THE INTESTINAL FLORA IN MOUSE TYPHOID INFECTION.

By LESLIE T. WEBSTER, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, July 21, 1922.)

During a study of mouse typhoid caused by a strain of *Bacillus pestis caviæ*, it was found that in the course of epizootics[1] as well as among any series of mice receiving the bacilli artificially by mouth[2] a certain number proved refractory to infection. The mere presence of the organism, then, is not sufficient in every case to induce mouse typhoid; other factors, properly related to the specific agent, are necessary. Consequently, as the first step in an analysis of these factors, we studied the intestinal flora of laboratory mice to detect, if possible, variation of flora associated with different degrees of infectivity, as well as any alteration in the normal flora following ingestion of the pathogenic bacilli.

Technique.

The mice employed were obtained from the stock breeding room. The regular diet of bread and milk was continued. Various quantitative methods for determining the intestinal flora led to the following technique.

Stool Cultures.—If the mouse, held in the usual way, 2 or 3 hours after feeding, was rubbed gently on the abdomen, the feces were voided. It was then possible to transfer a specimen to a sterile mortar with a platinum loop without fear of contamination. An emulsion was made with 5 cc. of saline and from this, subsequent higher dilutions were prepared. It was found impracticable to weigh the feces, so all counts are rough approximations of the total number of bacteria actually present. They do show accurately, however, the numerical ratio between various species of bacteria present in any given stool.

[1] Amoss, H. L., *J. Exp. Med.*, 1922, xxxvi, 25, 45.

[2] Webster, L. T., *J. Exp. Med.*, 1922, xxxvi, 71.

Intestinal Cultures.—The mouse was asphyxiated with chloroform, opened, and the intestines removed to a sterile Petri dish. Approximately 1 cm. each of duodenum, ileum, cecum, colon, and sigmoid was then sectioned, slit longitudinally, and placed with sterile instruments in a test-tube containing 5 cc. of saline and a few glass beads. After thorough mixing, subsequent higher dilutions were prepared from these emulsions. An attempt was made to choose sections of intestine containing similar amounts of fecal material, but this variable and the uncertainty of the standard section of 1 cm. make the rough total estimates only of value in showing the numerical ratio between the various species of bacteria present in small and large intestine.

Determination of the Normal Flora.

For a preliminary survey of the flora, stool cultures from 50 normal mice were examined, as well as intestinal autopsy cultures from eight normal mice. Optimum media for general use were determined and there was gained a general insight into the species of bacteria associated with the intestinal tract.

Primary inoculation of fecal and intestinal suspensions into plain broth, dextrose broth, dextrose broth fermentation tubes, and milk, at hydrogen ion concentrations from pH 3 to 9, was tried with subcultures at varying intervals of time, to plates of plain agar, dextrose and lactose agar, agar at pH 3 to 9, with and without brilliant green or gentian violet. No specificity or inhibition phenomena proved sharp enough for practical use in this work. Therefore, subsequently, the suspensions, at various dilutions, were streaked directly to agar plates, pH 7.4, containing 0.5 per cent dextrose, and incubated 24 hours. 18 hour broth cultures picked from these plates were then examined microscopically and subcultured into semisolid carbohydrate media, lead acetate broth, litmus milk, gelatin, and media for the indole and Vosges-Proskauer reactions. Cultures falling into the Salmonella group were titrated against a serum prepared from the stock mouse strain of *Bacillus pestis caviæ*. The final identity of each colony, therefore, was based upon the above technique. While it is realized that, for a most exact identification, this procedure should

be repeated at least twice for each strain, the frequent recurrence of species and the large amount of material examined made any such refinement of method impracticable and unnecessary.

For the cultivation of anaerobes, kidney ascitic fluid media,[3] potato media,[4] cooked meat media,[5] and glucose blood agar plates were used. Non-spore-bearing anaerobes were not isolated. Since direct plating did not yield as good results as primary incubation, each suspension was inoculated into meat medium, incubated 3 days, heated 20 minutes at 80°C., inoculated into potato medium, incubated 2 days, and then plated. Numerical estimation was not possible by this method. The plates were incubated 4 days in Brown anaerobe jars,[6] colonies were seeded to potato media, and after 48 hours, the pure cultures were examined microscopically and transferred to inspissated serum, litmus milk, and peptone carbohydrate media for identification.

From the above 58 mice, the following microorganisms were recognized. Spirochetal forms and vibrios were abundant in direct smears from cecum and colon; *Trichomonas muris*[7] was occasionally seen in direct smears from the cecum; yeasts, variable in number, were usually present. Cocci, white and yellow, some of which liquefied gelatin, were present in small numbers, usually in the upper part of the intestinal tract. The following Gram-positive bacilli were isolated. *Bacillus acidophilus* and aerobic colonies of *Bacillus bifidus* were invariably found; *Bacillus lactis citreum*, *Bacillus cereus*, *Bacillus subtilis*, and *Bacillus siccus* were occasionally present. Gram-negative bacilli were identified as follows: *Bacillus coli communis*, *Bacillus coli communior*, and *Bacillus acidi lactici* were frequently found; *Bacillus alkaligenes*, *Bacillus lactis aerogenes*, and *Bacillus neapolitanus* were occasionally present. One colony belonging to the Salmonella group was seen. Spore-bearing anaerobes were rarely found in normal stools. *Bacillus bifidus* was invariably present,

[3] Gates, F. L., and Olitsky, P. K., *J. Exp. Med.*, 1921, xxxiii, 51.

[4] Avery, O. T., and Morgan, H. J., *Proc. Soc. Exp. Biol. and Med.*, 1921–22, xix, 113.

[5] Robertson, M., *J. Path. and Bact.*, 1915–16, xx, 327.

[6] Brown, J. H., *J. Exp. Med.*, 1921, xxxiii, 677.

[7] Wenrich, D. H., *J. Morphol.*, 1921, xxxvi, 119.

TABLE I.

Mouse No.	Region.	Smear.			Cocci.	*B. acidophilus* and *B. bifidus.**	Colon† group.	Anaerobes.
		Spirochetes.	Vibrios.	Yeasts.				
9	Duodenum.	−	−	−	550§	90,000§	500§	−
	1:5; 1:50‡							
	Ileum.	−	−	−	125	720,000	1,500	−
	1:5; 1:50‡							
	Cecum.	++	++	+	5,000	18,000,000	300,000	−
	1:500; 1:5,000‡							
	Colon.	+	+	+	500	390,000	7,500	−
	1:50; 1:500‡							
	Sigmoid.	+	+	+	500	400,000	7,500	−
	1:50; 1:500‡							
10	Duodenum.	−	−	−	500	4,000	—‖	−
	Ileum.	−	−	−	500	60,000	5,000	−
	Cecum.	++	++	+	500	1,000,000	1,500,000	−
	Colon.	+	+	+	200	150,000	500,000	−
	Sigmoid.	+	+	+	—	10,000	1,000,000	−
11	Duodenum.	−	−	−	50	1,600	—	−
	Ileum.	−	−	−	100	25,000	—	−
	Cecum.	+	+	?	5,000	2,500,000	—	−
	Colon.	+	+	?	—	250,000	—	−
	Sigmoid.	−	−	−	10,000	100,000	—	−
12	Duodenum.	−	−	−	1,500	2,500	100	−
	Ileum.	−	−	−	—	120,000	150	−
	Cecum.	+	+	?	150,000	2,000,000	—	−
	Colon.	+	+	?	5,000	250,000	—	−
	Sigmoid.	+	+	?	2,000	3,000	—	−
13	Duodenum.	−	−	−	500	1,000	—	−
	Ileum.	−	−	−	1,000	104,000	4,000	−
	Cecum.	++	++	+	12,500	75,000	200,000	−
	Colon.	+	+	+	—	15,000	50,000	−
	Sigmoid.	−	−	−	500	100,000	150,000	−

* Aerobic colonies only.

† *B. coli communis*, *B. coli communior*, and *B. acidi lactici*.

‡ These same dilutions were employed for each animal.

§ The figures indicate the number of colonies per 5 cc. of original emulsion.

‖ The dashes indicate less than 50 or 500 colonies per 5 cc. of original emulsion according to dilution.

TABLE I—*Concluded.*

Mouse No.	Region.	Smear.			Cocci.	*B. acidophilus* and *B. bifidus.**	Colon† group.	Anaerobes.
		Spirochetes.	Vibrios.	Yeasts.				
14	Duodenum.	−	−	−	100	1,500	—	−
	Ileum.	−	−	−	1,500	10,000	250	−
	Cecum.	−	−	−	5,000	1,000,000	—	−
	Colon.	−	−	−	5,000	375,000	—	−
	Sigmoid.	−	−	−	20,000	100,000	—	−
15	Duodenum.	−	−	−	5,000	5,000	—	−
	Ileum.	−	−	−	2,000	12,500	350	−
	Cecum.	++	++	+	5,000	500,000	500	−
	Colon.	+	+	+	2,500	200,000	10,000	−
	Sigmoid.	−	−	−	—	—	—	−
16	Duodenum.	−	−	−	2,000	30,000	—	−
	Ileum.	−	−	+	2,500	50,000	—	−
	Cecum.	+	+	+	5,000	2,700,000	—	−
	Colon.	+	+	+	2,500	240,000	—	−
	Sigmoid.	−	−	−	1,000	700,000	—	−

Bacillus welchii was occasionally seen, and rarely a few unidentified Gram-negative forms grew out on the anaerobic plates.

This list of protozoa and bacteria is misleading unless the various species are related by numerical data. The following quantitative method brought out sharply the predominating types and gave a more simple indication of the flora.

Eight mice, 4 months old, fed on a bread and milk diet, were autopsied by the technique described above. Cultures were taken from duodenum, ileum, cecum, colon, and sigmoid. Suspensions were then diluted sufficiently to give discrete colonies on the various plates: duodenal and ileal contents 1:5 and 1:50, cecal contents 1:500 and 1:5,000, and colon and sigmoid contents 1:50 and 1:500. This method, while eliminating entirely organisms present in very small numbers, gave striking and characteristic plates.

From Table I it may be seen that the predominating organisms in the intestinal tract of white mice fed on a bread and milk diet are (1) spirochetes, vibrios, and yeasts, most plentiful in the cecum;

(2) micrococci, usually white, liquefying and non-liquefying, distributed in small numbers throughout the intestine; (3) the colon group, consisting of *Bacillus coli communis*, *Bacillus coli communior*, and *Bacillus acidi lactici*, more frequent than the cocci, most plentiful in the lower bowel; and (4) *Bacillus acidophilus* and *Bacillus bifidus*, invariably present, considerably outnumbering all other species, throughout the intestinal tract.

Intestinal Flora and Infectivity.

Having gained this general knowledge of the normal flora, we were then able to study its rôle in mouse typhoid infection.

Experiment.—Thirty-three mice, weighing approximately 18 gm. each, were divided into Series A and B, and placed in two separate cages. Series A (sixteen mice) was continued on the usual bread and milk diet for 5 days; Series B (seventeen mice) was fed, for 5 days, on raw beef muscle and water. On the 6th day each of the thirty-three mice was placed in a separate jar and stool cultures were taken (Table II). On the 7th day the animals of Series A were given by stomach tube 0.5 cc. of an 18 hour broth culture of *B. pestis caviæ* diluted 1:100; sixteen of Series B received the same treatment; No. 17 of Series B was reserved as a control. The bread and milk diet was continued and administered daily to Series A throughout the duration of the experiment (60 days) while eight of Series B were changed from the meat diet to bread and milk 3 days after inoculation and the remaining eight of Series B were changed to bread and milk diet 7 days after inoculation. Stool cultures were taken on Series A at short intervals during the 60 days of observation. Table II compares the floræ of Series A and B fed on bread and milk and meat diets respectively. Table III gives the protocols of Series A, and Text-fig. 1 compares the duration of life of Series A and B.

From Table II it is apparent that the mice fed on bread and milk showed floræ in which *Bacillus acidophilus* was the predominating organism, far outnumbering the colon group, and that when the diet was changed to meat and water, the floræ changed character almost immediately. The Gram-negative bacilli increased rapidly and became the predominating group, with numerous putrefactive, protein-splitting, and liquefying organisms evident. *Bacillus welchii* was usually present. *Bacillus acidophilus* was greatly reduced in number and was frequently absent. Table II does not show this contrast in such an extreme form as two duplicate series which were fed raw meat for a longer period of time.

Text-fig. 1 shows no striking difference in the course of disease or duration of life in the two series (A and B). Animals with the

TABLE II.

Effect of Diet on Intestinal Flora.

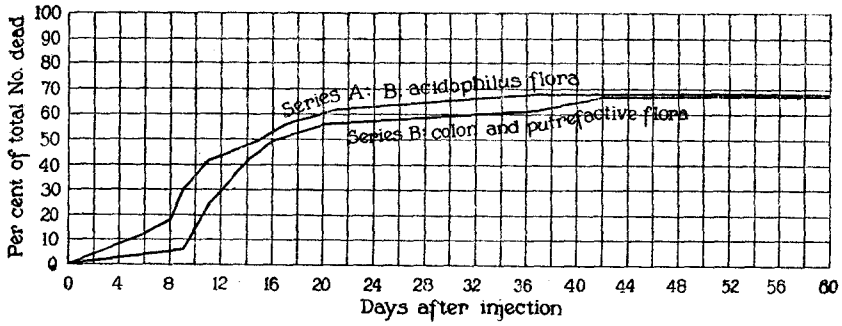
Series A.					Series B.				
Mouse No.	Cocci.	*B. acidophilus* and *B. bifidus* (aerobic).	Colon group.*	*B. welchii.*	Mouse No.	Cocci.	*B. acidophilus* and *B. bifidus* (aerobic).	Colon group.†	*B. welchii.*
1	—‡	2.5§	0.25	—	1	—	—	0.12	+
2	5.0	3.0	—	—	2	—	—	25.0	+
3	0.05	—	—	—	3	1.0	—	—	+
4	—	6.0	—	—	4	—	—	7.5	+
5	—	2.5	—	—	5	—	0.12	0.25	+
6	—	2.0	—	—	6	1.0	—	0.05	+
7	0.5	1.0	—	—	7	1.0	2.0	4.5	+
8	—	6.0	—	—	8	1.0	1.5	36.0	+
9	—	5.0	0.25	—	9	—	—	—	+
10	0.5	5.0	—	—	10	—	0.05	6.0	+
11	3.0	2.5	—	—	11	—	1.5	15.0	+
12	—	0.33	—	—	12	0.25	—	1.0	+
13	0.25	10.0	—	—	13	—	0.5	0.2	+
14	0.2	0.5	—	—	14	0.5	5.0	25.0	+
15	—	20.0	0.05	—	15	0.1	—	—	+
16	0.5	1.5	—	—	16	0.25	—	25.0	+
					17	0.02	—	3.0	+

* *B. coli, B. coli communis, B. coli communior,* and *B. acidi lactici.*

† *B. coli communis, B. coli communior, B. neapolitanus,* and *B. diffluens.*

‡ The dashes indicate less than 500 colonies per 5 cc. of original emulsion.

§ The figures indicate the number of colonies expressed in millions per 5 cc. of original emulsion.



TEXT-FIG. 1. Comparison of the duration of life of the mice of Series A and B.

TABLE III.

Protocols of Series A.

Mouse No.	Length of time after inoculation.	Cocci.	*B. acidophilus* and *B. bifidus* (aerobic).	Colon group (*B. coli* and *B. acidi lactici*).	Anaerobes.	*B. pestis caviæ.*
	days					
1	1	——*	4.0†	——	——	0.05
	2	——	1.0	——	——	——
	3	——	2.0	——	——	——
	6	——	∞	——	——	0.0001
	8	——	0.0004	——	——	0.002
	14	——	0.2	0.05	——	0.04
	20	——	+(?)	+(?)	——	+(?)
D.‡§‖	22					
2	1	2.0	10.0	——	——	——
	2	——	9.0	——	——	——
	3	0.10	50.0	——	——	——
	8	——	∞	∞	——	——
D.§‖	11					
3	1	0.05	6.5	——	——	——
	2	——	6.0	——	——	——
	3	——	0.5	——	——	——
	6	3.0	12.5	——	——	——
	8	0.2	12.5	——	——	——
	14	——	——	——	——	∞
D.§‖	15					
4	1	——	6.0	——	——	0.05
	2	——	12.0	0.5	——	——
	3	——	20.0	0.1	——	——
	6	——	2.0	0.1	——	0.5
D.§‖	8					
5	1	——	10.0	——	——	——
	2	——	10.0	——	——	——
	3	1.0	20.0	——	——	——
	6	——	10.0	——	——	——
	8	——	1.0	——	——	——
	14	——	50.0	5.0	——	——
	20	——	——	+(?)	——	——
D.§	35					

* The dashes indicate less than 500 colonies per 5 cc. of original emulsion.

† The figures indicate the number of colonies expressed in millions per 5 cc. of original emulsion.

‡ D. indicates dead, S. survived.

§ Autopsy lesions typical of mouse typhoid.

‖ Heart's blood culture positive.

TABLE III—*Continued.*

Mouse No.	Length of time after inoculation.	Cocci.	*B. acidophilus* and *B. bifidus* (aerobic).	Colon group (*B. coli* and *B. acidi lactici*).	Anaerobes.	*B. pestis caviæ.*
	days					
6	1	—	5.0	—	—	—
	2	—	12.5	—	—	—
	3	—	20.0	—	—	—
D.§‖	9	—				
7	1	—	1.2	—	—	—
	2	—	10.0	—	—	—
	3	—	25.0	—	—	—
	8	—	0.1	0.0001	—	0.001
	14	—	∞	∞	—	∞
D.§‖	17					
8	1	0.5	25.0	—	—	—
	2	—	35.0	—	—	—
	3	—	40.0	0.1	—	—
	6	0.5	2.0	0.005	—	—
	8	—	—	0.01	—	—
D.§‖	9					
9	1	—	50.0	7.5	—	—
	2	—	25.0	30.0	—	—
	3	—	15.0	7.5	—	—
	6	—	2.0	0.02	—	—
	8	—	—	0.0002	—	0.00002
	14	—	0.2	0.0005	—	0.0005
	20	+(?)	+(?)	+(?)	—	—
S.	60					
10	1	—	10.0	—	—	—
	2	—	5.0	—	—	—
	3	—	2.0	—	—	—
D.§‖	6					
11	1	—	—	—	—	—
	2	3.0	—	—	—	—
	3	—	—	—	—	1.0
	6	0.1	—	—	—	1.0
	14	—	—	—	—	—
	20	+(?)	—	+(?)	—	—
S.	60					

TABLE III—*Concluded.*

Mouse No.	Length of time after inoculation.	Cocci.	*B. acidophilus* and *B. bifidus* (aerobic).	Colon group (*B. coli* and *B. acidi lactici*).	Anaerobes.	*B. pestis caviæ.*
	days					
12	1	—	20.0	—	—	—
	2	—	2.0	—	—	—
	3	—	1.0	—	—	—
	6	—	1.0	—	—	—
	8	—	0.2	—	—	0.25
	14	—	10.0	—	—	5.0
	20	+(?)	+(?)	+(?)	—	+(?)
S.	60					
13	1	—	25.0	—	—	—
	2	0.5	12.5	—	—	—
	3	0.05	10.0	0.04	—	—
	6	—	4.0	—	—	—
	8	—	0.2	—	—	—
D.§‖	11					
14	1	—	1.5	—	—	—
	2	—	20.0	0.15	—	—
	3	—	10.0	—	—	—
	14	—	10.0	—	—	—
	20	+(?)	+(?)	—	—	—
S.	60					
15	1	—	20.0	0.01	—	—
	2	—	4.0	—	—	—
	3	—	20.0	—	—	0.5
D.§‖	6					
16	1	1.5	16.0	—	—	—
	2	10.0	6.0	—	—	—
	3	5.0	10.0	0.02	—	—
	6	0.001	0.1	0.1	—	0.001
	8	0.005	0.2	—	—	0.005
	20	+(?)	+(?)	+(?)	—	—
S.	60					

acidophilus flora were as susceptible to the dose of mouse typhoid bacilli as were the animals with a colon and putrefactive flora. Two repetitions of this experiment gave similar results.

Finally, in the protocols of Series A (Table III) it may be seen that the surviving mice showed the same floræ as those of the susceptible mice which succumbed to the infection after varying intervals of time. In additional series of control mice, numbering about 75 in all, this same fact was constantly observed.

DISCUSSION.

Metchnikoff and his collaborators, studying the rôle of the intestinal flora, were impressed by the small numbers and few species of microbes inhabiting the gastrointestinal tract of mice and rats.[8, 9] Change of flora following change of diet was also emphasized by them,[10, 11, 12] as well as by the more recent work of Torrey[13] and Rettger and Cheplin[14] who showed that in rats, dogs, and man, the intestinal flora bears a definite relation to the diet of the host, that lactose and dextrin have the power of stimulating growth of the aciduric bacteria, and that *Bacillus acidophilus*, administered *per os*, is capable of replacing the putrefactive and colon group.

While these facts have been very generally recognized, little credence has attended Metchnikoff's doctrine of orthobiosis, in which he stresses the importance of a putrefactive intestinal flora as a predisposing factor to gastrointestinal infection, as well as to many diseases of old age. Direct experimental evidence for this hypothesis has been meager;[11] nevertheless, Rettger, by attempting to alter the flora in this class of diseases, and as a prophylactic measure, seems to concede its validity.[15]

Our experiments show that in mice the intestinal flora plays no part in host susceptibility to mouse typhoid infection and that it does not affect the outcome of this disease.

[8] Metchnikoff, Weinberg, Pozerski, Distaso, and Berthelot, *Ann. Inst. Pasteur*, 1909, xxiii, 937.

[9] Tsiklinsky, *Ann. Inst. Pasteur*, 1914, xxviii, 441.

[10] De Gasperi, F., *Centr. Bakt., Ite Abt., Orig.*, 1911, lvii, 519.

[11] Belonovsky, J., *Ann. Inst. Pasteur*, 1907, xxi, 991.

[12] Distaso, A., and Schiller, J., *Compt. rend. Soc. biol.*, 1914, lxvi, pt. 1, 179.

[13] Torrey, J. C., *J. Med. Research*, 1918–19, xxxix, 415.

[14] Rettger, L. F., and Cheplin, H. A., A treatise on the transformation of the intestinal flora with special reference to the implantation of *Bacillus acidophilus*, New Haven and London, 1921.

[15] Rettger, L. F., and Cheplin, H. A., *Arch. Int. Med.*, 1922, xxix, 357.

CONCLUSIONS.

The normal flora of laboratory mice at The Rockefeller Institute, fed on a bread and milk diet, was determined. *Bacillus acidophilus* and *Bacillus bifidus* outnumber the *Bacillus coli*, *Bacillus acidi lactici*, and *Bacillus coli communior* group about twenty-five to one. White and yellow cocci which may or may not liquefy gelatin are occasionally noted; spirochetal and vibrio forms and yeasts are usually seen in stained preparations. This flora does not change when mice are artificially infected *per os* with a strain of mouse typhoid bacilli (*Bacillus pestis caviæ*) and is the same in the animals which resist the infection as in those which succumb.

Mice fed on a meat diet and showing a colon, *Bacillus diffluens*, and *Bacillus welchii* flora do not differ in susceptibility to mouse typhoid from the normal mice fed on bread and milk and showing the above *acidophilus* flora.