THE INTESTINAL MICROFLORA OF HENS AS INFLUENCED BY VARIOUS CARBOHYDRATES IN A BIOTIN-DEFICIENT RATION[1,2]

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The chicken is an animal of great economic importance and is widely used in the assay of certain vitamins, but little is known about its intestinal flora. The first study of the microbial flora of the chicken's intestinal tract was conducted by Kern (1897). His investigations embraced not only the chicken and other gallinaceous birds, but many of the common wild birds. Other work on the microflora of chickens has been reported by Rahner (1901), King (1905), Gage (1911), Menes and Rochlin (1929), and Emmel (1930). All of these workers agreed that *Escherichia coli* and *Aerobacter aerogenes* made up the greatest proportion of the intestinal flora. Other types of microorganisms commonly found in the chicken's intestines by these workers were lactobacilli, micrococci, pseudomonads, bacilli, sarcinae, clostridia (in two instances), and yeasts. Unfortunately, none of these studies was quantitative, and some were not too exact from a qualitative standpoint.

The type of dietary carbohydrate has a marked effect on the intestinal flora of animals. Early studies on the subject are well reviewed by Rettger and Cheplin (1921), who presented further evidence concerning the influence of carbohydrate upon the intestinal microflora. Later studies have confirmed and enlarged upon these earlier reports (Evenson, 1947). From the standpoint of vitamin synthesis, the type of carbohydrate in the diet is an important factor. Elvehjem and Krehl (1947) have reviewed this subject, hence an account of specific reports on the role of carbohydrates upon intestinal biosynthesis of vitamins will be unnecessary. Elvehjem and Krehl state, "One might place the carbohydrates in the following decreasing order in their favorable effect on vitamin requirement: dextrin, starch, lactose, glucose, sucrose, although this varies with different vitamins."

The work presented in this paper was carried out over a period of six months in connection with a nutritional investigation (Couch *et al.*, 1948) that was performed to determine the effects of different carbohydrates upon the intestinal synthesis of biotin by chickens. The microbiological work had as its objective the determination of the influence of various carbohydrates upon the numbers and kinds of microorganisms in the intestinal tract.

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METHODS

Animals. The animals employed were Single Comb White Leghorn pullets which had been reared on the experiment station range. Twenty-four pullets were placed in individual battery laying cages equipped with raised screen bottoms in order to minimize coprophagy. An all-mash laying ration was fed these hens 4 weeks prior to the start of the experiment. The pullets were divided into six groups of four each, and placed on six different diets, five of which were composed of purified ingredients (Couch *et al.*, 1948). The five synthetic rations were numbered B31, B32, B33, B34, and B35. They contained as their sole carbohydrate sucrose, dextrin, lactose plus sucrose, sucrose, and whey plus sucrose (the weight of the whey was adjusted to give the same percentage of lactose as in B33), respectively. Ration B34 contained added biotin and was considered nutritionally adequate; the other diets were biotin-deficient. The sixth group of birds was fed the same all-mash laying ration that they had received during the pre-experimental 4-week period.

Concurrent with the bacteriological work, Couch and collaborators made a nutritional study of the pullets. They kept an account of the egg production and hatchability, the abnormalities of the embryos and hatched chicks, the weekly weight gains of the hens, and the biotin content of the yolks and whites of some of the eggs produced.

Microbiological determinations. Fecal droppings for bacteriological analyses were collected on large pieces of clean waxed paper which were placed beneath the raised screen bottoms. As soon as a dropping was deposited upon the paper, it was removed with an alcohol-flamed spatula and put in a sterile wide-mouthed bottle. All fecal samples were collected between the hours of 7:45 and 9:30 A.M.

As soon as four to six samples were obtained (usually within a 0.5-hour period), the feces were prepared for analyses. One-half gram of each sample was weighed to the nearest milligram on a small piece of clean waxed paper and placed in a 49.5-ml (sterile tap water) 6-ounce dilution bottle containing a half-inch layer of glass beads. These initial 1:100 dilutions were kept on ice for a short time until preparations were completed for their microbiological analyses. The first dilutions were vigorously shaken until all visible clumps were dispersed. Serial decimal dilutions were then made in sterile tap water up to a 10^{-8} or 10^{-10} dilution and inoculations made into the following media: (1) 0.5 per cent tryptone, 0.3 per cent yeast extract, 0.5 per cent glucose, 1.5 per cent agar for aerobic plate counts; (2) "Thioglycolate medium" (Baltimore Biological Laboratories) for total dilution counts (steamed and cooled prior to inoculation); (3) carrot-liver extract ("CL") agar shake tubes (Garey *et al.*, 1941) for an indication of numbers of lactic acid bacteria; (4) "SF" broth of Hajna and Perry (1943) for dilution counts of enterococci; (5) Difco eosin methylene blue agar ("EMB") for coliform plate counts; and (6) potato-glucose agar acidified to pH 3.5 with citric acid (according to *Standard Methods for the Examination of Dairly Products*, 1941) for the enumeration of yeasts. Cultures prepared with media (1), (2), (3), and

(5) were incubated at 37 C; those with medium (4) were incubated at 45 C; and medium (6) cultures were incubated at room temperature. Cultures prepared with media (3) and (4) were incubated for 3 days, those with medium (6) for 5 days, and cultures made with other media were incubated for 2 days before counts were made. Five tubes per dilution were used for all dilution counts, and the most probable numbers of organisms in these samples were determined from the table in *Standard Methods for the Examination of Water and Sewage* (1946). All plates and shake tubes were run in duplicate. Dry weight determinations were made of each sample so that the final count could be expressed on a dry weight basis; this eliminated some of the variations encountered.

Occasional gram stains were made from smears of the 1:100 dilution and examined. No direct counts were made, however, since it was felt that such counts would contribute little to the objective of this investigation.

At the conclusion of the 6-month nutritional study it was possible to sacrifice the animals in order to obtain information on the distribution of the microorganisms within different segments of the intestinal tract. Two hens from each ration group were sacrificed by decapitation, and a posterior ventral incision was made so that the entire intestinal tract could be laid out. Representative 0.5-g samples were removed from the duodenum (a mixture of ascending and descending loops), ileum (from the middle 6 inches), cecal pouches, and colon. These samples were handled in the manner already described for fecal droppings, except that dry weight determinations were not made because many of the samples were insufficient in amount for such a purpose after the aliquot for microbiological procedures had been obtained.

Statistical analysis. Because of extreme variation in the results secured, the data on fecal dropping counts were analyzed statistically according to Snedecor (1946) and Torrie (1948). The data secured from samples taken at different levels of the intestinal tract were believed inadequate for statistical treatment.

For convenience in handling each individual count was converted to its respective logarithm to the base 10 (the mantissa was carried out to the second place). An analysis of variance for counts obtained on each medium was made, and the F value was determined. Since the coliform flora of hens on ration B31 was so obviously different from that of hens on the other five rations, EMB counts of feces from these four pullets were omitted from the analysis of variance in order to allow a more valid test of significance between such counts from hens on the remaining rations. The following statistics were then calculated: (1) arithmetic mean ($\bar{x}$); (2) standard deviation (s); (3) standard error of the mean ($s_{\bar{x}}$); (4) coefficient of variation (*C. V.*); and (5) least significant difference (*LSD*) where the F value was significant at the 1 or 5 per cent level of probability.[4]

In plotting the data graphically, it was decided to use modal values of fecal

[4] Note that all statistics are computed from logarithms, hence their antilogarithms will not be exactly similar to like statistics calculated from natural numbers of individual microbial counts. However, this more convenient method will result in comparable values, i.e., the change will be proportionate (Torrie, 1948).

counts rather than arithmetic means. It was believed that modes would give a more representative picture for comparative purposes since extreme values would be eliminated.

RESULTS

Fecal droppings. The enumerative results of this portion of the experiment may be seen in table 1 and figure 1. Note the similarity of the agar plate and Brewer's dilution counts on feces of birds on the six rations. Differences in fecal counts between hens on the various diets appear to be most prominent in carrot-liver agar shake (CL), SF dilution, eosin methylene blue agar plate (EMB), and yeast counts. The two lactose-containing diets (B33 and B35) appear to stimulate the development of lactic acid and coliform bacteria as indicated by their CL and EMB counts. Ration B31, which contained sucrose as its sole carbohydrate, was very depressing to coliforms. Enterococci appeared to be favored most by the grain and B35 rations. Dextrin (ration B32) favored intestinal coliforms but was somewhat depressing to yeasts.

Analyses of variance for the counts on feces of hens made with the six media indicate that differences between counts from birds on the six rations (five for coliforms) are highly significant (the probability, P, of assuming a significant difference is 0.01 or greater, or, 1:100) in the case of CL, SF, and yeast counts; the differences were only significant ($P = 0.05$) for fecal counts of coliform organisms. Examination of table 2 reveals a high degree of variation between individual counts in any one ration group (within rations variation) for coliform determinations, as indicated by the high value (.246) for s. Also, the $C.\ V.$ for coliforms is very high (55.09 per cent), which would tend to throw some doubt upon the validity of the EMB F value. The remaining $C.\ V.$ values were fairly similar; $s_{\bar{x}}$ values were comparable except in the case of coliform counts.

From results of *LSD* calculations, CL counts on feces from hens on rations B33 and B35 were highly significantly different from the rest, but not from each other; no significant difference was found to exist between the four low-count CL enumerations. Enterococcus counts revealed two rations which yielded droppings of comparatively high counts: B35 and grain. Fecal SF counts from hens on these two rations were not significantly different from each other or from samples of B34-fed hens. However, they were highly significantly different from SF counts on feces of hens on ration B31, and significantly different from B32- and B33-fed pullets. The *LSD* of SF counts between rations B31 and B34 was significant, but not between B32- and B34-fed birds. Despite the wide differences shown in fecal coliform counts, the F value was significant only at the 5 per cent level of probability. Since EMB counts of samples from animals fed ration B31 were left out of the analysis of variance for coliforms, *LSD*'s between B31-fed chickens and any of the other ration groups could not be calculated. However, its difference of greater than 2.5 logarithms from the next lowest group (grain) would indicate that these counts differ highly significantly from coliform counts obtained from hens on any one of the other five diets; most of the $LSD_{.01}$'s for coliforms ranged from 2.2 to 2.3 + logarithm units. The two high-count coliform groups (B32 and B33) gave an *LSD* value which was not

TABLE 1

Summary of microbial counts of fecal droppings from hens

MEDIUM	RATION	AVERAGE	MODE	NO. OF SAMPLES
CL		(10^6)*	(10^6)	
	B31	6,675.0	4,160.0	18
	B32	3,684.0	3,199.7	17
	B33	109,014.4	128,777.0	13
	B34	2,936.6	2,548.5	17
	B35	52,225.8	49,084.0	13
	Grain	6,504.6	3,134.3	17
Brewer's dilutions		(10^6)	(10^6)	
	B31	2,573.0	1,550.8	16
	B32	1,559.2	2,012.4	16
	B33	1,453.1	929.5	12
	B34	1,603.2	897.7	15
	B35	4,812.7	1,604.0	11
	Grain	1,395.4	1,138.4	18
SF		(10^4)	(10^4)	
	B31	107.1	123.5	7
	B32	534.7	293.6	8
	B33	819.2	561.7	7
	B34	1,747.9	787.6	9
	B35	6,758.4	2,651.5	7
	Grain	4,348.6	2,469.3	8
Agar plate		(10^5)	(10^5)	
	B31	4,082.1	1,875.0	20
	B32	2,331.0	2,003.0	18
	B33	2,287.6	1,873.7	14
	B34	1,840.1	2,532.8	17
	B35	5,755.0	3,042.1	13
	Grain	10,127.4	5,159.0	19
EMB		(10^4)	(10^4)	
	B31	0.06	0.01	20
	B32	4,078.4	1,210.2	18
	B33	2,592.1	839.9	14
	B34	1,328.0	6.5	17
	B35	397.6	64.8	12
	Grain	78.9	3.5	18
Acidified potato-glucose agar		(10^3)	(10^3)	
	B31	2,785.0	2,785.0	7
	B32	382.8	16.6	7
	B33	3,994.4	3,163.1	6
	B34	4,896.0	3,847.0	7
	B35	5,160.5	3,386.6	6
	Grain	99.7	169.6	7

* All counts are expressed in numbers per gram of dry weight; the average moisture content was about 80 per cent.

significant. Neither B32- nor B33-fed birds yielded fecal droppings which were found to differ significantly in numbers of coliforms from those of chickens fed

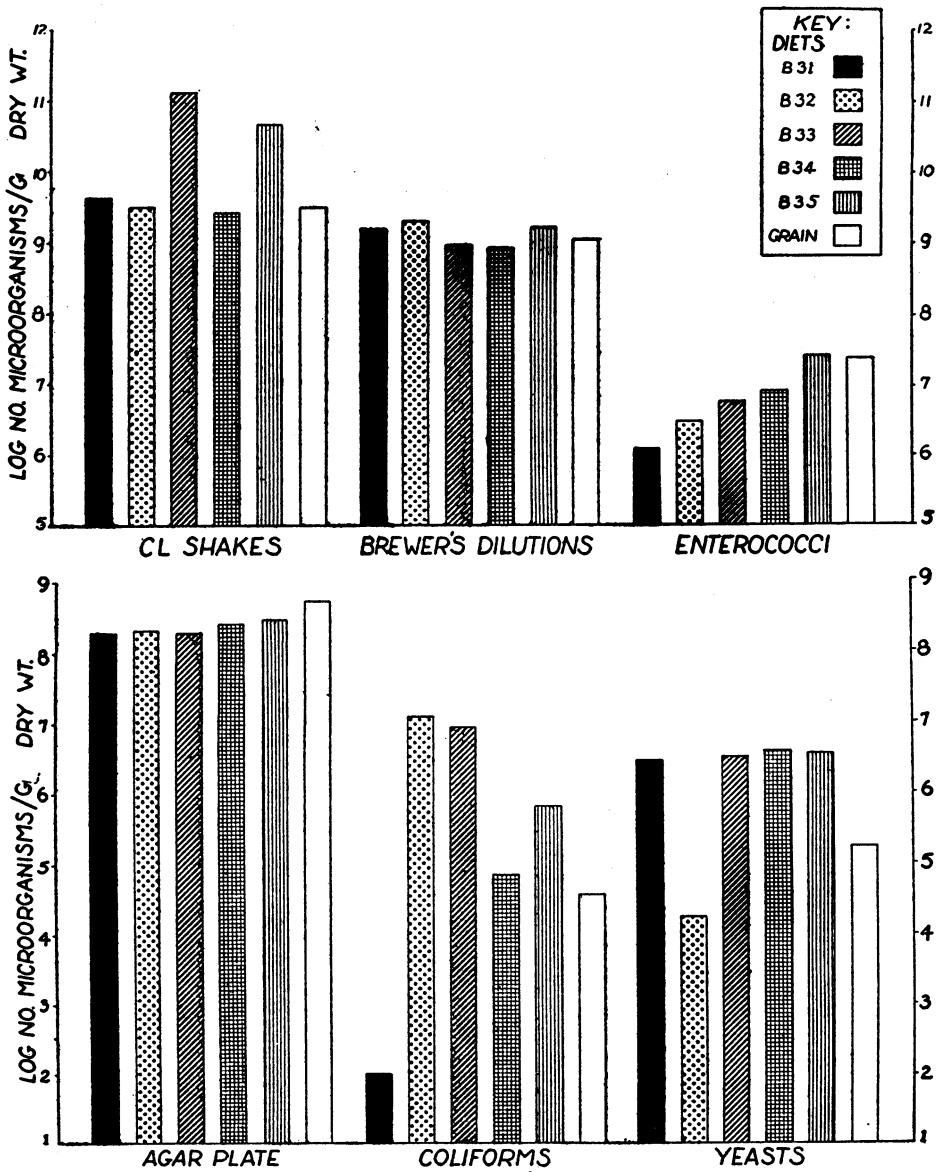

Figure 1. Fecal counts (modal values) from hens on various rations.

B35, but their *LSD*'s were significant when compared with B-34 hens. The following comparisons resulted in the finding of no significant differences in numbers of fecal coliforms: B34 and grain; B35 and grain; and B34 and B35. The grain and B32 rations were found to be least stimulatory to the growth of

yeasts. *LSD* calculations revealed that the difference between yeast counts of feces from hens fed the latter diets was not significant, and that fecal yeast counts on hens fed rations B31, B33, B34, and B35 were not significantly different from one another. However, the feces of chickens on any of the latter four rations had average yeast counts which differed significantly from those fed B32 or grain.

Difficulty was encountered in isolation of lactobacilli from the fecal samples, but enterococci could be isolated with ease from CL shakes and SF dilution tubes. Numerous gram stains of representative colonies appearing in CL tubes revealed cells morphologically similar to lactobacilli along with large cocci. Therefore, it was believed that CL counts, particularly at the higher dilutions, rep-

TABLE 2

Summary of statistical analyses of enumerative data from fecal droppings of hens on various rations

RATION	TYPE OF COUNT					
	Coliforms	Yeasts	Agar plate	Brewer's	Enterococci	CL agar shakes
B31	—	6.27[1]	8.10	8.78	5.76	9.35
B32	5.35	3.84	8.06	8.97	5.96	9.33
B33	5.55	6.35	7.88	8.59	6.16	10.58
B34	3.39	6.49	7.97	8.71	6.66	9.03
B35	4.74	6.55	8.33	9.06	7.42	10.21
Grain	3.56	4.40	8.30	8.44	7.07	9.33
STATISTICS[2]						
$\bar{x}$	4.46	5.61	8.11	8.75	6.51	9.58
s	2.46	1.02	0.73	0.84	0.88	0.86
$s_{\bar{x}}$	0.28	0.16	0.07	0.09	0.13	0.09
C. V.	55.09%	18.20%	8.94%	9.64%	13.57%	8.99%
F	2.725*	9.404**	0.944	1.124	4.040**	7.095**

[1] Average logarithm of the logarithm for each individual count.

[2] $\bar{x}$ = arithmetic mean; s = standard deviation; $s_{\bar{x}}$ = standard error of the mean; *C. V.* = coeficient of variation; F = F value (*–F significant at the 5 per cent point; **–F significant at the 1 per cent point).

resented a fairly accurate enumeration of lactic-acid-producing bacteria in the feces. When colonies appearing in such shake tubes were picked and transferred into CL broth, the gram-positive, rod-shaped bacteria rarely "came up" after incubation for as long as one week at 37 C. The cocci grew in nearly all cases. The few lactobacilli which were isolated were anaerobic to microaerophilic, and reduced litmus, produced acid, and coagulated skim milk within 24 to 36 hours at 37 C. Because of the difficulty in isolating and maintaining these lactobacilli, no further study was made of them. The enterococci were not studied to any great extent, but those encountered in the feces of young chicks have been partially characterized and found to be *Streptococcus faecalis* (Johansson and Sarles, 1948).

Coliform bacteria appeared to be predominately of the *E. coli* and inter-

TABLE 3

Summary of microbial counts at four levels of the intestinal tract

MEDIUM	LEVEL OF INTESTINE				
	Ration	Colon	Cecum	Ileum	Duodenum
CL		(10^6)	(10^6)	(10^6)	(10^6)
	B31	220	3,950	36.2	11.5
	B32	650	5,150	113.0	4.9
	B33	626	8,500	31.2	12.0
	B34	280	905	35.0	10.4
	B35	1,567	2,100	17.7	4.7
	Grain	1,725	14,000	12.5	0.4
Brewer's dilutions		(10^6)	(10^6)	(10^6)	(10^6)
	B31	192	2,950	28.0	79.0
	B32	721	6,350	185.0	9.3
	B33	54	177	24.9	12.0
	B34	262	560	9.0	0.2
	B35	2,315	1,140	1.2	0.9
	Grain	250	4,770	1.9	0.3
SF		(10^4)	(10^4)	(10^4)	(10^4)
	B31	1,100.0	239.0	280.0	0.02
	B32	2,300.0	350.0	300.0	18.7
	B33	—	—	—	—
	B34	—	—	—	—
	B35	57.0	4.1	80.0	17.5
	Grain	2.8	259.0	4.8	3.3
Agar plate		(10^5)	(10^5)	(10^5)	(10^5)
	B31	665	242	157.0	37.0
	B32	4,800	4,620	5,070.0	80.0
	B33	11	25	1.2	0.5
	B34	688	119	160.0	36.0
	B35	450	191	31.1	6.8
	Grain	90	490	145.0	4.0
EMB		(10^4)	(10^4)	(10^4)	(10^4)
	B31	0.008	0.55	0.008	0*
	B32	37.59	578.5	6.2	0.24
	B33	1.97	23.61	0.001	0.008
	B34	0.008	0.04	0.01	0.01
	B35	214.0	50.0	0.007	0.005
	Grain	0*	0.68	0*	0*
Acidified potato-glucose agar		(10^3)	(10^3)	(10^3)	(10^3)
	B31	80	15.0	94	8.8
	B32	2,220	287.0	4,600	59.0
	B33	103	16.0	10	10.0
	B34	4,200	137.0	53	149.0
	B35	460	10.7	111	19.0
	Grain	5	8.2	1	0.05

Each count is the average of two samples and is given on a fresh weight basis.

* Count of 0 means no colonies at the 10^{-2} dilution.

mediate types; only an occasional typical *A. aerogenes* colony was observed on EMB plates from any fecal sample.

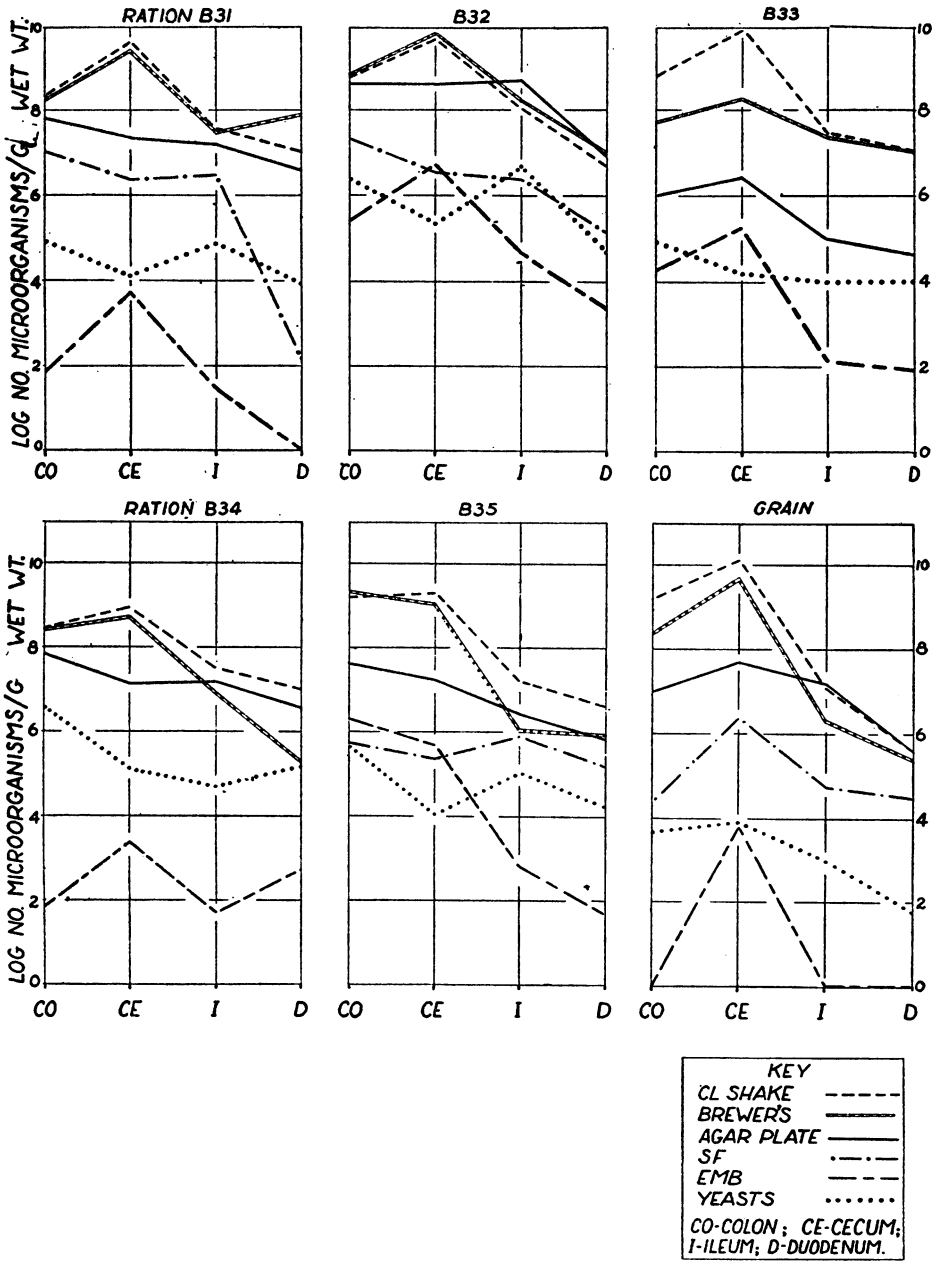

Figure 2. Intestinal flora at different levels from hens on various rations.

Seventy yeast cultures were isolated from the acidified potato-glucose agar plates. All grew well at 37 C, 14 produced a coral red pigment, 13 fermented dextrin, and 2 fermented lactose. However, those isolates able to ferment

lactose or dextrin did not necessairly come from fecal samples of birds consuming a lactose or dextrin diet.

It is believed that Brewer's dilution counts gave a fair indication of the anaerobic flora in fecal samples. Frequently, some of the highest dilution tubes evidenced growth indicative of a pure culture. Pleomorphic, gram-positive rods were isolated from such tubes but grew with difficulty and soon were lost.

Intestinal contents. Inspection of table 3 and figure 2 reveals an over-all increase in numbers of bacteria from the duodenum to the colon, and points to the cecum as the seat of the greatest concentration of microorganisms. In every case, CL counts reached a peak in the cecum and abruptly decreased between the cecum and duodenum. A difference in CL counts of intestinal contents between the lactose-fed hens and those from hens on the other rations was not apparent despite the fact that marked differences had been noted in the fecal droppings from these birds. However, on the basis of two samples for each ration, a difference is difficult to show, and might not be statistically significant even if it appeared to be obvious. Brewer's dilution counts paralleled CL counts at a lower level except in birds fed B32; in them Brewer's counts were slightly higher than CL counts.

Enterococcus counts were not obtained from hens on rations B33 and B34 because of incubator troubles. Only in birds on diet B31 was there a striking drop in numbers of enterococci from the colon to the duodenum. This decrease is but slight in the tract of hens fed B32; birds on B35 or grain showed no such decrease. Agar plate counts generally followed those obtained with CL and Brewer's media, but at a lower level, and with less differences between segments of the tract.

Coliforms increased in numbers going down the intestinal tract (from the duodenum to the cecum) except in hens on the "complete" synthetic ration (B34); in these birds coliforms were present in almost as great numbers in the duodenum as in the cecum, but the total numbers were comparatively low. No coliforms were detected in the duodenum of hens on B31, nor in the colon, ileum, or duodenum of hens on grain. Hens on dextrin (B32) maintained the richest flora of coliforms at all levels, whereas B31- and grain-fed birds possessed meager populations of these organisms.

Yeasts were found at all levels of the intestinal tract of hens on each ration. Only a very slight increase was found in samples from the duodenum to the colon. It is interesting to observe that pullets fed the dextrin ration had considerably more yeasts in their intestines than were found in fecal dropping samples. Only in hens fed the all-mash diet did yeast counts drop below 10^4 per gram at any level.

DISCUSSION

Fecal droppings. Several differences between the fecal flora of hens on the six rations might be considered important: (1) the extremely low numbers of coliforms in samples from hens on the sucrose-containing ration B31; (2) the relatively low numbers of yeasts in droppings from birds on the dextrin ration (B32);

and (3) the very high carrot-liver extract agar shake tube counts on fecal samples of pullets on rations B33 (lactose + sucrose) and B35 (whey + sucrose). Other differences were statistically significant, e.g., enterococcus counts and those between coliform counts of hens on rations other than B31 (sucrose).

The factor which interfered most with this study was that of variation between individual counts from hens on any one ration; even between counts from the same hen from week to week the variation was considerable. For example, the lowest coliform count obtained from hens on ration B32 was less than 100 per gram, whereas the highest was $49{,}200 \times 10^4$ per gram, dry weight (average $= 4{,}078.4 \times 10^4$; mode $= 1{,}210.2 \times 10^4$). Thus, statistical treatment of these data was necessary. The reason for such extreme variation is not known. At first it was believed that differences in water content of the fecal samples might increase the variation; hence all counts were expressed on the dry weight basis. This reduced the variation somewhat. A further step was instituted, and counts were determined on the basis of the dry organic matter content of the samples. This failed to alter appreciably the variation. A more likely explanation lies in the source of the fecal dropping taken for analysis. Cecal pouch contents are usually evacuated in the morning shortly after sunrise, and in the evening shortly before sunset. Therefore, a cecal dropping, which has a characteristic appearance, was not uncommon at the time of collecting (around 8:00 A.M.). The cecum appears to be the site of the greatest concentration of intestinal microorganisms. Therefore, a cecal sample, or a mixed cecal sample, would be expected to deviate in its microbial content from that of a usual colon sample. It is questionable that this can account for all of the variation observed. Other factors probably are involved, e.g., the time of day, time elapsed since last feeding, individual variation between birds under identical conditions, and experimental error.

Least significant differences, which were calculated only from counts on those media showing significant F values, give an indication as to the degree of significant difference that can be detected with the various media employed. For instance, at the 5 per cent point, the difference in coliform counts between samples from hens fed the six diets had to be approximately log 1.8 (nearly a 100-fold difference) to be considered significant. It was found, thus, that unless a difference is anywhere from 8-fold (for CL counts) to 100-fold (for EMB counts), it cannot be detected by the methods employed in this study.

The manner in which the data for fecal droppings were handled does not permit an inspection for trends. Only one definite trend was observed: a gradual increase in numbers of yeasts in feces from hens on ration B32 (dextrin) at the close of the study. At one point during this increase a tremendous development of red-pigmented yeasts was noticed, almost to the exclusion of the more common white varieties. However, the predominance of pigmented yeasts soon subsided to a point at which their occurrence was more sporadic, as found in the other samples, but the yeast flora of these dextrin-fed hens then remained at the higher level (note the high level of yeasts in the various levels of the tracts of these birds).

The results of Couch *et al.* (1948) suggested that the only birds receiving sufficient biotin, either by ingestion or biosynthesis, were those on rations B32, B34, and grain. Therefore, it must be concluded that the carbohydrate, dextrin, exerted a pronounced stimulatory effect upon microbial synthesis of biotin. The dextrin-fed hens possessed very high numbers of coliform organisms in their fecal droppings; coliforms are notably good biotin synthesizers (Landy and Dicken 1941; Landy *et al.*, 1942; Thompson, 1942; Burkholder and McVeigh, 1942). Also, these chickens were found to have relatively small numbers of fecal lactic acid bacteria, which are known to have high requirements for B vitamins. One might therefore conclude that dextrin encouraged the development of a biotin-synthesizing intestinal flora and discouraged the proliferation of a biotin-utilizing flora.

The stimulatory nature of dextrin on the synthesis of B vitamins in other animals is well known, but sucrose and lactose have usually been found to be of no particular value in establishing a vitamin-synthesizing flora in various experimental animals on purified rations (Elvehjem and Krehl, 1947). Hens fed lactose (B33) or whey (B35) also had a rich fecal coliform flora. However, they were found also to have large numbers of lactic acid bacteria. A competitive microbial equilibrium for biotin might have existed in these hens. In the case of whey, the minerals as well as lactose might have exerted some influence upon biotin-synthesizing bacteria (Daniel and Harvey, 1947). Sucrose was extremely depressing to coliform organisms; the majority of determinations resulted in the finding of fewer than 100 coliforms per gram in fecal droppings from these hens. It is difficult to understand why sucrose-fermenting coliforms such as *A. aerogenes* and *E. coli* var. *communior* did not establish themselves in these birds.

Pullets on the grain and B34 rations appeared to have a more balanced fecal flora, i.e., no one group of microorganisms was strikingly high or low in numbers.

There is already fair evidence of biotin synthesis in animals (McElroy and Jukes, 1940; Wegner *et al.*, 1941; Nielsen *et al.*, 1942; Mitchell and Isbell, 1942; McGregor *et al.*, 1947); and in humans (Gardner *et al.*, 1943, 1945, 1946; Oppel, 1942). It can be assumed, then, that the hens on the purified biotin-deficient diet containing dextrin as the sole carbohydrate were able to synthesize and assimilate adequate amounts of biotin; the source of such biotin is apparently from metabolic activities of intestinal microorganisms, perhaps those belonging to the coliform group.

One unknown quantity is that of strain difference among biotin-synthesizing microorganisms. Chickens kept on the two lactose-containing diets may have had few strains of coliforms able to synthesize appreciable amounts of biotin. Chemical and biological antagonisms should be considered also when evaluating the over-all influence of the intestinal flora on the nutrition of the host.

The carrot-liver extract agar of Garey *et al.* (1941) was by no means selective. However, lactic acid bacteria were present in such large numbers that, in effect, a lactic count was obtained as the result of dilution. The difficulty in isolation of lactobacilli might be indicative of large numbers of the anaerobic type, *Lacto-*

bacillus bifidus. It is not known whether the Brewer's medium dilution counts indicated actual numbers of anaerobic bacteria in the samples except that anaerobes could be isolated from this medium. Agar plates incubated aerobically also provided little qualitative information; colonies of sarcinae, micrococci, actinomycetes, bacilli, coliforms, and other types of microorganisms were encountered; it is doubtful that lactobacilli developed in these plates as prepared.

Intestinal contents. A brief survey of these data reveals increasingly greater numbers of intestinal microorganisms from the duodenum to the cecum with a slight decrease from the latter segment to the colon. This agrees with the early work on the intestinal flora of chickens and other birds (Rahner, 1901; King, 1905; Gage, 1911).

The duodenum of all hens studied contained very few coliform organisms. In hens on rations B31 or grain no coliforms were found in the 10^{-2} dilution of duodenum contents (recorded as zero). Yeasts were found in relatively large numbers in the duodenum of all birds except those consuming the grain mash, and increased the least of any of the organisms involved in this study from the duodenum to the colon. Enterococci also were encountered in relatively high numbers in the duodenum; these results agree to some extent with those of Kendall and Haner (1924), who claimed that *Micrococcus ovalis* (*Streptococcus faecalis*) is present in the largest numbers in the duodenum of the human intestine. Agar plate, Brewer's dilution, and CL counts indicated approximately the same concentration of microorganisms in the duodenum.

The flora of the ileum was very similar to that of the duodenum except that it appeared to contain slightly greater numbers of bacteria. No coliforms were found in the ileum of hens on the grain diet.

Colon counts were somewhat lower than cecal counts and slightly higher than ileum counts. The importance of the colon in the chicken in relation to intestinal synthesis is uncertain. It is a very small segment of the intestinal tract wherein fecal material resides for but a brief time before evacuation. Vitamins elaborated in this segment may be very incompletely absorbed by the host before being passed out of the tract. On the other hand, the cecum, which in the avian species has two large lobes, is a reservoir which is emptied usually twice a day; it may thus be of great importance in the synthesis and absorption of biotin as well as other B vitamins. Cecal counts were found to be the highest of any segment of the tract in this work. This is particularly true in the case of CL, Brewer's dilution, and coliform counts. Coliforms were found only in the ceca of birds on grain. The latter finding may have a bearing on the role of the cecum in intestinal synthesis because of the ability of coliforms to synthesize a wide variety of B vitamins, including biotin.

Birds on diet B32 (dextrin) generally had the greatest numbers of microorganisms at all levels of the intestinal tract as well as the least "spread" between the different counts. The latter might be indicative of a more heterogeneous flora in these birds.

It was hoped that the study of the microflora at different levels of the intestines

of these hens would yield results that might lend themselves, in part at least, to an explanation of the findings of the biochemists (Couch *et al.*, 1948). It is believed that the high concentration of bacteria in the cecum points to this organ as the focus of intestinal B vitamin synthesis, and we might assume that biotin was synthesized in the ceca of hens on the dextrin-containing ration. Several good studies on the rat indicate, in fact, that the cecum is the site of the greatest synthesis of B vitamins: Guerrant *et al.*, 1935; Mitchell and Isbell, 1942; Taylor *et al.*, 1942; Day *et al.*, 1943; McGregor *et al.*, 1947. The ingenious method of Mitchell and Isbell revealed the rat's cecum to absorb the greatest proportion of the various B vitamins synthesized therein by bacteria. Is the chicken similar to the rat in this respect?

Digestive enzymes might be intimately concerned with the stimulatory nature of dextrin and the depressing effect of sucrose and lactose toward biosynthesis of vitamins. Dextrin may be incompletely attacked by digestive enzymes, thus permitting a residual amount of fermentable carbohydrate to reach the cecum; this carbohydrate may be vital for vigorous vitamin synthesis. Sucrose and lactose, which are relatively soluble disaccharides, may be completely assimilated by the time peristalsis brings the ingesta into the cecum, and the consequent lack of carbohydrate may decrease the vitamin-synthesizing activities of cecal organisms.

SUMMARY AND CONCLUSIONS

The type of dietary carbohydrate was found to influence the microflora of fecal droppings from laying pullets ingesting a purified biotin-deficient ration. Dextrin was found to stimulate the development of considerable numbers of coliform bacteria. Lactose-containing diets likewise encouraged a fecal coliform flora, but lactic acid bacteria proliferated extensively in the intestines of hens fed such a diet. The most marked effect noted in case of the sucrose diet was a depressing action on fecal coliforms.

In general, intestinal microorganisms were found to increase in numbers from the duodenum to the cecum; yeasts and enterococci increased the least. Birds on the dextrin-containing diet appeared to have the greatest numbers of microorganisms at all levels of the intestinal tract as well as the least "spread" between the different counts. The cecum was found to be the site of the greatest concentration of intestinal microorganisms.

An analysis of variance was successfully applied to the data from fecal dropping samples. This revealed the presence of certain differences that were further detected by least significant difference calculations.

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