable quantities of solid food although weaning was not necessarily complete. From the above results, it is clear that this just preceded the establishment of the anaerobes in the intestinal tract and the decline in coliform numbers.

Appearance of volatile fatty acids in the large intestine of baby mice. The ceca and large intestines of baby mice of differing ages were removed and homogenized in 5 ml of charcoal water. Volatile acids were extracted, and samples were in-

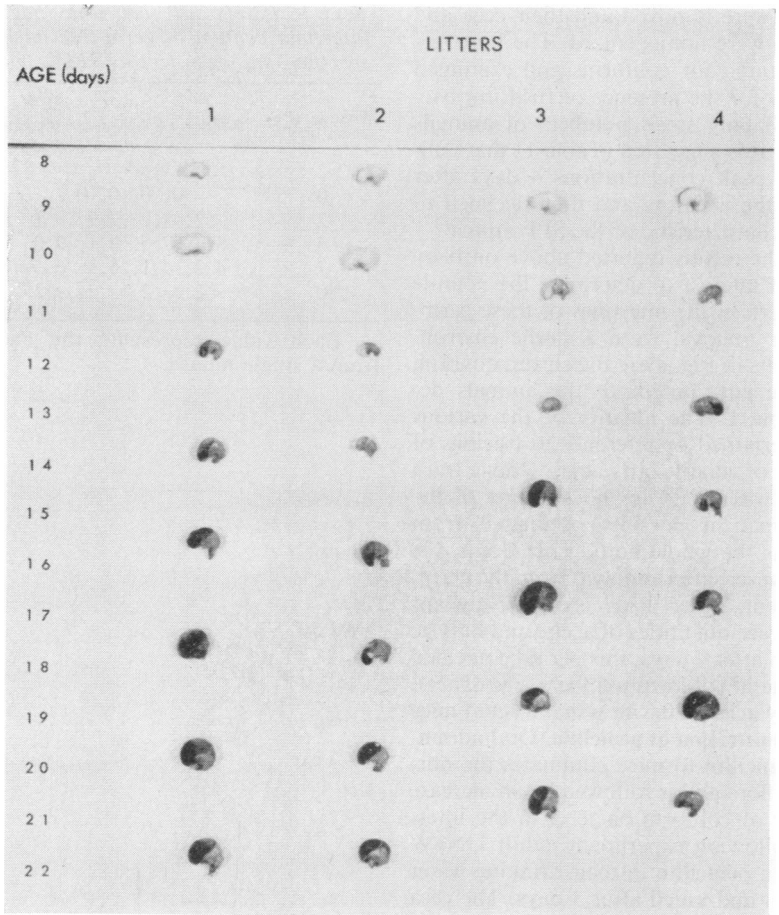

FIG. 2. *Appearance of solid food particles in the stomachs of young UNSW mice. The solid diet provided contained 10% charcoal.*

jected into a gas-liquid chromatograph. Quantitative estimates of butyric acid concentrations are shown in Fig. 3. No butyric acid was found in the intestine of very young mice; it was present in significant amounts when the animals were 12 to 14 days old. The concentration of the acid rapidly increased, reaching a maximum at about 18 days of age; this correlates well with the appearance of the fusiform bacteria in high numbers. The other major acid, acetic acid, was detectable much earlier, significant amounts being present at 7 days. The concentration gradually increased to a maximum at about day 16. The concentrations of the acid shown in Fig. 3 cannot be compared with other fatty acid levels described in the literature. In the present studies, the homogenate contained both intestinal contents and tissue. As a result, the values shown are presumably less than the absolute concentrations of acid present in the intestinal lumen of these baby mice.

Propionic, isobutyric, isovaleric and valeric acids were in lower concentrations than acetic and butyric acids in the adult animal. These acids all appeared at about day 16 in the baby mice and reached maximum concentration in a very short time; i.e., at less than day 15 none was detectable but all were present at maximum concentration at 16 days.

Development of the intestinal microflora in germ-free mice after removal from a sterile environment. When germ-free mice are placed in an SPF rodent colony, the gastrointestinal tract is colonized with bacteria in a similar fashion to that of newborn mice. Coliform bacteria establish in very high numbers initially but then decline as strictly anaerobic bacteria appear in the intestinal content. This was illustrated in the following experiment. Germ-free mice were taken from their isolators and placed in the breeding room of the SPF colony. At different periods, randomly se-

lected animals were removed and their ceca and large intestines were homogenized. The homogenates were cultured for coliforms and examined microscopically for the presence of fusiform bacteria. Although only small numbers of animals were available, it is suggested (Table 1) that coliforms reached peak concentrations 4 days after removal from the isolators and then declined to the low levels characteristic of the SPF animal.

Because of the results reported above on baby mice, it was of interest to determine the volatile fatty acid pattern in the intestines of these germ-free mice after removal from a sterile environment. The results in Fig. 4 are the chromatograph tracings of the gut content of the animals described in Table 1. The identity of the various peaks was ascertained by reference to tracings of pure standards of volatile fatty acids. Only a trace of acetic acid was found in the intestine of the germ-free animal; it possibly originated from mouse tissue as the whole homogenized gut was extracted. However, after removal from the germ-free environment, other acids progressively appeared. Significant quantities of acetic and butyric acids were seen after 7 days; this period coincided with the decline in coliform numbers (*see above*).

Volatile fatty acids in the intestinal tract of mice after oral administration of penicillin. Oral administration of penicillin to mice eliminates the normal anaerobic flora and is followed by an increase in the number of coliform bacteria in the intestine. In the following experiment, adult UNSW mice were given penicillin in their drinking water (0.3 g per liter) and killed after 3 days. The ceca of these animals were removed, homogenized, and cultured for coliform organisms. The homogenate was steam-distilled, and the volatile fatty acids were collected. Samples of the acids were injected into a gas-liquid chromatograph; two representative tracings are shown in Fig. 5.

The level of coliforms in the control animal was low (10^4 per g); as anticipated, it reached high levels in the treated mice (ca. 10^{10} per g). Acetic, propionic, isobutyric, butyric, isovaleric, and valeric acids were all present in the control animal; acetic and butyric acid were in highest con-

TABLE 1. *Levels of coliform bacteria in the intestinal content of germ-free mice placed in a specific pathogen-free (SPF) mouse colony*

Time in SPF room (day)	Coliform level[a] ($\log_{10}$ per g)	Presence of fusiform bacteria
0	0, 0, 0, 0	−
2	<2, <2, 7.9, 8.8	−
4	8.4, 7.5, 9.1, 9.0	±
7	4.2, 4.1, 6.3, 6.5	++
<80	<2, <2, <2, <2	+++

[a] Each value represents the bacterial count from a single mouse.

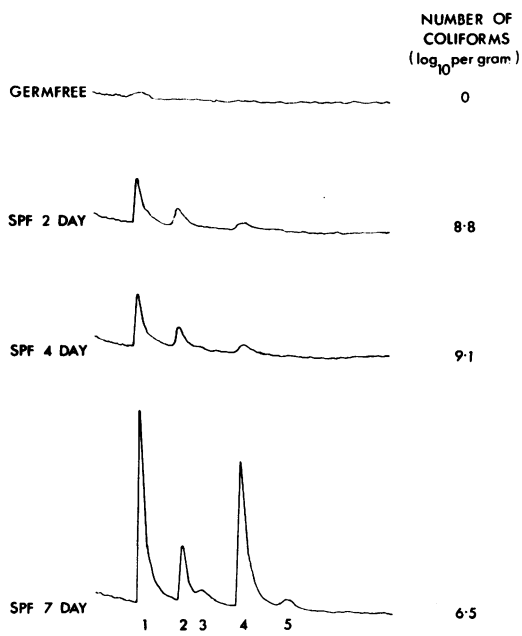

FIG. 4. *Gas chromatographic separation of volatile fatty acids in the large intestines of germ-free mice after differing periods of association with a specific pathogen-free mouse colony. Peaks: 1, acetic acid; 2, propionic acid; 3, isobutyric acid; 4, butyric acid; 5, isovaleric acid.*

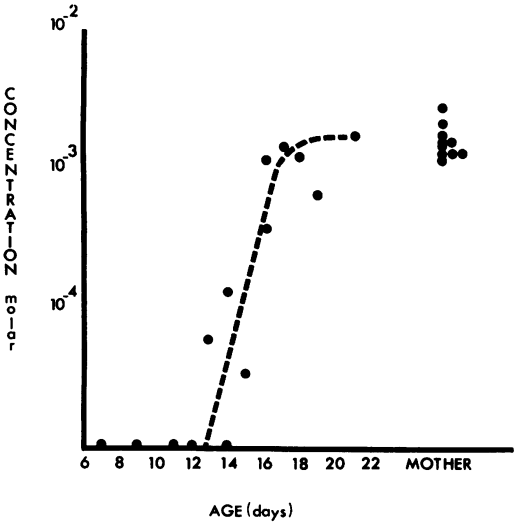

FIG. 3. *Appearance of butyric acid in the large intestine of young UNSW mice.*

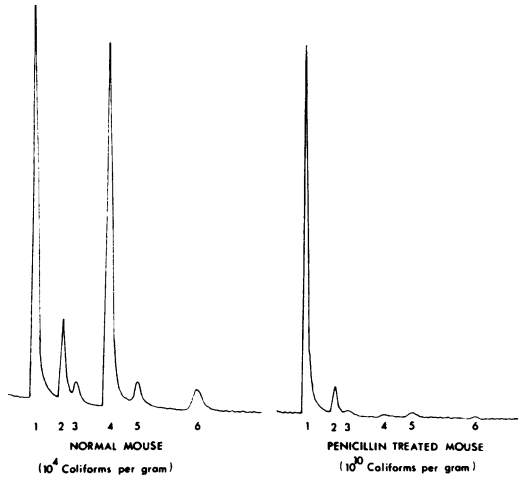

FIG. 5. *Gas chromatographic separation of volatile fatty acids in the large intestine of a normal adult mouse and a mouse treated with penicillin. Peaks: 1, acetic acid; 2, propionic acid; 3, isobutyric acid; 4, butyric acid; 5, isovaleric acid; 6, valeric acid.*

centrations. This contrasted sharply from the fatty acid profile of the antibiotic-treated animals. In these, only acetic acid was present in concentrations comparable to the control. A small amount of propionic acid was detected as were trace amounts of isobutyric and isovaleric acids. However neither butyric nor valeric acids were found. Microscopic examination of the intestinal contents of these animals indicated that, although large numbers of fusiform-shaped bacteria were present in normal untreated mice, these organisms had been eliminated from the penicillin-treated animals.

DISCUSSION

The mouse gastrointestinal tract serves as an excellent model for the study of interactions between bacterial populations within a normally stable ecosystem. Many species of organism are present, some in exceedingly high numbers, while inbalances in these populations may have deleterious effects on the host. Thus, as others have shown (3), the animals may become highly susceptible to oral infection by intestinal bacterial pathogens. The bacteria which appear to dominate the ecology of the large bowel in rodents are a group of strictly anaerobic tapered-fusiform rods which have recently been cultured by special techniques; although no detailed taxonomic studies have been reported, it appears they may belong to the genera *Eubacterium*, *Fusobacterium*, or, indeed, may be a new species (6).

Conditions which do not permit the growth of these fusiform bacilli lead to great fluctuations in the flora of the gut and allow establishment of vast numbers of members of the *Enterobacteriaceae*. Thus, treatment of mice with streptomycin results in the elimination of fusiform bacteria and lowers the lethal dose of *Salmonella typhimurium* from 10^6 to ca. 1 organism (3). Again, when mice are fed with a chemically defined liquid diet, the thick layers of fusiforms in the mucosal epithelium are eliminated. This leads to an increase in numbers of lactose-fermenting coliforms from 10^4 to 10^9 per g (T. D. Wilkins and W. R. Long, Bacteriol. Proc., p. 113, 1971).

In the present study, similar fluctuations in coliform numbers have been demonstrated in the gastrointestinal tracts of young mice as they approach weaning. This has been reported previously in a different SPF mouse colony when it was suggested that the precipitating factor was the ingestion of solid food by the young animals (10). Our experiments now seem to confirm that the change in the intestinal microflora is just preceded by ingestion of food particles. Presumably the solid food has a profound effect on the local environment, making it possible for the strict anaerobes to establish in large numbers. This could be due to provision of new nutrients or, more likely, the creation of highly reduced conditions.

The main purpose of this investigation was to demonstrate that certain changes of the intestinal milieu, probably involving the fusiform bacilli, were associated with the spontaneous decline of coliforms in the natural situation. The volatile acids were examined primarily, as other workers have suggested that these agents may play an extremely important role in the intestinal lumen (2, 3, 11). For reasons mentioned below, the material of most interest was butyric acid. This compound was absent in the very young animal and only appeared as the fusiforms established. By comparing Fig. 1 and 3, it will be obvious that this acid was first detected a short time before the bacteria were found in significant numbers. However, it must be stressed that the fusiform population is represented in terms of a percentage of the intestinal flora, assessed microscopically; under these circumstances it is clear, because of the vast numbers of organisms present, a figure of only 1% for the fusiforms could still represent an absolute concentration of 10^8 per g. The only other volatile acid present in high concentration, acetic acid, approaches its maximum a significant time before any drop in coliform number is observed. The other acids are all present in much lower levels than acetic and butyric and appear at about the same time as the latter.

It appears, therefore, that certain of the volatile acids, especially butyric, are produced by fusiform

bacteria and may influence the concentrations of other bacteria in the intestine. This is, of course, only circumstantial evidence; in an attempt to confirm it, two other models in which changes in numbers of coliforms could be correlated with the fusiform organisms were examined. In the first, adult germ-free mice were placed in a room with mice which had a fully developed intestinal microflora. The changes in the flora in these animals exactly paralleled those of the young mice approaching weaning. At first, the intestine was colonized by miscellaneous gram-positive rods and cocci as well as very high levels of coliforms. However, after a short interval, the intestine was colonized by anaerobes, presumably derived from neighboring animals. This led to the expected reduction in coliform numbers to the low level characteristic of SPF mice. The chromatographic traces of intestinal contents of animals killed at differing periods after removal from the sterile environment dramatically indicate that the coliform level only drops when significant quantities of butyric acid are present.

The final experiments questioned the fatty acid patterns of normal adult mice and mice which had been administered penicillin orally. Under these conditions, it has been shown that the fusiform flora is completely eliminated and coliforms invade the large bowel in high numbers (14). Once again butyric acid was implicated in this imbalance in the flora. Although virtually no butyric acid was detected in animals given penicillin (and containing exceedingly large numbers of coliforms), the level of acetic acid was only minimally decreased. This suggests that the latter acid plays only a minor role in the control of coliform levels.

The above experiments confirm the importance of certain volatile fatty acids as a major factor controlling bacterial populations within the intestinal tract (3). How they act is not yet fully resolved. It is interesting to record that over 30 years ago Bergeim (1, 2) investigated mechanisms involved in the destruction of microorganisms in the gastrointestinal tract; in these, he demonstrated that acetic and butyric acids had marked effects on the in vitro growth of *Escherichia coli* and a yeast in concentrations that might be expected in the intestinal tract. Furthermore, he showed that by varying the diet the concentrations of acids could be altered, leading to significant changes in the intestinal flora. It is also pertinent to record that the high levels of volatile fatty acids present in the rumen fluid of cattle and sheep may have antibacterial activity. Thus, starvation of both species of animals leads to reduction of the acid level and has been shown to allow the growth of salmonellae and *E. coli* in the rumen. It has been suggested that this may explain why the holding of cattle in yards before slaughter predisposes these animals to *Salmonella* and *E. coli* infection (4, 7).

Experiments on the growth of *Shigella* species in vitro with cultures of human *Bacteroides* strains suggest that acetic and propionic acids may be important in restricting the former organism; however, it is unlikely that conditions in these cultures resemble conditions within the intestinal lumen (8). Nonetheless, this report again stresses the importance of the environmental *p*H in determining the antibacterial activity of short-chain fatty acids. As others have suggested (11), butyric acid exerts a greater antibacterial effect than acetic acid at the normal *p*H of the intestine (6.0 to 7.0) because more acid is in the undissociated state. Furthermore, there is evidence to suggest that toxicity of acids also tends to increase with the length of the carbon chain. Although little is known of the identity of these organisms, similar morphological types are classified on their ability to produce butyric acid as a major product (12). As culture techniques have been developed for the strictly anaerobic fusiform bacteria, it is now intended to monocontaminate germ-free mice to determine the number of species of bacteria involved in the production of the fatty acids described above. Levels of volatile fatty acids in the human intestinal content are also being investigated to determine whether they are present in sufficient quantities to exert antibacterial effects.

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