Effect of Terramycin on Fecal Microflora of Rats. I. Interrelation of Diet and Terramycin.* (19938)

JOSEPH V. SCALETTI, WALTER L. KULP, AND ROBERT C. CLEVERDON.
(Introduced by Edgar Zwilling.)
From the Department of Bacteriology, University of Connecticut, Storrs, Conn.

The relationship of intestinal microorganisms to the well-being of the host has been extensively studied. Earlier experiments were designed to show the effect of various food substances on altering the intestinal flora from proteolytic saprophytes considered harmful to the host, to a supposedly beneficial saccharolytic flora(1-8). Supporting the original work of Cooper(9), much has been found to indicate that the intestines of man and of animals harbor bacteria capable of synthesizing nutrilites essential for the host. The growth-promoting effects of dietary sulfonamides and antibiotics have been extensively studied from this point of view(10-19). Few studies however, concerned the interrelation of dietary antibiotics and composition of diet upon intestinal flora of animals(20-23).

Considerable disparity exists in reports of changes in microflora, even using a single dietary antibiotic in comparable rations. The work here reported is a portion of the studies conducted to establish statistical significance of changes in microflora due to terramycin and in the influence of ration upon these changes. This was considered necessary in order to validate findings of changes in microflora as detected by available methods involving inherent fluctuations.

Methods and materials. Experimental animals. Thirty-six mature albino female rats were used.† Each animal was housed in a Hendrix cage, and received 30 g of appropriate ration every other day. In order to prevent spilling, deep 60 g opal ointment jars firmly fastened to each cage were used as feeding cups. Water was supplied *ad libitum*. The compositions of the rations fed are listed in Table I. Eighteen rats were placed on experiment April 1952. Of these, 6 received a "basal" ration, 6 received a "high protein low carbohydrate" ration and 6 received a "high carbohydrate low protein" ration. Of the 6 receiving each type of ration, 3 rats received in addition terramycin at the rate of 100 mg per kg of ration.‡ The other 18 rats were placed on experiment June 1952 and the above scheme was repeated.

Bacteriological examination. The feces of each animal were examined bacteriologically, first 2 days after initiation of treatment, and weekly thereafter for 5 weeks. Freshly excreted feces were taken from the 3 rats in each experimental group. A one gram sample of the composite feces was ground with sand in a sterile mortar and pestle, emulsified with 9 ml sterile 0.85% NaCl and the emulsion added to 90 ml of sterile water. From this initial suspension, serial dilutions were made. (Regular examination of this suspension for existence of protozoan infestation was discontinued after establishing that none of the animals examined was infected.) An estimate of the total viable anaerobes was made using B.B.L. eugon agar(23-25) incubated anaerobically at 37°C for 48 to 56 hours(27). A second set of similar pour plates was prepared using the same medium but incubated at 37°C for 48 to 56 hours to obtain aerobic count.

Lactobacillus acidophilus was enumerated in Difco tomato juice agar(28), incubated at 37°C for 48 hours in an atmosphere of approximately 10% CO_2. Total *Lactobacillus* counts as detected on the selective medium of Rogosa(29) are not reported pending identification of the isolations obtained in this laboratory. *Escherichia coli* population was enumerated on Tergitol-7 agar(30) to which was added 0.004% triphenyl tetrazolium chloride (T.T.C.)(31). Inoculation was made by spreading 0.1 ml amounts of the ap-

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‡ Crystalline terramycin hydrochloride donated by the Chas. Pfizer and Co., Brooklyn, N. Y.

TABLE I. Composition of Rations.

	Basal ration (lb)	High protein, low carbohydrate, (lb)	High carbohydrate, low protein (lb)
Basic ingredients*	5.125	5.125	5.125
Casein	14.705	26.470	4.875
Dextrin	30.170	18.405	40.000
Total	50	50	50
Protein, %	24	45.9	9.10
Carbohydrate, %	73.8	51.9	88.7

* Basic ingredients for 200 lb of finished ration:

Alfalfa meal 17%	5 lb	$MnSO_4$	11.2 g
Dried butyl solubles (by 500)	4	Niacin	2
NaH_2PO_4	5.5	Vit. A dry 5000 I.U./g	80
Limestone	3.2	" D " 2000 "	20
Choline chloride (25%)	.5	Thiamin	40
B_{12} (Proferm) 12 mg	2	Pyridoxine	50 mg

TABLE II. Mean Values of Logs of All Enumerations of Total Anaerobes, Aerobes, *L. acidophilus*, *E. coli*, *Proteus spp.* and Fecal Streptococci, in Feces of Albino Rats on 3 Diets With and Without Terramycin. Statistical data include stand. error, ration difference, duplication of experiment and significant probabilities.

	Organisms					
Exp. groupings	Total anaerobes count × 10^6	Total aerobes count × 10^6	*L. acidophilus* count × 10^5	*Proteus spp.* count × 10^2	*E. coli* count × 10^3	Fecal streptococci count × 10^3
Rations						
Basal	2.850±.101	2.628±.092	2.748±.223	3.833±.247	3.872±.296	3.132±.237
Protein	2.741±.152	2.303±.177	3.022±.216	3.576±.255	3.500±.189‖	2.466±.317
Carbohydrate	2.770±.134	2.518±.113	2.594±.223§	3.058±.349	2.976±.199*	2.929±.286
Terramycin						
(—)	3.192±.072†	2.870±.109†	3.548±.094†	2.596±.163‡	3.382±.135	1.901±.164
(+)	2.382±.090	2.096±.108	2.028±.155	4.381±.206	3.517±.246	3.784±.215
Replication						
I	2.919±.110*	2.508±.104	3.018±.157	3.335±.245	2.976±.212†	3.328±.249*
II	3.982±.099	2.548±.116	2.558±.195	3.642±.231	3.924±.147	2.357±.219

* $P < .05$. † $P < .01$. ‡ $P < .001$. § 2 missing values calculated (60–2 = 58).
‖ 1 missing value calculated (60–1 = 59).

propriate dilution with a bent glass rod. The plates were incubated aerobically for 12 to 18 hours at 37°C. The differentiation of *E. coli* from other enterobacteria, including *Proteus spp.* is based on the inability of *E. coli* to reduce T.T.C. to a deep red formazan compound, whereas other coliforms regularly do so. Colonies of *E. coli* appear on this medium as large yellow colonies with deep orange centers. *Proteus spp.* were enumerated on urea ricinoleate medium(32) and incubated aerobically at 37°C for 24 hours.

All poured and smeared plates of appropriate dilutions were prepared in duplicate and the colonies arising counted with a Quebec colony counter and hand tally. Fecal streptococci were detected using Difco sodium azide medium(33). Five tubes of medium were inoculated with each appropriate dilution and incubated at 45.5°C for 48 hours in a double walled incubator equipped with a fan. When no growth was apparent in any of the tubes after 2 days incubation, the tubes were re-incubated for an additional 24 hours. From the numbers of positive tubes, the most probable number of each sample was determined from the tables of Prescott(34).

Statistical analysis. The data were subjected to standard statistical methods(35). Table II includes the mean values of the logarithms of the real numbers ± their standard errors and degree of variability of the

TABLE III. Mean Logs of 6 Weekly Values Including L.S.D. Among Subgroups of All Animals on Different Rations With and Without Terramycin.

Organisms	Experiment	Basal		High protein, low carbohydrate		High carbohydrate, low protein		L.S.D. for† sub groups
		Terramycin						
		—	+	—	+	—	+	
Total anaerobes	I	3.288	2.560	3.330	2.382	3.379	2.578	
× 10^6	II	2.856	2.694	3.246	2.007	3.050	2.074	
	Mean	3.072	2.627	3.288	2.194	3.214	2.326	.440
Total aerobes	I	2.808	2.179	2.915	1.936	2.790	2.424	
× 10^6	II	2.829	2.694	2.978	1.385	2.900	1.959	
	Mean	2.819	2.436	2.946	1.660	2.845	2.191	.720
L. acidophilus	I	3.586	2.262	3.875	2.823	3.163	2.397*	
× 10^5	II	3.382	1.762	3.726	1.664	3.556	1.260*	
	Mean	3.484	2.012	3.800	2.244	3.360	1.829	1.102
E. coli	I	3.186	3.695	2.923	3.360	2.345	1.844	
× 10^3	II	3.490	5.118	3.914	3.806*	3.938	3.277	
	Mean	3.338	4.406	3.413	3.583	3.391	2.560	.872
Proteus spp.	I	3.053	4.381	2.269	4.295	2.141	3.872	
× 10^2	II	2.986	4.911	3.489	4.255	1.645	4.572	
	Mean	3.020	4.646	2.877	4.275	1.893	4.222	1.131
Fecal strepto-	I	2.815	4.373	2.072	4.351	1.726	4.630	
cocci × 10^3	II	2.073	3.264	.748	2.693	1.970	3.392	
	Mean	1.222	3.819	1.410	3.522	1.848	4.011	1.210

* One missing value calculated (Snedecor 1946).
† L.S.D. computed at the 5% level of probability.

following experimental groupings: (a) among rations, (b) between composited treated and untreated animals, and (c) between replicates. Only the significant probabilities are included in Table II. Table III shows the two values making up each subgroup (mean logs of all enumerations) and the subgroup means. The least significant difference (L.S.D.) at the 5% level probability between subgroup means is also included in Table III.

Results and discussion. The individual groups of microorganisms studied will be discussed in the order of their appearance in the Tables II and III.

Total anaerobic count. The type of ration had no noticeable effect on the total anaerobic count, although the addition of terramycin to any diet caused a significant decrease ($P < 0.01$). This effect was of the same order without regard to the type of ration as evidenced by the absence of a significant interaction between ration and terramycin.

Total aerobic count. The total aerobic count was also reduced in numbers ($P < 0.01$) by the addition of terramycin to any ration. The type of ration *per se* had no effect on the total numbers of aerobes, nor was there an apparent interaction between diet used and effect of terramycin.

L. acidophilus. Contrary to the reports of many investigators, the ability of dextrin to maintain a high aciduric intestinal flora was not demonstrated in this work(3,7). This finding is in agreement with Nath(36). A statistically significant decrease ($P < 0.01$) in *L. acidophilus* count resulted from the addition of terramycin to any of the 3 rations; these reductions were comparable regardless of rations.

E. coli. No change in *E. coli* count was observed from any ration, or from the mean effect of terramycin. A decrease in bacterial numbers ($P < 0.10$) was noted with the terramycin supplemented high carbohydrate low protein ration; an increase ($P < 0.05$) in the case of the terramycin supplemented basal ration, and no significant change in the case of the terramycin supplemented high protein low carbohydrate ration.

Proteus spp. The type of diet was found to affect the concentration of *Proteus spp.* A higher count was obtained with the basal diet than with the high carbohydrate low protein ration ($P < 0.05$), but the count with high

protein low carbohydrate was not statistically higher than the basal diet. The addition of terramycin to any diet resulted in a comparable increased *Proteus* count. ($P < 0.05$). Thus it is seen that an increase in *Proteus* numbers was encountered regardless of the numbers of *E. coli*, which is contrary to the findings of Seiburth(26).

Fecal streptococci. No interaction between diet and terramycin and no effect of diet *per se* were found to alter the count of fecal streptococci. The inclusion of terramycin, however, in any diet increased the numbers of fecal streptococci ($P < 0.001$).

Summary. 1. A study was made of changes in fecal microflora of albino rats engendered by changes in composition of diet, and by incorporation of terramycin in the 3 diets employed. The data were statistically analyzed as to ration effect, drug effect, interaction between ration and diet and reproducibility of experiments. 2. Diets *per se,* showed little or no effect in modifying the counts of total anaerobes and aerobes, *L. acidophilus,* or fecal streptococci. Nor was there a statistically significant interaction between terramycin and diet in these groups of organisms. Terramycin caused significant reductions of anaerobes, aerobes, and *L. acidophilus* and a marked increase of fecal streptococci regardless of ration. 3. The use of tergitol-7-agar containing T.T.C. was described for enumeration of *E. coli* in feces. The counts of *E. coli* were not affected by diet or terramycin *per se,* but a high degree of interaction between diet and terramycin obtained. The action of terramycin on the *E. coli* count was dependent on the type of ration fed, especially as evidenced by significant differences between terramycin supplemented high carbohydrate low protein ration and terramycin supplemented basal ration. 4. The high carbohydrate low protein diet resulted in a much lower count of *Proteus spp.* than either of the other 2 rations. Supplementation of all rations with terramycin resulted in a marked increase in numbers of *Proteus spp.* It was especially noted that the effect of terramycin was independent of the ration fed in this case.

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Effect of Actidione on Growth and Respiration of *Myrothecium verrucaria*.* (19939)

ALMA T. WALKER AND FREDERICK G. SMITH. (Introduced by R. M. Melampy.)
From the Department of Botany and Plant Pathology, Iowa State College, Ames.

Actidione produced by *Streptomyces griseus*(1) is one of the most active antibiotics toward yeasts and filamentous fungi(2,3). As with other fungicides, however, there is little or no evidence on the nature of its action. Since penicillin and streptomycin are known to interfere with respiratory metabolism(4), it was desirable to compare the effects of actidione on fungus growth and respiration. This was done by measuring the inhibition of germination and of spore and mycelial respiration of *Myrothecium verrucaria* under various conditions.

Methods. Strain QM460 of *Myrothecium verrucaria* from the Quartermaster General Laboratories was cultured at 30°C on Whatman No. 2 filter paper on a modified Fries medium of Sinden, Mix and Siu(5). Point inoculation was used for routine transfer to avoid sector variants and spore suspension inoculation was used for rapid production of spores of uniform age (Mandels and Norton)(6). Mycelial pellets, 0.2 to 0.5 mm in diameter were produced in a 24-hour growth period at 30°C on a rotary shaker, essentially by the technic of Darby and Goddard(7). Percentage germination was measured at 30°C on glass slides in drops of the Mandels and Norton(6) nutrient solution. Counts of 300 to 500 spores were taken after 4 to 5 hours from 4 fields in each of 3 drops. Controls regularly germinated 99 to 100% so that corrections for natural mortality were not required. Actidione† solutions were prepared in serial dilution with a dose ratio of 1.1 which provided 4 or 5 levels of inhibition between 1 and 99%. LD50 values were determined graphically from dosage response curves on log-probability coordinates. *Respiration measurements* were made in air in standard Warburg apparatus at 30°C using the "direct" method(8) for CO_2 with correction for bound CO_2. Two to 4 mg dry weight of washed spores or mycelium was used per flask. Spores were suspended in nutrient solution(6) containing

TABLE I. Effect of Actidione on Germination and on Respiration of Spores and Mycelium of *M. verrucaria*.

	No. of exp.	LD50 values in p.p.m. Mean ± S.E.
Germination	5	2.71 ± .20
Spore resp.* (O_2 consump.)	14	.74 ± .14
Mycelium resp.* (O_2 consump.)	4	8.03 ± .17

* Period of maximum sensitivity: ca. 30-60 min. for spores, 2nd or 3rd hr for mycelium.

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† Actidione (reagent grade) was kindly supplied by J. H. Ford, The Upjohn Co., Kalamazoo, Mich.