INTESTINAL BACTERIAL POPULATION IN RELATION TO ANTIBIOTIC GROWTH STIMULATION IN POULTRY

by

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ABSTRACT

Battery experiments were conducted with one-day-old broiler chicks and poults to study the importance of intestinal bacterial populations in explaining the mechanism(s) by which antibiotics stimulate growth. To practical rations containing high levels of broad and narrow spectrum antibiotics, the following compounds and bacterial preparations were added: (a) ristocetin, (b) polymixin, (c) sulfa drugs, (d) sialic acid, (e) yeast or yeast extract, (f) water-soaked barley, and (g) fresh, dried and autoclaved feces. In addition, birds in some experiments were given weekly or bi-weekly crop inoculations of an E. coli broth or its fractions.

Significant responses ($P < .01$) occurred from feeding growth-stimulating-type antibiotics in most instances. Numbers of intestinal micrococci, enterococci, and lactobacilli were generally depressed in the presence of dietary antibiotics, whereas, the E. coli population varied.

Among the E. coli treatments, only lysed cells were effective in significantly ($P < .01$) improving growth, and growth improvement was similar and non-additive to the response obtained from broad or narrow spectrum antibiotics.

Sulfasuxadine had no effect on weight of chicks at four weeks, whereas the significant ($P < .01$) growth depression which occurred from feeding sulfaguanadine was significantly improved from feeding ristocetin in combination with either zinc bacitracin or terramycin.

In three of five experiments, in which zinc bacitracin promoted a highly significant growth response ($P < .01$) over the basal, the addition of polymixin resulted in a further significant response ($P < .05$) over that obtained from zinc bacitracin fed singly.

Enzymes of yeast, yeast extract, or those from water-soaked barley were ineffective in mediating antibiotic responses in chicks.

Daily feeding of fresh feces from hens receiving an antibiotic-free ration significantly depressed ($P < .01$) growth. However, growth was restored equivalent to the basal-fed birds by addition of sialic acid and bacitracin, but not by addition of either compound alone. Fresh feces which were dried at 100° F. for 72 hours, or dried and autoclaved under 15 pounds pressure for 30 minutes, had no effect on chick growth.

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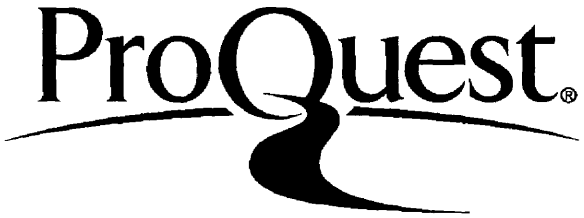

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INTRODUCTION

Since the isolation of antibiotics and the recognition that these compounds stimulate early growth of chicks, research investigators have made many attempts to determine the exact mechanism(s) by which they exert their beneficial effect.

Among the several theories which have been advanced to explain this action of antibiotics, the concept of quantitative or qualitative shifts in microbial population within the intestinal tract of the animal has received widespread attention.

Several investigators including Schottelius (1899), Balzam (1937), and Reyniers et al. (1949) have attempted to raise birds under germ-free conditions in order to study possible relationships between intestinal microflora and growth. While the results of the germ-free approach have not been particularly rewarding, they have made researchers aware of the importance of the part microorganisms may play in eliciting the growth effect from antibiotics.

Characteristic chemical and spectral differences between "broad-spectrum" (tetracyclines) and "narrow-spectrum" (penicillin and bacitracin) antibiotics may possibly cause differences in their growth stimulating mechanism(s). Since bacitracin is a complex polypeptide molecule that is not absorbed into the blood stream, the primary effect in growth stimulation must result from its action in the digestive tract. Tetracycline-type antibiotics, on the other hand, are absorbed into the blood to some extent and so could act systemically as well as enterically. In addition, the "broad-spectrum" antibiotics, as represented by the tetracyclines,

inhibit both gram-positive and gram-negative organisms whereas, "narrow-spectrum" antibiotics, such as bacitracin and penicillin, act more specifically against the general disease-producing gram-positive forms.

The research described in this thesis is concerned with the study of the effects of feeding both types of antibiotics, singly, or in combination with various microbial inhibitory agents, bacterial preparations, or enzymes. Particular emphasis has been placed on the changes in Escherichia coli in relation to observed growth differences among the various treatments.

REVIEW OF LITERATURE

Antibiotic mechanism of action

Despite the well established knowledge, Moore et al. (1946), Stokstad et al. (1949), and Reed and Couch (1950) that antibiotics increase growth rate and enhance feed utilization in poultry, the exact mechanism(s) by which these compounds function to improve performance has not been discovered since their initial use in feeds in 1946. General concepts which have developed to explain this mechanism include:

1. An effect on the physiology of the animal.
2. Inhibition of harmful organisms in the intestinal tract which are competing with the host animal for nutrients.
3. An increase in beneficial intestinal organisms which aid in increasing assimilation of nutrients.
4. An effect on sub-clinical diseases.

The concept of meaningful bacteriological changes within the gut from antibiotic feeding has received extensive attention since it was demonstrated early that antibiotics are more growth stimulating when fed to birds in contaminated quarters than those fed in new poultry houses. Coates et al. (1951, 1952), Bird et al. (1952), Hill et al. (1953), Jacobs et al. (1953), and Waibel et al. (1954). Also the bacterial population of the gut has been found to change significantly (Price and Zolli, 1959) when antibiotics are fed at levels as low as four grams per ton of feed.

Germ-free studies

Several investigators have studied the effect of microflora on animal performance in an attempt to correlate possible interrelationships between intestinal microbial population and rate of growth.

Schottelius (1899, 1902, 1908, 1913) raised both normal and germ-free chicks, but due to lack of essential nutrients in the ration, growth was abnormally slow in the germ-free birds. He concluded erroneously, therefore, that chicks could not be raised in a germ-free environment.

Cohendy (1912) also attempted to raise chicks in a germ-free environment. Lack of knowledge of the complete nutrient requirements of starting chicks resulted in his inability to keep the birds alive for more than five weeks. Balzam (1937), in a study on the effect of intestinal microflora on the vitamin requirement of birds, raised five germ-free chicks for a period of two months. Since growth and freedom from vitamin deficiencies were similar on germ-free and conventionally reared birds, he concluded that the intestinal flora of chickens exerted no measurable effect on feed utilization.

Moore et al. (1946) reported a growth response from feeding sulfasuxadine, streptothricin, and streptomycin. Since streptomycin appeared to depress the total number of intestinal coliform and micrococci bacteria, including Escherichia coli and enterococci, while at the same time increasing lactobacilli, this raised the question of enteric changes as an explanation for antibiotic action.

Experimental evidence presented by Coates et al. (1952) in England, lent strong support to the idea that an "infectious agent" must be present in order to obtain growth stimulation in chicks from dietary antibiotics. This work encouraged others to initiate studies with antibiotics.

While the initial germ-free chick studies conducted by Reyniers et al. (1949) were beset with mechanical and other difficulties, these researchers were able to successfully rear bacteria-free chicks for a

160-day period on an autoclaved commercial chick starting mash. Unfortunately, these workers did not attempt to determine the relative microorganism populations of the gut of the normal test birds, so only limited information was obtained.

Subsequent studies by Luckey et al. (1955) using limited numbers (4 or 5 birds per treatment) of day-old, New Hampshire chicks and Beltsville White poults showed inconsistent results. A feeding level of 46 mg. per kg. of procaine penicillin produced a significant growth response in four-week-old germ-free poults, while lower levels of oxytetracycline and procaine penicillin promoted only slight non-significant growth increases.

In further studies along the same lines with germ-free chicks, Gordon et al. (1957-58) attempted to correlate 34 to 37 day cecal bacterial counts in penicillin-fed birds with those in controls. No differences in the microflora population of the cecum were observed, with the possible exception of a decrease in streptococci count in the antibiotic-fed chicks. A comparison between untreated germ-free chicks and antibiotic-fed conventional birds showed similar characteristics; such as reduced weight of small intestine and ileocecal tonsil, unchanged weight of spleen and adrenals, and increased lymphocyte concentrations of the thymus. Thirty-five day weights of germ-free birds were also similar to those of the conventionally reared antibiotic-fed Leghorn chicks.

Forbes et al. (1958) recognizing the disadvantages of the limited numbers of birds employed in the Reynier and Luckey studies, re-examined the effect of antibiotics in two separate lots of approximately 25 germ-free poults at 14 days of age. Penicillin fed at 45 mg. per kgm. and

oleandomycin fed at 30 mg/kgm. produced significant growth responses in conventionally reared birds. Germ-free poults grew as rapidly as the conventionally reared antibiotic-fed birds but did not respond to antibiotic treatment. These workers concluded that their experiments supported the theory that growth response from antibiotics is due to their action on the microflora of the gut.

At the First International Conference on Antibiotics in Agriculture, Freerksen (1955) reviewed the numerous speculations concerning mechanisms of antibiotic growth stimulation in domestic animals and poultry up to that time. His own work in Germany supported the concept that microflora changes in the gut were involved in antibiotic growth stimulation, though not necessarily through increases in total numbers of organisms. Rather, he favored the theory that antibiotics act on certain mutant strains of organisms which produce toxins in the host. He also pointed out exactly contrary relationships where decomposed E. coli organisms are used therapeutically in human medicine.

Identification of intestinal organisms

Since it is known that bacteria, or their metabolic products, play essential roles in many biochemical reactions, much study has been directed toward the types of bacteria predominating in the normal intestinal tract of animals and poultry.

Kern (1897) identified 88 species of intestinal organisms in poultry and found 32 different motile bacilli, 28 various micrococci, 8 sarcinae, as well as 20 species of other bacteria.

In an early study of microflora of the chicken's digestive tract, King (1905) established that E. coli was the predominating microorganism

in the gut, but that it rarely occurred in the duodenal region. Clostridium welchii (or perfringens) was found to occur infrequently.

The findings of Gage (1911) suggested that about sixty percent of the intestinal bacteria were of the gram-negative type; however, some difficulty was encountered in interpretation because much of the fiber and other non-bacterial debris stained gram positive. In these studies, which involved birds in dirt floor pens, the predominating organisms were E. coli with a few diplococci (enterococci) present. Gage noted the E. coli always predominated, but appeared to vary with age and changes in environmental conditions.

It was noted by Menes and Rochen (1929) that the same species of microorganisms flourished throughout the intestinal tract, but that quantitative differences occurred depending upon the site. Their explanation for this apparent uniformity of population was based on the likelihood of lactic acid production, with its associated reduction of putrefactive organisms. They considered the lactic acid flora to be Streptococcus faecalis, E. coli, and Lactobacillus plantarum.

Emmel (1930) confirmed King's observation that the coliform type of organism (E. coli, Aerobacter aerogenes) constituted about 65 percent of the bacterial population of the intestinal tract of chickens. Twenty of the chickens which he had under observation showed a decrease in intestinal E. coli while suffering from an enteritis outbreak.

More recently Schumacher and Hauser (1941) and Yacowitz and Bird (1953) have verified the predominance of E. coli as the principal organism in the intestinal tract of poultry. In addition, evidence has been presented by Couch et al. (1948), Driesens (1951), and Sieburth et al. (1952)

that coliform bacteria have the ability to synthesize folic acid, riboflavin, biotin and niacin as well as other nutritional factors. The chick's requirement for protein was reported by Machlin et al. (1952) to be 19 percent with a diet containing chlortetracycline and 21 percent if the antibiotic were omitted. Further studies along these lines led Weakley et al. (1953) to conclude that bacitracin also spared protein for chick growth. Groschke and Evans (1950) observed a sparing effect on water-soluble vitamins in chicks fed chlortetracycline while a vitamin-sparing effect from antibiotics was also shown for riboflavin, niacin and folic acid by Biely and March (1951). Insofar as fat-soluble vitamins are concerned, Burgess et al. (1951) noted improved utilization of vitamin A and carotene due to penicillin feeding and investigations by Ross and Yacowitz (1954) showed a decreased vitamin D requirement for normal bone deposition in the presence of dietary penicillin.

Effects of antibiotics on intestinal microflora

Since growth responses appeared to be related to the presence of particular organisms, Johansson et al. (1948) and Johansson and Sarles (1949) initiated studies of microflora changes in the presence of bacitracin and penicillin. They showed that the ceca are an important site for the synthesis of B vitamins and probably other unknown nutritional entities. Rhodes et al. (1954) showed that coliform organisms accumulated in the ceca of antibiotic-fed birds. However, Dixon and Thayer (1951) demonstrated that growth responses could occur with penicillin and aureomycin in chicks whose ceca had been removed.

The early work of Groschke and Evans (loc. cit.) suggested that antibiotic growth responses were mediated by changes in intestinal

microflora which reduced competition from unfavorable bacteria. Sieburth et al. (1951) observed a reduction in cecal clostridia in turkey poults from the feeding of 100 grams per ton of penicillin. The total cecal enterococci, however, appeared to be quite variable.

March and Biely (1952) and Elam et al. (1953) suggested that antibiotic growth responses may be due to decreasing the incidence of enterotoxemia in birds -- primarily by reducing the number of clostridia and lactobacillus-type organisms in the gut.

Romoser et al. (1952a) found that A. aerogenes increased sharply upon penicillin feeding. This led to further work (1952b, 1953) in which lyophilized E. coli and A. aerogenes were fed to chicks in the presence and absence of penicillin. Addition of these cultures increased the response to the antibiotic 64 to 84 percent; however, no measurable response occurred from the feeding of the bacterial cultures in the absence of penicillin.

Work along the same lines was conducted by Anderson et al. (1952a,b; 1953a,b) in which normal- and abnormal-appearing strains of E. coli were isolated from cecal contents and fed to chicks. The killed coliform cells were without effect, but the live cells and their broth filtrate gave growth responses similar to penicillin in chicks. Poults, on the other hand, responded to penicillin and live cells, but not to the filtrate. Addition of micrococci depressed growth while anaerobic bacteria did not.

Further studies with chicks by Anderson et al. (1956) related growth responses from chlortetracycline to its effect in reducing organisms of the enterococci-type. Feeding of ten percent (volume/weight) of a high

concentration (28×10^9 cells) of a mixed enterococcus culture caused a highly significant depression in growth. Addition of 40 grams per ton of chlortetracycline reduced the numbers of enterococci and lactobacilli and tended to maintain the coliform count at approximately that of the control group, while weight was restored equivalent to the control birds.

Bogdonoff *et al.* (1957) obtained an added growth effect in broilers from bi-weekly crop inoculations of mixed cultures of *E. coli* and *A. aerogenes* in the presence of the 200 gram per ton level of several antibiotics. Addition of approximately 6.8×10^{12} coliform cells per ml. *per se* had no effect on growth or feed efficiency.

Using pooled samples of the entire digestive tract of chickens, Price and Zolli (1959) were able to show significant increases in coliform populations in the presence of four grams per ton of oleandomycin. These microorganisms counts correlated positively with increased growth at nine weeks, but not at five weeks. Pooled samples of proteus, micrococcus, lactobacilli, enterococci, yeasts, molds or clostridia, as well as total aerobic and anaerobic populations from all segments of the tract were not significantly changed due to antibiotic feeding.

Non-bacterial growth effects from antibiotics

Whereas, the antimicrobial concept as a mechanism of antibiotic action has received considerable support, some evidence points to additional modes of action. For example, Stokstad and Jukes (1950) observed that alkali-treated chlortetracycline residues contained growth-stimulating properties but were inactive from a microbiological standpoint. Additional work by Elam *et al.* (1951) lent further proof that more than one mechanism of growth stimulation by antibiotics might be operating.

They observed that autoclaved penicillin contained no measurable antimicrobial effect and did not appear to alter intestinal microflora, yet stimulated growth when fed or injected.

The effect of various microorganism inhibiting agents

Since the establishment by Moore et al. (1946) of tolerance levels of sulfasuxadine and the observation that this drug reduced coliform population in the ceca of the chick, interest has developed in using bacterial inhibiting compounds to study microflora changes in the gut, or in vitro inhibition of disease-producing microorganisms.

Peppler et al. (1950) fed 1.0 and 0.5 percent polymixin D in a charcoal adsorbate to chicks and noted a marked depression in coliform population which persisted for several days following removal of the compound. Streptococcus faecalis appeared to be the most resistant type of bacteria to the drug while lactic-acid-producing bacteria were unaffected.

Ristocetin, a new antibiotic possessing activity against gram-positive bacteria and mycobacteria, but not against the gram-negative species, was studied extensively in vitro by Grundy et al. (1956-57). Ristocetin B proved three to four times as active in vitro as ristocetin A against several disease-producing gram positive forms and resistance to the drug was not readily acquired. Since doses of greater than 100 ug. per ml. were required to inhibit E. coli, this organism remained relatively unaffected in therapeutic studies. Intravenous or intramuscular doses in rabbits gave measurable blood levels for at least eight hours, while infections of Streptococcus pyogenes, Staphylococcus aureus and Diplococcus pneumoniae in mice were readily controlled.

Goebel and Barry (1957), in investigating the biochemical properties of E. coli (strain K-235) reported that in a culture medium this organism elaborated colominic acid which had properties similar in many respects to those of sialic acid. Goebel reported that sialic acid has interested medical scientists in recent years because of its effect on viruses. When it is combined in its native state with certain proteins and sugars, the sialic-acid-containing complex interferes with the adherence of certain viruses, such as the influenza virus, to living cells.

In a study of the effect of coprophagy and refection, Barnes et al. (1959) observed a sparing effect of several vitamins when rats were fed a slowly digested carbohydrate such as dextrin. Closely associated with this observation was the slight growth increase noted upon thiamin supplementation and a further marked increase in growth when penicillin was added to a dextrin-type ration. However, when coprophagy was prevented neither thiamin nor penicillin promoted increased weight. This suggested possible involvement of fecal entities in sparing essential nutrients.

Mameesh et al. (1959) fed 0.1 and 1.0 percent raw hen feces mixed with the whole diet. In the presence of both levels of fecal contamination he observed a consistent growth response in four-week chicks to fifty ppm. of terramycin. Penicillin at the same level, on the other hand, gave a growth response in only one of six trials in the presence of fecal contamination.

Gut physiology studies

Several investigators have attempted to correlate increased feed consumption, decreased thickness of gut wall and differences in feed transit

time with antibiotic responses in poultry as a possible mechanism of growth stimulation. Whereas, increased feed consumption does occur due to antibiotic feeding, the added percentage of feed consumed does not, in most instances, equal the magnitude of response observed during the early period of growth stimulation. Measurements of intestinal walls have shown reduced thickness and lower overall weight of the cleaned intestine in antibiotic fed birds as compared to birds receiving the control ration. In addition, tracer studies have indicated that antibiotics may have an effect on the amount of time required for passage of feed through the alimentary tract; however, the meaning of these changes has not been established.

Gordon et al. (1957-58) showed a highly significant decrease in small intestine weight of chicks at 37 days ($P < 0.01$) from feeding fifty mg. per kgm. of penicillin. In the absence of antibiotics, germ-free chicks also showed a highly significant decreased weight of small intestine compared to conventionally reared birds.

Several investigators (Busse, 1952; Busse and Spiess, 1952; Schaumann et al. 1952) have shown that many drugs can affect the motility of the intestinal tract -- in most cases inhibiting peristalisis to some extent. However, Nakatsuka et al. (1952) found that penicillin or chlortetracycline in concentrations as low as 1:10,000 to 1:5,000 stimulated contractions of isolated frog intestine. Wolterink et al. (1958) observed a reduction in transit time of radioactive P^{32} from the crop to the intestine due to the feeding of reserpine plus dienestrol diacetate to White Rock cockerels. However, a comparison of treated with control birds showed that treatment with the drugs resulted in a 44 percent faster absorption of P^{32} from the duodenal area.

Hillerman et al. (1953), in a series of tests involving young and mature turkeys and chickens, showed an increase in average transit time for feed in the presence of fifty ppm of dietary penicillin. A capsule containing two-tenths gram of ferric oxide per bird was found to be an acceptable tracer for providing good coloration without affecting the consistency of the feces. Under the conditions of these experiments, average passage time was two hours, 57 minutes for birds receiving the control ration and three hours, 15 minutes for the antibiotic-fed birds.

On the other hand, Jukes et al. (1956) using carmine or chromic oxide in the feed as tracers found significant decreases in time of feed transit ranging from 10 to 35 minutes from feeding ten ppm of penicillin or aureomycin. In these cage experiments, both heavy and light breed chicks were tested with observations being made at five-minute intervals for appearance of the marker in the feces.

Further studies along the same lines led Jukes et al. (1956) to conclude that average weight of the intestinal tract was less for antibiotic-fed chicks than for controls. Microscopic examination of serial sections of the duodenum of birds receiving ten ppm of penicillin revealed, on the average, a significantly smaller diameter, thinner tunica propria layer, and shorter villi than those of controls.

General Experimental Procedures

Similar procedures were employed for each of the fourteen experiments outlined in this section, except where discussed under an individual trial.

Randomization of chicks to replicate pens was made as follows: one-day-old Cobb's strain of White Rock broiler cockerels, as well as White Leghorn De Kalb chicks or turkey poults hatched at Michigan State University were individually weighed and distributed into consecutive weight ranges, such as 36-38, 39-41 grams, etc., with each weight range having a three-gram spread. Birds from highest and lowest weight ranges were discarded and an equal number of birds from all remaining weight ranges were allotted to each battery pen. This method was employed to minimize any possible effect that initial weight differences might have on performance. Each experiment involved 36 or 40 birds per treatment.

Lots were randomly assigned to replicate pens, except that antibiotic-free treatments were placed in top pens to reduce likelihood of these birds receiving antibiotic. This was felt to be necessary, since the bacteriological tests conducted on intestinal microflora could be markedly influenced by relatively small quantities of these compounds sifting into pens below. One replicate of each treatment was assigned to a fluorescent-lighted deck level in each of four electrically-heated, starting battery brooders having raised wire floors. This procedure was followed in order to equalize between lots any possible effects due to position of batteries in the room. In experiments conducted beyond four weeks, birds were transferred to finishing batteries using the same method of placement to avoid antibiotic sifting into the basal feed.

Batteries were thoroughly cleaned between experiments and temperature regulated at 96-98° F. at least 24 hours before arrival of the birds. Temperature was reduced 5° F. weekly and discontinued at the fourteenth day when practical. Feed and water were supplied ad libitum throughout the experiment and feed was also placed on paper on the floor of all pens for the first three days.

Group weights were recorded at the end of the second week and individual weights were obtained for statistical analysis at the end of the fourth or eighth week. Analysis of variance and Duncan's (1955) multiple range and multiple F tests were employed to determine differences between lots. Feed consumption was recorded for each replicate of each treatment and used to calculate feed efficiency based on weight gains.

Chicks on all experiments received a practical broiler-starter mash, or starter and then broiler-finishing mash from the sixth week to the eighth week. Turkeys received a practical turkey starting mash for the first four or seven weeks. Composition and calculated analysis of these all-mash rations are shown in tables 1a and 2a, respectively.

Representative birds were sacrificed from each treatment for bacteriological examination 48 to 72 hours after final weights were obtained. Contents of the digestive tracts were removed aseptically and placed in physiological buffer solution at 35° F. without shaking within a few minutes after slaughter.

The Mallmann-Peabody (1957) drop plate method for determining intestinal E. coli was employed in each test. Each area of the digestive tract in experiment 1 was studied for microorganism population and an area two inches above the cecum was studied in all other experiments.

When studied, total gram negative intestinal population was determined by culturing on laurel sulfate agar. Azide agar was used to obtain total gram positive intestinal population. Dextrose azide broth and eosin violet azide (E.V.A.) broth were used to determine total micrococci and enterococci intestinal populations, respectively. Trypsin digest agar was used as media in determining intestinal lactobacilli population.

Procedures for preparation of these various media and culturing techniques for the organisms are given in the Difco Manual.* Composition of the media used in culturing the various microflora are shown in table 1c.

Differentiation tests, as found in Standard Methods (1955) were employed to verify the purity of E. coli as coliform organisms cultured by the Mallmann-Peabody method. A table of most probable numbers provided statistical basis for total micrococci and enterococci intestinal populations.

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*Difco Manual of dehydrated culture media and reagents for microbiological and clinical laboratory procedures. Difco Laboratories, Inc., Detroit 1, Michigan.

EXPERIMENT I

The possibility of an association of intestinal E. coli population with bacitracin growth stimulation in poultry prompted an initial experiment. Its purpose was to determine if crop inoculations with a broth culture of E. coli would affect performance in the presence of broad or narrow spectrum antibiotics. Broad spectrum antibiotics are known to inhibit intestinal coliform population, whereas narrow spectrum antibiotics do not. The effects of oral inoculation with these organisms was studied with respect to growth, feed utilization, carcass analysis, distensibility of intestinal wall and digestive tract E. coli population.

Four replicate lots of one-day-old Broad Breasted Bronze turkey poults were fed the supplemented all-mash starter ration for a 28-day test period. (Table 1) Equal numbers of males and females were reared together and fed 200 grams of zinc bacitracin or terramycin per ton of feed continuously throughout the test period. In addition, trypticase soy broth or a culture of E. coli in trypticase soy broth was administered directly into the crop of the birds by automatic syringe at bi-weekly intervals. The broth culture of E. coli was adjusted on the basis of light transmission as follows:

(1) One loopful of a stock culture* of E. coli was transferred to ten ml. of sterile trypticase soy broth and incubated at 37° C. for 18 hours.

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*Stock cultures of E. coli provided through courtesy of Commercial Solvents Corp., Terre Haute, Indiana.

(2) By use of a photoelectric meter, the cultured medium was adjusted from approximately 10 to 25 percent light transmission by addition of sterile broth.

(3) 0.2 ml. of the 18-hour culture was added to each 100 ml. of soy broth and incubated, with shaking, for an additional 18 hours at 37° C. to produce approximately 6.8×10^{12} organisms per ml.

The bi-weekly dosage of broth or E. coli in broth was increased from one ml. per bird the first week (starting at three days of age) to four ml. at the fourth week -- by one ml. increments.

The birds were maintained on the experimental rations and moved to finisher batteries at the end of the fourth week. A determination of differences in gut distensibility due to treatment was made at the end of the seventh week. Ten cm. sections of intestine immediately anterior to the ceca were suspended in a 37° C. water bath and perfused by manometer with 37° C. water containing a red vegetable dye.

Results and Discussion

The results are shown in tables 1, 2, 3, and 4.

An analysis of variance (table 4) revealed that significant growth responses occurred with the addition of either broad or narrow spectrum antibiotics. Crop inoculation of E. coli was ineffective in mediating or affecting growth response to either type of antibiotic. A preliminary test of the E. coli population of the various areas of the digestive tract revealed no marked differences between treatments, based on examination of a single representative poult per lot. While no specific pattern of E. coli population was evident, there was a general increase

in E. coli nearest the cloaca for all treatments with very few E. coli noted in the crop or duodenum.

Carcass analysis of homogenous samples of dressed eviscerated birds revealed no significant differences in protein, water, or ether extract due to the various treatments.

Slight differences in distensibility of gut were noted, with antibiotic-fed birds having somewhat greater water-holding capacity at a given water pressure. These differences, while consistent, did not approach significance even at the five percent level of probability due to the limited numbers of birds involved.

TABLE 1

THE EFFECT OF BI-WEEKLY CROP INOCULATIONS OF E. COLI IN THE PRESENCE OF ANTIBIOTICS IN THE FEED ON POULT GROWTH TO FOUR WEEKS OF AGE

Lot	Antibiotic additions to basal (gms per ton)	Weekly crop inoculum: Broth	Weekly crop inoculum: Broth** plus E. coli	Average poult wts. at 4 wks. (gms)	Poults alive of 40 started	Feed/gain
1	None	+		594	37	2.15
2	None		+	573	34	2.19
3	200 zinc bacitracin	+		678	32	2.12
4	200 zinc bacitracin		+	683	35	2.02
5	200 terramycin	+		665	37	2.07
6	200 terramycin		+	678	37	2.02

* Trypticase soy broth

** Approximately 6.8 X 10^{12} organisms/ml

TABLE 2

RELATIVE CONCENTRATIONS OF E. COLI IN DIGESTIVE TRACT OF TURKEY POULTS

Treatment no.	Antibiotic Supplement	Crop inoculum: Broth*	Crop inoculum: Broth plus E. coli**	Coliform count X 10^4 per gram at four wks. of age: Crop	Duod.	2" above Cecum	Cecum	Feces
1	None	+	-	81	0.2	270,000	65,400	537,000
2	None	-	+	5.	10.	12,100	19,800	80,070
3	200 gms/ton zinc bacitracin	+	-	TNC***	TNC	TNC	703,000	TNC
4	200 gms/ton zinc bacitracin	-	+	58.	92.	TNC	750,000	192,000
5	200 gms/ton terramycin	+	-	TNC	140.	97,200	108,000	459,000
6	200 gms/ton terramycin	-	+	None	None	None	22,900	44,000

* Trypticase soy broth
** Approximately 6.8 X 10^{12} organisms/ml
*** Too numerous to count

TABLE 3

FRESH CARCASS ANALYSIS* OF FOUR-WEEK-OLD TURKEY POULTS

Lot	Antibiotic addition to the basal	Weekly crop inoculum		Ether extract (%)	Moisture (%)	Protein (%)
		Broth	Broth plus E. coli			
1	None	+		4.26	73.03	19.69
2	None		+	3.37	73.43	19.38
3	200 zinc bacitracin	+		5.40	71.85	20.00
4	200 zinc bacitracin		+	5.40	72.55	19.75
5	200 terramycin	+		5.76	71.42	20.19
6	200 terramycin		+	5.66	72.12	19.63

* Average values from replicated determinations of homogenous samples of eviscerated dressed carcass

TABLE 4

ANALYSIS OF VARIANCE OF WEIGHTS OF POULTS AT FOUR WEEKS OF AGE.
Experiment I

Source of variation	Degrees of freedom	Mean square	F values Calculated	P = 0.05	P = 0.01
Total	211				
Subclass	23	24,961	2.29		1.91
Lot	5	83,894	7.71**		3.14
Replicate	3	12,639	1.16	2.67	
L X R	15	7,780	0.72	2.10	
Error	165	10,877			

Comparison among lots at 1 percent level of probability

Lot	2	1	5	6	3	4
Wt. in grams	573	594	665	678	678	683

** Significant (P < .01)

EXPERIMENT II

In view of the negative findings with whole broth cultures in the previous experiment, a second test was conducted to determine if a fraction of an E. coli broth culture might alter the growth pattern of poultry fed a broad or narrow spectrum antibiotic.

A broth culture similar to that employed in the first trial was fractionated as follows: The 36-hour incubated culture was centrifuged at 3600 r.p.m. for 45 minutes and the supernatant saved. The centrifuged E. coli cells were then triple-washed by centrifugation in physiological saline and combined with a volume of saline equivalent to the original broth volume.

Twelve lots of one-day-old Cobb's White Rock chicks were allotted as previously described into four replicates of ten chicks each. Equal numbers of cockerel and pullet chicks per pen were fed the supplemented chick starter ration (Table 1a) continuously for a 28-day period (Table 5).

Crop inoculations of trypticase soy broth, E. coli in broth, supernatant of an E. coli culture, or centrifuged E. coli cells in saline were administered weekly. Dosage was increased from one ml. per bird the first week (starting at three days of age) to four ml. at the fourth week -- in one ml. per week increments.

One representative cockerel chick was selected from each lot for determination of the E. coli population. Limited numbers of E. coli occur in the anterior regions of the digestive tract, due probably to the high acidity. On the other hand, the excessively high numbers of E. coli ordinarily observed in the cecum make interpretation of this

population of questionable value. Therefore, E. coli population was determined in the area approximately two inches above the cecum, since it was felt that this location would represent an area which would give meaningful differences in population, if E. coli were involved in growth stimulation from antibiotics.

Results and Discussion

Effects of treatments on intestinal E. coli population and bird performance are shown in tables 5 and 6.

Weekly inoculations into the crop of chicks of the several fractions of E. coli culture were ineffective in changing growth rate in the presence of either zinc bacitracin or terramycin. Only zinc bacitracin treatments promoted a significant growth response (Table 6) over the controls and no relationship between improved growth and E. coli population was evident. In addition, none of the various coliform-crop inoculation treatments tempered or increased antibiotic responses. The fact that the terramycin-fed birds did not grow significantly faster than the controls did not appear to be associated with intestinal E. coli, since microorganism population did not vary markedly between treatments.

It is interesting to note that despite the lack of significant growth response from the broad spectrum type, feed utilization was improved from feeding either antibiotic. Furthermore, crop inoculation of the live coliforms in broth or coliform cells was associated with maximum improvements in feed conversion in the presence of bacitracin, but not when terramycin was fed. This effect may be coincidental, or may indicate associative effects between E. coli and zinc bacitracin.

TABLE 5

THE EFFECT OF WEEKLY CROP INOCULATIONS OF FRACTIONS OF E. COLI CULTURES IN THE PRESENCE OF ANTIBIOTICS ON FOUR WEEK CHICK GROWTH AND INTESTINAL E. COLI POPULATION

Lot	Antibiotic additions to the basal (gms)	Broth*	Broth plus E. coli**	Supernatant of E. coli broth***	Saline washed E. coli cells****	Average chick weights at 4 wks. (gms)	Chicks Alive of 40 started	Feed/ gain	E. coli X 10^4 population 2 inches above cecum
1	None	+				424	39	2.12	1.0
2	None		+			419	40	2.12	420.0
3	None			+		414	38	2.05	1.0
4	None				+	411	39	1.96	No growth
5	200 zinc bacitracin	+				477	37	1.84	1.0
6	200 zinc bacitracin		+			471	39	1.76	1.0
7	200 zinc bacitracin			+		460	39	1.88	0.5
8	200 zinc bacitracin				+	469	38	1.79	78.0
9	200 terramycin	+				441	40	1.84	No growth
10	200 terramycin		+			452	39	1.94	1.0
11	200 terramycin			+		449	39	1.85	No growth
12	200 terramycin				+	421	39	1.92	No growth

* Trypticase soy broth
** Approximately 6.7 X 10^{12} organisms/ml
*** Centrifuged at 3600 r.p.m. for 45 minutes
**** Cells triple washed in physiological saline

TABLE 6

ANALYSIS OF VARIANCE OF FOUR WEEK WEIGHTS. Experiment II

Source of variation	Degrees of freedom	Mean square	F values Calculated	P = 0.05	P = 0.01
Total	465				
Subclass	33	5,542	2.08**		1.74
Lot	11	22,058	3.98**		3.64
Replicate	3	9,363	1.69		
Sex	1	333,273	125.62**		6.70
S X L	11	1,866			
S X R	3	2,093			
S X R X L	33	2,317			
Error	370	2,653			

Comparison among lots at 5 percent level of probability

Lot	4	3	2	12	1	9	11	10	7	8	6	5
4 wk. wt.	411	414	419	421	425	441	449	452	460	469	471	477

at 1 percent level of probability

Lot	4	3	2	12	1	9	11	10	7	8	6	5
4 wk. wt.	411	414	419	421	425	441	449	452	460	469	471	477

** Significant ($P < 0.01$)

EXPERIMENT III

Since the findings of trials I and II did not support the concept of E. coli involvement per se in mediating responses to broad or narrow spectrum antibiotics, a third trial was conducted. In this test an attempt was made to reduce the numbers of intestinal microorganisms to a minimum. It was theorized that, if an equivalent antibiotic response occurred in the presence and absence of high E. coli population, the likelihood of E. coli contributing to the antibiotic mechanism of action could be discounted.

Accordingly, nine lots of Cobb's White Rock one-day-old cockerels were allotted, as previously described, into four replicate pens of eight chicks each. Antibiotic, sulfaguanadine, and/or ristocetin were added to the broiler basal diet (Table 1a) as shown on the experimental design (Table 7) and fed for a 28-day period. Sulfaguanadine is effective in reducing gram negative forms, such as E. coli, while in vitro and in vivo work with ristocetin by Grundy et al. (1956-57) showed this compound to be highly effective in reducing the common disease-producing gram-positive organisms.

One representative bird was selected from each experimental lot at the fourth week for determination of intestinal E. coli population.

Results and Discussion

Neither zinc bacitracin nor terramycin produced a significant improvement in weight gains (Table 8). While there was a significant growth-depressing effect due to the presence of sulfaguanadine in the ration (Table 7), addition of either antibiotic or ristocetin partially

compensated for this though it did not entirely restore performance equivalent to the basal group.

Ristocetin plus sulfaguanadine promoted a significant growth increase over "sulfa" alone, which was not further improved by addition of either type of growth-stimulating antibiotic. This suggested that ristocetin may have exerted a maximum depressing effect on harmful gram-positive organisms which were competing with or inhibiting the host in some way, and that bacitracin or terramycin did not further reduce these detrimental microorganisms.

It was noted that bacitracin restored the intestinal E. coli population more effectively than did terramycin in the presence of the E. coli-depressing sulfa drug and that the increased population was associated with significantly greater growth.

The observation that terramycin per se as compared to bacitracin effected a reduction in E. coli population without causing a growth depression, suggested possible differences in mode of action of broad and narrow spectrum antibiotics in mediating growth.

It was an interesting observation that feed utilization was markedly depressed by addition of the sulfa drug, and that under these conditions zinc bacitracin was more effective in improving efficiency of feed utilization than was terramycin.

Results, insofar as intestinal E. coli population was concerned, were not clear cut in this particular trial. However, it appeared that sulfaguanadine acted to inhibit intestinal coliforms and that ristocetin counteracted this depressing sulfa effect directly or indirectly, and thereby permitted increases in E. coli bacteria which were associated with increased growth.

TABLE 7

THE EFFECT OF SULFAGUANADINE IN THE PRESENCE OF CERTAIN ANTIBIOTICS ON CHICK GROWTH AND INTESTINAL E. COLI POPULATION

Lot	Antibiotic additions to the basal (gms/ton)	Sulfaguanadine addition to the basal (%)	Average chick wt. at 4 wks. (gms)	Chicks Alive of 32 started	Feed/ gain	E. coli X 10^4 population 2 inches above cecum
1	None	None	401	31	1.89	68.0
2	200 zinc bacitracin	None	434	27	1.78	55.0
3	200 terramycin	None	425	29	1.74	3.0
4	None	0.5	313	27	2.07	8.0
5	200 zinc bacitracin	0.5	362	31	1.82	101.0
6	200 terramycin	0.5	345	31	1.98	5.5
7	90 ristocetin*	0.5	363	30	1.91	2.7
8	200 zinc bacitracin + 90 ristocetin	0.5	379	31	1.84	TNC**
9	200 terramycin + 90 ristocetin	0.5	404	31	1.83	290.0

* Abbott Laboratories - A non-growth stimulating antibiotic which inhibits gram-positive organisms including staphlococci, enterococci, and clostridia.

** TNC - Too numerous to count at any dilution

TABLE 8

ANALYSIS OF VARIANCE OF WEIGHTS OF FOUR-WEEK-OLD CHICKS
Experiment III

Source of variation	Degrees of freedom	Mean square	F values Calculated	P = 0.05	P = 0.01
Total	267				
Subclass	35	13,352	3.55**		1.94
Lot	8	45,808	12.16**		4.91
Replicate	3	4,000	1.06	8.54	
L X R	24	3,702	0.98	2.27	
Error	197	3,766			

Comparison among lots at 5 percent level of probability

Lot	4	6	5	7	8	1	9	3	2
4 wk. wt.	313	346	362	363	379	401	404	425	434

at 1 percent level of probability

Lot	4	6	5	7	8	1	9	3	2
4 wk. wt.	313	346	362	363	379	401	404	425	434

** Significant ($P < 0.01$)

EXPERIMENT IV

Based on the findings in experiment II, in which zinc bacitracin caused a significant improvement in chick growth, whereas terramycin did not, it seemed advisable to verify these results and possibly learn if sex differences occurred with respect to growth performance or intestinal E. coli population.

One-day-old, sexed, cockerels and pullets, hatched from a White Leghorn X De Kalb cross at Michigan State University, were separately allotted, as previously described, into six lots, each containing four replicates of ten chicks each.

To the broiler basal diet shown in table 1a, 200 grams per ton of zinc bacitracin or terramycin were added. These rations were fed continuously for the 28-day test period.

One cockerel and one pullet representative of each experimental lot were selected for intestinal E. coli determination at the fourth and sixth week.

Results and Discussion

Of the two antibiotics tested, only zinc bacitracin promoted a significant growth response (tables 10 and 11), and the improvement was noted in both sexes reared separately. No differences in intestinal E. coli counts were evident at the fourth week. However, bacitracin-fed birds were the only ones showing coliform population in the gut areas two inches above the cecum at six weeks. While evidence did not strongly support involvement of E. coli in zinc bacitracin-fed chicks, an association may have existed. Apparently, the intestinal E. coli population

was variable and probably was affected by age and sex as well as other physiological factors (i.e. pH, location in the digestive tract, differences in diet).

Associated with the growth response to bacitracin was a slight improvement in feed utilization in both sexes, which was not observed in the terramycin-fed lots.

Insofar as chickens were concerned, bacitracin, at high levels contained a mechanism of growth-promoting action which was not consistently evident with terramycin throughout these particular experiments. In five experiments (II, III, IV, IX and XII) where the two types of antibiotics were compared directly on the same basal ration, zinc bacitracin produced significant responses four times, while terramycin promoted a response in only one instance. In each experiment, intestinal E. coli population was higher at four or six weeks in the bacitracin-fed birds than in the terramycin-fed birds (table 12). Furthermore, in the experiment where terramycin gave significant growth increases, intestinal E. coli population was markedly increased over those experiments showing no responses.

TABLE 9

THE EFFECT OF NARROW-SPECTRUM AND BROAD-SPECTRUM ANTIBIOTICS ON INTESTINAL E. COLI POPULATION AND WEIGHT OF FOUR-WEEK-OLD COCKERELS AND PULLETS REARED SEPARATELY

Lot	Sex	Antibiotic addition to the basal (gms/ton)	Average chick wt. at 4 weeks (gms)	Chicks alive of 40 started	Feed/ gain	E. coli X 10^4 population 2 inches above cecum	
						4 wks.	6 wks.
1	Cockerel	None	308	38	1.91	4	N.C.**
2	Pullet	None	274	38	1.88	4	N.C.
3	Cockerel	200 zinc bacitracin	344	40	1.88	4	1135.
4	Pullet	200 zinc bacitracin	305	38	1.85	4	186
5	Cockerel	200 terramycin	318	39	1.92	4	N.C.
6	Pullet	200 terramycin	275	37	1.91	4	N.C.

* White Leghorn breed
** No count, any dilution

TABLE 10

ANALYSIS OF VARIANCE OF FOUR-WEEK-OLD COCKEREL CHICKS. Experiment IV

Source of variation	Degrees of freedom	Mean square	F values Calculated	P = 0.05	P = 0.01
Total	116				
Subclass	11	4,669	3.38**		2.43
Lot	2	13,816	10.00**		4.82
Replicate	3	2,068	1.50	2.70	
L X R	6	2,900	2.11	2.19	
Error	105	1,382			

Comparison among lots at 5 percent level of probability

Lot	1	5	3
grams wt.	308	317	344

at 1 percent level of probability

Lot	1	5	3
Grams wt.	308	317	344

** Significant (P < 0.01)

TABLE 11

ANALYSIS OF VARIANCE OF FOUR-WEEK PULLET CHICKS
Experiment IV

Source of variation	Degrees of freedom	Mean square	F values Calculated	P = 0.05	P = 0.01
Total	112				
Subclass	11	3478	3.58**		2.43
Lot	2	12,168	12.53**		4.82
Replicate	3	259	0.27	8.56	
L X R	6	2,192	2.26	2.19*	2.99
Error	101	971			

Comparison among lots at 1 percent level of probability

Lot	2	6	4
Grams wt.	273	275	305

* Significant ($P < 0.05$)

** Significant ($P < 0.01$)

TABLE 12

EFFECT OF SPECTRUM OF ANTIBIOTIC ON FOUR-WEEK-OLD CHICK WEIGHT RESPONSE AND INTESTINAL E. COLI POPULATION

Exp. no.	Sex	Average chick weights (gms) Control	Zinc bacitracin	Terramycin	$E. coli \times 10^4$ 2 inches above cecum Control	Zinc bacitracin	Terramycin
II	Mixed	424	477***	441	1	1	N.C.*
III	Cockerels	401	434	425	68	55	3
IV	Cockerels	308	344***	318	N.C.	1135**	N.C.
	Pullets	274	305***	275	N.C.	186**	N.C.
IX	Cockerels	445	507***	463	23	14	2
XII	Cockerels	534	605***	593***	5	190	58

* N.C. No count at any dilution
** Six week count
*** Significantly greater than control ($P < 0.01$)

EXPERIMENT V

This experiment was conducted to determine if a relationship between amolytic or proteolytic enzymes and intestinal E. coli population might be involved in mechanism(s) of antibiotic action in stimulating chick growth.

Six lots of one-day-old Cobb's White Rock cockerels were allotted, as previously described, into each of four replicate pens containing ten chicks each.

Two hundred grams per ton of zinc bacitracin was added to the basal rations described below and fed continuously for the 28-day test period. In order to evaluate the enzymatic effect from a barley diet, all rations were made iso-caloric and isoprotein with the basal by adjusting barley, corn, stabilized animal fat, and dehulled soybean meal in the broiler basal diet shown in Table 1a. All other ingredients remained unchanged.

A midwestern type of certified barley seed of the Moore variety was passed through a hammer mill having a 1/8 inch screen and added to the modified basal as described above. In addition, a portion of the barley was coarsely ground, soaked in an equal weight of cold tap water in a stainless steel tank for twelve hours, and then dried in thin layers at room temperature with frequent turning. After drying, the soaked barley was passed through a hammer mill using a 1/8 inch screen and then added to the modified basal ration.

Intestinal E. coli organisms were determined in one representative bird from each lot at the fourth week.

Results and Discussion

The growth pattern observed in this trial indicated that the mechanism of growth stimulation from bacitracin differed from the enzymatic effect due to soaking barley, since a significant growth response occurred from soaking the barley ($P < .05$) and a highly significant ($P < .01$) added effect occurred when bacitracin was added to that diet (table 14).

Further support for a different mechanism of bacitracin action as compared with barley liberated enzymes was the similarity of absolute growth noted (92 and 94 grams) when bacitracin was added to each type of barley diet.

Since both the magnitude of growth response and improvement in feed utilization was greater on the soaked barley basal when compared with the corn-type ration (table 13), bacitracin may have had an indirect enzymatic effect of enhancing the growth of intestinal microorganisms capable of producing enzymes. These enzymes might then aid in the utilization of nutrients which would ordinarily be unavailable in barley.

TABLE 13

THE EFFECT OF WATER-SOAKED BARLEY IN THE PRESENCE OF ZINC BACITRACIN ON FOUR-WEEK-OLD CHICK WEIGHTS AND INTESTINAL POPULATION

Lot	Antibiotic additions to the basal (gms/ton)	Grain portion of basal kind	Grain portion of basal treatment	Average chick wts. at 4 weeks (gms)	Chicks alive of 40 started	Feed/ gain	E. coli X 10^4 population 2 inches above cecum
1	None	corn	None	362	39	1.91	1.
2	200 zinc bacitracin	corn	None	410	39	1.90	1792.
3	None	barley*	None	309	39	1.98	107.
4	200 zinc bacitracin	barley	None	401	38	1.78	309
5	None	barley	Soaked**	340	35	1.90	14.
6	200 zinc bacitracin	barley	Soaked	434	38	1.81	645.

* Midwest variety - certified Moore variety

** Barley was coarsely ground and soaked in equal weight of cold tap water in stainless steel tank for twelve hours. Soaked grain was air-dried in thin layers and passed through 1/8" screen in a hammer-mill.

TABLE 14

ANALYSIS OF VARIANCE OF FOUR-WEEK-OLD CHICK WEIGHTS. Experiment V.

Source of variation	Degrees of freedom	Mean square	F values		
			Calculated	P = 0.05	P = 0.01
Total	227				
Subclass	23	20,722	4.80**		1.88
Lot	5	82,962	19.09**		3.11
Replicate	3	7,245	1.67	2.65	
L X R	15	2,763	0.64	2.10	
Error	204	4,331			

Comparison among lots at 5 percent level of probability

Lot	3	5	1	4	2	6
4 wk. wts.	309	340	362	401	410	434

At 1 percent level of probability

Lot	3	5	1	4	2	6
4 wk. wts.	309	340	362	401	410	434

** Significant ($P < 0.01$)

EXPERIMENT VI

Whereas, the evidence thus far did not strongly support the involvement of coliforms in the mechanism of antibiotic action, increased intestinal E. coli population in the presence of bacitracin supplementation was generally observed in the previous five trials.

The findings of Moore et al. (1946) indicate that sulfasuxadine is not growth depressing at the 0.5 percent level as is sulfaguanadine. It was, therefore, deemed advisable to re-investigate the effect of zinc bacitracin on growth in relation to intestinal E. coli in the presence of certain bacterial-inhibiting compounds.

Eight lots of Cobb's White Rock, one-day-old cockerels were allotted, as previously described, into four replicate pens of ten chicks each and fed for a 27-day test period. To the basal chick starting diet shown in table 1a, 50 grams per ton of polymixin B sulfate and 0.5 percent sulfasuxadine were added and fed continuously. The polymixin compound was employed since it is effective against gram negative intestinal organisms, while sulfasuxadine is extremely effective in inhibiting gram-negative E. coli as well as some gram-positive forms (Moore et al., 1946).

One representative bird from each experimental lot was selected for intestinal E. coli determination at the fourth week.

Results and Discussion

Sulfasuxadine, while not growth inhibitory in itself, did depress intestinal E. coli population when fed in combination with bacitracin (table 15). This phenomenon suggested that reduction of intestinal E. coli is not, in itself, a factor in the mechanism of bacitracin action, but that large increases in the organisms may have been related to optimum

performance. Certainly, the improved growth pattern and increased feed efficiency as well as coliform count were strongly suggestive of a close relationship between this organism and chick performance.

Since polymixin alone did not stimulate growth or affect feed conversion, but appeared to increase intestinal E. coli, the possibility existed that bacitracin may have provided the "trigger mechanism" necessary to obtain a growth response. It can be theorized that the antibiotic causes the release of intestinal enzymes, which act on the nutrients present to improve assimilation and subsequent utilization by the bird.

There was an apparent synergism between polymixin and zinc bacitracin in supporting a large increase in intestinal E. coli organisms. The increased organism population was also associated with the maximum growth observed and was significantly greater, at the five percent level of probability than the highly significant ($P = < 0.01$) response from antibiotic alone (table 16).

TABLE 15

THE EFFECT OF SULFASUXADINE IN THE PRESENCE OF ANTIBIOTICS ON CHICK GROWTH AND INTESTINAL E. COLI POPULATION

Lot	Antibiotic additions to the basal (gms/ton)	Sulfasuxadine addition to the basal (%)	Average chick wts. at 27 days (gms)	Chicks alive of 40 started	Feed/ gain	E. coli X 10^4 population 2 inches above cecum
1	None	None	452	40	1.71	9.0
2	200 zinc bacitracin	None	506	37	1.64	2.0
3	None	0.5	461	39	1.74	53.0
4	50 polymixin*	None	469	40	1.71	37.0
5	50 polymixin	0.5	473	38	1.70	2.0
6	50 polymixin + 200 zinc bacitracin	None	537	39	1.58	1979.0
7	200 zinc bacitracin	0.5	491	40	1.71	1.0
8	50 polymixin + 200 zinc bacitracin	0.5	514	38	1.73	5.0

* Antibiotic effective against gram positive disease producing organisms.
Provided by courtesy of Chas. Pfizer and Co., Inc. - potency 7150 U/mg

TABLE 16

ANALYSIS OF VARIANCE OF CHICK WEIGHTS AT 27 DAYS. Experiment VI

Source of variation	Degrees of freedom	Mean square	F values Calculated	P = 0.05	P = 0.01
Total	310				
Subclass	31	11.301	4.51**		1.79
Lot	7	33,893	13.51**		2.73
Replicate	3	2,613	1.04	8.54	
L X R	21	5,013	2.00**		1.97
Error	248	2,508			

Comparison among lots at 5 percent level of probability

Lot	1	3	4	5	7	2	8	6
27 day wt.	452	461	469	473	491	506	514	537

at 1 percent level of probability

Lot	1	3	4	5	7	2	8	6
27 day wt.	452	461	469	473	491	506	514	537

** Significant ($P < 0.01$)

EXPERIMENT VII

The observation in experiment VI of possible synergistic action between bacitracin and polymixin in increasing intestinal E. coli, with the associated added growth above normal antibiotic response, prompted re-evaluation of this apparent effect.

Oleandomycin, having a narrow spectrum of microbial activity similar to penicillin (Price et al. 1959) was consequently compared with two levels of zinc bacitracin. Low levels (1 or 5 grams per ton) were compared with high levels of antibiotic (200 grams per ton) for an eight and one-half week period to learn if associative effects existed between intestinal E. coli population and performance and, if so, did it persist beyond four weeks. Each growth-promoting antibiotic was fed singly, or in combination with polymixin, at approximately a four to one ratio throughout the experiment. However, due to the pronounced growth-depressing effect of oleandomycin at the higher level, lots 8 and 9 (table 16) were discontinued at the end of the fourth week.

Nine lots of one-day-old Cobb's White Rock cockerels were allotted, as previously described, into four replicate pens of ten chicks each and fed the supplemented broiler-starter ration (table 1a) through the fourth week. At that time, Lots 1 through 7 were transferred to finisher batteries, as previously described, and were fed the supplemented broiler-finisher ration (table 1a) for the remaining four and one-half weeks.

One representative bird from each experimental lot was selected for intestinal E. coli determination at the fourth week and one at the ninth week.

Results and Discussion

Polymixin, in combination with the high level of bacitracin, again promoted a maximum growth response which was significantly greater at the five percent level of probability (table 18) than the highly significant response obtained over the basal with bacitracin alone. This improved performance was not, however, associated with an increase in intestinal E. coli count, based on the single sample cultured. Whereas, increased weight gains occurred when polymixin was added to each growth-stimulating antibiotic, with the exception of the growth-depressing higher level of oleandomycin, an analysis of variance (table 18) revealed that these differences were not significant.

It was noted that except for an absence of organisms in the control lots no marked differences in intestinal E. coli occurred in any of the experimental lots, despite the highly significant early growth responses from the antibiotics. Since the higher level of oleandomycin showed a count of intestinal E. coli similar to the other lots, but a highly significant growth depression, mechanism of action in depressing growth was probably not due to reduction in E. coli alone. This suggests that high levels of this particular antibiotic may inhibit vital metabolic processes by blocking essential enzyme functions, or by increasing intestinal microorganisms which act in a toxic manner toward the host.

At eight and one-half weeks, significant responses to all antibiotic treatments had disappeared (table 20). This was in accordance with the view held by many investigators that antibiotics exert their greatest growth stimulating effect during the early growing period and

tend to lose their effectiveness in late growth. No marked increases occurred in intestinal E. coli due to bacitracin supplementation at the ninth week, except in the presence of the low level (table 19). This microflora difference could not be associated with growth, since lots showing equivalent growth had a lower intestinal E. coli population.

TABLE 17

THE EFFECT OF A LOW AND HIGH LEVEL OF GROWTH STIMULATING ANTIBIOTIC IN THE PRESENCE OF POLYMIXIN ON CHICK GROWTH AND INTESTINAL E. COLI POPULATION AT 4 WEEKS.

Lot	Antibiotic additions to the basal (gms)	Polymixin addition to the basal (gms/ton)	Average chick wts. at 4 wks. (gms)	Chicks alive of 36 started	Feed/ gain	E. coli X 10^4 population 2 inches above cecum
1	None	None	469	35	1.99	No growth
2	5 zinc bacitracin	None	512	35	1.76	No growth
3	5 zinc bacitracin	1.0	530	36	1.79	10.0
4	200 zinc bacitracin	None	531	34	1.76	41.0
5	200 zinc bacitracin	50.0	552	35	1.88	0.1
6	1 oleandomycin*	None	521	31	1.69	7.0
7	1 oleandomycin	1.0	538	36	2.39	1.0
8	100 oleandomycin	None	372	33	2.04	3.0
9	100 oleandomycin	25.0	375	36	2.01	No growth

* Growth stimulating antibiotic provided by courtesy of Chas. Pfizer and Co., Inc. potency 1.0 gm/lb

TABLE 18

ANALYSIS OF VARIANCE OF FOUR-WEEK-OLD CHICK WEIGHTS. Experiment VII

Source of variation	Degrees of freedom	Mean square	F values Calculated	F values P = 0.05	F values P = 0.01
Total	310				
Subclass	35	43,176	7.54**		1.74
Lot	8	164,979	28.83**		2.60
Replicate	3	8,820	1.54	2.65	
L X R	24	6,870	1.20	1.57	
Error	240	5,722			

Comparison among lots at 5 percent level of probability

Lot	8	9	1	2	6	3	4	7	5
4 wk. wt.	371	375	469	512	521	530	531	538	552

at 1 percent level of probability

Lot	8	9	1	2	6	3	4	7	5
4 wk. wt.	371	375	469	512	521	530	531	538	552

** Significant ($P < 0.01$)

TABLE 19

THE EFFECT OF A LOW AND HIGH LEVEL OF GROWTH STIMULATING ANTIBIOTIC IN THE PRESENCE OF POLYMIXIN ON CHICK GROWTH AND INTESTINAL E. COLI POPULATION AT 60 DAYS

Lot	Antibiotic additions to the basal (gms/ton)	Polymixin addition to the basal (gms/ton)	Average bird wts. at 60 days (gms)	Chicks alive of 36 started	Feed/ gain	E. coli X 10^4 population 2" above above cecum
1	None	None	1547	31	2.45	No growth
2	5 zinc bacitracin	None	1589	33	2.37	124.0
3	5 zinc bacitracin	1.0	1613	35	2.38	1.5
4	200 zinc bacitracin	None	1551	31	2.45	3.7
5	200 zinc bacitracin	50.0	1522	30	2.57	1.5
6	1 oleandomycin	None	1558	28	2.34	41.0
7	1 oleandomycin	1.0	1581	33	2.39	9.7

TABLE 20

ANALYSIS OF VARIANCE OF EIGHT WEEK BROILER WEIGHTS. Experiment VII

Source of variation	Degrees of freedom	Mean square	F values	
			Calculated	P = 0.05
Total	220			
Subclass	27	25,317		
Lot	6	27,990	1.03	1.54
Replicate	3	18,834	1.14	3.70
L X R	18	25,506	0.77	8.55
Error	166	24,513	1.04	2.06

No significant differences among lots at the 5 percent level of probability.

EXPERIMENT VIII

The possibility of synergistic action between bacitracin and polymixin (Experiments VI and VII) in affecting intestinal E. coli population and growth, suggested that other microorganisms, their enzymes, or their metabolic products which might be found in the intestine, could combine favorably to enhance antibiotic growth stimulation and shed light on possible mechanism(s) involved.

In the following experiment, one percent of live yeast cells of Saccaromyces cerevisiae or 0.3 percent of an acid hydrolysate of killed cells of S. cerevisiae were fed singly and in combination with bacitracin or bacitracin plus polymixin.

One-day-old Cobb's White Rock cockerels were allotted, as previously described, into eight lots, each containing four replicate pens of ten chicks each. The basal broiler-starter (table 1a) was supplemented with the levels of bacitracin, polymixin, yeast, or yeast extract as shown in the experimental design (table 21) and fed continuously for the 26-day test period.

Since intestinal E. coli involvement as a basis for explaining the growth-stimulating mechanism of bacitracin appeared uncertain, relative populations of other intestinal microorganisms were cultured aseptically, as described previously, from the area approximately two inches above the cecum. A representative bird was selected from only the control and bacitracin-fed groups for intestinal microorganism count, since yeast treatments were ineffective in stimulating growth.

Results and Discussion

Neither live cells of S. cerevisiae nor an acid hydrolysate of killed cells were effective in altering the growth rate of the chicks, which indicated that yeast cells or their metabolic products are not directly involved in the mechanism by which bacitracin stimulates growth. Bacitracin alone produced highly significant increases in 26-day weights (table 22), but further increases were not obtained in this trial by addition of polymixin.

No noticeable differences were observed in intestinal E. coli count from bacitracin supplementation in this test. However, based on the sample cultured, there were increased numbers of total gram-positive organisms in the antibiotic-fed birds. Total numbers of micrococci, enterococci-type organisms, lactobacilli and the total gram-negative population were decreased in the presence of bacitracin (table 23). These findings are in agreement with Anderson et al. (1956) who showed a decrease in enterococci and lactobacilli-type organisms from feeding chlortetracycline.

TABLE 21

THE EFFECT OF ANTIBIOTIC IN THE PRESENCE OF LIVE AND KILLED YEAST ON CHICK GROWTH AT TWENTY-SIX DAYS

Lot	Antibiotic addition to the basal (gms/ton)	% Yeast addition to the basal		Average chick wts. at 26 days (gms)	Chicks alive of 40 started	Feed/ gain
		live*	Killed**			
1	None	None	None	449	39	1.73
2	200 zinc bacitracin	None	None	506	39	1.66
3	None	1.0	None	455	35	1.70
4	200 zinc bacitracin	1.0	None	502	33	1.67
5	200 zinc bacitracin + 50 polymixin	1.0	None	512	36	1.66
6	None	None	0.3	443	36	1.71
7	200 zinc bacitracin	None	0.3	516	35	1.65
8	200 zinc bacitracin + 50 polymixin	None	0.3	505	38	1.73

* Saccaromyces cerevisiae
** Acid hydrolysate of Saccaromyces cerevisiae

TABLE 22

ANALYSIS OF VARIANCE OF 26-DAY CHICK WEIGHTS. Experiment VIII

Source of variation	Source of freedom	Mean square	F values		
			Calculated	P = 0.05	P = 0.01
Total	290				
Subclass	31	11,812	2.88**		1.79
Lot	7	35,777	8.73**		2.70
Replicate	3	15,958	3.89*		2.65
L X R	21	3,232	0.79		1.62
Error	228	4,096			

Comparison among lots at 1 percent level of probability

Lot	6	1	3	4	8	2	5	7
26-day wt.	443	449	455	502	505	506	512	516

Same comparison at 5 percent level of probability

* Significant ($P < 0.01$)

** Significant ($P < 0.05$)

TABLE 23

THE EFFECT OF ANTIBIOTIC TREATMENT ON RELATIVE POPULATION ON INTESTINAL BACTERIA IN FOUR-WEEK CHICKS

Lot	Antibiotic addition to the basal (gms/ton)	Organisms in intestinal contents 2 inches above cecum*					
		Total gram positive[1] (10^4/gm)	Total cocci[2] (per gm)	Entero-cocci[3] (per gm)	Lacto-bacilli[4] (10^4/gm)	Total gram negative Population[5] (10^4/gm)	E. coli[6] (10^4/gm)
1	None	2934	790	790	55.	75.	21
2	200 zinc bacitracin	TNC**	None	None	4.	24.	2

Culturing media:
1 Dextrose azide agar -- Total gram-positive population
2 Dextrose azide broth -- Gram-positive cocci population
3 E.V.A. broth -- Gram-positive enterococci (confirming test)
4 Trypsin digest agar -- Gram-positive lactobacilli population
5 Laurel sulfate agar -- Total gram-negative population
6 Mallmann-Peabody agar - Gram-negative E. coli

* Average of two replicate birds, average counts calculated from best dilutions
** TNC -- Too numerous to count at any dilution

EXPERIMENT IX

The likelihood that other intestinal microorganisms, in addition to E. coli, might be involved in the mechanism by which antibiotics stimulate growth prompted this experiment. In addition to evaluating certain intestinal gram-positive and gram-negative bacteria, a series of several confirming tests for E. coli was also conducted.

Accordingly, five lots of one-day-old Cobb's White Rock cockerels were allotted, as previously described, into four replicate pens of ten chicks each. The starter basal (table 1a) was supplemented with antibiotic as shown in the experimental design (table 24) and fed continuously for a 28-day period.

A representative bird was selected from each experimental lot from which to culture intestinal microorganisms from an area approximately two inches above the cecum. These included total gram-positive bacteria, total micrococci, enterococci and lactobacilli, as well as total gram-negative population and the E. coli fraction of this group. In addition, five confirming tests were made on several representative E. coli colonies from each of the experimental lots which had been incubated by the Mallmann-Peabody (1957) drop plate method.

Results and Discussion

Significant growth response occurred from bacitracin supplementation alone or in combination with polymixin, but not from terramycin (table 25). No marked change in intestinal E. coli was observed from the feeding of either type of growth-stimulating antibiotic alone, based on the sample cultured. However, addition of polymixin in combination

with both broad and narrow spectrum compounds was associated with a marked increase in E. coli, but with no apparent effect on the growth pattern. While some of the previous experiments indicated that an optimum E. coli population may be associated with growth responses, this particular test (table 26) did not support their involvement in explaining the mechanism of antibiotic action. On the other hand, antibiotic supplementation resulted generally in a depression of the total gram-positive forms, as well as enterococci and lactobacillus groups, though reduction in numbers was not associated with increased growth in the presence of terramycin.

Results of the five confirming tests for the cultured intestinal E. coli (table 27) strongly indicated the presence of pure strains of coliforms and added support to the validity of this method of culturing.

TABLE 24

THE EFFECT OF POLYMIXIN IN THE PRESENCE OF NARROW AND BROAD SPECTRUM ANTIBIOTICS ON CHICK GROWTH AT FOUR WEEKS

Lot	Antibiotic addition to the basal (gms/ton)	Polymixin addition to the basal (gms/ton)	Average chick wts. at 4 weeks (gms)	Chicks alive of 40 started	Feed/ gain
1	None	None	445	33	1.79
2	200 zinc bacitracin	None	507	39	1.66
3	200 zinc bacitracin	50	524	40	1.64
4	200 terramycin	None	463	36	1.78
5	200 terramycin	50	470	35	1.70

TABLE 25

ANALYSIS OF VARIANCE OF FOUR-WEEK CHICK WEIGHTS. Experiment IX

Source of variation	Degrees of freedom	Mean square	F values Calculated	P = 0.05	P = 0.01
Total	181				
Subclass	19	96,889	34.30**		2.00
Lot	4	37,993	13.40**		3.45
Replicate	3	4,704	1.66	2.67	
L X R	12	1,501	.53	2.34	
Error	143	2,825			

Comparison among lots at 1 percent level of probability

Lot	1	4	5	2	3
4 wk. wt.	445	463	470	507	524

Significance between lots at 5 percent level of probability did not differ from significance at 1 percent level of probability

** Significant ($P < 0.01$)

TABLE 26

THE EFFECT OF POLYMIXIN IN THE PRESENCE OF GROWTH STIMULATING ANTIBIOTICS ON RELATIVE POPULATION OF INTESTINAL BACTERIA IN FOUR-WEEK CHICKS

Lot	Growth stimulating antibiotic addition to the basal (gms/ton)	Polymixin addition to the basal (gms/ton)	Organisms X 10^4 in intestinal contents 2" above cecum*					
			Total gram positive (10^4/gm)	Total cocci (per gm)	Entero-cocci (per gm)	Lacto-bacilli (10^4/gm)	Total gram negative population (10^4/gm)	E. coli (10^4/gm)
1	None	None	6246	2400	270	4201	161	23
2	200 zinc bacitracin	None	None	1300	None	None	49	14
3	200 zinc bacitracin	50	1.8	2400	None	None	1045	823
4	200 terramycin	None	373	None	None	None	169	1.9
5	200 terramycin	50	2154	2200	20	None	2836	2836

* One representative bird per lot, average counts calculated from best dilution 1:100, 1:1,000, 1:10,000, 1:100,000

TABLE 27

RESULTS OF CONFIRMING TESTS TO DETERMINE PURITY OF INTESTINAL E. COLI POPULATION CULTURED BY THE MALLMANN-PEABODY DROP PLATE METHOD

Test no.	Bacteriological tests*	Replicates of organisms cultured from each lot of Experiment VI Substrate	Reactions	Results
1	Voges-Proskauer Differential	Incubated 24 hour colonies transferred from Mallmann-Peabody agar	Positive test (ruby red color) indicates presence of organisms other than E. coli. Negative test (no color change indicates presence of E. coli organisms	-
2	Sodium citrate	Same	Positive test (growth) indicates presence of aerobacter organism. Negative test (no growth) indicates presence of E. coli organisms	-
3	Indole differential	Same	Positive test (dark red color) indicates presence of E. coli. Negative test (no color change) indicates organisms other than E. coli.	+
4	Methyl Red	Same	Positive test (distinct red) indicates presence of E. coli. Negative test (no color change) indicates organism other than E. coli	+
5	Lactose broth fermenting	Same	Gas formation indicates presence of coliform organisms	+

* Standard Methods for the Examination of Water, Sewage, and Industrial Wastes, Tenth Edition, 1955

EXPERIMENT X

Despite the apparent variability in intestinal E. coli population, it was felt that additional work should be done to accumulate more information on the probability of their involvement in antibiotic action. Since interfering debris made counting of intestinal population of total gram-positive and total gram-negative forms difficult to interpret these bacteriological tests were not included.

The work of Perdue et al. (1958) suggested possible differences in growth effect and tolerance to erythromycin compared with penicillin which could be reflected in differences in intestinal microflora population of animals fed different narrow spectrum antibiotics. This prompted the following experiment in which performance and certain intestinal microorganism population were determined.

Six lots of one-day-old Cobb's White Rock cockerels were allotted, as previously described, into four replicate pens of ten birds each. The basal diet (table 1a) was supplemented with antibiotics as shown in the experimental design (table 28) and fed continuously for a 28-day period.

One representative bird was selected from each experimental lot for intestinal bacteria determination at the fourth week. Among the gram-positive forms, total micrococci and enterococci were determined, while the E. coli population among the gram-negative species was counted.

Results and Discussion

Highly significant growth responses were obtained from the addition of bacitracin or erythromycin to the ration (table 29). The further addition of polymixin to the bacitracin diet results in a large increase

in intestinal E. coli, which was also associated with a slight non-significant increase in growth.

Whereas, erythromycin alone promoted a significant growth increase, equivalent to that from bacitracin, further supplementation with polymixin resulted in a suppression of growth and a pattern of intestinal microorganism population resembling that from the bird fed the basal ration. This may have indicated a suppressing effect from the combination of these two compounds on certain gut microorganisms that were a prerequisite for optimum antibiotic response in the chick.

Total intestinal micrococci and enterococci populations were depressed below the basal lot by feeding polymixin or zinc bacitracin (table 30), though it was noted that depression of these organisms which occurred when polymixin was fed singly was not associated with a growth increase. Erythromycin, on the other hand, appeared ineffective in altering these gram positive organisms, yet promoted a significant growth response in the chicks.

TABLE 28

THE EFFECT OF POLYMIXIN IN THE PRESENCE OF GROWTH STIMULATING ANTIBIOTICS ON CHICKS AT FOUR WEEKS

Lot	Antibiotic addition to the basal (gms/ton)	Polymixin addition to the basal (gms/ton)	Average chick wts. at 4 weeks (gms)	Chicks alive of 40 started	Feed/ gain
1	None	None	554	37	1.70
2	None	50	562	38	1.68
3	200 zinc bacitracin	None	606	37	1.70
4	200 zinc bacitracin	50	625	39	1.65
5	100 erythromycin*	None	604	37	1.72
6	100 erythromycin	25	569	35	1.66

* Erythromycin thiocyanate, a growth stimulating antibiotic provided through courtesy of Abbott Laboratories, North Chicago, Illinois, potency 10 gms. per pound

TABLE 29

ANALYSIS OF VARIANCE OF FOUR-WEEK-OLD CHICK WEIGHTS. Experiment X

Source of variation	Degrees of freedom	Mean square	F values Calculated	P = 0.05	P = 0.01
Total	222				
Subclass	23	10,985	2.75**		1.91
Lot	5	31,136	7.79**		3.14
Replicate	3	12,114	3.03*	2.67	
L X R	15	4,042	1.01	1.76	
Error	176	3,996			

Comparisons among lots at 5 percent level of probability

Lot	1	2	6	5	3	4
4 wk. wt.	554	562	569	604	606	625

at 1 percent level of probability

Lot	1	2	6	5	3	4
4 wk. wt.	554	562	569	604	606	625

* Significant ($P < 0.05$)

** Significant ($P < 0.01$)

TABLE 30

THE EFFECT OF POLYMIXIN IN THE PRESENCE OF GROWTH STIMULATING ANTIBIOTICS ON RELATIVE POPULATION OF SELECTED INTESTINAL BACTERIA IN FOUR-WEEK-OLD CHICKS

Lot	Growth stimulating antibiotic addition to the basal (gms/ton)	Polymixin addition to the basal (gms/ton)	Organisms in intestinal contents 2" above cecum* (Gram positive) Total cocci (per gm)	(Gram positive) Entero-cocci (per gm)	(Gram negative) E. coli (10^4/gm)
1	None	None	24,000 +	24,000 +	9
2	None	50	2400	790	6
3	200 zinc bacitracin	None	24,000 +	24,000 +	14,571
4	200 zinc bacitracin	50	3500	2800	53
5	100 erythromycin	None	24,000 +	24,000 +	49
6	100 erythromycin	25	24,000 +	24,000 +	7

* One representative bird per lot, average counts calculated from best dilution 1:100, 1:1,000, 1;10,000.

EXPERIMENT XI

The findings of Barnes et al. (1959) that rats fed penicillin increase their growth if they have access to feces but not in the absence of coprophagy suggested possible relationships between the ingesting of fresh droppings and the mechanism of response to broad and narrow spectrum antibiotics.

In order to find what associative effects might occur, an experiment was conducted in which fresh feces were fed to chicks daily in the presence and absence of antibiotics.

Ten lots of Cobb's White Rock one-day-old cockerels were allotted, as previously described, into four replicate pens of nine chicks each and fed for a 27-day test period. To the basal chick starting diet (table 1a) antibiotics were added as shown in table 31 and the chicks were given access to fresh fecal material in the following manner. Fresh feces were collected daily from caged-layer hens receiving an antibiotic-free diet. The fecal material was diluted in an equal volume of physiological saline solution and poured in a strip on top of the mash each morning. The moisture content of the feces was 82 percent, based on the 72-hour 90° C. oven-dried sample tested which agrees with the work of Rosenberg and Palafox (1956). On this basis of feeding each chick had the opportunity to receive a daily intake of approximately 0.5 to 0.75 grams of fecal material on a dry basis during the test period.

One representative bird from each experimental lot was selected for intestinal bacteria determination at the fourth week. Total micrococci, enterococci and E. coli were cultured, as previously described.

Results and Discussion

In each instance, presence of fresh fecal material in the diet caused a highly significant growth depression (table 31). This was not completely compensated for by the addition of antibiotics. Aureomycin restored growth more nearly equivalent to the basal than did zinc bacitracin (table 31) and this improvement in growth was significant at the five percent level of probability (table 32). This suggested that the fecal material may have contained growth-depressing organisms, which are more effectively inhibited by wide spectrum antibiotics, or combinations of compounds having wide spectrums of activity rather than by those having narrow spectrums of activity.

The presence of fresh fecal material also caused a depression in feed utilization in each instance (table 33) despite the presence of antibiotic treatment. It should be pointed out that antibiotic supplementation resulted in an improvement in feed efficiency over non-antibiotic-fed birds. Apparently, the antibiotics exerted part of their action by inhibiting these depressing microorganisms, or substances produced by certain microbes that interfere with nutrient assimilation.

Bacterial populations of micrococci, enterococci and E. coli were changed, some forms markedly so, in each experimental lot which had received fecal material. A depression in the gram-positive population was particularly noted among the micrococci organisms (table 34). A significant ($P < .01$) depression in growth occurred from feeding bacitracin in combination with fresh fecal material, compared to the bacitracin-fed lot receiving no feces. Since intestinal E. coli population was not depressed from feeding feces, intestinal E. coli per se are probably not

an important consideration in the mechanism by which antibiotics stimulate growth. Rather it appeared that a balance between populations of beneficial intestinal microorganisms, including *E. coli* and an inhibition of certain detrimental forms, were an important mechanism that permitted increased growth and improved feed conversion.

TABLE 31

THE EFFECT OF FRESH HEN FECES AND POLYMIXIN ON ANTIBIOTIC RESPONSE IN CHICKS AT TWENTY-SEVEN DAYS

Lot	Antibiotic addition to the basal (gms/ton)	Polymixin addition to the basal (gms/ton)	Feces daily*	Average chick wts. at 27 days (gms)	Chicks alive of 36 started	Feed/ gain
1	None	None	-	536	35	1.80
2	None	None	+	458	34	2.31
3	200 zinc bacitracin	None	-	632	35	1.65
4	200 zinc bacitracin	None	+	495	35	2.04
5	200 zinc bacitracin	50	-	615	35	1.64
6	200 zinc bacitracin	50	+	499	36	1.89
7	200 aureomycin**	None	-	595	36	1.72
8	200 aureomycin	None	+	531	36	1.92
9	200 aureomycin	50	-	602	35	1.74
10	200 aureomycin	50	+	539	36	1.88

* Daily feeding of fresh feces from caged layers receiving an antibiotic-free ration. One-half feces and one-half physiological saline solution added on top of feed each morning. Based on 20 percent dry matter content of feces, birds had access to approximately one-half to three-quarters of a gram of dry fecal material daily during the test period.

** Aurofac-10 a growth stimulating antibiotic. Potency 10 grams per pound.

TABLE 32

ANALYSIS OF VARIANCE OF 27-DAY CHICK WEIGHTS. Experiment XI

Source of variation	Degrees of freedom	Mean square	F values Calculated	P = 0.05	P = 0.01
Total	352				
Subclass	39	29,727	6.59**		1.68
Lot	9	117,004	25.93**		2.49
Replicate	3	2,655	0.59	8.54	
L X R	27	3,643	0.81	1.70	
Error	274	4,512			

Comparison among lots at 5 percent level of probability

Lot	2	4	6	8	1	10	7	9	5	3
27 day wt.	458	495	499	531	536	539	595	602	615	632

at 1 percent level of probability

27 day wt.	458	495	499	531	536	539	595	602	615	632

** Signifncant ($P < 0.01$)

TABLE 33

ANALYSIS OF VARIANCE OF 27-DAY CHICK FEED EFFICIENCIES.
Experiment XI

Source of variation	Degrees of freedom	Mean square	F values		
			Calculated	P = 0.05	P = 0.01
Total	39	0.0449			
Lot	9	0.1656	19.03**		3.06
Error	30	0.0087			

Comparison among lots at 5 percent level of probability

Lot	5	3	7	9	1	10	6	8	4	2
27-day feed eff.	1.64	1.65	1.72	1.74	1.80	1.88	1.89	1.92	2.04	2.31

at 1 percent level of probability

Lot	5	3	7	9	1	10	6	8	4	2
25-day feed eff.	1.64	1.65	1.72	1.74	1.80	1.88	1.89	1.92	2.04	2.31

** Significant ($P < 0.01$)

TABLE 34

THE EFFECT OF DIETARY FECES IN THE PRESENCE OF GROWTH STIMULATING ANTIBIOTICS ON RELATIVE POPULATION OF SELECTED INTESTINAL BACTERIA IN FOUR WEEK CHICKS

Lot	Growth stimulating antibiotic addition to the basal (gms/ton)	Polymixin addition to the basal (gms/ton)	Feces*	Organisms in intestinal contents 2" above cecum** Gram positive Total cocci (per gm)	Gram positive Entero-cocci (per gm)	Gram negative $E_{.}$ coli (10^4 per gm)
1	None	None	-	16000	9200	None
2	None	None	+	24000+	9200	143.0
3	200 zinc bacitracin	None	-	1300	790	3
4	200 zinc bacitracin	None	+	16000	2800	1857.0
5	200 zinc bacitracin	50	-	1400	1400	2
6	200 zinc bacitracin	50	+	24000+	16000	13
7	200 aureomycin	None	-	2400	790	2
8	200 aureomycin	None	+	24000+	24000+	101.0
9	200 aureomycin	50	-	16000	16000	6
10	200 aureomycin	50	+	24000+	24000+	22

* Daily feeding of fresh feces from hens receiving antibiotic-free ration. One part feces, one part physiological saline solution by volume added on top of mash each morning

** One representative bird per lot, average counts calculated from best dilution 1:100, 1:1,000, 1:10,000

EXPERIMENT XII

A logical next step in the work was to attempt to learn if the growth depression in chicks caused by fresh fecal material was due to organisms contained only in fresh feces, those sensitive to drying, or a substance produced by the organisms themselves.

An experiment was conducted in which fresh fecal material from caged layers on an antibiotic-free diet was fed as described in the previous experiment. In addition, fecal material from the same hens was dried in thin layers at 100° F. for 72 hours, passed through a hammer mill without a screen and added to the broiler-starter ration (table 1a) at the level of one percent. Finally, a portion of the dried, ground fecal material was autoclaved for thirty minutes under fifteen pounds of pressure and also added to the ration at one percent. This provided the birds with an amount of dried fecal material approximately the same as those receiving the fresh fecal material on a dry matter basis.

Ten lots of Cobb's White Rock one-day-old cockerels were allotted, as previously described, into four replicates of ten chicks each. Zinc bacitracin and terramycin were added singly and in combination with the various fecal material treatments (table 35) and fed continuously for a 28-day test period.

One representative bird was selected from each lot for certain intestinal microorganism determinations at the fourth week (table 37).

Result and Discussion

There was a highly significant ($P < .01$) depression in chick growth from feeding fresh fecal material to the birds receiving the basal diet, or the diet containing bacitracin (table 36). On the other hand, feeding

of either dry or autoclaved fecal material had no effect on the growth pattern of control or antibiotic-fed chicks. This indicates that the agent involved may be living organisms, rather than a toxic substance liberated by organisms.

The chicks which received terramycin in addition to fresh fecal material grew at the same rate as the control birds and were significantly heavier than the bacitracin-fresh feces lot. This phenomenon, which was observed in the previous test, added support to the concept that a wide spectrum of antimicrobial activity may be necessary to offset the depressing effect of certain substances contained in fresh feces.

Feed efficiency of birds fed fresh feces was depressed to approximately the same extent as in birds receiving the fresh fecal material in the preceding test. This may indicate the presence of toxins which block vital enzymes concerned with nutrient assimilation.

Feeding fresh fecal material also resulted in marked increases in intestinal micrococci, enterococci and E. coli, but when antibiotics were incorporated into the fecal-containing ration, these microorganisms were depressed in numbers similar to the basal group in most instances (table 37). It was uncertain whether the greater microorganism inhibiting effect noted from dry as compared with autoclaved fecal material in each instance was real or coincidental for it did not effect the growth of the birds in any of the lots.

The presence of sharply increased numbers of both intestinal E. coli and total micrococci in lot 5, where growth was depressed, supported the concept that the balance of intestinal microorganisms, rather than absolute numbers, is a controlling factor in governing chick performance.

TABLE 35

THE EFFECT OF FEEDING FECES IN THE PRESENCE OF BROAD AND NARROW SPECTRUM ANTIBIOTICS ON FOUR-WEEK CHICK GROWTH

Lot	Antibiotic addition to the basal (gms/ton)	Fecal treatment				Average chick wts. at 4 wks.	Chicks alive of 40 started	Feed/ gain
		None	Wet*	Dry**	Autoclaved***			
1	None	+				564	39	1.77
2	None		+			497	39	1.92
3	None			+		561	40	1.79
4	None				+	534	38	1.76
5	200 zinc bacitracin		+			529	37	1.88
6	200 zinc bacitracin			+		615	36	1.68
7	200 zinc bacitracin				+	605	39	1.70
8	200 terramycin		+			564	37	1.82
9	200 terramycin			+		606	37	1.75
10	200 terramycin				+	593	40	1.67

* Daily feeding of fresh feces from caged layers receiving an antibiotic-free ration. One-half feces and one-half physiological saline solution added on top of feed each morning.

** Feces from above source dried at 100° F. passed through hammer mill and added to ration at the 1 percent level.

*** Feces from above source dried at 100° F. passed through hammer mill, autoclaved for thirty minutes at 15 pounds pressure, and added to ration at the 1 percent level.

TABLE 36

ANALYSIS OF VARIANCE OF FOUR-WEEK CHICK WEIGHTS. Experiment XII

Source of variation	Degrees of freedom	Mean square	F values		
			Calculated	P = 0.05	P = 0.01
Total	380				
Subclass	39	17,965	4.17**		1.66
Lot	9	56,209	13.03**		2.48
Replicate	3	5,680	1.32	2.64	
L X R	27	6,582	1.53*	1.50	1.80
Error	302	4,313			

Comparison among lots at 5 percent level of probability

Lot	2	5	4	3	8	1	10	7	9	6
4 wk.	497	529	534	561	564	564	593	605	606	615

at 1 percent level of probability

Lot	2	5	4	3	8	1	10	7	9	6
4 wk.	497	529	534	561	564	564	593	605	606	615

* Significant ($P < 0.05$)

** Significant ($P < 0.01$)

TABLE 37

THE EFFECT OF FRESH, DRIED, OR AUTOCLAVED FECES ON RELATIVE POPULATION OF CERTAIN INTESTINAL BACTERIA IN FOUR-WEEK CHICKS

Lot	Antibiotic addition to the basal	Fecal treatment*				Organisms in intestinal contents 2" above cecum**			
						Gram positive		Gm.negative	Av. 4 wk.
		None	Wet$_1$	Dry$_2$	Autoclaved$_3$	Total cocci (per gram)	Enterococci (per gram)	E.coli (10^4/gm)	weights (gms.)
1	None	+				490	210	5	564
2	None		+			24,000 +	3,500	478	497
3	None			+		1,800	1,800	12	561
4	None				+	9,200	5,400	162	534
5	200 zinc bacitracin		+			24,000 +	450	9,969	529
6	200 zinc bacitracin			+		390	None	88	615
7	200 zinc bacitracin				+	1,800	140	190	605
8	200 terramycin		+			24,000 +	24,000 +	373	564
9	200 terramycin			+		330	45	111	606
10	200 terramycin				+	24,000 +	24,000 +	58	593

* (1) Daily feeding of fresh feces from caged layers receiving an antibiotic-free ration. One part feces, one part physiological saline solution by volume added on top of mash each morning.
(2) Feces from above hens dried at 100° F., ground through hammer mill and added to ration at one percent level.
(3) Dried, ground feces from above hens autoclaved for 30 minutes at 15 pounds of pressure and added to ration at 1 percent level.

** One representative bird per lot, average counts calculated from best dilution 1:100, 1:1,000, 1:10,000.

EXPERIMENT XIII

Since Goebel and Barry (1957) reported that sialic acid, effective against certain disease-producing viruses, closely resembled colominic acid, which is produced by E. coli, an experiment was conducted to evaluate possible antibiotic-related effects this compound might have on chick performance. In order to test for possible effectiveness against the growth-depressing entity in fresh chicken fecal material, sialic acid was fed singly, or with zinc bacitracin, in the presence or absence of fresh feces.

Eight lots of Cobb's White Rock one-day-old cockerels were allotted, as previously described into four replicates of ten chicks each and fed the supplemented basal chick starter ration (table 1a) continuously for a 28-day period (table 38).

Two representative birds each were selected at the fourth week from lots 1, 2, 5 and 6 for certain intestinal bacteria determinations, as previously described.

The possibility that the mechanism of antibiotic action might be closely related to the amount of feed consumed, as well as feed transit time in the digestive tract, prompted a comparison of certain gut effects between control and antibiotic-fed birds.

Thirty randomly selected chicks from each of Lots 1 and 5, which were normally full-fed beforehand, were fed mash containing a ferric oxide tracer from 8:00 a.m. until 11:00 a.m. and then fed the normal-colored mash for the following three-hour period. Each bird received approximately 0.1 gram of FeO in the mash. This proved adequate as a

marker and did not affect the consistency of the droppings. Measurements were made of the rate of movement of normal colored feed through the tract in three hours following tracer feed. Additional measurements included live weight, length of tract from gizzard to cloaca, full and empty weights of the intestine and weight of intestinal contents on each treatment (table 41).

Results and Discussion

Fresh fecal material significantly depressed chick weights below the basal at four weeks when fed alone or in combination with zinc bacitracin, and/or sialic acid (table 39). The addition of either the acid or the antibiotic in the presence of fecal material significantly improved chick growth at the five percent level of probability, but it required the addition of both compounds to produce growth equivalent to birds receiving the basal diet. Apparently, the sialic acid was partially effective in counteracting the deleterious effects of fresh fecal material, though the optimum level may have been higher than that employed in this study. The acid was ineffective in further enhancing the highly significant growth effect from bacitracin in the absence of fresh fecal material.

Counts of intestinal populations of two birds per lot showed relatively good agreement between replicates for total micrococci, enterococci, and lactobacillus organisms, with an indication that the numbers increased in the presence of fecal material and were decreased when bacitracin was added to the diet (table 40). On the other hand, although E. coli increased when fecal material was fed, wide variations between replicates made the counts of the bacitracin-fed lots difficult to interpret.

Since the antibiotic-fed lot gained 63 grams more, on the average, and consumed only 76 grams more feed on the average than the controls (table 41), all the increased gain from bacitracin cannot be accounted for on the basis of increased feed consumption alone. This appeared true since the antibiotic prompted an eleven percent added gain from only eight percent additional feed intake.

In this experiment, average weight of intestine, as well as feed transit time, was less for control birds than for antibiotic-fed chicks, though the individual variations among the limited number of samples determined revealed that these differences were not significant. Large numbers of birds would have been required to measure whether differences between gut capacity and feed transit rate would be meaningful in explaining mechanism of antibiotic growth stimulating action.

TABLE 38

THE EFFECT OF DIETARY FECAL CONTAMINATION ON ANTIBIOTIC RESPONSE IN THE PRESENCE OF SIALIC ACID IN FOUR-WEEK-OLD CHICKS

Lot	Zinc bacitracin addition to the basal (gms/ton)	Sialic acid* addition to to the basal (mgms/ton)	Feces** daily	Average chick wts. at 4 wks. (gms)	Chicks alive of 40 started	Feed/ gain
1	None	None	-	509	40	1.76
2	None	None	+	434	40	1.91
3	None	20	-	523	40	1.72
4	None	20	+	469	38	1.83
5	200	None	-	566	39	1.70
6	200	None	+	472	39	1.92
7	200	20	-	560	40	1.66
8	200	20	+	489	40	1.87

* Sialic acid provided through courtesy of Dr. Saul Roseman, University Hospital, Ann Arbor, Mich.

** Daily feeding of fresh feces from caged layers receiving an antibiotic-free ration. One-half feces and one-half physiological saline solution added on top of feed each morning.

TABLE 39

ANALYSIS OF VARIANCE OF FOUR-WEEK-OLD CHICK WEIGHTS. Experiment XIII

Source of variation	Degrees of freedom	Mean square	F values		
			Calculated	P = 0.05	P = 0.01
Total	315				
Subclass	31	22,465	4.39**		1.76
Lot	7	82,398	16.10**		2.72
Replicate	3	4,555	0.89	8.54	
L X R	21	5,046	0.99	1.84	
Error	262	5,118			

Comparison among lots at 5 percent level of probability

Lot	2	4	6	8	1	3	7	5
4 wk. wt.	434	469	472	489	509	523	560	566

at 1 percent level of probability

Lot	2	4	6	8	1	3	7	5
4 wk. wt.	434	469	472	489	509	523	560	566

** Significant ($P < .01$)

TABLE 40

THE EFFECT OF ZINC BACITRACIN ON RELATIVE POPULATION OF CERTAIN INTESTINAL BACTERIA IN FOUR-WEEK-OLD CHICKS

Lot	Zinc bacitracin addition to the basal (gms/ton)	Feces*	Replicate	Organisms in intestinal contents 2" above cecum**				Av. 4 wk. chick weight
				Gram positive			Gram negative	
				Total cocci[1] (per gm)	Entero-cocci[2] (per gm)	Lacto-bacillus[3] (10^4/gm)	E. coli[4] (10^4/gm)	
1	None	-	1	5,400	700	3,632	0.3	509
			2	2,200	1,400	1,939	0.9	
2	None	+	1	24,000 +	24,000 +	54,873	86.	434
			2	24,000 +	24,000 +	76,716	382.	
5	200	-	1	1,700	1,300	52	1.6	566
			2	5,400	1,700	874	1748.	
6	200	+	1	790	490	3,176	27.	472
			2	2,400	2,400	1,317	5882.	

* Daily feeding of fresh feces from hens receiving an antibiotic-free ration. One-part feces, one-part physiological saline solution by volume added on top of mash each morning.

** Two representative birds per lot, average counts calculated from best dilution 1:100, 1:1,000, 1:10,000.

1,2 Counts based on table of most probable numbers using 5 tubes, Standard Methods for the Examination of water, sewage, and industrial wastes. Tenth edition, 1955.

3,4 Mallmann-Peabody (1957) Drop Plate method.

TABLE 41

MEASUREMENT OF FEED TRANSIT TIME AND GUT CAPACITY OF ANTIBIOTIC-FED CHICKS AT 30 DAYS OF AGE

Lot	Zinc bacitracin addition to the basal (gms/ton)	Number birds per measurement	Av. wt. of birds	Feed/ gain	Av. length of intestine from gizzard to cloaca (Cm)	Passage of test feed from gizzard to cloaca in 3 hrs.* (Cm)	Wt. of intestine from gizzard to cloaca Full (gms)	Wt. of intestine from gizzard to cloaca Empty (gms)	Intestinal contents (gms)
1	None	30	568	1.76	123	32.5	41.5	20.7	20.8
5	200	30	631	1.70	123	36.3	45.6	22.1	23.5

* Birds which were normally full-fed beforehand were fed ferric oxide tracer feed (0.5 gm FeO/100 gms feed) from 8:00 a.m. until 11:00 a.m. and then fed normal-colored feed from 11:00 a.m. until 2:00 p.m. All birds were sacrificed at 2:00 p.m. and simultaneous measurements from each treatment were made on movement beyond gizzard of normal colored feed.

EXPERIMENT XIV

A final experiment was conducted in which lysed cells of E. coli were included in the diet of broiler chicks. It was theorized that if an active growth principal, which might explain antibiotic action, exists in these microorganisms within the digestive tract, the cellular membrane of the live organism may represent an effective barrier against its utilization.

Accordingly, ten lots of Cobb's White Rock one-day-old cockerels were allotted, as previously described, and fed the supplemented basal chick starter ration (table 1a) continuously for a 27-day period as shown in the experimental design (table 42).

Fresh fecal material, as described in experiment XI, was included in the daily ration of lots 3 and 6 to learn whether the cellular contents of the microorganisms would exert a counteracting effect on the fecal growth depressing entity.

One representative bird from each experimental lot was selected for determination of certain intestinal microorganisms at the fourth week.

Results and Discussion

A highly significant growth response occurred from inclusion of the lysed E. coli cells* to the basal diet and this response was equivalent to each of the antibiotic responses observed (table 43). The fact that growth from lysed E. coli cells in combination with bacitracin,

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*Lysed E. coli cells provided through the courtesy of Commercial Solvents Corp. Method of preparation shown on table 1d.

penicillin or terramycin proved non-additive, lends support to the concept that cellular material within the organism is the active principal responsible for growth stimulation in each instance. This indicates that the cellular contents must be liberated, probably by the death of the organism, before growth stimulation occurs.

An indication that identical mechanisms are operating in bacitracin and lysed E. coli cells is shown by the similarity of growth depression when either material is fed in combination with the fresh feces.

In view of the small amount (approximately 0.9 grams) of lysed E. coli cellular material fed each bird, the 48-gram-average growth response noted above the basal represents an improvement involving more than simply additional feed intake. For example, both growth and feed efficiency are improved by addition of the cellular material, since for each one gram of added feed ingested, approximately one gram of added gain resulted. This represents a feed efficiency of about 1.0 as contrasted with 1.82 for the basal lot.

Insofar as intestinal microorganism population is concerned, the only noticeable pattern evident is the marked increase of micrococci, enterococci, and possibly lactobacillus in the absence of antibiotic, or in the presence of feces-induced growth depression (table 44). It should be pointed out though, that intestinal population of these particular organisms remained high in the lot, receiving lysed E. coli cells. Live intestinal E. coli population, on the other hand, showed no particular pattern among the experimental lots. This would also tend to indicate that the organisms per se are not an important consideration in mediating antibiotic stimulated growth.

TABLE 42

THE EFFECT OF FEEDING LYSED *E. COLI* AND FRESH FECAL MATERIAL ON ANTIBIOTIC RESPONSE IN FOUR-WEEK-OLD CHICKS

Lot	Antibiotic addition to the basal (gms/ton)	Lysed *E. coli* addition to the basal* (0.1%)	Feces**	Average chick wts. at 4 wks.	Chicks alive of 40 started	Feed/ gain
1	None	-	-	466	40	1.82
2	None	+	-	514	39	1.75
3	None	+	+	423	40	2.00
4	200 zinc bacitracin	-	-	547	39	1.70
5	200 zinc bacitracin	+	-	531	39	1.70
6	200 zinc bacitracin	+	+	432	40	1.93
7	200 procaine penicillin	-	-	536	39	1.71
8	200 procaine penicillin	+	-	532	39	1.68
9	200 terramycin	-	-	523	40	1.74
10	200 terramycin	+	-	522	40	1.74

* Provided through courtesy of Commercial Solvents Corporation, Terre Haute, Indiana Method of preparation described in table 1d.

** Daily feeding of fresh feces from hens receiving an antibiotic-free diet. One-half feces and one-half physiological saline solution by volume strip-fed on top of mash each morning.

TABLE 43

ANALYSIS OF VARIANCE OF 27-DAY-OLD CHICK WEIGHTS. Experiment XIV

Source of variation	Degrees of freedom	Mean square	F values		
			Calculated	P = 0.05	P = 0.01
Total	394				
Subclass	39	22,895	3.91**		1.67
Lot	9	80,896	13.83**		2.48
Replicate	3	11,823	2.02	2.64	
L X R	27	4,791	0.82	8.54	
Error	316	5,849			

Comparison among lots at 5 percent level of probability

Lot	3	6	1	2	10	9	5	8	7	4
27-day wt.	423	432	466	514	522	523	531	532	536	547

at 1 percent level of probability

Lot	3	6	1	2	10	9	5	8	7	4
27-day wt.	423	432	466	514	522	523	531	532	536	547

** Significant ($P < 0.01$)

TABLE 44

THE EFFECT OF ANTIBIOTICS, FECAL MATERIAL AND LYSED E. COLI ON RELATIVE POPULATIONS OF CERTAIN INTESTINAL BACTERIA IN FOUR-WEEK-OLD CHICKS

Lot	Antibiotic addition to the basal (gms/ton)	0.10% Lysed E. coli addition to the basal*	Feces**	Organisms in intestinal content 2" above cecum			
				Gram positive			Gram negative
				Total micrococci (per gm)	Entero cocci (per gm)	Lacto-bacillus (10^4/gm)	E. coli (10^4/ gm)
1	None	-	-	24,000 +	24,000 +	739	393
2	None	+	-	24,000 +	24,000 +	1507	1
3	None	+	+	5,400	5,400	4643	2515
4	200 zinc bacitracin	-	-	320	260	3	2
5	200 zinc bacitracin	+	-	330	230	21	5
6	200 zinc bacitracin	+	+	24,000 +	24,000 +	63	593
7	200 procaine penicillin	-	-	130	110	1	1738
8	200 procaine penicillin	+	-	270	220	4	806
9	200 terramycin	-	-	3,500	3,500	114	100
10	200 terramycin	+	-	5,400	4,700	22	72

*

** See footnote, table 37

GENERAL DISCUSSION

The specific manner in which antibiotics stimulate growth has been a subject of extensive investigation during the past decade. From the numerous tests conducted several theories have been proposed to explain the action of antibiotics. According to Bird (1956), at least four mechanisms may be involved.

1. Direct effect on the metabolism or physiology of the animal.
2. Sparing of certain essential dietary nutrients.
3. Reduction of toxic entities or sub-clinical disease in the bird or animal.
4. Stimulation of beneficial microorganisms in the digestive tract.

Much circumstantial evidence has been presented in favor of each theory, though no positive proof of a single major action has been shown. Therefore, the possibility still exists that multiple physiological phenomenon may be operating in effecting the increased weight gain and improved feed utilization usually observed from feeding antibiotics to animals.

Certainly, the germ-free approach represents a realistic method of establishing whether the growth effect of antibiotics is a direct one of increasing uptake of certain essential nutrients, or of altering intestinal wall structure more favorably for nutrient assimilation. Luckey (1956) pointed out that some differences may occur in the gross morphology, nutritional requirement, and chemical composition of germ-free birds as compared with conventionally-reared chicks, but that these

differences do not detract from the general similarity noted. For example, Luckey found that a mature Bantam chicken, which has been reared to an egg-laying state in a germ-free environment compared favorably in most physiological measurements with a normal Bantam bird. The major differences noted in the germ-free bird were smaller proventriculus and adrenals, thinner intestinal and ceca walls, less lymphatic tissue and a larger thymus than the conventionally reared bird. Gordon (1952) made further studies on the morphology of germ-free birds and found no effect from feeding antibiotics in the absence of microorganisms, though he did suggest that feeding penicillin to conventionally-reared chicks tended to change these birds morphologically so that they resembled germ-free chicks. Gordon also found that neither terramycin nor streptomycin caused physiological changes, although the terramycin-fed birds did show marked increases in biotin, folic acid and vitamin B_{12} in cecal contents compared with the control chicks.

It appears then, that antibiotics do exert an influence on the morphology of certain specific tissues in the bird. However, interpretation of these physiological alterations is difficult. From a strictly physiological standpoint, the effect of dietary antibiotics in thinning the wall of the intestine suggests the possibility that this may improve absorption of hydrolyzed amino acids, simple sugars, and fatty acids which are in direct contact with the villi of the duodenal mucosa. On this basis, improvement in growth rate from antibiotic feeding would be due to the physiological function leading to the transportion of more essential nutrients across the less resistant, thinner membrane.

On the other hand, certain intestinal microorganism populations may increase the amount of several water-soluble vitamins within the digestive tract of the bird. But it is unlikely that added vitamins per se explain the growth-stimulating effect of antibiotics since usually dietary vitamin levels have been adequate for maximum growth. Since total protein, balance of essential amino acids, as well as vitamin and mineral levels were all adequate with respect to known requirements, it was felt that likelihood of a nutrient-sparing effect in the experiments described was not great. The possibility of antibiotics reducing the incidence of biochemical degradation of nutrients within the digestive tract, on the other hand, may offer an explanation of a mechanism of their action. If, for example, organic, inorganic, or mixed organic-inorganic oxidation: reduction potentials for enzymes were changed by the presence of an antibiotic, the availability of the nutrients present in the gut might conceivably be altered due to differences in enzyme efficiency.

Results of several tests reported in this thesis (table 45a,b) are generally in agreement with those shown in the literature insofar as an antibiotic-depressing effect on the gram-positive microorganisms is concerned. However, all tests (Exp. XII, XIII) did not show reduction in total gut micrococci or enterococci from antibiotic feeding. Nor was an antibiotic growth increase noted in each instance (Exp. IX) where these bacterial populations were reduced.

Since many of the micrococci forms of bacteria are disease producers in animals, they may be indicative of adaptive forms capable of depressing optimum performance. Staphlococcus, streptoccoccus, gonococcus,

pneumococcus and diplocci-type organisms are examples of those which might logically be suspected as capable of reducing growth potential, feed utilization, or livability when present in the digestive tract. It is conceivable, too, that measurable populations of these organisms in the gut, even though they be present in sub-clinical numbers, could produce toxins or antimetabolites which could interfere with normal assimilation of nutrients, or block vital enzymes.

The growth-depressing effect observed from feeding fresh fecal material in trials XI, XII, XIII and XIV, demonstrated the presence of a fraction, which is effective in extremely small amounts and is sensitive to heat and drying at 100° F. (approx. 38° C.) or less. Jordan and Burrows (1947) state that the thermal death point of bacteria is dependent on moisture and time, and that most vegetative forms are killed at 55° to 58° C. in ten minutes under moist heat. Despite the fact that the temperature at which the fecal material was dried was below the thermal death point for most vegetative forms, the extended drying time (72 hours) probably proved lethal for most organisms. Since no growth depression resulted from dried fecal material, live microorganisms, rather than products of their metabolism are responsible for the observed growth retarding effect. The noticeable increase in intestinal micrococci and enterococci in the lots receiving fresh fecal material (table 44) suggests that these may be the organisms responsible for depressing growth, though the birds receiving terramycin and fecal material showed a significant growth improvement, despite the marked increase in these particular gram-positive forms.

On the other hand, depression of lactobacillus population in the intestine noted from antibiotic feeding may reflect adjustments in gut pH to a more nearly ideal medium for maximum absorption of some hydrolyzed nutrients. Proteins, for example, are precipitated from solution at different isoelectric pH points and, therefore, will probably be attached by the appropriate proteolytic enzymes much more readily when in solution.

In contrast to the concept of depressing harmful intestinal bacteria, many investigators have taken the view that certain beneficial microorganisms in the digestive tract are directly, or indirectly, involved in the mechanism by which antibiotics stimulate growth. Numerous investigators (loc. cit.) have observed that the coliform bacteria comprise sixty percent or more of the bacterial population of the intestinal tract, and some have associated these types of organisms with antibiotic action in mediating growth patterns.

The principal objective outlined in the experiments of this thesis has been to learn if E. coli is directly, or indirectly, involved in explaining the mechanism of antibiotic action. It is apparent from experiment I and II that introduction of live E. coli organisms via crop inoculation does not provide a satisfactory explanation. Furthermore, attempts to learn the effects of antibiotic action when reducing gut E. coli by drug inhibition were not particularly fruitful in elucidating a specific pathway of antibiotic function.

Trials III and VI did indicate that zinc bacitracin was effective in restoring depleted intestinal E. coli population and growth due to sulfa drug inhibition. The depressing action of sulfonamides on E. coli is due to their antimetabolite-like action whereby the sulfonamide drug is

preferentially substituted for p-aminobenzoic acid in the enzyme system of E. coli, thereby depriving the organism of essential growth factors. Since zinc bacitracin effectively reduces the growth inhibition on E. coli caused by sulfaguanadine (Exp. III), part of the growth stimulating effect of the antibiotic in the bird may be due to its action in blocking certain unknown antimetabolites.

In experiment VI, an apparent synergism on growth was observed from combining polymixin with zinc bacitracin and this was associated with a marked increase in intestinal coliform population (table 46). Additional experiments, however, indicated that no particular pattern could be ascribed for these organisms due to treatment, notwithstanding the improved growth noted from feeding the combination of drugs. Further, when intestinal E. coli from all experiments are considered (table 47) no pattern emerges that can be associated with antibiotic-stimulated growth responses. Any beneficial effect of microorganisms in improving chick growth must be an indirect one in which the organisms act to provide necessary enzymes that may be missing from the digestive tract, or are present only in limited amounts. If, for example, these digestive enzymes are sub-optimal in amount or concentration in the absence of a specific microorganism, antibiotic action might be explained on the basis of increased enzyme action on those nutrients not normally available.

No completely satisfactory explanation exists concerning the action of enzymes, however, the most probable postulate is that given by Michaelis and Menton (1913) and currently supported. According to their theory, action of enzymes is explained on the basis of "active centers" on the surface of the enzyme molecule. The specific effect is described by the following equations.

Enzyme + substrate $\rightleftharpoons$ enzyme substrate complex

Enzyme substrate complex $\rightleftharpoons$ enzyme + products of enzyme action

While the available evidence points favorably toward involvement of the cellular material from E. coli in explaining the mechanism of bacitracin action in stimulating chick growth (table 42), it appears that if the action is enzymatic there is a certain specificity of enzyme action. This is borne out by the fact that neither the cellular yeast material, with its multiplicity of enzymes, nor the enzymes liberated from soaked barley were effective in altering bacitracin-stimulated growth.

The significant growth response noted from inclusion of cellular E. coli material, but not to crop inoculated live organisms, may possibly be explained by the concept that the cell membrane of E. coli represents an effective barrier against nutrient contact with certain enzyme surfaces. Since narrow spectrum antibiotics, such as zinc bacitracin or penicillin, do not depress intestinal E. coli, their numbers may be increased in direct proportion to the microflora which these compounds inhibit. Increased numbers of live E. coli will then result in larger numbers of dead coliforms in the intestine. The cell membrane of these dead organisms may not be as much of a barrier against movement of specific enzymes which could be involved in antibiotic stimulated growth as is the cell membrane of live organisms. Fruton and Simmonds (1953) state that extracts of E. coli contain a multiplicity of enzymes, including phospho trans acetylase and aspartase. Aspartase is required in the Kreb cycle exergonic conversion of fumarate to aspartate. Conceivably then, more effective conversion of protein to amino acids with less loss of energy

from certain of these exergonic enzyme reactions offers a possible explanation for the mechanism of antibiotic action.

On the other hand, experiment XI, XII and XIII support the concept that antibiotics and antiviral compounds reduce the growth-depressing effects of fresh feces. This may explain their mechanism of action. Since birds normally have access to droppings, growth would in most instances be depressed below a certain attainable level due to effect of fecal microorganisms on the host animal's metabolism. However, when the organisms are weakened or destroyed by antibiotic action, the growth inhibition is removed.

The possibility also exists that antibiotics may stimulate growth due to an effect on the appetite, since antibiotic-fed birds consumed more feed than did non-antibiotic-fed chicks in all experiments except II, III and VII. While it is conceivable that the increased feed intake observed in antibiotic-fed birds may provide more opportunity for building tissue than in control fed birds, both groups were consuming feed far in excess of maintenance requirements. Furthermore, the increased efficiency of added gains usually observed from antibiotic feeding cannot be explained on the basis of increased intake alone. It would, therefore, seem that factors in addition to increased feed intake are operating in effecting growth increases from antibiotic substances.

From these studies, it appears that further work is indicated in the area of enzymes as the explanation for the major action of antibiotics in stimulating growth in poultry and farm animals. In vitro experiments should be conducted to resolve the specific nature of the enzyme(s) present in cellular material from E. coli, which has the capacity to

influence chick growth. In addition, further tests involving feeding of fecal material should add to our knowledge of certain specific harmful microorganisms that are probably depressing performance in the poultry house. Finally, the area of virus inhibition on bird performance requires further investigation. Titration of various levels of anti-viral agents, or those compounds showing promise, should prove fruitful in unlocking some of the many secrets of the digestive tract as yet unknown to us.

TABLE 45a

ASSOCIATION OF ANTIBIOTIC FEEDING AND CERTAIN GRAM-POSITIVE INTESTINAL MICROORGANISM POPULATIONS

Intestinal organisms	Experiment number											
	VIII		IX			X			XI			
	C_1	B_2	C	B	T_3	C	B	E_4	C	B	A_5	
Total micrococci (per gm)	790	N.G.*	2400	2400	N.G.	2400	240	240	160	13	24	
enterococci (per gm)	790	N.G.	270	N.G.	N.G.	2400	240	240	92	8	8	
lactobacilli (10^4 per gram)	55	4	4201	N.G.	N.G.	-	-	-	-	-	-	
4 wk. chick wt. (grams)	449	506	445	507	463	554	606	604	536	632	595	

1 C - Control

2 B - zinc bacitracin

3 T - terramycin

4 E - erythromycin

5 A - aureomycin

* N.G. - No growth any dilution

\- No test conducted

TABLE 45b

ASSOCIATION OF ANTIBIOTIC FEEDING AND CERTAIN GRAM-POSITIVE INTESTINAL MICROORGANISM POPULATIONS

Intestinal organisms	Experiment number XII C	XII B	XII T	XIII C	XIII B	XIV C	XIV B	XIV P_6	XIV T
Total micrococci (per gram)	490	1800	24,000+	2200 5400	5400 1700	24,000	320	130	3500
enterococci (per gram)	210	140	24,000 +	1400 700	1700 1300	24,000	260	110	3500
lactobacilli (10^4/gm)	-	-	-	1939 3632	874 52	739	3	1	114
4 wk chick wt. (gms.)	564	610	600	509	566	466	547	536	523

6 - P-procaine penicillin
- No test conducted

TABLE 46

EFFECT OF COMBINATIONS OF BACITRACIN AND POLYMIXIN ON FOUR-WEEK CHICK WEIGHTS

Exp. no.	Control		Zinc bacitracin		Zinc bacitracin + polymixin	
	Wt.	Intestinal E. coli*	Wt.	Intestinal E. coli	Wt.	Intestinal E. coli
VI	452	9	506	2	537	1979
VII	469	N.G.**	531	41	552	0.1
IX	445	23	507	14	524	823
X	554	9	606	14,571	625	53
XI	536	N.G.	632	3	615	2

* Approximate *E. coli* population two inches above cecum, counts X 10^4 per gram of intestinal contents

** No growth at any dilution

TABLE 47

EFFECT OF VARIOUS ANTIBIOTIC TREATMENTS ON INTESTINAL *E. COLI* POPULATION AND GROWTH OF FOUR-WEEK-OLD CHICKS

Exp. no.	E. coli population (10^4/gm) two inches above cecum											
	Control		Zinc bacitracin		Penicillin		Erythromycin		Terramycin		Aureomycin	
	Count	Wt.	Count	Wt.	Count	Wt.	Count	Wt.	Count	Wt.	Count	Wt.
I	270,000	594	TNC	678					97,200	665		
II	1	424	1	477					N.G.	441		
III	68	401	55	434					3	425		
IV	4	291	4	325					4	297		
V	1	362	1792	410								
VI	9	452	2	506								
VII	N.G.	469	41	531								
VIII	21	449	2	506								
IX	23	445	14	507					2	463		
X	9	554	14,571	606			50	604				
XI	N.G.	536	3	632							2	595
XII	5	564	190	615					58	600		
XIII	0.3) 0.9)	509	16,204 1,748	566								
XIV	393	466	2	547	1738	536			100	523		

TNC Too numerous to count at any dilution
N.G. No growth any dilution

TABLE 48

SUMMARY OF DRUG EFFECTS ON BIRD WEIGHT

Exp. No.	Effect of dietary additives on weight			
	Increase Over basal	Decrease below basal	No effect	Increase when combined with antibiotic
I	B, T		CC	
II	B		T,CC,CS,CW	
III		S.G.	B, T	S.G.,(S.G.+R)
IV	B		T	
V	B, S.Ba	Ba		
VI	B		S.S.,P	P
VII	B, OL_1	OL_2		P
VIII	B		Y,YE,P	
IX	B		T, (B+P)	P
X	B,E		P, (E+P)	P
XI	B, A	FF	P, (A+P)	
XII	B, T	FF	FD, FA	
XIII	B	FF	S	S
XIV	B, LC,PP,T	FF		

B - Zinc bacitracin, 200 gms/ton
T - Terramycin, 200 gms/ton
CC - Crop inoc. of E. coli broth
CS - Supernatant of an E. coli broth
CW - Washed cells of an E. coli broth
CL - Lysed E. coli, 0.1%
S.G. - Sulfaguanadine, 0.5%
S.Ba - Water soaked barley
Ba - Barley (midwest variety)
S.S. - Sulfasuxadine, 0.5%
P - Polymixin, 25-50 gms/ton
OL - Oleandomycin, 1.0 gm/ton
OL_2 - Oleandomycin, 100 gms/ton
Y - Live yeast, 1.0%
YE - Yeast extract, 0.3%
E - Erythromycin, 100 gms/ton
FF - Fresh hen feces--daily
FA - Fresh hen feces autoclaved, 1.0%
FD - Fresh hen feces dried, 1.0%
A - Aureomycin, 200 gms/ton
S - Sialic acid, 20 mg/ton
PP - Proc. penicillin, 200 gms/ton
R - Ristocetin, 90 gms/ton

CONCLUSIONS

Fourteen battery experiments were conducted with one-day-old Broad Breasted Bronze poults and one-day-old chicks. A study was made to determine the importance of certain selected gram-positive and gram-negative intestinal bacteria in explaining the mechanism of antibiotic action in stimulating growth.

When practical starting rations, containing high levels of growth stimulating antibiotics, were supplemented with ristocetin, polymixin, sulfa drugs, sialic acid, yeast or yeast extract, water-soaked barley, fresh, dried or autoclaved feces, or fed in combination with weekly or bi-weekly crop inoculations of an E. coli broth, or its fractions, results were obtained which permit the following conclusions:

1. Certain antibiotics (zinc bacitracin, penicillin, aureomycin and terramycin, as well as oleandomycin and erythromycin) continued to prove effective in stimulating growth of poultry. In most instances, the 200 gram per ton level significantly improved early (0 - 4 week) growth in battery-raised chicks and poults.
2. Zinc bacitracin (narrow spectrum) proved consistently more effective in stimulating increased early growth in battery-raised chicks than did terramycin (broad spectrum). This indicates that differences exist in their mechanisms of action with respect to their effect on the birds' physiology, or their intestinal microflora population, and that these differences are important in mediating growth responses to antibiotics in chicks.

3. Certain heat-sensitive microorganisms, which are present in fresh but not in dried fecal material, significantly depressed chick growth. This growth-depressing effect was moderated to some extent by dietary antibiotics. Growth was restored equivalent to the basal when antibiotic was combined with polymixin or polymixin and sialic acid.
4. Based on crop inoculation of live organisms, the presence of increased numbers of intestinal E. coli per se was found not to be involved in the mechanism by which antibiotics stimulate growth in poultry. However, the cellular material contained within these particular bacteria appeared to exert growth-stimulating properties in much the same manner as does zinc bacitracin.
5. Possible mechanisms, by which antibiotics stimulate growth in poultry may be as follows:
    a. Inhibitory effect of antibiotics on certain harmful microorganisms normally found in fresh poultry feces, but not in dried feces.
    b. Action of antibiotics increasing permeability of the cell membrane of intestinal E. coli, and possibly other gut bacteria, to effect release of specific enzymes that are normally sub-optimal or missing in the host animal.
    c. Increased appetite due to antibiotic feeding, whereby greater intake of feed nutrients above maintenance requirements results in increased body tissue synthesis.

TABLE 1A

Experimental rations

Ingredients	Turkey starter	Chick broiler-starter	Chick broiler-finisher
	%	%	%
Yellow corn, ground	30.96	46.43	59.009
Oats, pulverized	--	1.00	1.00
Wheat standard middlings	10.00	1.00	1.00
Soybean oil meal (50% prot.)	33.18	31.32	21.31
Dehydrated alfalfa meal (17% prot.)	5.00	2.00	2.00
Stabilized animal fat	3.79	6.94	4.43
Fish meal (55% prot.)	5.00	2.00	2.00
Meat and bone scraps (50% prot.)	5.00	2.50	2.50
Dried corn fermentation solubles	--	2.00	2.00
Dried whey (50% delactosed)	3.00	2.00	2.00
Dicalcium phosphate	1.38	0.50	0.50
Ground limestone	1.79	1.50	1.50
Salt (iodized)	0.30	0.30	0.30
Trace mineral mix*	0.10	0.10	0.10
Methionine	--	0.059	--
Arsanilic acid mixture /	--	0.025	0.025
Butylated hydroxy toluene (antioxidant)	--	0.01	0.01
Vitamin mixture	0.51(1)	.316(2)	0.316(3)
	100.00	100.000	100.000

* Trace mineral mixture supplied the following per pound of ration: 27.2 mg. manganese, 0.54 mg. of iodine, 9.07 mg. of iron, 0.91 mg. of copper, 0.45 mg. of zinc and 0.09 mg. of cobalt.

1 Vitamin mixture supplied the following per pound of ration: 2,270 I.U. of vitamin A, 671 I.C.U. of vitamin D_3, 6.0 mcgm. of vitamin B_{12}, 2.0 mg. of riboflavin, 9.0 mg. of niacin, 4.0 mg. of pantothenic acid, 112 mg. of choline, 0.5 mg. of folic acid and 10 mg. ofalpha tocopherol acetate.

2,3 Vitamin mixture supplied the following per pound of ration: 1818 I.U. of vitamin A, 300 I.C.U. of vitamin D_3, 6.0 mcgm. of vitamin B_{12}, 1.0 mg. of riboflavin, 8.08 mg. of niacin, 2.0 mg. of pantothenic acid and 169 mg. of choline.

/ Abbott Laboratories Pro-Gen containing 20% arsanilic acid.

TABLE 1B

Calculated analyses of starter rations

		Turkey starter	Chick broiler-starter	Chick broiler-finisher
Crude protein	%	28.00	24.00	20.00
Arginine	%	1.39	1.36	1.314
Methionine	%	.55	.50	0.337
Cystine	%	.38	.32	0.320
Lysine	%	1.50	1.23	1.045
Tryptophane	%	.26	.25	0.241
Crude fat	%	5.90	9.70	7.16
Crude fiber	%	3.80	2.50	2.75
Productive energy (Cal/lb)		840	1020	1025
Calorie-Protein ratio		30	42	51
Salt	%	0.30	0.30	0.30
Calcium	%	2.09	1.21	1.21
Phosphorus	%	1.10	0.66	0.65
Manganese	mg/lb	27.00	27.00	27.00
Iron	mg/lb	8.08	8.08	8.08
Copper	mg/lb	0.91	0.91	0.91
Iodine	mg/lb	0.54	0.54	0.54
Zinc	mg/lb	0.027	0.027	0.027
Cobalt	mg/lb	0.09	0.09	0.09
Vitamin A	IU/lb	6733	4118	4118
" D_3	ICU/lb	681	300	300
" B_{12}	mcg/lb	7.3	6.0	6.0
Riboflavin	mg/lb	3.87	2.576	2.50
Niacin	mg/lb	43.90	21.41	21.41
Pantothenic acid	mg/lb	8.27	6.02	6.02
Choline	mg/lb	916	744	744
Folic acid	mg/lb (supplemental)	0.5	--	--
Arsanilic acid	mg/lb	--	23.5	23.5
Vitamin E	mg/lb (supplemental)	10	--	--

TABLE 1C

Composition of Media

Mallmann-Peabody agar
(Selective for *Escherichia coli*)

	Gms/1000 ml.
Peptone	10.0
Lactose	5.0
Bile salts #3	1.5
NaCl	5.0
Laurel sulfate	0.1
Agar	15.0
Water to bring to	1000.0
Indicator*	

* 1 ml. per liter of a 1.6 percent bromcresol purple indicator

Laurel tryptose agar
(Selective for gram-negative population)

	Gms/1000 ml.
Tryptose*	20.00
Lactose	5.00
Dipotassium phosphate	2.75
Monopotassium phosphate	2.75
Sodium chloride	5.00
Agar	15.00
Water to bring to	1000.00

* Peptone, Difco Lab.

Dextrose azide agar
(Selective for gram-positive population)

	Gms/1000 ml.
Beef extract	4.50
Peptone	15.00
Dextrose	7.50
Sodium chloride	7.50
Sodium azide	0.20
Agar	15.00
Water to bring to	1000.00

Ethyl violet azide broth
(Enterococci verification)

	Gms/1000 ml.
Peptone	20.00
Dextrose	15.00
NaCl	5.00
K_2HPO_4	2.70
KH_2PO_4	2.70
Sodium azide	0.40
Ethyl violet	0.00083
H_2O (to bring to 1000 ml)	

Trypsin digest agar
(Selective for lactobacilli)

	Gms/1000 ml.
Trypsinized milk	20.00
Tomato juice	10.00
Peptone	8.00
Dextrin	4.00
Dextrose	4.00
Agar	13.00
Water (to bring to 1000 ml)	

Dextrose azide broth
(Selective for micrococci)

	Gms/1000 ml.
Beef extract	4.50
Peptone	15.00
Dextrose	7.50
Sodium chloride	7.50
Sodium azide	0.20
Water (to bring to 1000 ml)	

TABLE 1d

LYSED E. COLI CELL PREPARATION

Culture and Inoculum

E. coli strain isolated from the intestine of a two-pound broiler had been lyophilized and stored at -10° C. A transfer was made from a lyophilized vial to a trypticase soy agar (Baltimore Biological Laboratories) slant and incubated 18 hours at 37° C. The slant was washed with three ml. of physiological saline at pH 6.8 and transferred to a sterile tube. The density of the suspension was adjusted to 25 percent light transmission using a spectrophotometer (Bausch and Lamb) at a wave length of 25 mu.

Seed Preparation

Four 500-ml. wide-mouth Erlenmeyer shake flasks containing 100 ml. of trypticase soy broth at pH 7.3 was inoculated with five ml. of each of the washed cell suspension. Flasks were placed on a rotary shaker (Gump) and incubated for 24 hours at 37° C. The entire contents of the four flasks were then evenly divided and used to inoculate two six-liter inoculum flasks containing 1500 ml. each of trypticase soy broth. The flasks were placed on a reciprocating shaker for thirty hours at 37° C.

Fermentation

Contents of the above two six-liter flasks were used to inoculate the "B" tank containing 166 liters of trypticase soy broth at pH 7.2. Antifoam (silicone-type) was added at a concentration of 150 ppm two hours post inoculum to prevent excessive foaming. Aeration was at an undetermined rate along with agitation. After 24 hours the liquid was dropped to a fifty-gallon epoxy resin lined drum and stored at 5° C. Final pH was 8.3. Sterility

checks on the various stages were made on eosin methylene blue agar. No contamination was apparent at any stage.

Harvest

Cells were recovered using a Sharples centrifuge to yield 7.5 pounds wet weight. Cells were frozen and thawed twice and lyophilized. Lysed cells were dried at 20 to 25° C in order to retain enzyme activity.

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