MICROORGANISMS IN THE INTESTINAL TRACT OF NORMAL CHICKENS[1]

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One of the objectives of studies on the intestinal flora of animals is to elucidate any interrelationships that may exist between the microorganisms and the host. Recently there has been renewed interest in the significance of the intestinal flora in relation to vitamin biosynthesis. These studies have been reviewed in detail by several authors (Najjar and Barrett, 1945; Elvehjem, 1946, 1948; Johansson and Sarles, 1949).

There have been few studies of the intestinal flora of normal chickens. Kern (1897) studied the intestinal microflora of the stomach and intestinal contents of 22 species of birds. He concluded that the following species were obligate intestinal forms: "*Bacterium coli commune*," "*Bacillus vegetus*," "*Pseudomonas granulata*," "*Bacillus defessus*," and "*Bacterium verrucosum*." Kern believed that there were no obligate stomach species, that the stomach flora was merely a reflection of the food eaten, and hence was variable. A few years later, Rahner (1901) studied the microflora of the contents of various levels of the intestinal tract of chickens at various ages. He reported that "*B. coli gallinarum*" first appeared in two-day-old chicks whereas gram-positive rods and cocci appeared only in chicks four to five days of age. Rahner concluded that "*B. coli*" was the only obligate intestinal form. He did not detect any anaerobes. King (1905) also studied the intestinal microflora of different levels of the intestinal tract of chickens. His results were in agreement with those of Rahner in that "*B. coli*" was found in small numbers, if at all, in the duodenum but in the lower levels of the tract it was present in large numbers, and reached a maximum in the cecum. Gage (1911) reported that "*B. coli*" made up 60 per cent of the intestinal flora, whereas gram-positive cocci were found to constitute 30 per cent of the flora. He made the interesting observation that the ceca of newly hatched chicks were filled with gas, but was unable to isolate any anaerobes. Menes and Rochlin (1929) studied the intestinal microflora of hens, geese, and turkeys. They concluded that the flora was identical at all levels of the tract. The predominating inhabitants were *Escherichia acidi-lacti*, *Streptococcus faecalis*, and *Lactobacillus beijerincki*. Emmel (1930) reported that 50 per cent of the flora of two-week-old chicks and hens was made up of *Escherichia coli* and *E. coli* var. *communior*. He also detected considerable numbers of aerobic spore formers, but very few anaerobes.

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These early studies did not establish on a quantitative basis the kinds and numbers of microorganisms that make up the so-called normal intestinal flora of the chicken at the various levels of the intestinal tract. They did not provide the information needed as a foundation for combined studies by nutritionists and microbiologists.

The results of an investigation of the influence of various carbohydrates on the numbers and kinds of microorganisms in the intestinal tract of the chicken have been published by Johansson *et al.* (1948). The changes produced in the cecal flora of chicks infected with *Eimeria tenella* have been reported by Johansson and Sarles (1948). These studies showed again the need for knowledge of the normal flora of normal chickens.

The work reported in this paper represents an attempt to determine the kinds and numbers of certain groups of bacteria present at various levels of the intestinal tract of the normal chicken. It is hoped that this information will help to establish a foundation for future studies on the biological significance of the intestinal flora of the chicken.

METHODS

Animals and rations. The chickens used throughout the course of this work were the offspring of New Hampshire males crossed with Single Comb White Leghorn females. After the chicks hatched they were kept without food or water for 24 hours. The chicks were than placed in wire battery cages and given food and water *ad libitum*.

The initial ration used was starter mash no. 45 of Halpin *et al.* (1944). At the age of 10 weeks this ration was supplemented with a mixture of corn, wheat, and oats made up so as to supply 3 parts of corn, 2 parts of wheat, and 1 part of oats. At maturity, all the chickens were placed on a slightly different grain mash (Robblee *et al.*, 1948).

Two chicks were picked at random from the group for sampling at 3- to 4-day intervals up to the age of 45 days; at 7- to 14-day intervals up to the age of 30 weeks; and at irregular intervals thereafter up to the age of 1 year. In addition, 20-day-old chick embryos and chicks that had just hatched and had not yet been given food or water were also sampled.

Preparation of samples. Each chick was killed by decapitation and the intestinal tract from the gizzard to the cloaca was exposed. The specific segments to be sampled were set off by ligatures. The segments of the tract sampled were the duodenum, the ileum (approximately 6 inches of the middle of the small intestine), the cecal pouches, and the colon. The contents of each segment were squeezed under aseptic conditions into sterile petri dishes, which were immediately refrigerated. The time required between the killing of the chicken and the refrigeration of all samples varied between 5 to 10 minutes.

When all samples had been obtained, each was thoroughly mixed, and a 0.5-gram sample was weighed out on a piece of waxed paper. With very young chicks it was often impossible to obtain half-gram samples and the amount available had to be weighed and used. The weighed sample was thoroughly mixed in a 6-ounce bottle containing 49.5 ml of sterile tap water and a 6- to 8-mm layer of

small glass beads. Occasional samples were mixed for various lengths of time in sterilized stainless steel containers of a Waring blender. From these 1:100 dilutions, serial dilutions up to 10^8 were made in sterile tap water. From these dilutions inoculations were made as rapidly as possible into the tubes and petri dishes required for each cultural procedure.

Media and cultural procedures. (a) For aerobic agar plate counts the following medium was used: tryptone (Difco) 0.5 per cent, yeast extract (Difco) 0.3 per cent, glucose 0.5 per cent, and agar 1.5 per cent. The medium was adjusted to pH 6.8 to 7.0.

(b) For anaerobic agar plate counts the same medium as in (a) was used. Anaerobic conditions were obtained by incubating the plates in modified McIntosh and Fildes jars with a 100 per cent hydrogen atmosphere.

(c) Eosin methylene blue agar (Difco) plates were used for coliform plate counts.

(d) Potato glucose agar acidified to pH 3.5 was used for yeast plate counts (*Standard Methods for the Examination of Dairy Products*, 1941).

(e) "SF" broth of Hajna and Perry (1943) was used for dilution counts of enterococci.

(f) Carrot liver agar "shake" tubes (Garey *et al.*, 1941) were used to enumerate lactic acid bacteria.

(g) For spore counts, the 1:100 dilution of each sample was heated in a water bath at 80 C for 11 minutes and then rapidly cooled. From this heat-shocked sample suitable dilutions were made and agar plates of medium "a" (tryptone glucose yeast extract agar) were prepared. A duplicate series of plates of the same medium was also prepared and incubated anaerobically in a 100 per cent hydrogen atmosphere.

All the plates in cultural procedures (a), (b), (c), (d), and (g) were prepared in duplicate by the usual "pour" plate method. The "shake" tubes in cultural procedure (f) were also prepared in duplicate. The "SF" broth cultures in procedure (e) were prepared by using 5 tubes per dilution. The "SF" broth cultures were incubated at 45 C whereas the potato glucose agar plates were incubated at room temperature (20 to 25 C). The remaining cultures, procedures (a), (b), (c), (f), and (g), were incubated at 37 C. The potato glucose agar plates were incubated for 5 days; the "SF" broth cultures and the carrot liver agar shake tubes were incubated for 3 days; and the remaining cultures, procedures (a), (b), (c), and (g), were incubated for 2 days.

Colony counts were made of suitable dilutions of the agar plates and agar shake tubes, with the aid of a Quebec colony counter. The most probable numbers of enterococci as shown by "SF" broth cultures were calculated according to the M. P. N. table in *Standard Methods for the Examination of Water and Sewage* (1946). Since it was impossible to obtain enough intestinal contents from young chicks for dry weight determination, all counts are expressed on a wet weight basis.

Statistical analysis.[3] An analysis of variance was made of the counts obtained

[3] The authors acknowledge with thanks the aid and advice of the University of Wisconsin Computing Service in performing the statistical analyses.

in each cultural procedure except the anaerobic agar plates; the latter counts were so similar to the aerobic agar plate counts that there seemed to be no need for a separate statistical analysis. All statistics were computed with the logarithms of the counts; the mantissa was carried out to two significant numbers. The logs of the counts from chicks up to 141 days of age were included so as to make use of counts obtained from two separate chickens on each day. The eosin methylene blue agar plate counts and enterococcus dilution counts obtained from duodenal contents were omitted from the statistical analysis because in many cases the numbers were so low that no precise counts were obtained. This made the comparison of the counts secured on the contents in the remaining levels of the tract more accurate. F values were determined for variation among levels,

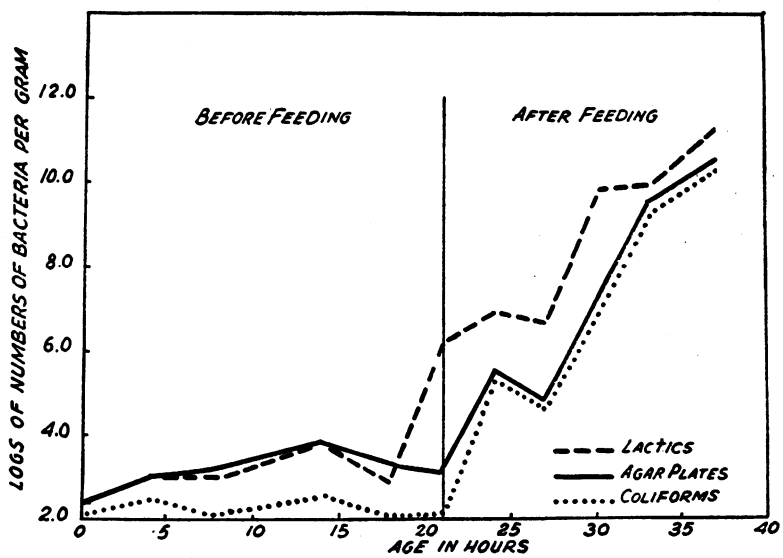

Figure 1. The cecal microflora of young chicks at various ages.

for variation among days, and for the interaction between levels and days. Least significant differences (LSD) were calculated for the F values that were significant.

RESULTS

The cecal microflora of chicks from the time of hatching until 37 hours of age. In order to follow the establishment of each group of bacteria in the cecal contents of chicks that had just hatched, samples were obtained at frequent intervals after hatching until the chicks were first given food, and for 16 hours after that time. Only three counts were determined: aerobic agar plate counts; coliform (EMB) plate counts; and carrot liver agar shake tube counts. The results of this study are summarized in figure 1. At each sampling period, three chicks were killed and the cecal contents pooled and mixed. One sample was taken from this mixture for analysis. The 0-hour age interval was used to designate a sample taken from the mixture of the cecal contents of three chicks that were still in the shell.

No coliforms were detected in the cecal contents during the 21 hours that the chicks were not fed. The agar plate counts showed low numbers of organisms (between 312 to 4,500 per gram) during the first 21 hours. The carrot liver agar shake tube counts paralleled the agar plate counts during the first 18 hours, but then went up to 1.5 million per gram at 21 hours, although the chicks had not yet consumed any food. After the chicks were fed, the counts obtained with all three cultural procedures increased up to the 37-hour sampling period—16 hours after food had been provided. At this time the three counts were of the order of magnitude characteristic of mature chickens. It should be pointed out that the carrot liver agar shake tubes showed gas formation whenever growth appeared. In older chicks, gas was evident only in the lower dilutions, and not in the higher dilutions. This would indicate that the non-gas-producing bacteria, probably lactic acid bacteria, were present in older chicks in higher numbers than the gas producers. In the very young chicks, however, the gas-producing bacteria were present in greater numbers than the lactic acid bacteria. The kinds of gas producers present were not identified, but it is certain that they were not coliforms.

The Intestinal Microflora of Chickens from Hatching to Maturity

After the initial establishment of the cecal microflora had been observed, samples were taken from four levels of the intestinal tracts of chickens from the age of 1 day through 200 days. Two chicks were sampled at each age interval and the arithmetic means of the counts on each chick obtained with each cultural procedure were used in plotting the graphs. Throughout this work, no yeasts or molds were detected in plates inoculated with dilutions of intestinal contents as low as 1:50. Anaerobic agar plate counts were very similar to the aerobic agar plate counts and therefore only the aerobic agar plate counts are plotted on the graphs. The data for four counts are plotted for each level of the intestinal tract studied and are shown in figures 2 to 5.

The cecal pouches. The numbers of all groups of bacteria were highest in the cecal pouches (figure 2). It can be seen that the numbers of all groups of bacteria showed an initial decline during the first 4 weeks. The numbers of enterococci decreased gradually as the chicks became older, but the other counts showed no clear trends in numbers at any other time. It is interesting to note that the coliform counts seem to follow the agar plate counts quite closely, suggesting that coliforms make up most of that part of the population determined by aerobic agar plate counts. The isolation and identification of organisms growing in the agar plates verified this observation. It is also interesting to observe that the lactic counts were considerably higher than the agar plate counts. The enterococcus counts were lower than the coliform counts at all times.

The colon. The numbers of all groups of bacteria were lower in the colon (figure 3) than in the cecal pouches. None of the groups showed a discernible trend in numbers as the chickens grew older. The coliforms in the colon contents did not appear to constitute so large a portion of the population determined by the agar plate counts as in the cecal pouches. The agar plate counts were much closer to the lactic counts than in the cecal pouches. In the colon, the coliform

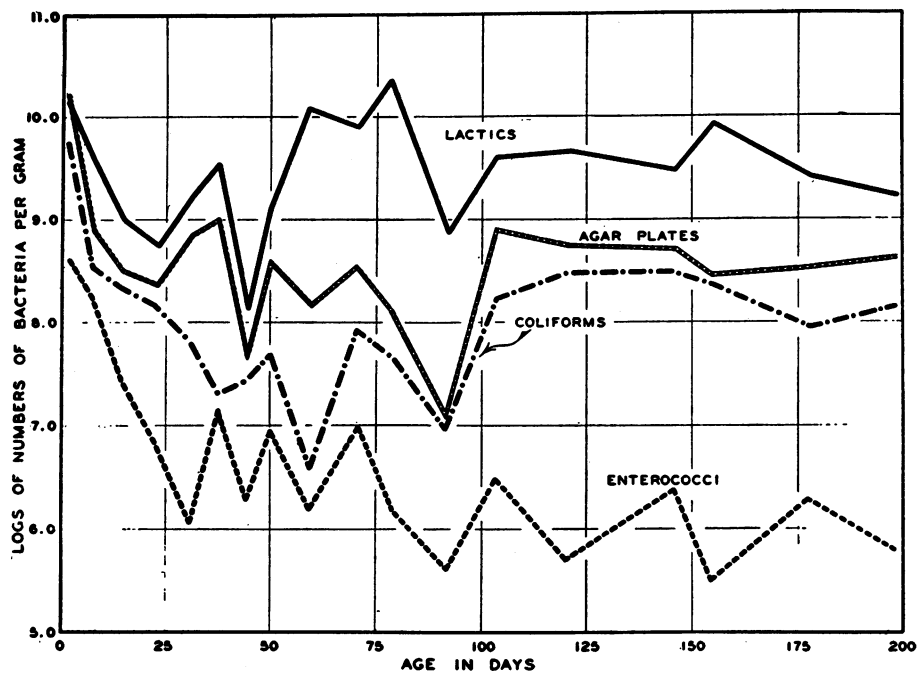

Figure 2. Flora of the cecal pouches.

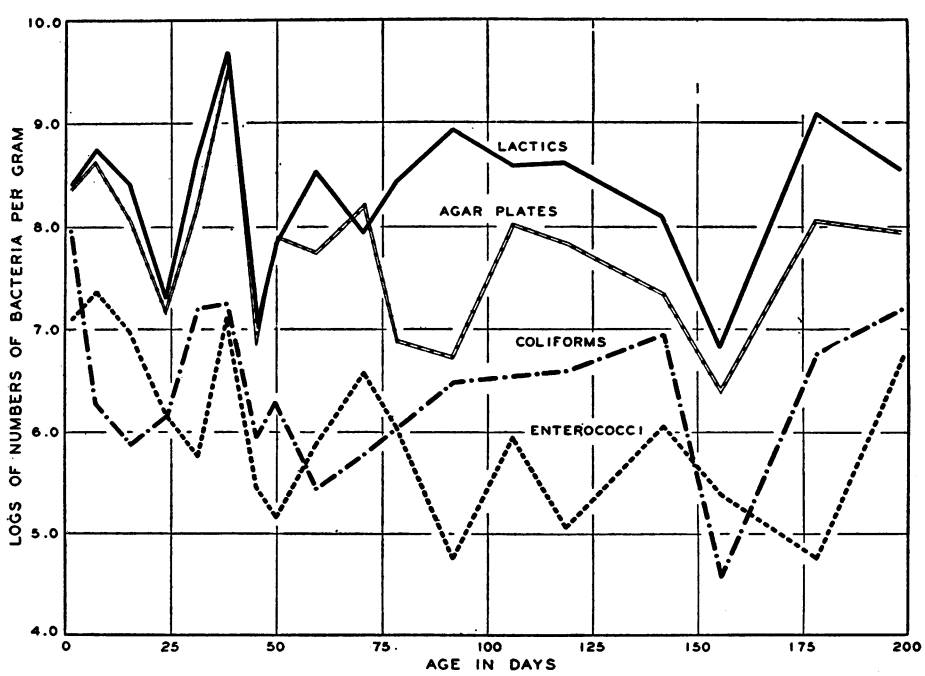

Figure 3. Flora of the colon.

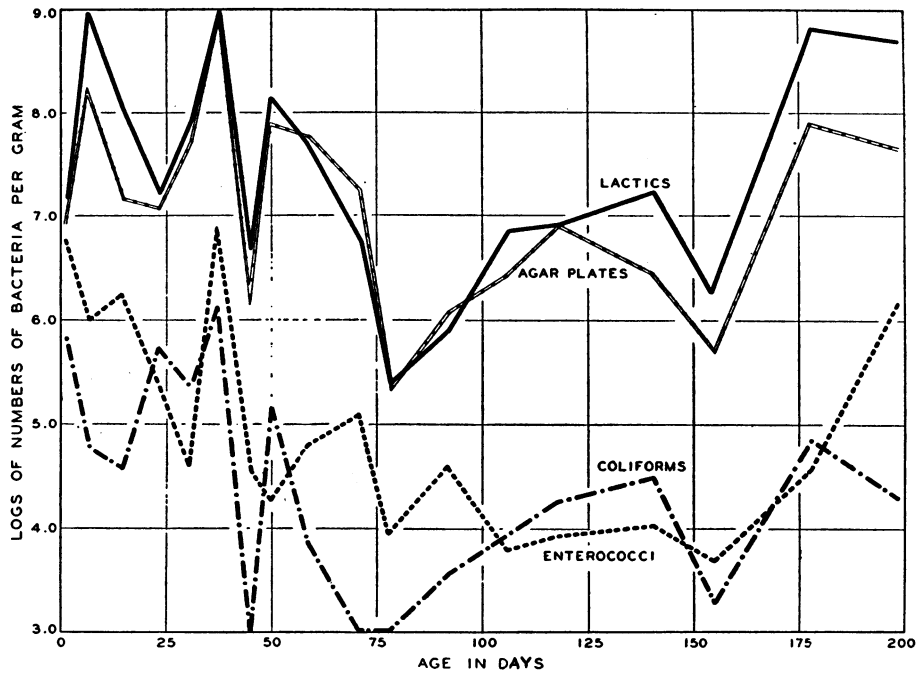

Figure 4. Flora of the ileum.

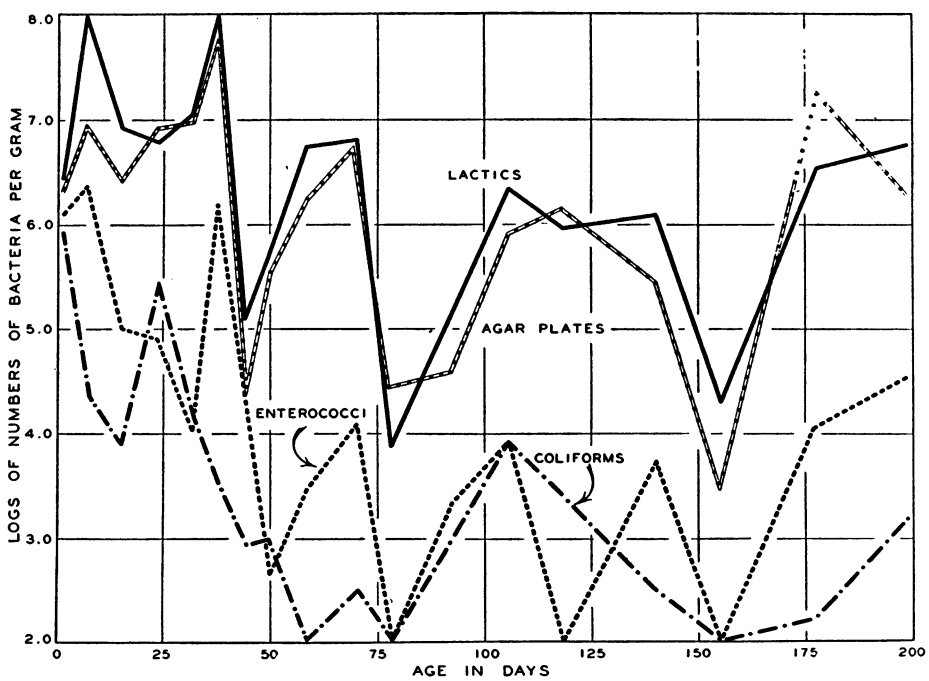

Figure 5. Flora of the duodenum.

and enterococcus counts were more similar than in the cecal pouches, where the numbers of coliforms were considerably higher than the numbers of enterococci.

The ileum. In the ileum (figure 4) the numbers of all groups of bacteria were lower than in the cecal pouches. It can be seen that the numbers of all groups of bacteria decreased gradually until about 11 weeks. The counts then leveled off, except for two high readings at 178 and 199 days. Coliforms made up a very small portion of that part of the population determined by agar plate counts; the agar plate counts and lactic counts were practically identical. The numbers of enterococci and coliforms were very similar.

The duodenum. As can be seen in figure 5, the numbers of all groups of bacteria were lowest in the duodenal contents. The numbers of all groups of bacteria decreased gradually as the chicks grew older until about 11 weeks, and then leveled off, except for two high readings at 178 and 199 days. As in the ileum, coliforms made up a very small portion of that part of the population determined by agar plate counts, whereas the lactic counts and agar plate counts were very similar.

Aerobic and anaerobic agar plate spore counts were made of the contents of the gizzard, duodenum, ileum, cecal pouches, and colon. All counts were of the same order of magnitude at all levels of the tract (1 to 6 $\times$ 10^5 bacteria per gram, wet weight). This seems to indicate that the spores were transient forms which passed through the intestinal tract of the chicken.

It can readily be seen in figures 2 to 5 how jagged the lines for each count appear on the graphs. The day-to-day variation encountered was very great. At each sampling period two birds were sacrificed and the difference in counts between birds was sometimes as great as 1,000-fold. This great variation makes it difficult to discover any trends or to evaluate the results secured. For this reason a statistical analysis of the results was believed necessary.

Variation among levels of the tract. The results of analyses of variance for the counts obtained with various media are summarized in table 1. It is interesting that in spite of the great variation in counts within each level of the tract, the differences among all counts at different levels of the intestinal tract were highly significant (significant at the 1 per cent level of significance). Least significant differences were calculated for the variation among levels. Since the carrot liver agar shake tube counts showed interaction between levels and days, a least significant difference for levels could not be determined for this count. Variation of the counts of the other three cultural procedures was not significantly different among levels on different days, so that least significant differences for levels could be calculated. It was found that the arithmetic means of the aerobic agar plate counts (expressed as logarithms) of the contents of the four levels of the intestinal tract were significantly different from one another. A difference of log 0.28 was needed to detect a significant change at the 5 per cent level of significance. Similarly the arithmetic means of the coliform plate counts (expressed as logarithms) of the contents of the three levels of the intestinal tract were significantly different from one another. A difference of log 0.40 was needed to detect a significant change at the 5 per cent level of significance. The arithmetic means of the entero-

TABLE 1

Analysis of variance of the counts from different levels of the intestinal tract of chickens at various ages

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARE	F VALUE
1. Carrot liver agar shake tube counts				
Among days	14	46.7464	3.3390	1.6152 N. S.
Between chickens within days	15	31.0095	2.0673	
Among levels	3	159.6993	53.2331	115.2731*
Days × levels	42	43.1833	1.0282	2.2265*
Levels × chickens within days	45	20.7808	0.4618	
Total	119	301.4193		
2. Aerobic agar plate counts				
Among days	14	61.9472	4.4248	4.0906*
Between chickens within days	15	16.2259	1.0817	
Among levels	3	104.5522	34.8507	124.6448*
Days × levels	42	19.2551	0.4585	1.6398 N. S.
Levels × chickens within days	45	12.5832	0.2796	
Total	119	214.5636		
3. Coliform ("EMB" medium) plate counts				
Among days	14	41.2963	2.9497	1.8614 N. S.
Between chickens within days	15	23.7703	1.5847	
Among levels	2	196.2331	98.1166	173.0146*
Days × levels	28	23.7640	0.8487	1.4966 N. S.
Levels × chickens within days	30	17.0129	0.5671	
Total	89	302.0766		
4. Enterococcus ("SF" medium) dilution counts				
Among days	14	58.1692	4.1549	5.6216*
Between chickens within days	15	11.0872	0.7391	
Among levels	2	46.4555	23.2278	90.8045*
Days × levels	28	9.5610	0.3415	1.3350 N. S.
Levels × chickens within days	30	7.6737	0.2558	
Total	89	132.9466		

N. S.—not significant.

* Significant at the 0.01 level of significance.

coccus dilution counts (expressed as logarithms) of the contents of the three levels of the intestinal tract were also significantly different from one another.

Here a significant difference of log 0.27 was needed to detect a significant change at the 5 per cent level of significance.

Variation among days. Comparing the counts obtained on different days, it was found that there was a significant difference among the counts obtained on different days with the aerobic agar plates and the enterococcus dilution tubes (both significant at the 1 per cent level of significance). The significant difference among days for the enterococcus counts is probably due to the decrease in these counts with age, observed in the contents of the cecal pouches and ileum (figures 2 and 4). The significant difference among days for the aerobic agar plate counts does not seem to be reflected in any over-all trends. Least significant differences

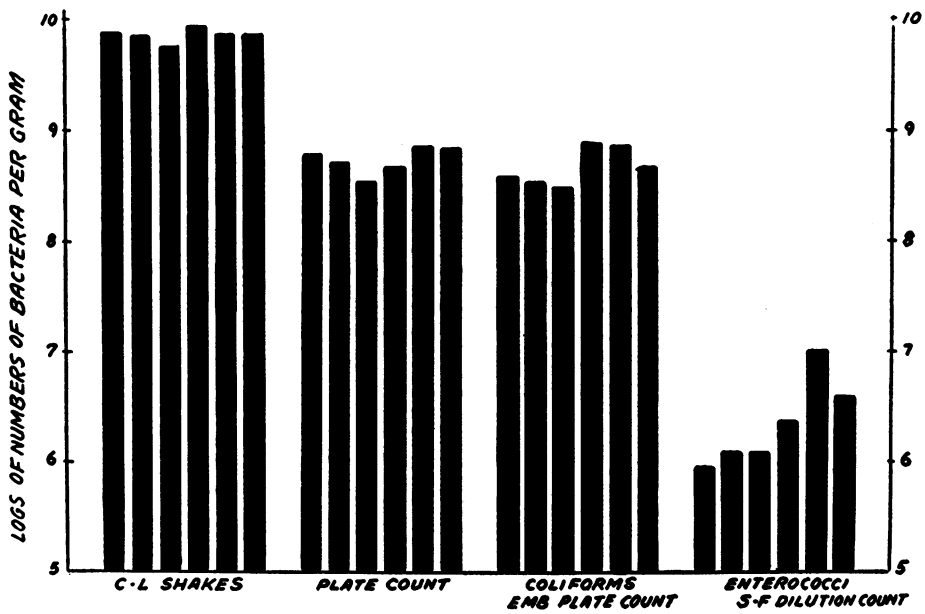

Figure 6. Samples of cecal contents.

were calculated for the variation among days in the aerobic agar plate counts and the enterococcus dilution counts. A difference of log 1.11 was required to detect a significant difference in the aerobic agar plate counts, and a difference of log 1.06 was required to detect a significant difference in the enterococcus dilution counts.

In order to check on the accuracy of the enumerative techniques employed, a cecal sample was taken and divided into six equal portions. Three replicates of this mixed sample were mixed with glass beads, and three were mixed in a Waring blender. The same cultural procedures as described earlier were used to determine the numbers of bacteria in the cecal sample. The results are summarized in figure 6. It will be seen that there is good agreement between the carrot liver agar shake tube counts, and between the aerobic agar plate counts, and fairly good agreement between the enterococcus dilution counts made on replicates of cecal samples. In each case the first three counts (from left to right) were

obtained from samples mixed with glass beads, and the last three counts were obtained from samples mixed in a Waring blender for one minute. The Waring blender counts were slightly higher than counts obtained from samples mixed with glass beads. This was especially true in the enterococcus counts, perhaps because of the breaking up of chains.

Replicate samples of colon contents were tested in the same manner used for cecal samples with essentially similar results. It appears, then, that the variations encountered between different birds are not due to inherent inaccuracies in the enumerative technique; apparently there is that much variation between the animals. When one considers the number of factors influencing the mixed flora of the intestinal tract, it is reasonable to expect great variation, for at any time when samples might be taken it is clear that variation in any one of these factors —such as the amount of contents in the tract—might profoundly influence the numbers of bacteria that will be determined by plating or by dilution count procedures.

The intestinal microflora of chickens that had been reared on range was found to be similar to that of chickens raised in battery cages. The relationships of the numbers of the different groups of bacteria to one another at each level of the intestinal tract were similar to those obtained with chickens raised in cages. However, eosin methylene blue agar plates from the cecal contents of range chickens showed roughly 10 to 25 per cent of the colonies to be the *Aerobacter aerogenes* type, whereas similar plates from cecal contents of chicks kept in battery cages rarely showed any *A. aerogenes* colonies. The presence of the *Aerobacter* organisms is no doubt due to the availability of plant and soil sources of contamination of the food consumed by chicks on range.

Isolation and identification of cultures from some of the principal groups of bacteria found after the normal flora had become established in the intestine of the chicken provided information of value in arriving at an explanation of the quantitative data.

Coliforms. Two hundred and fifty cultures were isolated and all were identified as typical *Escherichia coli* cultures. Apparently *E. coli* makes up the entire coliform population. Furthermore, the eosin methylene blue agar medium used served as a very good measure of the numbers of coliforms, for higher numbers of coliforms were not found when any other medium was used.

Lactic acid bacteria. It was found that the carrot liver agar medium used in these studies served as a measure of the numbers of lactic acid bacteria that were present in the samples. With samples of the contents of the duodenum, ileum, and cecal pouches, the carrot liver agar shake tube counts represented a good estimate of the numbers of lactobacilli. The lactic counts of the colon samples, however, are higher than the numbers of lactobacilli that are present. Lactobacilli were found to be the most numerous group of bacteria in most areas of the intestinal tract. Since the vitamin requirements of these organisms are known to be quite extensive, it is likely these bacteria play an important role in the vitamin economy of the host. Hence a detailed study of this group of intestinal bacteria was undertaken and will be reported separately.

The "SF" broth was found to be a very selective medium. Only enterococci

were found to grow in this medium and so the counts obtained for enterococci were considered quite reliable. It was found, however, that enterococci appeared in the carrot liver medium in higher numbers than would be indicated from the "SF" dilution counts obtained from the same sample. This was true in samples of the contents of the colon and cecal pouches. Thus, it appears that the "SF" dilution counts for enterococci were lower than the actual numbers of enterococci that were present in the contents of the colon and cecal pouches. One hundred and five cultures of enterococci were isolated from "SF" broth and carrot liver agar tubes. All cultures were identified as *Streptococcus faecalis*. Apparently this organism makes up the entire enterococcus population.

Anaerobes. Re-examination of the anaerobic flora of the normal chicken has confirmed the earlier report of Johansson and Sarles (1948) that the principal obligate anaerobe is *Clostridium perfringens*. Attempts to isolate other anaerobes, particularly putrefactive anaerobes, have not been successful.

DISCUSSION AND SUMMARY

Quantitative studies on the intestinal microflora of normal chickens at various ages have revealed that newly hatched chicks harbor very few microorganisms in their intestinal tracts. This remains true for the 21-hour period following hatching, during which the chicks were not fed, except for the carrot liver agar shake tube counts, which showed a significant increase in numbers at the twenty-first hour after hatching. Gas-producing microorganisms were present at the twenty-first hour in high numbers (1,500,000 per gram), but these microorganisms were not isolated in pure culture. These organisms were not coliforms, since no coliforms were detected on the eosin methylene blue agar plates inoculated with the same samples as were the carrot liver agar shake tubes. After the chicks were given food and water the numbers of all groups of bacteria increased very rapidly until 16 hours after the chicks were fed, when the numbers reached a peak.

Qualitative studies of the establishment of the microflora would serve to complement the quantitative results obtained. Of special interest would be the identification of the organisms that become established in the cecal pouches of chicks before they consume any food. It would also be valuable to determine the conditions which permit the lactobacilli to establish themselves as the most numerous species of bacteria in the contents of the intestinal tract and at what time they become established.

The results show that the "normal numbers" of bacteria became established in the contents of the duodenum, ileum, cecal pouches, and colon after the chicks had been given food and water for only 16 hours (within 40 hours after hatching). The numbers of bacteria of all groups studied were found to be highest in the contents of the cecal pouches and progressively lower in the contents of the colon, ileum, and duodenum.

A statistical analysis of the quantitative results showed that the counts obtained with each cultural procedure were significantly different among the different levels of the intestinal tract. It was found that at each level of the intestinal tract the arithmetic means of the aerobic agar plate counts, the coliform plate

counts, and the enterococcus dilution counts were significantly different from the corresponding arithmetic means obtained with each cultural procedure at any other level of the intestinal tract.

In comparing the differences in counts obtained with each cultural procedure on different days without regard to levels of the intestinal tract, it was found that only the enterococcus dilution counts and the aerobic agar plate counts showed significant differences among days. The differences in the enterococcus dilution counts among days are probably a reflection of the decrease in counts observed with this cultural procedure in the contents of the cecal pouches and ileum.

To determine whether the enumerative techniques would give consistent results, experiments were conducted in which one sample was divided into several replicates. In these cases, the replicate counts obtained with all cultural procedures showed very good agreement. This indicates that the techniques employed to obtain counts on the intestinal flora of each of two chickens on any one day contributed very little variation to the results obtained.

The relationships of some of the groups of bacteria to one another were characteristic of each level of the intestinal tract. Larger numbers of coliforms in proportion to the total agar plate counts were found in cecal pouch contents than in colon contents. In the ileum and duodenum it was found that the coliforms made up a very small portion of that part of the population determined by the agar plate counts. The agar plate counts were much lower than the lactic counts made on the cecal pouch contents. However, in the colon, ileum, and duodenum, the lactic counts and agar plate counts were very similar. In addition, it was found that the enterococcus and coliform counts were very similar in the duodenum, ileum, and colon. In the cecal pouches, the numbers of enterococci were lower than the numbers of coliforms. The anaerobic agar plate counts paralleled the aerobic agar plate counts so closely in all cases that no significant differences could be observed between the two counts.

Unidentified species of lactobacilli appear to be the most numerous group of bacteria in all levels of the intestinal tract of the chicken, with the exception of the colon.

Escherichia coli was found to be the predominant coliform, but was of numerical significance only in the contents of the colon and cecal pouches. *Streptococcus faecalis* was present in large numbers only in the cecal pouches and colon.

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