LD2441

ABSTRACT

EFFECTS OF ANTIBIOTICS ON
INTESTINAL MICROFLORA, GROWTH AND FEED EFFICIENCY
OF THE CHICKEN

By

Dennis Wilson Murphy

Three experiments were conducted to determine the microbiological basis for antibiotic growth promotion in the chicken. These studies departed from classical methods in two important respects. Entire gut segments, as opposed to chyme or feces, were sampled. Further, a model was developed that allows for continuous monitoring of simultaneous growth, feed efficiency, and bacterial population responses, and provides a method to evaluate cause-and-effect relationships in the three variables by regression analysis. The model also accounts for antibiotic, dose level, and time differences in bacterial responses, as they relate to growth in the antibiotic supplemented chick.

Experiment one showed that inclusion of entire gut segments in microbiological studies tends to reduce variation in bacterial counts, as reflected in chyme samples. This experiment also demonstrated that the study of the microflora of the small intestine reveals more consistent, more pronounced, and more often significant bacteriological responses than do similar studies addressed to more distal segments of the gut to include the caeca and large intestine.

Experiment two studied the response of lactobacilli, coliforms enterococci and clostridia to varying levels of Bacitracin MD and

Chlortetracycline. These responses, as they varied with time were related to accompanying growth responses to determine cause-and-effect relationships. Analyses of the relationship of each of these populations to growth indicate that none of them figure significantly in the common growth-stimulating properties of Chlortetracycline and Bacitracin MD.

Experiment three repeated the analyses of lactobacilli, coliforms enterococci, and clostridia and was expanded to examine the role of small intestine strict anaerobes in antibiotic growth responses. Results of experiment three, with respect to lactobacilli, coliforms, enterococci and clostridia, again suggest that none of these groups contributes materially to growth promotion, as influenced by antibiotics. Anaerobic populations shared similar responses in both Chlortetracycline and Bacitracin MD supplemented chicks. Regressions of growth responses on anaerobic population showed that anaerobic reductions contribute substantially to improved growth in Bacitracin supplemented birds, and that the general effect also obtains in Chlortetracycline supplemented birds.

Results of the three experiments have not defined a specific bacterial population that can, in itself, account for the growth stimulation with Bacitracin or Chlortetracycline. By process of elimination however, we were able to demonstrate that lactobacilli, coliforms, enterococci, and clostridia responses to varying selection are not constant in the face of accompanying, constant, growth stimulation. We were able to demonstrate a common Bacitracin MD/Chlortetracycline action on anaerobes of the small intestine, and to discern

a common relationship of this action to positive growth effects with both antibiotics.

The results of these experiments have important implications for two reasons. They account for the discrepancies observed in literature concerning the roles of lactobacilli, coliforms, enterococci and clostridia in antibiotic growth responses. Further, if subsequent studies continue to confirm the findings with respect to these populations, they may be eliminated from further consideration as significant participants in growth promotion with antibiotics.

Results of these experiments also demonstrate that the anaerobic populations of the small intestine figure most prominently in the common growth effects dissimilar antibiotics share. Further study, and more careful speciation of these populations, may result in a precise definition of growth depressing intestinal bacteria, and may allow the development of more efficacious, and safer, drugs to promote the growth of poultry.

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Poultry Science

1975

ACKNOWLEDGMENTS

The author wishes to express his gratitude to Dr. Timothy S. Chang for his support and guidance throughout the study. Dr. Howard Zindel, Dr. Donald Polin and Dr. Theo Coleman of the Michigan State University department of Poultry Science made substantial and helpful contributions to the preparation of the dissertation.

Dr. Gordon Carter of the department of Microbiology and Public Health, and Dr. Allan Trapp of the department of Pathology provided valuable assistance and advice, and helped with the preparation of the final draft.

The author is grateful to Mrs. Deborah Currigan and Mrs. Deborah Flint for their help in the laboratory. Mr. John Beck, Mr. Kenneth Klippen, Mr. Terry Wing and Mr. Dennis Dodson provided much-needed counsel and support, both in the mechanics of the experiment, and in the statistical analysis of data.

I am grateful to the personnel of the Michigan State University Poultry Science Teaching and Research Center. Mr. Paul Bowen, Mr. Victor Thurlby, Mr. William Blair and Mr. Roger Jacobs provided invaluable assistance in the care of chicks reared at the center.

Mr. Sulo Hulkonen has given generously of his time and talents in the preparation of plates and transparencies. To these, and to the many others who, by their encouragement and willingness to help, aided in the completion of this work, the author is truly grateful.

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INTRODUCTION

Several years of study have been devoted to defining the biological basis for antibiotic growth promotion in chickens. To date, no consensus of opinion is available. However, some general trends and principles have been defined.

All growth promoting antibiotics exert their effects by acting upon normal bacterial populations of the gut. Several studies with germfree birds, and with defined-flora chicks, have demonstrated this fact abundantly. Further, defined flora chick studies have shown that particular members of the normal intestinal microflora may depress chick growth, and that these members respond either qualitatively (physiologically), or quantitatively, to antibiotic selection. These responses may then be translated into improved chick growth and feed efficiency.

Bacterial populations of the gut, as usually defined, are broadly classified, and are very generally characterized with respect to their effects on chick growth, as influenced by dietary antibiotics. Clostridium perfringens and Streptococcus faecalis are normally associated with growth depression; and show measureable responses to antibiotic supplementation. Other groups, particularly lactobacilli, (e.g. L. acidophilus), and coliforms (e.g. E. coli and A. aerogenes) are often associated with improved chick growth, and with corresponding increases in the gut of antibiotic fed chicks. However, the evidence

accumulated with respect to each of these "bad" and "good" bacteria can, and often is, contradicted in various reports in the literature. The sum of our knowledge of the role and importance of each in the growth of antibiotic fed birds is, in the final analysis, circumstantial and inconclusive.

Several studies of the gut of antibiotic fed birds have shown that the small intestine is the organ of the gut that most dramatically responds to antibiotic supplementation. The antibiotic fed chicks' gut is thinner than normal, it weighs less, absorbs nutrients more readily, and has fewer reticuloendothelial cells and smaller regional lymph nodes than conventional birds. The condition of the antibiotic supplemented chicks' gut approaches, more or less, the condition of that of germfree birds.

Two independent lines of investigation then converge. Antibiotics act by influencing certain members of the normal gut flora, and the small intestine of antibiotic fed birds shows marked, nutritionally significant, changes. Whereas the study of antibiotic effects on microflora of the small intestine would seem to follow logically from these two lines of evidence, the small intestine has been largely ignored by microbiologists for various reasons to be discussed later.

Other studies have addressed themselves to characterizing normal microflora of the chicken, as it varies with age and site. Several authors have shown that the microflora of each segment of the gut is distinctive, and does not necessarily reflect the microflora of other habitats in the gastrointestinal tract. Rather, normal microflora varies linearly from the crop, small intestine caeca, large intestine and feces.

Normal flora also varies with the age of the bird. The gut of the newly hatched chick is essentially sterile, but is soon colonized by a succession of bacterial species that, after a period of adaptation, eventually stabilize at characteristic adult levels. Acquiring this stable, normal, association of bacteria normally takes up to six weeks. The normal microflora of the gut, in addition to varying with organ and age, also varies considerably from the center of the lumen, in chyme, to the absorptive surfaces of the mucosa. Large, characteristic, firmly attached bacterial communities exist in mucin, or attached to cells of the mucosal epithelium. Ordinarily these populations are not considered in studies based on intestinal contents or feces.

Much work remains to be done to characterize what the normal microflora of the chicken consists of. Only recently has it become possible to recover and culture a significant proportion of the numerous and important strict anaerobic bacteria of the gut. Anaerobic intestinal microbiology is still in it's infancy but has shown that we know amazingly little about the ecology of normal gut bacteria, or of their relationship to the host.

The several independent, but relevant studies outlined above indicate that antibiotics work by acting on normal bacterial populations of the gut, and that morphological changes are most pronounced in the small intestine. A few members of the normal flora of the chicken have been studied intensively. While some species are generally regarded as "good" or "bad" their real significance to the growth of antibiotic fed birds is not convincingly demonstrated. Normal bacterial populations of the gut vary linearly, temporally,

and radially. Each of these sources of variation are important and should be taken into account in studies of antibiotic effects on normal gut bacteria, as they relate to growth. In the past, the several independent lines of evidence relating to the intestinal microbiology of the chick, antibiotic growth effects, and morphological and physiological changes in the small intestine, have not complemented each other. As a result, we know little more about how and why antibiotics stimulate chick growth than we did twenty-five years ago. Eventually, the answer will depend on an appreciation of and synthesis of the concepts outlined above.

The three experiments described in this thesis attempt such a synthesis. Experiment one was a pilot trial to determine the responses of several bacterial populations to antibiotic selection as it varied from the small intestine, to the caeca and large intestine. It also included the entire respective segment of the gut in an attempt to represent both wall-associated bacteria and those in contents of the gut. Neither of these approaches has been previously reported. In fact, all bacterial response, growth studies have been based on chyme or feces alone, and usually of the posterior segments of the gut, or feces. Experiment one also attempted to determine the response of each of six bacterial populations to continuous antibiotic selection, as it varies with the age and growth of the chick. This is the first continuous response study, to the authors knowledge.

Experiments two and three were designed to relate the responses of several bacterial populations in the small intestine to varying levels of chlortetracycline (CTC) and bacitracin MD (B-MD), to accompanying growth and feed efficiency responses. Repeated, simultaneous

determinations of bacterial responses, growth responses and feed efficiency responses were employed to detect cause-and-effect relationships between these variables, and to determine common relationships within and between chlortetracycline and bacitracin supplemented chicks.

The results of the three experiments suggest that the methods and interpretations derived are important to the further study of biological mechanisms by which antibiotics stimulate growth and improved feed efficiency in chickens.

Bacitracin methylene disalicylate and Aureomycin (R) are referred to in the thesis as B-MD and chlortetracycline (CTC), respectively. All bacterial populations and population differences are represented as base $_{10}$ logarithms and denote densities of bacteria, per gram wet weight. A key to the bacterial population classifications employed is included in the appendices.

OBJECTIVES

The general objective of all three experiments was to determine a method to evaluate the importance and role of each of several bacterial populations in growth promotion with antibiotics. A method was developed, refined, and applied to several populations to determine their relative contribution to growth, as influenced by antibiotics.

Experiment one was a pilot study and was conducted to determine the practicability of including entire gut segments in bacteriological studies, to determine and quantify the responses of several bacterial populations to antibiotic selection as it varied with time, and with site in the gut, and to relate observed bacterial responses to positive growth responses in the antibiotic fed chick.

Experiment two expanded the objectives of experiment one and attempted to develop a quantitative model to study and evaluate the relationship of bacterial population responses in the small intestine to corresponding growth effects. Several populations were subjected to varying degrees and types of antibiotic selection. Their responses, as they varied with time, antibiotic and dose level, were related to accompanying growth responses in antibiotic fed chicks. Lactobacilli, coliforms, enterococci, and clostridia were tested in this model to determine whether they participate actively, or passively, in growth responses in the antibiotic supplemented bird .

Experiment three expanded the model of the experiment two to include anaerobes, a higher degree of B-MD selection pressure, and more comprehensive growth and feed efficiency response data. Experiment three attempted to confirm the relationship of each of the above populations to positive nutritional responses, and to evaluate the importance of anaerobic responses to nutritional responses, as they vary with antibiotic, level, and time.

LITERATURE REVIEW

Anatomy and Physiology of the Alimentary Tract

The entire alimentary tract of the chicken extends from the pharynx to the cloaca. The intestine ranges from the anterior duodenum to the cloaca. The length of the entire intestine is approximately sixty centimeters in twenty-day old birds and one hundred-forty centimeters in adults. The intestinal mucosa includes crypts of Lieberkuhn, and is covered with flat, leaflike villi. Lamina propria, muscularis mucosae, submucosa and outer muscular layers lie beneath the mucosa. The caeca of chickens are large, paired and blind structures of about six inches in length, situated at the junction of the small and large intestine. Muscular ileo-caecal valves control filling and evacuation of the caeca. The histology of the caeca resembles that of the small intestine, except the villi are shorter. The large intestine is relatively short, with no demarcation between the colon and rectum, as in mammals. It empties directly into the cloaca (Sturkie, 1965).

The chicken alimentary tract is a continuum of complex and specialized environments, each with its peculiar functions and biological associations. Temperature in the gut does not vary substantially in different sites, but approximates 40.5^{o} C. in all organs. As regard function, anterior segments (esophagus,

crop, proventriculus and gizzard) are concerned with transport, storage, preconditioning of the food mass, and initial processes of digestion. Some nutrients (volatile fatty acids) are absorbed in the anterior segments, particularly the proventriculus and crop. However, the greatest proportion of nutrients are absorbed from the small intestine, chiefly the jejunum and ileum.

The redox potential of the gut varies with site. The crop, proventriculus and gizzard are fairly aerobic, having redox potentials of approximately +100 mv. The jejunum, ileum, large intestine and caeca are strictly anaerobic. Small intestine redox potential is -100 mv., while that of the caeca and large intestine drops to -200 mv.(Moore, et al 1969). Acidity and degrees of anaerobiosis are inversely related. Anterior, relatively aerobic, segments of the alimentary tract are acidic, with pHs of from two to six. More distal segments, from the duodenum to the cloaca, are neutral in pH, and are strictly anaerobic.

Intestinal Microflora of the Chicken

Reports of numbers and types of bacteria in the gut of normal chickens vary considerably, depending on organs reported, culture media and methods used, bacterial classification schemes, and the age and diets of the birds studied. This review will follow general trends in numerically important bacterial populations, by organ, and as they vary with age of the bird. In the literature review, and throughout the thesis, bacterial population densities are expressed as base 10 logarithms, and represent numbers per gram wet weight.

Normal Crop Microflora

Compared to the intestine and caeca, the bacterial populations of the crop are extremely simple, largely because of the low (4-6) pH of this organ, which selects against many would-be colonizers. Acidity also helps regulate the colonizing flora of more distal segments of the alimentary tract. Lactobacilli are the most common bacteria in the crop and maintain normal densities of from seven to nine. Enterococci and coliforms occur regularly--both at densities of five. Micrococci, yeasts and staphylocci occur irregularly, and in low numbers in the crop (Jayne-Williams and Fuller, 1971).

Normal Small Intestine Microflora

The small intestine microflora of the new-hatched chick derives from that of the caeca. Bacterial populations fluctuate, both qualitatively and quantitatively in early life. In from two days to six weeks, depending on the group, populations colonize and stabilize at characteristic adult levels and deviate little from that point on (Smith, 1965 a,b; Shapiro and Sarles, 1949; Huhtanen and Pensack, 1965 a,b; Timms, 1968). A substantial proportion of the total small intestine microflora, lactobacilli in particular, are intimately associated with, and may be attached to, the mucosal epithelium (Fuller, et al., 1971; Fuller, 1972). While normal populations are stable and characteristic, starving for a period of twenty four hours may essentially depopulate the small intestine (Moore, 1969).

Total Bacteria: Estimates of total bacterial populations in the small intestine vary with different reports. Barnes (1972) reports low numbers of bacteria in the small intestine of young chicks, which then increase with age to levels of less than 8 at two weeks. Similarly, Lev and Briggs (1956 a,b) examined small intestine contents of newly hatched chicks, microscopically, and report no bacteria present immediately post-hatch. Total adult populations then increased to 6.5 to 7.5. On the other hand, Huhtanen and Pensack (1965 a,b) report total populations from 9.1 to 10.0 at four weeks of age. They also report that anaerobes occur ten times more frequently than aerobes.

Lactobacilli: Lactobacilli constitute the largest single component of the total small intestine microflora. Further, a large proportion of them attach to the mucosal epithelium (Fuller and Turvey 1971). Estimates of their numbers vary considerably. Barnes (1972) states they seldom exceed densities of 4. Similarly, Barnes et al. (1972) report adult population levels of 5. In contrast, Shapiro and Sarles (1949) and Eyssen (1962) report normal lactobacilli populations of 7.0 and 7.3, respectively.

Enterococci: Enterococci are a remarkably constant bacterial associate, both of the small intestine, and elsewhere in the gut. They colonize from the caeca, and constitute nearly 100% of the newly hatched chick's small intestinal microflora. They increase from near 0 to 8 in the first twenty-four hours on feed, then drop to characteristic adult levels of approximately 5 (Huhtanen and Pensack, 1965 a,b; Barnes, 1972; Barnes et al., 1972; Smith, 1962, 1965 a.b; Shapiro and Sarles, 1949).

Coliforms: The distribution and numbers of coliforms in the small intestine closely resemble those of enterococci. Coliforms colonize the small intestine immediately post-hatch, increase to 8 in the first twenty-four hours on feed, and then stabilize at characteristic adult levels of 4.5 to 5.0 (Barnes, 1972; Barnes et al., 1972; Shapiro and Sarles, 1949; Smith, 1962; 1965.

Clostridia: Clostridia (usually Clostridium perfringens) occur sporadically and in low numbers in the normal small intestine. If seen at all, its numbers seldom exceed 3.8 and are usually much lower. Increases in small intestine clostridia may accompany enteric infections (Barnes, 1972; Barnes et al., 1972; Smith, 1962; 1965).

Normal Caecal Microflora

The normal microflora of the caeca differs from that of other sites in the gut in many respects. They are the first site of the gut in which bacteria proliferate in the new-hatched chick. Owing to their structure and intermittent stasis they allow proliferation of slow-growing bacteria, provide a unique nitritional substrate of chyme, feces and urine, and are absolutely anaerobic. The caeca support the largest and most diverse bacterial populations in the gut, in which strict anaerobes figure most importantly (Jayne-Williams and Fuller, 1971). The caecal microflora is the most studied of various sites in the chicken alimentary tract. Still, many normal associates (anaerobes) of the caecal flora have yet to be cultivated *in vitro*, are poorly understood, and are only beginning to be characterized.

Total Anaerobes: Very dense populations of a variety of anaerobes have been reported in the caeca of new-hatched, unfed, chicks. While total numbers vary little from day one, the species profile of caecal anaerobes may vary with age, as new, normal associates are acquired from the environment (Barnes, 1972). Estimates of total caecal bacteria range from 7 to 12 (Huhtanen and Pensack, 1965; Barnes et al., 1972; Shrimpton, 1966; Barnes, 1972; Goldberg, 1964 and Moore, 1969). Shrimpton (1966) reports *Bacteroides fragilis* is the most common caecal anaerobe while Barnes (1972) noted a qualitative shift, with age, from *Peptostreptococcus* to Gram-positive filamentous forms, to bifidobacteria.

Clostridia: Clostridia (chiefly Cl. perfringens) constitute a proportionately small, but numerically large and constant, representative of caecal anaerobes. Several authors report high populations of clostridia from day one, ranging from 5 to 9. Total numbers of caecal clostridia remain fairly constant over the life of the bird (Barnes, 1972; Lev et al., 1946; Lev and Briggs, 1946 a,b; 1957 a,b; Shrimpton, 1966 and Barnes et al., 1972).

Lactobacilli: Lactobacilli, as elsewhere in the gut, constitute an important fraction of the total caecal microflora. Lactobacilli colonize the caeca soon after hatching, increase rapidly to 11 when feed is provided, and maintain stable adult population levels of 9.0-9.5 (Barnes et al., 1972; Shapiro and Sarles, 1949).

Coliforms and Enterococci: Coliforms and enterococci are more numerous in the caeca than elsewhere in the gut. Both colonize the caeca immediately post-hatch, proliferate to levels of 8-9 in the first twenty-four hours on feed, and then decline to stable population levels of 7 and 8, respectively (Huhtanen and Pensack, 1965 a, b; Barnes et al., 1972; Shapiro and Sarles, 1949; Lev and Briggs, 1956 a,b; Lev et al. 1957. All populations stabilize at adult levels by six weeks (Timms, 1968). Avian enterococci strains exhibit distinctive biochemical properties. Nowlan and Deibel (1967) propose the name Streptococcus avium to include all chicken enterococci.

Normal Bursa of Fabricius Microflora

The microflora of the Bursa of Fabricius differs from that of adjacent (large intestine and cloaca) segments of the gut. Coliform bacteria predominate, followed in occurrence by lactobacilli and enterococci (Fuller, 1973).

Normal Microflora of Feces

Smith (1962, 1965 a, b) reports fecal microflora are generally representative of the flora of the large intestine. As such, fluctuations in bacterial populations of more anterior segments of the gut, particularly the small intestine, may not be reflected in fecal microflora. Types and numbers of bacteria in chicken droppings vary considerably, in that they are mixtures of feces proper, caecal feces and urine (Jayne-Williams and Fuller, 1971).

Emmel (1930) reports that coliforms are the best-represented bacterial population in feces and constitute 65% of the total microflora. Clostridia occur commonly and usually amount to 10% of the total, while Bacteroides sp. and staphylococci occur sporadically.

Physiologic Effects of Intestinal Microflora

Pasteur first proposed the use of germfree animals to determine various physiologic effects of normal intestinal microflora on the host. Since the 1950's, germfree animals have been used extensively to determine effects on growth, nutrient absorption, immune response, intestinal morphology, the mode of action of antibiotics, and the specific effects of normal intestinal bacteria both in monoinfected gnotobiotic animals and in those with selected normal bacteria (Lev et al., 1962).

Studies with germfree chickens have repeatedly demonstrated that intestinal bacteria are not essential to the nutritional well-being of the host (Lamanna, 1972). In fact, conventional birds' growth may be depressed from germfree levels by from 15% to 25% (Lev and Forbes, 1959; Gordon et al., 1958). Growth-depressing effects of intestinal bacteria include inflammation and thickening of the gut wall, reduced half-life of intestinal epithelial cells, and changes in several immune parameters.

Gordon et al. (1958) and Gordon and Bruckner-Kardoss (1959) report the gut of conventional birds is thicker than that of germ-free counterparts. Most of the thickening occurs in the lamina propria, the layer immediately below the mucosal epithelium. Draper (1958) and Lindblad et al. (1954) showed that this thickening results in reduced absorptive rates of lysine and calcium, respectively.

Inflammatory processes should be accompanied by appropriate immune responses. Thorbecke (1959) demonstrated that germfree birds have fewer plasma cells and secondary nodules, and lower gamma globulin levels than conventional birds. The effect was confirmed by Gordon et al. (1958) and Gordon and Bruckner-Kardoss (1959), who noted a reduction in Schallen leucocytes and lymphcytes in germfree versus conventional birds. The germfree animal is, compared to normal animals, immuno-incompetent. The difference in immune parameters between germfree and conventional animals is reduced by antibiotic supplementation. Moore (1969) suggests antibiotics may have undesirable effects in that they tend to lower the normal immune response.

Wagner and Wastmann (1959) and Wastmann et al. (1960) introduced Clostridium perfringens and Streptococcus faecalis into germfree birds, singly and in combination. Both organisms caused a proliferation of reticuloendothelial cells and increased the thickness of the lamina propria. Antibiotic supplementation partially reversed the effect. Similarly, Forbes et al. (1959) infected germfree chicks with various combinations of Escherichia coli, Lactobacillus lactis and Streptococcus liquefaciens, with and without Clostridium perfringens. The three former species had no effect on growth on gnotobiotic chicks, whereas Clostridium significantly reduced growth. Lev and Forbes, (1959) obtained similar results. In both studies, antibiotic supplementation reduced intestinal clostridial populations and partially reversed the associated growth depression.

Clostridium perfringens produces enterotoxins, collagenase, lecithinase and hemolysin, all of which irritate the gut and cause thickening of the lamina propria. Penicillin supplementation either eliminates Clostridium from the gut of germfree birds, or alters its toxicity (Lev and Forbes, 1959; Lev et al., 1956; Lev and Briggs, 1957). Streptococcus faecalis reduces fat absorption in monoinfected germfree birds, particularly in the fourth day of life. These organisms split bile salt, yielding deconjugated lithocholic and cholic acids. Aside from the destruction of needed bile, bile acids reduce the lifespan of mucosal epithelial cells and elevate serum cholesterol levels. The net result is steatorrhea and thickening of the gut wall, and reduced growth (Eyssen and De Somer, 1963 a,b; 1965; Edwards and Boyd, 1963; Gustaffson and Norman, 1962; and Rosenberg, 1969).

Lactobacilli are an important constituent of the total flora of all sites in the chicken alimentary tract, particularly of the small intestine. Further, lactobacilli are intimately associated with the mocosal epithelium and are often attached to its cells. This cell attachment reduces the half-life of mucosa cells from four to two days. Germfree birds infected with avian strains of Lactobacillus acidophilus show this effect clearly. The net effect of rapid cell turnover translates to a reduction of growth, relative to germfree birds, of 8% (Khoury et al, 1969). Antibiotic supplementation reduces the conventional/germfree growth rate difference.

Nutritional Effects of Intestinal Microflora

There are from ten million to one hundred billion live bacteria in every gram of intestinal contents, from the jejunum to the cloaca. These bacteria include at least five major classifications, over two hundred species, and thousands of strains. Bacterial cell mass accounts for up to twenty-five per cent of the dry weight of the caeca and large intestine. Each of the bacterial populations in the gut interacts with its bacterial associates, the nutritional substrate, to include host cells and chyme, and with the host. The demonstrated effects of normal intestinal microflora in conventional versus germ-free birds stimulated many studies to determine which bacteria significantly affect the nutrition of the chicken, and how.

Bacteria and Vitamin Economy: Effects of intestinal bacteria on the vitamin economy of the chicken have been exhaustively investigated. Many common enteric bacteria have absolute requirements for vitamins and may compete with the host for them. On the other hand, many others synthesize essential vitamins which are utilized by the chicken (Jayne-Williams and Fuller, 1971). Abdel-Salaam and Leong (1938) batch-cultured normal caecal bacteria, to include lactobacilli, enterococci, clostridia and coliforms, and found that *in vitro* thiamine production increased initially, and then was sharply reduced. Similar associations were later demonstrated to produce several B-complex vitamins to include thiamine, riboflavin, pantothenic acid and inositol. At the same time vitamin C was destroyed (Young and James, 1942).

Mitchell and Isbell (1942) found that pantothenic acid, thiamine and riboflavin are cell-bound and are therefore unavailable to the host. Pyridoxine, biotin and folic acid diffuse rapidly from synthesizing cells and may be available to the host. CTC supplementation causes coliform bacteria to increase in the gut. Monson, et al. (1954) found that these bacteria synthesize large amounts of folic acid *in vitro*. Subsequent studies confirmed that increases in coliforms from antibiotic supplementation spared folic acid requirements in birds maintained on folic acid-deficient diets. Non-antibiotic supplemented birds have large populations of lactobacilli, which are usually reduced by supplementation. Further, lactobacilli have strict requirements for thiamine. Wiseman et al. (1956, a,b) report that reductions in lactobacilli in antibiotic supplemented birds are accompanied by substantial increases in *Enterobacter aerogenes*, and that these bacteria synthesize large amounts of B-complex vitamins. Other examples of vitamin-cross feeding among normal bacterial associaaes are vitamin K synthesis by *Bacteroides fragilis* and *Veillonella alcalescens* and utilization of it by *Bacteroides melaninogenicus*, and nicotinic acid production by aerobes and utilization of it by anaerobes (Bryant, 1972; Mitchell and Isbell, 1942)

March and Biely (1967) reviewed several years of investigations of the role of normal bacteria in the vitamin economy of chickens, and concluded that, with modern rations and management practices, there is no sound evidence for a significant effect of bacteria in the total vitamin economy of the chicken.

Bacteria and Protein Utilization: Several investigators have shown that the normal microflora of the gastrointestinal tract reduces protein utilization from germfree levels, and that the effect is accompanied by a thickening of the intestinal wall. Further, these effects can be partially reversed by antibiotic supplementation (Machlin et al., 1952; Slinger et al., 1952; Weakley et al., 1953; Heth and Bird, 1962).

Bacteria reportedly have no effect on intestinal proteases. Many intestinal bacteria do, however, produce ureases which split uric acid to produce ammonia (Delluva et al., 1968).

Specific nutritional effects have been attributed to enterococci and clostridia. Enterococci deaminate L-arginine to yield ammonia. They also have been shown to decarboxylate tyrosine with tyramine production resulting. Tyramine is a vasoconstrictor which in turn impairs nutrient utilization (Huhtanen and Pensack, 1965 b).

Bacteria and Fat Utilization: Enterococci and clostridia reduce fat absorption from the gut. Enterococci are the commonest bacteria in the small intestine of young chicks. They interfere with fat absorption by splitting bile salts. Fecal fat excretion is increased and growth is depressed as a result (Cole and Boyd, 1967; Eyssen and De somer, 1963 a,b; 1965, 1967). Pensack (1963) exaggerated the malabsorption-of-fats syndrome by adding a filtrable, infectious agent to S. faecalis cultures fed young chicks. Lactobacilli, coliforms and staphylococci do not effect fat utilization in moni-infected gnotobiotic chicks (Cole and Boyd, 1967).

Bacteria and Metabolizable Energy: Intestinal bacteria generally reduce metabolizable energy and increase intestinal weight. Antibiotics improve metabolizable energy by their actions on several bacterial populations in the gut. The net effect of antibiotics on intestinal bacteria, metabolizable energy and growth is an increase in uptake of calorigenic nutrients (Nelson et al, 1963; March et al., 1972).

Effects of Antibiotics on Intestinal Microflora

Many investigators have studied effects of dietary antibiotics on several members of the normal gastrointestinal microflora of the chicken. Methods, to include dose levels of antibiotics fed, organs sampled and populations included, vary with reports. As a result, reports are not consistent with respect to particular bacterial populations. However, all previous reports of antibiotic effects are based on contents of various segments of the gastrointestinal tract, to the exclusion of the gut wall, or on feces. The following review will summarize various reports of CTC and bacitracin effects.

CTC Effects:

a. Total aerobes and anaerobes: Rosenberg et al. (1952) reported that CTC supplementation increased total anaerobes and total aerobes in the chicken gut. However, total populations, particularly of anaerobes, are generally either unaffected by CTC, or are reduced with supplementation (Eisenstark and Sanford, 1953; Sieburth et al., 1954; Barnes and Goldberg, 1962; Anderson et al., 1953 a,b.c; March and Biely 1952; Goldberg et al., 1964). Clostridia are nutritionally significant members of the strictly anaerobic microflora and are either unchanged (numerically) with CTC supplementation, or are decreased (Smyser et al., 1952; Sieburth et al., 1951).

b. Lactobacilli: Lactobacilli are important members of the total microflora of all segments of the gastrointestinal tract of the chicken and, as such, are generally included in antibiotic

supplementation bacterial response studies. The response of lactobacilli to CTC varies considerably with site in the gut, and with reports. Eyssen et al. (1962) report that CTC reduces lactobacilli of the crop and small intestine, while Sieburth and Roth (1954) and Rhodes et al. (1954) report substantial reductions in lactobacilli numbers at all sites in the gut. Lactobacilli reductions with CTC are confirmed by Anderson et al. (1956 and March and Biely, (1952).

On the other hand, other reports show lactobacilli increase with CTC supplementation (Anderson et al., 1951; 1952 a,b,c; 1953 a.b; Rosenberg et al., 1952; Dixon and Thayer, 1951, Wiseman and Sarles, 1956).

c. Coliforms: Most authors agree that coliforms increase with CTC supplementation (Sieburth and Roth, 1952, Sieburth et al. 1951; 1954; Anderson et al., 1951; 1956; Rivera and Sborov, 1951; Rhodes et al., 1954; Hauser et al., 1956), or that their numbers are uninfluenced by CTC (March and Biely, 1952; Barnes and Goldberg, 1962; Williams et al., 1951; Anderson et al., 1953). This general effect is important in that it stimulated the many studies of vitamin synthesis reported under nutritional effects of intestinal microflora, above. More importantly, the use of CTC for growth promotion has been linked to the spread of antibiotic resistant bacteria in the environment. Because of this, the use of antibiotics for growth promotion has been discontinued in the United Kingdom (Swann, 1969) and may soon be in the United States.

d. Enterococci: Rosenberg et al. (1952) reported gut enterococci (Lancefield Group D Streptococcus spp.) increased with CTC supplementation. Other studies show their numbers are unaffected by

CTC (Eisenstark and Sanford, 1953; Eyssen and De Somer, 1967). Barnes (1958) observed that, while numbers of enterococci were not influenced by CTC supplementation, cellular physiology of CTC supplemented strains were. Normal, proteolytic, enterococci were supplanted by non-proteolytic strains. When CTC was discontinued, normal types quickly reestablished themselves in the gut. Other workers note reductions in gut enterococci with CTC supplementation (Anderson et al., 1951; 1953; 1956; Sieburth et al. 1954). These authors also note an enterococci/lactobacilli and coliforms antagonism. Relative numbers of enterococci and the other two groups are inversely related.

e. Yeasts: Yeasts (Torulopsis molishianus) generally increase with antibiotic supplementation. However, their increase is accompanied by increases in yeast antagonists, principally Proteus mirabilis, which compensate for the effect (Sieburth and Roth, 1954, Rosenberg et al., 1952).

Bacitracin Effects: Bacitracin generally, and bacitracin methylene disalicylate particularly, was introduced for purposes of growth promotion much later than CTC (Chang, 1974). Whereas reports of CTC effects on intestinal microflora are plentiful, bacitracin effects are rarely reported in the literature.

Eyssen and De Somer (1967) report that intestinal enterococci are reduced in bactracin supplemented chickens. Eyssen et al. (1962) were unable to demonstrate any effect of bacitracin on lactobacilli

in the gut. Rhodes et al. (1954) observed that bacitracin almost eliminated lactobacilli from the intestine. Further, Tortuero (1973) observed a time effect in lactobacilli intestinal counts. In bacitracin supplemented birds, lactobacilli were decreased initially, increased above control levels, and again decreased in repeated sampling over nine days.

Barnes and Impey (1972) studied effects of bacitracin on strict anaerobes of the caeca. Bacteroides spp., Bifidobacterium spp., Propionibacterium spp. and Peptostreptococcus spp. were sensitive to bacitracin, whereas Eubacterium is resistant.

Variables in Antibiotic Growth Response

Reports of antibiotic growth effects generally range from 3% to 15%, but vary considerably in time and space. Any stimulatory effect from antibiotic supplementation depends on the presence of antibiotic sensitive, growth depressing bacteria in the gut. These, particularly clostridia, occur most frequently in old and dirty quarters. Antibiotic growth effects are correspondingly increased as the degree of environmental contamination increases (Bird et al., 1952; Coates et al., 1951 a,b).

Several investigators report a decline in effect with continued use of a particular antibiotic over prolonged periods. They attribute the loss of effect to two causes--improvement in performance of controls as a result of antibiotic related environmental decontamination, and emerging resistance among growth depressing bacteria (Brewer, 1975; Mc Ginnis et al., 1958, Nelson et al., 1963a, b; Libby and Shaible, 1955). In contrast, others continue to observe the same positive growth effect with continued use of particular antibiotics (Heth and Bird, 1962; Coates and Davies, 1959; Peter et al., 1966). The difference in various reports is likely due to the rate at which antibiotic sensitive, growth depressing bacteria are being reintroduced into a particular environment. Environmental sanitation and degrees of growth promotion with antibiotics are consistently, and inversely, related.

Source, Structure and Action of CTC and Bacitracin

CTC: CTC (chlortetracycline) is one of a family of related four-ringed antibiotics which are distinguished by radicals on the basic structure. Other forms are oxytetracycline (terramycin) dimethyltetracycline and tetracycline. Chlortetracycline is a fermentation product of Streptomyces aureofaciens. Its spectrum includes both Gram-positive and Gram-negative bacteria. Chlortetracycline acts by interfering with t-RNA attachment to ribosomes and, as a result, it blocks polypetide synthesis. All tetracyclines are readily absorbed from the intestine. However, absorptive rate and dose level are inversely related. Also, calcium and other divalent ions form insoluble complexes with tetracyclines, and neutralize their effect. Excretion of tetracyclines is principally via the kidneys. Large quantities may also be excreted in bile, so the drug may be recycled in the intestine (Davis et al., 1970; Marine and Sellers, 1965).

Bacitracin: Bacitracin is a cyclic polypeptide produced by a strain of Bacillus subtilis. It acts by causing accumulation of cell wall precursor nucleotides, in a manner similar to penicillin. However, unlike penicillin, it does not produce spheroblasts so the cidal mechanisms of the two antibiotics probably differ. Bacitracin also blocks induced enzyme synthesis. The antibacterial spectrums of bacitracin is restricted to Gram-positive organisms. It is particularly potent against staphylococci and streptococci. Bacitracin is not absorbed from the gut (Davis et al., 1970; Marine and Sellers, 1965).

METHODS AND PROCEDURES

Experiment One:

A. Treatment Design

Treatment	Birds
CTC 50 g./t.	70
Control	70
Total	140

B. Bacterial Sampling

Bacterial Population	[1]Samples	[2]Organs	Reps.	[3]Trts.	Total Cts.
Total Anaerobes	7	3	2	2	84
Total Aerobes	7	3	2	2	84
Lactobacilli	7	3	2	2	84
Coliforms	7	3	2	2	84
Enterococci	7	3	2	2	84
Anaerobic Spores	7	3	2	2	84
Total Counts					504

[1] pooled five-bird samples were examined at 1,7,14,21,28,42 and 70 days of age
[2] small intestine, caeca and large intestine
[3] CTC at 50 g./t., and controls

C. Weight Determinations

Days of Age	Controls	CTC 50 g./t.
21	10	10
28	10	10
42	10	10
70	6	8
81	8	7
Total	44	45

Birds, Management and Diets

One hundred-forty female Single Comb White Leghorn chicks were obtained from Rainbow Trail Hatchery, St. Louis, Michigan, at one day of age. Equal numbers of chicks were randomly assigned to each of two treatment (to include control) groups. Chicks were housed in brooder batteries at the MSU Department of Poultry Science for the entire ten weeks of the experiment. Feed and water were provided ad libitum. MSU chick starter ration 71-7 (Table 25) was fed from one to forty-two days of age. MSU chick grower ration 71-5 (Table 26) was fed from forty-three to seventy days.

Antibiotic Supplement

Control chicks were fed rations 71-7 and 71-5. Treatment chicks received the same ration, to which was added fifty grams per ton of feed of CTC activity. Antibiotic supplementation was continuous from day one to the termination of the experiment.

Bacteriological Sampling

A. Collection and Preparation of Samples: Five each of treatment and control chicks were randomly selected for examination at five ages, as outlined above. Each five-bird sample was replicated twice each day, to give a total of ten treatment and ten control birds for each age group. Chicks were sacrificed by cervical dislocation. Immediately thereafter, the skin was wetted with a phenolic disinfectant and the body cavity was opened as aseptically as possible.

Three regions of the alimentary tract were isolated and removed to sterile, chilled containers of distilled water. The small intestine, to include duodenum, jejunum and ileum, the large intestine and the caeca of each five bird treatment group were pooled for bacteriological counts. Entire organs, including contents, were macerated on a Waring blender and were then serially diluted in distilled water for subsequent counts.

B. Bacterial Populations, Culture and Count Methods: Six bacterial populations were characterized for each of the three organs in treatment and control chick pools. Bacterial populations, culture media and incubation conditions were:

1. Total Anaerobes: Total anaerobes were grown on Brain-Heart Infusion agar (Difco). Incubation proceeded forty-eight hours at 37°C. Anaerobiosis was maintained in an anaerobic jar with a commercial gas-generating pack (BBL), and palladium catalyst.
2. Total Aerobes: Total aerobes were grown on Brain-Heart Infusion agar (Difco). Incubation proceeded twenty-four hours at 37° C.
3. Lactobacilli: Lactobacilli were grown on acidified Rogosa's SL agar (Difco). Incubation proceeded forty-eight hours at 37° C., anaerobically. Anaerobiosis was maintained, as above.
4. Coliforms: Coliforms were grown on Eosin-Methylene Blue agar (Difco). Incubation proceeded twenty-four hours at 37° C.

5. Enterococci: Enterococci were grown on Ethylene Violet-Azide medium (Difco), to which 25% agar (w/w) was added. Incubation proceeded aerobically for twenty-four hours at 37° C.

6. Anaerobic Spore Formers: After the above five populations were plated, dilution tubes were heated to 80° C. for twenty minutes to kill vegetative cells. Surviving spores were then plated onto Wynne's agar (Difco). Plates were incubated anaerobically for forty-eight hours, as above.

Statistical Analyses

A. Bacterial Population Effects: Estimates of bacterial numbers, by bacterial type, organ, treatment and age were obtained by averaging two replicate counts determined at each of seven ages, for treatment and control birds. The significance of treatment differences was tested by the Wilcoxon matched-pair, signed rank analysis (Siegel, 1956). This method provided a test of two variables of interest--the constancy (repeatability) of a treatment difference over time, and a weighted estimate of the relative importance of deviations from control values in repeated determinations.

B. Treatment Weight Differences: Relative growth rates of control and treatment birds were a secondary concern of experiment one. Weight data collected reflect this emphasis and are, accordingly, limited. As a result, two modifications of the ordinary "t" test were required to accommodate heterogeneous variance components and

unequal coefficients of variation. The adjustments of Lohrding (1969) and Gill (1971) were required in the former case, and that of Aspin and Welch (1949) in the latter.

Experiment Two:

A. Treatment Design

Treatment Design	Number of Chicks
Control	150
CTC 50 g./t.	150
CTC 100 g./t.	150
CTC 200 g./t.	150
Bacitracin 50 g./t.	150
Bacitracin 100 g./t.	150
Total	900

B. Bacterial Sampling

Bacterial Population	[1]Samples	[2]Reps.	Trts.	Total Cts.
Lactobacilli	8	5	6	240
Coliforms	8	5	6	240
Enterococci	8	5	6	240
Clostridia	8	5	6	240
Total Counts				960

[1] birds were examined at 1,7,14,21,35,42 and 49 days
[2] counts were made from each of five birds, per treatment, per day (30 individual bird examinations per day)

C. [1]Weight and Feed Efficiency

Treatment	Individual Weight Determinations by Age (Days) 1	14	28	42	52	Total
Control	150	100	100	100	100	550
CTC 50 g./t.	150	100	100	100	100	550
CTC 100 g./t.	150	100	100	100	100	550
CTC 200 g./t.	150	100	100	100	100	550
Bacitracin 50 g./t	150	100	100	100	100	550
Bacitracin 100 g./t.	150	100	100	100	100	550
Total						3300

[1] feed consumption was determined at 14,28,42 and 52 days of age.

Birds, Management and Diets

Nine hundred unsexed White Mountain strain Hubbard broiler chicks were used in experiment two. They were obtained from a commercial hatchery at one day of age. One hundred-fifty chicks were randomly assigned to each of six treatment groups, and to one control group. Chicks were reared at the MSU Poultry Science Teaching and Research Center in contiguous, wire partitioned, concrete floor pens. Prior to placement of chicks, all pens were thoroughly cleaned, disinfected and fumigated. Wood shaving litter was provided and feed and water were available ad libitum. TC broiler starter ration (Table 27) was obtained from a commercial feed mill and was fed the entire seven and one-half weeks of the experiment.

Antibiotics Fed

Respective treatment groups were fed either CTC or bacitracin MD. CTC was added at levels of fifty, one-hundred and two-hundred grams per ton; bacitracin was added at fifty and one-hundred grams per ton levels. All levels of both antibiotics were fed continuously from one to fifty-three days of age. In addition, a commercial coccidiostat, amprolium-plus, was added to control and antibiotic-supplemented rations at the recommended level of one pound concentrate per ton of finished feed.

Bacteriological Sampling

A. Collection and Preparation of Samples: Birds were collected and examined, as outlined under "Bacterial Sampling", above. Five birds from treatment and control groups were randomly selected at weekly

intervals from one to eight weeks of age. Sacrifice and organ collection followed the procedure of experiment one, except that only the distal two-thirds of the small intestine (jejunum and ileum) were included. Each of the thirty birds selected on a given day were examined individually, rather than as treatment pools.

B. Bacterial Populations, Culture and Count Methods: Four bacterial populations (lactobacilli, coliforms, enterococci and sulfite-reducing clostridia) were characterized. The former three groups were grown on the same media as in experiment one. Sulfite-reducing clostridia were distinguished from the more general anaerobic spore populations of experiment one. These were grown on sulfite-polymyxin-sulfadiazine (SPS) agar (Difco). Anaerobiosis for lactobacilli and clostridial counts was achieved with CO_2 and an anaerobic incubator, rather than the anaerobic jar previously employed.

Weight and Feed Efficiency Determinations

Individual chick weights were determined at 0 (one day), 2,4,6 and 7 1/2 weeks of age. Day old weights were determined for the entire 150 bird treatment group. Subsequent weights were determined for random 100 bird samples in each of the six treatment groups. Feed consumption and feed efficiency determinations accompanied weight determinations.

Statistical Analyses

A. Bacterial Counts: Five individual bird counts, per treatment, were averaged, for each bacterial population, in each age group. Treatment effects, expressed as $\log_{10}$ bacteria per gram differences

from controls, were analyzed by the Wilcoxon matched-pair, signed ranks method. Count differences between varying levels of each antibiotic were analyzed similarly.

B. Treatment Weight Differences: One-way analysis of variance was employed to detect treatment effects in each of four successive growth periods (0-2 weeks, 2-4 weeks, 4-6 weeks and 6- 7 1/2 weeks).

C. Growth and Antibiotic-Related Bacterial Count Effects: The relationship of bacterial population effects and accompanying growth responses in antibiotic supplemented chicks was tested by regression analysis. Bacterial population effects and growth effects were both related to control values. The former were expressed as log $_{10}$ per gram count differences from controls. The latter were expressed as percent differences in body weight gains of antibiotic supplemented versus control chicks. Growth stimulation from antibiotic supplementation is supposed to depend on effects on intestinal bacteria. Therefore, bacterial count differences in antibiotic supplemented birds were treated as independent variables in the analyses, while accompanying growth responses were treated as dependent variables.

This type analysis depends on repeated point comparisons of dependent and independent variables, and gains strength proportionate to the number of covariate values determined. The design of experiment two provided bacterial count data for seven consecutive weeks, but only four consecutive bi-weekly growth response determinations. Missing growth data were then extrapolated from growth curves to provide seven covariates for regression analysis.

Experiment Three:

A. Treatment Design

Treatment	Birds male	Birds female	Replicates	Total
Control	25	25	2	100
CTC 50 g./t.	25	25	2	100
CTC 100 g./t.	25	25	2	100
CTC 200 g./t.	25	25	2	100
Bacitracin 50 g./t.	25	25	2	100
Bacitracin 100 g./t.	25	25	2	100
Bacitracin 200 g./t.	25	25	2	100
Total Birds				700

B. Bacterial Sampling

Bacterial Population	[1]Samples	[2]Reps.	Trts.	Total Cts.
Total Anaerobes	5	2	7	70
Lactobacilli	5	2	7	70
Coliforms	5	2	7	70
Enterococci	5	2	7	70
Clostridia	5	2	7	70
Total Counts				350

[1] birds were examined at 12, 20, 27, 34 and 41 days

[2] one male and one female were pooled from each treatment pen; two replicate treatment pools were examined separately

C. Weight and Feed Efficiency

Treatment	[1] Samples male	female	Reps.	Total
Control	5	5	2	20
CTC 50 g./t.	5	5	2	20
CTC 100 g./t.	5	5	2	20
CTC 200 g./t.	5	5	2	20
Bacitracin 50 g./t.	5	5	2	20
Bacitracin 100 g./t.	5	5	2	20
Bacitracin 200 g./t.	5	5	2	20

[1] Pen weights, by sex, treatment and replicate, were recorded at 12, 20, 27 and 34 days; individual weights by sex, treatment and replicate were determined at day 41. Feed consumption was recorded simultaneously.

Birds, Management and Diets

Three hundred-fifty each of male and female White Mountain strain Hubbard broiler chicks were used in experiment three. Twenty-five birds of each sex were randomly assigned to each of fourteen floor pens, to include two replicates of each of seven treatments, including controls. TC broiler ration (Table 27) was provided ad libitum to forty-two days of age.

Antibiotics Fed

Commercial feed-grade CTC and bactracin MD were added to treatment rations singly, and at levels of 50, 100 and 200 grams per ton of antibiotic activity in the finished feed.

As in experiment two, amprolium-plus was included in all diets at the manufacturer-specified level.

Bacteriological Sampling

A. Collection and Preparation of Samples: One male and one female were randomly selected from each pen at 12, 20, 27, 34 and 42 days of age. Sacrifice and collection of the distal segments of the small intestine (jejunum and ileum) followed the procedures of experiment two. Treatment/replicate pairs were combined to yield a total of fourteen organ pools per day. The two counts thus obtained per treatment were then averaged to obtain bacterial counts. Anaerobic diluting solution (ADS) was used as a diluent rather than distilled water, in order to facilitate recovery of fastidious, strictly anaerobic bacteria.

B. Bacterial Populations, Culture and Count Methods: Five bacterial populations (strict anaerobes, lactobacilli, coliforms, enterococci and sulfite-reducing clostridia) were characterized and enumerated. Anaerobic count plates were prepared immediately after organ collection, maceration and dilution. Standardized inocula of appropriate gut dilutions were introduced into sterile, pre-reduced bottles of reinforced clostridial medium (RCM), under a continuous stream of oxygen-free CO_2. The bottles were then stoppered anaerobically and were incubated at 37° C. for seventy-two hours. Counts of lactobacilli, coliforms, enterococci and clostridia were made as in experiment two. The pour-plate technique was adapted for anaerobic counts in bottles. Counts of other populations were made by the drop-plate method of Miles and Misra (1938).

Weight and Feed Efficiency Determinations

Weight and feed efficiency determinations followed the design outlined above in "Weight and Feed Efficiency". Pretreatment weights of all chicks were recorded at day one, prior to pen assignment. Thereafter, pen weights, by sex, treatment and replicate, were determined at 12, 20, 27 and 34 days of age. Individual weights were again recorded at 42 days of age, at the termination of the experiment. Weight and feed consumption determinations were made concurrently.

Statistical Analyses

A. Bacterial Population Counts: Replicate two-bird pool counts were averaged for statistical analyses. Treatment effects (expressed as $\log_{10}$ count differences from controls) were analyzed by the Wilcoxon match-pair, signed rank method. Sulfite-reducing clostridia were encountered so infrequently in experiment three that antibiotic treatment effects on their numbers could not be analyzed.

B. Treatment Weight Gains: Mean body weight differences in treatment and control chicks were analyzed by "t" tests at one and forty-one days of age. Interim pen weight growth effects were tested by Chi-square analyses. Two methods were employed in Chi-square tests of treatment growth effects. Individual levels of each antibiotic were compared with controls (i.e. CTC 100 g./t. gains versus control gains).

Further, combined weight gains of birds on three levels of each antibiotic were compared to corresponding control gains (i.e. CTC 50, 100, and 200 g./t. gains versus control gains). All test statistics were derived from expected values for treatment and control gains,

combined.

C. Relating Bacterial Population Changes to Growth and Feed Efficiency Effects: Three variables--bacterial count differences, growth response and feed efficiency response, were determined simultaneously in experiment three. The influences of bacterial population changes on growth and feed efficiency were determined by regression analysis of corresponding covariates, bacterial shifts being treated as independent variables and growth and feed efficiency responses being treated as dependent variables. As in experiment two, bacterial effects were expressed as $\log_{10}$ differences from control values and growth and feed efficiency responses were expressed as percent differences from control values.

RESULTS

Experiment One

Tables 1 through 6 summarize treatment responses of total anaerobes, total aerobes, lactobacilli, coliforms and anaerobic spores, respectively, to CTC 50 g./t. selection. Each of the table values is the average of two five-organ pools. The entire study included 120 birds--60 each of control and antibiotic supplemented chicks.

Control counts, as $\log_{10}$ transformations of bacteria per gram wet weight, fall within the range of normal values, as previously reported in the literature. The responses of intestinal bacteria to CTC selection varied with age, bacterial population, and with anatomical site from the small intestine, caeca and large intestine.

Total anaerobes (Table 1) tended to increase with CTC supplementation. However, the increases were irregular and varied from site to site in the gut. Mean treatment responses (as $\log_{10}$ differences from controls) ranged from +0.2 in the small intestine, to +0.1 and +0.5 in the caeca and large intestine, respectively. None of the treatment responses of total anaerobes to CTC were significant. Mean treatment responses, though slight, were consistently positive in all sites of the gut.

Total aerobe responses (Table 2) to CTC selection, as reflected in mean responses, varied from the small intestine, where they were

reduced, to increases in the caeca and large intestine. The responses of this group to CTC tended to be more consistent than corresponding anaerobe effects. Neither small intestinal decreases, nor increases in the caeca and large intestine attained significance at the $P < .05$ level. Further, organ treatment responses, though they differed in net effects, did not differ substantially from control values.

Lactobacilli responses to CTC (Table 3) were positive in all sites of the gut. Relative increases, as reflected in count differences, were most consistently positive, and were most pronounced, in the small intestine, followed by caecal and large intestinal responses, respectively. Both small intestinal and caecal responses were significant ($P < .05$). Large intestinal responses were slighter, more irregular, and failed to attain significance. Mean treatment responses varied in degree from the small intestine, to the caeca and large intestine, respectively.

Coliform responses to CTC selection are summarized in Table 4. Organ differences in response are apparent, and range from net $\log_{10}$ decreases (-0.7) in the small intestine, to increases of +0.3 and +0.2 in the caeca and large intestine, respectively. None of the coliform responses attained significance.

Enterococci responses to CTC are summarized in Table 5. Organ differences are apparent, though mean responses over 70 days are small. Effects in the small intestine were more consistent, and were more pronounced than corresponding effects in the caeca and large intestine, as reflected in mean responses of +0.3, +0.2 and 0.0, respectively.

Anaerobic spore responses (Table 6) were consistently positive in all sites of the gut. As with total anaerobes, lactobacilli, and

enterocci, the mean response was most pronounced in the small intestine, versus the caeca and large intestine. The mean treatment response of anaerobic spores in the small intestine (+1.1) was significant ($P < .05$), whereas corresponding effects in other sites were not. Further, treatment responses of anaerobic spores in the large intestine are notable for their lack of consistency, as compared to responses in the small intestine and caeca.

Total anaerobe, lactobacilli, enterococci and anerobic spore increases with CTC selection were consistent from the small intestine to the caeca and large intestine. Effects ranged from 0.0 (anaerobic spores in the caeca) to +1.1 (anaerobic spores in the small intestine). Coliform and total aerobe net responses varied from the small intestine, where they were reduced, to the caeca and large intestine, where they increased. Three significant treatment responses were observed in the small intestine, as increases in lactobacilli, enterococci and anaerobic spores. Only one significant effect (lactobacilli increase) was observed in the caeca, while none of the treatment responses in the large intestine attained significance.

The relative importance of small intestinal, versus caecal and large intestinal, effects is illustrated in Table 7. When consistent effects (total anaerobes, lactobacilli, enterococci and anaerobic spores) are compared, they are most pronounced in the small intestine. Further, increases in populations of the small intestine are more frequently significant than are corresponding increases in more distal sites of the gut. The variability of treatment responses, as reflected in C. V. %S, also tends to be less in the small intestine than elsewhere in the gut. This suggests that CTC effects on intestinal bacteria are

Table 1. [1]Chloretetracycline effects on total anaerobes in three sites of the gut

	Small Intestine			Caeca			Large Intestine		
Age (Days)	Control	CTC 50 g./t.	[2] d	Control	CTC 50 g./t.	d	Control	CTC 50 g./t.	d
7	10.6	10.0	-0.6	10.8	11.3	+0.5	8.7	11.3	+2.6
14	6.8	7.5	+0.7	8.8	8.8	0.0	8.3	8.3	0.0
21	7.2	6.7	-0.5	8.2	8.4	+0.2	8.3	8.0	-0.3
28	7.3	7.5	+0.2	8.8	8.8	0.0	8.3	8.3	0.0
42	6.7	7.5	+0.8	7.3	7.6	+0.3	7.2	7.5	+0.3
70	7.0	7.4	+0.4	7.8	7.5	-0.3	7.3	7.4	+0.1
Average Count	7.6	7.8		8.6	8.7		8.0	8.5	
[3]Mean Response			+0.2			+0.1			+0.5

[1]
[2]All counts are expressed as base 10 logs of bacteria per gram wet weight
[2]log 10 treatment difference (CTC 50 g./t. minus control)
[3]average treatment response expressed as log 10

Table 2. [1]Chlortetracycline effects on total aerobes in three sites of the gut

	Small Intestine			Caeca			Large Intestine		
Age (Days)	Control	CTC 50 g./t.	[2]d	Control	CTC 50 g.lt.	d	Control	CTC 50 g.lt.	d
7	9.8	9.1	-0.7	10.8	11.0	+0.2	8.2	10.1	+1.9
14	7.0	7.1	+0.1	7.5	7.8	+0.3	7.8	7.7	-0.1
21	6.8	4.9	-1.9	7.4	6.9	-0.5	7.7	7.0	-0.7
28	7.0	7.1	+0.1	7.5	7.8	+0.3	7.8	7.7	-0.1
42	6.7	7.5	+0.8	6.2	6.8	+0.6	6.2	7.0	+0.8
70	6.3	5.8	-0.5	6.9	7.5	+0.6	6.9	6.7	-0.2
Average Count	7.3	6.9		7.7	8.0		7.4	7.7	
[3]Mean Response			-0.4			+0.3			+0.3

[1]all counts are expressed as base 10 logs of bacteria per gram wet weight
[2]log 10 treatment difference (CTC 50 g.lt. minus control)
[3]average treatment response expressed as log 10

Table 3. [1]Chlortetracycline effects on lactobacilli in three sites of the gut

	Small Intestine			Caeca			Large Intestine		
Age (Days)	Control	CTC 50 g./t.	[2]d	Control	CTC 50 g./t.	d	Control	CTC 50 g./t.	d
7	7.9	9.1	+1.2	8.4	8.2	-0.2	8.4	8.1	-0.3
14	6.4	7.4	+1.0	7.5	8.3	+0.8	7.5	7.8	+0.3
21	7.3	6.9	-0.4	7.8	8.1	+0.3	7.8	8.2	+0.4
28	6.9	7.4	+0.5	7.5	8.2	+0.7	7.5	7.8	+0.3
42	6.9	7.3	+0.4	7.6	7.5	-0.1	7.6	7.5	-0.1
70	7.2	7.7	+0.5	7.6	8.0	+0.4	7.6	7.3	-0.3
Average Count	7.1	7.6		7.7	8.1		7.7	7.8	
[3]Mean Response			+0.5			+0.3			+0.1

[1] all counts are expressed as base 10 logs of bacteria per gram wet weight
[2] $\log_{10}$ treatment difference (CTC 50 g./t. minus control)
[3] average treatment response expressed as log 10

Table 4. [1]Chlortetracycline effects on coliforms in three sites of the gut

	Small Intestine			Caeca			Large Intestine		
Age (Days)	Control	CTC 50 g./t.	[2]d	Control	CTC 50 g./t	d	Control	CTC 50 g./t.	d
7	8.3	8.5	+0.2	7.9	10.8	+2.9	8.0	9.3	+1.3
14	5.1	3.3	-1.8	7.5	6.8	-0.7	6.6	6.7	+0.1
21	5.4	3.5	-1.9	7.1	6.8	-0.3	6.6	5.7	-0.9
28	5.1	3.3	-1.8	7.5	6.8	-0.7	6.6	6.7	+0.1
42	3.0	4.0	+1.0	6.4	6.8	+0.4	6.6	6.7	+0.1
70	4.1	4.4	+0.3	6.4	6.6	+0.2	6.0	6.2	+0.2
Average Count	5.2	4.5		7.1	7.4		6.7	6.9	
[3]Mean Response			-0.7			+0.3			+0.2

[1] all counts are expressed as base 10 logs of bacteria per gram wet weight
[2] $\log_{10}$ treatment difference (CTC 50 g./t. minus control)
[3] average treatment response expressed as $\log_{10}$

Table 5. [1]Chlortetracycline effects on enterococci in three sites of the gut

	Small Intestine			Caeca			Large Intestine		
Age (Days)	Control	CTC 50 g./t.	[2]d	Control	CTC 50 g./t.	d	Control	CTC 50 g./t.	d
7	5.9	6.0	+0.1	7.3	6.7	-0.6	6.5	6.5	0.0
14	3.7	3.9	+0.2	5.5	5.9	+0.4	4.7	4.9	+0.2
21	4.0	3.7	-0.3	5.1	5.1	0.0	5.1	4.5	-0.6
28	3.7	3.8	+0.1	5.5	5.8	+0.3	4.7	4.9	+0.2
42	3.0	3.9	+0.9	4.9	5.7	+0.8	5.0	5.3	+0.3
70	3.1	3.9	+0.8	3.9	4.1	+0.2	4.5	4.3	-0.2
Average Count	3.9	4.2		5.4	5.6		5.1	5.1	
[3]Mean Response			+0.3			+0.2			0.0

[1] all counts are expressed as base 10 logs of bacteria per gram wet weight
[2] $\log_{10}$ treatment difference (CTC 50 g./t. minus control)
[3] average treatment response expressed as $\log_{10}$

Table 6. [1] Chlortetracycline effects on anaerobic spores in three sites of the gut

	Small Intestine			Caecca			Large Intestine		
Age (Days)	Control	CTC 50 g./t.	[2]d	Control	CTC 50 g./t	d	Control	CTC 50 g./t.	d
7	4.2	4.4	+0.2	8.2	3.7	-4.5	6.5	3.5	-3.0
14	3.9	6.0	+2.1	3.9	6.8	+2.9	4.7	6.7	+2.0
21	6.0	7.3	+1.3	8.2	8.4	+0.2	5.1	7.8	+2.7
28	4.5	5.2	+0.7	4.3	4.4	+0.1	4.7	4.4	-0.3
42	4.5	4.8	+0.3	5.3	5.5	+0.2	5.0	5.5	+0.5
70	4.3	6.2	+1.9	5.5	6.8	+1.3	4.5	5.7	+1.2
Average Count	4.6	5.7		5.9	5.9		5.1	5.6	
[3]Mean Response			+1.1			0.0			+0.5

[1] all counts are expressed as base 10 logs of bacteria per gram wet weight
[2] $\log_{10}$ treatment difference (CTC 50 g./t. minus control)
[3] average treatment response expressed as $\log_{10}$

Table 7. Variability of bacterial responses to CTC with site in the gut

Bacterial Population		Small Intestine			Caeca			Large Intestine		
		X	S.E.	C.V.%	X	S.E.	C.V.%	X	S.E.	C.V.%
[1] Anaerobes		+0.2	.24	295	+0.1	.11	280	+0.5	.44	214
Aerobes		-0.4	.34	120	+0.3	.16	133	+0.3	.38	310
[1] Lactobacilli	**	+0.5	.23	112	** +0.3	.17	137	+0.1	.13	320
Coliforms		-0.7	.53	187	+0.3	.55	450	+0.2	.29	350
[1] Enterococci	**	+0.3	.18	153	+0.2	.19	235	0.0	.13	---
[1] Anaerobic Spores	**	+1.1	.33	74	0.0	1.01	---	+0.5	.82	404

[1] average log $_{10}$ treatmdnt response, as compared to control values, based on 12 determinations, six age groups, and 60 each of treatment and control birds.

[2] coefficient of variation, expressed as a per cent of the mean response

[1] bacterial populations for which consistent treatment responses were observed in the small intestine, caeca and large intestine

**treatment response significant ($P < .05$)

more consistent, are more pronounced and are less variable in the small intestine than are corresponding effects in the caeca and large intestine.

This concept was developed further, as illustrated in Table 8. Net responses of each of the six bacterial populations to CTC were accorded a rank of from one to three, depending on the relative magnitude of their responses in the small intestine, caeca and large intestine. Resulting response rank sums for all six populations indicate the relative degree of effect bacterial populations showed to CTC, as it varies with site in the gut. Rank sums of 7, 14.5 and 14.5 were obtained for all six populations in the small intestine caeca, and large intestine, respectively. Response summations for the four populations with consistent response signs were 5.0, 10.0 and 9.0 in the small intestine, caeca and large intestine, respectively. Results of experiment one strongly suggest that the selective actions of CTC on several components of normal intestinal flora are exerted most rigorously in the small intestine, rather than in more distal sites of the gut.

Table 9 summarizes growth responses of control and CTC supplemented chicks, from 21 to 81 days of age. In three comparisons, significant ($P < .05$) weight increases accompanied CTC supplementation. Conversely, 29 and 42 day weights of control chicks exceeded those of treatment birds. Generally, however, CTC supplementation improved the growth of supplemented chicks, though the response varied in repeated comparisons.

Table 8. Comparative treatment responses in three sites of the gut

Bacterial Population	Small Intestine [1] d	[2] response rank	Caeca d	response rank	Large Intestine d	response rank	Three-Organ Net Response
Anaerobes	+0.2	2	+0.1	3	+0.5	1	+0.3
Aerobes	-0.4	1	+0.3	2.5	+0.3	2.5	+0.1
Lactobacilli	* +0.5	1	* +0.3	2	+0.1	3	+0.3
Coliforms	-0.7	1	+0.3	2	+0.2	3	-0.1
Enterococci	* +0.3	1	+0.2	2	0.0	3	+0.2
Anaerobic Spores	* +1.1	1	0.0	3	+0.5	2	+0.5
[3] Response Rank Sum (all populations)		7.0		14.5		14.5	
[4] Response Rank Sum (consistent effect populations)		5.0		10.0		9.0	

[1] average log $_{10}$ difference from control counts, averaged from sixty birds, 7-70 days of age

[2] d values for each population and organ were ranked by degree of response to CTC. Values of 1 were assigned to largest organ responses, etc.

[3] a reflection of degree of response by bacterial populations, as it varies with site in the gut

[4] rank sum of anaerobes, lactobacilli, enterococci and anaerobic spores

* treatment effects, as reflected in count differences from controls, significant ($P<.05$)

Table 9. Growth response to antibiotic supplementation

Age (Days)	Control Wt. (g.)	CTC 50 g./t. Wt. (g)	percent response
21	166.2	183.8	* +10.6
29	268.0	246.7	* - 7.9
42	450.7	417.4	- 7.3
70	735.9	790.8	* + 7.4
81	860.9	908.5	* + 5.5

* weights differ significantly ($P < .05$)

Experiment Two

Bacterial Responses:

Each of the four bacterial populations studied (lactobacilli, coliforms, enterococci and clostridia) was monitored continuously to determine the responses of each to continuous antibiotic selection, and to detect antibiotic and treatment level differences in the response of each. The response of each population to CTC and B-MD selection is presented, by population, below.

Lactobacilli responses to CTC and B-MD selection are summarized in Table 10. Control lactobacilli counts were fairly constant in repeated determinations and averaged (log $_{10}$ per gram wet weight) 7.67 over the 49 day experimental period. Responses of lactobacilli, as reflected in log $_{10}$ count differences from controls, ranged from increases of +1.51 (CTC gO g./t. at 42 days), to reductions of -1.58 (B-MD 50 g./t. at 28 days). The range of observed effects translates to an approximately 1,000 fold difference in counts, from extreme lows to highest counts.

Responses of lactobacilli to antibiotic selection differed from CTC to B-MD, both in the direction of their population shifts, and in the constancy of the responses in repeated determinations. CTC at all three levels tended to slightly increase intestinal lactobacilli, as compared to controls. The increase was most pronounced at 50 g./t., and diminished in both constancy and net effect as CTC selection pressure was increased to 100 and 200 g./t.

Fifty and one hundred grams per ton of B-MD essentially precluded lactobacilli in the small intestine for up to 21 days of age. Thereafter, their numbers tended to be substantially, and consistently, reduced from control numbers. Forty-two day count differences between B-MD treatments and controls may be more a reflection of unusually low control counts than of real numerical increases in lactobacilli in B-MD supplemented chicks. In any event, B-MD clearly exerted a strong suppressing effect on intestinal lactobacilli. This is even more evident when the TFTC values of 50 and 100 g./t. B-MD groups at 7 and 14 days of age are taken into account. Reductions of lactobacilli were consistent with both levels of B-MD, and appear to be dose related. Reductions, as mean treatment responses, and in statistical significance, were more pronounced at 100 g./t. than at 50 g./t.

Coliform responses to antibiotic selection are summarized in Table 11. All levels of both CTC and B-MD resulted in increased coliform counts, as compared to controls. Within levels of CTC, increasing dose levels of from 50 to 100 to 200 g./t, produced correspondingly dose related increases in coliforms. Further, the constancy (as response sign differences) of the effect increased with increasing dose levels of CTC selection pressure. However, none of the coliform increases were sufficient to attain significance at the $P < .05$ level.

Similarly, coliforms increased with 50 and 100 g./t. B-MD selection. As with CTC, the response (as mean treatment response) appeared to be dose related. Increases in B-MD supplemented chicks were significant ($P < .05$) at the 100 g./t. level.

Table 10. Responses of intestinal lactobacilli to antibiotic selection

Age (Days)	Control count	CTC 50 g./t. response	CTC 100 g./t. response	CTC 200 g./t. response	B-MD 50 g./t. response	B-MD 100 g./t. response
7	7.56	TFTC	+1.36	TFTC	[1] TFTC	TFTC
14	7.99	+0.49	-0.74	-0.21	TFTC	TFTC
21	7.07	-0.09	-0.60	-0.04	-0.78	-0.81
28	8.35	+0.18	-0.04	+0.65	-1.58	-1.20
35	8.32	+0.36	-0.62	-0.75	-1.36	-1.04
42	6.84	+1.51	+0.83	+1.36	+1.44	+0.35
49	7.56	-1.11	+0.16	-0.15	-0.22	-1.23
Average Count	7.67	7.91	7.72	7.83	7.13	6.84
Mean Treatment Response		+0.22	+0.05	+0.14	-0.50	* -0.79

[1] reductions in lactobacilli in B-MD supplemented chicks at 7 and 14 days were greater than -0.56 and -3.5, respectively, as deduced from lowest dilutions at which no lactobacilli were counted.

* Response significant ($P < .05$)

Table 11. Responses of intestinal coliforms to antibiotic selection

Age (Days)	Control count	CTC 50 g./t. response	CTC 100 g./t. response	CTC 200 g./t. response	B-MD 50 g./t. response	B-MD 100 g.lt. response
7	TFTC	----	----	----	----	----
14	5.67	+1.00	-0.78	+0.27	+0.33	+1.27
21	6.57	-0.83	-1.52	-0.33	-1.05	-1.09
28	5.96	-0.07	+1.36	+0.23	-0.08	+0.88
35	5.54	-0.04	+1.81	+2.06	+1.54	+2.12
42	5.50	+0.50	+1.30	+1.72	+1.56	+1.73
49	6.09	+0.15	+0.40	-0.48	+0.67	+0.94
Average Count	5.89	5.91	6.24	6.47	6.35	6.70
Mean Treatment Response		+0.12	+0.43	+0.58	+0.50	* +0.80

* Response significant (P<.05)

Small intestine enterococci responses, as reflected in treatment counts and corresponding control counts, appeared to vary with antibiotic, and dose level. Enterococci generally appeared to be quite refractory to selection with either antibiotic (Table 12). The net effect of varying levels of each antibiotic, as reflected in mean treatment responses, differed between dose levels with CTC and B-MD. A slight, though consistent increase in enterococci with B-MD 100 g./t. was significant ($P < .05$).

Clostridia occurred sporadically in both control and treatment groups. Further, their average counts, when they were obtained, were very low, relative to other bacterial populations studied. (Table 13) shows that clostridia occurred most frequently in controls, and, where detected, were less common in antibiotic supplemented chicks. So few counts of this population were obtained, however, that no statistical interpretation of treatment effects could be derived from the data.

Table 12. Responses of intestinal enterococci to antibiotic selection

Age (Days)	Control count	CTC 50 g./t. response	CTC 100 g./t. response	CTC 200 g./t. response	B-MD 50 g./t. response	B-MD 100 g./t. response
7	6.05	TFTC	-1.15	-1.38	-0.55	+0.07
14	6.36	+0.50	-0.53	-1.01	+0.06	+0.59
21	6.20	-0.14	-1.05	-0.28	-0.53	-0.11
28	6.27	+0.62	+0.92	+0.51	+0.30	+0.80
35	7.50	-0.18	-0.30	+0.21	+0.14	+0.10
42	6.94	-0.26	+0.57	+0.47	-0.10	+0.60
49	6.96	-0.12	+0.08	-0.09	-0.26	+0.15
Average Count	6.61	6.78	6.40	6.39	6.48	6.93
Mean Treatment Response		+0.07	-0.21	-0.22	-0.13	* +0.31

* Response significant ($P < .05$)

Table 13. Responses of intestinal clostridia to antibiotic selection

Age (Days)	Control count	CTC 50 g./t. response	CTC 100 g./t. response	CTC 200 g./t. response	B-MD 50 g./t. response	B-MD 100 g./t. response
7	TFTC	----	----	----	----	----
14	2.67	-0.42	TFTC	TFTC	TFTC	TFTC
21	2.33	TFTC	TFTC	TFTC	TFTC	TFTC
28	TFTC	----	----	----	----	----
35	2.41	-0.45	-0.16	-0.90	TFTC	TFTC
42	2.03	+0.82	+0.11	+1.27	TFTC	TFTC
49	TFTC	----	----	----	----	----
Average Count	2.36	2.31	2.33	2.41	1.52	

NUTRITIONAL EFFECTS

Growth: Growth responses of treatment and control chicks are summarized in Table 14. All treatment groups showed improved growth as compared to control. Antibiotic type, and level differences, were observed however. Chlortetracycline effects were significant ($P<.05$) in the first and third growth periods. Bacitracin effects differed in that they attained significance in the first two growth periods. No treatment responses from 6 to 7 1/2 weeks of age differed significantly from controls.

Low, intermediate and high levels of CTC supplementation produced net (7 1/2 weeks) weight increases of 24, 35 and 146 grams, respectively. The growth responses to both levels of B-MD, and to 200 g./t. CTC, tended to be fairly constant in repeated determinations, as reflected in responses (R) and response changes from previous performance (R.C.) Responses of 50 and 100 g./t. levels of CTC varied in repeated determinations, and appeared to be maximal in the 4 to 6 week growth period. After six weeks, growth responses to these levels of CTC diminished to below corresponding growth in control chicks. All levels of both CTC and B-MD appeared to contribute substantially to the growth of supplemented chicks. B-MD at 50 and 100 g./t., and CTC at 200 g./t.,

Table 14. Growth responses of antibiotic supplemented chicks

Age (weeks)	Control B.W.[1]	CTC 50 g./t. B.W.	R[2]	R.C.[3]	CTC 100 g./t. B.W.	R	R.C.	CTC 200 g./t. B.W.	R	R.C.	B-MD 50 g./t. B.W.	R	R.C.	B-MD 100 g./t. B.W.	R	R.C.
0	*41[a]	41[a]			41[a]			41[a]			41[a]			41[a]		
2	263[a]	266[b]	+ 3		263[b]	0		271[b]	+ 8		269[b]	+ 6		276[b]	+13	
				0			+10			+21			+7			+18
4	759[a]	762[a]	+ 3		769[a]	+10		788[a]	+ 29		772[b]	+13		790[b]	+31	
				+35			+51			+24			+27			-14
6	1383[a]	1421[b]	+38		1444[b]	+61		1436[b]	+ 53		1423[a]	+40		1400[a]	+17	
				-14			-26			+93			+ 9			+53
7 1/2	1864[a]	1888[a]	+24		1899[a]	+35		2010[a]	+146		1915[a]	+51		1934[a]	+70	

1 Average body weight, in grams
2 Antibiotic response, expressed as gram difference from controls
3 Response change from previous period
* Average body weights with different superscripts differ significantly from control ($P < .05$). Note: Differences, as reflected in mean weights, represent drug differences, to include three levels of CTC and two levels of B-MD.

produced constant, positive, responses to 7 1/2 weeks of age, whereas responses to 50 and 100 g./t. levels of CTC appeared to diminish after six weeks of continuous supplementation. Net treatment effects may be the result of carryover of weight from previous high performance periods, or may be the result of continuous growth stimulation.

Bacterial Response/Growth Response Interactions:

Twenty-one regression analyses of bacterial responses and accompanying growth responses in CTC and B-MD supplemented chicks are summarized in Table 15. All levels of both CTC and B-MD stimulated chick growth, as compared to controls. Bacterial responses in lactobacilli, coliforms, and enterococci were in some cases consistent (coliforms) within and between antibiotics, and in other cases (lactobacilli, and enterococci) were inconsistent. The several analyses, when compared by group, indicate the relative contribution each bacterial population makes to observed growth responses with CTC and B-MD.

Lactobacilli: All three levels of CTC and both levels of B-MD improved growth, but the mean response of lactobacilli differed between the two antibiotics. Lactobacilli were slightly increased in CTC fed birds, but were substantially reduced in B-MD supplemented chicks. Regression analyses within levels of CTC suggest that lactobacilli increases in CTC 50 g./t. supplemented chicks contributed materially to corresponding positive growth responses of the group. The same relationship at higher (100 and 200 g./t.) levels of CTC showed that the contribution of lactobacilli to growth in CTC fed chicks is inconsistent, and is peculiar to low (50 g./t.) levels of

CTC selection.

The response of lactobacilli to B-MD selection, and the accompanying growth responses in these two treatment groups, suggests that lactobacilli numbers do not relate positively (contribute to) accompanying positive growth responses in B-MD supplemented chicks.

Lactobacilli, by virtue of their inconstant relationships to growth of CTC supplemented chicks, and by virtue of their inconsistent relationships to growth of CTC and B-MD supplemented birds, appear not to contribute materially to common positive growth stimulating properties of CTC and B-MD. Lactobacilli responses to antibiotic selection appear, on the contrary, to be unrelated to growth.

Coliforms: Dose related increases in coliforms with all levels of CTC and B-MD accompanied corresponding dose-related growth responses. However, regression analyses of coliform/growth responses in CTC supplemented chicks failed to demonstrate a consistent positive relationship. Similarly, varying dose levels of B-MD again revealed that the relationship of coliform numbers to growth in B-MD supplemented chicks is not constant or consistent with growth responses with increased dose levels. Taken together, the analyses of coliform and growth responses in CTC and B-MD supplemented chicks suggest that coliforms do not contribute materially to positive growth stimulation with these antibiotics.

Enterococci: Enterococci numbers were reduced slightly in CTC supplemented chicks, and B-MD 50 g./t. supplemented chicks. They tended to increase, however, with B-MD at 100 g./t. Regression analyses of enterococci/growth responses within and between CTC and B-MD show inconsistent relationships of enterococci responses to growth

responses. This inconsistent relationship to growth, in the face of constant, linear growth responses with increasing levels of CTC and B-MD, suggests that enterococci do not figure significantly in common growth stimulating properties of CTC and B-MD.

We interpret the findings of the several regression analyses of experiment two as suggesting that neither lactobacilli, coliforms or enterococci contribute to the constant, linear growth responses observed with varying levels of CTC and B-MD. One significant relationship (Lactobacilli and CTC 50 g./t. growth responses) was observed. This observation, if taken at face value, would support the conclusions of other investigators that increases in lactobacilli that may accompany antibiotic supplementation, are casually related to accompanying growth responses. Results of B-MD growth/lactobacilli responses, and 200 g./lt. CTC growth/lactobacilli response analyses tend to refute this conclusion.

Table 15. [1]Relation of growth to bacterial responses in the antibiotic fed chick

Bacterial Population	CTC				B-MD		
	50 g./t.	100 g./t.	200 g./t.	50+100+200 g./t.	50 g./t.	100 g./t.	50+100 g./t.
Lactobacilli							
[2] Bact. Resp.	+0.34	+0.05	+0.09	+0.14	-1.42	-1.85	-1.64
[3] Gr. Resp.	+1.56	+2.34	+7.07	+3.69	+3.93	+4.70	+4.31
[4] R	*+ .87	+ .28	- .13	+ .07	- .43	- .44	- .44
[5] C.V.	.75	.75	.75	.43	.75	.75	.53
Coliforms							
Bact. Resp.	+0.12	+0.45	+0.53	+0.37	+0.57	+0.91	+0.74
Gr. Resp.	+1.56	+2.34	+7.07	+3.69	+3.93	+4.70	+4.31
R	+ .35	+ .37	- .73	- .06	+ .56	- .58	- .13
C.V.	.75	.75	.75	.43	.75	.75	.53
Enterococci							
Bact. Resp.	-0.14	-0.21	-0.20	-0.18	-0.13	+0.31	+0.09
Gr. Resp.	+1.56	+2.34	+7.07	+3.69	+3.93	+4.70	+4.31
R	+ .12	+ .04	- .20	- .09	- .39	- .43	-.26
C.V.	.75	.75	.75	.43	.75	.75	.53

[1] All bacterial and growth values are mean treatment responses, as determined from 7 to 49 days of age
[2] Mean bacterial response, expressed as log $_{10}$ difference from controls
[3] Mean growth response, expressed as percent growth response, relative to controls
[4] Regression coefficient (Bacterial responses independent; Growth responses dependent)
[5] Critical value of R ($P<.05$)

Feed Efficiency: Changes in cumulative feed conversions of CTC and B-MD supplemented chicks, as compared to controls, are summarized in Table 16. CTC and B-MD generally improved feed conversions from control values, as would be expected. Antibiotic differences, irrespective of dose level were obvious however. Both 50 and 100 g./t. levels of B-MD consistently improved feed utilization of chicks to which they were fed. In contrast, 200 g./t. of CTC substantially reduced the efficiency of this treatment group, as evidenced by consistently poorer performance (R-positive) than controls. Low level (50 g./t.) CTC showed a negligible net effect on conversions while 100 g./t. CTC improved conversions of chicks to which it was fed.

These results, when examined together with growth response data, suggest some interesting interactions. B-MD at both levels employed, produced both good and consistent responses in both growth and improvement in feed conversions. High level (200 g./t.) CTC, while showing substantial growth effects, appears to detract from the efficient conversion of feed. Low and intermediate CTC levels are consistent, and intermediate in their effects on growth and feed conversion. Fifty and 100 g./t. of CTC produced dose-related improvements in conversions and growth rates.

Table 16. [1]Antibiotic effects on feed conversion.

Age (weeks)	Control	CTC 50 g./t.		CTC 100 g/t.		CTC 200 g./t.		B-MD 50 g./t.		B-MD 100 g./t.	
	F.C.	F.C.	R.	F.C.	R.	F.C.	R.	F.C.	R.	F.C.	R.
2	1.24	1.27	+.03	1.22	-.02	1.39	+.15	1.20	-.04	1.18	-.06
4	1.77	1.76	+.01	1.83	+.06	1.85	+.08	1.62	-.15	1.77	N.C.
6	2.00	1.95	-.05	1.85	-.15	2.03	+.03	1.85	-.15	1.96	-.04
7 1/2	1.96			1.93	-.03	1.98	+.02	1.95	-.01	1.93	-.03
Response Sum			-.01		-.16		+.28		-.35		-.13
Response Mean			-.00		-.04		+.07		-.09		-.03

[1] Cumulative, to 7 1/2 weeks
[2] Response, as hundredths, as compared to controls

EXPERIMENT THREE

Bacterial Responses:

Tables 17, 18, 19 and 20 summarize treatment responses of anaerobes, lactobacilli, coliforms and enterococci, respectively, to CTC and B-MD selection.

Anaerobes: Total anaerobe populations were substantially and consistently reduced by all levels of CTC and B-MD (Table 17). However, counts at 12 days of age show a general lack of response. Thereafter, anaerobes were reduced from control levels. The net effect of antibiotic selection, as reflected in mean treatment responses, was significant ($P < .05$) with B-MD at levels of 100 and 200 g./t. Note the general "bowing" tendency of anaerobe responses to antibiotic selection, from 12 to 41 days of age. The results suggest a delayed initial response (12 days of age), followed by a sharp reduction in anaerobes from 20 to 34 days of age, and a recovery or adaptation to antibiotic selection from 35 - 41 days of age. This variable time response is important, as it relates to growth responses in the respective treatment groups. Regression analyses, to follow, will relate the variable response of bacteria to corresponding variation in growth responses of antibiotic supplemented birds.

Lactobacilli: All antibiotic treatments were accompanied by reductions in small intestinal lactobacilli. The effects, as reflected in mean treatment responses, were significant ($P < .05$) with CTC at

200 g./t., and with all levels of B-MD. As with total anaerobes, the treatment responses of lactobacilli varied with time and tended generally to diminish by 41 days.

Coliforms: Coliform increases, as compared to controls, generally accompanied antibiotic supplementation with CTC and B-MD, at all levels. Relative treatment differences varied considerably in repeated determinations, however, and none of the net effects were significant. Counts at 20 days unobtainable, due to a technical error.

Enterococci: Enterococci responses (Table 20) showed marked differences between B-MD and CTC, irrespective of dose level. Although slight decreases in enterococci accompanied CTC supplementation, the net response was slight and varied in repeated determinations. The small reductions in enterococci with CTC failed to achieve significance. On the other hand, B-MD, at all three levels employed, substantially (approximately 10-fold) reduced enterococci. Effects on B-MD 50 and 100 g./t. levels on enterococci were significant ($P < .05$).

Reinforced clostridial medium is a rich general supportive medium that does not distinguish bacterial populations. While strict anaerobes were not speciated, representative colony types from control and antibiotic supplemented birds were picked and Gram strained to determine Gram reaction and morphology. Treatment differences were obvious.

Control birds' intestines yielded Gram-positive rods and cocci initially, with occasional Gram-negative rods seen. At three and four weeks, Gram positive cocci clearly dominate the intestine microflora. At six weeks, small filamentous, weakly straining Gram-negative rods appeared.

Gram-positive cocci predominated in bacitracin supplemented birds, throughout the study. Gram-variable and Gram-positive rods also occurred in one hundred and two hundred gram per ton supplemented birds but were a distinct minority.

CTC supplemented birds' flora was remarkable for its diversity of forms. Gram-positive rods and cocci, and Gram-negative rods were all well represented at twelve days. All groups continued to figure importantly in total anaerobic counts throughout the study. However, Gram-positive rods were most common.

Control and CTC supplemented birds' anaerobic flora is fairly diverse. The flora of bacitracin supplemented birds differs in that it is consistently dominated by Gram-positive cocci.

Level-for-level total anaerobes were ten times more numerous in CTC supplemented than in B-MD supplemented birds.

Table 17. [1]Responses of intestinal anaerobes to antibiotic selection

Age (Days)	Control Count	[2]Treatment Responses					
		CTC 50 g./t.	CTC 100 g./t.	CTC 200 g./t.	B-MD 50 g./t.	B-MD 100 g./t.	B-MD 200 g.1t.
12	11.79	+0.57	+1.26	+2.35	-0.05	+0.13	+0.10
20	13.09	-0.87	-0.29	-3.68	-1.91	-3.35	-4.53
27	11.54	-0.06	-0.71	-2.65	-2.22	-2.78	-3.06
34	11.66	-3.06	-2.16	-0.23	-1.48	-2.45	-3.35
41	9.56	-0.63	-0.06	-1.06	+0.17	-0.73	-0.33
Average Count	11.53	10.72	11.14	10.47	10.43	9.69	9.29
Mean Treatment Response		-0.81	-0.39	-1.05	-1.10	*-1.84	*-2.23

1 Control counts are $\log_{10}$ transformations of bacteria per gram wet weight
2 Treatment responses are $\log_{10}$ differences (antibiotic supplemented minus control)
* Treatment response significant ($P < .05$)

Table 18. [1]Responses of intestinal lactobacilli to antibiotic selection

Age (Days)	Control Count	[2]Treatment Responses					
		CTC 50 g./t.	CTC 100 g./t.	CTC 200 g./t.	B-MD 50 g./t.	B-MD 100 g./t.	B-MD 200 g./t.
12	9.25	-1.82	+0.35	+0.50	-1.34	-0.40	-3.49
20	11.16	+0.31	+0.62	-2.62	-2.54	-3.46	-4.37
27	10.37	+0.98	-1.07	-2.56	-2.53	-3.53	-3.65
34	11.04	-4.16	-1.85	-0.64	-3.91	-2.64	-3.33
41	9.06	-0.79	-0.96	-0.62	+0.29	-0.75	-0.87
Average Count	10.18	9.08	9.59	8.99	8.17	8.02	7.03
Mean Treatment Response		-1.10	-0.58	* -1.19	* -2.01	* -2.16	* -3.14

1 Control counts are log $_{10}$ transformations of bacteria per gram wet weight
2 Treatment responses are log $_{10}$ differences (antibiotic supplemented minus control)
* Treatment response significant ($P < .05$)

Table 19. [1]Responses of intestinal coliforms to antibiotic selection

Age (Days)	Control Count	[2]Treatment Responses					
		CTC 50 g./t.	CTC 100 g./t.	CTC 200 g./t.	B-MD 50 g./t.	B-MD 100 g./t.	B-MD 200 g./t.
12	8.10	-0.17	+0.36	+3.24	+0.11	-1.08	-0.16
20	TFTC	-----	-----	---- -	---- -	-----	-----
27	6.75	+1.19	0.00	-0.87	+0.41	+1.70	-0.15
34	6.48	-0.63	-0.04	+0.10	-1.14	+0.85	+0.69
41	6.34	+3.00	+1.01	+0.47	+1.05	+1.23	+2.25
Average Count	6.92	7.77	7.27	7.65	7.03	7.59	7.58
Mean Treatment Response		+0.85	+0.35	+0.74	+0.11	+0.68	+0.66

1 Control counts are log $_{10}$ transformations of bacteria per gram wet weight
2 Treatment responses are log $_{10}$ differences (antibiotic supplemented minus control)

Table 20. [1]Responses of intestinal enterococci to antibiotic selection

Age (Days)	Control Counts	[2] Treatment Responses					
		CTC 50 g./t.	CTC 100 g./t.	CTC 200 g./t.	B-MD 50 g./t.	B-MD 100 g./t.	B-MD 200 g./t.
12	8.47	-0.84	+0.80	-0.39	-2.27	-2.36	-3.07
20	5.65	+0.88	+0.47	+0.69	-0.13	-0.78	+0.34
27	5.95	+0.39	-0.05	+0.51	-1.09	-0.89	-0.05
34	6.72	-1.01	-0.46	-0.13	-1.45	-0.14	-0.29
41	8.47	-1.13	-0.82	-0.94	+0.04	-1.39	-0.88
Average Count	7.05	6.71	7.04	7.00	6.07	5.92	6.26
Mean Treatment Response		-0.34	-0.01	-0.05	* -0.98	* -1.11	-0.79

[1] [2] Control counts are $\log_{10}$ transformations of bacteria per gram wet weight
Treatment responses are $\log_{10}$ differences (antibiotic supplemented minus control)
* Treatment response significant ($P < .05$)

Growth Responses:

Growth responses of antibiotic supplemented chicks, from 1 to 41 days of age, are summarized in Table 21. All levels of both CTC and B-MD stimulated chick growth in a dose-related fashion. Generally, however, more substantial and consistent gains were observed in B-MD supplemented chick than in their CTC supplemented counterparts.

Individual 1 and 41 day weights did not differ significantly between antibiotic supplemented and control chicks. However, interim and final weight gains with all three levels of B-MD were consistently positive and were significant ($P<.05$) if subjected to the same analytical procedure as bacterial responses were (Wilcoxon matched pair, signed rank analysis). CTC growth responses, on the other hand, varied in repeated determinations as did their accompanying bacterial responses.

Two general effects are denoted at the bottom of Table 21. Final response (%) shows final weight differences from controls. The responses are both dose-related and fall within the range of normal expected values for these antibiotics. The second value (mean treatment response (%) is the average of incremental growth responses, as determined at 12, 20, 27, 34 and 41 days of age. Incremental growth response values are important in that they show the relative stimulus antibiotics provide at different ages in the growing chick, and allow an estimate of how much and at what age a particular level of a compound is stimulating the growth of a bird.

An examination of incremental growth responses illustrates important differences in the effects a B-MD and CTC exert on growth rate

in the early life of the chick. Growth stimulation is not a constant, either in effect or lack of it, or in degree throughout the early life of the chick.

B-MD growth stimulation is consistently positive from 1 to 41 days of age, irrespective of the level supplied in the diet. Further, maximal responses are seen from 13 to 20 days of age, and tend to diminish thereafter, to 41 days. CTC growth stimulation effects fluctuated erratically in repeated determinations, though net effects, as reflected in final bird weights, tend to mask this variability. The constancy or variability of B-MD and CTC growth stimulation in the growing chick, when analyzed together with the accompanying bacterial responses, allows for the basis of a cause and effect determination for each of several bacterial populations which, in return, may or may not influence the growth response of antibiotic supplemented birds.

Table 21. Chick growth responses to antibiotic supplementation

Age (Days)	Control Weight	CTC 50 g./t.		CTC 100 g./t.		CTC 200 g./t.		B-MD 50 g./t.		B-MD 100 g./t.		B-MD 200 g./t.	
	Wt.	[1] Wt.	[2] R%	Wt.	R%	Wt.	R%	Wt.	R%	Wt.	R%.	Wt.	R%
1	37[a]	36[a]		37[a]		37[a]		37[a]		37[a]		38[a]	
2- 12	196[a]	195	0.00	202a	+3.77	195a	-0.63	200a	+2.52	202a	+3.77	207[a]	+6.29
13-20	394[a]	382	-5.56	416[a]	+8.08	417[a]	+12.12	418[a]	+10.10	427[a]	+13.64	429[a]	+12.12
21-27	635[a]	635	+5.00	651[a]	-2.49	651[a]	-2.90	665[a]	+2.49	697[a]	+4.56	681[a]	+4.56
28-34	922[a]	913	-3.14	947[a]	+3.14	962[a]	+8.36	967[a]	+5.23	983[a]	+5.92	990[a]	+7.67
35-41	1215[a]	1237	+10.58	1248[a]	+2.73	1281[a]	+8.87	1275[a]	+5.12	1291[a]	+5.12	1285[a]	+0.68
[3] Final Response (%)		+1.81		+2.72		+5.43		+4.94		+6.26		+5.76	
[4] Mean Treatment Response (%)			+1.38		+3.05		+5.16		*+5.09		*+6.60		*+6.26

[1] Grams, adjusted for sex ratio
[2] percent incremental growth, compared to controls
[3] 41 day treatment weight differences, as percent of control weight
[4] mean growth response for five growth periods to 41 days
* treatment response significant ($P<.05$)

Bacterial Response/Growth Response Interactions:

Table 22 summarizes the results of 32 regression analyses of bacterial responses in total anaerobes, lactobacilli, coliforms and enterococci, and corresponding growth responses in CTC and B-MD supplemented chicks. The object of the analyses was to determine how variation in bacterial populations affected the growth of chicks as influenced by antibiotic supplementation. In this approach, experiment three and experiment two differ from previous studies which have limited themselves to comparisons of mean bacterial population counts and mean growth responses, as reflected in final weights. Previous studies have attempted by this method to determine whether a particular type of intestinal bacterium is beneficial or not, and whether a particular antibiotic will produce a change in its numbers.

Two criteria were established to qualify populations for serious consideration as agents of growth promotion, as influenced by antibiotic supplementation:

1. regression analyses should, by their signs, indicate consistent relationships between bacterial responses and accompanying growth responses. The nature of the relationship should, further, be consistent within and between antibiotics.
2. the strength of the bacterial response/growth response should, hopefully, be significant, or closely approach significance.

Anaerobe Responses and Growth:

All levels of both B-MD and CTC produced similar net effects with respect to anaerobe populations (reduced) and growth (increased). Anaerobes, more than any other population studied, satisfy the criteria detailed above.

Anaerobe responses, and growth, in CTC supplemented birds were negatively correlated at 50 and 200 g./t. levels. The combined analysis of all levels of CTC with anaerobes was consistent in that it is negative also. Regressions of individual levels of B-MD showed consistent negative correlations between anaerobe numbers and growth responses. Further, the combined level B-MD analysis of anaerobes and growth response is negative and significant ($P < .05$).

The atypical relationship of anaerobes and growth in CTC 100 g./t. supplemented birds detracts from the otherwise consistent relationships observed. Generally, however, results of anaerobe analyses suggest that CTC and B-MD act similarly on this population in the small intestine, and that their common actions may contribute materially to common growth effects observed with various levels of both antibiotics.

Lactobacilli Responses and Growth:

Regression analyses within levels of CTC showed inconsistent lactobacilli/growth response relationships. Whereas lactobacilli appeared to contribute to the positive growth response of CTC 100 g./t. supplemented chicks, the same relationship did not obtain at higher and lower levels of CTC.

Results of lactobacilli/growth response analyses in B-MD supplemented chicks suggest that lactobacilli may tend to detract from positive growth stimulation with this antibiotic, or at least that lactobacilli do not participate positively in growth responses with B-MD. Taken together, the results of CTC and B-MD lactobacilli/growth response analyses suggest that lactobacilli responses in antibiotic supplemented chicks are not causally related to accompanying growth responses. Lactobacilli responses, by virtue of their marked inconsistency between antibiotics, cannot be regarded as common, active, participants in growth responses with both CTC and B-MD. This inconsistency supports the findings of experiment two with respect to this population, and its role in growth stimulation, as influenced by antibiotic selection.

Coliform Responses and Growth:

Coliform increases, as reflected in bacterial responses, accompanied positive growth responses with all levels of both CTC and B-MD. The causal relationship of coliform responses to growth responses did not, however, suggest a constant (common) relationship either within levels of each antibiotic, or between antibiotics.

Whereas coliform increases correlated significantly with growth responses in CTC 50 g./t. supplemented chicks, the increasing CTC selection pressure of 100 and 200 g./t. obliterated the effect. Further, coliform response/growth response relationships in B-MD supplemented chicks were inconsistent between levels of B-MD. Given the inconstant causal relationships of coliform and growth responses within and between CTC and B-MD, we cannot infer that coliform increases in antibiotic supplemented birds contribute substantially to

accompanying growth responses. Their population fluctuations are irregular with respect to controls, and their tendency to increase appears to be more a coincidental than a causal relationship to antibiotic growth promotion. The inconstancy of coliform response/growth response relationships in experiment three confirms the findings of experiment two.

Enterococci Responses and Growth:

The responses of enterococci to CTC and B-MD differed substantially, though both antibiotics reduced their populations from control levels. B-MD, at all levels, reduced intestinal enterococci approximately 100-fold from control levels. CTC effects on this group were slight by comparison.

Regression analyses of enterococci and growth responses in CTC supplemented chicks showed weak and inconsistent causal relationships. On the other hand, enterococci responses correlated positively, and consistently, with growth responses at all levels of B-MD supplementation. General (combined CTC and B-MD levels) relationships and growth responses in CTC and B-MD supplemented birds differed between antibiotics.

As with lactobacilli and coliforms, comparison of the enterococci/growth response relationships in CTC and B-MD supplemented birds were remarkable for their lack of consistency. Based on the results of these analyses, and corroborating results in experiment two, enterococci responses in antibiotic supplemented chicks do not appear to be causally related to accompanying growth responses.

Table 22. [1] Relation of growth to bacterial responses in the antibiotic fed chick

Bacterial Population	CTC				B-MD			
	50 g./t.	100 g./t.	200 g./t.	50+100+200 g./t.	50 g./t.	100 g./t.	200 g./t.	50+100+200 g./t.
Anaerobes								
[2] Bact. Resp.	-0.81	-0.39	-1.05	-0.75	-1.10	-1.84	-2.23	-1.72
[3] Gr. Resp.	+1.38	+3.05	+5.16	+3.61	+5.09	+6.60	+6.20	+5.99
[4] R	- .03	+ .17	- .37	- .20	- .29	- .65	- .71	*- .62
[5] C.V.	.88	.88	.88	.51	.88	.88	.88	.51
Lactobacilli								
Bact. Resp.	-1.10	-0.58	-1.19	-1.25	-2.01	-2.16	-3.14	-2.43
Gr. Resp.	+1.38	+3.05	+5.16	+3.61	+5.09	+6.60	+6.26	+5.99
R	+ .29	*+ .90	- .13	+ .30	- .17	- .55	- .87	*- .52
C.V.	.88	.88	.88	.51	.88	.88	.88	.51
Coliforms								
Bact. Resp.	+0.85	+0.35	+0.74	+0.66	+0.11	+0.68	+0.66	+0.48
Gr. Resp.	+3.13	+0.23	+3.45	+3.30	+3.83	+4.85	+4.83	+4.50
R	*+ .99	+ .74	- .14	+ .31	- .22	+ .59	- .73	- .23
C.V.	.95	.95	.95	.58	.95	.95	.95	.58
Enterococci								
Bact. Resp.	-0.34	-0.01	-0.05	-0.20	-0.98	-1.13	-0.79	-0.89
Gr. Resp.	+1.38	+3.05	+5.16	+3.61	+5.09	+6.60	+6.26	+5.99
R	- .40	+ .51	- .06	- .23	+ .66	+ .34	+ .29	*+ .61
C.V.	.88	.88	.88	.51	.88	.88	.88	.51

[1] All bacterial and growth values are mean treatment responses as determined from 12 to 41 days of age
[2] Mean treatment response, expressed as $\log_{10}$ difference from controls
[3] Mean growth response, expressed as percent growth response, relative to controls
[4] Regression coefficient (Bacterial responses independent; Growth responses dependent)
[5] Critical value of R ($P < .05$)
* Correlation significant ($P < .05$)

Feed Conversion Responses:

Table 23 summarizes feed conversion treatment responses of experiment three. All levels of CTC, and 50 and 100 g./t. of B-MD improved feed conversions, as compared to controls. However, high level (200 g.t.) B-MD appeared to detract from efficient conversion generally, and particularly in the 35-41 day period.

Generally, incremental feed conversion responses fluctuated more in repeated comparisons with controls than did accompanying growth responses. All improvements in feed conversion, as reflected in mean response to 41 days, were within the range of effects normally associated with CTC and B-MD supplementation.

Table 23. Antibiotic effects on feed conversion

Age (Days)[1]	Control	CTC 50 g./t.		CTC 100 g./t.		CTC 200 g./t.		B-MD 50 g./t.		B-MD 100 g./t.		B-MD 200 g./t.	
	F.C.	F.C.	R[2]	F.C.	R	F.C.	R	F.C.	R	F.C.	R	F.C.	R
1-12	1.43	1.36	+ 7	1.39	+ 4	1.35	+ 8	1.37	+ 6	1.37	+ 6	1.37	+ 6
13-20	1.61	1.63	- 2	1.65	- 4	1.56	+ 5	1.68	- 7	1.53	+ 8	1.61	0
21-27	1.84	1.78	+ 6	1.92	- 8	1.84	0	1.69	+15	1.89	- 5	1.87	- 3
28-34	1.87	1.76	+11	1.81	+ 6	1.77	+10	1.63	+24	1.79	+ 8	1.77	+10
35-41	2.07	2.06	+ 1	2.06	+ 1	1.96	+11	2.18	-11	2.11	- 4	2.30	-23
Growth	1178	1201		1211		1244		1238		1254		1247	
Consumption	2133	2127		2189		2165		2169		2240		2286	
Final Conversion	1.81	1.72		1.81		1.74		1.75		1.79		1.83	
[3] Cum. Resp.		+23		+1		+34		+27		+13		-10	
[4] Mean Response		+ 4.6		+0.2		+ 6.8		+ 5.4		+ 2.6		- 2.0	

[1] Feed conversion, by growth period
[2] Treatment response, as hundredths of conversion factors
[3] Summation of conversion responses in five growth periods
[4] Average conversion response in antibiotic supplemented chicks

Bacterial Response/Feed Conversion Interactions:

Table 24 summarizes four regression analyses that attempted to relate bacterial responses to feed conversion responses in CTC and B-MD supplemented chicks. Anaerobes, lactobacilli and enterococci were reduced by all levels of both antibiotics. Corresponding improvements (overall) in feed conversions (except with B-MD 200 g./t.) accompanied these reductions. Tests to determine causal relationships between the two variables were inconclusive with respect to anaerobes and coliforms.

Lactobacilli response/feed conversion response relationships were consistent, though not significant. Lactobacilli appeared to detract from the feed efficiency of chicks in both CTC and B-MD supplemented treatment groups.

Similarly, consistent causal relationships were observed between enterococci populations and feed conversions in CTC and B-MD supplemented chicks. Enterococci appeared to impair efficient conversion of feed as influenced by both antibiotics. As with the lactobacilli, the effect with CTC and B-MD was not sufficiently strong to attain significance.

Anaerobes and coliforms showed no consistent relationship to feed conversion responses of CTC and B-MD supplemented chicks. However, both lactobacilli and enterococci appeared to be consistently, though not significantly related to reductions in feed efficiency in antibiotic supplemented chicks.

Table 24. [1]Relation of feed conversion to bacterial response in the antibiotic fed chick

Bacterial Population	CTC (50,100,200 g./t. inclusive)	B-MD (50,100,200 g./t. inclusive)
Anaerobes		
[2] Bact. Resp.	-0.75	-1.52
[3] Conv. Resp.	+2.16	+1.52
[4] R	+0.06	-0.22
[5] C.V.	0.51	0.53
Lactobacilli		
Bact. Resp.	-0.96	-2.30
Conv. Resp.	+2.16	+1.52
R	-0.22	-0.48
C.V.	0.51	0.53
Coliforms		
Bact. Resp.	+0.65	+0.48
Conv. Resp.	+2.75	+1.72
R	+0.05	* -0.76
C.V.	0.58	0.58
Enterococci		
Bact. Resp.	-0.14	-1.06
Conv. Resp.	+2.16	+1.52
R	-0.39	-0.36
C.V.	0.51	0.53

[1]Conversion and bacterial response values are means, as determined from 12 to 41 days of age

[2]Bacterial response, expressed as $\log_{10}$ difference from controls

[3]Improvements in feed conversion, expressed as percent of control conversions

[4]Regression coefficient (Bacterial responses independent; Conversion responses dependent)

[5]Critical value of R ($P < .05$)

DISCUSSION

Germfree bird studies have amply demonstrated that growth promoting antibiotics act by selective pressures on normal gastrointestinal microflora of the chicken. Similarly, many other studies have shown that the small intestine of antibiotic fed and germfree birds reflected, indirectly, positive growth effects, and that the small intestine changes markedly, and in several respects, with antibiotic supplementation. It absorbs nutrients more efficiently than the small intestine of conventional (no antibiotic) birds. The small intestine of antibiotic fed birds is thinner, lighter, and the half-life of its mucosal epithelium is doubled. Further, studies of the immune responses of antibiotic fed, germfree and conventional chicks have shown that conventional birds' small intestines exhibit signs of a chronic, low level inflammation, which is alleviated by antibiotic supplementation, and which is absent in germfree birds.

These independent and converging lines of evidence with germfree antibiotic fed and conventional birds suggest that antibiotics act principally on bacterial populations of the small intestine, rather than on those of the caeca or large intestine, much less feces. Despite the strong evidence for antibiotic effects in the small intestine, most previous studies have addressed themselves to bacterial responses in the flora of the caeca and feces of antibiotic fed chicks.

This emphasis derives from two faulty, but prevalent assumptions.

First, it assumes that the flora of distal segments of the gut is an accurate measure of the flora of the small intestine. Smith (1965 a, b); Timms (1968); and Huhtanen and Pensack (1965 a,b) have shown repeatedly that this is not the case, and that each of the several sites of the gut have their own distinctive microflora. Further, previous studies have assumed that intestinal contents alone, or feces, reflect the entirety of the intestinal microflora. The recent studies of Fuller and Turvey (1971) Fuller, 1972), and of Schaedler et al. (1965); Savage (1972); Dubos and Schaedler, (1960) and Dubos et al. (1963) strongly refute this assumption. They demonstrated the presence of large, characteristic (autocthonous) bacterial populations in the gut which are firmly attached to and intimately associated with the mucosal epithelium of the gut, to include the small intestine. They have further shown that, by virtue of their modes of attachment, that these bacteria are not included in chyme samples mechanically expressed from segments of gut. Our studies have not discounted the potential effects of chyme bacteria in the nutritional response of antibiotic supplemented birds. We have however, by including the entire gut segment in our samples, attempted to represent the entire intestinal microflora, and to measure its responses to antibiotic selection. Our studies do not distinguish between chyme populations, and those which are characteristic of the absorptive surface of the gut, but have attempted to measure significant responses of the entirety of intestinal microflora, irrespective of their radial distribution in the gut.

We have assumed with this approach that the entire microflora of

the intestine is influenced, directly or indirectly, by antibiotic selection, and that both chyme and wall-associated population responses should be considered as nutritionally important. We have further assumed that the response of the several bacterial populations of the gut will be reflected in numerical population shifts from control levels. This approach admittedly ignores evidence for subtle qualitative responses to antibiotic selection, as demonstrated in enterococci by Eyssen and De Somer (1965, 1963 a,b).

All of our interpretations of bacterial responses, and their relationships to growth, must then carry the qualification that we did not discern physiological strain differences which may have nutritional significance, and which are not recognized in our results.

The three experiments described in the thesis depart in some important respects from previous reports, both in methods and interpretation. In other respects, for instance count, dilution, and culture methods, and population descriptions, they follow traditional usages.

With respect to population descriptions we have characterized large and diverse populations under general headings as total aerobes total anaerobes, lactobacilli, coliforms, enterococci, clostridia and anaerobic spore formers. (See Appendix). Various culture media and methods were tried and adapted to our purpose as the studies proceeded. Lactobacilli were rather precisely defined by the use of acidified Rogosa SL medium. This selective medium has been shown to effectively preclude other bacterial associates in the gut and, at the same time, will support luxuriant growth of all important members of the Lactobacillus genus in the gut (Rogosa et al., 1951 a,b). Characterization of

enterococci was also precise, as confirmed by biochemical tests of bacteria recovered on Ethylene Violet Azide plates. This medium, as a broth, was developed by Litsky et al. (1953, 1955) for isolation and enumeration of fecal enterococci in water supplies. It has not been used previous to this work to characterize intestinal enterococci. Early comparisons of its efficacy versus that of Azide Dextrose medium and m-enterococcus medium showed it to be markedly superior to these media, both in its selective and growth supporting properties. Similarly, sulfite-reducing clostridia, as characterized in experiments two and three, are precise Genus descriptions. The SPS medium of Angelotti et al. (1962) proved satisfactory for selecting for clostridia, and for distinguishing sulfite reducing (e.g. Cl. perfringens) forms.

The general classification of coliforms translates as all members of the family Enterobacteriaceae. In the chicken, these are represented primarily by Escherichia coli and Aerobacter (Enterobacter) aerogenes, but also include Proteus, Pseudomonas and other less common members of the family. All grew equally on Eosin Methylene Blue medium of Holt-Harris and Teague (1916).

Enumeration and characterization of total anaerobes varied from experiment one to three. Whereas Brain-Heart-Infusion medium supported large populations of bacteria in experiment one, the Reinforced Clostridial Medium of Hirsch and Grinsted was substituted in experiment three in an attempt to recover a more representative sample of strict anaerobes of the small intestine. Preliminary studies with this medium did confirm that it would support laboratory strains of various

Bacteroides species. However, since even the most specialized anaerobic laboratories do not claim to have recovered and cultured all of the strict anaerobes of the gut, we with our methods, certainly do not infer this from our results.

These studies differ most from previous related studies in the data treatment, collection, analysis and interpretation methods we developed. A review of bacterial population studies in the chicken show that individual, unrepeated estimates of numbers of a particular population are notoriously variable. Similarly, repeated counts vary considerably with time. We attempted in various ways to deal with time and bird variation in bacterial populations estimates in order to detect treatment related, consistent and significant bacterial responses.

In experiment one, each data point (population estimate) is the average of ten birds per treatment, per organ, per day. This design was intended to obscure individual differences, and to reveal treatment responses in the several populations, as they varied with time. Control and antibiotic supplemented birds' bacterial populations fluctuate considerably with time, independently of treatment responses to antibiotic. To evaluate the consistency of a populations' response to continuous antibiotic selection, treatment and control count differences, rather than absolute counts, were analyzed, after being transformed into $\log_{10}$ values. We were thus able to minimize individual bird variation, and non-treatment related fluctuations in the populations of each bacterial group studied. As bacterial counts are much less precise and repeatable than other biological parameters,

a correspondingly imprecise analytical method had to be applied to treatment data. The nonparametric rank sign analyses, though unsophisticated, are legitimate and appropriate, and were consistent with the nature of our data, and with the objectives of the experiments--to discern treatment related, consistent, population responses to continuous selection pressure, as it varied over time. This analysis, despite its apparent simplicity, imposes rigorous conditions for significance. It depends on the repeatability of a response with time, and accounts for the relative degree of response over extended periods of time. We feel then that the populations whose responses did attain significance as determined by this method did respond to their respective selection pressures in a substantial way, rather than in an incidental one.

Experiments one, two and three represent the first attempts to quantify bacterial responses to antibiotic selection in the chicken, and to subject these responses to statistical analysis.

The collection, interpretation and analysis of growth and feed efficiency data also differ from most previous studies of antibiotic growth effects in the chicken. Most reports of antibiotic growth effects cite final bird weights and treatment differences. They may also include isolated observations of bacterial count differences that accompany increased final weights. They do not however, usually cite incremental weight gains, or indicate by how much, and when, an antibiotic supplemented chick is being improved over controls. Our data include both final weight differences, as are usually reported, and more importantly, incremental growth differences, as determined at weekly intervals. Gain response data is more meaningful than total weight data because our results, and previous reports, have shown repeatedly that growth stimulation with a particular antibiotic is not constant, but that it varies with the age of the bird. Experiment two and three results show, as expected, that antibiotic supplemented birds weigh more than controls. The inclusion of incremental growth data show important response differences however, both within and between antibiotics. For instance, in experiment two 50 g./t. CTC showed almost no stimulatory effect from one to twelve days of age, but that its most pronounced stimulus was observed from 12 to 35 days of age. Thereafter, treatment chicks' growth (35-42 days) was less than that of controls.

On the other hand, B-MD growth stimulation was consistently positive in each of four consecutive growth periods, but the degree

of stimulation was not constant from week to week, but rather resembled a bell-shaped response curve, when related to control growth responses.

The recognition of variation in growth stimulating effects with antibiotics, with time, is essential to determining the relationship between bacterial responses in a given time period and accompanying growth responses, as influenced by antibiotic selection. This two-response method allows a meaningful systhesis of bacterial and growth effects with antibiotic supplementation, and lends itself to a cause-and-effect evaluation method with regression analysis. Experiments two and three are the first attempts to emphasize the time-variable, simultaneous, growth and bacterial responses in antibiotic fed chicks and to relate the two variables causally. In experiment three, bacterial responses were similarly related to accompanying feed conversion responses, to determine the effect antibiotics exert on each parameter, and to determine causal relationships between the response of a particular population, and accompanying feed conversion responses.

Responses in bacterial populations, chick growth, and feed conversions were quantified as $\log_{10}$ treatment responses, percent treatment growth responses, and percent feed conversion responses, respectively.

This relationship, under statistical analysis, accords equal significance to the response of each variable. Whether $\log_{10}$ changes in bacterial counts can be equated to percent nutritional responses in a one-to-one relationship remains for further investigation to determine. Our results in experiments two and three suggest the

method has merit, and though imprecise, is useful in determining the influence bacterial responses exert on accompanying nutritional responses.

Experiment one demonstrated that, generally, the microflora of the small intestine responds most predictably, and markedly, to antibiotic selection, and that, conversely, the influence of antibiotics on caecal and large intestine populations is minimal. This finding is consistent with the nutritional and physiological changes observed in the small intestine. It also argues strongly against the emphasis of studies addressed to posterior segments of the gut, and which attempt to relate bacterial responses there to positive growth effects. Eyssen and De Somer (1963) and Jayne-Williams and Fuller (1971) have inferred that the microflora of the small intestine, by virtue of its relative size, and the rapidity of movement of chyme through it, does not exert a substantial effect on the condition of the small intestine, as influenced by antibiotics. This concept has prevailed since 1963, as reflected by the de-emphasis of the small intestine in current literature. It is unfortunate, and we feel the emphasis on posterior gut flora in the antibiotic fed chick is the main reason we know little more about how antibiotics influence bacterial flora and growth than we did twenty five years ago. Inclusion of the entire gut, measurement of several bacterial responses over time, and a comparison of response differences in various organs has shown in experiment one that, though the small intestine flora is small by comparison with the caeca and large intestine, and is more variable, that it is substantial. When experiment one emphasized treatment responses, and eliminated non-treatment time differences, we

showed clearly that small intestinal microflora figure importantly in antibiotic growth promotion, and that the study of the small intestine deserves far more attention than has been accorded it. Small intestine responses, versus caecal and large intestine responses, as reflected in response rank sums of 9.0, 12.0 and 15.0, clearly show the relative degree of response in each organ. Further, the significance of bacterial responses (three in the small intestine versus one in the caeca and none in the large intestine) argues strongly the importance of the small intestine's bacterial response, as it relates to growth responses with antibiotics. Experiment one also demonstrated that inclusion of entire gut segments, versus chyme, in bacteriological studies is a legitimate method, and may well reduce variation in counts as previously reported.

Experiments two and three related the responses of lactobacilli, coliforms, enterococci and anaerobes of the small intestine to accompanying growth responses in the antibiotic fed bird. All of the relationships were determined by regression analysis. Before we procede further with a discussion of our results and the interpretation of them, a review of some basic concepts is necessary.

1. Various levels of CTC and B-MD both produce consistent growth responses in conventionally reared chicks.
2. The two antibiotics are inherently dissimilar. CTC is broad spectrum and generally selects both Gram-positive and Gram negative target organisms in the gut. B-MD on the other hand, exerts its effects on Gram-positive microflora.
3. Bacterial responses to varying levels of each antibiotic are not constant with time, as determined in repeated response measurements.
4. Growth responses, and feed efficiency responses, to varying levels of each antibiotic are, like bacterial responses, variable and not constant with repeated determinations.
5. Since various levels of two dissimilar produce dose related, growth responses in supplemented chicks, and since it is established that they promote growth by actions on intestinal bacteria, then they likely produce similar growth related effects in particular populations of the total intestinal microflora.
6. Conversely, by the very nature of the two antibiotics, they will, with respect to some bacterial populations, produce dissimilar responses, in the face of consistent growth effects.

7. A comparison of bacterial response, growth response relationships, as determined by regression analysis, will reveal apparent relationships between growth and bacterial response, both within varying levels of each antibiotic, and between antibiotics.

8. With respect to particular populations, especially lactobacilli, coliforms, enterococci and clostridia, the bacterial response, growth response relationships should be consistent within and between antibiotics, if they are important in growth responses.

9. Consistent relationships between bacterial responses and accompanying nutritional responses suggest which of the several gut populations are most responsible for common antibiotic growth effects. Conversely, inconsistent bacterial response nutritional response relationships should indicate which effects are peculiar to a particular level of a particular antibiotic and which therefore, are incidental to main growth effects.

10. By comparing the bacterial response, nutritional response relationships of several bacterial populations under varying types and degrees of antibiotic selection we may suggest which of the populations are deserving of further, more detailed study and which ought to be accordingly de-emphasized.

11. Discussions of the importance of lactobacilli, coliforms, enterococci and clostridia in antibiotic growth promotion have dominated the literature for twenty five years. We propose, by the methods employed in experiments two and three, to begin to explain reasons for contradictions in the literature and to

examine in a meaningful, quantitative way, the role "good" or "bad" (or indifferent) that each of these populations assumes in the antibiotic fed bird. We hope also to begin to pinpoint the elusive microbial populations of the chicken which are responsible for positive growth effects, or for the lack of them, in antibiotic fed birds, and to attempt an explanation for the variable response with time observed in the field.

Experiment two bacterial response/growth response regression analyses suggest that none of the four populations studied contribute in a meaningful way to observed growth effects in antibiotic supplemented chicks. Lactobacilli/growth regressions showed glaring inconsistencies both within and between antibiotics. B-MD almost eliminated this population from the gut for three weeks, despite accompanying positive growth effects in this period. Conversely, slight lactobacilli increases accompanied positive growth responses in CTC supplemented chicks. CTC at 50 g./t. resulted in a significant (P .05), positive relationship between lactobacilli increases, and corresponding growth effects in this treatment group. This result, taken alone, would tend to support the literature claiming positive roles for intestinal lactobacilli, in both conventional and antibiotic supplemented birds. The consistency of lactobacilli/growth responses does not obtain with higher levels of CTC, nor with B-MD (all of which contributed more substantially to growth than did CTC at 50 g./t.). The lactobacillus growth relationship appears then to be peculiar to low level CTC alone, and does not apply to antibiotic growth promotion generally.

Coliform bacteria generally increased with all levels of both CTC and B-MD, and their net increases correspond to net positive growth effects. The regression analyses within and between CTC and B-MD are notable for their lack of consistency. Coliform responses to antibiotics, and the relationship of that response to growth then appeared to be incidental in nature, rather than causal.

Enterococci responses to CTC and B-MD were slight, suggesting that this population was resistant to high levels of both antibiotics

from the outset of the experiment. This suggestion, with respect to B-MD particularly, is supported by the consistent, though slight increases in enterococci in B-MD 100 g./t. supplemented birds. Regression analyses of enterococci/growth responses in CTC and B-MD supplemented chicks suggest this population does not contribute materially to positive growth effects, as shared by the two antibiotics.

Clostridial counts were obtained so infrequently in both experiment two and three that no regressions of their responses with growth were possible. Empirically however, it is difficult to accord a population which, when present in detectable numbers, constitutes .0001% of the total population, much nutritional significance. We do not feel, despite the abundant literature to the contrary, that clostridia or their responses to antibiotic selection, figure significantly in antibiotic growth effects.

Experiment three results confirmed and expanded the results of experiment two. Lactobacilli, coliforms and enterococci showed the same inconsistency of relationship to growth that was observed in experiment two. As a result, we conclude that none of these populations in the small intestine figure importantly in general growth responses to antibiotic supplementation.

In terms of consistency of response/response relationships, anaerobes agree most closely. Except for the case of CTC at 100 g./t., all anaerobe response/growth response relationships were negative. Further, general (combined level) CTC and B-MD relationships were consistent, in that both were negative. The strength of the anaerobe growth response relationship in combined B-MD level analysis was significant, and closely approached significance at the 200 g./t.

level.

Anaerobes, when compared to lactobacilli, coliforms and enterococci showed the most consistent bacterial response/growth response relationships within and between antibiotics. Contrasted with this consistency, we observed marked discrepancies in the relationships of lactobacilli, coliforms and enterococci. The results allow a reasonable assumption that lactobacilli, coliform and enterococci responses to antibiotic selection, as they relate to accompanying growth responses in the chick, are coincidental, rather than causal. CTC anaerobe analyses failed to achieve significance. However, they were the only population to agree generally within levels of CTC, and with B-MD relationships. Further study, to include a more precise characterization of anaerobic populations, and more frequent monitoring of growth and bacterial responses in anaerobes should be valuable in further elucidating the role of this large and heterogeneous population in antibiotic growth effects. We based our analyses and our conclusions, on a limited data base (5 sets of covariates). Further work with simultaneous variables should provide more precise estimates of cause and effect relationships between anaerobic population responses to antibiotic selection, and of the contribution of those responses to accompanying growth effects.

Experiment three results also allowed a limited estimate of the relationship of bacterial responses to accompanying feed efficiency responses in the antibiotic supplemented chick. Both lactobacilli and enterococci appeared to detract from the efficiency of antibiotic fed chicks. While none of the lactobacilli or enterococci analyses yielded significant results, the nature of their relationships, as indicated by the sign of correlation coefficients, to feed efficiency was consistent between CTC and B-MD supplemented chicks.

The effect of enterococci on feed conversion has been previously reported as the malabsorption of fats syndrome (Huhtanen and Pensack, 1965 b.), and has been explained in detail by Khoury et al (1969); The relationship of lactobacilli populations to feed efficiency is not explored or reported in the literature. Some authors however, (Tortuero, 1973) have reported a lactobacilli enterococci antagonism in the gut which would suggest lactobacilli, by their indirect effects on enterococci, may improve feed efficiency.

The results of experiments one, two and three offer an explanation of many of the conflicting reports of bacterial responses with antibiotic supplementation, and suggest which of the classical populations (lactobacilli, coliforms, enterococci, and clostridia) tend to contribute to, detract from, or are indifferent to antibiotic selection, as it stimulates the growth of chicks.

Counts of all populations varied in repeated determinations, and varied with antibiotic, dose level, and with time. Many of the responses of these populations suggest that they were initially sensitive to antibiotics employed, and responded to them with sharp

reductions in their numbers. More importantly, we saw repeatedly that responses to continuous selection pressure were not constant. Lactobacilli in particular offer an example of variable response to B-MD. In experiments two and three, lactobacilli numbers were substantially reduced initially, but then adapted or acquired resistance to B-MD and, by 42 days of age, had reestablished themselves in the gut at near nontrol levels. One-point observations of any population under selection pressure are likely to be, and have been misleading and confusing. The response of a population varies in several important respects to include antibiotic type, dose level, and exposure time. We may, if we choose, select from our hundreds of individual bacterial counts each and every one of the various, and similarly arrived at, results that have filled and confused the literature for the past twenty five years. The continuous response data obtained in these experiments explain the many contradictory reports and in many instances have shown that a particular population may be seen to increase, or decrease, or to converge on control levels over time, and that many of them do just this as they adapt themselves to the altered environment, as influenced by antibiotics. We would suggest then that further studies of antibiotic effects on bacterial populations account for time variation in response, and that the adaptation of bacterial populations be taken into account by continuously monitoring their responses over time.

Experiments one, two and three are, in a sense, irreverent of the work of classical specialists in nutrition, anaerobic microbiology anatomy and of the several other disciplines that have touched upon the role of antibiotics and intestinal microflora. The results obtained in these experiments would, in themselves, not satisfy the exacting criteria of any of the contributing disciplines. We do feel that by attempting to recognize the many factors involved in antibiotic effects on intestinal microflora as they relate to growth, and by attempting an interdisciplinary synthesis of the specialized knowledge of the various specialties, that we have, on the one hand offered a service in that much of the contradiction in literature can be reconciled and, on the other hand that we have proposed a method and a conceptual model which will eventually lead to a precise understanding of the role of the several populations in the gut to antibiotic growth promotion in the chicken.

CONCLUSIONS

1. In experiment one, traditional cultural techniques and bacterial classifications showed that the response of intestinal bacteria to antibiotic supplementation may show regional differences from the small intestine, large intestine and caeca. Further, inclusion of the entire intestine, including contents, does not obscure treatment effects on intestinal bacteria, and in fact, may clarify them.

2. Whereas degrees of growth stimulation with antibiotics are a function of dose level, accompanying bacterial population changes in the small intestine may or may not be consistent with increasing dose levels of a particular antibiotic, or between two dissimilar antibiotics.

3. The response of coliforms, enterococci, lactobacilli and clostridia differs with varying levels of CTC and B-MD despite consistent growth effects. In experiment two, the differential response was both qualitative and quantitative. In experiment three, it was qualitatively consistent, but differed quantitatively.

4. Neither growth nor bacterial population changes with antibiotic supplementation are constant over time. The growth accelerator effect of antibiotic supplementation may persist after incremental growth responses disappear, and give net increases in total body weight. Incremental growth responses, and bacterial population responses, to

antibiotic supplementation, approach control levels by six weeks.

5. Modifications of technique yielded very high counts in experiment three. Varying levels of CTC and B-MD gave consistent growth effects, and consistent bacterial effects, with respect to lactobacilli (reduced), coliforms (increased), enterococci (reduced) and total anaerobes (sharply reduced).

6. Regression analyses of incremental growth on accompanying bacterial population changes in the gut showed inconsistent relationships with lactobacilli, enterococci and coliforms, when CTC and bacitracin results were compared. The relationship of total anaerobic population shifts and growth was consistent with CTC and bacitracin, significantly so in the latter. The common growth promoting property of CTC and B-MD appeared to lie in their common action on strict anaerobes, and to be independent of population fluctuations of lactobacilli, coliforms and enterococci. Clostridia were encountered so infrequently in the small intestine, their role in antibiotic growth response could not be ascertained. However, results of experiments two and three suggest it is minimal.

7. Regressions of relative feed efficiency on antibiotic-related bacterial population shifts showed consistent effects with enterococci. Their numbers in the small intestine were consistently, and inversely, related to feed efficiency. Lactobacilli also appeared to detract from feed efficiency in CTC and B-MD supplemented birds. The relationships of coliforms and total anaerobes and feed efficiency were inconsistent in CTC and B-MD supplemented birds.

8. Anaerobic populations were not speciated. However, they were characterized by morphology and Gram reaction. Large numbers of Gram-positive cocci (peptostreptococci?) were a constant feature of control and antibiotic supplemented birds. Gram-positive anaerobic rods (Butyribacterium, Eubacterium?) varied in occurrence from control. CTC supplemented and B-MD supplemented birds. Gram-negative anaerobic rods (Bacteroides, Fusobacterium?) were encountered rarely, and most often in control birds. CTC and B-MD common effects on small intestine anaerobes most likely influence Gram-positive anaerobic rods, to include Eubacterium,Fusobacterium and Butyribacterium spp.

APPENDIX

Table 25. Formulation of MSU chick starter ration 71-7

	Parts/1,000
Corn, #2, yellow	578.6
Soybean meal (49%)	262.0
Alfalfa, dehydrated	56.0
Distiller's dried solubles	15.0
Methionine hydroxy analogue	.9
Corn oil	36.0
Limestone	5.0
Dicalcium phosphate	30.0
Salt, iodized	3.0
Choline chloride (50%)	3.5
[1] Vitamin mix	5.0
[2] Mineral mix	5.0
Ethoxyquin	125 mg.

[1] Vitamin mix supplies (per Kg. of diet) Vitamin A-10,000 I.U.; Vitamin D-3--1,000 I.C.U.; Vitamin E--10.0 I.U.; Menadione sodium bisulfate--2.0 mg.; Niacin--100.00 mg.,; Pyriodoxine--6.0 mg.; Biotin--150 mcg; Folacin--3.0 mg.; Vitamin B_{12}--5.0 mcg.; Distiller's dried solubles to 5.0 parts per 1,000.

[2] Mineral mix supplies (per Kg. of diet) Manganese--55.0 mg.; Magnesium--500 mg.; Iron--80 mg.; Copper--4.0 mg.; Zinc--80 mg.; Selenium--1.0 mg.; Distiller's dried solubles to 5.0 parts per 1,000.

Table 26. Formulation of MSU chick grower ration 71-5.

	parts/1,000
Corn, #2, yellow	650.0
Soybean meal (49%)	192.0
Distiller's dried solubles	20.0
Methionine hydroxy analogue	.5
Corn oil	30.0
Limestone	5.0
Dicalcium phosphate	30.0
Salt, iodized	3.0
Choline chloride (50%)	3.5
[1] Vitamin mix	5.0
[2] Mineral mix	5.0

[1] Vitamin mix supplies (per Kg. of diet) Vitamin A--10,000 I.U.; Vitamin D_3 --10 I.U.; Menadione sodium bisulfate--2.0 mg.; Niacin--100.0 mg.; Pyridoxine--6.0 mg.; Biotin--150 mcg.; Folacin--3.0 mg. Vitamin B_{12} --5.0 mcg.; Riboflavin--10.0 mcg.; Distiller's dried solubles to 5.0 parts per 1,000.

[2] Mineral mix supplies (per Kg. of diet) Manganese--55.0 mg.; Magnesium--500 mg.; Iron--80 mg.; Copper--4.0 mg.; Zinc--80 mg.; Selenium--0.1 mg.; Distiller's dried solubles to 5.0 parts per 1,000; Ethoxyquin supplied at 125 mg. Kg. of diet.

Table 27. Formulation of TC broiler starter ration

	pounds / ton
Corn	1148
Soybean meal (49%)	370
Fish meal (60%)	150
Meat and bone meal	80
Corn gluten meal	90
Brewer's grain	40
Fat	100
Calcium	12
Salt	2
Vitamin and mineral premix	5

Table 28. Formulation of the reinforced clostridial medium of Hirsch and Grinsted (1954)

	per liter
Yeast extract	.3 g.
Peptone	10.0 g.
Meat extract	10.0 g.
Glucose	10.0 g.
Sodium acetate	5.0 g.
Cysteine hydrochloride	.5 g.
Soluble starch	1.0 g.
Agar	10.0 g.
Resazurin (1% solution)	4.0 ml.

KEY TO BACTERIAL POPULATION CLASSIFICATIONS

1. Total Anaerobes (Experiment One): All anaerobic and facultative bacteria, irrespective of taxonomic subdivisions.

2. Total Aerobes (Experiment One): All aerobic and facultative bacteria, irrespective of taxonomic subdivisions.

3. Lactobacilli (Experiments One, Two and Three): Members of genus Lactobacillus to include L. acidophilus, L. fermentii, L. casei, L. brevis, and others.

4. Coliforms (Experiments One, Two and Three): All members of family Enterobacteriaceae; chiefly Escherichia coli; also Enterobacter (Aerobacter) aerogenes, Proteus spp., and Pseudomonas spp., and others.

5. Enterococci(Experiments One, Two and Three): Lancefield Group D. streptococci to include Streptococcus faecalis, S. avium (nov. sp.), S. liquefaciens, S. zymogenes, S. durans, and others.

6. Anaerobic Spore Formers (Experiment One): Heat resistant facultative and anaerobic Bacillaceae, principally Clostridium perfringens (welchii).

7. Strict Anaerobes (Experiment Three): All anaerobic and facultative bacteria which require oxygen-precluding methods for recovery; principally Bacteroides fragilis.

8. Sulfite-reducing Clostridia (Experiment Three): Vegetative cells and viable spores of hydrogen sulfide producing Clostridium spp., principally Cl. perfringens (welchii).

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