THE DEVELOPMENT OF THE BACTERIAL FLORA IN THE GASTROINTESTINAL TRACT OF MICE*

By RUSSELL W. SCHAEDLER, M.D., RENÉ DUBOS, PH.D., AND RICHARD COSTELLO, PH.D.

(From The Rockefeller Institute)

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The findings to be reported in this and the following papers reveal that mice and other mammals normally harbor an extensive bacterial flora not only in the large intestine, but also in the stomach and small intestine. Although this flora plays an essential role in the development and well being of its host, its exact composition is not known. In fact, several of its most important and numerous components are usually overlooked in bacteriological studies because they have exacting growth requirements, and because their susceptibility to the antagonistic activity of other microbial species makes it difficult to cultivate them *in vitro*.

We shall describe in the present paper: (*a*) the methods used in our laboratory for quantitative bacteriological studies of the gastrointestinal tract; and (*b*) the development of the gastrointestinal flora as observed in newborn mice until the time of weaning.

Materials and Methods

Experimental Animals.—Extensive studies of the gastrointestinal flora have been carried out during the past 6 years with socalled Swiss mice of the NCS colony, maintained in our laboratory under the conditions described earlier (1–3); mice from other colonies, raised under conventional conditions, were also used for comparative studies.

The animals were housed, and allowed to mate, in stainless steel cages, with autoclaved wood shavings as litter. The drinking water, given *ad lib.*, was acidified with HCl to control bacterial contamination as described earlier. In certain experiments, the same end was achieved by adding an organic silver compound in a dilution of 1 part of silver per million. The food also given *ad lib.* consisted either of pasteurized pellets (supplied by Dietrich and Gambrill, Frederick, Maryland) or of a semisynthetic complete diet containing 15 per cent casein (described in reference 4). It is worth emphasizing that both the pellets and the casein diets had been so treated as to eliminate all bacterial contaminants except a few spores.

Preparation of Specimens for Bacteriological Examination.—Stool specimens were collected from mice on sterile paper between 9:00 a.m. and 10:30 a.m. Approximately 0.1 gm of stool was emulsified in 5 ml of sterile diluent (charcoal water) by 4 minutes agitation on a mechani-

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cal shaker; the diluent was prepared by adding 1 gm norite A to 1 liter of tap water and filtering over filter paper. The charcoal water diluent was distributed in tubes and autoclaved. Animals were sacrificed under chloroform anesthesia in the morning; either the whole digestive organs, or selected portions of them, were homogenized in teflon grinders in 5 ml of sterile charcoal water. The tests were carried out on the whole organ, including its contents.

Bacteriological Techniques.—The suspensions obtained from either stools or organ specimens were further diluted in charcoal water in several 10-fold steps. Calibrated loopfuls of each dilution were then spread on the surface of various selective agar culture media, as described below. Attempts were made to obtain dilutions of each specimen yielding separated and countable colonies. The conditions of incubation are described for each type of culture medium. The numbers of colonies obtained per gram of stool or of organ were calculated from those obtained per loopful of the appropriate dilution.

Selective Media.—Enterococci, coliform bacilli, and other Gram-negative bacilli are the bacterial species of the intestinal tract which are most commonly studied. These organisms multiply rapidly and abundantly *in vitro*, and their enumeration therefore presents no problem. However, when these organisms are present in large numbers, as is usually the case, the very rapidity of their growth constitutes a source of difficulties for the enumeration of other more delicate and nutritionally more exacting bacterial species. The development of culture media and incubation techniques suitable for the enumeration of the bacterial species which occur in the gastrointestinal tract demands, therefore, that emphasis be placed not only on the nutritional quality of the media but also on their selectivity. As will be noted, all media described below contain inhibitors which prevent or at least retard the development of certain bacterial species while allowing the multiplication of others. In other words, our objective has not been necessarily to achieve the most abundant growth, but rather to provide selective conditions under which the desired species would develop *in vitro* despite the presence of antagonistic organisms in the specimen under study.

The following formulas are used at present in our laboratory for the preparation of selective media which permit quantitative bacteriological analysis of fecal material and of the various organs of the digestive tract.

(*a*) *Base medium for anaerobic lactobacilli, anaerobic streptococci, clostridia, bacteroides, and flavobacteria:*

Trypticase (Baltimore Biological Laboratories, Baltimore)	10 gm
Proteose peptone No. 3 (Difco Laboratories, Inc., Detroit)	5 "
Dextrose	5 "
Yeast extract (Difco Laboratories)	5 "
Tris buffer 7 to 9 (Sigma Chemical Company, St. Louis, Missouri)	3 "

Hemin*	10 ml
Cystine‡	10 "
Agar	15 gm
Distilled water	1000 ml

* Hemin, 100 mg of hemin + 99 ml H_2O + 1 ml 5 N NaOH.
‡ Cystine, 4 gm cystine + 99 H_2O + 8 ml 5 N NaOH.

(*b*) *Medium for anaerobic lactobacilli and anaerobic streptococci:*
To 1000 ml of the base medium described under (*a*) add

NaCl	10 gm
Neomycin	20 mg

To be incubated anaerobically at 37.5°C.

(*c*) *Medium for bacteroides and clostridia:*
To 1000 ml of the base medium described under (*a*) add

Placenta powder (Nutritional Biochemicals Corp., Cleveland)	2 gm
Neomycin	20 mg

To be incubated anaerobically at 37.5°C.

(*d*) *Medium for flavobacteria:*
To 1000 ml of the base medium described under (*a*) add
7 ml of 0.5 per cent tyrothricin in ethyl alcohol
To be incubated aerobically at 37.5°C.

(*e*) *Medium for Escherichia coli and other members of the coliform group:*
To tergitol 7 agar (Difco Laboratories) add
10 ml of 0.4 per cent tetrazolium chloride per liter of medium after autoclaving
To be incubated aerobically at 37.5°C.

(*f*) *Methylene blue medium for enterococci:*

Proteose peptone 3 (Difco Laboratories)	10.0 gm
Tryptose (Difco Laboratories)	10.0 "
Dextrose	5.0 "
Saccharose	10.0 "
K_2HPO_4 (anhydrous)	4.0 "
KH_2PO_4 (anhydrous)	1.5 "
NaCl	5.0 "
1 per cent sodium azide	5.0 ml
Glycerine	2.5 "
Methylene blue*	4.0 "
Agar (Baltimore Biological Laboratories)	15.0 gm
Distilled water	1000.0 ml

* Methylene blue stock solution, 1 gm methylene blue in 100 ml acetone and 100 ml of 95 per cent alcohol. Keep stoppered and refrigerated. Add 4 ml per liter of medium.
To be incubated aerobically at 37.5°C.

(*g*) *General medium for lactobacilli:*

Trypticase (Baltimore Biological Laboratories)	12.0 gm
Yeast extract (Difco Laboratories)	5.0 "
Dextrose	10.0 "
KH_2PO_4 (anhydrous)	2.0 "

K_2HPO_4 (anhydrous)	1.5 "
$(NH_4)_2 SO_4$	1.0 "
Cystine*	2.5 ml
1 per cent oleic acid in 10 per cent triton (WR-1339)	40.0 "
1 per cent sodium azide	10.0 "
Agar	15.0 gm
Distilled water	1000.0 ml

Autoclave; then add 8 ml of 1 per cent $K_2Cr_4O_7$ liter of media.

* Cystine, 4 gm cystine + 100 ml H_2O + 7.5 ml N HCl.

The conditions of incubation differ depending upon the group of lactobacilli to be favored.

1. To bring out the rhizoid forms (2), the agar plates should be incubated at 37.5°C. under ordinary atmospheric conditions.

2. Other lactobacilli grow best in an atmosphere containing CO_2 (approximately 6 to 10 per cent), as provided by a candle jar.

Incubation Under Controlled Atmospheric Conditions.—The growth *in vitro* of the bacterial species which predominate in the gastrointestinal tract is conditioned not only by their exacting nutritional requirements and by their susceptibility to the antagonistic effect of other organisms *in vitro*, but also by the composition of the gaseous atmosphere. Most of them can grow under anaerobic conditions, and some indeed are strictly anaerobic. Some require CO_2 in the atmosphere and others appear to be somewhat inhibited by this gas.

Even though growth on the surface of agar media is not usually considered to provide optimum conditions for the strictly anaerobic species, we have resorted to this technique because it proves best suited to the differentiation and enumeration of the viable organisms present in the gastrointestinal tract. One of the practical difficulties posed by this choice came from the necessity to use numerous chambers in which large numbers of agar plates could be incubated in atmospheres of different gaseous composition. It may be helpful to describe briefly the equipment developed in our laboratory from parts readily available commercially.

The anaerobic incubation chambers are stainless steel tanks of commercial milking machines.[1] The tanks we have used were obtained from DeLaval Separator Company, Poughkeepsie, New York. The complete unit consists of a pail (No. 04226), a cover (No. 06403), and a flat gasket (No. 06409). Each of the tanks is large enough to hold over 100 standard Petri dishes. The units can be used as purchased. However, their operation was improved by fitting the covers with a compound vacuum-pressure gauge and a ¾ inch bellows type vacuum valve (Kinney Manufacturing Company, Boston, Massachusetts, No. 056035). A tubular manifold using four of the aforementioned vacuum valves was also constructed. With this apparatus three tanks can be evacuated simultaneously with a large pump. For anaerobic conditions, the chambers are evacuated to 29 to 30 inches five times; after each evacuation the chamber is filled with nitrogen. After the fifth evacuation, nitrogen and CO_2 are admitted. The tanks are maintained under a 10 inch vacuum to assure a firm seating of the cover and gasket (Fig. 1).

[1] We wish to express our thanks to our former colleague Dr. Paul Quie for having suggested the use of these tanks.

FIG. 1. Illustrates two of the adapted milk cans used as anaerobic chambers connected to the manifold for evacuation of air and subsequent filling with nitrogen and carbon dioxide. The heavy rubber tube on the left of the picture connects to a large vacuum pump.

In the left foreground is the cover as received from the manufacturer. On the right is a cover which has been fitted with a large vacuum valve and guage.

This unit can accommodate 1, 2, or 3 chambers each of which holds over 100 Petri dishes.

RESULTS

The Development of the Bacterial Flora in the Gastrointestinal Tract of Mice.—(*a*) *NCS mice:* As far as can be judged, the mammalian fetus is essentially free of cultivable microorganisms at the time of birth. Evidence for this fact has been provided by the regularity with which germfree animals can be obtained by cesarean section. However, several bacterial species become established throughout the gastrointestinal tract shortly after birth, in fact almost as soon as suckling begins.

The development of this indigenous gastrointestinal flora *in vivo* was followed by sacrificing NCS mice at daily intervals after birth, and plating multiple dilutions of organ homogenates on the various culture media described above. Many experiments of this type with NCS mice, carried out over a period of 4 years, have yielded such uniform results that it is legitimate to present them in the form of a few general statements (Table I).

Lactobacilli, anaerobic streptococci (those which have been typed were of

Group N), and flavobacteria appeared within 24 hours after birth, and their populations soon reached high levels in the stomach, the small intestine, and the large intestine.

The lactobacilli and anaerobic streptococci were always more numerous in the stomach and large intestine than in the small intestine. For both species the maximum population was reached around the 12th day of the animal's life,

TABLE I

Development of the Flora in the Digestive Tract of NCS Mice

	Age of Mice in Days									
	2	4	6	8	10	12	14	16	18	30
Stomach										
Lactobacilli and strept. (N)	3*	5	6	8	8	9	9	9	9	9
Flavobacteria	3	5	6	4	2	0	0	0	0	0
Coliforms (SLF) and enterococci	0	0	0	0	±	±	±	0	0	0
Bacteroides	0	0	0	0	0	0	0	0	0	0
Small Intestine										
Lactobacilli and strept. (N)	3	4	5	7	7	8	8	8	8	8
Flavobacteria	3	5	6	8	8	±	±	0	0	0
Coliforms (SLF) and enterococci	0	0	0	0	±	±	±	0	0	0
Bacteroides	0	0	0	0	0	0	0	0	0	0
Large Intestine										
Lactobaclili and strept. (N)	3	5	6	8	9	9	9	9	9	9
Flavobacteria	3	4	7	8	±	0	0	0	0	0
Enterococci	1	2	2	2	8	9	8	3	3	3
Coliforms (SLF)	0	0	0	0	9	9	9	3	3	3
Bacteroides	0	0	0	0	0	0	0	±	8	9

* Figures indicate the logarithms of the approximate numbers of colonies recovered per gram of organ homogenate.

but it was approximately 10-fold greater for the lactobacilli than for the streptococci. Most striking was the fact that the population of the two species remained at an approximately constant level from then on, at least as long as the animals were maintained under favorable physiological conditions.

The flavobacteria also invaded the whole gastrointestinal tract, and reached their maximum population around the 10th day of life. In contrast to the lactobacilli and anaerobic streptococci, however, they were more numerous in the small intestine than in the stomach and the large intestine. Furthermore,

their colonization of the gastrointestinal tract was extremely transient; they disappeared abruptly and lastingly after the 12th day.

An abrupt change occurred in the flora of the whole gastrointestinal tract at the time of the disappearance of the flavobacteria. Enterococci which had been present in very low numbers and only in the large intestine multiplied explosively in this organ where they reached population levels up to 10^9 living organisms per gram of tissue; they were also found occasionally in the stomach and small intestine. The slow lactose fermenting coliform bacilli (designated hereafter as SLF) which had not been detected during the first 10 days of life also multiplied explosively around that time, especially in the large intestine. However, this proliferation did not last long. Within a very few days, the populations of enterococci and SLF coliforms fell precipitously from 10^9 per gram of tissue to 10^3 or less, and they persisted at these or even lower levels throughout the animal's life span, as long as the animals remained in a favorable physiological state.

The invasion of the gastrointestinal tract by the strict anaerobic Gram-negative bacilli of the bacteroides group presented some unusual features. Two types of these organisms, differing in colonial size, appeared on the 15th to 16th day after birth, and they immediately multiplied extensively reaching levels of the order of 10^9 organisms per gram of tissue. They persisted at such high levels, but remained almost exclusively localized in the large intestine. Only on rare occasions were they recovered from the stomach.

(*b*) *Mice from colonies other than NCS:* Studies similar to those described above have been carried out with other colonies of albino mice: in particular, CFW, a Swiss mouse produced by Carworth Farms Laboratory, New City, New York, and ICR, produced by Millerton Farms, Millerton, New York. Although many of the findings in these animals were similar to those made in NCS mice, they differed in important details. *Escherichia coli* and other coliform bacilli appeared early after birth, but at erratic times; they rapidly reached very high numbers, but these populations tended to decrease within a week after reaching their maximum levels.

SUMMARY

Selective culture media, and equipment for anaerobic incubation of large numbers of specimens, have been developed to facilitate the quantitative enumeration of the various aerobic and anaerobic bacterial species present in the gastrointestinal tract.

The evolution of this flora has been followed in young mice from several colonies by cultivating homogenates of the different parts of the gastrointestinal tract at daily intervals from the time of birth to the time of weaning.

It has been found that the lactobacilli and anaerobic streptococci become established immediately after birth and persist in large numbers, not only in

the large intestine but also in the stomach and in the small intestine. In contrast, the anaerobic bacilli of the bacteroides group become established only after the 16th day; they multiply only in the large intestine but persist in this organ in very large numbers. Other bacterial species become established at different periods of time after birth, exhibit characteristic anatomic localizations, and greatly fluctuate in numbers. In general, the populations of enterobacilli and enterococci decrease precipitously after having reached a maximum level shortly after the beginning of colonization.

BIBLIOGRAPHY

1. Dubos, R., and Schaedler, R. W., The effect of the intestinal flora on the growth rate of mice, and on their susceptibility to experimental infections, *J. Exp. Med.*, 1960, **111**, 407.
2. Schaedler, R. W., and Dubos, R., The fecal flora of various strains of mice. Its bearing on their susceptibility to endotoxin, *J. Exp. Med.*, 1962, **115**, 1149.
3. Dubos, R., Schaedler, R. W., and Costello, R., Composition, alteration, and effects of the intestinal flora, *Fed. Proc.*, 1963, **22**, 1322.
4. Schaedler, R. W., and Dubos, R., Effect of dietary proteins and amino acids on the susceptibility of mice to bacterial infections, *J. Exp. Med.*, 1959, **110**, 921.