Mode of Action of High Dietary Copper in Promoting Growth of Weanling Pigs.

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ABSTRACT

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The mechanisms by which copper improves growth performance of growing swine have not been elucidated. Copper has antimicrobial properties and may affect the gut microflora to improve growth performance. The primary objective of the first experiment was to determine if feeding a diet containing 250 ppm supplemental copper improves growth performance of germ-free pigs, as typically observed for conventional pigs. A second experiment was conducted to determine the effects of 250 ppm copper and 100 ppm chlortetracycline on microbial growth and fermentation patterns of gastrointestinal microflora of weanling pigs in vitro.

In the first experiment, 10 germ-free and 10 conventional weanling pigs were fed one of two experimental diets containing either 16 ppm or 283 ppm copper for a three-week trial. Several criteria were measured including: growth performance, hematology, plasma and liver copper, zinc and iron, and plasma ceruloplasmin.

In the second experiment, microbes present in gut contents and gut wall from five sections of the gastrointestinal tracts of 4 weanling pigs were used to inoculate culture tubes of anaerobic growth media containing one of four treatments (control, 250 ppm copper, 100 ppm

chlortetracycline, 250 ppm copper + 100 ppm chlortetracycline). Culture tubes were incubated at 38°C for 24 hours and microbial growth, media pH, ammonia-nitrogen and organic acid production were determined.

Results from the first experiment indicated that feeding the high copper diet improved growth rate of conventional pigs and reduced growth rate of germ-free pigs. However, this difference in growth rate was confounded by an apparent development of a moderate copper toxicity in germ-free pigs receiving the high copper diet. Germ-free pigs fed a high copper diet had a higher concentration of copper in liver and plasma and a lower concentration of liver iron compared to conventional pigs receiving the same diet, and lower hemoglobin, hematocrit and erythrocyte number compared to germ-free pigs fed the control diet.

Microbial growth, ammonia-nitrogen and organic acid production were dramatically reduced in vitro when 250 ppm copper was present in the media compared to control and chlortetracycline treated microbes. However, the reduction in microbial growth confounded differences between treatments for ammonia-nitrogen and organic acid production.

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INTRODUCTION

Addition of growth promoting levels of copper (125 to 250 ppm) above the copper requirement (4 to 6 ppm) to diets for growing swine is a generally accepted practice in today's swine industry. When copper is added to swine diets at levels up to 250 ppm, it is an equally effective and less expensive growth promoting agent compared to many broad spectrum antibiotics typically added to swine diets at subtherapeutic levels.

The addition of high dietary levels of copper originated in 1955 when Barber and coworkers (1955a) demonstrated that inclusion of 250 ppm supplemental copper resulted in an improvement in growth performance of growing swine. Since that time, substantial evidence has been reported in the scientific literature to verify the growth promoting effect of copper but very little is known about how copper exerts its action to produce this beneficial response.

At least three potential modes of action have been postulated for the growth promoting effects of subtherapeutic levels of antibiotics when included in diets for growing swine. These mechanisms could also apply to copper since copper has antimicrobial properties (Sollman, 1957). The growth promoting effects of antimicrobials may be mediated through 1) a metabolic effect, 2) a disease-control effect or 3) a nutrient-sparing effect (Hays, 1969). There is evidence to support each of these hypotheses, but most of the attention has focused on the influence of antimicrobials on the microbial population of the gastrointestinal tract in regard to a nutrient-sparing effect.

It is known that antimicrobial agents (including copper) generally reduce the microbial population of the gut and also cause shifts in the

microbial population. However, if these phenomena are to be nutritionally significant, it appears that the overall metabolism of gut microbes must be altered in such a way as to elicit a growth promoting response.

Studies have shown that when virginiamycin and spiramycin are added to diets for growing swine, utilization of glucose and amino acids as substrates for microbial metabolism is reduced compared to that of pigs fed diets containing no antimicrobial agents. As a result, the availability of these nutrients to the host appears to be a primary contributing factor to the total improvement of growth performance observed when these two antibiotics are added to diets for growing swine (Vervaeke et al., 1979; Dierick et al., 1986a; 1986b). Perhaps copper also exerts its beneficial growth effects in this manner.

Not only may the gut microbial population and metabolism be altered by antimicrobials to spare nutrients, but it may also be altered so that natural, endogenously produced, growth depressing bacterial toxins (e.g., ammonia) are reduced to provide an improved growth response. When antibiotics are fed to animals, portal ammonia concentration is reduced (Dintzis and Hastings, 1953; Silen et al. 1955) and when ammonia absorption is reduced, growth performance is improved (Holtzman and Visek, 1965). Since copper is an inhibitor of sulfhydryl enzymes such as urease, ammonia production may be significantly reduced to require less energy expenditure in the detoxification of absorbed ammonia via the urea cycle.

Due to the antimicrobial properties of copper and the limited data regarding the effects of copper on microbial populations and metabolism in the gastrointestinal tract of the pig, this study was designed to

examine the relationship between high copper feeding and the gut microflora of the weanling pig. Several response criteria were measured to identify this relationship in an attempt to understand potential mechanisms by which copper exerts its growth promoting effects in growing pigs.

REVIEW OF LITERATURE

I. ESSENTIALITY OF COPPER

A. Introduction

Copper is an element with atomic number 29, and an atomic weight of 63.546. Copper has two natural isotopes, copper-63 and copper-65 and occurs in nature as the metal in the +1 and +2 valence states. Copper is commonly found in the ecosystem associated with sulfide deposits, particularly in igneous rocks (NRC, 1977).

Copper is one of fifteen naturally occurring trace elements known to be essential for animal life. However, copper, like all heavy metals, is also potentially toxic when ingested in sufficient quantities for an extended period of time. Biochemical mechanisms regulate the uptake and elimination of copper in order to maintain copper homeostasis during periods of dietary excess or deficiency so that adverse effects on the animal's health are avoided.

B. Copper Absorption and Excretion

Approximately 25 to 40 percent of copper consumed is absorbed depending upon dietary source and amount (O'Dell, 1984). Copper homeostasis is regulated by controlling the rate of absorption. Sulfur-rich proteins in the mucosal cells, which are identical or closely related to the thionine moiety of metallothionine, regulate the intestinal absorption of copper as well as zinc (Bremner and Davies, 1976). Copper is absorbed from the stomach and along all portions of the small intestine, especially in the upper small intestine (VanCampen and Mitchell, 1965). Primary route of copper excretion occurs via the bile and intestinal tract (O'Dell, 1984).

C. Bioavailability of Copper from Copper Sources and Interaction with Other Nutrients

Like most trace elements, the biological availability of copper depends to some extent upon its chemical form and its interaction with other dietary components. Copper carbonate and copper sulfate are more available forms of copper than copper sulfide, copper oxide and the copper in copper wire, which is largely unavailable (Chapman and Bell, 1963; Bowland et al., 1961). Copper assimilation is reduced by phytate which forms a very stable complex with copper (Vohra et al., 1965; Davis et al., 1962). Ascorbic acid, when fed at high levels, reduces copper retention by reducing intestinal absorption (Van Campen and Gross, 1968). Furthermore, several inorganic elements greatly influence copper absorption, retention and distribution in the body. These elements include: calcium, cadmium, zinc, iron, lead, silver, molybdenum and sulfur (Underwood, 1977). Although copper absorption mechanisms are poorly understood, these elements appear to compete with copper for protein-metal binding sites to inhibit copper absorption.

D. Intermediary Metabolism of Copper

When copper is absorbed from the intestine and enters the blood plasma, approximately 10 percent is loosely bound to serum albumin and amino acids and the remainder is bound tightly to ceruloplasmin for distribution to the various tissues of the body. Copper uptake by tissues tends to be greater from ceruloplasmin than from the cupric ion form (O'Dell, 1984). Ceruloplasmin serves as a direct donor of copper to metalloenzymes by entering the heart and to a lesser extent the kidney, brain and liver intact (Hsieh and Frieden, 1975; Owen, 1975; Linder and Moor, 1977). Copper also readily traverses into erythrocytes (Bush et al., 1956).

E. Copper Storage Sites

The main copper storage organ in the body is the liver where copper is incorporated into the mitochondria, microsomes, nuclei and the soluble fractions of the parenchymal cells, depending on age, strain and copper status of the animal (Milne and Weswig, 1968; Thiers and Vallee, 1957). Copper may be stored in those sites or released for incorporation into erythrocuprein (synthesized in normoblasts of bone marrow; Bush et al., 1955), ceruloplasmin (synthesized in liver; Markowitz et al., 1955) or other cellular enzymes containing copper. Brain also contains appreciable concentrations of copper, while less copper is present in heart, spleen, kidneys and blood (NRC, 1977).

F. Copper Requiring Enzymes

At the cellular level, copper is primarily involved with copper proteins of which many are enzymes with oxidative functions. Copper is a very versatile cofactor in specific enzymatic reactions. Several copper containing enzymes include: tyrosinase, lactate dehydrogenase, ascorbic acid oxidase, uricase, monoamine oxidase, dopamine-β-hydroxylase and cytochrome oxidase (Underwood, 1977). Cytochrome C oxidase and superoxide dismutase are good indicators of copper status (Phan and Mahler, 1976; Williams et al., 1975).

G. Physiological Functions of Copper

Copper has a variety of physiological functions. Copper is involved in iron metabolism, cross-linking of connective tissue, neurotransmitters and brain function, lipid metabolism, pigmentation and keratinization of hair and wool, intestinal integrity, reproduction and immunology.

1. Iron metabolism

Copper is vital in facilitating iron absorption and mobilization. In a copper deficiency, serum iron levels are generally low and iron levels in the intestinal mucosa and liver are higher than normal, resulting in a hypochromic anemia (Underwood, 1977). Since the oxidation rate of Fe (II) by oxygen limits the rate of iron mobilization, which is catalyzed by a ferroxidase, and since there are at least two plasma ferroxidases (ferroxidase I (ceruloplasmin) and ferroxidase II), copper plays a role in absorption and mobilization of iron through its function in ceruloplasmin (O'Dell, 1984).

2. Cross-linking of connective tissue

Lysyl oxidase is a copper metalloenzyme which catalyzes the oxidation of specific lysyl and hydroxylysyl residues in soluble collagen and elastin (O'Dell, 1976). Without adequate copper, lysyl oxidase activity is low and the cross-links in collagen and elastin are not formed. This results in fragile bone and other skeletal abnormalities (Teague and Carpenter, 1951). Furthermore, aortic rupture has been shown in copper deficient chicks (O'Dell et al., 1961) and in pigs (Carnes et al., 1961) due to reduced elastin content of the aortas of pigs (Weissmann et al., 1963) and chicks (Starcher et al., 1964) and less cross-linking groups due to depressed lysyl oxidase activity of the aorta.

3. Neurotransmitters and brain function

Concentrations of at least two brain neurotransmitters, dopamine and norepinephrine, are reduced by copper deficiency (O'Dell, 1984). Copper deficiency appears to reduce the catalytic function of dopamine-β-hydroxylase in the adrenergic pathways and appears to

adversely affect a structural component of the dopaminergic system through defective myelination (O'Dell, 1984).

4. Lipid metabolism

Copper deficiency results in an increase of serum triglycerides, phospholipids and cholesterol; a decrease in the ratio of mono-unsaturated to saturated C_{16} and C_{18} fatty acids in subcutaneous adipose tissue; and a decrease in the C_{18}, $\Delta 9$ desaturase enzyme (Wahle and Davies, 1975; Petering et al., 1977). The site of copper involvement in the desaturase reaction may be in the terminal component of the microsomal electron transport chain (Wahle and Davies, 1975). Copper deficiency causes a rapid release of cholesterol from the liver to the plasma due to a high zinc:copper ratio (Klevay, 1973).

5. Pigmentation and keratinization of hair and wool

Failure of pigmentation of hair or wool (achromotrichia) among rats, rabbits, guinea pigs, cats, dogs, cattle and sheep, but not pigs, probably results in impaired conversion of tyrosine to melanin by copper-containing polyphenyloxidases in a copper deficiency (Underwood, 1977). Changes in hair or wool growth occur when these same species are fed copper deficient diets. Copper deficiency adversely affects the typical physical characteristics of wool which are dependent on the disulfide groups to provide cross-linkages or bonding of keratin (Underwood, 1977). Inadequate copper also disrupts the alignment or orientation of the hair or wool keratin in the fiber (Underwood, 1977). Furthermore, a lack of copper can interfere with the arrangement of the polypeptide chain in keratin synthesis (Underwood, 1977).

6. Intestinal integrity

In copper-deficient cattle, diarrhea (often called peat scours or teart scours) is usually a secondary symptom due to excess molybdenum intake (Miller, 1979). However, diarrhea is not a manifestation of copper-deficient swine (Miller et al., 1979). Copper-deficient cattle have reduced cytochrome oxidase activity in intestinal epithelium as well as villous atrophy (Fell et al., 1975).

7. Infertility

Copper deficiency results in reproductive failure due to fetal death and resorption in rats and guinea pigs, reduced egg production and hatchability in chickens, depressed estrus in cattle and abortion in sheep (Underwood, 1977).

8. Immunology

Superoxide dismutase is a zinc, copper and manganese dependent enzyme and is important in the microbiocidal systems of phagocytes (Miller et al., 1979) . This enzyme is important when a bacterial stimulus causes normal phagocytes to undergo a respiratory burst which produces a very destructive superoxide radical. Superoxide dismutase converts the superoxide radical to less destructive hydrogen peroxide, which is subsequently converted to water via glutathione peroxidase.

H. Copper Requirement of Swine

The copper requirement for adequate growth in the pig is 4 to 6 ppm to 90 kg body weight (NRC, 1979). Work by Ullrey et al. (1960) showed no treatment differences for growth rate and feed utilization efficiency when baby pigs were fed diets containing 6, 16 or 106 ppm of

copper. More definitive studies by Okonkwo et al. (1979) and Hill et al. (1983) indicate that the copper requirement of the baby pig is about 5 ppm. Most swine diets contain much higher levels of copper than the requirement since the two major dietary ingredients include cereal grains and leguminous or oilseed meals (protein supplement) which contain 4-8 ppm copper and 15-30 ppm copper, respectively (NRC, 1979).

I. Consequences of Feeding High Copper Diets to Growing Swine

Although the copper requirement is 4 to 6 ppm, addition of supplemental copper up to 250 ppm in a normal swine ration results in an increase in rate of weight gain and feed utilization efficiency in growing pigs (Barber et al., 1955a, 1955b, 1955c). However, in some instances, addition of 250 ppm of copper can produce toxicosis, depending upon the dietary levels of zinc and iron. When copper toxicosis occurs, growth performance is often reduced (Bass et al., 1956). In copper toxicosis, anemia also develops but can be prevented by iron supplementation, (Ritchie et al., 1963; Wallace et al., 1960) and skin lesions may occur but are alleviated by zinc supplementation (Ritchie et al., 1963).

Copper appears to be involved in the desaturase reaction of fatty acid metabolism because in copper-deficient rats there was a lower monounsaturated:saturated ratio and less desaturase activity in liver microsomes than in rats with adequate copper status (Wahle and Davies, 1975). It appears that the level of copper fed influences fatty acid composition and physical properties of backfat since higher concentrations of oleic acid and lower concentrations of stearic acid

in backfat lowered the mean melting point of whole backfat by 10°C (Moore et al., 1969).

Taylor and Thomke (1964) fed 250 ppm copper to pigs and showed that high dietary levels of copper produced softer backfat with a lower melting point. Bowland and Castell (1964, 1965) also observed that high dietary copper levels softened backfat. Copper supplementation at 280 ppm caused a significant increase in the proportion of unsaturated fatty acids in the outer and inner layer of backfat, and in perinephric fat at 26, 47, and 70 kg body weight (Elliot and Bowland, 1968). In addition, a corresponding decrease in the proportion of saturated fatty acids was observed while no difference in fatty acid composition was found at 90 kg live weight. The increases in unsaturated fatty acids was due to increases in 16:1 and 18:1 fatty acids with decreases in 16:0 and 18:0 saturated fatty acids. Other workers have attributed the softness of depot fat arising from copper supplementation to changes to increases in major unsaturated fatty acids (Elliot and Bowland, 1968; 1969; 1970). Moore et al. (1969) reported that depot fat from pigs fed 250 ppm copper, had a higher concentration of oleic acid and a lower concentration of stearic acid. Castell et al. (1975) reported a lower amount of stearic acid, higher palmitoleic acid and a higher oleic:stearic acid ratio. Several researchers have shown that depot fat softness arising from high copper feeding was caused by an increase in the proportion of unsaturated fatty acids and a concomitant decrease in the amount of saturated fatty acids (Amer and Elliot, 1973; Myres and Bowland, 1973; Ho and Elliot, 1973, 1974; Ho et al., 1975; Castell et al., 1975). Ho and Elliot (1973) suggested that the increase in

18:1 and the decrease in 18:0 fatty acids in depot fat of pigs fed high copper diets was related to an enhancing effect of copper on specific activities of hepatic and adipose stearoyl-CoA desaturase systems but later implied the involvement of the entire fatty acyl desaturase system (Ho and Elliot, 1974). Later, these authors suggested that unsaturated fatty acid increases were caused by the role of copper in desaturation reactions as a component of some cuproprotein enzyme system or metalloprotein (Ho et al., 1975).

II. GROWTH PROMOTING EFFECTS OF HIGH COPPER DIETS FED TO SWINE

A. Introduction

A considerable amount of research has been conducted in feeding high dietary levels of copper to stimulate growth and efficiency of gain in growing pigs. In 1955, Barber et al. observed improved growth response from pigs fed a normal diet supplemented with 250 ppm of copper as copper sulfate. Since that time, British researchers have observed a consistent 8% improvement in daily weight gain and a 5.5% improvement in feed efficiency by feeding supplemental copper sulfate at the 250 ppm level. However, U.S. researchers have not observed the same consistency in growth promotion when high copper diets were fed, as noted in reviews by Braude (1967) and Wallace (1967). The mode of action of copper on improving growth performance is still not well understood (Underwood, 1977) and little research has been conducted in this area.

B. Effect of High Copper Feeding on Growth Performance

Numerous studies have shown that supplementing the diets of growing pigs with copper (in the form of copper sulfate) at levels up

to 250 ppm, results in improved growth rate and feed efficiency (Braude, 1945; Barber et al., 1955a, 1955b, 1955c; Bowler et al., 1955; Barber et al., 1960; Allen et al., 1961; Barber et al., 1961; Lucas et al., 1962a; Wallace, 1967; Braude et al., 1970; Young and Jamieson, 1970; Braude and Ryder, 1973; Ho and Elliot, 1973). The beneficial effect from copper sulfate feeding has been shown to be due to the copper and not the sulfate radical (Barber et al., 1957; Bowland et al., 1961; Hawbaker et al., 1959).

Despite the reports of beneficial effects of high levels of copper feeding in the U.S. and Europe, other researchers observed no beneficial response (Lucas and Calder, 1957a; Teague and Grifo, 1966; Livingstone and Livingston, 1968; Parris and McDonald, 1969; Elliot and Bowland, 1970; Amer and Elliot, 1973). Gipp et al. (1973), reported that body weight gain was not consistently affected by feeding 250 ppm copper in corn-soy diets during the starter, grower, finisher stages or during the entire growth period. Ho et al. (1975), observed that the presence of high dietary levels of copper reduced average daily gain and feed efficiency. Canadian workers have also shown inconsistent benefits, with most of the studies showing no improvement in growth rate or efficiency by feeding high levels of copper (Castell and Bowland, 1968; Drouliscos et al., 1970; Young et al., 1970; Castell et al., 1975). Nevertheless, some Canadian workers reported beneficial effects with 250 ppm copper in starter diets of pigs up to 50 kg body weight (Beames and Lloyd, 1965; Young and Jamieson, 1970). Improvement in growth performance peaks and then plateaus when growing pigs fed high copper diets reach 50-60 kg in body weight, and a decline in

response after this time is due to an accumulation of copper in the liver (Lucas and Calder, 1957b; Bunch et al., 1963; Bellis, 1961). Miller et al. (1969) and the NCR-42 Committee on Swine Nutrition (1974) reported that growth rate during the growing phase was improved by feeding copper up to 250 ppm in the diet, but removing the copper after eight weeks did not affect subsequent growth performance. Elliot and Amer (1973) observed an improvement in growth rate in growing pigs fed high copper diets only up to 23, 46, or 69 kg body weight, which was not statistically significant, but upon removal of copper from the diet, growth was depressed. Withdrawal of copper from the diet when the pigs reached 46 or 57 kg in body weight did not affect performance but allowed liver copper concentration to return to normal levels by slaughter weight (Teague and Grifo, 1966).

C. Proposed Mechanisms of Growth Promotion When High Copper Diets Are Fed to Growing Swine

The mechanism of action relative to the effects of high copper feeding is not known but several suggestions have been made.

1. Bacteriostatic effects of copper

The bacteriostatic properties of copper in regard to the gut microflora (Sollman, 1957) have been implicated due to the "antibiotic-like" response observed when feeding high copper diets to swine. In other words, copper may alter microbial populations so that growth of desirable organisms that synthesize vitamins or amino acids is stimulated, by depressing undesirable organisms which compete with the host for nutrients, or both. In one study, Hawbaker et al. (1961) showed that lactobacilli, total aerobic, total anaerobic and

streptococci fecal counts were reduced, and molds, yeasts and coliforms were increased when high copper (as $CuSO_4$) diets were fed. These authors noted that copper sulfate increased molds and yeasts much like that reported for broad spectrum antibiotics (Pappenfort and Schnall, 1951). Similar effects of high copper feeding (as copper sulfate) on fecal flora counts were observed by Bunch et al. (1961), where lactobacilli, total aerobic and total anaerobic counts were reduced but molds' and yeasts' counts were reduced. Miller et al. (1969) reported a lower total fecal bacterial count in feces from pigs fed diets containing 250 ppm copper than feces of pigs fed a combination of oxytetracycline and neomycin sulfate. Fuller et al. (1960) observed a reduction in the numbers of streptococci and a shift in the relative proportion of three lactobacilli species when pigs were fed high copper diets. However, Smith and Jones (1963) did not observe any differences in fecal flora counts when high copper diets were fed.

Based on some of these reports, it is likely that copper, analogous to antibiotics, does alter the intestinal flora and this effect may be primarily responsible for the growth promoting effect observed when swine are fed diets containing high levels of copper. However, it must be realized that differences in fecal flora counts reported, represent only about 10% of the intestinal population. The gastrointestinal tract of man and animals has an oxidation-reduction potential of the contents below -250 mv which makes this an extremely anaerobic environment (Moore, 1969). Because of this extremely anaerobic environment, many of the bacteria present cannot be cultured with current anaerobic techniques. Therefore, approximately 90% of the

intestinal population of microbes has not been described and is essentially unknown (Moore, 1969).

2. Effects of high copper feeding on digestive and metabolic efficiency

Several workers have reported that copper supplementation at high dietary levels exerts its pronounced effects on growing pigs by increasing digestive and/or metabolic efficiency (Lucas et al., 1962a; Bunch et al., 1963; Wallace, 1967; Drouliscos et al., 1970). Other researchers attribute the effect of high copper feeding to a stimulatory effect on feed consumption (Mitchell, 1953; Barber et al., 1962; Braude, 1965; Young et al., 1970).

a. Relationship of high copper and dietary protein source and level on protein digestion and utilization

By dosing young pigs with copper, an improvement in the digestibility of proteins has been observed and appears to be a result of an increase in cupric ion concentration, increasing pepsin activation and producing more peptic hydrolysis of proteins (Kirchgessner and Giessler, 1961; Kirchgessner et al., 1976).

Protein level and source appear to influence the effectiveness of high level copper feeding in young growing pigs. British researchers have continually observed beneficial effects of feeding barley-fish meal diets containing 150 to 250 ppm copper as copper sulfate (Barber et al., 1955a, 1955b, 1955c, 1957; Bowler et al., 1955; Bellis, 1961; Braude and Ryder, 1973). Pigs fed corn-soybean meal basal diets, typically used in the U.S., have not produced consistent growth performance responses to copper supplementation and copper levels above

250 ppm have been shown to be toxic (Wallace et al., 1960). However, Bunch et al. (1961) fed 22% protein corn-soybean meal diets containing copper levels up to 250 ppm and observed an improvement in growth performance but there was a linear decrease in hemoglobin levels. Canadian workers observed a 2.2 to 1.7% improvement in growth and a 2.0 to 2.2% improvement in feed efficiency from pigs when 125 and 250 ppm copper, respectively, were supplemented in these diets (Castell et al., 1975). Omole and Bowland (1974) fed pigs diets that were isonitrogenous and isocaloric, utilizing either soybean meal, low glucosinolate rapeseed meal or a combination of these two protein sources, along with 10, 135 and 210 ppm copper, and observed no differences on feed intake, gain, or feed efficiency. Pigs fed dried skim milk diets containing 250 ppm copper during both the growing and finishing phases, gained at the same rate as pigs fed a corn-soybean meal diet with or without copper supplementation, but pigs fed dried skim milk diets had carcasses with a higher fat content (Gipp et al., 1973). Edmonds and Baker (1984) have observed that when high levels of dried whey are added to a low-protein antibiotic fortified corn-soybean meal diet, and fed to weaning pigs, weight gain and feed efficiency are greatly improved. Edmonds and Baker (1984) showed that adding 250 ppm copper (from $CuSO_4 \cdot 5H_2O$) greatly improved rate and efficiency of gain, particularly during the first week postweaning, and an additive response for rate of weight gain was observed when 25% dried whey and 250 ppm copper were both added to a corn-soybean meal diet. There may be a unique relationship between the amount of copper and the amount of dried whey present in the diet to elicit such a response.

b. Effect of high copper feeding on lipid metabolism

Myres and Bowland (1973) measured plasma free fatty acids in both the fed and fasted state in pigs receiving 250 ppm or no supplemental copper. In the fasted state, pigs fed 250 ppm copper had lower levels of free fatty acids than pigs receiving no supplemental copper, with the opposite effects occurring in the fed state.

c. Mineral interrelationships relative to the effectiveness of high copper feeding

Certainly, mineral interrelationships with copper would also determine the effectiveness of high level copper feeding. Zinc, iron, cadmium, molybdenum, sulfur, calcium, lead, silver and phytate either directly or indirectly influence the absorption and utilization of copper due to competition between metals for functional sites on enzymes and formation of metal complexes with protein, such as metallothionein (Underwood, 1977). In other words, an unusually high intake of one element can adversely affect the utilization of other elements. Ascorbic acid also chelates copper to reduce copper absorption (Underwood, 1977). Variations in growth response observed when pigs are fed high levels of copper may be due to the levels of iron and zinc in the basal diet (Underwood, 1977). Copper is known to compete with iron for absorption binding sites and with zinc for metal-binding protein. Zinc and iron have been shown to have a significant impact on copper absorption. When pigs were fed 250 ppm copper sulfate, they developed skin lesions similar to parakeratosis (O'Hara et al., 1960). DeGoey et al. (1971) observed a reduction in the incidence of parakeratosis in pigs fed a basal diet supplemented

with 100 ppm iron or basal plus 100 ppm zinc. Supplemental zinc has been shown to reduce liver copper accumulation from high dietary levels of copper (Hoefer et al., 1960; Wallace et al., 1960; Ritchie et al., 1963). Liver iron levels were lowered in pigs fed 250 ppm copper (Bunch et al., 1963). When toxic levels of copper are fed (750 ppm), high levels of zinc (500 ppm) reduce copper retention in the liver and reduce tissue damage. When 750 ppm iron was added to a 750 ppm copper diet, anemia was less severe than with only 750 ppm copper, less copper accumulated in the serum and tissue damage was reduced (Suttle and Mills, 1966).

Several forms of copper such as copper sulfate, copper oxide, copper chloride, and copper carbonate are effective growth promoters (Wallace, 1967), while copper sulfide is not an effective growth promotant because the copper sulfide is insoluble and thus, copper is not available to perform its role as a growth promotant (Cromwell et al., 1978).

Molybdenum and sulfate in combination, prevent tissue buildup of copper in ruminants (Dick, 1953, 1954; Ammerman and Marcilese, 1968) but are ineffective in swine (Kline et al., 1971). Sulfide, however, appears to prevent excessive liver accumulation of copper in pigs fed high copper diets (Kline et al. 1973). Cromwell et al. (1978) fed sodium sulfide to growing-finishing pigs and found a reduction of copper uptake in the liver, with no apparent interference with the growth promoting effects of copper in pigs fed high copper diets. However, when these authors fed copper sulfide to pigs, no improvement in performance was noted, nor was there an increase in liver copper

levels. Sulfide, either in the form of sodium sulfide (Kline et al., 1973; Cromwell et al., 1978) or ferrous sulfide (Prince et al., 1979; Lima et al., 1981) are effective in preventing copper accumulation in the liver in pigs fed 250 ppm copper. A 2:1 ratio of sulfide:copper reduced liver copper to approximately the same level as observed in control pigs, but didn't interfere with the growth promotion effects of copper. Elemental sulfur is only about half as effective as sulfide in reducing liver copper accumulation. Prince et al. (1979) indicated that sulfide acts to prevent uptake of copper by liver tissue, apparently by reduced absorption, rather than by increasing mobilization of copper from the liver. This suggests that copper exerts its action in the digestive tract of the pig. Williams et al. 1981, suggested that a portion of the copper in the gut may be rendered insoluble by the inclusion of sulfide or sulfur in the diet, but this does not appear to be of sufficient magnitude to account for the decreased liver stores and does not explain the reason for sulfide interaction with the growth promoting effects of copper.

d. Other proposed mechanisms of growth promotion when high copper diets are fed

Braude (1967) suggested other possible modes of action as fungicidal action, anthelmintic activity, or a reaction of microbial-produced hydrogen sulfide in the intestine with copper to provide copper sulfide. Newton (1984) suggested that feeding high copper diets to young, growing swine may reduce the detrimental effects of naturally occurring phenol and polyphenol compounds present in plant products which depress growth rate and feed efficiency.

III. EFFECTS OF FEEDING HIGH LEVELS OF COPPER IN COMBINATION WITH ANTIBIOTICS ON GROWTH PERFORMANCE

Since both high levels of copper and antibiotics frequently improve growth performance in the young pig, studies have been conducted to ascertain if a synergistic effect on growth stimulation could be obtained. Stahly et al. (1980) fed a diet supplemented with 250 ppm copper with or without 55 ppm chlortetracycline or 27.5 ppm of virginiamycin to early weaned pigs. When either copper or antibiotics were fed singly, growth rate increased by 17 to 22 percent and feed/gain improved by 5 to 9 percent but the combination of copper and antibiotics resulted in a 23 to 32 percent improvement in growth rate and 7 to 14 percent improvement in feed to gain ratios compared to pigs receiving the control diet. Similar additive responses were observed when copper was fed in combination with carbadox or with a combination of penicillin, chlortetracycine and sulfamethazine (Mahan, 1980). Beames and Lloyd (1965) reported an additive response in early weaned pigs fed a combination of copper and tylosin.

It is less clear if there is a complementary effect from feeding copper in combination with antibiotics to older, growing-finishing pigs. An additive trend was reported by Barber et al. (1978) with copper and virginiamycin but Lima et al. (1981) failed to show any additive response from the combination of copper and chlortetracycline or the combination of copper and virginiamycin compared to single additions of these antimicrobial agents.

No differences in growth performance were observed when higher copper diets were fed in combination with tylosin (Lillie et al., 1977;

Beames, 1969), zinc bacitracin or emtryl (Barber et al., 1965). Furthermore, when copper sulfate ($CuSO_4 \cdot 5H_2O$) was added at a level of 0.1% to diets containing dried skim milk, white fish meal or extracted soybean meal as protein sources, there was no synergistic effect with zinc bacitracin, tylosin, or formosulphathiazole (Livingstone and Livingston, 1968). However, feeding a combination of high copper and chlortetracycline improved growth performance compared to feeding chlortetracycline alone (Wallace, 1967). Similarly, Hawbaker et al. (1959) observed an additive response to weight gain when high copper was fed in combination with oleandomycin or oxytetracycline. In contrast, Braude (1967) observed no additive effect on growth performance when copper sulfate and oxytetracycline were fed in combination. Finally, Cromwell et al. (1978) summarized Kentucky, Ohio, and Canadian studies where there was an additive effect in growth response from feeding high levels of copper sulfate along with antibiotics and suggested that copper may have a broader spectrum of antibacterial activity or that copper may have a different mode of action than the antibiotics studied.

Because of the evidence that copper is "antibiotic-like" in its growth promoting effects and appears to influence the intestinal microflora, it is essential to understand the current knowledge of the mode of action of antibiotics to gain insight into possible modes of action of high copper feeding to swine.

IV. ANTIBIOTICS IN LIVESTOCK FEEDS AND THEIR MODE OF ACTION

Antibiotics have been widely used as feed additives in livestock feeds for the past 30 years because of their beneficial effects on

improving growth rate and feed conversion as well as reducing mortality and morbidity from clinical or subclinical infections.

Many antibiotics have been used in livestock production and these feed additives vary in their effectiveness as growth promotants. Several commonly used antibiotics include: chlortetracycline, oxytetracycline, penicillin, sulfamethazine, bacitracin, tylosin and others.

It is generally accepted that antibiotics have an ability to suppress or inhibit the growth of certain microorganisms in their growth promoting effects. Antibiotics differ in a variety of ways such as in their chemical composition, bacterial spectrum and absorption and excretion patterns (Hays, 1969). These characteristics certainly influence bactericidal and bacteriostatic properties and their effectiveness against systemic infections (Hays, 1969).

When considering the mechanisms by which antibiotics improve growth performance, three general modes of action have been postulated: (1) a metabolic effect, (2) a nutrient-sparing effect and (3) a disease-control effect (Hays, 1969).

A. Metabolic Effect

The metabolic effect hypothesis suggests that antibiotics directly affect the rate or pattern of the metabolic processes in the host animal, which is also related to the disease-control effect hypothesis because the rate of metabolism may be related to systemic infections (Hays, 1969). Water and nitrogen excretion are affected by feeding chlortetracycline, (Braude and Johnson, 1953). Tetracycline inhibited fatty acid oxidation by the mitochondria in rat liver homogenates

(Brody et al., 1954). Phosphorylation and oxidation reactions requiring magnesium ions were inhibited by tetracycline (Weinberg, 1957). Tetracyclines have also been shown to inhibit protein synthesis (Hash et al., 1964). Although antibiotics do appear to alter metabolic processes, the contribution of metabolic effects relative to other contributing factors probably does not account for a majority of the beneficial growth response typically observed because of the magnitude of animal responses relative to normal tissue levels of antibiotics when added to the diet at growth-promotant levels (Hays, 1969).

B. Nutrient Sparing Effect

Hays (1969) noted that the nutrient-sparing effect hypothesis is based on the assumption that antibiotics may reduce the dietary requirement for certain nutrients by:

1) stimulating growth of desirable organisms that synthesize vitamins or amino acids.
2) depressing organisms that compete with the host for nutrients.
3) increasing the availability of nutrients by chelation mechanisms.
4) improving absorptive capacity of the intestinal tract.

Certain intestinal organisms synthesize vitamins and amino acids which are needed by both the host animal and other intestinal microorganisms, and these microbes in turn, compete with the host animal for these nutrients (Hays, 1969). Visek (1978) proposed that it is not likely that antibacterials exert a vitamin-like action or provide chemical moieties essential for metabolism.

Streptomycin stimulated growth of some yeasts (Moore et al., 1946) and penicillin increases the number of coliforms (except E. coli)

(Anderson et al., 1952). Since these organisms are important for synthesizing dietary essential nutrients, the supplementation of diets with antibiotics would increase the synthesis of these nutrients.

Since lactobacilli require amino acids in similar relative proportions as the pig, and since the level and source of protein supporting maximum growth in pigs is also near optimum for multiplication of lactobacilli in the intestinal tract (Kellogg et al., 1964), lactobacilli are considered to be an example of organisms which compete with the host animal for dietary needs. Certain antibiotics cause a reduction in the number of lactobacilli (March and Biely, 1952; Anderson et al., 1952; Johansson and Sarles, 1949). Furthermore, it appears that the most effective, routinely used growth promotants are those antibiotics that are the most effective in reducing the number of these intestinal organisms (Kellogg et al., 1966).

Jukes (1955) and Taylor (1957) have indicated that the metabolism of intestinal microbes is changed rather than the relative number of each microbial species when antibiotics are fed.

When an antibiotic is fed to pigs, the intestinal wall is thinner than the intestinal wall of pigs fed diets without antibiotics (Braude et al., 1955). This same effect was observed in chicks (Eyssen and DeSomer, 1963a, 1963b; Pepper et al., 1953) and when fed the intestinal contents of infected chicks, the intestinal wall was thicker (Coates, et al., 1955). Hill et al. (1957) and Taylor and Harrington (1955) reported similar effects in chicks and pigs. A reduction in intestinal thickness implies a potential for improved absorption due to inhibition of organisms which damage or produce toxins that damage intestinal tissue (Taylor, 1957).

Glucose absorption was increased when pigs were fed diets containing antibiotics which supports the basis for improved nutrient utilization from feeding antibiotics (Catron et al., 1953). Several researchers have also shown that less protein is required by pigs for maximum performance when pigs are fed diets containing antibiotics (Catron et al., 1952; Burnside et al., 1954; Beacom, 1959a, b). Several researchers have shown an association between diet and antibiotic response where the response to antibiotics is generally greater when antibiotics are added to the inadequate diet (Lucas and Calder, 1957b; Braude and Johnson, 1953; Stokstad, 1954; Burnside et al., 1949).

Visek (1978) noted that the growth improvement observed when feeding diets containing antibiotics to swine is not well correlated with differences in bacterial counts in specific intestinal sections or for bacterial counts of the entire gut. This also appears to be true in pigs fed copper-supplemented diets (Smith and Jones, 1963; Miller et al., 1969; Hawbaker et al., 1961; Fuller et al., 1960; Bunch et al., 1961). As a result, Visek (1978) suggested that a change in bacterial metabolism may be nutritionally more important for the animal than a change in the relative number of microbial species. Vervaeke et al. (1979) studied the production rates of volatile fatty acids (VFA) and lactate when virginiamycin and spiramycin were added to incubated stomach, ileal and cecal contents of pigs. These authors observed a decrease in organic acid production and a concommitant sparing of a measurable quantity of glucose upon addition of these two antibiotics to stomach and ileal contents. When virginiamycin was added to cecal

contents organic acid production was also reduced, but spiramycin increased organic acid production. Furthermore, these authors, using in vivo data, calculated that the sparing of carbohydrates by incorporating these two antibiotics in diets for swine resulted in more net energy available for growth, and this estimated increase in net energy accounted for a majority of the growth improvement typically observed when these antibiotics are fed. This carbohydrate sparing action observed for these two antibiotics may also explain the improvements in growth of pigs fed other antimicrobials, particularly copper.

For carbohydrates not digested and absorbed in the small intestine, these energy sources can be readily fermented to volatile fatty acids in the cecum and colon and contribute 5 to 28 percent of the pig's energy requirement (Friend et al., 1963a; 1963b; Farrall and Johnson, 1972; Kass et al., 1980). Imoto and Namioka (1978) measured the production of acetate, propionate and butyrate in the large intestine of the pig when either a high or low carbohydrate diet was fed. Mean total VFA absorption rates were 0.85 and 0.65 moles per day for the low carbohydrate and high carbohydrate diets, respectively. This amount of VFA absorption is equivalent to 230 and 190 kcal of energy for the high and low carbohydrate diets respectively, which correspond to 11.6 and 9.6% of the metabolizable energy for maintenance. Rerat (1978) indicated that digestion of carbohydrate in the pig large intestine represents only 10 percent of digestible energy intake. Thus, VFA production is important as an energy source as it is absorbed from the lower digestive tract. Perhaps copper increases VFA production in the lower digestive tract to improve the efficiency of

energy utilization of carbohydrates not digested and absorbed in the upper intestinal tract.

Several researchers have suggested that improved growth performance is due mainly to an increase in food intake because even though total nutrients retained is greater for animals fed antibiotics, the percentage retained between those animals fed antibiotics and those which are not, are similar (Meade and Forbes, 1956; Wallace et al., 1954; Bush et al., 1959).

C. Disease-Control Effect

Finally, the third proposed mode of action for antibiotics is a disease-control effect by suppression of organisms causing clinical or subclinical disease (Hays, 1969). Ninety seven percent of the microbial population within the alimentary tract of man and animals is made up of obligate anaerobes (Moore and Holdeman, 1972) and these anaerobes are significant causes of disease (Keusch, 1974; Klainer and Beisel, 1969). Many studies lend support for the disease-control effect as a major factor in improved growth performance when animals are fed antibiotics. In general, the degree of response to antibiotics is inversely related to the well-being of animals. Antibacterial agents are generally more effective growth promotants in rearing conditions where hygiene is poor, compared to new or isolated environments (Libby and Schaible, 1955), but not in every situation (Coates and Harrison, 1969). Healthy pigs fed well balanced diets and reared in a sanitary environment responded less to antibiotic supplementation then those pigs that were not (Speer et al., 1950; Hays and Speer, 1960).

Visek (1978) postulated that antibiotics reduce microbial production of growth depressing toxins from bile acids and nitrogenous substances.

In the absence of bacteria, portal blood ammonia concentrations are about 25% of portal ammonia concentrations in conventional animals (Warren and Newton, 1959) and are reduced in conventional animals fed antibiotics (Stahl, 1963; Warren and Newton, 1959). Urea and bile acid hydrolysis occur in the gastrointestinal lumen of conventional animals but not in germ-free animals and their hydrolytic products are reduced or abolished by antibiotics (Dintzis and Hastings, 1953; Kornberg and Davies, 1955; Levenson et al., 1959; Delluva et al., 1968; Norman and Widstrom, 1964; Walser and Bodenlos, 1959; Visek et al., 1959).

It appears that the main source of ammonia in the body occurs when bacteria act on nitrogenous substances in the intestinal lumen (Folin and Denis, 1912; Phillips et al., 1952). Bacteria provide a major contribution to the portal ammonia concentration through deamination of ingested protein and urea hydrolysis as observed when antibiotics are fed (Dintzis and Hastings, 1953; Silen et al., 1955). Ammonia is recognized as a toxin in warm-blooded animal species (Phillips et al., 1952; Visek, 1964; 1972). Ammonia is one of the microbial products that increases the wet weight, alters nucleic acid synthesis and increases protein in the intestinal mucosa (Topping and Visek, 1977). Normal ammonia concentrations range between 8 to 10 mM (140 to 180 ppm) in the intestinal lumen (Prior et al., 1974; Wrong et al., 1965; Wilson et al., 1968a, b) which is several times the concentration required to destroy cells (Dang and Visek, 1968), alter the rate of nucleic acid

synthesis by mammalian cells (Zimber and Visek, 1972) increase mycoplasma infections in controlled experiments in laboratory animals (Broderson et al., 1976) and to depress the immune response (Fridlyand, 1959). Copper is an inhibitor of sulfhydryl enzymes such as urease and a reduction of urease activity has been observed in the gastrointestinal lumen of birds fed copper-supplemented diets (Stokstad et al., 1953; Visek et al., 1959).

Products of hydrolysis produced from bile acids by bacterial enzymes also have detrimental consequences because they change micelle formation, lipid absorption, intestinal histology and calcium absorption (Combe et al., 1976). They depress growth when fed in small amounts but there are no studies describing the effects of growth promoting concentrations of antibacterial agents upon bile acid metabolism. The microbial flora modify lipids and bile acids and has been described by Eyssen and DeSomer (1963a, 1963b, 1965) where transitory malabsorption of fats and carbohydrates in chicks fed casein-sucrose diets was observed along with decreased feed efficiency. Malabsorption of fats and carbohydrates was reduced and feed efficiency was improved by feeding 20 ppm of virginiamycin. It has been suggested that intestinal flora diminish the absorption of alimentary fat by modifying the intestinal mucosa (Sprinz, 1962) or by producing toxic unconjugated derivatives of bile acids (Dawson and Isselbacher, 1960). Feeding of 0.2% lithocholic acid to chicks caused malabsorption of fats, impaired efficiency of feed utilization and a profound increase in liver size with a proliferation of bile ductules (Leveille et al., 1962). The metabolites of bile acid metabolism, which are not produced

in germ-free animals, need further investigation in antibiotic supplemented conventional animals. According to Norman and Widstrom (1964), bile acids are excreted in a conjugated form, with more than 90% of these bile acids being conjugated with taurine. In the small intestine, most of the bile acids are also conjugated, whereas the bile acids in the cecum, colon and feces are almost all unconjugated (Norman and Widstrom, 1964). Since bile acids in the feces of germ-free rats and rats treated with antibiotics are almost exclusively conjugated, the hydrolysis of taurine and glycine conjugates has been ascribed to the action of microbial enzymes. Combe and coworkers (1976) concluded that the presence of "non-pathogenic" bacterial flora in the digestive tract has to be considered as a factor having an unfavorable action on the digestion and absorption of dietary lipids and that action on the absorption of long and saturated fatty acids (palmitic and stearic acids) is particularly marked. These authors further suggest that the microflora acts principally at this level through intermediary bile acid metabolism.

V. MORPHOLOGICAL AND PHYSIOLOGICAL CHARACTERISTICS OF THE GERM-FREE ANIMAL

It is essential to understand the morphological and physiological differences between germ-free and conventional animals in order to determine the association between the microflora and the systemic functions of the host. Early workers in germ-free research examined the ability of germ-free animals to survive, grow and reproduce (Pleasants, 1968). Most studies with germ-free animals involve laboratory rodents, primarily rats and mice, because of their

acceptability as experimental models as well as the ease of managing these animals under germ-free conditions. Morphological and physiological data are lacking for the larger animal species (ruminants and pigs) because of size limitations, quantity of feed required and increased cost. Chickens have been used somewhat more extensively in germ-free research.

A. Survival, Growth and Reproduction of Germ-Free Animals

Pleasants (1968) reviewed species differences on their abilty to survive, grow and reproduce in a germ-free environment. Germ-free rats and mice live longer than their conventional counterparts and also live longer than germ-free rabbits and guinea pigs. Mortality of germ-free guinea pigs and rabbits is lower than conventional animals in early life but is reversed for these animals later in life. Cause of mortality for all of these species is generally attributed to enlargement and distention of the cecum. This is particularly responsible for increased mortality among rabbits and guinea pigs due to the greater organ size for these species even in the conventional state. Dogs, cats and monkeys have been maintained for more than 10 months with no apparent problem. Lambs and kids have been maintained for 4 months but diet modifications were required to prevent altered mineral metabolism and kidney lesions.

Pleasants (1968) classified the various species used in germ-free research based on relative growth rates compared to conventional animals of the same species.

Germ-Free Growth Equal to or Superior to Conventional Growth	
Chicken	Japanese quail
Monkey	Pig
Germ-Free Growth Similar to Conventional Growth	
Rat	Turkey
Mouse	Calf
Dog	Burro
Cat	
Germ-Free Growth Less Than Conventional Growth	
Guinea pig	Kid
Rabbit	Lamb

Most of the species that rely on microbial fermentation in their gastrointestinal tracts for nutritive sustenance, perform submaximally in a germ-free environment. However, germ-free ruminants were maintained only during the pre-ruminant period and were fed milk diets rather than dry feedstuffs.

Landy and Ledbetter (1966) reported that conventionally reared miniature pigs weighed more at 3 weeks of age than their germ-free counterparts, but by 10 weeks, the germ-free pigs surpassed the conventional pigs in body weight. Waxler and Drees (1972) and Miller et al. (1982) observed a similar growth rate for germ-free and conventional pigs up to 3 weeks of age but no data were collected after 3 weeks. Miniats and Valli (1973) reported that conventional pigs grew about 50% faster than germ-free pigs up to 8 weeks of age but conventionally reared pigs were allowed to nurse the sow while germ-free pigs were fed a condensed cow's milk diet supplemented with iron.

Pleasants (1968) noted that germ-free rats and mice have been maintained through 28 generations with no loss of reproductive capacity. Germ-free rabbits have been maintained through three generations but fertility declined with each successive generation and was finally lost due to the sensitivity of the species to inbreeding. Germ-free chickens and Japanese quail have been reproduced but not for extended periods of time. No data are available on reproductive efficiency for the larger animal species because of the physical limitations of rearing these animals through one generation.

B. Differences in Organs, Tissues and Systems Between the Germ-Free and Conventional Animal

Since active defense against microbial action involves tissues, organs and systems which serve other functions, these may be altered in form or functional capacity of the germ-free animal.

1. Organ weights of germ-free pigs

When organ weights of germ-free pigs were expressed as mg/100 gm of body weight to compensate for differences in growth rates between germ-free and conventional pigs, the mandibular lymph nodes, adrenals, heart, spleen, kidneys, thyroid, stomach and small intestine of germ-free pigs weighed less than those organs from conventional pigs (Waxler and Drees, 1972).

2. Systemic characteristics of the germ-free animal

Several systemic differences have been described when germ-free and conventional rats were compared (Pleasants, 1968; Gordon, 1968). The following characterisitcs were expressed on the basis of percentage reduction of the germ-free level below the conventional level.

Basal metabolic rate	20%
Iodine uptake by thyroid	50%
Cardiac output (ml/min/kg BW)	32%
Blood flow (mg/100 g BW)	22%
Arterial blood flow to liver	50%

3. Characteristics of the gastrointestinal tract of germ-free animals

The gastrointestinal tract of germ-free rats is very different in its characteristics compared to conventional rats. Gordon (1968) reviewed several unique characteristics of the germ-free rat. The intestinal wall is thinner and the gastrointestinal tract is less well filled in the germ-free rat. The wet weight of the intestinal wall is reduced and the dry weight is increased when expressed per unit of body weight for rats in the germ-free state. Lamina propria is reduced in the germ-free rat and is probably the main cause of reduced weight of the small intestine. The proportion of intestinal epithelium is normal or slightly reduced. Mucosal surface area is reduced by about 30% and corresponds well to the reduction in dry matter per unit length of the intestinal wall. Renewal rates of the epithelium, lamina propria and Peyer's patches of the intestine are reduced in germ-free rats. A reduction in lamina propria may also be a cause for the reduced DNA content of the intestinal wall. An enlarged and distended cecum is usually observed and both wet and dry weight of the cecum wall is increased due to an increase in mucosa, submucosa and muscle tissue in the germ-free condition. Colon appearance is similar to conventional rodents. Finally, alkaline phosphatase is elevated and acid phosphatase is reduced in the epithelial lining of the cecum.

Germ-free rats generally consume 10% more feed and slightly more water than their conventional counterparts and cecal contents and feces are softer in consistency.

The digestive tract of germ-free pigs differs from conventional pigs by its lower weight, reduced thickness and cellularity of lamina propria and muscularis, poorer development of the associated lymph tissue and by the liquid contents of the cecum and colon (Miniats and Valli, 1973). Most of these characteristics are similar to that described for germ-free rodents except that the cecum of the germ-free pig is not enlarged.

4. Digestive functions

Since several characteristics of the gastrointestinal tract are different in a germ-free environment, and since gastrointestinal microflora play a role in digestive functions and in the nutrition of the animal, it is important to understand these differences when comparing germ-free and conventional animals.

Gordon (1968) summarized research involving various factors related to digestive functions in the germ-free rat. The upper gastrointestinal tract pH of the germ-free rat is the same as the conventional rat but the lower tract of the germ-free rat is more alkaline. Osmolarity of the gastrointestinal tract of the germ-free rat is generally lower and the oxidation-reduction potential of the cecal contents is 250-300 mV more positive than conventional rats.

In Gordon's (1968) review, he noted that germ-free rats excrete more fecal nitrogen which decreases the coefficient of nitrogen utilization. However, there is a 3 to 4 fold increase in nitrogen in

cecal contents. More specifically, the quantity of amino acids (primarily tyrosine, threonine and methionine which are characteristic of mucoproteins) are 50 to 100 times higher and there is also an increase in mucoproteins, hexosamines and urea, but histidine levels are lower in cecal contents of germ-free rats.

More bilirubin is excreted in feces but no urobilin is found in urine or feces of germ-free rats.

Gordon (1968) also described changes in lipid digestion and metabolism from reports by various researchers. Less fecal fatty acids were excreted in the germ-free rat and most of these fatty acids are unsaturated. Cyclical and branched chain fatty acids, which are characteristic of bacterial synthesis, were not found. Less total bile acids were excreted, and of those excreted, most was taurocholic acid which indicates a profound influence of the intestinal microflora on bile acid metabolism. Germ-free rats excreted more cholesterol while liver and serum cholesterol were increased, and systemic cholesterol metabolism was reduced.

In regard to intestinal digestive enzymes, Gordon (1968) summarized research studies which indicated that feces of germ-free rats had higher levels of trypsin and invertase. Carbohydrate digestive enzymes were somewhat different in the germ-free rat. Disaccharidases (maltase, invertase, lactase, trehalase and cellobiase) were increased, and slightly more amylase was excreted in feces of the germ-free rat. In germ-free chicks, protease (trypsin fraction) and amylases were increased in the cecum, colon and cloaca while the reverse was observed for lipase.

Reddy et al. (1965a) observed that plasma iron and copper levels were lower, liver iron and copper concentrations were higher, while spleen, kidney and total body iron and copper levels were lower for germ-free rats compared to their conventional counterparts. Further evidence that copper and iron metabolism is influenced by the gut microflora was reported by Reddy et al. (1965b) with germ-free rabbits. Hypochromic anemia was observed in germ-free rabbits as evidenced by low hemoglobin and hematocrit levels. These rabbits had low plasma iron but normal copper levels and total iron binding capacity was increased. Iron and copper concentrations were lower in spleen and kidneys but higher in the liver of the germ-free rabbits. When these anemic rabbits were conventionalized, these symptoms were alleviated. Gordon (1968) reviewed evidence which indicates that the oxidation-reduction potential in cecal contents is more positive in germ-free compared to conventional animals. If this is also true for the intestinal contents, the availability of certain nutrients (such as iron) may be improved.

When germ-free and conventional pigs were fed a sterile condensed milk diet from birth to 4 weeks of age, higher levels of hemoglobin and hematocrit were observed for germ-free pigs compared to conventional pigs (Miller et al., 1982). Waxler and Drees (1972) noted the same relationship for hemoglobin and hematocrit in germ-free pigs receiving a condensed milk diet compared to conventionally reared baby pigs. Furthermore, mean corpuscular volume, mean corpuscular hemoglobin and serum iron tended to be greater for the germ-free pigs (Miller et al., 1982).

Finally, Gordon (1968) indicated that calcium metabolism is somehow altered in the germ-free rat since a high incidence of urinary calculi has been observed. Tissue levels of manganese in germ-free rats are similar to those of conventional rats while the amount of sodium in the cecum is 6 to 10 times greater in germ-free rats due to the greatly enlarged cecum in the germ-free rats. However, the concentration of sodium in the cecum of germ-free rats was similar to conventional rats. Conversely, the total amount of potassium in germ-free and conventional rat ceca is comparable but its concentration is much lower in the germ-free rats.

Germ-free rats require all known vitamins needed by their conventional counterparts. When deficiency experiments were conducted, no major differences in deficiency symptoms were observed between conventional or germ-free rats. However, vitamins such as thiamin, biotin, folic acid and vitamin K which are synthesized by gut microflora and contribute to meeting the animal's nutrient requirements in the conventional state, must be supplemented to a properly fortified diet to insure presence of adequate levels after autoclaving (Zimmerman and Wostmann, 1963; Wostmann et al., 1962).

5. Effect of germ-free environment on humoral and cellular defense mechanisms

Wostmann (1968) reviewed research involving humoral defense mechanisms in the germ-free animals. In general, these animals have an antibody production system that is quantitatively smaller, deficient in potential antibody forming cells and only partially accessible depending upon dietary and environmental conditions. Sterilized diets

for germ-free animals which may have been heavily contaminated with microorganisms prior to sterilization can be antigenic. This system responds well to antigenic stimulation but prolonged stimulation may be required to overcome the original deficiency in size and cellularity.

Bauer (1968) summarized the effects of rearing animals in a germ-free environment on cellular defense mechanisms. Macrophage morphology, distribution, phagocytic capacity or delivery of antigenic foreign material to the regional lymph nodes are not altered in germ-free animals. Conventional mice have a greater capacity for macrophages to process antigens due to continuous exposure to microbial flora even though these functions are equally developed in germ-free mice. Absence of microflora stimulation causes a delayed onset of immune response in germ-free lymphatic tissue but slower digestion and release of immunogenic fragments results in a more sufficient response in conventional animals.

Germ-free pigs have lower total leukocyte counts due to lower absolute lymphocyte counts compared to conventional pigs (Waxler and Drees, 1972).

6. Importance of gut microflora to growth

When antibiotics are added to growing livestock diets, an improvement in growth performance is observed (Hays, 1969) . In attempts to understand the cause of this phenomenon, it was postulated that the microflora of the alimentary tract may interfere with an animal's ability to develop its full growth potential and that antibiotics (or any dietary constituent) capable of eliminating such organisms, or discouraging their establishment, might have a beneficial effect on growth.

Several studies have demonstrated that dietary antibiotic supplementation had no effect on growth rate of germ-free chicks. Lev and Forbes (1959) introduced a strain of colstridium welchii to germ-free chicks and a growth depression was observed. However, when these monocontaminated chicks were given dietary procaine penicillin, growth depression was alleviated. No growth stimulation was observed when germ-free chicks and turkeys were fed antibiotics at a level of 50 mg/kg of diet (Luckey, 1952). Luckey et al. (1955) later reported that low levels of antibiotics, 25 mg/kg of oxytetracycline and 11 mg/kg of procaine penicillin, produced a slight improvement in growth rate but these data are based upon a limited number of birds. A growth promoting effect in conventional but not germ-free turkeys was observed when 45 mg/kg of penicillin or 30 mg/kg or oleandomycin were added to the diet (Forbes et al., 1958). Germ-free chicks grew 15 to 25 percent faster than conventional chicks fed the same autoclaved casein-starch or soybean meal-corn diet containing 25 mg potassium penicillin or 45 mg procaine penicillin (Forbes and Park, 1959). When chlortetracycline, penicillin and streptomycin were injected into chick embryos under sterile conditions, there was no direct growth promoting action of the antibiotic on tissues of the host (Jukes and Williams, 1953; Stokstad, 1954). Furthermore, Whitehair and Thompson (1956) observed a lack of growth improvement when antibiotics were fed to pigs under germ-free conditions.

MATERIALS AND METHODS

I. EFFECT OF HIGH-COPPER FEEDING TO WEANLING PIGS TO GERM-FREE AND CONVENTIONAL ENVIRONMENTS ON GROWTH PERFORMANCE AND VARIOUS PHYSIOLOGICAL PARAMETERS.

A comparative study of the effects of feeding a supra-nutritional level of dietary copper to weanling pigs reared in germ-free and conventional environments was conducted to examine potential differences in growth performance; portal and peripheral blood glucose, amino acids and ammonia levels; intestinal weight and morphology; organ weights; hematology; and tissue trace element concentrations. The study utilized two types of rearing environments, a germ-free environment and a conventional environment. Pigs were managed similarly in each environment and fed one of two autoclaved experimental diets.

A. Management of Germ-Free Pigs

One litter of 10 (3 gilts, 7 boars) crossbred pigs (Hampshire x Yorkshire-Duroc-Landrace) was delivered by caesarotomy on day 112 of gestation and placed in rearing isolators containing the necessary equipment for the 7-week germ-free portion of the trial. Refer to Appendix B for a detailed description of isolator preparation and procedures used in obtaining and successfully rearing these germ-free pigs.

Rearing isolators were marked and identified as I, II and III. Isolator I and III contained 3 pigs each (1 gilt, 2 boars) while isolator II contained 4 pigs (1 gilt, 3 boars). On day 1, all pigs were ear notched (#1 through #10) and needle teeth were clipped using side cutters.

Pigs were bottle fed with evaporated milk (Carnation Company, Los Angeles, CA) three times daily at 6 am, 2 pm and 9 pm for 4 weeks. Nutrient analysis for the evaporated milk (Leveille et al., 1983) and a comparison to nutrient composition of sow's milk (Pond and Houpt, 1978) is shown in Table 1. Average daily milk consumption per pig for weeks 1 through 4 was as follows:

Week 1 235 ml/pig/day

Week 2 467 ml/pig/day

Week 3 617 ml/pig/day

Week 4 359 ml/pig/day

Total milk consumed per pig during the 28 day feeding period averaged 11,746 ml/pig which was approximately 54% of the amount consumed when pigs are nursing the sow (Hitchcock, 1973). Daily milk consumption was reduced during week 4 in order to encourage water and feed consumption before weaning, as well as to standardize pig weights with the conventionally reared pigs before imposing dietary treatments.

Iron supplementation was provided by injecting 200 mg iron as iron dextran intramuscularly when pigs were 3 days of age.

Rectal swabs were taken from one pig in each isolator on days 6, 21, 39 and 49 of the trial and monitored for anaerobe and aerobe bacterial growth in plated culture media. All isolators were germ-free up to day 21 when Streptococcus faecalis was isolated from isolator III. Isolators I and II remained germ-free for the duration of the trial.

On day 16, pigs were provided ad libitum access to water and creep feed (autoclaved control diet) and water. Composition and nutrient

Table 1. Nutrient Analysis of Evaporated Cow's Milk and Comparison to Nutrient Analysis of Sow's Milk.

Nutrient	Evaporated Cow's Milk[a]		Sow's Milk[b]	
	As is /100 g	Dry Matter/100 g	As is/100 mg	Dry Matter/100 g
Dry matter, g	26	100	19	100
Energy, kcal	134	516	150	790
Protein, g	6.8	26	5.5	29
Fat, g	7.6	29	7.5	39
Carbohydrate, g	10	39	5.0	26
Ash, g	1.6	6.0	1.0	5.3
Linoleic acid, g	0.17	0.65	0.75	4.0
Amino Acids				
Lysine, mg	540	2080	440	2316
Methionine, mg	171	659	83	434
Cystine, mg	63	243	72	376
Isoleucine, mg	412	1588	220	1158
Threonine, mg	307	1183	220	1158
Tryptophan, mg	96	370	66	347
Arginine, mg	247	951	275	1447
Minerals				
Calcium, mg	261	1005	220	1158
Phosphorus, mg	202	778	160	842
Sodium, mg	106	408	30	158
Potassium, mg	303	1167	90	474
Magnesium, mg	24	92	20	105
Iron, mg	0.19	0.73	0.10	0.53
Iodine, µg	16	62	--	--
Copper, µg	90	347	20	105

Table 1. Nutrient Analysis of Evaporated Cow's Milk and Comparison to Nutrient Analysis of Sow's Milk.

	Evaporated Cow's Milk[a]		Sow's Milk[b]	
Nutrient	As is /100 g	Dry Matter /100 g	As is/100 mg	Dry Matter/100 g
Zinc, mg	0.77	3.0	0.50	2.6
Vitamins				
Vitamin D, IU	79	304	10	53
Vitamin E, IU	0.20	0.77	0.14	0.74
Thiamin, mg	0.04	0.15	0.07	0.34
Riboflavin, mg	0.31	1.2	0.36	1.9
Pyridoxine, µg	50	193	20	105
Vitamin B_{12}, µg	0.16	0.62	0.14	0.74
Niacin, mg	0.19	0.73	0.74	3.9
Folic acid, µg	8.0	31	390	2053
Biotin, µg	8.0	31	1.4	7.4
Pantothenic acid, mg	0.64	2.5	0.41	2.1
Ascorbic acid, mg	1.9	7.2	15	79

[a]Nutrient composition of evaporated cow's milk (Leveille et al., 1983).

[b]Nutrient composition of sow's milk (Pond and Houpt, 1978).

analysis of the creep diet was the same as the experimental control diet which is shown in table 2. Pigs consumed approximately 31 g of creep feed/pig/day.

Average environmental temperature was maintained at 33°C during week 1, 30°C during week 2 and 3, 28°C during week 4 and was maintained at 27°C for the duration of the trial.

Pigs were weaned on day 28 of age, weighed and fed their assigned experimental diets for 3 weeks. Each group of 3 pigs in isolator I and III were fed the control diet containing 16 ppm copper while the 4 pigs in isolator II were fed a high copper diet containing 250 ppm supplemental copper as copper sulfate (Table 2).

Experimental diets were formulated based on practical swine starter diets which are generally a corn-soybean meal-20% dried whey mixture. Furthermore, this type of diet tends to evoke a greater growth response from supplemental copper than other types of starter diets (Edmonds and Baker, 1984). However, sterilization of solid diets by steam results in partial loss of the nutritive value of proteins, particularly through its effects on lysine, methionine and cystine (Rice and Beuk, 1953), loss of certain B vitamins and also of Vitamins A and E (Zimmerman and Wostman, 1963).

A series of preliminary 3-week growth performance trials with weanling pigs were conducted to compare the effect of vitamin and amino acid supplementation between autoclaved and non-autoclaved diets and to characterize the effects of autoclaving diets on growth performance and nutrient balance. The results of these trials are shown in Appendix A. Diets were formulated to meet or exceed NRC requirements (NRC, 1979)

and were fortified with 1% synthetic L-lysine HCl to elevate the lysine level to 1.82% in each diet before diets were autoclaved.

Diet composition and analysis is shown in Table 2. Average initial weight of pigs receiving the control diet was 5.15 kg while the initial weight of high copper-fed pigs was 5.51 kg. Feed and water were provided ad libitum and each pre-weighed pan of autoclaved feed and sterilized water bottle were recorded when transferred into the isolators in order to calculate average daily feed and water consumption. Procedures used to sterilize and transfer feed and water and to transfer into isolators are discussed in detail in Appendix A.

B. Management of Conventional Pigs

One litter of 5 (2 gilts, 3 boars) and one litter of 10 (7 gilts, 3 boars) crossbred Hampshire x Yorkshire-Landrace-Duroc pigs were allowed to nurse the sows for 3 days in order to obtain adequate colostrum before weaning from the sow and changing their diet to canned evaporated cow's milk. Prior to weaning, pigs were ear notched, needle teeth were removed and supplemental iron was provided by intramuscular injection of 200 mg iron dextran per pig at birth. When pigs were 3 days of age, they were removed from sows and placed in stainless steel rearing pens. Two pigs shared each pen measuring 0.75 m^2 (except for one pen where 3 pigs were reared together) and were paired based on size and physical condition.

The litter of 10 pigs developed diarrhea when nursing the sow and 4 out of the 10 pigs were somewhat smaller and dehydrated when weaned.

Pigs were bottle fed with evaporated milk 3 times daily at 7 am, 3 pm and 10 pm analogous to the germ-free pigs. Average daily milk

consumption per pig for weeks 1 through 4 was as follows:

Week 1	179 ml/pig/day
Week 2	355 ml/pig/day
Week 3	570 ml/pig/day
Week 4	602 ml/pig/day

Total milk consumed per pig during the 28 day feeding period averaged 11,941 ml/pig which was approximately the same as the germ-free pigs. During the 28 day period while pigs were receiving the evaporated milk diet, incidence and severity of diarrhea was monitored at each feeding. When diarrhea persisted among individual pigs, quantity of milk fed was slightly reduced and oral neomycin was administered to those pigs affected until diarrhea was under control. Because of the persistence of the diarrhea problem among individual pigs, a greater quantity of milk was fed to the conventional pigs during week 4 to allow them to compensate for their poorer condition, as a result of the diarrhea, in order to equalize initial weights of the conventional and germ-free pigs before imposing dietary treatments.

All pigs were provided ad libitum access to water and creep feed (autoclaved control diet) identical to that provided for the germ-free pigs. Refer to Table 2 for diet composition and nutrient analysis. Pigs consumed 23 g/pig/day of the creep feed until weaning.

Average room temperature was maintained at 31°C during week 1 and 29°C throughout the remainder of the trial. Supplemental heat was provided by heat lamps located over each pen during the first 4 weeks of age which elevated the temperature in the pig's sleeping area to

Table 2. Diet Composition and Calculated Nutrient Analysis (Experiment 1).

		Dietary Treatment (%)	
Ingredient	Int. Ref. No.	Control	High Copper
Corn, ground shelled	4-02-935	50.90	50.80
Soybean meal, 44%	5-04-604	25.00	25.00
Dried whey	4-01-182	20.00	20.00
Mono-dicalcium phosphate	6-01-080	0.75	0.75
Calcium carbonate	6-01-632	1.00	1.00
Salt	6-04-152	0.35	0.35
Vitamin-trace mineral premix[a]		0.50	0.50
Vitamin E-selenium premix[a]		0.50	0.50
L-lysine HCl	5-08-022	1.00	1.00
$CuSO_4 \cdot 5H_2O$	6-01-719	--	.10
Total		100.00	100.00
Calculated Analysis			
Metabolizable energy, kcal/kg		3103	3100
Crude protein, %		18.20	18.20
Lysine, %		1.83	1.83
Calcium, %		0.81	0.81
Phosphorus, %		0.62	0.62

[a]Refer to Appendix A (Table 28) for the amount of vitamins and trace minerals supplied per kg of diet.

35°C during this time period. Heat lamps were removed when pigs were weaned at 28 days of age and room temperature was maintained at 29°C throughout the duration of the trial.

At weaning, pigs were weighed and the 10 most uniform pigs were allotted by sex, litter and weight to one of the two experimental diets. Five pigs with an average initial weight of 5.97 kg were moved to a stainless steel pen measuring 1.50 m^2 with a wire mesh floor and fed the autoclaved control diet, while another group of 5 pigs averaging 6.03 kg in initial weight were moved to another stainless steel pen (dimensions of 1.50 m^2) and fed the autoclaved experimental diet containing 250 ppm supplemental copper. Both stainless steel pens were of the same dimensions and located in the same room where pigs were previously fed the milk diet. Diet composition and nutrient analysis is shown in Table 2. Feed and water were provided ad libitum and the amount of feed fed was weighed and recorded before adding to the feeders in order to calculate average daily feed consumption.

C. Collection of Data and Laboratory Analyses

1. Determination of growth performance criteria

At the completion of the 7-week trial, pigs were weighed and individual weights were recorded. Any feed remaining in feeders was weighed and subtracted from total feed fed during the 3-week experimental period. Average daily weight gain, average daily feed consumption and feed utilization efficiency was calculated. Individual average daily gain values were statistically analyzed using Federer-Zelen factorial analysis for unbalanced data (Gill, 1978). Since average daily feed consumption and feed utilization efficiency values were pen values, and since there were no replications, these parameters could not be analyzed statistically.

2. Collection and preparation of blood samples for laboratory analyses

Peripheral blood samples were taken by vena puncture from the anterior vena cava prior to anaesthetizing each pig. Before all remaining blood and tissue samples were collected, each pen or isolator of pigs was fed a meal 3 1/2 hours before anaesthetizing pigs to obtain blood and tissue samples. After 3 1/2 hours, pigs were anaesthetized by injecting iv approximately 6 cc of sodium pentobarbital (Sodium Pentobarbital Injectable, Butler Co., Columbus, OH) and state of anaesthesia was closely monitored to maintain a constant level of sedation. A vertical incision was made on the ventral side, approximately 25 cm from the posterior portion of the sternum with a scalpel. The portal vein was identified and approximately 20 ml of blood was drawn and placed in heparinized plastic tubes used to harvest plasma. An additional 10 ml of blood was placed in tubes containing EDTA for hemotology determinations. After mixing blood with heparin (both portal and peripheral blood), the blood samples were placed in a centrifuge and spun for 15 minutes at 2000 x g. Plasma was harvested and stored temporarily in coolers containing ice for subsequent laboratory analyses. Plasma samples for glucose, ammonia and ceruloplasmin determinations were analyzed immediately while plasma samples for amino acid and trace element determinations were frozen for later analyses. Hematology determinations were performed immediately. All data were analyzed statistically using Federer-Zelen factorial analysis of variance for unbalanced data (Gill, 1978).

a. Hematological determinations

Hemoglobin was determined by the cyanmethemoglobin method of Crosby et al. (1954). Hematocrit was measured by the micro-method described by McGovern et al. (1955). Reticulocytes were enumerated per 1000 erythrocytes in blood smears stained with methylene blue on the hemocytometer. For leukocytes, blood was diluted using Turk's solution as a diluent and total leukocyte counts were determined with a hemocytometer. Differential leukocyte counts were determined by preparing a blood smear with Wright's stain and counting and differentiating 100 leukocytes.

b. Plasma trace elements

Plasma copper, iron and zinc concentrations were determined using atomic absorption spectrophotometry (Model 951, Instrument Laboratory, Inc., Lexington, MA). Copper and zinc concentrations were measured by diluting the plasma samples with appropriate amounts of distilled deionized water to obtain plasma zinc concentrations in the range of 0.1 to 1.0 ppm and copper concentrations in the range of 0.25 to 2.0 ppm. Plasma iron was determined by first deproteinizing the plasma with 20% tricarboxylic acid. After centrifugation the deproteinized supernatant was diluted and measured in the range of 1.0 to 5.0 ppm. Wavelengths used for maximum absorption were 213.9 nm, 324.7 nm and 248.3 nm for zinc, copper and iron, respectively.

c. Plasma ceruloplasmin determinations

Plasma ceruloplasmin oxidase activity was measured with fresh samples according to the procedure of Smith and Wright (1974).

This procedure involves adding 2.4 ml of buffer (containing 0.8M sodium acetate AR which contained 3.1g EDTA disodium salt/liter and was adjusted with acetic acid to pH 6.5) to 0.5 ml plasma. Samples were incubated at 37°C for 5 minutes and the substrate p-phenylenediamine dihydrochloride was added and samples were mixed. Change in absorbance was recorded at 540 nm wavelength on a spectrophotometer (Beckman-Gilford Spectrophotometer, Model 2400, Beckman Instruments, Inc., Fullerton, CA and Gilford Instrument Laboratories, Inc., Oberlin, OH). A blank sample was used to correct for substrate autooxidation in the absence of plasma.

d. Plasma ammonia determinations

Plasma ammonia concentrations were determined by using an ammonia diagnostic kit (Sigma Diagnostics, P.O. Box 14508, St. Louis, MO 63178). This procedure is based upon the reductive amination of 2-oxoglutarate using glutamate dehydrogenase and NADH. The decrease in absorbance at 340 nm on a spectrophotometer (Beckman-Gilford Spectrophotometer, Model 2400, Beckman Instruments, Inc., Fullerton, CA and Gilford Instrument Laboratories, Inc., Oberlin, OH), due to the oxidation of NADH, is proportional to the plasma ammonia concentration.

e. Plasma glucose determinations

Plasma glucose concentrations were determined using a glucose diagnostic kit (Sigma Diagnostics, P.O. Box 14508, St. Louis, MO 63178). This procedure is based upon the hexokinase method where glucose is converted to glucose-6-phosphate by hexokinase. This reaction is coupled with the subsequent reduction of NADP to NADPH by

the action of glucose-6-phosphate dehydrogenase. In the presence of NADPH, phenazine methosulfate (PMS) is reduced to PMSH which is responsible for the reduction of iodonitrotetrazolium chloride (INT) to form INTH which is measured colorimetrically on a spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, OH) at 520 nm. The concentration of INTH is proportional to the plasma glucose concentration.

f. Plasma amino acid determinations

Two milliliters of plasma supernatant were added to each labelled Cortex centrifuge tube and 0.2 ml of 1 mM norleucine-SB-(4 pyridyl:ethyl)-l cysteine was added as an internal standard to each tube and tubes were mixed on a vortex. Next, 0.2 ml of 50% sulfosalicylic acid was added to each tube to deproteinize the plasma proteins. Tubes were then mixed and placed on ice for 30 minutes. Finally, samples were centrifuged at 20,000 x g for 20 minutes, supernatant was removed with a Pasteur pipet and placed in storage vials. Samples were frozen until amino acid analyses could be performed.

When amino acid analyses could be performed, plasma samples were thawed and filtered through 0.5 μm filter paper and were diluted 1:3 with 0.01 N HCl, mixed and put into vials. Individual plasma amino acids were determined by ion exchange chromotography using a lithium citrate buffer system and post column derivitization with ninhydrin.

3. Preparation of intestinal sections for histological determinations

Three-5 cm intestinal sections were isolated by tying a piece of suture material at each end of the intestinal section to be excised.

Once isolated, approximately 7 ml of a solution consisting of formalin-mercuric chloride was injected with a 23 gauge, 2.5 cm needle and syringe to uniformily distend each intestinal section. The formalin-mercuric chloride solution was used to quickly fix the living tissue before atrophy could occur. Each of the three intestinal sections were excised and placed in a labelled beaker containing the fixing solution. Intestinal segments were removed starting with the duodenum near the pylorus and extending down the duodenum 5 cm, the most posterior portion of the ileum (adjacent to the ileal-cecal valve) and a section of jejunum approximately 155 cm cranially to the ileal-cecal valve. All samples were then taken to the laboratory for further preparation. Tissues were fixed for approximately 24 hours. Ends of the intestinal loops were then cut off, washed with tap water, trimmed and stored in 80% alcohol. Tissue buttons were then placed in labelled paraffin blocks, sliced and mounted on slides for examination under the microscope.

a. Intestinal histological determinations

Histologic differences due to dietary treatment and environment were determined by taking linear measurements of intestinal wall structures. Comparisons of three locations of the small intestine (duodenum, jejunum, ileum) were made by measuring villus height, villus width, crypt depth, depth of the submucosa and total thickness using an ocular micrometer. Three representative samples from each intestinal section of each pig were examined, and 25 measurements for each parameter were made and then pooled. Mean values were calculated for each parameter for each pig and analyzed statistically using

Federer-Zelen factorial analysis of variance for unbalanced data (Gill, 1978).

4. Body organ weights and preparation of organs and cecal contents for laboratory analyses

The pigs were then euthanized by giving an overdose of sodium pentobarbital. Various organs were then removed, weighed and put in plastic whirlpak bags and frozen for later laboratory analyses. Organs evaluated include:

Liver	Heart	Lungs
Kidneys	Thyroid	Gall bladder
Spleen	Thymus	Stomach-empty
Adrenals	Pancreas	Small intestine-empty
		Large intestine-empty

Cecal contents were frozen in plastic whirl pak bags for ammonia and urea determinations.

a. Determination of ammonia and urea in cecal contents

Ammonia and urea nitrogen determinations were conducted for cecal contents by using diagnostic kits for ammonia and urea (Sigma Diagnostics, P.O. Box 14508, St. Louis, MO 63178). Frozen cecal content samples were thawed, weighed and diluted with water. After centrifugation, supernatant was deproteinized with trichloracetic acid and change in absorbance (compared to blanks) was recorded after reagents were added to the sample. The change in absorbance was proportional to the ammonia or urea concentrations. The procedure was verified by comparing values obtained with those of Combe et al. (1965) and were very similar.

II. INFLUENCE OF COPPER AND CHLORTETRACYCLINE ON MICROBIAL FERMENTATION PATTERNS AND MICROBIAL POPULATION GROWTH IN FIVE SECTIONS OF THE GASTROINTESTINAL TRACT IN VITRO.

A. Preparation of Culture Tubes

Prior to the start of the experiment, 1250 glass culture tubes (18 mm O.D. x 150 mm long, 29 ml) obtained from (VWR Scientific Inc., Subsidiary of Univar, P.O. Box 3200, San Francisco, CA 94119) were sterilized by autoclaving at 121°C for 30 minutes. Rubber stoppers were immediately placed in the glass culture tubes after sterilization to minimize contamination.

B. Preparation of Media

Two types of media were formulated and prepared to conduct this in vitro study:

1. anaerobic dilution medium.
2. non-selective anaerobic growth medium.

Several reagents were prepared prior to mixing of the two types of media. First, two mineral buffer solutions were made and eventually added to the media. Chemical composition of these mineral buffer solutions is shown in Table 3.

Next, an 8 percent Na_2CO_3 solution was prepared by weighing 24 g Na_2CO_3 and dissolving in 300 ml distilled water. Sodium carbonate solution was then poured into a 1000 ml round bottom flask and the flask was sealed with a cotton plug. The flask containing the Na_2CO_3 solution was then autoclaved at 121°C for 30 minutes, cooled and pipetted into sterile test tubes in 10 ml quantities after equilibrating with CO_2 gas for 30 minutes.

An ascorbic acid solution (2.38%) was prepared by weighing 7.125 g of ascorbic acid and adding to 300 ml of distilled water. Ascorbic acid was used as the reducing agent for both the dilution and growth media even though it is a less powerful reducing agent than cysteine HCl and other reducing agents containing sulfdryl groups, because it allowed the copper to remain in solution unlike these other reducing agents. After pouring the solution into a 1000 ml round bottom flask, the ascorbic acid solution was slowly boiled under CO_2 gas and a #7 rubber stopper was used to seal the flask as the CO_2 gas was removed. The rubber stopper was fastened in place with wire that was tightly twisted over the rubber stopper and around the neck of the flask to avoid the stopper from popping out during the autoclaved process (which would allow contamination by microbes and oxygen). After the ascorbic acid solution was sterilized by autoclaving at 121°C for 30 minutes, the flask was allowed to cool. The wire holding the rubber stopper in place and the rubber stopper were removed, and the contents of the flask were immediately placed under CO_2 gas. Ascorbic acid solution was then pipetted in 10 ml quantities into sterile test tubes for later use. The pH was adjusted to neutrality by adding 10 N NaOH prior to boiling under CO_2 and sterilization.

Once these solutions were made, the two types of media used in this experiment were prepared.

1. Anaerobic dilution media

One thousand milliliters of anaerobic dilution medium was prepared in 500 ml quantities by mixing ingredients shown in Table 4 with distilled water to provide a total volume of 465 ml. After adjusting the pH to approximately 6.75 by adding a few drops of 10N

Table 3. Composition of Mineral Buffer Solutions Added to Dilution Media and Non-Selective Growth Media (g/l distilled H_2O).

	Mineral Buffer 1	Mineral Buffer 2
Distilled H_2O	988.00	977.96
K_2HPO_4	6.00	--
KH_2PO_4	6.00	6.00
NaCl	--	12.0
$MgSO_4 \cdot 7H_2O$	--	2.45
$CaCl_2 \cdot 2H_2O$	--	1.59

Table 4. Composition of Anaerobic Dilution Media

Compound	(ml)
Distilled H_2O	427.00
Mineral Buffer 1	18.75
Mineral Buffer 2	18.75
Resazurin	0.50
Total	465.00

NaOH, media was added to 1000 ml round bottom flask and boiled slowly under CO_2 gas (which was reduced by passing through a hot copper column) to remove the soluble oxygen present in the media. As the media cooled, a #7 rubber was used to seal the flask as the CO_2 gas was removed. Rubber stoppers were held in place by using wire to keep the flask sealed during sterilization (autoclaving at 121°C for 30 minutes) and during subsequent cooling prior to dispensing into sterile culture tubes. Once cooled, the rubber stoppers were removed and the media was placed under CO_2 gas. Previously prepared sodium carbonate and ascorbic acid solutions were added from sterile culture tubes to each flask containing 465 ml of dilution medium, (under CO_2 gas) in 25 and 10 ml quantities, respectively, to provide a final volume of 500 ml of media in each flask. Carbon dioxide gas was then bubbled into the media through a glass pipette to mix the Na_2CO_3 and ascorbic acid with the media for 10 minutes. Medium was then dispensed into sterile test tubes in 18 ml quantities, under CO_2 gas, and tubes were tightly sealed with rubber stoppers.

2. Non-selective anaerobic growth media

Composition of non-selective anaerobic growth media is shown in Table 5. A total of 5500 ml of anaerobic growth media was prepared in 465 ml quantities and the pH was adjusted to 6.75 with 10 N NaOH.

After boiling growth media under CO_2 gas, flasks were sealed with rubber stoppers and autoclaved for 30 minutes at 121°C. Once the media was cooled to room temperature, 25 ml of Na_2CO_3 were added to each flask of media and mixed while under CO_2 gas. Media was then dispensed, in 8 ml quantities, into 640 sterile culture tubes. Next, 0.2 ml of previously prepared ascorbic acid was added to media in each

Table 5. Composition of Non-Selective Anaerobic Growth Media.

Ingredient	(%)
Distilled H_2O	91.48
Glucose	0.05
Starch	0.05
Cellobiose	0.05
Urea	0.10
Trypticase	0.50
Mineral buffer 1	3.75
Mineral buffer 2	3.75
Yeast extract	0.10
Resazurin	0.10
Na_2CO_3	0.05
Ascorbic acid	0.02
Total	100.00

tube. Each of four treatment solutions (H_2O, $CuSO_4 \cdot 5H_2O$, chlortetracycline, $CuSO_4 \cdot 5H_2O$ + chlortetracycline) were filter sterilized through 0.45 μm pore filters (Nalge Co., Division of Sybron Corp., Rochester, NY 14602). Each treatment solution was added in 0.3 ml quantities, to each of 160 tubes containing media to provide a total volume of 8.5 ml of media in each of the 640 culture tubes. One hundred sixty culture tubes of each treatment contained either the control (distilled H_2O), 250 ppm copper, 100 ppm chlortetracycline, or 250 ppm copper and 100 ppm chlortetracycline. The addition of ascorbic acid and treatments to culture tubes was made at least 12 hours before the start of the experiment. All tubes were labelled according to treatment, source of inocula, replicate and pig number. An additional 8 culture tubes containing each of the treatments were prepared to serve as blanks used for determining optical density readings, initial pH, ammonia, and organic acid profiles.

C. Inoculation of Culture Media

Four littermate crossbred pigs (2 gilts, 2 barrows) were weaned at 15 days of age and fed a typical corn-soybean meal-20% dried whey starter diet containing no supplemental copper or antibiotic (Table 6). These pigs did not receive creep feed or sow feed while nursing the sow so that they had not been exposed to antibiotics or high copper diets prior to the experiment. Two of the 4 pigs were fed the starter diet for 3 weeks while the other 2 pigs received the diet for 4 weeks. To obtain microbes from the gut contents and gut wall, each pair of pigs (1 gilt and 1 barrow) were euthanized by giving an overdose of sodium pentobarbital and were then exsanguinated before removing the digestive tract. Pigs were euthanized in pairs to allow

Table 6. Diet Composition and Calculated Nutrient Analysis (Experiment 2).

Ingredient	Int. Ref. No.	(%)
Corn, ground shelled	4-02-935	51.61
Soybean meal, 44%	5-04-604	25.00
Dried whey	4-01-182	20.00
Mono-dicalcium phosphate	6-01-080	0.75
Calcium carbonate	6-01-632	1.10
Salt	6-04-152	0.35
Vitamin-trace mineral premix[a]		0.50
Vitamin E-selenium premix[a]		0.50
L-lysine HCl	5-08-022	0.19
Total		100.00
Calculated Analysis		
Metabolizable energy, kcal/kg		3127
Crude protein, %		18.26
Lysine, %		1.20
Calcium, %		0.85
Phosphorus, %		0.60

[a]Refer to Appendix A (Table 28) for the amount of vitamins and trace minerals supplied per kg of diet.

better management of inoculating tubes and collecting data. As a result, the first pair of pigs averaged 7.6 kg in body weight while the second pair of pigs, euthanized one-week later averaged 10.3 kg in body weight.

Once the digestive tract was removed from each pig, each of five sections were removed by first removing the mesentery and stretching out the gastrointestinal tract, followed by using monofilament line to separate and tie each section from the other sections. The five sections studied and removed were: stomach, a 15 cm section of the first half of the small intestine obtained at 305 cm caudally from the pyloric valve, a 15 cm section caudally of the second half of the small intestine obtained at 245 cm cranially from the ileal cecal valve, cecum, and a 15 cm section of the colon obtained 90 cm cranially from the rectum. Average gastrointestinal length was 1050 cm. Once the sections were removed, carcasses were disposed and gut sections were brought to the laboratory for further preparation.

Two grams of gut contents from each gut section were weighed and immediately put into previously prepared culture tubes containing 18 ml of anaerobic dilution media while under CO_2 gas. Once the stoppers were replaced, the inocula and medium were thoroughly mixed by shaking tubes after inoculation. A 2 x 2 cm section of each intestinal site was measured, cut and thoroughly washed with sterile physiological saline solution to remove any gut contents associated with the gut wall. Each piece of intestinal tissue was then placed into dilution tubes, while under CO_2, and mixed vigorously to inoculate the media. After the contents and tissues were placed in dilution tubes, and

mixed, tubes were allowed to stand for 1 hour to allow the particulate matter or tissues to settle.

Each dilution tube was then reopened, placed under CO_2 gas and an additional 2 ml of filter sterilized water (filtered through a 0.45 µm pore filter) was added and mixed to provide an additional 2 ml volume of fluid necessary to provide enough innocula to inoculate all culture tubes. Using a 1 ml pipette, 1 ml of inocula from each microbe source was then added to one of sixteen treatment tubes (4 treatments of 4 replicates/treatment).

D. Data Collection and Laboratory Analyses

1. Determination of microbial population growth

Once all of the treatment tubes were inoculated from each pig, initial optical density readings were recorded on a spectrophotometer, Spectronic 70, (Bausch and Lomb Analytical Systems Division, 820 Linden Ave., Rochester, NY 14625) by zeroing the meter using a blank tube containing each of the four treatment media, but no inocula, to adjust for color differences. After initial readings were recorded, tubes were incubated at 38°C for 4 hours until the next reading was taken. This process continued in 4 hour intervals for a 24 hour period when the last reading was recorded.

2. Determination of pH

At the end of the 24 hour incubation period, all treatment tubes were used to measure final pH with a pH meter Model PHM64a (The London Company, Westlake, OH). Each set of blank tubes, corresponding to each treatment were also measured for pH, which served as a reference for the initial pH of the media before inoculation and incubation. After the pH was measured and recorded, all tubes were acidified by adding

0.25 ml of concentrated HCl to arrest microbial growth and fermentation. Samples were then immediately frozen until preparation and analyses could be performed for ammonia nitrogen and volatile and non-volatile organic acids.

Changes in pH, after incubation, were statistically compared using a completely randomized design, 3-factor analysis of variance for balanced data, with 2 fixed factors (microbe source and treatment) and 1 random factor (pigs) (Gill, 1978).

3. Determination of ammonia nitrogen

Ammonia nitrogen was measured according to the semi-automated method described in A.O.A.C. (1984) with an automatic analyzer where a NH_3-salicylate complex is read in a flow cell at 660 nm wavelength. Samples were prepared for analysis by thawing and adding 0.5 ml of 9N H_2SO_4 to 2.5 ml of fermented media. Samples were mixed and then 6 ml of distilled, deionized water was added, mixed and centrifuged at 43,500 xg for 15 minutes (Sorvall Superspeed Centrifuge Model RC2-B, Sorvall, Inc., Norwalk, CT 06852) in Corex glass centrifuge tubes. The supernatant was then transfered to disposable plastic tubes for analysis on the Technicon Auto-analyzer with appropriate standards.

Data were statistically analyzed as previously described for pH changes.

4. Determination of fatty acid butyl esters

Both volatile and non-volatile fatty acids were determined simultaneously by using the procedure of Salanitro and Muirhead (1975) which is a quantitative method for gas chromatographic analysis of short-chain monocarboxylic and dicarboxylic acids in fermentation

media. One ml of each fermented media sample was placed in culture tubes, frozen in an alcohol-dry ice bath, and placed in a continuous freeze drier for 12 hours. Chloroform (0.8 ml) and 0.2 ml of 1-butanol, saturated with anhydrous HCl were added to the dry salts of acids. After mixing with a Vortex, tubes were tightly capped and heated at 80°C for 2 hours. Tubes were then cooled to room temperature and 0.2 ml of trifluoroacetic anhydride (TFA) was added to each tube and allowed to react for 1 hour. The TFA was used to react with hydroxy acids forming the trifluoroacetyl esters and to react with any excess butanol in the mixture. Samples were then washed twice with 1 ml aliquots of distilled water to remove excess TFA reagent, and the water layer was discarded. The 1 ml chloroform layer, which contained the butyl esters, was placed in vials and sealed. Two microliters of each sample was injected and analyzed by gas chromotography (Hewlett Packard Model 5840A, Route 41, Avondale, PA 19311) by using a 183 cm x 0.32 cm custom packed column packed with Chromosorb W (80100 mesh, HP DMCS, AW) coated with 10% Dexsil 300 GC (Supelco Inc., Supelco Park, Belefonte, PA 16823).

Data were statistically analyzed as previously described for pH changes.

RESULTS AND DISCUSSION

I. EFFECT OF HIGH COPPER FEEDING TO WEANLING PIGS IN GERM-FREE AND CONVENTIONAL ENVIRONMENTS ON GROWTH PERFORMANCE AND VARIOUS PHYSIOLOGICAL PARAMETERS

A. Growth Performance

Germ-free pigs grew at a faster rate ($P<.001$) than conventionally reared pigs (Table 7). Landy and Ledbetter (1966) reported that conventional miniature pigs were heavier at three weeks of age than their germ-free counterparts, but by 10 weeks of age, the conventional pigs weighed slightly more than the germ-free pigs. A diet by environment interaction ($P<.07$) was observed for average daily gain (ADG) (Table 7) indicating that feeding the high copper diet improved ADG in conventionally reared pigs but decreased ADG in germ-free pigs. In other words, feeding 250 ppm supplemental copper appears to allow the conventional pig to grow at a rate more like that of the germ-free pig by reducing antagonistic effects of microbiota in the conventional environment. The reduction in ADG for germ-free pigs fed the high copper diet may have resulted from the development of a moderate copper toxicity (Sections B, C, D, E). In regard to the development of a moderate copper toxicity state in the germ-free pigs receiving the copper supplemented diet, Ritchie et al. (1963) demonstrated that conventional pigs receiving diets containing supplemental copper in excess of 250 ppm, without iron or zinc supplementation, developed a copper toxicity state. Bunch et al. (1963) and Suttle and Mills (1966) reported similar results. In a copper toxicity, liver copper levels may range between 800-2500 ppm on a dry matter basis (Wallace, 1968)

with a concommitant decrease in liver iron (Bunch et al., 1963; Suttle and Mills, 1966). Hemoglobin and hematocrit are reduced (Hoefer et al., 1960; Ritchie et al., 1963), serum copper levels are increased and feed intake and growth rate are depressed (Suttle and Mills, 1966).

Table 7. Effect of Diet and Environment on Growth Performance.

	Treatment Combination[b]				
Parameter[a]	GCo	GCu	CCo	CCu	MSE[e]
Avg. initial wt., kg	5.27	5.51	5.97	6.03	-
Avg. final wt., kg	10.20	9.60	8.10	8.57	-
ADG, g[c,d]	223	195	102	126	102
ADFI, g	520	500	320	340	-
F/G	2.19	2.54	3.12	2.82	-

[a]ADG=average daily gain.

ADFI=average daily feed intake.

F/G=feed/gain.

[b]G=germ free, C=conventional, Co=control diet,

Cu=250 ppm copper diet.

[c]Environment effect (P<.001).

[d]Environment x diet interaction (P<.07).

[e]MSE = mean square error.

No statistical comparison could be made for average daily feed intake (ADFI) and feed efficiency (F/G). However, germ-free pigs tended to consume more feed than conventionally reared pigs, and feeding the high copper diet tended to depress ADFI in germ-free pigs but increase ADFI for conventional pigs (Table 7). Similarly,

germ-free pigs tended to be more efficient in converting feed to body weight gain than conventional pigs, and feeding the high copper diet tended to increase F/G in germ-free pigs but decrease F/G in conventional pigs (Table 7). During the course of the trial, the conventional pigs required an extended period of time following weaning, to adjust to the experimental diets, which may have confounded differences in ADG observed between germ-free and conventional pigs.

B. Hematology

Hemoglobin and hematocrit values were higher (Table 8) for germ-free pigs than conventional pigs ($P<.001$), which is consistent with reports by Waxler and Drees (1972) and Miller et al. (1982). Perhaps a lower blood volume, which has been observed in germ-free animals (Gordon et al., 1963), may be responsible for a higher concentration of hemoglobin and hematocrit observed in germ-free pigs in this study. No differences between germ-free and conventional pigs were noted for erythocyte number.

Hematocrit ($P<.01$) and erythrocyte count ($P<.06$) were reduced and hemoglobin tended to be reduced by feeding the high copper diet (Table 8). Hemoglobin and hematocrit values are generally reduced by feeding high copper diets (Bunch et al., 1961; Wallace et al., 1960; Ritchie et al., 1963) but not in every case (Kline et al., 1972; Castell and Bowland, 1968). Gipp et al. (1974) showed that the hypochromic microcytic anemia induced by high dietary copper was due to an impairment of iron absorption. A decrease in hemoglobin and hematocrit and a reduction in plasma and liver iron concentrations (Section C and E), may have resulted in a reduction in erythrocyte count due to an

Table 8. Effect of Diet and Environment on Hematology.

Parameter	Treatment Combination[a]				Statistical Significance[c]			
						P value[d]		
	GCo	GCu	CCo	CCu	MSE[b]	E	D	DxE
Hemoglobin, g/dl	11.6	11.1	10.3	10.0	0.30	.001	NS	NS
Hematocrit, %	36.2	34.4	32.9	31.6	1.41	.001	.01	NS
Erythrocytes, cells x $10^4/mm^3$	489	480	513	466	911	NS	.06	NS
Leukocytes, cells/mm^3	9704	9140	15840	12474	12.6	.01	NS	NS
Segmented neutrophils, %	35.7	26.4	50.5	49.3	50.8	.001	NS	NS
Band neutrophils, %	0.10	4.38	1.00	0.80	4.71	NS	.06	.04
Lymphocytes, %	54.3	48.4	39.4	43.3	45.5	.01	NS	NS
Monocytes, %	5.83	19.1	6.10	4.90	4.13	.001	.001	.001
Eosinophils, %	2.90	3.25	2.30	1.00	1.55	.02	NS	NS
Basophils, %	0.40	0.38	0.70	0.70	0.40	NS	NS	NS
Nucleated erythrocytes, %	0.00	0.75	0.40	0.10	0.13	NS	NS	.009

[a]G=germ-free, C=conventional, Co=control diet, Cu=250 ppm copper diet.

[b]MSE=mean square error.

[c]E=environment effect, D=diet effect, ExD=environment-diet interaction.

[d]P value=level of statistical significance, NS=not significantly different (P>.10).

antagonistic effect of the high copper diet on normal iron utilization. In other words, feeding the high copper diet to pigs may have resulted in a reduction of iron absorption which could reduce iron incorporation into reticulocytes resulting in a reduction of the number of mature erythrocytes present.

Germ-free pigs had fewer leukocytes ($P<.01$) than conventional pigs (Table 8) which is in agreement with previous reports (Waxler and Drees, 1972; Miller et al., 1982). Since leukocytes are involved in the immune system, it would be expected that total leukocyte number would be lower in germ-free pigs because of the lack of infectious agents in the environment. Feeding a high dietary level of copper also tended to reduce total leukocyte number.

Differentation of leukocytes is used in diagnosis of immunological status of the body. Leukocytes are typically differentiated into the various constituent classes which include: 1) segmented (mature) neutrophils, 2) band (immature) neutophils, 3) lymphocytes, 4) monocytes, 5) eosinophils, 6) basophils.

Germ-free pigs had a lower proportion of mature (segmented) neutrophils ($P<.001$) than conventional pigs which is reflected by the lack of antigenic stimulation of pigs reared in a germ-free environment (Table 8). Neutrophils (microphages) participate in the initial stages of inflammation and destroy invading agents by phagocytosis (Schalm, 1965). In severe infections, immature (band) neutrophils appear in the peripheral blood but are not capable of participating in phagocytosis of antigens. Waxler and Drees (1972) observed an increase in the number of neutrophils but had no explanation for this occurence.

Feeding high copper diets to pigs in the germ-free environment increased the percentage of immature (band) neutrophils but decreased the percentage of these cells in the conventional pig ($P<.04$). In conditions of stress, there is an increase in ACTH which causes a decrease in eosinophils and lymphocytes but an increase in neutrophils (Gordon, 1955). Perhaps during the last few days of the trial, germ-free, high copper fed pigs were too crowded in their isolator so that stress (increased ACTH) stimulated a rapid rate of neutrophil production which had not matured before blood samples were obtained.

It is unclear why the percentage of lymphocytes was increased ($P<.01$) for germ-free pigs compared to conventional pigs as observed in this study (Table 8). Lymphocytes are involved in antibody formation and decrease during the initial phases of infection, but return to normal levels during convalescence (Schalm, 1965). Lymphocytes originate from the thymus, spleen and lymph nodes and are widely distributed throughout the body (Schalm, 1965). Waxler and Drees (1972) indicated that lymph nodes of pigs raised in the presence of microbial flora are much more active than in germ-free pigs and that the degree of activity of the lymph nodes is generally correlated with the lymphocyte counts of the peripheral blood. These researchers consequently observed a lower percentage of lymphocytes in germ-free pigs compared to conventional pigs.

Percentage of monocytes (Table 8) was dramatically increased for germ-free pigs fed the high copper diet ($P<.001$), and this resulted in germ-free pigs having a higher percentage of monocytes ($P<.001$) compared to conventional pigs. There was also a diet by environment interaction ($P<.001$) indicating that feeding the high copper diet

increased the percentage of monocytes in germ-free pigs but decreased the percentage of these cells in conventional pigs fed the same diet (Table 8). As indicated in Sections C, D, and E, a moderate copper toxicity developed in germ-free pigs fed the high copper diet. Copper poisoning has been shown to result in the development of a hemolytic crisis when liver copper concentrations are high (Schalm, 1965). In this situation, when pigs in this condition are stressed, copper is released from the liver into the blood resulting in rapid hemolysis of erythrocytes (Gordon and Luke, 1957). As previously mentioned the germ-free pigs receiving the high copper diet may have been subjected to stress during the last few days of the trial and this, coupled with a high concentration of copper in the liver may have resulted in a hemolytic crisis. As a result, more monocytes may have been produced to remove the erythrocyte remnants resulting in the response observed in this trial.

C. Plasma Copper, Zinc and Iron Concentrations

Germ-free pigs had higher peripheral and portal plasma concentrations of copper, zinc and iron ($P<.001$) than their conventional counterparts (Table 9). Reddy et al. (1965b) observed a reduction in hemoglobin, hematocrit and plasma iron and an increase in plasma total iron binding capacity in germ-free rabbits fed either of two steam sterilized diets which proved adequate when fed to conventional rabbits. When these germ-free animals were conventionalized, these parameters returned to normal. These authors concluded that iron availability was reduced in the absence of gut microflora. In rats, however, there were only slight indications that

iron is less available in the absence of gut flora (Reddy et al., 1965a). These researchers reported that germ-free rats had lower spleen and kidney iron concentrations but liver iron levels were increased compared to conventional rats receiving the same diet. Hemosiderin and ferritin iron concentrations were similar for rats reared in both environments. Copper levels followed the same pattern as for iron. Furthermore, Geever and Levenson (1964) observed dense foci of an iron containing pigment in the tunica propria of conventional but not germ-free pigs, suggesting that gut microflora may influence iron metabolism.

More copper, zinc and iron appeared to be absorbed by germ-free pigs ($P<.001$) than by conventional pigs as indicated by portal element concentration (Table 9) and liver copper, zinc and iron concentrations (Table 11). Wostmann and Bruckner-Kardoss (1966) and Phillips et al. (1959) showed that the cecal contents of germ-free rats and guinea pigs have a more positive oxidation-reduction potential than their conventional counterparts. If this is also true for the intestinal contents of germ-free animals, then the availability of certain nutrients (such as copper, zinc and iron) may be affected. Perhaps a more positive oxidation-reduction potential in the intestinal contents of germ-free pigs allowed greater copper, zinc and iron absorption observed in this study.

Feeding the high copper diet increased peripheral plasma copper ($P<.001$) (Table 9). In general, plasma copper appears to be a poor criterion from which to assess copper status in the pig since Buntain (1961) observed no differences in plasma copper levels between normal pigs and those dying from copper toxicity. Other reports substantiate

Table 9. Effect of Diet and Environment on Plasma Copper, Zinc and Iron.

	Treatment Combination[a]					Statistical Significance[c] P Value[d]		
Parameter	GCo	GCu	CCo	CCu	MSE[b]	E	D	ExD
Peripheral plasma								
Cu, µg/dl	184	270	119	149	861	.001	.001	.06
Zn, µg/dl	84	90	62	63	141	.001	NS	NS
Fe, µg/dl	159	83	131	156	576	.06	.03	.001
Portal plasma								
Cu, µg/dl	187	252	126	157	763	.001	.001	NS
Zn, µg/dl	106	113	66	67	348	.001	NS	NS
Fe, µg/dl	167	98	133	145	590	NS	.02	.003

[a]G=germ-free, C=conventional, Co=control diet, Cu=250 ppm copper diet.
[b]MSE=mean square error.
[c]E=environment effect, D=diet effect, ExD=environment-diet interaction.
[d]P value=level of statistical significance, NS=not significantly different (P>.10).

Table 10. Effect of Diet and Environment on Portal and Peripheral Blood Plasma Ceruloplasmin.

	Treatment Combination[a]					Statistical Significance[c] P value[d]		
Parameter	GCo	GCu	CCo	CCu	MSE[b]	E	D	ExD
Ceruloplasmin, ΔA/min/ml								
Portal plasma	.463	.606	.338	.264	.014	.001	NS	.07
Peripheral plasma	.478	.660	.339	.300	.020	.001	.29	.11

[a]G=germ-free, C=conventional, Co=control diet, Cu=250 ppm copper diet.
[b]MSE=mean square error.
[c]E=environment effect, D=diet effect, ExD=environment-diet interaction.
[d]P value=level of statistical significance, NS=not significantly different (P>.10).

Table 11. Effect of Diet and Environment on Liver Copper, Zinc and Iron (DM basis).

	Treatment Combination[a]					Statistical Significance[c] P Value[d]		
Parameter	GCo	GCu	CCo	CCu	MSE[b]	E	D	ExD
Cu, ppm	63	1911	15	537	7436	.001	.001	.001
Zn, ppm	436	969	105	104	13124	.001	.001	.001
Fe, ppm	155	93	126	112	773	NS	.01	.01

[a]G=germ-free, C=conventional, Co=control diet, Cu=250 ppm copper diet.
[b]MSE=mean square error.
[c]E=environment effect, D=diet effect, ExD=environment-diet interaction.
[d]P value=level of statistical significance, NS=not significantly different (P<.10).

the inadequacy of plasma copper levels as an index of copper status (Castell and Bowland, 1968; Parris and McDonald, 1969; Kline et al., 1972). However, Suttle and Mills (1966) showed that feeding dietary levels of 425 to 750 ppm markedly increased serum copper levels. Furthermore, Underwood (1977) indicated that copper intakes of 100 ppm copper or more are necessary to produce a significant elevation of plasma copper.

Portal plasma copper levels were higher ($P<.001$) for pigs fed the diet containing 250 ppm supplemental copper (Table 9). Copper in the form of water-soluble salts ($CuSO_4$) is more available than copper in copper oxides or copper wire (Underwood, 1977). Copper absorption appears to be regulated in accordance with body copper need at low dietary copper levels (Crampton et al., 1965; Gitlin et al., 1960), but at high dietary copper levels, absorption appears to parallel the dose until a tissue saturation point is reached at which additional copper intake results in no further absorption. High dietary levels of calcium, zinc and iron have been demonstrated to reduce copper absorption. Since copper in the form of copper sulfate is a highly available source of copper and since copper absorption increases when pigs are fed a high dietary level of copper, it would be expected that portal copper concentrations would be greater than that of pigs fed the control diet. The dietary levels of calcium, zinc and iron used in this study (Table 2), were not high enough to interfere with copper absorption.

Finally, an environment x diet interaction ($P<.06$) was observed for peripheral and portal plasma copper indicating that feeding the high copper diet increased peripheral and portal copper concentrations

more dramatically in the germ-free pigs than in conventional pigs (Table 9). This relationship suggests that the presence of gut microflora alters copper metabolism as previously suggested by Reddy et al. (1965a).

D. Plasma Ceruloplasmin Activity

Ceruloplasmin, measured as change in absorbance/minute/ml of plasma was higher ($P<.001$) in portal and peripheral plasma of germ-free pigs than their conventional counterparts (Table 10). Since there was an apparent increase in copper absorption creating a moderate copper toxicity in germ-free pigs (discussed in Sections C and E), there appeared to be a concommitant increase in plasma ceruloplasmin in order to accomodate the increased copper load entering the plasma.

Germ-free pigs fed the high copper diet had higher levels of portal plasma ceruloplasmin but when conventional pigs were fed this same diet, portal ceruloplasmin decreased ($P<.07$). It appears that the gut microflora greatly influences the absorption and metabolism of copper and other trace elements.

E. Liver Copper, Zinc and Iron Concentrations

In this study, germ-free pigs had higher liver copper and zinc concentrations than conventional pigs ($P<.001$), while liver iron levels were not different (Table 11). Since the liver is the primary storage organ for copper and zinc following absorption (Underwood, 1977), and since it appears that germ-free pigs absorbed more copper and zinc, as indicated by increased portal concentrations of these elements (Table 9), then a higher concentration of liver copper and zinc would be expected.

Feeding the high copper diet increased liver copper and zinc ($P<.001$) and increased the concentration of these elements more dramatically in germ-free pigs than their conventional counterparts ($P<.001$) (Table 11). In contrast, liver iron concentrations were reduced by feeding the high copper diet ($P<.01$) and liver iron was reduced more dramatically in germ-free than in conventional pigs ($P<.01$).

Feeding a high copper diet typically results in increased copper levels in the liver (Wallace, 1967). Copper is known to compete with iron for absorption binding sites and with zinc for metal-binding protein (Miller et al., 1979). Therefore, an abnormally high intake of one element can adversely affect utilization of other elements. When high dietary levels of copper have been fed, zinc deficiency symptoms have been alleviated (Wallace et al., 1960; Hoefer et al., 1960; Ritchie et al., 1963). Perhaps copper is less inhibitory to zinc absorption in the germ-free pig than in the conventional pig due to the absence of gut microflora. Bunch et al. (1963) observed that high copper feeding decreased liver iron concentrations which could be alleviated by liberal supplementation of these diets with iron (Suttle and Mills, 1966). Again, as for copper and zinc, the decrease in liver iron was not as dramatic in the presence of the gut microflora, which may suggest that absorption and metabolism of these trace elements are markedly affected by the gut environment.

F. Plasma Ammonia Concentrations

Peripheral plasma ammonia concentrations were reduced ($P<.07$) and tended to be reduced in the portal plasma of pigs fed the high copper diet (Table 12). An inhibitory effect of copper on urease

activity would support the decrease in plasma ammonia concentration in the conventionally reared pigs, but since germ-free animals (pigs) would not have any bacterial urease activity (Kornberg and Davies, 1955; Levenson et al., 1959), copper might be acting in another capacity to elicit the response observed.

Germ-free pigs had lower levels of portal ammonia ($P<.001$) than conventional animals apparently due to a lack of bacterial urease activity (Table 12).

G. Concentration of Ammonia and Urea in Cecal Contents

In this study (Table 13), germ-free pigs had a higher concentration of urea ($P<.001$) and a lower concentration of ammonia-nitrogen ($P<.001$) in cecal contents compared to conventionally reared pigs. these values are consistent with those reported by Combe et al. (1965) when comparing these two response variables between germ-free and conventional rats. Cecal contents of germ-free rats contain a greater amount of non-precipitable nitrogen and very little ammonia compared to conventional rats (Combe and Sacquet, 1966; Levenson and Tennant, 1963). It appears that nitrogenous compounds are degraded to a lesser extent in germ-free animals than in conventional animals. Germ-free rats excrete more fecal nitrogen in their feces than their conventional counterparts (Levenson and Tennant, 1963; Evrard et al., 1964) and urea accounted for more than 25 percent of the nitrogen in feces of germ-free rats, while conventional rats had no measurable urea in feces.

Feeding the high copper diet had no effect on urea or ammonia-nitrogen concentrations in cecal contents of either germ-free or conventional pigs (Table 13). Chachulowa (1964) observed a

Table 12. Effect of Diet and Environment on Portal and Peripheral Plasma Ammonia Concentration.

	Treatment Combination[a]				Statistical Significance[c]			
						P Value[d]		
Parameter	GCo	GCu	CCo	CCu	MSE[b]	E	D	ExD
Peripheral NH_3, µg/ml	1.26	1.01	1.39	1.14	0.08	NS	.07	NS
Portal NH_3, µg/ml	3.48	3.27	8.69	7.29	4.20	.001	NS	NS

[a]G=germ free, C=conventional, Co=control diet, Cu=250 ppm copper diet.

[b]MSE=mean square error.

[c]E=environment effect, D=diet effect, ExD=environment-diet interaction.

[d]P value=level of statistical significance, NS=not significantly different (P>.10).

Table 13. Effect of Diet and Environment on Urea and Ammonia Nitrogen Concentrations in Cecal Contents (dry matter basis).

	Treatment Combination[a]				Statistical Significance[c]			
						P Value[d]		
Parameter	GCo	GCu	CCo	CCu	MSE[b]	E	D	ExD
Urea in cecal contents, mg/g	0.99	1.16	0.20	0.15	0.03	.001	NS	NS
Ammonia in cecal contents, mg/g	0.30	0.31	0.57	0.56	0.03	.005	NS	NS

[a]G=germ free, C=conventional, Co=control diet, Cu=250 ppm copper diet.

[b]MSE=mean square error.

[c]E=environment effect, D=diet effect, ExD=environment-diet interaction.

[d]P value=level of statistical significance, NS=not significantly different (P>.10).

reduction in cecal ammonia when copper (as copper sulfate) or chlortetracycline was included in the diet but this effect was not observed in this study.

H. Plasma Glucose Concentrations

Peripheral glucose levels (P<.08) and portal glucose levels (P<.01) were higher for germ-free pigs compared to conventional pigs (Table 14). Portal plasma glucose levels were also increased (P<.07) by supplementing the diets with 250 ppm copper.

Table 14. Effect of Diet and Environment on Portal and Peripheral Plasma Glucose.

	Treatment Combination[a]				
Parameter	GCo	GCu	CCo	CCu	MSE[e]
Peripheral glucose, mg/dl[b]	91.8	88.4	70.2	85.8	199
Portal glucose, mg/dl[c,d]	148	226	84.5	109	32.6

[a]G=germ-free, C=conventional, Co=control diet, Cu=250 ppm copper diet.

[b]Environment effect (P<.08).

[c]Environment effect (P<.01).

[d]Diet effect (P<.07).

[e]MSE = mean square error.

I. Plasma Amino Acid Concentrations

Germ-free pigs had lower peripheral amino acid concentrations (P<.05) with the exception of methionine and isoleucine, which tended to be lower, and higher cystine (P<.001), histidine (P<.02) and arginine (P<.003) concentrations, compared to peripheral amino acid levels conventional pigs (Table 15). This same relationship existed,

Table 15. Effect of Diet and Equipment

Table 15. Effect of Diet and Environment on Peripheral Plasma Amino Acid (PPAA) Concentrations (μM).

	Treatment Combinations[a]					Statistical Significance[c] P Value[d]		
PPAA	GCo	GCu	CCo	CCu	MSE[b]	E	D	ExD
Aspartate	74	64	189	187	6872	.02	NS	NS
Threonine	564	312	589	998	40759	.004	NS	.006
Serine	369	241	464	531	16774	.01	NS	NS
Glutamate	249	226	471	545	21101	.002	NS	NS
Glutamine	197	157	317	324	4502	.001	NS	NS
Glycine	1398	727	1380	1532	62464	.007	.06	.005
Alanine	731	361	806	946	45838	.008	NS	.03
Valine	335	156	713	902	63941	.001	NS	NS
Cystine	91	83	42	48	100	.001	NS	NS
Methionine	48	31	48	49	157	NS	NS	NS
Isoleucine	174	114	182	192	2543	NS	NS	NS
Leucine	281	168	311	305	6239	.05	NS	NS
Tyrosine	191	85	239	216	1605	.001	.006	.05
Phenylalanine	106	85	207	152	1029	.001	.03	NS
Lysine	210	57	157	253	4792	.06	NS	.003
Histidine	44	24	10	12	182	.02	NS	NS
Arginine	180	108	95	79	1004	.003	.02	.09

[a]G=germ free, C=conventional, Co=control diet, Cu=250 ppm copper diet.

[b]MSE=mean square error.

[c]E=environment effect, D=diet effect, ExD=environment x diet interaction.

[d]p value=level of statistical signficance, NS=not significantly different (P>.10).

Table 16. Effect of Diet and Environment on Portal Plasma Amino Acid (PoPAA) Concentrations (μM).

	Treatment Combinations[a]				Statistical Significance[c]			
						P Value[d]		
PoPAA	GC	GCu	CCO	CCu	MSE[b]	E	D	ExD
Aspartate	94	75	129	140	286	.001	NS	NS
Threonine	559	450	605	891	36482	.02	NS	.06
Serine	455	418	450	429	12391	NS	NS	NS
Glutamate	371	316	557	647	8407	.001	NS	NS
Glutamine	230	113	203	244	3094	NS	NS	.05
Glycine	1497	746	1527	1252	43457	.02	.001	.04
Alanine	1300	574	1130	1175	76672	NS	.03	.01
Valine	408	302	342	419	6256	NS	NS	.04
Cystine	129	137	85	104	789	.02	NS	NS
Methionine	57	33	44	43	87	NS	.02	.03
Isoleucine	264	177	204	232	1670	NS	NS	.01
Leucine	404	258	348	375	5032	NS	NS	.03
Tyrosine	231	111	209	190	966	.09	.001	.005
Phenylalanine	159	125	145	156	855	NS	NS	NS
Lysine	349	118	104	136	11389	.05	.08	.03
Histidine	87	49	108	86	312	.007	.005	NS
Arginine	298	132	311	212	3620	NS	.001	NS

[a]G=germ-free, C=conventional, Co=control diet, Cu=250 ppm copper diet.

[b]MSE=mean square error.

[c]E=environment effect, D=diet effect, ExD=environment x diet interaction.

[d]P value = level of statistical significance, NS=not significantly different (P>.10).

but to a lesser extent for portal plasma amino acids (Table 16). Germ-free pigs had lower aspartate ($P<.001$), threonine ($P<.02$), glutamate ($P<.001$), glycine ($P<.02$) and histidine ($P<.007$), and tended to have less tyrosine in portal blood plasma than conventional pigs. However, germ-free pigs had higher cystine ($P<.02$) and lysine ($P<.05$) concentrations than conventional pigs. Herskovic et al. (1967) showed that germ-free mice absorb amino acids more efficiently than conventional animals, and as a result, it would be expected that portal amino acid concentrations would be elevated in the germ-free animal after a meal feeding.

Feeding the high copper diet decreased, and in some cases tended to decrease portal, amino acid levels in the germ-free pig but increased or tended to increase plasma amino acid levels in the conventional pigs fed the same diet (Table 16). Glycine and tyrosine however, were reduced by feeding the high copper diet to pigs in both environments ($P<.001$). High copper feeding also tended to reduce portal serine, histidine and arginine levels in pigs in both environments. This same relationship existed for peripheral amino acid concentrations with the exception of aspartate, leucine, tyrosine, phenylalanine and arginine which tended to be lower for pigs fed the high copper diet in both environments (Table 15).

J. Organ Weights

In this experiment, germ-free pigs had a smaller thyroid ($P<.001$), liver ($P<.001$) stomach ($P<.01$), small intestine ($P<.001$) and large intestine ($P<.001$) but larger spleen ($P<.003$), lungs ($P<.03$), heart ($P<.01$) and gall bladder ($P<.02$) than conventional pigs (Table 17). No differences were noted for thymus, adrenals, kidneys or

Table 17. Effect of Diet and Environment on Mean Organ Weights (g/kg body weight).

	Treatment Combination[a]					Statistical Significance[c]		
						P Value[d]		
Organ	GCo	GCu	CCo	CCu	MSE[b]	E	D	DxE
Thymus	2.69	1.61	2.72	1.76	0.351	NS	.002	NS
Adrenals	0.13	0.16	0.17	0.14	0.001	NS	NS	.04
Thyroid	0.07	0.08	0.10	0.09	0.001	.001	NS	NS
Spleen	2.03	1.75	1.44	1.49	0.069	.003	NS	NS
Lungs	12.0	11.4	10.7	10.8	0.742	.03	NS	NS
Heart	5.60	5.86	5.24	5.15	0.131	.006	NS	NS
Kidneys	6.65	7.21	6.51	6.57	0.621	NS	NS	NS
Liver	24.4	26.2	34.4	35.9	5.824	.001	NS	NS
Pancreas	1.97	1.98	2.14	2.18	0.072	NS	NS	NS
Gall Bladder	0.63	0.46	0.38	0.37	0.020	.02	NS	NS
Stomach	9.17	8.62	9.83	9.75	0.382	.007	NS	NS
Small Intestine	39.1	36.7	50.6	53.6	32.59	.001	NS	NS
Large Intestine	12.4	16.9	24.4	25.3	18.27	.001	NS	NS

[a]G=germ free; C=conventional, Co=control diet, Cu=250 ppm copper diet.

[b]MSE=mean square error.

[c]E=environment effect, D=diet effect, ExD=environment-diet interaction.

[d]P value=level of statistical significance, NS=not significantly different (P>.10).

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pancreas weights between germ-free and conventional pigs. Waxler and Drees (1972) examined differences in organ weights between germ-free, monocontaminated and conventional pigs and noted that germ-free pigs had a smaller thyroid and spleen but larger adrenals, heart, kidney, stomach and small intestine than conventional pigs when expressed on the basis of mg/100 g body weight. Pigs monocontaminated with E. coli had larger adrenals, thyroid, stomach and small intestine than germ-free pigs but a smaller spleen, and a larger kidney, stomach and small intestine than conventional pigs (Waxler and Drees, 1972). Waxler and Drees (1973) and Kruml et al. (1969) did not observe differences in thymus weight of germ-free and conventional pigs. Miniats and Valli (1978) noted that the weight of the gastrointestinal tract, as a percentage of body weight, for germ-free pigs was less than for conventional pigs. However, in both reports, conventional pigs grew faster and weighed more at the termination of these experiments, which is contrary to the results observed in this experiment (see discussion on growth performance in Section A). As a result, even though organ weights were expressed on a body weight basis, large differences in body weight of pigs, along with small changes in organ weights, could explain some discrepancies between organ weights observed in this study compared to those reported by other workers. Germ-free pigs were heavier in this experiment while conventional pigs were heavier in the other two studies previously discussed which could explain these conflicting findings.

A smaller thyroid gland as observed in this study might be expected since basal metabolic rate in germ-free rats is approximately 20% of that in conventional rats (Deplaces et al., 1963; Levenson et

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al., 1966) and iodine uptake by the thyroid of germ-free rats is approximately 50% of that in conventional rats (Levenson et al., 1966). Since basal metabolic rate is reduced in germ-free rats, germ-free pigs may also have a lower basal metabolic rate which could explain the smaller thyroid observed for germ-free pigs in this study.

The gastrointestinal tract is one organ in direct contact with environmental microbes and wet weight of the germ-free pig gastrointestinal tract was reduced compared to that of conventional pigs. This relationship is in agreement with other germ-free pig studies (Waxler and Drees, 1972; Miniats and Valli, 1973) and with other germ-free animal studies (Gordon, 1968) except for the cecum of the rat and mouse which is greatly enlarged relative to their conventional counterparts (Gordon et al., 1966). This reduction in intestinal weight of germ-free pigs appears to be primarily due to a reduction in lamina propria (Gordon and Bruckner-Kardoss, 1959; 1961b).

Thymus was the only organ affected by feeding the high copper diet (Table 17). Thymus weights were reduced ($P<.002$) by feeding the high copper diet in both environments. Although no other significant differences due to diet were observed, liver, kidney, heart and adrenal glands tended to increase in germ-free pigs fed the high copper diet, as a percentage of body weight. Miller et al. (1967) observed that iron-deficient pigs had larger livers, kidneys, hearts, spleen and adrenal glands, as a percentage of body weight, than pigs receiving an iron adequate diets.

A diet by environment interaction (Table 17) was observed for adrenal gland weights ($P<.04$). The heavier adrenal weights of the germ-free pigs receiving the high copper diet may have been due to

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stress from overcrowding in that isolator during the last week of the trial, but adrenal weights were reduced for conventional pigs fed the high copper diet.

K. Intestinal Morphology

Results from this study (Table 18) indicate that germ-free pigs had longer ileal villi ($P<.05$), tended to have longer duodenal villi (400 μm vs. 346 μm), but had shorter jejunal villi ($P<.02$) than their conventional counterparts. Miniats and Valli (1973) observed longer jejunal villi in the intestine of germ-free pigs but did not examine duodenum or ileum sites. Measurements of villus height and observations on villus shape provide a reasonable estimate of villus enterocyte numbers (Hampson, 1986). Wright (1982) reported that in normal, finger-like villi, (analogous to villi in the germ-free intestine) villus height and cell population are highly correlated, but correlations between villus height and cell population are lower for more complex shaped villi relative to cell population (Creamer, 1964). Since there is a slower turnover of epithelial cells in the germ-free small intestine, there may be a greater number of mature cells at any time, and thus, potentially higher amounts of enzymes associated with them (Coates and Fuller, 1977). Therefore, even though it may seem that the villi should be shorter for the germ-free pig, a slower cell replacement rate allowing more mature cells to be present at any given time, would explain the longer ileal and duodenal villi, observed in germ-free pigs in this experiment (assuming that villi height is highly correlated with cell number). If there are more mature cells in the germ-free small intestine, resulting in higher amounts of enzymes associated with them, then the increase in absorption of xylose and

Table 18. Effect of Diet and Environment on Villus Height, Villus Width, Crypt Depth, Submucosa Depth and Total Intestinal Thickness of the Duodenum, Jejunum and Ileum (microns).

	Treatment Combination[a]				Statistical Significance[c]			
						P Value[d]		
Parameter	GCo	GCu	CCo	CCu	MSE[b]	E	D	ExD
Duodenum								
Villus Height	496	304	367	325	5623	NS	.01	.05
Villus Width	140	127	160	175	271	.001	NS	.07
Crypt Depth	172	171	357	315	1098	.001	NS	NS
Submucosa Depth	144	184	205	231	2269	.03	NS	NS
Total Thickness	862	770	978	944	8425	.01	NS	NS
Jejunum								
Villus Height	386	339	375	446	1465	.02	NS	.004
Villus Width	128	101	124	122	120	NS	.01	.03
Crypt Depth	147	103	177	184	443	.001	.08	.02
Submucosa Depth	188	130	168	166	1865	NS	NS	NS
Total thickness	812	645	833	828	6820	.02	.04	.06
Ileum								
Villus Height	406	363	312	333	3964	.05	NS	NS
Villus Width	132	120	124	128	80	NS	NS	.07
Crypt Depth	145	111	175	193	447	.001	NS	.02
Submucosa Depth	155	128	147	185	3700	NS	NS	.09
Total thickness	871	699	830	918	18046	NS	NS	.09

[a]G=germ free, C=conventional, Co=control diet, Cu=250 ppm copper diet.

[b]MSE=mean square error.

[c]E=environment effect, D=diet effect, ExD=environment-diet interaction.

[d]P value=level of statistical significance, NS=not significantly different (P>.10).

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amino acids observed by Heneghan (1963) and Herkovic et al. (1967) in germ-free rats, and the increase in portal glucose concentration observed in this study may be partially explained.

Kenworthy and Allen (1966) showed that when germ-free pigs were exposed to the "bacterial environment", villi became stunted, fused and clubbed. Width of villi (measured at the base of the villi) was reduced in the duodenum ($P<.001$), tended to be reduced in the jejunum (115 μm vs. 123 μm) but was not different in the ileum of germ-free pigs compared to conventional pigs (Table 18). Since the conventional pigs in this study had shorter villi (duodenal and ileal), there was also a likely reduction in enterocyte number even though villi were more irregular than that observed for the germ-free pigs. This apparent reduction in enterocyte number could either be a result of an increased rate of cell loss or a reduction in crypt cell production rate.

Feeding high copper diets reduced villus height in the duodenum ($P<.01$) and to a greater extent in the germ-free pigs ($P<.05$) (Table pigs when fed the high copper diet, but increased when conventional pigs were fed this same diet ($P<.004$). This same relationship tended to also exist for the ileal site. Apparent increases in jejunal and ileal villus height (enterocyte number) of conventional pigs fed the high copper diet may have been a result of local alterations in the luminal environment caused by copper. In other words, copper may have reduced the accumulation of toxic bacterial metabolites shown to cause villi shortening (Kenworthy, 1976).

Crypt depth gives a general indication of the rate of crypt cell production (Hampson, 1986). In other words, crypt cell production

increases as additional cells are recruited to the crypt epithelium to cause it to elongate. As crypt cells migrate upward, they become villus enterocytes and this cycle takes 2 to 4 days to occur in three-week old, unweaned conventional pigs (Moon, 1971). During their migration, these cells become increasingly differentiated and their absorptive capacity develops (Kinter and Wilson, 1965). Measurement of crypt depth therefore gives a general indication of the likely maturity and functional capacity of enterocytes of the villi.

In this experiment, crypt depth was decreased ($P<.001$) in germ-free pigs at each site along the small intestine (Table 18) indicating a slower rate of enterocyte production which is consistent with work by Abrams et al. (1963) and Lesher et al. (1964). The slower turnover of epithelial cells in the small intestine of germ-free pigs implies that there is a greater number of mature cells present at any time, and thus, potentially higher amounts of enzymes associated with them.

When high copper diets were fed, crypt depth decreased in the germ-free pig but increased in the conventional pig in the jejunum and ileum ($P<.02$). The small intestinal mucosa is the most rapidly regenerating tissue in the body (LeBlond and Walker, 1956). Any tissue undergoing such a rapid turnover needs a readily available supply of nutrients. Perhaps high copper feeding improves the amount of readily available nutrients so that cell turnover is reduced in the germ-free pig but microflora in the conventional pig utilize some of these nutrients and cell turnover is increased.

Depth of the submucosa was less in the duodenum ($P<.03$) and tended to be reduced in the jejunum (159 μm vs. 167 μm) and ileum (141 μm vs.

166 μm) of germ-free pigs compared to their conventional counterparts (Table 16). The submucosa (containing the larger blood vessels, lymphatics and neural complexes) is a loose connective arrangement paralleling that of the lamina propria and accomodates contractions of the adjacent muscularis mucosa. Since germ-free animals have a thinner intestinal wall due to a reduction in connective tissue (Coates and Fuller, 1977; Gordon, 1968; Minats and Valli, 1973) then a reduction in thickness of the submucosa would also be expected for the intestinal tract of germ-free pigs in this experiment because of its connective tissue constituents.

Total intestinal thickness of the duodenum ($P<.01$) and jejunum ($P<.02$) was reduced in the germ-free pigs and tended to be reduced (785 μm vs. 874 μm) in the ileum (Table 18). This is in agreement with other reports when germ-free and conventional animals were compared (Coates and Fuller, 1977; Gordon, 1968; Miniats and Valli, 1973).

Feeding the high copper diet reduced total intestinal thickness of the jejunum of both germ-free and conventional pigs ($P<.04$) but more dramatically in the germ-free pigs ($P<.06$). This same trend existed for the duodenal site. High copper feeding tended to increase total thickness of the ileum of conventionally reared pigs but tended to be reduced in germ-free pigs ($P<.09$). In general, feeding high copper diets to germ-free pigs reduced total intestinal thickness but not to the extent of that in the conventional pigs. This implies that gut thickness may not play a role in the mechanism of action of copper since conventional pigs appeared to grow at a faster rate when supplemental copper was included in the diet even though total gut wall thickness appeared to be not appreciably different.

II. INFLUENCE OF COPPER AND CHLORTETRACYCLINE ON MICROBIAL FERMENTATION PATTERNS AND MICROBIAL GROWTH IN FIVE SECTIONS OF THE GASTROINTESTINAL TRACT IN VITRO.

A. Microbial Growth

Microbial growth, measured by change in optical density of the inoculated media per unit time, was greater for the control (Co) and 100 ppm chlortetracycline (CTC) treated microbes, with each microbe source, compared to either the 250 ppm copper (Cu) or 250 ppm copper and 100 ppm chlortetracycline (Cu + CTC) treated microbes at 16 hours ($P<.01$) and 24 hours ($P<.01$) of incubation (Table 19 and Figures 1-5). Microbial growth was not different between Co and CTC treated microbes within microbe source except for cecal contents at 16 ($P<.01$) and 24 hours ($P<.01$) of incubation (Table 19 and Figures 1-5). Similarly, no difference was noted for microbial growth within microbe source when the Cu treatment was compared to the Cu + CTC treatment of 16 and 24 hours of incubation (Table 19 and Figures 1-5). Within each microbe source (except for 1st half small intestine contents and cecal contents) there appeared to be a general tendency for CTC to slightly reduce growth compared to growth of Co treated microbes and for the Cu + CTC treated microbes to grow at a slightly reduced rate compared to microbes incubated with the Cu treatment (Figures 1-5).

Within the Co and CTC treatments, microbial growth was increased at 16 hours ($P<.001$) and at 24 hours ($P<.001$) of incubation compared to the initial optical density readings at 0 hours (Table 20). However, microbial growth within these two treatments was not different between 16 hours and 24 hours of incubation (Table 20). Within the Cu treatment, microbial growth occurred, and peaked at 16 hours of

Table 19. Comparison of Average Optical Density Readings (As a Measure of Microbial Growth) and Standard Deviations Within Microbe Source, Between Treatments, at 16 and 24 Hours of Incubation.

Incubation Time	16 hours				24 hours			
Treatment[a]	Co	Cu	CTC	Cu + CTC	Co	Cu	CTC	Cu + CTC
Microbe Source:								
Stomach contents	1.71 ± .36[b]	0.44 ± .11[c]	1.65 ± .34[b]	0.50 ± .05[c]	1.99 ± .03[b]	0.22 ± .08[c]	1.73 ± .46[b]	0.23 ± .03[c]
Stomach wall	1.22 ± .20[b]	0.23 ± .04[c]	1.02 ± .37[b]	0.29 ± .14[c]	1.56 ± .24[b]	0.08 ± .03[c]	1.50 ± .25[b]	0.08 ± .16[c]
Sm. int. contents (1)	1.69 ± .38[b]	0.34 ± .05[c]	1.71 ± .32[b]	0.40 ± .13[c]	1.79 ± .31[b]	0.17 ± .06[c]	1.85 ± .20[b]	0.16 ± .06[c]
Sm. int. wall (1)	1.54 ± .36[b]	0.22 ± .04[c]	1.38 ± .27[b]	0.27 ± .08[c]	2.00 ± .01[b]	0.04 ± .02[c]	1.88 ± .20[b]	0.02 ± .01[c]
Sm. int. contents (2)	1.84 ± .22[b]	0.31 ± .03[c]	1.75 ± .44[b]	0.34 ± .03[c]	1.84 ± .15[b]	0.11 ± .03[c]	1.92 ± .14[b]	0.11 ± .03[c]
Sm. int. wall (2)	1.45 ± .23[b]	0.17 ± .18[c]	1.19 ± .34[b]	0.20 ± .03[c]	1.69 ± .40[b]	0.01 ± .10[c]	1.59 ± .37[b]	0.03 ± .01[c]
Cecal contents	1.97 ± .07[b]	0.38 ± .54[c]	1.40 ± .01[b]	0.30 ± .27[c]	1.95 ± .05[b]	0.22 ± .45[d]	1.38 ± .05[b]	0.08 ± .38[c]
Cecal wall	1.83 ± .21[b]	0.17 ± .18[c]	1.71 ± .39[b]	0.17 ± .04[c]	2.00 ± .11[b]	0.01 ± .11[c]	1.98 ± .05[b]	0.03 ± .01[c]
Cecal contents	1.96 ± .01[b]	0.42 ± .06[c]	2.00 ± .01[b]	0.41 ± .10[c]	1.94 ± .06[b]	0.27 ± .05[c]	1.98 ± .03[b]	0.08 ± .11[c]
Colon wall	1.80 ± .23[b]	0.19 ± .17[c]	1.57 ± .51[b]	0.18 ± .03[c]	2.00 ± .01[b]	0.02 ± .13[c]	2.00 ± .01[b]	0.02 ± .02[c]

[a]Co=control, Cu=250 ppm copper, CTC=100 ppm chlortetracycline, Cu + CTC=250 ppm copper + 100 ppm chlortetracycline.

[b,c,d]Means with different superscripts within microbe source within incubation time are significantly different (P<.01).

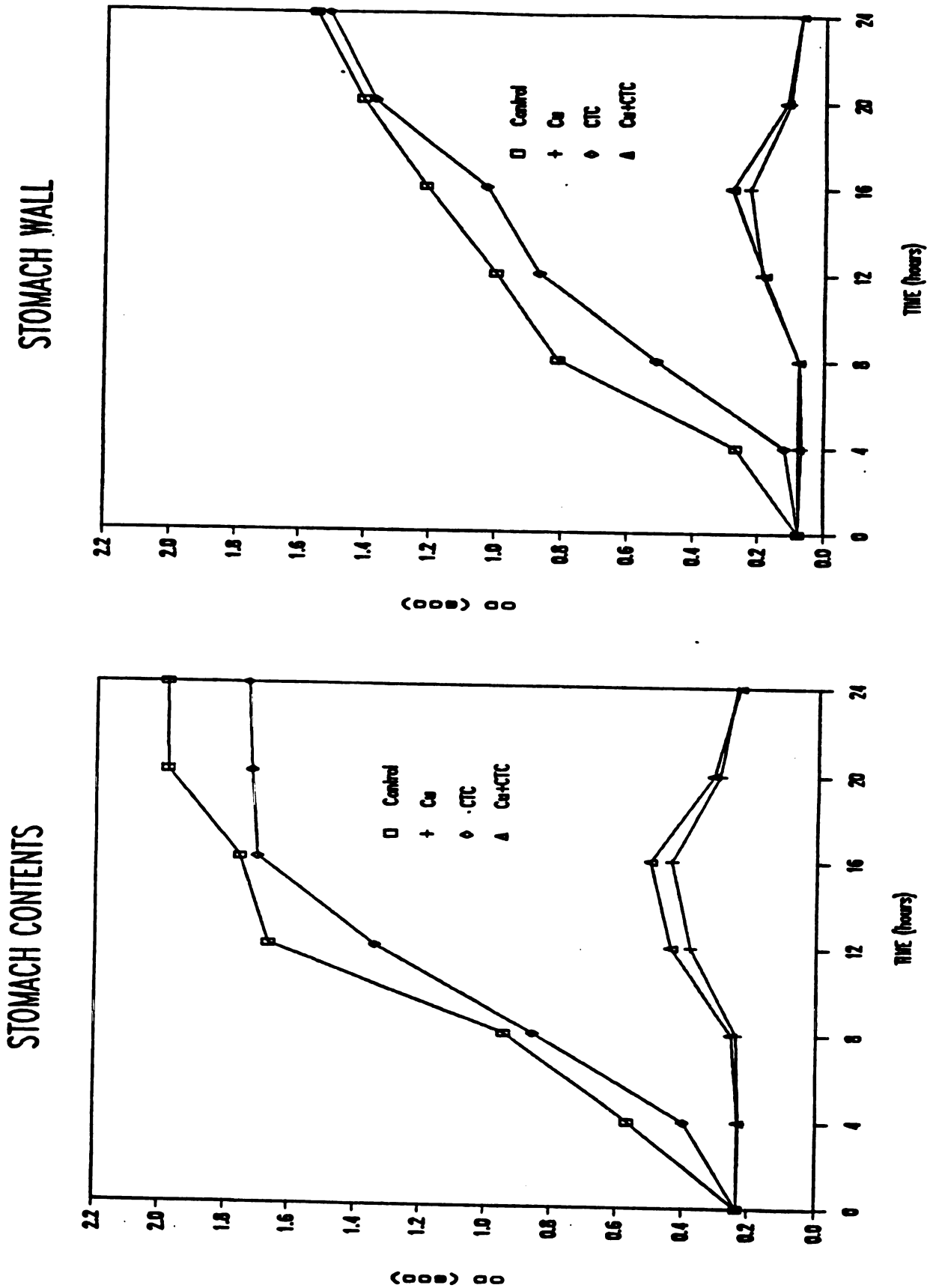

Figure 1. Influence of Copper and Chlortetracycline on Growth of Microflora from Stomach Contents and Wall During a 24 Hour Incubation Period.

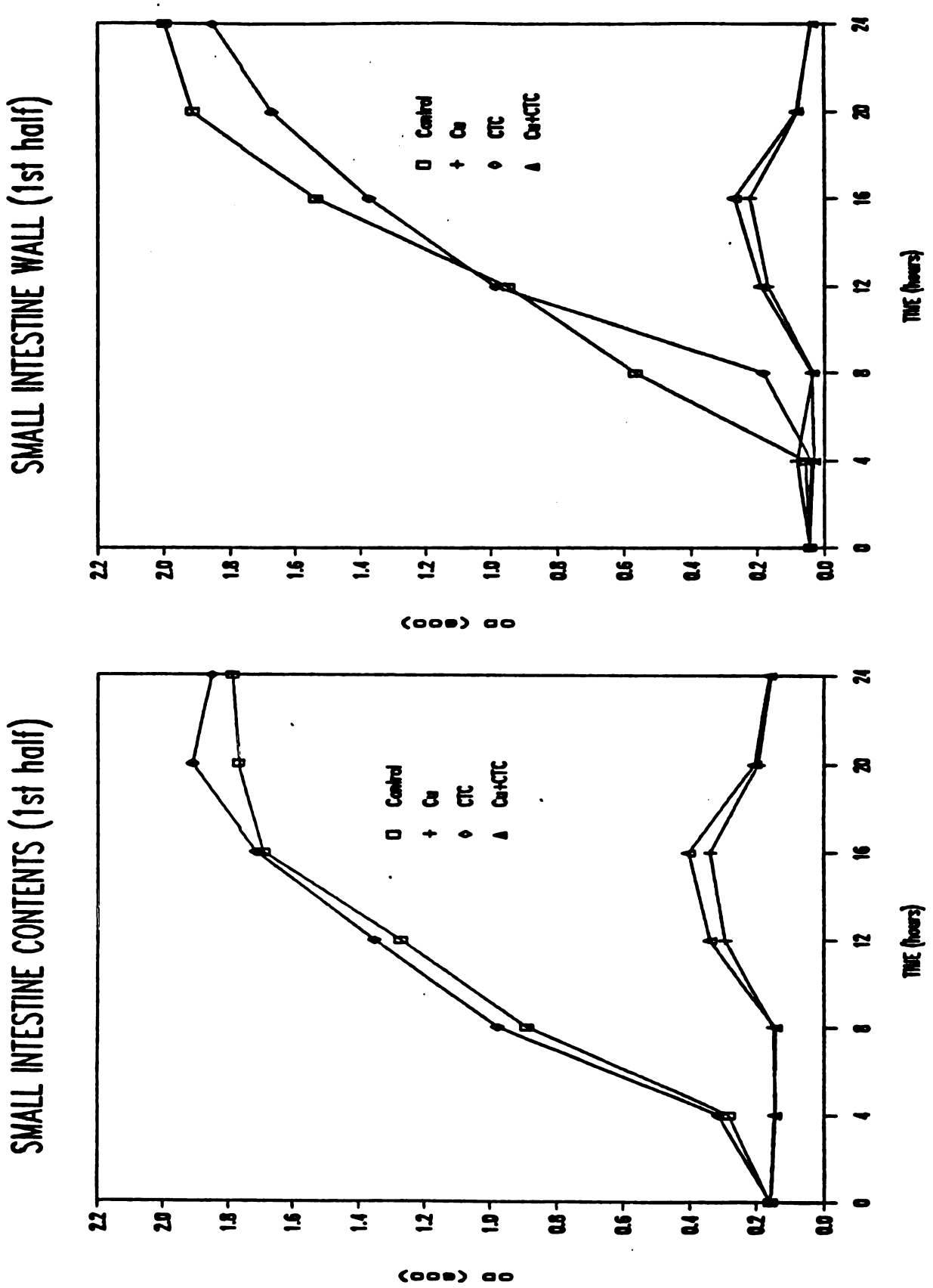

Figure 2. Influence of Copper and Chlortetracycline on Growth of Microflora from Small Intestine Contents and Wall (1st half) During a 24 Hour Incubation Period.

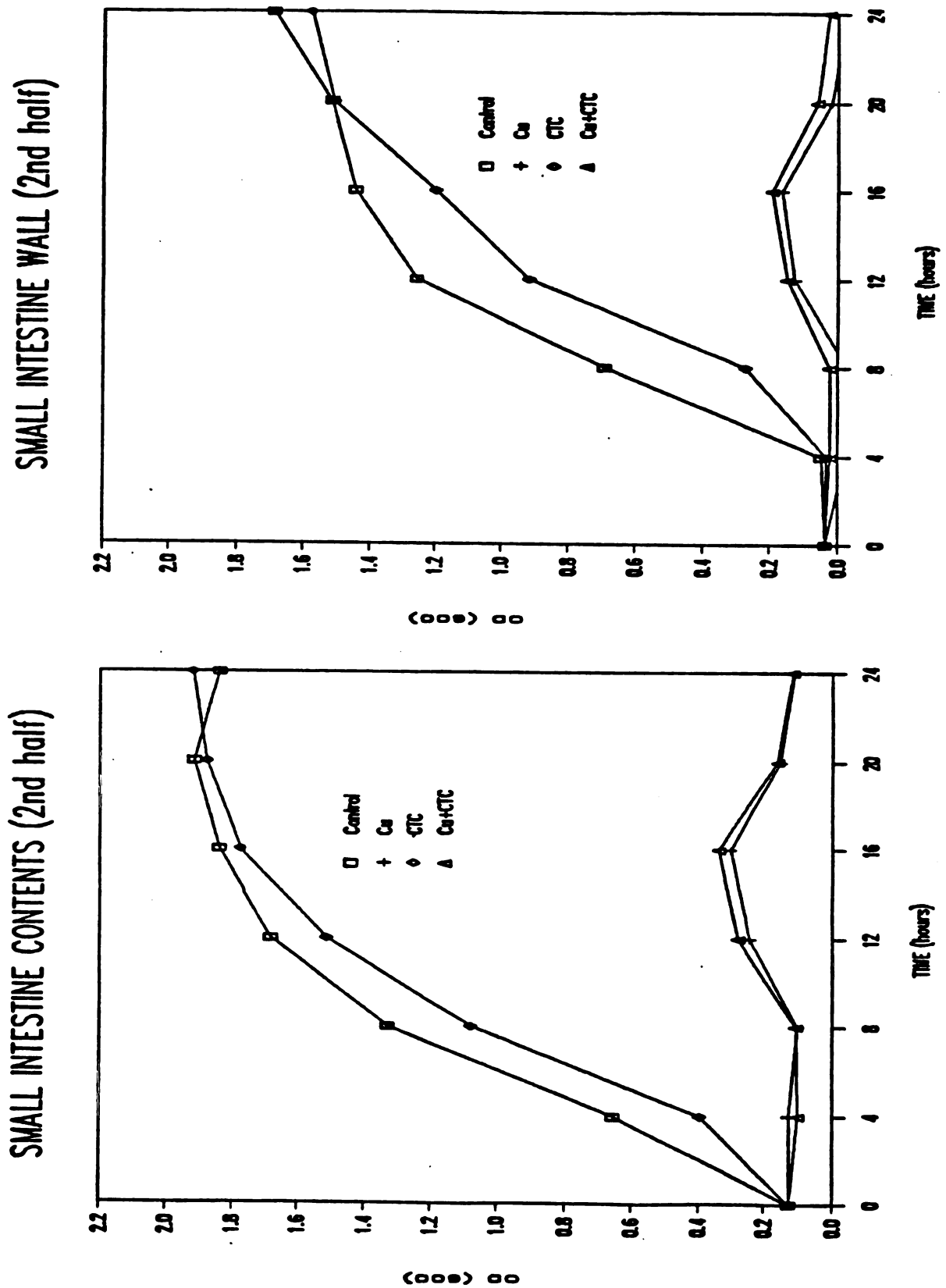

Figure 3. Influence of Copper and Chlortetracycline on Growth of Microflora from Small Intestine Contents and Wall (2nd half) During a 24 Hour Incubation Period.

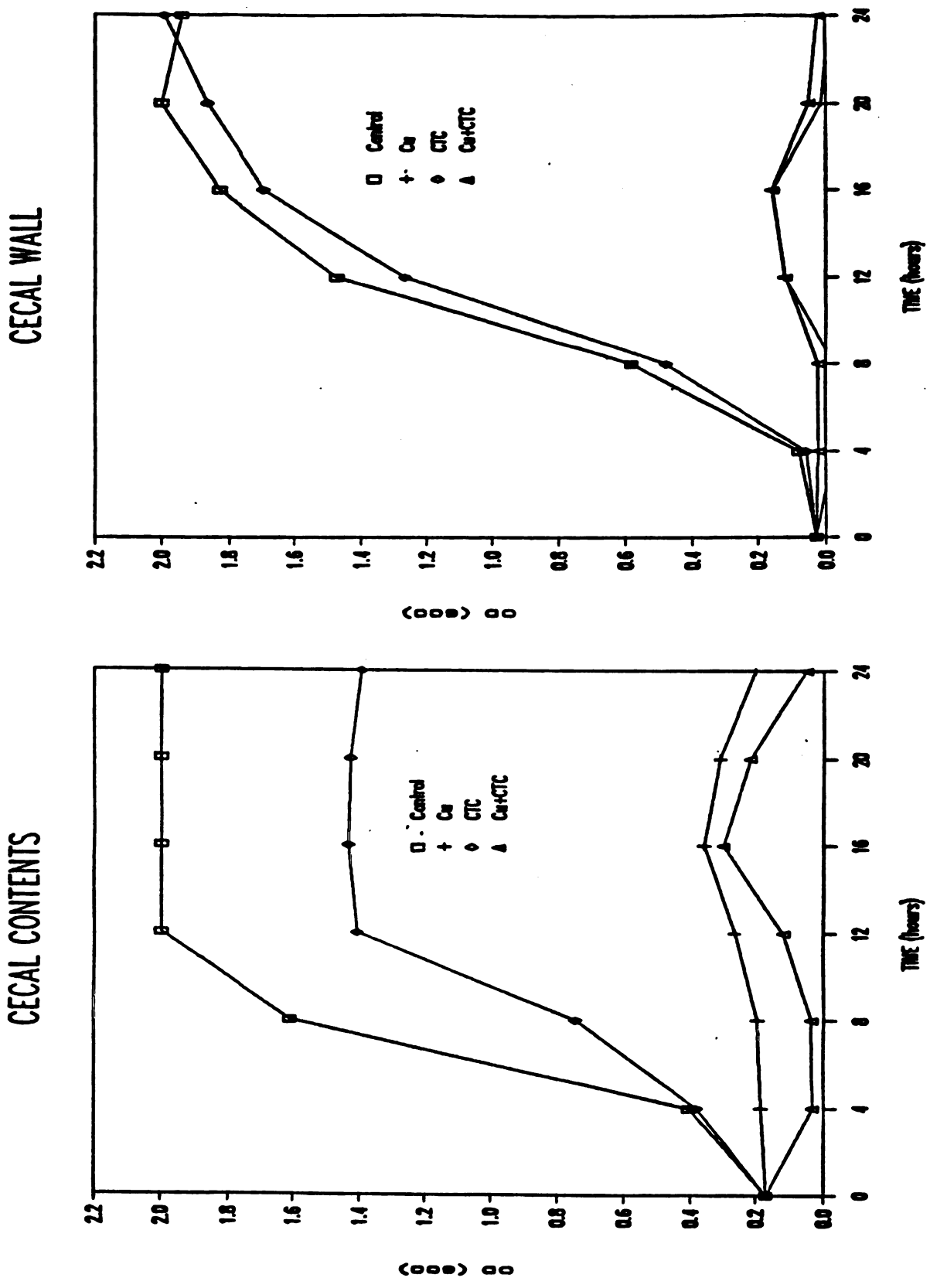

Figure 4. Influence of Copper and Chlortetracycline on Growth of Microflora from Cecal Contents and Wall During a 24 Hour Incubation Period.

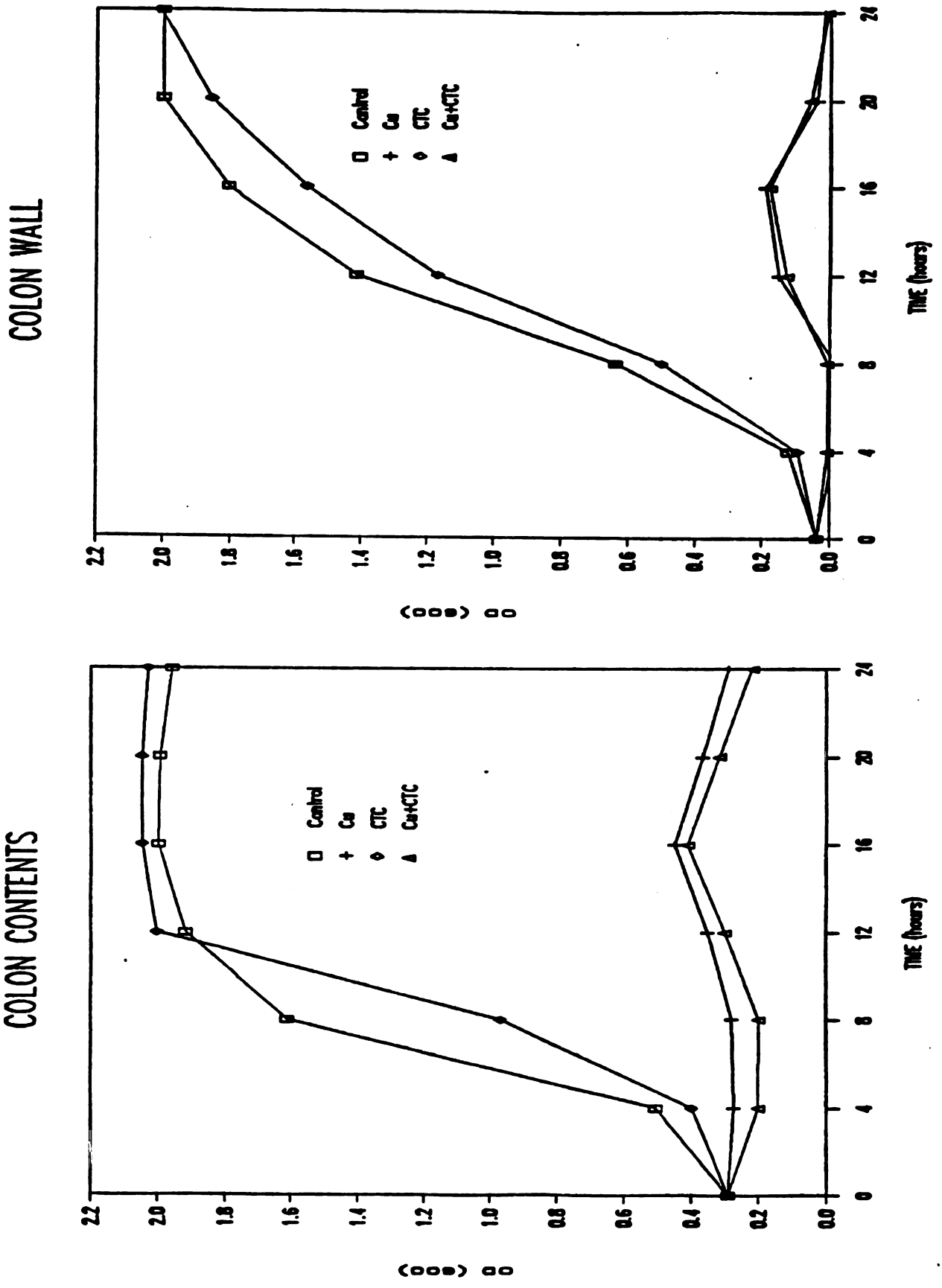

Figure 5. Influence of Copper and Chlortetracycline on Growth of Microflora from Colon Contents and Wall During a 24 Hour Incubation Period.

Table 20. Comparison of Average Optical Density Readings (As a Measure of Microbial Growth) and Standard Deviations at 0, 16 and 24 Hours of Incubation Within Microbe Source, Within Treatments.

Treatment[a]	Co			CTC		
Incubation Time (hrs)	0	16	24	0	16	24
Microbe Sources:						
Stomach contents	0.23 ± .11[b]	1.71 ± .36[e]	1.99 ± .03[c]	0.25 ± .07[b]	1.65 ± .34[c]	1.73 ± .46[c]
Stomach wall	0.08 ± .07[b]	1.22 ± .20[c]	1.56 ± .24[c]	0.07 ± .07[b]	1.02 ± .37[c]	1.50 ± .25[c]
Sm. int. contents (1)	0.16 ± .07[b]	1.69 ± .38[c]	1.79 ± .31[c]	0.14 ± .05[b]	1.71 ± .32[c]	1.85 ± .20[c]
Sm. int. wall (1)	0.04 ± .02[b]	1.54 ± .36[c]	2.00 ± .01[c]	0.05 ± .01[b]	1.38 ± .27[c]	1.88 ± .20[c]
Sm. int. contents (2)	0.13 ± .03[b]	1.84 ± .22[c]	1.84 ± .15[c]	0.12 ± .04[b]	1.75 ± .44[c]	1.92 ± .14[c]
Sm. int. wall (2)	0.04 ± .01[b]	1.45 ± .23[c]	1.69 ± .40[c]	0.05 ± .01[b]	1.19 ± .34[c]	1.59 ± .37[c]
Cecal contents	0.18 ± .07[b]	1.97 ± .07[c]	1.95 ± .05[c]	0.19 ± .09[b]	1.40 ± .01[c]	1.38 ± .05[c]
Cecal wall	0.04 ± .02[b]	1.83 ± .21[c]	2.00 ± .11[c]	0.03 ± .01[b]	1.71 ± .39[c]	1.98 ± .05[c]
Colon contents	0.29 ± .15[b]	1.96 ± .01[c]	1.94 ± .06[c]	0.28 ± .12[b]	2.00 ± .01[c]	1.98 ± .03[c]
Colon wall	0.04 ± .01[b]	1.80 ± .23[c]	2.00 ± .01[c]	0.04 ± .01[b]	1.57 ± .51[c]	2.00 ± .01[c]

[a]Co=control, CTC=100 ppm chlortetracycline.

[b,c]Means with different superscripts within microbe source within treatment are significantly different ($P<.05$).

Table 20. Comparison of Average Optical Density Readings (As a Measure of Microbial Growth) and Standard Deviations at 0, 16 and 24 Hours of Incubation Within Microbe Source, Within Treatments.

Treatment[a]	Cu			Cu + CTC		
Incubation Time (hrs)	0	16	24	0	16	24
Microbe Sources:						
Stomach contents	0.21 ± .07[b]	0.44 ± .11[c]	0.22 ± .08[b]	0.23 ± .06[b]	0.50 ± .05[c]	0.23 ± .03[b]
Stomach wall	0.08 ± .02[b]	0.23 ± .04[c]	0.08 ± .03[b]	0.08 ± .18[b]	0.29 ± .14[b]	0.08 ± .16[b]
Sm. int. contents (1)	0.15 ± .05[b]	0.34 ± .05[c]	0.17 ± .06[b]	0.16 ± .07[b]	0.40 ± .13[c]	0.16 ± .06[b]
Sm. int. wall (1)	0.04 ± .03[b]	0.22 ± .04[c]	0.04 ± .02[b]	0.03 ± .01[b]	0.27 ± .08[c]	0.02 ± .01[b]
Sm. int. contents (2)	0.15 ± .03[b]	0.31 ± .03[c]	0.11 ± .03[b]	0.12 ± .06[b]	0.34 ± .03[c]	0.11 ± .03[n]
Sm. int. wall (2)	0.05 ± .18[b]	0.17 ± .18[b]	0.01 ± .10[b]	0.03 ± .02[b]	0.20 ± .03[c]	0.03 ± .01[b]
Cecal contents	0.17 ± .07[b]	0.38 ± .54[b]	0.22 ± .45[b]	0.18 ± .36[b]	0.30 ± .27[b]	0.08 ± .38[b]
Cecal wall	0.05 ± .16[b]	0.17 ± .18[b]	0.01 ± .11[b]	0.04 ± .02[b]	0.17 ± .04[c]	0.03 ± .01[b]
Colon contents	0.28 ± .01[b]	0.42 ± .06[b]	0.27 ± .05[b]	0.30 ± .03[b]	0.41 ± .10[b]	0.08 ± .11[b]
Colon wall	0.05 ± .17[b]	0.19 ± .17[b]	0.02 ± .13[c]	0.04 ± .04[b]	0.18 ± .03[c]	0.02 ± .02[b]

[a]Cu=250 ppm copper, Cu+CTC=250 ppm copper+100 ppm chlortetracycline.

[b,c]Means with different superscripts with microbe source within treatment are significantly different (P<.05).

incubation compared to initial optical density readings ($P<.01$) for microbes from the stomach contents (SC), stomach wall (SW), small intestine contents (1st half) (SI_1C), small intestine wall (1st half) (SI_1W) and small intestine contents (2nd half) (SI_2C), but not for microbes associated with the small intestine wall (2nd half) (SI_2W), cecal contents (CeC), cecal wall (CeW), colon contents (CoC) or colon wall (CoW) as shown in Table 20. However, after 16 hours of incubation, optical density readings declined to initial levels ($P<.01$) at 24 hours of incubation for microbes from SC, SW, SI_1C, SI_1W and SI_2C sources, and tended to decline for microbes from SI_2W, CeC, CeW, CoC and CoW (Table 20). For each microbe source incubated in Cu media, there was an 8 hour lag phase where little, if any, growth occured, which may have been due to an attempted adaptation of microbes to the adverse effects of Cu in the media. This lag phase appeared to be particularly marked for the microorganisms associated with the CeC and CoC sources (Figures 4 and 5).

Similarly, when optical density readings were determined at 0, 16 and 24 hours, within the Cu + CTC treatment for each microbial source, growth did occur and peaked at 16 hours of incubation for microbes from the SC, SI_1C, SI_1W, SI_2C, SI_2W, CeW and CoW, ($P<.01$) and growth tended to occur for SW, CeC and CoC microbe sources (Table 20). It appears that after maximum growth was obtained at 16 hours of incubation, the attempts of the microbes to compensate for the apparent adverse environment created by copper, were not adequate, and bacterial cell lysis occurred, since optical density readings at 24 hours of incubation were approximately the same as the initial optical density readings (Table 20). Changes in optical density readings during

incubation may be due to either an increase in bacterial cell number, caused by multiplication, or an increase in cell size.

Studies have been conducted to examine the influence of high levels of copper on specific bacterial species in digesta and feces of pigs. Kellogg et al. (1966) observed that when pigs were fed 250 ppm supplemental copper, growth rate was improved, compared to pigs fed a control diet and associated with this growth improvement, they observed a lower number of lactobacilli, total aerobes and total anaerobes. When Fuller et al. (1960) fed diets supplemented with 250 ppm copper, they observed no difference in the number of lactobacilli, and coli-aerogenes compared to microbes of pigs fed a control diet, but they did observe a decrease in the total number of streptococci and a shift in the species of lactobacilli (decrease in L. acidophilus and a corresponding increase in L. brevis and L. cellobiosus). Smith and Jones (1963) studied the effects of 250 ppm and 1000 ppm supplemental copper on the bacterial flora of the alimentary tract of the pig and observed no differences in E. coli, lactobacilli, streptococci, Cl. welchii, bacteroides or yeasts compared to control pigs. However, the minimum inhibitory concentration (MIC) for microbes grown in reinforced clostridial medium (RCM) was as follows:

	MIC of copper (ppm)[a]			
Test medium	Lactobacilli	Streptococci	E. coli	Cl welchii
RCM agar	10	200	200	19
RCM broth	100	200	200	50
Aqueous suspension of pig feed (30% wt./vol.)	400	400	400	100

[a]Smith and Jones (1963).

As these workers observed, a higher concentration of copper was required to inhibit growth of these organisms in liquid than on solid media. These workers suggested that the bactericidal effectiveness of copper sulfate is decreased in the alimentary tract by other factors that are not present in an aqueous suspension of pig feed (e.g., digestive secretions), which would explain their results. Therefore, the detrimental effects of copper on in vitro microbial growth observed in this study, may not be nearly as dramatic in vivo, but copper does appear to affect microbial population growth at all sites along the digestive tract in the absence of factors in the gut that reduce the bactericidal effectiveness of copper.

Based on these data, it appears that 100 ppm chlortetracycline reduced microbial growth only in cecal contents as compared to the control treated microbes and the presence of 250 ppm copper, singly, or in combination with 100 ppm CTC, dramatically reduced growth of microbes when optical density readings were compared with those of the control treated microbes at 16 and 24 hours of incubation. Apparently, the level of 250 ppm copper, which consistently promotes growth when included in diets for the young pig, is toxic to the gastrointestinal microflora in both the gut contents and the gut wall along all sections of the digestive tract. Bacterial growth is inhibited in vitro, but based on work reported by Smith and Jones (1963), the presence of other factors in the gut environment may reduce the bactericidal effectiveness of copper in vivo, as observed in this study.

B. Fermentation Changes in pH of Growth Media

Since the buffer system of the media used in this study was designed to prevent large changes in pH which would severely affect

microbe viability, a large change in acid or base production would have to occur from the microbial fermentation process before changes in pH could be observed. In other words, a large increase in amine production caused by the fermentative action of bacterial amino acid decarboxylases on amino acids or a release of these highly basic amines from bacterial cells upon lysis could cause a small increase in the pH of the growth medium (Prins, 1977). Similarly, a large increase in fatty acid production from microbial fermentation of carbohydrate sources could result in a small reduction in media pH.

An initial pH of 6.54 was measured in the anaerobic growth media and when microbes from each source were incubated in media containing either the Co or CTC treatments, pH was generally reduced ($P<.05$) by 0.03 and 0.05 pH units, respectively, compared to an increase in pH ($P<.05$) by 0.09 and 0.06 units for Cu and Cu+CTC treatments, respectively, regardless of microbe source (Table 21). Perhaps an increase in total organic acid production for microbes incubated in the Cu and CTC media (Tables 23 and 24) was sufficient to cause this small increase in pH over the initial level, and an apparent bacterial cell lysis after 16 hours of incubation (which may explain the reduction in optical density readings shown in Table 20) when microbes were incubated in Cu and Cu+CTC media, may have liberated highly basic amines to cause the small increase in pH observed in this study.

C. Net Ammonia Nitrogen Changes

In this study, net ammonia nitrogen change after 24 hours of incubation was generally increased for Co and CTC treated microbes, within microbe source, compared to a net reduction in ammonia nitrogen for Cu and Cu+CTC treated microbes ($P<.05$) (Table 22).

Table 21. Comparison of Treatment Means for Change in Media pH After a 24-Hour Incubation Period, Within Microbe Source.

	Treatment[a]			
Microbe Source	Co	Cu	CTC	Cu+CTC
Stomach contents	-0.05[b]	+0.09[d]	-0.07[b]	+0.05[c]
Stomach wall	-0.03[b]	+0.10[d]	-0.05[b]	+0.06[c]
Small intestine contents (1st half)	-0.07[b]	+0.09[d]	-0.07[b]	+0.06[c]
Small intestine wall (1st half)	-0.05[b]	+0.11[d]	-0.05[b]	+0.07[c]
Small intestine contents (2nd half)	-0.04[b]	+0.10[d]	-0.07[b]	+0.06[c]
Small intestine wall (2nd half)	-0.04[b]	+0.11[d]	-0.07[b]	+0.07[c]
Cecal contents	+0.01[b]	+0.10[d]	+0.00[b]	+0.06[c]
Cecal wall	-0.03[b]	+0.09[c]	-0.04[b]	+0.06[c]
Colon contents	+0.02[b]	+0.08[d]	+0.01[b]	+0.05[c]
Colon wall	-0.06[b]	+0.08[c]	-0.07[b]	+0.06[c]
Overall	-0.03[b]	+0.09[d]	-0.05[b]	+0.06[c]

[a]Co=control, Cu=250 ppm copper as $CuSO_4 \cdot 5H_2O$, CTC=100 ppm chlortetracycline, Cu+CTC=250 ppm copper as $CuSO_4 \cdot 5H_2O$ and 100 ppm chlortetracycline.

[b,c,d]Mean changes between treatments, within microbe source, with different superscripts are significantly different ($P<.05$), Std. Error = 0.02.

Table 22. Comparison of Treatment Means for Net NH_3-N Change (mg) After a 24 Hour Incubation Period, Within Microbe Source.

	Treatment[a]			
Microbe Source	Co	Cu	CTC	Cu+CTC
Stomach contents	+0.73[b]	-0.25[c]	-0.17[c]	-0.23[c]
Stomach wall	+0.66[b]	-0.27[c]	+0.30[b]	-0.26[c]
Small intestine contents (1st half)	+0.65[b]	-0.22[c]	+0.54[b]	-0.28[c]
Small intestine wall (1st half)	+0.61[b]	-0.23[c]	+0.74[b]	-0.29[c]
Small intestine contents (2nd half)	+1.16[b]	-0.20[c]	+0.88[b]	-0.30[c]
Small intestine wall (2nd half)	+0.90[b]	-0.12[cd]	+0.17[c]	-0.33[d]
Cecal contents	+2.59[b]	-0.13[c]	+1.95[d]	-0.24[c]
Cecal wall	+0.82[b]	-0.14[c]	+1.13[b]	-0.23[c]
Colon contents	+3.35[b]	-0.07[c]	+2.50[d]	-0.20[c]
Colon wall	+0.66[b]	-0.13[c]	+0.77[b]	-0.23[c]
Overall	+1.21[b]	-0.17[c]	+0.88[b]	-0.26[c]

[a]Co=control, Cu=250 ppm copper, CTC=100 ppm chlortetracycline, Cu+CTC=250 ppm copper + 100 ppm chlortetracycline.

[b,c,d]Means within microbe source, among treatments with different superscripts are significantly different ($P<.05$), Std. Error = 0.27.

In the large intestine of the pig, there is considerable bacterial deamination of amino acids (Michel, 1966; Fauconneau and Michel, 1970). Urea also diffuses from the body tissues into the intestinal lumen and is a readily available source of nitrogen for those bacteria having urease activity (Ratcliffe, 1985). Microflora have an important role in the recycling of endogenous nitrogen secreted into the gut as urea (Richards, 1972), but hydrolysis of endogenous urea alters turnover rate of intestinal cells and ammonia production in the animal has a deleterious effect Visek et al. (1972). Alvares et al. (1964a, 1964b) and Harbers et al. (1963) with chicks, and Visek et al. (1959) with rats, showed that 100 ppm of chlortetracycline or 3 ppm copper inhibit urease activity in vitro. Chachulowa (1964) observed a reduction in cecal ammonia production in pigs fed diets containing copper (as copper sulfate) or chlortetracyline.

D. Volatile and Non-Volatile Fatty Acid Production

1. Comparison of the relative quantities of volatile and non-volatile fatty acids produced in the gastrointestinal tract.

a. Volatile fatty acids

The relative proportions of VFA's found in fermentation media are shown in Tables 23A, 23B, 23C and 23D. Of the organic acids measured, acetic acid and formic acid contributed the greatest proportion of the total, followed by lower amounts of valeric, butyric and caproic acid. Propionic, isobutyric and isovaleric acid were found primarily in the cecum and colon sites. Isocaproic acid was not found in any of the incubated media samples. For microbes incubated in Cu and Cu+CTC media, acetic acid was the primary VFA produced with smaller quantitites of butyric, isovaleric, valeric and

Table 23A. Comparison of Volatile Fatty Acid (Formic and Acetic Acid) Production (μmoles/ml) Between Microbe Sources and Treatments at 24 Hours of Incubation.

Volatile Fatty Acid	Formic Acid				Acetic Acid			
Treatment[a]	Co	Cu	CTC	Cu + CTC	Co	Cu	CTC	Cu + CTC
Microbe Source:								
Stomach contents	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	6.40 ± .67	10.2 ± 4.3	4.25 ± 1.5
Stomach wall	16.7 ± 3.4	0.0 ± 0.0	10.9 ± 1.7	0.0 ± 0.0	25.5 ± 5.0	7.41 ± 1.1	7.90 ± 1.0	3.75 ± .79
Sm. int. contents (1)	15.9 ± 3.7	0.0 ± 0.0	10.7 ± 1.1	0.0 ± 0.0	19.5 ± 2.7	6.43 ± .22	11.9 ± 1.2	4.60 ± 2.1
Sm. int. wall (1)	13.2 ± 3.7	0.0 ± 0.0	13.8 ± 2.1	0.0 ± 0.0	11.5 ± 3.3	6.32 ± .54	10.4 ± 1.1	4.15 ± 1.6
Sm. int. contents (2)	14.3 ± 3.2	0.0 ± 0.0	14.2 ± 2.7	0.0 ± 0.0	11.9 ± 3.5	5.50 ± .91	10.1 ± 2.1	4.55 ± 1.7
Sm. int. wall (2)	14.5 ± 3.7	0.0 ± 0.0	10.9 ± 3.2	0.0 ± 0.0	10.2 ± 2.5	3.49 ± .96	8.63 ± .90	4.13 ± 1.5
Cecal contents	6.39 ± .67	0.0 ± 0.0	13.5 ± 4.2	0.0 ± 0.0	16.2 ± 3.1	6.25 ± .79	15.7 ± 1.9	4.93 ± 2.0
Cecal wall	13.1 ± 4.1	0.0 ± 0.0	12.5 ± 2.2	0.0 ± 0.0	12.4 ± 2.0	9.15 ± 3.2	11.2 ± .87	4.80 ± 2.0
Cecal contents	8.68 ± 1.6	0.0 ± 0.0	10.9 ± .70	0.0 ± 0.0	25.9 ± 3.2	7.48 ± .78	14.9 ± .41	8.08 ± .58
Colon wall	12.5 ± 3.3	0.0 ± 0.0	13.3 ± 2.9	0.0 ± 0.0	12.5 ± 2.2	5.99 ± 1.1	11.5 ± 2.0	4.80 ± .94

[a]Co=control, Cu=250 ppm copper, CTC=100 ppm chlortetracycline, Cu + CTC=250 ppm copper + 100 ppm chlortetracycline.

Table 23B. Comparison of Volatile Fatty Acid (Propionic and Isobutyric Acid) Production (μmoles/ml) Between Microbe Source and Treatment at 24 Hours of Incubation.

Volatile Fatty Acid	Propionic				Isobutyric			
Treatment[a]	Co	Cu	CTC	Cu + CTC	Co	Cu	CTC	Cu + CTC
Microbe Source:								
Stomach contents	0.0 ± 0.0	0.0 ± 0.0	0.53 ± .10	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Stomach wall	0.0 ± 0.0	0.0 ± 0.0	0.15 ± .06	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Sm. int. contents (1)	0.0 ± 0.0	0.0 ± 0.0	0.73 ± .13	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Sm. int. wall (1)	0.0 ± 0.0	0.0 ± 0.0	0.28 ± .05	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Sm. int. contents (2)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Sm. int. wall (2)	0.0 ± 0.0	0.0 ± 0.0	0.63 ± .05	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Cecal contents	9.80 ± 1.6	0.0 ± 0.0	8.35 ± 2.8	0.0 ± 0.0	1.00 ± .11	0.06 ± .01	0.92 ± .21	0.0 ± 0.0
Cecal wall	3.80 ± .14	0.0 ± 0.0	3.73 ± 1.4	0.0 ± 0.0	0.19 ± .10	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Cecal contents	10.1 ± 3.3	0.7 ± .14	7.63 ± 1.0	0.0 ± 0.0	1.51 ± .36	0.06 ± .02	1.05 ± .19	0.04 ± .02
Colon wall	4.45 ± 1.1	0.0 ± 0.0	3.70 ± .22	0.0 ± 0.0	0.05 ± .03	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

[a]Co=control, Cu=250 ppm copper, CTC=100 ppm chlortetracycline, Cu + CTC=250 ppm copper + 100 ppm chlortetracycline.

Table 23C. Comparison of Volatile Fatty Acid (Butyric and Isovaleric Acid) Production (μmoles/ml) Between Microbe Source and Treatment at 24 Hours of Incubation.

Volatile Fatty Acid	Butyric				Isovaleric			
Treatment[a]	Co	Cu	CTC	Cu + CTC	Co	Cu	CTC	Cu + CTC
Microbe Source:								
Stomach contents	0.02 ± .01	0.03 ± .01	0.13 ± .15	0.02 ± .01	0.0 ± 0.0	0.09 ± .01	0.0 ± 0.0	0.0 ± .01
Stomach wall	0.03 ± .01	0.05 ± .03	0.08 ± .04	0.02 ± .01	0.0 ± 0.0	0.07 ± .02	0.0 ± 0.0	0.05 ± .01
Sm. int. contents (1)	0.18 ± .17	0.02 ± .01	0.03 ± .01	0.02 ± .01	0.0 ± 0.0	0.05 ± .01	0.0 ± 0.0	0.02 ± .01
Sm. int. wall (1)	0.02 ± .01	0.02 ± .01	0.07 ± .06	0.02 ± .01	0.0 ± 0.0	0.04 ± .02	0.0 ± 0.0	0.03 ± .01
Sm. int. contents (2)	0.41 ± .35	0.01 ± .00	0.07 ± .05	0.02 ± .01	0.0 ± 0.0	0.03 ± .01	0.0 ± 0.0	0.04 ± .01
Sm. int. wall (2)	0.22 ± .15	0.02 ± .01	0.02 ± .01	0.02 ± .01	0.0 ± 0.0	0.06 ± .01	0.0 ± 0.0	0.04 ± .02
Cecal contents	2.39 ± .15	0.15 ± .02	3.54 ± 1.0	0.08 ± .12	1.39 ± .19	0.05 ± .01	2.14 ± .22	0.05 ± .01
Cecal wall	0.45 ± .19	0.04 ± .01	0.23 ± .17	0.02 ± .01	0.15 ± .02	0.08 ± .04	1.91 ± .14	0.04 ± .01
Cecal contents	4.27 ± 1.2	0.16 ± .03	2.91 ± 1.0	0.13 ± .08	1.79 ± .15	0.09 ± .01	1.96 ± .25	0.03 ± .01
Colon wall	0.82 ± .34	0.03 ± .00	0.28 ± .11	0.02 ± .02	0.16 ± .04	0.07 ± .01	0.27 ± .13	0.05 ± .01

[a]Co=control, Cu=250 ppm copper, CTC=100 ppm chlortetracycline, Cu + CTC=250 ppm copper + 100 ppm chlortetracycline.

Table 230. Comparison of Volatile Fatty Acid (Valeric and Caproic Acid) Production (μmoles/ml) Between Microbe Source and Treatment at 24 Hours of Incubation.

Volatile Fatty Acid	Valeric				Caproic			
Treatment[a]	Co	Cu	CTC	Cu + CTC	Co	Cu	CTC	Cu + CTC
Microbe Source:								
Stomach contents	1.40 ± .27	1.05 ± .14	1.98 ± .69	0.84 ± .23	0.10 ± .03	0.19 ± .01	0.18 ± .04	0.16 ± .03
Stomach wall	1.15 ± .15	0.74 ± .21	0.99 ± .07	0.80 ± .41	0.34 ± .08	0.25 ± .05	0.23 ± .10	0.16 ± .01
Sm. int. contents (1)	1.81 ± .66	0.89 ± .15	0.96 ± .23	0.75 ± .28	0.28 ± .10	0.17 ± .03	0.13 ± .01	0.11 ± .02
Sm. int. wall (1)	0.86 ± .12	0.92 ± .31	0.85 ± .07	0.65 ± .17	0.21 ± .08	0.15 ± .03	0.21 ± .09	0.13 ± .03
Sm. int. contents (2)	1.07 ± .51	0.56 ± .14	0.86 ± .38	0.62 ± .36	0.13 ± .02	0.12 ± .03	0.11 ± .02	0.12 ± .05
Sm. int. wall (2)	0.88 ± .26	0.65 ± .26	0.37 ± .17	0.60 ± .30	0.19 ± .08	0.16 ± .02	0.21 ± .07	0.13 ± .02
Cecal contents	2.50 ± .31	0.73 ± .09	2.19 ± .51	0.74 ± .54	0.11 ± .02	0.16 ± .02	0.18 ± .01	0.13 ± .01
Cecal wall	0.97 ± .37	0.90 ± .40	1.00 ± .15	0.64 ± .26	0.10 ± .03	0.22 ± .10	0.11 ± .01	0.14 ± .03
Cecal contents	3.79 ± .64	0.91 ± .23	2.25 ± 1.0	0.95 ± .08	0.15 ± .07	0.20 ± .06	0.21 ± .05	0.20 ± .02
Colon wall	0.89 ± .35	0.69 ± .11	1.11 ± .36	0.56 ± .17	0.10 ± .03	0.18 ± .02	0.10 ± .01	0.11 ± .01

[a]Co=control, Cu=250 ppm copper, CTC=100 ppm chlortetracycline, Cu + CTC=250 ppm copper + 100 ppm chlortetracycline.

caproic acid. No formic or propionic acid were produced, and isobutyric acid was produced only in small quantities in the cecum and colon contents.

b. Non-volatile fatty acids

For the non-volatile fatty acids, lactic, oxaloacetic and succinic acids were the only acids produced (Tables 24A and 24B). No pyruvic, oxalic, methyl malonic, malonic or fumaric acids were detected. Of these organic acids, lactic acid was the most predominant and high concentrations were found in media containing microbes from the stomach and small intestine. Oxalacetic and succinic acids were produced in greater amounts in the stomach and small intestine compared to concentrations of the acids in the lower gastrointestinal tract.

Friend et al. (1963a,b) found that the total organic acids in the stomach amounted to approximately 30-92 meq/l in early weaned pigs. Of this, lactic acid comprised over 90 percent of the molar concentration when pigs were fed a whey diet, but this proportion declined to 50 percent when pigs were fed primarily cereal diets. Propionic acid comprised approximately half of the remaining organic acids with lesser concentrations of butyric and valeric acids. These authors also noted that in older pigs on cereal diets, formic acid comprised 3.5-4.5 percent of organic acids, which was greater than the amount of butyric acid production pigs fed a cereal diet and greater than the amount of propionic acid produced in pigs fed a whey diet. However, when diets contain more digestive or fermentable carbohydrate, there is considerably more fermentation in the stomach and less in the large intestine (Mason and Just, 1976).

Table 24A. Comparison of Non-Volatile Fatty Acid (Lactic and Oxaloacetic Acid) Production (μmoles/ml) Between Microbe Source and Treatment at 24 Hours of Incubation.

Non-Volatile Fatty Acid	Lactic Acid				Oxaloacetic Acid			
Treatment[a]	Co	Cu	CTC	Cu + CTC	Co	Cu	CTC	Cu + CTC
Microbe Source:								
Stomach contents	12.9 ± 2.0	0.19 ± .04	13.3 ± 2.3	0.22 ± .06	0.53 ± .18	1.06 ± .18	2.25 ± .38	0.75 ± .10
Stomach wall	0.76 ± .19	0.22 ± .07	0.47 ± .12	0.21 ± .13	2.77 ± .42	1.18 ± .31	2.86 ± .46	0.87 ± .26
Sm. int. contents (1)	16.1 ± 1.9	0.20 ± .07	18.5 ± 2.1	0.12 ± .03	2.76 ± .64	1.33 ± .26	0.41 ± .08	2.20 ± .77
Sm. int. wall (1)	0.21 ± .10	0.18 ± .08	0.27 ± .11	0.15 ± .08	2.43 ± .88	0.98 ± .15	3.17 ± .29	0.69 ± .27
Sm. int. contents (2)	13.8 ± 1.3	0.20 ± .07	18.2 ± 1.2	0.28 ± .05	0.44 ± .12	0.97 ± .18	2.49 ± .11	1.28 ± .23
Sm. int. wall (2)	0.24 ± .08	0.15 ± .05	0.40 ± .12	0.22 ± .03	0.44 ± .19	0.96 ± .05	2.61 ± .21	0.69 ± .29
Cecal contents	0.20 ± .03	0.21 ± .04	0.38 ± .04	0.31 ± .01	0.20 ± .02	0.96 ± .02	0.21 ± .04	0.64 ± .20
Cecal wall	0.90 ± .04	0.22 ± .05	0.67 ± .09	0.12 ± .05	0.30 ± .11	1.44 ± .24	1.23 ± .02	0.78 ± .28
Cecal contents	0.29 ± .08	0.21 ± .05	0.34 ± .10	0.23 ± .06	0.21 ± .08	0.99 ± .13	0.23 ± .11	1.28 ± .13
Colon wall	0.40 ± .11	0.29 ± .09	0.67 ± .22	0.15 ± .09	0.41 ± .17	0.99 ± .10	0.28 ± .08	0.71 ± .19

[a]Co=control, Cu=250 ppm copper, CTC=100 ppm chlortetracycline, Cu + CTC=250 ppm copper + 100 ppm chlortetracycline.

Table 24B. Comparison of Non-Volatile Fatty Acid (Succinic Acid) Production (μmoles/ml) Between Microbe Sources and Treatments at 24 Hours Incubation.

Non-Volatile Fatty Acid	Succinic Acid			
Treatment[a]	Co	Cu	CTC	Cu+CTC
Microbe Source:				
Stomach contents	1.01 ± .18	0.23 ± .06	0.52 ± .08	0.16 ± .06
Stomach wall	2.12 ± .03	0.26 ± .08	1.05 ± .25	0.25 ± .10
Sm. int. contents (1)	1.41 ± .22	0.24 ± .07	1.24 ± .16	0.24 ± .12
Sm. int. wall (1)	1.15 ± .13	0.23 ± .10	1.26 ± .20	0.23 ± .15
Sm. int. contents (2)	1.25 ± .13	0.25 ± .07	1.27 ± .23	0.22 ± .13
Sm. int. wall (2)	0.69 ± .10	0.20 ± .06	0.90 ± .25	0.18 ± .09
Cecal contents	0.11 ± .02	0.21 ± .06	0.09 ± .03	0.16 ± .07
Cecal wall	0.08 ± .03	0.22 ± .05	0.07 ± .04	0.15 ± .04
Colon contents	0.24 ± .10	0.20 ± .05	0.08 ± .02	0.35 ± .20
Colon wall	0.18 ± .09	0.21 ± .04	0.04 ± .03	0.20 ± .15

[a]Co=control, Cu=250 ppm copper, CTC=100 ppm chlortetracycline, Cu + CTC=250 ppm copper + 100 ppm chlortetracycline.

In the small intestine, Friend et al. (1963a,b) reported that the total organic acid concentration was lower in the small intestine than in any other part of the alimentary tract. These authors consistently found formic acid in the small intestine which was not present in the large intestine, and only present in the stomach of older pigs. Considerable quantitites of lactic acid were found in the small intestine but lactic acid contributed a smaller proportion of the total organic acids than in the stomach. Of the remaining organic acids, acetic acid was found along with some propionic acid.

Friend et al. (1963a,b) found levels of total organic acids to be up to 300 meq/l in the cecum and colon which is the highest of any site of the gastrointestinal tract. Of the total amount of organic acids, acetic acid was present in the greatest quantity, followed by propionic acid, butyric acid, lactic acid and valeric acid. These authors observed little difference in levels or proportions of organic acids between the cecum and colon. Argenzio and Southworth (1974) observed similar levels (150-250 meq/l) of total VFA's. Clemens et al. (1975) observed low VFA production in stomach and small intestine but a dramatic increase in VFA production in cecum and colon. Argenzio and Southworth (1974) measured VFA transport across the intestinal mucosa and observed a high capacity for VFA transport in the cecum and colon but relatively low transport of VFA's in other types of gut mucosa. However, Barcroft et al. (1944) and Elsden et al. (1946) showed that VFA's are present in the stomach and small intestine, as well as the large intestine, and these VFA's are absorbed.

Imoto and Namioka (1978) observed that 31 percent of the acetate was absorbed from the large intestine of the pig when a low

carbohydrate diet was fed while 43 percent was absorbed when a high carbohydrate diet was fed. Only 1 to 3 percent of propionic and butyric acid was absorbed. When Imoto and Namioka (1983) added acetate to diets of swine at 0, 5 and 10 percent of metabolizable energy intake, the efficiency of acetate utilization was calculated to be between 56 and 59 percent. Since acetate was the predominant VFA measured in this study and since its energetic efficiency is relatively high, it could be a contributing factor to any improvement in energy utilization from feeding a high copper diet to growing swine.

2. Total volatile fatty acid production

Total volatile fatty acid (VFA) production between treatments, within microbe source was compared by determining the sum of the eight volatile fatty acids detected and measured after 24 hours of incubation in fermentation media inoculated with microbes from each of ten microbe sources. These microbe sources include the stomach contents (SC), stomach wall (SW), small intestine contents (1st half) (SI_1C), small intestine wall (1st half) (SI_1W), small intestine contents (2nd half) (SI_1C), small intestine wall (2nd half) (SI_2W), cecal contents (CeC), cecal wall (CeW), colon contents (CoC) and colon wall (CoW).

In general, the control (Co) and 100 ppm chlortetracycline (CTC) treatments resulted in a greater production of VFA's ($P<.05$) compared to that of microbes incubated in the 250 ppm copper (Cu) and the copper-chlortetracycline (Cu+CTC) treated media (Table 25). This relationship was evident for microbes obtained from SI_1W, SI_2C, SI_2W, CeW and CoW. For SW, SI_1C and CoC microbe sources, total VFA production was reduced for the CTC treatment compared to that for the

Table 25. Comparison of Treatment Means for Total Volatile Fatty Acid Production[a] (μmole/ml of media) After a 24 Hour Incubation Period, Within Microbe Source.

	Treatment[b]				
Microbe Source	Co	Cu	CTC	Cu+CTC	Overall $\bar{x}$
Stomach contents	1.52^{c}	7.74^{cd}	13.0^{d}	5.32^{c}	6.89
Stomach wall	43.7^{c}	8.50^{d}	20.2^{e}	4.77^{d}	19.3
Small int. contents (1st half)	38.3^{c}	7.54^{d}	24.5^{e}	5.48^{d}	18.9
Small int. wall (1st half)	25.8^{c}	7.44^{d}	25.0^{c}	4.97^{d}	15.8
Small int. contents (2nd half)	27.8^{c}	6.22^{d}	26.0^{c}	5.33^{d}	16.3
Small int. wall (2nd half)	25.6^{c}	4.35^{d}	21.0^{c}	4.90^{d}	14.0
Cecal contents	39.7^{c}	7.39^{d}	46.5^{e}	5.92^{d}	24.9
Cecal wall	31.1^{c}	10.4^{d}	30.6^{c}	5.64^{d}	19.4
Colon contents	53.3^{c}	9.59^{d}	41.7^{e}	9.44^{d}	28.5
Colon wall	31.4^{c}	6.96^{d}	30.2^{c}	5.54^{d}	18.5
Overall $\bar{x}$	31.8^{c}	7.61^{d}	27.9^{c}	5.73^{d}	

[a]Total volatile fatty acid production = the sum of of the quantity (μmoles) of formic, acetic, propionic, isobutyric, butyric, isovaleric, valeric and caproic acids, per ml of media containing microbes from each of 10 microbe sources.

[b]Co=control, Cu=250 ppm copper, CTC=100 ppm chlortetracycline, Cu+CTC=250 ppm copper + 100 ppm chlortetracycline.

[c,d,e]Means within microbe source, among treatments with different superscripts are significantly different ($P<.05$).

Std. Error = 14.2

Co treatment (P<.05) and increased for microbes of the SC and CeC microbes incubated in CTC treated media compared to the control treatment (P<.05). However, for all microbe sources, Cu and Cu+CTC treated microbes produced less VFA's compared to Co and CTC treated microbes (P<.05). This apparent reduction in VFA production was confounded with a severe depression in microbial growth (Table 19 and Figures 1-5) when microbes were incubated for 24 hours in the Cu and Cu+CTC treated media.

When microbe sources were compared within treatments, no differences were noted between microbes obtained from contents and wall at the same intestinal site, between microbes from gut contents, or gut wall at each intestinal site for the Cu and Cu+CTC treatments. For the Co and CTC treatments, total VFA production was greater for microbes of contents from the cecum and colon (and SI_1C of the control treatment) than for microbes of the wall at these intestinal sites (P<.05). However, for the Co and CTC treatments, more VFA's were produced by the SW microbes than by SC microbes (P<.05).

When microbes from gut contents of each intestinal site were compared, within treatments, the lowest amount of total VFA production occurred from SC microbes compared to other microbe sources exposed to the Co and CTC media (P<.05). For the Co treatment, CoC microbes produced the highest amount of VFA's (P<.05) compared to cecum and small intestine content sources. These results are consistent with work reported by Friend et al. (1963a,b). This same relationship was also true for CTC treated microbes except that total VFA production from CeC microbes was as great as that for CoC microbes (P<.05).

For microbes associated with the gut wall at each intestinal site, were compared within treatment, total VFA production was the greatest for SW microbes ($P<.05$), compared to that by CeW and CoW microbes, which produced more total VFA's than SI_1W and SI_2W microbes ($P<.05$) within the Co and CTC treatments. In general, microbes of the stomach are relatively low in number and produce a limited amount of VFA's (Friend et al., 1963a; 1963b) because the highly acidic environment is prohibitive to the establishment of a large bacterial population and as a result the bacterial species that are capable of tolerating this environment are highly specific (Clarke, 1977b). Species of Lactobacillus, Bifidobacterium and Streptococcus are the primary microflora of the stomach (Smith and Jones, 1963) and lactic acid is the primary organic acid produced (Friend et al., 1963a; 1963b). Perhaps the relatively high amount of fermentable carbohydrate present in the growth media and the higher pH of the fermentation media (6.7) allowed more microbial fermentation and VFA production than typically observed in vivo.

3. Total non-volatile fatty acid production

The sum of the quantity of non-volatile acids (lactic, oxaloacetic and succinic acids) per ml of media are presented in Table 26. In general, total non-volatile fatty acid production (NVFA) was greater for microbes incubated in Co and CTC treated media compared to that for Cu and Cu+CTC treated microbes ($P<.05$). As for the total VFA production, these differences in NVFA production were confounded by the dramatic reduction in microbial growth when microbes were incubated in Cu and Cu+CTC media (Table 19 and Figures 1-5). No differences in NVFA production were noted between treatments within CeC, CeW, CoC and CoW

Table 26. Comparison of Treatment Means for Total Non-Volatile Fatty Acid Production[a] (μmoles/ml of media) After a 24 Hour Incubation Period, Within Microbe Source.

	Treatment[b]				
Microbe Source	Co	Cu	CTC	Cu+CTC	Overall $\bar{x}$
Stomach contents	14.6[c]	1.48[d]	16.0[e]	1.13[d]	8.30
Stomach wall	5.66[c]	1.65[d]	4.37[e]	1.33[d]	3.25
Small int. contents (1st half)	19.0[c]	1.75[d]	20.1[c]	2.56[d]	10.9
Small int. wall (1st half)	3.78[c]	1.39[d]	4.70[c]	1.01[d]	2.72
Small int. contents (2nd half)	15.4[c]	1.41[d]	21.9[e]	1.78[d]	10.1
Small int. wall (2nd half)	1.37[c]	1.30[c]	3.91[d]	1.09[c]	1.91
Cecal contents	0.51[c]	1.38[c]	0.68[c]	1.11[c]	0.91
Cecal wall	1.26[c]	1.88[c]	1.96[c]	1.04[c]	1.53
Colon contents	0.76[c]	1.40[c]	0.64[c]	1.86[c]	1.16
Colon wall	1.00[c]	1.48[c]	1.00[c]	1.06[c]	1.13
Overall $\bar{x}$	6.33[c]	1.51[d]	7.53[c]	1.40[d]	

[a]Total non-volatile fatty acid production = the sum of the quanity (μmoles) of lactic, oxaloacetic and succinic acids per ml of media, containing microbes from each of 10 microbe sources.

[b]Co=control, Cu=250 ppm copper, CTC=100 ppm chlortetracycline, Cu+CTC=250 ppm copper + 100 ppm chlortetracycline.

[c,d,e]Means within microbe source, among treatments with different superscripts are significantly different (P<.05).

Std. Error = 0.55

microbe sources, which is contrary to that observed for VFA production (Table 25). Microbes from SC and SI_2C produced more NVFA's when incubated in CTC media than that produced from those microbes in the Co media ($P<.05$). However, the inverse was true for Co and CTC treatments within the SW microbe source ($P<.05$).

When microbes of gut wall and contents were compared for each intestinal site, microbes from SC, SI_1C and SI_2C produced more NVFAs than their respective SW, SI_1W and SI_2W microbe sources within the Co and CTC treatments ($P<.05$). Only the microbes of CeW produced more NVFA's than microbes of the CeC within the CTC treatment ($P<.05$). No differences between gut wall and gut content microbe sources were noted within either the Cu or Cu+CTC treatments.

When total NVFA production of microbes from gut contents of each intestinal site were compared, within treatment, microbes of SC, SI_1C and SI_2C produced more NVFA's than CeC and CoC microbes within the Co and CTC treatments ($P<.05$). This is likely due to the greater lactic acid production typically observed in the upper digestive tract compared to more total VFA production in the lower digestive tract. No differences in total NVFA production were noted between gut content sources within the Cu treatment but for the Cu+CTC treatment, more total NVFA's were produced from SI_1C microbes than for SC and CeC microbes ($P<.05$).

As for gut contents, microbes of SW, SI_1W and SI_2W produced more total NVFA's than for CeW and CoW sources within the CTC treatment ($P<.05$). Microbes from SW produced more total NVFA's than SI_1W microbes which produced more total NVFA's than SI_2W, CeW and CoW microbes within the Co treatment ($P<.05$). No differences for NVFA

production were noted between gut wall sources within the Cu and Cu+CTC treatments.

In general, more VFA's are produced in the lower digestive tract than in the upper digestive tract and the reverse is true for NVFA production. Volatile fatty acid and NVFA production were reduced by adding Cu and Cu+CTC to fermentation media, but this reduction in VFA production was confounded with lower microbial growth and mass which greatly influence the quantity of substrate fermented. Copper may inhibit organic acid production and thus spare carbohydrate as observed for the antibiotics Virginiamycin and Spiramycin (Vervaeke et al., 1979) but further studies are required to test this hypothesis.

SUMMARY

A. Feeding a diet containing 250 ppm supplemental copper increased growth rate of conventional pigs but decreased growth rate of germ-free pigs compared to pigs receiving a control diet in each environment.

B. In the absence of microflora, the effects of feeding a high copper diet on copper, iron and zinc metabolism in the pig were greatly exaggerated compared to that observed for pigs in the conventional environment. In other words, high copper feeding increased copper concentrations in liver and plasma, increased plasma ceruloplasmin, decreased liver and plasma iron concentrations, and hematocrit and erythrocyte count were reduced more dramatically for germ-free pigs fed the high copper diet compared to conventional pigs fed the same diet. Furthermore, these effects in the germ-free pigs receiving the high copper diet, were of the magnitude typically observed when pigs develop a moderate copper toxicity and may explain the apparent reduction in growth rate of these pigs compared to germ-free pigs receiving the control diet. These results suggest that gut microflora have a significant impact on absorption and retention of copper, iron and zinc in the pig.

C. Germ-free pigs had a lower percentage of leukocytes and mature (segmented) neutrophils than conventional pigs due to a lack of antigenic stimulation of pigs reared in the germ-free environment. Feeding the high copper diet increased the percentage of immature (band) neutrophils in germ-free pigs but decreased the percentage of these cells in conventional pigs fed the high copper diet.

Germ-free pigs had a higher percentage of lymphocytes than conventional pigs. The percentage of monocytes was dramatically increased by feeding the high copper diet to germ-free pigs but was reduced in conventional pigs fed the same diet.

D. Peripheral plasma ammonia concentrations were reduced, and portal plasma ammonia concentrations tended to be reduced in pigs fed the high copper diet. Germ-free pigs had lower levels of ammonia than conventional animals due to a lack of bacterial urease. Similarly, germ-free pigs had higher concentrations of urea, and lower concentrations of ammonia in cecal contents of germ-free pigs compared to cecal contents of conventional pigs. However, no effect of copper was noted on urea or ammonia-nitrogen concentrations.

E. Germ-free pigs had a smaller thyroid, liver, stomach, small intestine and large intestine but a larger spleen, lungs, heart and gall bladder than conventional pigs. No differences were noted for thymus, adrenals, kidneys or pancreas weights between germ-free and conventionally reared pigs. Feeding the high copper diet reduced thymus weight.

F. Total thickness of the wall of the duodenum and jejunum was reduced for pigs in the germ-free environment compared to conventional pigs. Feeding the high copper diet reduced total intestinal thickness of the jejunum, tended to reduce total thickness of the duodenum, and tended to reduce thickness of the ileum of germ-free pigs but increased total thickness of the ileum of conventional pigs.

G. When microflora from gut contents and gut wall from five gut sections were incubated in an anaerobic growth media, the presence of 250 ppm copper dramatically reduced microbial growth and, for microbes obtained from the lower digestive tract, inhibited growth, compared to microbes from these same sources grown in the control or 100 ppm chlortetracycline medium. After 16 hours of incubation, microbes appeared to die when incubated in media containing 250 ppm copper which apparently was too toxic to support microbial growth. These results verify that copper, at the 250 ppm level, does affect microbial growth in vitro, but in vivo, the bactericidal effectiveness of copper may be reduced by other factors present in the gut which were not present in the growth media. Furthermore, a level of 250 ppm copper appears to reduce or inhibit microbial growth along all gastrointestinal sites.

H. A small reduction in pH of the growth media after 24 hours of incubation (compared to initial pH) of microbes from all microbe sources, within the control and 100 ppm chlortetracycline treated microbes, may have been due to acid production during carbohydrate fermentation. When 250 ppm copper and the copper-chlortetracycline combination were present in growth media, pH increased compared to initial pH.

I. Ammonia-nitrogen production of microbes was greatest in the control treated media, tended to be less when microbes were incubated in 100 ppm chlortetracycline media, but both the control and chlortetracycline treated microbes produced significantly higher amounts of ammonia nitrogen than microbes treated with 250

ppm copper or the copper-chlortetracycline combination. Most ammonia-nitrogen production occurred in the lower sections of the digestive tract. Differences between treatments in regard to ammonia production were confounded with the mass of microbes present at 24 hours of incubation but a negative net ammonia change was noted for the media containing copper which may indicate that bacterial urease activity was inhibited.

J. Total volatile and total non-volatile fatty acid production was greatest for microbes incubated for 24 hours in media containing the control and 100 ppm chlortetracycline treatments. When 250 ppm copper was present in media, volatile and non-volatile fatty acid production was reduced but this reduction was confounded by a reduction in microbial growth and less microbial mass. Microbes incubated in media containing 250 ppm copper or the copper-chlortetracycline combination treatments, produced no formic or propionic acid which were produced by microbes incubated in control and chlortetracycline media. Of the volatile fatty acids measured, acetic and formic acid were produced in the greatest quantities particularly by the cecum and colon microbes. Lactic acid was the predominant non-volatile fatty acid produced and was produced in the greatest quantities in the stomach and small intestine.

APPENDIX A

APPENDIX A

A series of five preliminary trials were conducted to determine the effect of autoclaving a typical corn-soybean meal-20% dried whey starter diet on growth performance and nutrient balance. Effectiveness of sterilization was also evaluated to determine the minimum time needed for complete sterilization.

Sterilization of solid diets by steam results in loss of nutritive value of proteins through its effects on lysine, methionine and cysteine (Rice and Beuk, 1953), loss of certain B vitamins and vitamins A and E (Zimmerman and Wostmann, 1963), formation of potential antagonists to important nutrients or metabolites (Reyniers et al., 1946) and the formation of potentially antigenic products. Theoretically, some loss could also occur by leaching, but a comparison of iron and copper contents of diets before and after autoclaving showed no demonstrable loss by leaching (Reddy et al., 1965a, 1965b). Nutrient losses resulting from steam sterilization of diets can be minimized by careful control of temperature and sterilization time. Furthermore, nutrient losses from sterilization can be estimated and compensated for by supplementation of the original diet.

The results of these five studies presented in this appendix helped provide the basis for formulating diets for autoclaving and use in the germ-free experiment contained in this dissertation.

I. EFFECTIVENESS OF STERILIZATION OF CORN-SOYBEAN MEAL-DRIED WHEY STARTER DIETS BY AUTOCLAVING AND THE EFFECT OF AUTOCLAVING DIETS, WITH OR WITHOUT VITAMIN SUPPLEMENTATION, ON GROWTH PERFORMANCE OF WEANLING PIGS.

A. Objective

The primary objectives of this trial were to 1) determine if processing a practical swine starter diet by autoclaving affects nutrient composition of the diet as measured by growth performance of weanling pigs, 2) determine if supplementing these autoclaved diets with a vitamin-trace mineral premix improves growth performance, 3) determine the effectiveness of sterilization by two methods and 4) determine the minimum time needed to provide adequate sterilization.

B. Methodology

Thirty-two crossbred weanling pigs averaging 7.5 kg in initial body weight were assigned to one of four experimental diets for a 3-week growth performance trial. Experimental diets consisted of a typical corn-soybean meal-20% dried whey starter diet formulated as shown in Table 27. Diet 1 was a non-autoclaved conventional diet which served as a control for comparison of growth performance of pigs fed autoclaved diets 2, 3 and 4. Diets 1 and 2 were identical in nutrient composition and were formulated to meet or exceed NRC (1979) recommendations for starter pigs. Diet 3 contained 150% of the vitamin-trace mineral premix and vitamin E-selenium premix while diet 4 contained 200% of each of these two ingredients compared to the levels added in diets 1 and 2. Refer to Table 28 for the amount of vitamins and trace minerals supplied per kg of diet. Pigs were weighed weekly and feed consumption was measured. Average daily gain was analyzed statistically using a one-way analysis of variance (Gill, 1978).

In order to determine the effectiveness of the autoclave procedure on sterilization of these diets, two types of containers were evaluated as well as the minimum length of time necessary for complete sterilization so that unnecessary nutrient loss could be avoided.

Table 27. Composition and Calculated Nutrient Analysis of Diets Used in Trial 1.

Ingredients (%)	Int. Ref. No.	Experimental Diet[a] 1	2	3	4
Ground shelled corn	4-02-935	51.61	51.61	51.11	50.61
Soybean meal, 44%	5-04-604	25.00	25.00	25.00	25.00
Dried whey	4-01-182	20.00	20.00	20.00	20.00
Mono-dicalcium phosphate	6-01-080	0.75	0.75	0.75	0.75
Calcium carbonate	6-01-632	1.10	1.10	1.10	1.10
Salt	6-04-152	0.35	0.35	0.35	0.35
Vitamin-TM premix		0.50	0.50	0.75	1.00
Vitamin E-Se premix		0.50	0.50	0.75	1.00
L-lysine HCl	5-08-022	0.19	0.19	0.19	0.19
		100.00	100.00	100.00	100.00
Calculated Nutrient Analysis					
ME, kcal/kg		3127	3127	3110	3093
Crude protein, %		18.26	18.26	18.22	18.17
Lysine, %		1.20	1.20	1.20	1.19
Calcium, %		0.85	0.85	0.85	0.85
Phosphorus, %		0.60	0.60	0.60	0.60

[a]Diet 1 (non-autoclaved control).

Diet 2 (autoclaved control).

Diet 3 (autoclaved control + 150% vitamin-trace mineral premix).

Diet 4 (autoclaved control + 200% vitamin-trace mineral premix).

Table 28. Amount of Vitamin and Trace Minerals Supplied Per Kg of Diet in Trial 1.

	Experimental Diet		
	0.50% level	0.75% level	1.00% level
Nutrient	1 and 2	3	4
Vitamin A, IU	3300	4950	6600
Vitamin D, IU	660	990	1320
Vitamin E, IU	16.5	24.8	33.0
Menadione sodium bisulfite, mg	2.20	3.30	4.40
Riboflavin, mg	3.30	4.95	6.60
Niacin, mg	17.6	26.4	35.2
D-pantothenic acid, mg	13.2	19.8	26.4
Choline, mg	110	165	220
Vitamin B_{12}, mg	19.8	29.7	39.6
Zinc, mg	75.0	113	150
Iron, mg	60.0	90.0	120
Manganese, mg	37.0	55.5	74.0
Copper, mg	10.0	15.0	20.0
Iodine, mg	0.20	0.30	0.40
Selenium, mg	.10	.15	.20

Diets were either placed in paper bags in 2.5 kg quantities or in aluminum pans in 2.5 kg quantities. Feed was evenly distributed to provide approximately a 4 cm thickness in either container and paper bags were sealed with autoclave tape. Before autoclaving feed, Kilit ampules, (BBL Microbiology Systems, P.O. Box 243, Cockeysville, MD 21030) containing Bacillus stearothermophilus spores, were inserted into the center of the feed samples and feed samples were autoclaved at 121°C for 10, 15, 20, 25 or 30 minutes. Upon completion of the sterilization procedure, ampules were removed and incubated at 60°C for 48 hours to determine the effectiveness of sterilization by monitoring the ampules for color change.

C. Results

1. Growth performance

Growth performance of pigs fed these experimental diets is shown in Table 29. Average daily gain for the 3-week period was reduced by approximately 54% when the control diet was autoclaved, and tended to be reduced even further upon addition of higher levels of vitamin-trace mineral premix. Although statistical analysis of average daily feed intake and feed efficiency could not be performed because of lack of replication, feed intake appeared to be reduced when the control diet was autoclaved (in comparison to pigs fed the non-autoclaved control diet), but not as greatly when the vitamin-trace mineral premix was added at higher levels. The apparent reduction in feed intake was probably caused by a reduction in palatability of diets after autoclaving. Feed efficiency tended to mirror the response observed for average daily gain observed. Because of the rapid

Table 29. Effect of Autoclaving and Addition of Supplemental Vitamin-Trace Mineral Premix to Starter Diets on Growth Performance.

	Dietary Treatment[a]			
Growth Parameter[b]	1	2	3	4
Avg. initial wt., kg	7.2	7.3	7.9	7.5
Avg. final wt., kg	10.8	9.2	9.3	8.9
ADG, g	254[c]	136[d]	98[d]	98[d]
ADFI, g	407	271	365	314
F/G	1.60	1.99	3.72	3.20

[a]Diet 1 (non-autoclaved control).

Diet 2 (autoclaved control).

Diet 3 (autoclaved control + 150% vitamin-trace mineral premix).

Diet 4 (autoclaved control + 200% vitamin-trace mineral premix).

[b]ADG=average daily gain.

ADFI=average daily feed intake.

F/G=feed/gain.

[c,d]Mean values for ADG with different superscripts are significantly different ($P<.01$). MSE = 1108.

reduction in growth observed by feeding the autoclaved diets, regardless of the level of vitamin-trace mineral premix present in the diet, a significant amount of protein (amino acid) destruction was suspected as being a greater contributing factor, when diets were subjected to steam sterilization. A reduction in growth response due to vitamin deficiency is generally a long-term effect and was not improved by providing a higher level of premix for the 3-week period. A lack of available amino acids generally produces an immediate growth depression analogous to that observed in this trial. As a result, a second trial was conducted to determine the effects of autoclaving diets fortified with supplemental lysine and methionine.

2. Effect of sterilization method and time on diet sterilization.

In regard to the effectiveness of sterilization of diets using two different (methods) containers for different lengths of time, the paper bag method proved undesirable. The paper bag method required a greater amount of time to package the diets and made it difficult to maintain a uniform thickness of feed, which consequently resulted in a lack of uniformity of heating, and ineffective sterilization, even with 35 minutes of autoclaving. This lack of uniformity of heated feed was undesirable not only from a sterility standpoint but also in regard to consistency of product quality. Furthermore, after autoclaving was completed and the feed cooled in the bags, the feed became very hard, adhered to the walls of the paper bags and was difficult to remove.

In contrast, the use of aluminum pans was much more desirable because of ease of packaging and maintenance of uniformity of thickness, which resulted in uniformity of heating and effective

sterilization. It was also much easier to remove the feed from the pan after the feed cooled from the autoclave process. Minimum effective sterilization was obtained by autoclaving feed for 25 minutes at 121°C. Shorter autoclaving times of 10, 15 or 20 minutes did not consistently provide complete sterilization as determined with the Kilit ampules, while autoclaving for greater than 25 minutes was unnecessary and avoided to minimize nutrient loss by autoclaving.

II. EFFECTIVENESS OF LYSINE AND METHIONINE SUPPLEMENTATION TO DIETS PRIOR TO AUTOCLAVING ON GROWTH PERFORMANCE.

A. Objective

As mentioned in the previous trial, supplementation of diets with vitamin-trace mineral premix did not alleviate the depression in growth performance when autoclaved diets were fed to starter pigs. Because of an immediate growth depression, it was suspected that amino acid destruction was the major contributing factor for this growth depression. Lysine and methionine were considered because lysine is the first limiting and methionine is the third limiting amino acid in a corn-soybean meal diet and because these two amino acids are markedly affected by autoclaving (Rice and Beuk, 1953). Therefore, the objective of this trial was to determine if supplementation of starter diets with 0.38% L-lysine·HCl and 0.10% D,L-methionine alleviates growth depression of pigs fed autoclaved diets.

B. Methodology

Sixteen crossbred weanling pigs from 2 litters averaging 8.1 kg in initial body weight were allotted by sex and weight to one of two dietary treatments for a three-week growth performance trial and housed

in two slotted-floor nursery pens. Diet composition and calculated nutrient analyses are shown in Table 30. Diets 5 and 6 were identical in nutrient composition but diet 6 was autoclaved. Individual pig weights were recorded on a weekly basis and feed consumption was measured by recording feed added to feeders. Average daily gain, average daily feed intake and feed efficiency were calculated and analyzed statistically using one-way analysis of variance (Gill, 1978).

After obtaining the results from the three-week growth performance trial (described in the following discussion), diet 6 was modified by adding non-autoclaved 0.38% L-lysine HCl to that already present in this autoclaved diet and is indicated as diet 8 in Table 30. Diet 7 served as a non-autoclaved control and had the same level of ingredients and calculated nutrient analyses as in diet 5 which was used in the previous three-week growth performance trial. Diets 7 and 8 were fed to the same groups of pigs as for diets 5 and 6, respectively, for an additional week to ascertain if lysine destruction is the primary nutrient affected by autoclaving to cause the poor growth performance observed when autoclaved diets are fed to starter pigs. Average daily gain, average daily feed intake and feed efficiency were calculated and analyzed statistically using one-way analysis of variance (Gill, 1978).

C. Results

When 0.38% L-lysine HCL and 0.10% D,L-methionine were added to the diets, autoclaving appeared to be 9.0% less deleterious to average daily gain and 5.26% less deleterious to feed/gain (Table 31) compared to growth performance of pigs fed autoclaved diets containing 0.19%

Table 30. Composition and Calculated Nutrient Analysis of Diets Used in Trial 2.

Ingredients	Int. Ref. No.	Experimental Diet[a] 5	6	7	8
Ground shelled corn	4-02-935	51.32	51.32	51.32	50.94
Soybean meal, 44%	5-04-604	25.00	25.00	25.00	25.00
Dried whey	4-01-182	20.00	20.00	20.00	20.00
Mono-dicalcium phosphate	6-01-080	0.75	0.75	0.75	0.75
Calcium carbonate	6-01-632	1.10	1.10	1.10	1.10
Salt	6-04-152	0.35	0.35	0.35	0.35
Vitamin-TM premix		0.50	0.50	0.50	0.50
Vitamin E-Se premix		0.50	0.50	0.50	0.50
L-lysine HCl	5-08-022	0.38	0.38	0.38	0.38
Non-autoclaved L-lysine HCl	5-08-022	--	--	--	0.38
D,L-methionine	5-03-086	0.10	0.10	0.10	0.10
		100.00	100.00	100.00	100.00
Calculated Nutrient Analysis					
ME, kcal/kg		3117	3117	3117	3104
Crude protein, %		18.24	18.24	18.24	18.20
Lysine, %		1.35	1.35	1.35	1.65
Calcium, %		0.85	0.85	0.85	0.85
Phosphorus, %		0.60	0.60	0.60	0.60

[a]Diet 5 (non-autoclaved control).

Diet 6 (autoclaved).

Diet 7 (non-autoclaved diet 5).

Diet 8 (autoclaved diet 6 + 0.38% non-autoclaved) L-lysine HCL.

Tab

Grow

Avg.

Avg.

ADG,

ADFI

F/G

[a]Die

Die

Die

Die

non-

[b]ADG=

ADF

F/G=

c,d[Me

(P<.

[e]Mean

Table 31. Effect of Autoclaving and Addition of Supplemental Lysine and Methionine to Starter Diets on Growth Performance (3 weeks) and the Effect of Addition of Non-Autoclaved Supplemental L-Lysine HCl to Diets for an Additional 7-Days on Growth Performance.

	Dietary Treatment[a]			
	3-Week Growth Performance		4th Week Growth Performance	
	5	6	7	8
Growth Parameter[b]	NAC+Lys+Met	AC+Lys+Met	NAC+Lys+Met	AC+Lys
Avg. initial wt., kg	8.3	8.5	9.6	9.2
Avg. final wt., kg	9.6	9.2	9.9	9.5
ADG, g	267[c]	155[d]	435[e]	451[e]
ADFI, g	639	411	1180	939
F/G	2.39	2.65	2.71	2.08

[a]Diet 5 (non-autoclaved + 0.38% L-lysine HCl + 0.10% D,L methionine).
Diet 6 (autoclaved + 0.38% L-lysine HCl + 0.10% D,L methionine).
Diet 7 (non-autoclaved + 0.38% L-lysine HCl + 0.10%, D,L methionine).
Diet 8 (autoclaved + 0.38% L-lysine HCl + 0.10% D,L methionine + 0.38% non-autoclaved L-lysine HCl).

[b]ADG=average daily gain.
ADFI=average daily feed intake.
F/G=feed/gain.

[c,d]Mean values for ADG with different superscripts are significantly different ($P<.03$). MSE = 5609.

[e]Mean values for ADG are not significantly different ($P>.10$). MSE = 20,614.

supplemental L-lysine HCl in the previous trial (Table 29). Average daily feed intake was depressed ($P<.03$), but to a lesser extent when 0.38% supplemental L-lysine HCl and 0.10%, D,L-methionine was included in the autoclaved diet compared to results in the previous trial. These results suggested that growth performance tended to improve by supplementing diets with lysine and methionine before autoclaving but these levels of lysine and methionine were not high enough to give growth performance similar to pigs fed the non-autoclaved control diet. Since lysine is the first limiting amino acid in these diets, and because methionine can be toxic when added at higher levels, and to verify that the depression in growth response was due to amino acids (lysine), the autoclaved diet (Diet 6) was reformulated and fortified with an additional 0.38% L-lysine HCl after autoclaving to provide a total of 1.65% lysine in the diet (Diet 8) at the end of the three week trial. It was estimated that 35% of the lysine in the diet was destroyed by autoclaving. Non-autoclaved L-lysine HCl was added at the level of 0.38% (0.30% actual lysine) in addition to that already present when the diet was originally mixed. If 35% of the lysine was destroyed by autoclaving, diet 6 would have an available lysine level of approximately 0.88% after autoclaving which would explain the poor growth performance observed in this trial. Consequently, the addition of non-autoclaved 0.38% L-lysine HCl to diet 6 would raise the level of available lysine to 1.18% (diet 8). The reformulated diet was fed along with the original diet 5 (indicated in Table 30 as diet 7) to the same pigs for an additional week to ascertain if the destruction of lysine was the major factor for poor growth with the autoclaved diet.

At the end of the 7-day feeding period, average daily gain for pigs fed diet 7 was 435 g/day while it was 451 g/day for pigs fed diet 8 containing 1.65% lysine (1.18% estimated available lysine) which would be similar to that present in the non-autoclaved diet. Feed efficiency was 2.71 and 2.02 kg feed/kg weight gain while average daily feed intake was 1.18 kg and 0.91 kg feed consumed per day for pigs fed diet 7 and 8, respectively. Although this feeding period was of short duration, it reconfirmed that the majority of growth depression observed was due to a reduction in availability of lysine, and that diets could be fortified with L-lysine HCl prior to autoclaving to give similar growth responses for a 3-week growth performance trial when autoclaved diets were fed to germ-free and conventional pigs in the germ-free experiment. Although a level of 1.65% lysine in the pre-autoclaved diet appeared to alleviate growth depression from the reduction of lysine availability during the last week of this trial, a third trial was conducted to determine the optimum level of supplemental L-lysine HCl needed in autoclaved diets to produce a growth response analogous to the non-autoclaved original diet used in this trial.

III. BIOAVAILABILITY OF LYSINE IN AUTOCLAVED CORN-SOYBEAN MEAL-DRIED WHEY STARTER DIETS.

A. Objective

In order to determine the optimum level of synthetic L-lysine HCl to add to pre-autoclaved diets to obtain growth performance similar to that of pigs fed a non-autoclaved diet containing approximately 1.35% lysine (Trial 2), this experiment was designed to examine the growth performance responses of pigs fed diets containing increasing levels of L-lysine HCl when diets were autoclaved for 25 minutes at 121°C.

B. Methodology

Four litters of crossbred weanling pigs (28) averaging 7.8 kg in initial body weight were allotted in pairs, based on sex, weight and litter, to one of 7 experimental diets for a three-week growth performance trial. Experimental diet composition and calculated nutrient analyses are shown in table 32. Diets were autoclaved for 25 minutes at 121°C. Weight gain was recorded for each pig and feed consumption was measured and recorded on a weekly basis in order to calculate average daily gain, feed efficiency and average daily feed intake. Data were analyzed statistically using orthogonal polynomial contrasts (Gill, 1978) to characterize the form of response and to estimate the level of supplemental lysine needed in autoclaved diets to obtain maximum growth rate.

C. Results

Cumulative 3-week growth performance data are shown in Table 33. No significant differences for average daily gain (ADG) were noted for first, second or third degree nonlinearity but the response was strongest ($P<.40$) for second degree (quadratic) nonlinearity. As a result, a prediction equation was developed for second degree nonlinearity which is $Y=83.49 + 102.16x_i - 26.88x_i^2$. The stationary point ($x_s$), where an estimate of the level of lysine at which maximum growth is expected to occur is $x_s=-\hat{\beta}_1/2\hat{\beta}_2=-102.16/2(-26.88)=1.90\%$ lysine. Therefore, a level of approximately 1.9% total lysine was added to experimental diets before autoclaving for use in the subsequent germ-free experiment. It appears that at levels higher than 2.0% total lysine, growth rate and feed consumption decline, perhaps due to an amino acid imbalance. Because

Table 32. Composition and Calculated Nutrient Analysis of Diets Used in Trial 3.

	Experimental Diet						
Ingredient (%)	Autoclaved Basal diet 9	Autoclaved Basal + .25% lys 10	Autoclaved Basal + .50% lys 11	Autoclaved Basal + .75% lys 12	Autoclaved Basal + 1.0% lys 13	Autoclaved Basal + 1.25% lys 14	Autoclaved Basal + 1.50% lys 15
Ground shelled corn	51.65	51.33	51.01	50.69	50.37	50.05	49.73
Soybean meal, 44%	25.00	25.00	25.00	25.00	25.00	25.00	25.00
Dried whey	20.00	20.00	20.00	20.00	20.00	20.00	20.00
Mono-dicalcium phosphate	0.75	0.75	0.75	0.75	0.75	0.75	0.75
Calcium carbonate	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Salt	0.35	0.35	0.35	0.35	0.35	0.35	0.35
Vitamin TM-premix	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Vitamin E-Se premix	0.50	0.50	0.50	0.50	0.50	0.50	0.50
L-lysine HCl	0.25	0.57	0.89	1.21	1.53	1.85	2.17
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Calculated Nutrient Analysis							
ME, kcal/kg	3128	3117	3107	3096	3085	3074	3063
Crude protein, %	18.27	18.24	18.21	18.18	18.15	18.12	18.09
Lysine, %	1.25	1.50	1.75	2.00	2.25	2.50	2.75
Calcium, %	0.81	0.81	0.81	0.81	0.81	0.81	0.81
Phosphorus, %	0.62	0.62	0.62	0.62	0.62	0.62	0.62

Table 33. Effect of Increasing Levels of Supplemental Lysine in Autoclaved Diets in Determining Optimal Lysine Supplementation as Measured by Growth Performance.

	Dietary Treatment						
Growth Parameter[a]	9	10	11	12	13	14	15
ADG, g[b]	145	187	215	226	155	142	145
ADFI, g	438	459	477	488	418	362	355
F/G	3.02	2.45	2.22	2.16	2.70	2.55	2.45

[a]ADG=average daily gain.

ADFI=average daily feed intake.

F/G=feed/gain

[b]Average daily gain was not significantly different between dietary treatments for first (linear), second (quadratic) or third (cubic) degree nonlinearity. Second degree (quadratic) nonlinearity ($P<.40$). MSE = 1102.

growth rate appeared to be affected more dramatically than feed consumption, feed efficiency is also worsened.

Average daily feed intake (ADFI) tended to follow the same pattern as for average daily gain while the feed efficiency (F/G) response appeared to be third degree (cubic) in nonlinearity (Table 33). Statistical analyses were performed only for average daily gain because it provides a more reliable estimate of lysine bioavailability than ADFI or F/G and because there were more observations for ADG (4) compared to ADFI and F/G (2) per treatment.

When comparing ADG, ADFI and F/G values for pigs receiving the autoclaved diet containing 2.0% lysine versus the non-autoclaved diet containing 1.35% lysine (Table 30 and 31), ADG appeared to be approximately 85%, ADFI was 76% and F/G was 111% of the growth response of pigs fed the control non-autoclaved diet.

Perhaps, some of the reduction in growth performance of pigs fed the autoclaved diets was due to a reduction in palatability which appears to be the case in all of the preliminary trials. Nevertheless, it appears that a major proportion of the growth depression of autoclaved swine diets is due to the apparent destruction or lowered bioavailability of lysine which can be corrected by proper fortification of diets with synthetic-lysine HCl prior to autoclaving. It also appears that approximately 35% of the lysine in the diet is destroyed by autoclaving which supports our findings in Trial 2.

IV. EFFECTS OF AUTOCLAVING AND SUPPLEMENTING DIETS WITH 250 PPM COPPER ON ENERGY, NITROGEN AND MINERAL BALANCE OF WEANLING PIGS.

A. Objective

Our previous studies, as well as work by Rice and Beuk (1953), have indicated that the bioavailability of certain amino acids (lysine,

methionine and cysteine is reduced by autoclaving. Furthermore, Zimmerman and Wostmann (1963) showed that certain B vitamins and A and E are reduced upon sterilizing conventional diets with steam even though our initial studies showed no benefit in growth performance from adding additional vitamin-trace mineral permix to autoclaved diets fed to pigs for a 3-week growth performance trial. Reddy et al. (1965b) compared the copper and iron content of diets before and after autoclaving and observed no demonstrable loss from leaching which may theoretically occur. Although the effects of autoclaving diets on energy utilization have not been studied, energy utilization is not likely to be affected and may be improved by heating. Because of the lack of knowledge of the specific effects of autoclaving conventional starter diets, this study was designed to quantitatively examine differences in energy, nitrogen and mineral utilization of autoclaved and non-autoclaved corn-soybean meal-dried whey starter diets with or without high levels of supplemental copper, when feed intakes are standardized.

B. Methodology

Sixteen crossbred pigs, 4 weeks of age, obtained from two litters and averaging 7 kg of initial body weight, were allotted by sex, litter and initial body weight to one of the four experimental diets (Table 34) in a 2 x 2 factorial arrangement of treatments for a 10-day balance trial. Pigs were housed in individual collection cages and fed twice daily an amount of their respective experimental diets equivalent to 2% of their average initial body weight. Pigs were removed from their collection cages at each feeding and fed a gruel mixture in individual feeding cages to alleviate problems associated

Table 34. Composition and Calculated Nutrient Analysis of Diets Used in Trial 4.

		Experimental diet			
		Non-autoclaved control	Non-autoclaved + $CuSO_4$ $5H_2O$	Autoclaved control	Autoclaved + $CuSO_4$ $5H_2O$
Ingredient (%)	Int. Ref. No.	16	17	18	19
Ground shelled corn	4-02-935	50.90	50.80	50.90	50.80
Soybean meal, 44%	5-04-604	25.00	25.00	25.00	25.00
Dried whey	4-01-182	20.00	20.00	20.00	20.00
Mono-dicalcium phosphate	6-01-080	0.75	0.75	0.75	0.75
Calcium carbonate	6-01-632	1.00	1.00	1.00	1.00
Salt	6-04-152	0.35	0.35	0.35	0.35
Vitamin-TM premix		0.50	0.50	0.50	0.50
Vitamin E-Se premix		0.50	0.50	0.50	0.50
L-lysine HCl	5-08-022	1.00	1.00	1.00	1.00
$CuSO_4 \cdot 5H_2O$	6-01-719	--	0.10	--	0.10
Total		100.0	100.0	100.0	100.0
Calculated Nutrient Analysis					
ME, kcal/kg		3103	3100	3103	3100
Crude protein, %		18.20	18.20	18.20	18.20
Lysine, %		1.83	1.83	1.83	1.83
Calcium, %		0.81	0.81	0.81	0.81
Phosphorus, %		0.62	0.62	0.62	0.62

with contaminating the collected feces. After a 5-day adjustment period, used to acclimate pigs to their respective diets, feeding schedule and environment, collection cages were cleaned to start the 5-day collection period. Concentrated hydrochloric acid was added to urine containers to eliminate microbial growth in the collected urine. At the end of the collection period, urine volumes were measured and a 100 ml sample was retained for subsequent laboratory analyses. Collected feces were dried, weighed and taken to the laboratory for analysis along with feed samples of each diet.

Gross energy of feed feces and freeze dried urine was determined by using an adiabatic bomb calorimeter (Parr Instrument Company, Moline, IL) standarized with benzoic acid. Nitrogen content of feed, feces and urine was determined by a semi-micro Kjeldahl method (AOAC, 1984). Calcium, iron, zinc and copper concentrations were determined by atomic absorption spectrophotometry (Model 951, Instrument Laboratory, Inc., Lexington, MA) while phosphorus determinations were conducted using colorimetric spectrometery (Gomori, 1942). Concentrations of these nutrients were then used to calculate daily intake and excretion of nutrients to determine daily nutrient balance. Data were analyzed using Federer-Zelen factorial one-way analysis of variance (Gill, 1978).

C. Results

1. Energy balance

Although daily feed intake was standardized across all treatments, some pigs would not consume all of their meal, particularly those pigs receiving the autoclaved diets because of apparent palatability differences. As a result, daily feed intake was different

Table 35. Effects of Autoclaving and Addition of 250 ppm Supplemental Copper to Diets on Energy Balance.

Energy Parameter[b]	Dietary Treatment[a] 17	18	19	20	MSE[c]	Statistical Significance P value[d] H[e]	D[f]	HxD[g]
GE, kcal/g	3.95	3.94	4.07	4.03	--	--	--	--
Daily feed intake, g	236.0	246.6	207.1	238.8	530	NS	.09	NS
Daily GE intake, g	931.7	971.9	842.5	961.5	8582	NS	NS	NS
DE, kcal/g	3.43	3.56	3.40	3.32	.005	.003	NS	.01
DE/GE x 100%	86.8	90.3	83.7	82.4	2.97	.001	NS	.02
Daily urine GE, kcal	27.2	23.6	21.1	19.2	--	--	--	--
ME, kcal/g	3.31	3.46	3.30	3.24	.003	.002	NS	.003
Daily N balance, g	3.82	4.37	1.86	2.99	.241	.001	.005	NS
Caloric correction, kcal	25.9	29.6	12.6	20.2	--	--	--	--
ME_N, kcal/g	3.20	3.34	3.24	3.15	.003	.02	NS	.002
ME_N/GE x 100%	81.1	85.0	79.7	78.4	2.07	.001	NS	.003

[a]Diet 17 (non-autoclaved control).
Diet 18 (non-autoclaved + 250 ppm supplemental copper as $CuSO_4 \cdot 5H_2O$).
Diet 19 (autoclaved control).
Diet 20 (autoclaved + 250 ppm supplemental copper as $CuSO_4 \cdot 5H_2O$).

[b]GE = gross energy.
DE = digestible energy.
ME = metabolizable energy
Daily N balance from Table 36.
Caloric correction = N balance (g) x 6.77 kcal/g N (Diggs et al., 1965).
ME_N = nitrogen corrected ME.

[c]MSE = mean square error.

[d]P value = level of statistical significance, NS = not significant (P>.10).

[e]H = heating effect (autoclaving at 121°C for 25 minutes).

[f]D = diet effect.

[g]HxD = heating - diet interaction.

across dietary treatments which confounds the true effects of nutrient balance. The non-autoclaved diets were higher in digestible energy (DE) (P<.003), metabolizable energy (ME) (P<.002) and metabolizable energy corrected for nitrogen balance (ME_N) (P<.02) compared to autoclaved diets (Table 35). However, pigs receiving these non-autoclaved diets tended to consume more gross energy although there was no statistical difference in gross energy intake. If intakes were standardized to 250 g of feed intake per day, and assuming that DE, ME and ME_N are proportional to the amount of GE consumed, DE values would be 3.61, 3.63, 4.10 and 3.48 kcal/g of feed consumed which would indicate that energy utilization of autoclaved diets is improved as might be expected, since heating of grain diets (i.e., as in a pelleting process) does improve energy utilization by the pig (Erickson et al., 1980; Seerley et al., 1962). Nevertheless, comparing numerical differences between the non-autoclaved and autoclaved control diet for DE, ME and ME_N, they are almost identical even though the pigs consumed less energy when the diets were autoclaved. There were no differences due to copper supplementation between the two diets, but there was a heating x diet interaction for DE, ME and ME_N (Table 35). In each case, adding copper to the non-autoclaved diet increased DE, ME and ME_N but decreased these parameters when copper was added to the autoclaved diets. This general relationship exists even when these parameters are adjusted to the same energy intake and is supported by energy balance data of non-autoclaved diets from preliminary experiment 5 which will be described in the next section.

It appears that autoclaving these diets has no effect on, or may increase, DE, ME and ME_N, and that copper supplementation increases

DE, ME and ME_N when included in non-autoclaved diets. However, these energy parameters are reduced when pigs are fed autoclaved diets containing supplemental copper.

2. Nitrogen balance

As for energy balance, differences in feed (nitrogen) intake ($P<.09$) tended to confound the true picture of nitrogen utilization in pigs fed these diets (Table 36). Daily nitrogen absorbed, apparent digestibility of nitrogen, daily nitrogen balance, net protein utilization and biological value of protein were higher ($P<.001$) when non-autoclaved diets were fed compared to pigs fed the autoclaved diets (Table 36). However, this same relationship would also exist if pigs consumed a standardized amount of nitrogen. These data support our previous observations in regard to improvements in growth performance of pigs when diets were supplemented with L-lysine HCl prior to autoclaving, and the reduction of growth performance when autoclaved diets were not fortified with lysine, compared to pig performance when non-autoclaved diets were fed. Copper supplementation increased daily nitrogen absorption ($P<.03$), net protein utilization ($P<.02$) and biological value of protein ($P<.05$) when both autoclaved and non-autoclaved diets were fed (Table 36). This same relationship exists when daily nitrogen intakes are standardized, which suggests that feeding high copper diets improves nitrogen utilization, perhaps by increasing peptic hydrolysis of proteins as shown by Kirchgessner et al. (1976) or by reducing the amount of bacterial urease activity and thus reducing the absorption of ammonia and production or urea, (Visek et al., 1959) which would produce such effects. This relationship tended to exist in a preliminary experiment (discussed in the next

Table 36. Effects of Autoclaving and Addition of 250 ppm Supplemental Copper to Diets on Nitrogen Balance.

Nitrogen Parameter[a]	Dietary Treatment[b] 17	18	19	20	MSE[c]	Statistical Significance P Values[d] H[e]	D[f]	HxD[g]
Daily N intake, g	7.18	7.60	6.10	7.41	4738	.09	.03	NS
Daily fecal N, g	1.18	0.89	1.61	1.94	--	--	--	--
Daily absorbed N, g	6.00	6.71	4.49	5.47	.441	.001	.03	NS
App. dig. of N, %	88.3	88.3	73.4	73.8	9.67	.001	.10	NS
Daily urine N, g	2.18	2.34	2.63	2.48	--	--	--	--
Daily N balance, g	3.82	4.37	1.86	2.99	.003	.002	NS	.003
Net Protein, Util., %	53.1	57.5	30.5	40.4	30.9	.001	.02	NS
Biol. Value of Protein	63.8	65.1	41.5	54.9	44.9	.001	.05	.10

[a]Net protein utilization, % = 100 (N balance ÷ N intake).
Biological value of protein = 100 (N balance ÷ N absorbed).

[b]Diet 17 (non-autoclaved control).
Diet 18 (non-autoclaved + 250 ppm supplemental copper as $CuSO_4 \cdot 5H_2O$).
Diet 19 (autoclaved control).
Diet 20 (autoclaved + 250 ppm supplemental copper as $CuSO_4 \cdot 5H_2O$).

[c]MSE = mean square error.

[d]P value = level of statistical significance, NS = not significant (P>.10).

[e]H = heating effect (autoclaving at 121°C for 25 minutes).

[f]D = diet effect.

[g]HxD = heating-diet interaction.

section) when daily feed intakes were identical, even though dried whey was not included in the diet. A heat treatment x copper interaction ($P<.003$) existed for daily nitrogen balance indicating that copper was much more effective (50% more effective) in improving daily nitrogen balance for pigs fed the autoclaved diets compared to the pigs fed the non-autoclaved diets. This could be possible since there is poorer amino acid availability of autoclaved diets due to amino acid destruction during the autoclave process which consequently results in less amino acids being absorbed. As a result, more amino acids are available to the gut microflora for deamination and urea production which may be inhibited by copper.

3. Mineral balance

a. Calcium and phosphorus

Effects of autoclaving and copper supplementation on mineral balance are shown in Table 37. Daily calcium retention was not affected by autoclaving or copper supplementation. Phosphorus retention was also not affected by autoclaving but was improved ($P<.04$) by adding 250 ppm supplemental copper to these diets.

b. Copper

Copper retention was reduced in pigs fed autoclaved diets ($P<.001$) and a heating x copper interaction was noted ($P<.002$) since high copper feeding increased copper retention of the non-autoclaved diet but decreased copper retention when present in autoclaved diets fed to these pigs (Table 37). A portion of the reduction in copper retention for pigs fed the autoclaved high-copper diets was no doubt due to the lower copper intake relative to fecal copper excretion compared to the non-autoclaved high-copper diet. Perhaps another cause for this reduction in copper retention is that autoclaving diets affects the availability of copper.

Table 37. Effects of Autoclaving and Addition of 250 ppm Supplemental Copper to Diets on Mineral Balance. Statistical Significance

Mineral	Dietary Treatment[a] 17	18	19	20	MSE[b]	P Value[c] H	D	HxD
Calcium								
Daily intake, g	2.17	1.92	1.57	1.84	.036	.004	NS	.02
Daily fecal, g	0.73	0.66	0.57	0.81	.030	NS	NS	.09
Daily urine, g	0.15	0.08	0.02	0.03	.004	.01	NS	NS
Daily balance, g	1.29	1.19	0.99	1.00	.014	.001	NS	NS
% Retention	60.0	61.8	63.2	54.3	38.6	NS	NS	NS
Phosphorus								
Daily intake, g	1.39	1.38	1.24	1.38	.019	NS	NS	NS
Daily fecal, g	0.65	0.56	0.54	0.59	.010	NS	NS	NS
Daily urine, g	0.01	0.01	0.08	0.04	.001	.001	.006	.02
Daily balance, g	0.73	0.81	0.62	0.75	.009	.10	.04	NS
% Retention	52.3	58.9	50.3	54.6	22.2	NS	.04	NS
Copper								
Daily intake, mg	3.87	70.01	3.37	58.65	.959	.001	.001	.001
Daily fecal, mg	3.02	49.00	2.74	54.58	3.52	.02	.001	.01
Daily urine, mg	0.09	0.43	0.09	0.72	.024	NS	.001	.09
Daily balance, mg	0.76	20.58	0.55	3.35	3.57	.001	.001	.001
% retention	19.63	29.40	16.21	5.67	24.0	.001	NS	.002

Table 37. Effects of Autoclaving and Addition of 250 ppm Supplemental Copper to Diet on Mineral Balance (Cont'd).

	Dietary Treatment[a]					Statistical Significance P Value[c]		
Mineral	17	18	19	20	MSE[b]	H	D	HxD
Zinc								
Daily intake, mg	28.6	25.0	22.2	22.9	3.83	.001	NS	.05
Daily fecal, mg	21.2	17.7	18.4	31.8	55.6	NS	NS	.04
Daily urine, mg	4.44	1.85	1.13	2.63	0.40	.002	NS	.001
Daily balance, mg	3.03	5.44	2.67	-11.6	50.7	.03	NS	.04
% retention	10.6	21.8	13.1	-49.7	886	.04	NS	.03
Iron								
Daily intake, mg	115.3	106.8	103.1	107.4	128	NS	NS	NS
Daily fecal, mg	88.9	81.9	76.3	94.7	184	NS	NS	.08
Daily urine, mg	1.41	1.56	1.62	2.26	0.42	NS	NS	NS
Daily balance, mg	24.9	23.3	25.2	10.40	40.2	.07	.02	.06
% retention	22.09	21.82	24.44	9.56	42.5	NS	.03	.04

[a]Diet 17 (non-autoclaved control).
Diet 18 (non-autoclaved + 250 ppm supplemental copper as $CuSO_4 \cdot 5H_2O$).
Diet 19 (autoclaved control).
Diet 20 (autoclaved + 250 ppm supplemental copper as $CuSO_4 \cdot 5H_2O$).

[b]MSE = mean square error.

[c]P value = level of statistical significance, NS=not significant (P>.10).

[d]H = heating effect (autoclaving at 121°C for 25 minutes).

[e]D = diet effect.

[f]H x D=heating-diet interaction.

c. Zinc

Zinc retention was reduced by autoclaving (P<.04) and a heating x diet interaction was noted (P<.03), where zinc retention increased in the non-autoclaved-high-copper diet but decreased when the same diet was autoclaved (Table 37). The bioavailability of zinc may have been reduced when the high copper diet was autoclaved but the reason for a loss of zinc from the pig (as indicated by the negative Zn retention of pigs fed diet 20) is not very apparent and may be a result of contamination of fecal samples.

d. Iron

Iron balance (P<.02) and iron retention (P<.03) was reduced (Table 37) by feeding the high copper diets and were reduced to a greater extent when diets were autoclaved (P<.06, P<.09, respectively). Reductions in iron balance and retention (and zinc balance and retention of pigs fed diet 20) from feeding the high copper diets may be expected, since copper, zinc, iron and other divalent metals are believed to compete for binding sites in the intestinal mucosa (Underwood, 1981). Thus, a high copper diet would reduce zinc and iron absorption by inhibiting the uptake of these elements to a greater extent than when a low copper diet (diet 17 and 19) are fed.

V. EFFECTS OF FEEDING A CORN-SOYBEAN MEAL STARTER DIET CONTAINING 250 PPM SUPPLEMENTAL COPPER ON ENERGY, NITROGEN AND MINERAL BALANCE.

A. Objective

In order to determine the influence of feeding high copper diets to weanling pigs on energy and nitrogen utilization when feed intake is standardized, a balance trial was conducted to partition

digestive and metabolic effects of energy and nitrogen as influenced by high copper feeding. The effects of a high copper diet on copper, iron and zinc balance were also determined.

B. Methodology

Twelve crossbred pigs, 50 days of age and averaging 20.4 kg in initial body weight, were assigned to one of two experimental diets (Table 38) for a 10-day balance trial. All pigs were fed 375 g of their respective experimental diets, twice daily for a 5-day adjustment period and during the subsequent 5-day collection period. Procedures used in conducting this trial are identical to those described for trial IV in this Appendix. Data were analyzed statistically by using one-way analysis of variance (Gill, 1978).

C. Results

1. Energy balance

No differences were noted for digestible energy (DE), metabolizable energy (ME), or metabolizable energy corrected for nitrogen (ME_N) when a diet containing 250 ppm supplemental copper was fed at the same daily intake level as the control diet (Table 39). There was a slight tendency for DE, ME and ME_N to be improved by high copper feeding as observed in Trial IV when pigs were fed the non-autoclaved diets.

2. Nitrogen balance

More nitrogen was absorbed ($P<.01$) when diet 22 was fed, which subsequently resulted in an increase in nitrogen balance ($P<.01$) and was not affected by the slight increase in daily nitrogen intake since net protein utilization was also improved ($P<.01$) compared to pigs fed the control diet 21 (Table 40). This increase in nitrogen

Table 38. Composition and Calculated Nutrient Analysis of Diets Used in Trial 5.

		Experimental Diet	
		Control	+ 250 ppm Cu
Ingredients (%)		21	22
Ground shelled corn	4-02-935	68.67	68.57
Soybean meal, 44%	5-04-604	27.40	27.40
Mono-dicalcium phosphate	6-01-080	1.13	1.13
Calcium carbonate	6-01-632	1.45	1.45
Salt	6-04-152	0.35	0.35
Vitamin-TM premix		0.50	0.50
Vitamin E-Se premix		0.50	0.50
$CuSO_4 \cdot 5H_2O$	6-01-719	--	0.10
Total		100.00	100.00
Calculated Nutrient Analysis			
ME, kcal/kg		3130	3127
Crude protein, %		18.1	18.1
Lysine, %		0.97	0.97
Calcium, %		0.87	0.87
Phosphorus, %		0.60	0.60

Table 39. Effects of Adding 250 ppm Supplemental Copper on Energy Balance.

	Dietary Treatment		Statistical Significance	
	Control	+250 ppm Cu		
Energy Parameter[a]	21	22	MSE[b]	P value[c]
GE, kcal/kg	4.02	4.02	--	--
Daily feed intake, g	750	750	--	--
Daily GE intake, g	3015	3015	--	--
Daily fecal GE, kcal	427.5	394.3	1980	NS
DE, kcal/kg	3.45	3.50	0.001	NS
ME/GE X 100%	85.8	86.9	2.18	NS
Daily urine GE, kcal	68.8	74.9	48.67	NS
ME, kcal/g	3.36	3.39	0.001	NS
Daily N balance, g	10.6	11.5	0.07	.01
Caloric correction, kcal	70.0	76.8	--	--
ME_N , kcal/g	3.27	3.29	0.001	NS
ME_N/GE x 100%	81.2	81.9	1.71	NS

[a] GE=gross energy.

DE=digestible energy.

ME=metabolizable energy.

Daily N balance from Table 40.

Caloric correction = N balance (g) x 6.77 kcal/g N (Diggs et al., 1965).

ME_N=nitrogen corrected ME.

[b] MSE=mean square error.

[c] P value = level of statistical significance, NS= not significant (P>.10).

Table 40. Effects of Adding 250 ppm Supplemental Copper on Nitrogen Balance.

	Dietary Treatment		Statistical Significance	
	Control	+250 ppm Cu		
Nitrogen Parameter[a]	21	22	MSE[b]	P value[c]
Daily N intake, g	19.80	20.55	--	--
Daily fecal N, g	3.72	3.57	0.14	NS
Daily absorbed N, g	16.1	17.0	0.14	.01
Apparent dig. of N, %	81.2	82.6	3.34	NS
Daily urine N, g	5.51	5.46	0.15	NS
Daily N balance, g	10.6	11.5	0.07	.01
Net protein util., %	53.4	56.1	1.61	.01
Biol. value of protein	65.8	67.9	3.46	NS

[a]Net protein utilization, % = 100 (N balance ÷ N intake).

Biological value of protein = 100 (N balance ÷ N absorbed).

[b]MSE=mean square error.

[c]P value=level of statistical significance, NS=not significant (P>.10).

utilization could be a result of increased peptic hydrolysis of proteins during digestion when in the presence of high cupric ion concentrations (Kirchgessner et al., 1976). There was also a tendency for copper supplementation to improve the biological value of protein.

3. Mineral balance

a. Copper

Daily copper intake was greater and daily fecal and urinary copper excretion was greater ($P<.01$) when pigs were fed diet 22 (Table 41). Although copper excretion increased by feeding the high copper diet, the amount of copper retained by the pig, relative to daily copper intake, was also increased ($P<.05$). These results are consistent with other reports (Underwood, 1977) where copper absorption increases as dietary copper increases until copper storage organs are saturated and homeostatic mechanisms then reduce the amount of copper absorbed relative to intake.

b. Zinc

Fecal zinc excretion was increased ($P<.05$) by feeding the high copper diet (diet 22) but zinc retention was not affected (Table 41).

c. Iron

No differences were observed for iron balance or retention when the copper supplemented diet was fed as compared to pigs fed the control diet (Table 41).

Table 41. Effects of Adding 250 ppm Supplemental Copper on Copper, Zinc and Iron Balance.

	Dietary Treatment		Statistical Significance	
Mineral	21	22	MSE	P value
Copper				
Daily intake, mg	14.3	214	--	--
Daily fecal, mg	12.5	168	110	.01
Daily urine, mg	0.22	2.08	0.10	.01
Daily balance, mg	1.64	43.4	110	.01
% retention	11.4	20.3	32.2	.05
Zinc				
Daily intake, mg	198	91.3	--	--
Daily fecal, mg	72.6	84.4	56.0	.05
Daily urine, mg	4.83	4.59	4.68	NS
Daily balance, mg	13.9	19.0	66.2	NS
% retention	15.2	17.6	62.2	NS
Iron				
Daily intake, mg	212	210	--	--
Daily fecal, mg	173	171	57.8	NS
Daily urine, mg	3.01	2.15	0.54	NS
Daily balance, mg	37.1	37.8	59.9	NS
% retention	17.5	18.0	13.4	NS

[a]MSE=mean square error.

[b]P value=level of significance, NS=not significant (P>.10).

APPENDIX B

APPENDIX B

Prior to the start of the gnotobiotic portion of the experiment, a great amount of preparation was required. The following discussion describes in detail, the various steps involved in preparing and sterilizing the isolators and equipment necessary to obtain and successfully rear one litter of germ-free pigs for the seven-week trial. In addition, the surgical procedure used to obtain the germ-free pigs by Caesarian-section, sterilization and transfer of milk, feed and water into isolators, and microbial culture procedures used to verify germ-free status are described.

I. PREPARATION OF REARING ISOLATORS FOR GERM-FREE RESEARCH

A. Description of Rearing Isolators

Three small ruminant stainless steel isolator units measuring approximately 122 x 60 x 91 cm and enclosed with vinyl film were used to provide adequate floor space, durability and ease of rearing during the seven-week trial. Two isolators housed three pigs providing 0.25 m^2 of floor space per pig while the third isolator accomodated four pigs and provided 0.19 m^2 of floor space per pig.

B. Initial Cleaning of Isolators

Isolator preparation was begun by washing the vinyl film of the isolator with warm water containing a quaternary ammonia detergent (Diversey Chemicals, Division of the Diversey Corp., Des Plaines, IL), placed along with dry towels inside the deflated isolator. Once the inside cap was placed on the outside of the entry port and the nipples of the vinyl film of the isolator were plugged with rubber stoppers, the isolators were inflated by attaching an air hose to the air intake

nipple on the plastic portion of the isolator. Both inside and outside of the vinyl film portion of the isolator were thoroughly washed, rinsed and dried with cloth towels to prevent accumulation of detergent residue. Any tape or soil remaining after initial washing was removed by applying Super Takeoff (Diversey Chemicals, Division of the Diversey Corportation, Des Plaines, IL) to a dry cloth towel and rubbing the soiled spot until clean. Once the vinyl film was cleaned satisfactorily, all cleaning materials were taken out of the isolators by removing the cap which sealed the outside of the entry port.

C. Installation of Gloves and Aluminum Rings

Yellow vinyl tape, C320 (Standard Safety Equipment Company, Palatine, IL) and fiberglass tape, #898 (Scotch Brand, Minnesota Mining and Manufacturing Co., St. Paul, MN), holding each of the six rubber gloves to the aluminum rings on each isolator was removed. Each of the six rings on each isolator was then removed from the preformed plastic grooves and were cleaned to remove all soil and corrosion. These aluminum rings were then replaced and new rubber gloves were properly oriented and attached to the rings. Rubber gloves were then secured to the rings by several wraps of the fiberglass tape followed by yellow vinyl tape to protect and insure a tight seal between the rib at the end of the rubber glove and the adjacent ring groove.

D. Testing for Leaks in the Plastic Isolator

The vinyl film portion of the isolator was tested for leaks by placing the inside cap on the outside of the entry port, inserting rubber stoppers in nipples of the plastic, and inflating the vinyl film with air from the air hose until it was approximately 75 percent

inflated. Vinyl film was further inflated with freon gas from a compressed gas cylinder until the gloves were fully extended. A halogen leak detector (Type: H-1- obtained from General Electric Co., Medical Systems Division, Milwaukee, WI 53201) was used to examine the entire vinyl portion of the isolator to identify small holes, cracks and leaks needing repair. Particular attention was given to the seams, rubber gloves, seal around compression rings (where gloves were attached to the isolator) and the base of the vinyl attached to the stainless steel frame of the isolator cage. When air leaks were identified, they were marked and later repaired when the vinyl portion was removed from the stainless steel frame. All holes and cracks identified with the halogen leak detector were repaired by first cleaning the vinyl surface with Poly Kleen (Schwartz Chemical Company, Inc., 50-01 Second St., L.I.C., NY) followed by sealing a small piece of vinyl over the hole or crack.

E. Final Cleaning of Isolators

Once all repairs were made to the vinyl portion of the isolator, both the inside and outside surfaces of the vinyl were cleaned by applying Poly Kleen to a dry cloth towel and polishing the surfaces to remove any finger prints, soil and detergent residue. All cleaning was done using rubber gloves to avoid getting oil from fingers during handling on the vinyl surface.

Before the vinyl could be reattached to the stainless steel portion of the isolator, the entire frame of the stainless steel isolator was disassembled and thoroughly cleaned with detergent, rinsed, and all rusted bolts, nuts and screws were replaced. The wire mesh floor material was wrapped in paper, autoclaved and placed at the

bottom of the isolator tank while two aluminum storage trays were attached with wire ties to the aluminum sheet located at the top of the stainless steel frame. When all components of the isolator were cleaned, dried and reassembled, the vinyl portion of the isolator was reattached to the stainless steel frame of the isolator by wrapping yellow vinyl tape several times (overlapping each previous wrap) to properly seal the vinyl to the metal frame. Steel support bars lined with adhesive weather stripping (3M Company, Minneapolis, MN) were used to give further support to the vinyl-metal attachment and to hold the yellow vinyl tape in place.

Following the attachment of vinyl to the metal frame, the isolators were reinflated with 75 percent air and 25 percent freon gas. A final check for leaks was conducted, as previously described, to identify any holes or cracks not identified in the initial test. If leaks were identified at this time, they were repaired by placing a piece of yellow vinyl tape over the location in question until no leaks were identified using halogen leak detector.

Once all isolator leaks were repaired, the isolator units were ready to be sterilized.

F. Preparation of Air Filters

For the air intake filter, a 43 x 137 cm section of 1.27 cm thick filter media (FM 004 Glass Fiber Media obtained from Owens Corning) was cut and wrapped tightly around the air filter frame. Wire was then wrapped around the air filter fiber media to hold it in place. A wire screen was then wrapped around the filter media to further secure the filter media to the filter frame and also to provide protection from potential damage to the glass fiber media. Mylar tape

was wrapped around both the top and bottom edges of the filter media and yellow vinyl tape was wrapped over the mylar tape to seal any leaks that may have been present between the filter media and the filter frame. The inlet and outlet filter connections were then covered with mylar film to prevent contamination before attachment to the sterilized isolator. All intake air filters were then sterilized in a dry heating oven for 4 hours at 150°C.

Oil bath exhaust filters were cleaned, checked for leaks and approximately 500 ml of mineral oil was added so that 50 percent of the filter was filled with oil.

G. Preparation of Sterilizing Cylinder

A 51 x 457 cm section of filter media (sufficient to provide four compete wraps around the sterilizing cylinder) was cut and applied in the same manner as previously described for the air intake filter. Filter media was held in place by two M-196 clamps at each end of the cylinder. A piece of wire screen measuring 112 x 51 cm was cut to cover the filter and overlap it by 3 cm on each end of the cylinder. This screen was then secured at each end of the cylinder with two M-200 clamps.

H. Sterilization of Equipment for Use in Isolators

Each of three wire baskets were lined with cloth towels and filled with the following equipment needed to accomplish various procedures within each isolator:

- 2 can openers
- 10 screw cap vials containing rectal swabs
- 1 ear notcher
- 1 side cutter

- 2 (5cc) syringes
- 4 (20 gauge, 3.8 cm) needles
- 10 pieces of 40 cm nylon cord
- 1 suction bulb and one 30 cm length piece of plastic tubing
- 1 feeder (Starcraft, Star Manufacturing Co., Oklahoma City, OK)
- 1 plastic waterer (Staley Livestock Products, A.E. Staley Mfg. Co., Decatur, IL)
- 4 (3.8 liter) plastic bottles with plastic caps

These baskets, plus 12 folded cloth towels, 2 pair of cloth gloves, 5 (300 ml) plastic milk bottles and 5 rubber nipples were placed inside of two sterilizing cylinders on a 22 x 61 cm perforated stainless steel sheet located at the bottom of the cylinder. This stainless steel sheet allowed circulation of steam in the bottom of the cylinder for maximum steam penetration and provided a flat surface to place equipment. All items were carefully packed within the cylinders to provide adequate steam circulation and glass screw cap vials were placed upside down to prevent steam from condensing.

When all materials were properly placed in the cylinders, the open end of the cylinder was covered with a sheet of .001"-1 mil mylar film (Standard Safety Equipment Company, Palatine, IL) and held in place with an "O" ring placed approximately one inch behind the retaining edge of the cylinder. After the mylar film was pulled tight and wrinkles were removed, the mylar film was attached to the cylinder using two wraps of mylar tape on the side of the "O" ring toward the open end of the cylinder. The first wrap completely sealed the edge of the mylar film to the edge of the cylinder while the second wrap

overlapped the first wrap by one half toward the "0" ring. Excess mylar film was then trimmed as close to the "0" ring as possible, away from the open end of the cylinder. The "0" ring was then removed and three additional wraps of mylar tape were applied, followed by four wraps of yellow vinyl tape to completely cover the mylar so that none was exposed. In order to insure that no creases, wrinkles and bubbles developed during tape application, tape was applied with a strong, even tension at all times.

The sealed cylinders, loaded with the previously described equipment, were placed in the autoclave (American Sterilizer Co., Erie, PA) and were ready to be sterilized. The autoclave door was closed and sealed tightly and a vacuum of 65 cm of mercury was imposed for 20 minutes. After the expired 20 minutes, the vacuum pump was turned off and the vacuum valve was closed prior to opening the steam valve. All materials located within the cylinders were sterilized at 121°C for at least one hour. At the completion of the sterilization process, steam was exhausted from the autoclave and another vacuum of 65 cm of mercury was imposed for 20 minutes to dry the contents of the cylinders. Finally, the vacuum was turned off and the autoclave door was opened, upon reaching neutral pressure, to allow the cylinders to cool before preparing to transfer cylinder contents into the isolators. After cooling, the mylar film on the cylinder should remain tight. If it were not tight, there likely was a leak in the mylar film or a leak in the tape attaching mylar film to the cylinder, and the autoclaving process was repeated after replacing the mylar film and tape.

I. Final Preparation and Sterilization of the Isolators

Plastic covers were placed on the presterilized air intake filters and secured with M-52 clamps followed by wrapping with yellow

vinyl tape. Both air intake and exhaust filters were attached to the isolator base with screws, and both filters on each isolator were attached to the isolator nipples and secured with M-16 clamps. Rubber stoppers were tightly inserted in the bottom of the stainless steel tank of each isolator and sealed with mylar film, held in place with yellow vinyl tape on the outside bottom of the tank.

Next, all items which could be sterilized with peracetic acid (i.e. any item with a smooth surface which could be completely exposed to peractic acid) were placed in the isolator. These items included:

- 2 inside plastic caps
- 2 rubber bands
- preautoclaved wire mesh floor
- 2 aluminum storage pans
- 1 long, blunt nosed forceps

Using a graduated cylinder, 20 ml of concentrated peracetic acid (40% aqueous peracetic acid obtained from F.M.C., Speciality Chemicals Division, Buffalo, NY) was added to 380 ml distilled water and a few flakes (approximately 0.1%) of Nacconal detergent (triethanolamine alky arye sulfonate) were added as a wetting agent. This 2% peracetic acid solution was then poured into a plastic spray bottle, mixed and attached to the spraying apparatus linked to a compressed nitrogen gas cylinder with a plastic hose. Compressed nitrogen gas was used because of its inert properties and as a source of pressure for applying the peracetic acid solution. The spray bottle containing peracetic acid was placed inside of the isolator and the flexible plastic tube connecting the gas cylinder to the spraying apparatus was inserted through a vinyl stopper located in one of the nipples on the outer cap

of the entry port. The outer cap was sealed to the entry port by five overlapping wraps of yellow vinyl tape.

Two operators entered their arms into the rubber gloves, one on each side of the isolator. One operator sprayed the gloves and equipment on the opposite side of the isolator while the other operator manipulated the gloves and equipment to insure adequate coverage by the acid. When the entire inside of the isolator and its contents were saturated with acid and the vinyl film was fully inflated, the spray bottle was placed in the entry port for removal from the isolator. The inside cap was then placed on the inside of the entry port and sealed with a rubber band. Next, the outside cap was removed and the spray bottle was detached from the plastic tubing, and reattached when the plastic tubing was pulled through the nipple of the outside cap. Finally, the entry port was sprayed and resealed by pulling the outside cap over the outside of the entry port, wrapped with yellow vinyl tape and nipples were plugged with vinyl stoppers after the entry port was sprayed and inflated.

Isolators remained sealed for at least one hour. In the meantime, the blower hose from the air pump was attached to the nipple on the air inlet filter with an M-52 clamp. After the required time had elapsed, one operator entered the rubber gloves and punctured the mylar on the air inlet and outlet filters. The operator then removed his hands from the gloves, started the blower and adjusted the air intake and exhaust flow clamps. Isolators were dried by the circulating air and were ready to receive animals at the end of 48 hours.

J. Transfer of Materials from Cylinder to Isolators

In order to transfer the required equipment into the isolator (as well as transferring autoclaved feed and any other equipment needed

later in the trial) the outer cap of the entry port was removed and the entry port was wiped dry with a paper towel to remove any debris from the previous transfer. A vinyl transfer sleeve was then attached to the entry port of the isolator and over the taped section of the cylinder by wrapping yellow vinyl tape around each component three times. Four hundred milliliters of the previously described 2% peracetic acid solution was mixed and some of the solution was sprayed through a nipple located in the center of each side of the vinyl sleeve until the sleeve was fully inflated and the entire inside of the exchange area was thoroughly saturated with peracetic acid. Vinyl stoppers were then inserted into the nipples and taped with yellow vinyl tape to hold stoppers in place when the inside cap was removed during the transfer process. After a 30 minute sterilization period following spraying of the exchange area, the operator entered the gloves of the isolator and removed the inside cap, while the second operator pushed the cylinder up to the entry port of the isolator. The operator working inside of the isolator then punctured the mylar film at the mouth of the cylinder with a pair of blunt end forceps. Materials present in the cylinder were then transferred into the isolator through the vinyl sleeve and entry port. When all of the cylinder contents were transferred, the inner cap was replaced and a rubber band was stretched around the inner cap to secure it. The transfer sleeve was then removed, the entry port was resprayed with peracetic acid and the outer cap was replaced and taped as previously described. Finally, peracetic acid was sprayed into the nipple of the outer cap until the inside cap was inflated. Vinyl stoppers were then inserted into the nipples and sealed with yellow vinyl tape.

When all of these preparations were completed, these isolators were ready to receive the baby pigs.

II. PREPARATION OF TRANSFER AND SURGICAL ISOLATORS FOR GERM-FREE RESEARCH

The comprehensive discussion of the steps involved in preparing the rearing isolators previously described also applied to preparation of the transfer and surgical isolators with a few of the following exceptions.

A. Description of Isolators

1. Transfer Isolator

The transfer isolator measured approximately 152 x 102 x 102 cm and was constructed entirely of flexible plastic with a 46 cm diameter entry port on one end. The vinyl isolator was placed on a paper covered, 1.2 cm thick plywood sheet located on top of a steel cart with wheels for ease of manipulation. The stainless steel entry port was attached to the plywood sheet by a bracket to give stability to the isolator. Air intake and exhaust nipples were located in the rear of the isolator for attachment to air filters when needed.

2. Surgical isolator

A vinyl surgical isolator measuring approximately 150 x 102 x 102 cm was attached to a steel rod frame which gave stability and was used to suspend the isolator with rope and pully from the ceiling of the surgical room. A 46 cm diameter entry port attached to the suspended frame was located at one end so that once pigs were removed from the uterus, they could be readily passed into the transfer isolator. A nipple was located above and behind the entry port and contained electrical wires embedded in a vinyl stopper for

electrocautery use during surgery. In addition, a 30 cm port containing a fiber glass ring with rubber bands attached and covered with a vinyl cap was located at the bottom of the isolator, which was eventually attached to the sow's skin for the surgical operation. Air intake and exhaust nipples were located in the rear of the vinyl isolator for attachment to air filter and outlet trap when needed.

B. Cleaning and Preparation of Isolators for Sterilization

Both transfer and surgical isolators were cleaned and checked for leaks in the same manner as described for the rearing isolators.

1. Transfer isolator

Four stainless steel cages were cleaned, covered with brown wrapping paper and sealed with autoclave tape for sterilization. These cages were autoclaved, as previously described, for at least one hour at 250°C. After cages were cooled, they were placed inside of the transfer isolator on a plastic mat and later the paper was removed before spraying with peracetic acid. These cages were later used to sort and hold the baby pigs when being transferred from the surgical isolator to the rearing isolators.

2. Surgical isolator

The 30 cm cap at the bottom of the surgical isolator was put in place after the fiberglass ring containing rubber bands was put in place for the surgery. The surgical isolator was ready for sterilization when a plastic mat and long, blunt end forceps were placed in the isolator.

C. Sterilization of Isolators and Preparation for Surgery

Both the transfer and surgical isolators were sprayed with 2% peracetic acid with the same procedure as described previously for the

rearing isolators. Sterilized, dry cloth towels and all surgical equipment were then transferred from cylinders to the surgical isolator. Next, the transfer isolator was attached and sealed to the surgical isolator by a vinyl transfer sleeve and the glove was sterilized with peracetic acid.

III. SURGICAL TECHNIQUE FOR OBTAINING GNOTOBIOTIC PIGS

A pregnant sow was anesthetized on the 112th day of gestation by injecting 15 ml of lidocaine hydrochloride (Lid-o-cain 2%, Butler Co., Columbus, OH) in the epidural space at the lumbosacral articulation, using an 18 gauge, 15.25 cm needle. This was followed by an intramuscular injection of 5 ml of promazine hydrochloride (Sparine, Wyeth Laboratories, Inc., Philadelphia, PA).

The sow was secured on the surgical table in lateral recumbency with the left side uppermost. The left flank area was then prepared for surgery. The hair was clipped and shaved, and the skin was scrubbed with Betadine Surgical Scrub (Purdue Frederick Co., Norwalk, CT). Betadine Solution (Purdue Frederick Co., Norwalk, CT) was then applied to the site, followed by 70% ethyl alcohol. Chloroform was next applied to the skin, and the area was dried with sterile towels.

The skin of the surgical site and the vinyl film covering the 12-inch port in the floor of the surgical isolator were sprayed with Vi-Drape Adhesive (Parke-Davis Co., Detroit, MI). When the adhesive was dry, the vinyl film of the isolator was placed over the surgical site, and the 12-inch port was secured in place with three cords. Each cord was tied to one side of the port and extended around the abdomen of the sow before being tied to the other side of the port.

Working through shoulder-length rubber gloves in either side of

the surgical isolator, the operators made a 9 inch incision through the vinyl film covering the 12-inch port located at the bottom of the surgical isolator and the skin of the sow with a thermocautery unit (Thermo Cautery, National Electric Instruments Div., Englehard Hanovia, Inc., Elmhurst, L.I., N.Y.). As the incision was made, the cut edges of the vinyl film and the skin and the handles of the forceps were secured to rubber bands extending through holes in the circumference of the 12-inch port. The forceps were placed approximately 1 inch apart throughout the length of the incision. The remainder of the abdominal well (muscle, fascia, and peritoneum) was incised with scissors.

A loop of the gravid uterus containing a fetus was pulled through the incision into the interior of the isolator. The uterine wall over the dorsal aspect of the head and neck of the fetus was incised with scissors, and the pig was pulled into the interior of the isolator. An umbilical clamp (Hollister Double-grip Disposable Cord-Clamp, Hollister, Inc., Chicago, IL) was placed on the umbilical cord near the body wall, and the cord was cut approximately one inch below the clamp. The pig was then passed into an attached transport isolator where it was dried and placed in a cage. The loop of the uterus was then passed back into the abdominal cavity of the sow, another loop was brought through the incision, and the process was repeated. When all the pigs had been removed, the sow was euthanatized by the intravenous injection of an overdose of sodium pentobarbital (Sodium Pentobarbital Injectable, Butler Co., Columbus, OH).

Following delivery of all the pigs, the inside cap was placed on the entry port of the transport isolator, and the sleeve connecting the

surgical and transport isolators was removed. The outside cap was placed on the entry port of the transport isolator, and the space between the two caps was sprayed with a solution of 2% peracetic acid.

The transfer isolator containing the baby pigs was moved to the room containing the rearing isolators and attached to each individual isolator by a plastic sleeve. The space within the plastic sleeve between the two isolators was sprayed with 2% peracetic acid for each transfer. Pigs were removed from cages and placed in rearing isolators after removing the inside cap. Pigs were evenly distributed between the rearing isolators based on sex, size and number. When all pigs were placed in their respective isolators, the outside caps were replaced and sealed after the entry ports were sprayed with 2% peracetic acid solution.

IV. PREPARATION AND TRANSFER OF MILK, WATER AND EXPERIMENTAL DIETS INTO REARING ISOLATORS

A. Preparation and Transfer of Milk

Twenty-354 ml cans of evaporated milk (Carnation Company, Los Angeles, CA) were prepared for transfer into each isolator by first removing all labels, glue and rust. Damaged cans were not used because of potential contamination. Each can was then rolled in 70% ethanol solution and placed under a fume hood to dry. When needed, cans were thoroughly sprayed with 2% peracetic acid solution and stacked on a stainless steel tray in the entry ports of the isolators after removing the outside cap. When the outer cap was replaced and sealed, the entry port was resprayed with 2% peracetic acid solution. All cans were transferred into isolators following a 30 minute time period after spraying. Any empty cans were then placed into the entry port for removal from the isolator.

B. Preparation and Transfer of Water

Six-2000 ml glass Pyrex flasks (American Sterilizer Company, Erie, PA) were filled with tap water and sealed with rubber disposable closures (American Sterilizer Company, Erie, PA). All sealed flasks were placed in the liquid autoclave (Wilmot Castle Co., Rochester, NY) and sterilized for at least one hour at 121°C. Flasks were then cooled and wiped with a paper towel saturated with 70% ethanol to remove any oil or debris. Two flasks were then thoroughly sprayed with 2% peracetic acid solution and placed in the entry port of each isolator. Entry ports were capped and sealed as previously described. After a 30 minute time period allowed for sterilization, the flasks were transferred into the isolator by removing the inside cap and water was poured into plastic storage containers for later use. Empty water flasks were then placed into the entry port and ready to be removed from the entry port after replacing the inside cap. These flasks were filled with water and autoclaved to repeat this process as needed.

C. Preparation and Transfer of Experimental Diets

An amount of experimental diet, in meal form, was placed in a labeled aluminum pan and weighed to measure 2.5 kg. This amount of diet measured 2.54 cm thick in the bottom of the pan which allowed satisfactory sterilization of the diet which was previously determined by incubating Kilit ampules (American Scientific Products, Industrial Division, McGaw Park, IL 60085) containing Bacillus sterothermophilus spores, subjected to this sterilization procedure. Prior to putting the diet in the pans, small holes (2 cm apart) were drilled in the bottom of the pens so that adequate steam penetration could be accomplished without the diet sifting out of the pan. Two 2.5 cm diameter metal pipes were attached to the top of one pan with nylon

cord to allow a 2.5 cm space between the bottom and top pan in each cylinder for adequate steam circulation. Pans were positioned in the center of the sterilizing cylinder. Cylinders were then sealed with mylar film, as previously described, and placed in the autoclave (American Sterilizer Co., Erie, PA). After a 20 minute, 65 cm of mercury vacuum, diets were sterilized by steam for 25 minutes at 121°C. Another 20 minute vacuum was then imposed to dry the diets. Cylinders were allowed to cool before transfer of diets into the isolators. Once diets were transferred into the isolators, the dried cooked slab of feed was broken into smaller pieces and reground using a mortar and pestle before putting into feeders.

V. MONITORING ISOLATORS FOR GERM-FREE STATUS

Standard aerobic and anaerobic microbial culture procedures were used to verify germ-free status of the pigs in the rearing isolators on day 6, 21, 39 and 49 of the experiment. Swabs contained in presterilized screw cap vials located in each isolator were used to sample the feces from the rectal area of one pig in each isolator and the feeding equipment within each isolator. Once the swabs were inoculated with feces and feed, they were replaced inside of the screw cap vials, which were sealed tightly, and passed out of the isolator through the entry port. The entry port was sprayed with 2% peracetic acid to resterilize the entry port following removal of the vials as previously described. These vials containing the swabs were then taken to the laboratory and media were inoculated with the swabs.

Three types of media were used to assess the presence of aerobic and anaerobic microbial growth. Thioglycolate broth was used to assess the presence of anaerobic organisms and plates were incubated at three

temperatures (25°, 27° and 56°C) when inoculated with a swab from an isolator. Plates were examined daily for 5 days and weekly thereafter for 3 weeks for presence of anaerobic bacterial growth. Blood agar plates were inoculated with swabs to determine the presence or absence of anaerobic and aerobic bacterial growth when plates were incubated at 25°, 37° and 56°C. Both aerobic and anaerobic plates were checked daily for 3 days and were discarded if negative after this time period. If there was microbial growth present in these plates, organisms were allowed to grow and were then identified. Mycoplasma broth was the third culture media used to assess the presence or absence of mycoplasma organisms when plates were inoculated with swabs and incubated at 37°C for 3 days. After 3 days, 0.1 ml of mycoplasma broth was transferred to plates containing mycoplasma agar and were incubated in jars at 37°C for 1 week. Plates were examined microscopically for growth and were discarded if negative.

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