IMPACT OF VIRGINIAMYCIN ON THE DEVELOPMENT OF INTESTINAL STRUCTURE AND FUNCTIONAL CAPACITY IN GROWING PIGS

BY

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THESIS

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Abstract

Antibiotics have been used in animal feed to promote weight gain, improve feed efficiency, and control disease since the 1950s. Concerns about antibiotic resistance have increased since the widespread use of in-feed antibiotics began, but a clear connection between antibiotic use in animals and adverse human health effects has not been established. Understanding the mechanisms behind antibiotic growth promoters is an important step in determining the safety of in-feed antibiotics. Our study tested the hypothesis that diets with virginiamycin would increase intestinal surface area and nutrient processing capacity compared with diets without virginiamycin. Littermate barrows with similar body weight (n=72; 23.0±1.3 kg; 9 weeks of age) were randomized to pens and pens were randomized to one of three treatment groups (0, 11, or 27.5 mg virginiamycin/kg corn-soybean diet). Within each pen, pigs were further randomized to a time point (7 or 14 days). Intestinal samples were obtained for assessment of gross morphology, histomorphology, immunohistochemistry, biochemical measurements, disaccharidase activity, and nutrient and ion transport. Analysis of variance was used to statistically analyze the data using the Mixed Model of SAS fitted with a split plot design (treatment as the whole plot and time as the subplot). Preplanned contrasts between virginiamycin and control groups were completed. Pigs fed diets supplemented with virginiamycin gained an average of 10% more weight per day with about 12% better feed conversion compared with control. Virginiamycin impacted intestinal functional capacity to a greater extent than structural indices. Glutamine and arginine transport were increased 2-fold in the jejunum of virginiamycin fed pigs compared with control (p=0.010, p=0.046, respectively). Ileal carbachol induced chloride secretion was lower at day seven, but it increased over time with virginiamycin resulting in increased secretion at day fourteen with virginiamycin compared with

control ($p=0.017$). Virginiamycin directly impacted proximal gut nutrient transport with a more delayed response in the distal gut by providing protection against loss in nutrient transport and secretory capacity. Increased nutrient transport was observed without increases in absorptive surface area indicating that increased functional capacity may be a mechanism by which antibiotic promote growth. Increasing our knowledge on the mechanisms behind antibiotic growth promoters will help direct government policy on antibiotic use in animals.

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Chapter 1: Introduction and Literature Review

Antibiotics have been used in animal feed to promote weight gain and feed efficiency for over 60 years. Antibiotics can be defined as naturally occurring substances produced by yeasts, molds, and other microorganisms that, at low concentrations, suppress or inhibit the growth of microorganisms (Cromwell, 2002). In 1949, it was discovered that "mash" fermented with *Streptomyces aureofaciens* increased the growth of chickens (Stokstad et al., 1949). The fermentation product was found to contain the antibiotic chlortetracycline, which was identified to be responsible for the growth response. Similar growth responses were demonstrated in pigs soon after (Cunha et al., 1949; Jukes et al., 1950; Lepley et al., 1950; Luecke et al., 1950). Further developments revealed a few parts per million (ppm) of antibiotics in diets were effective (Jukes, 1955) and, thus, began widespread use of in-feed antibiotics for growth promotion, feed efficiency, and disease control in livestock and poultry.

Since the widespread use of antibiotics began, there has been increased concern about the development of antibiotic resistance. Although it has been debated for years, in-feed antibiotics have been proposed to result in resistant bacteria with the potential to be transferred to human pathogens. The concern of antibiotic resistance has led to political involvement all over the world including the banning of in-feed antibiotic use in the European Union. Political focus in the United States has increased since the banning of antibiotic growth promoters in the European Union. Antibiotics continue to play a large role in animal health and production. Discussions of banning such substances have been increasing and it is important to understand the most safe and effective use of growth promoting antibiotics. An understanding of the mechanism(s) behind antibiotic growth promoters is essential to better assess antibiotic resistance and its potential impact on human health.

Antibiotic Growth Promoters

Antibiotics have been used as growth promoters for years because of the improvement in animal weight gain, feed efficiency, and performance. Antibiotics used for growth promotion are given at sub-therapeutic levels and are usually given for an extended period of time. Research demonstrating the effectiveness of antibiotics as growth promoters is well documented. Cromwell (2002) reported values from data compiled up to 1985 on antibiotic-feed pigs in reviews by Hays (1981) and Zimmerman (1986). Dietary supplementation with antibiotics improves average daily gain by an average of 16.4% and feed per unit of gain by 6.9% during the starter period (Cromwell, 2002). Antibiotics fed during the grower period resulted in an average increase of 10.6% in average daily gain and a 4.5% decreased in feed per unit of gain (Cromwell, 2002). Improvements averaged over the entire grower-finisher period were 4.2% for average daily gain and 2.2% for feed per unit of gain (Cromwell, 2002; **Figure 1.1**). In-feed antibiotics also improve sow performance and can increase farrowing rate up to ten percent (Zimmerman, 1986). Currently, over 81% of pig production sites for grower-finisher pigs use in-feed antibiotics and almost 33% of sites use in-feed antibiotics continuously (USDA, 2007).

Virginiamycin and Swine Performance

Several antibiotics are used for growth promotion in animals, but, for the purpose of this review, the focus will be on virginiamycin use in swine. Virginiamycin (trade name: Stafac®) has been used in animals as a growth promoter for many years. Virginiamycin was approved in 1975 for use in turkeys, swine, cattle, and chickens in the United States (FDA, 2004). Virginiamycin has been shown to improve average daily gain by 11%, 10.7%, and 5.7% in starter, grower, and finisher pigs, respectively, and feed per unit of gain by 5%, 6.6%, and 3.3%,

respectively (Hays, 1981). Zimmerman (1986) also reported improvements with virginiamycin in his review. In that report, virginiamycin improved average daily gain by 8% and feed per unit of gain by 3.6% in starter pigs and average daily gain by 2.5% and feed per unit of gain by 1.4% in grower-finisher pigs (Zimmerman, 1986).

Virginiamycin has continued to be an effective growth promoter in pigs. Lewis and Giesemann (1991) reported significant improvements in daily gain (5.8%) and feed efficiency (3.5%) when virginiamycin was feed at 11 mg/kg diet to grower pigs for six weeks but no significant differences when fed at 5.5 mg/kg diet for nine weeks during the finishing period. Schinckel (1993) found significant improvements in daily gain and feed conversion during growing-finishing periods when pigs were fed diets supplemented with 20 mg/kg of virginiamycin during the grower period and 10 mg/kg during the finisher period. Virginiamycin improved daily gain by 7.7% and 2.9 to 3.5% in grower and finisher periods, respectively, and improved feed per unit of gain by 6.5% and 2.4 to 3.1% in grower and finisher periods, respectively (Schinckel, 1993). Virginiamycin improved feed conversion in grower (1.4%; Gaines et al., 2005) and finisher pigs (4.6 to 10.4%; Gramm et al., 2006) when supplemented at 11 mg/kg diet. Gramm and colleagues (2006) also reported a trend toward improved weight gain (6.4 to 12%) in finisher pigs fed a diet supplemented with 11 mg/kg of virginiamycin. Percent improvements in weight gain are greater than those of feed per unit of gain. This may suggest that feed intake is increased in pigs consuming antibiotics, but improvements in weight gain and feed efficiency are observed without increases in daily feed intake (Gramm et al., 2006; Harper & Kornegay, 1983; Lewis & Giesemann, 1991; Schinckel, 1993; Stahly et al., 1980).

Dietary supplementation with virginiamycin also improves sow performance. Virginiamycin increases sow body weight at each farrowing, decreases sow weight loss from

farrowing to weaning, and decreases time between weaning to conception (Kantas et al., 1998; Kyriakis et al., 1992). Kyriakis and colleagues (1992) observed increases in milk fat content in sows fed virginiamycin. Virginiamycin, fed at levels of 20, 40, and 60 mg/kg diet from six months of age through three breeding cycles, increased both blood cholesterol and total lipids concentration in sows during each pregnancy, farrowing, and weaning and improved milk quality during the third lactation by increasing fat, protein, lactose, and total solids content (Alexopoulos et al., 1998). Virginiamycin fed at these three levels also increased litter size, birth weight, number of piglets weaned, and piglet weaning weight (Kantas et al., 1998; Kyriakis et al., 1992). Virginiamycin is effective in market pigs and at other stages of swine production.

Antibiotic Resistance

Concerns about bacterial resistance began to arise shortly after the performance benefits of in-feed antibiotics were discovered. Resistance became a new concept when descriptions of it were published in 1960 in Japan (Akiba et al., 1960). The increased concern about development of resistance started a movement against use of antibiotics in animal feed around 1967 (Jukes, 1972). Concerns about adverse human health effects associated with antibiotic use in animals were brought about worldwide when a report by The Joint Committee on the Use of Antibiotics in Animal Husbandry and Veterinary Medicine was issued to the British Parliament (Chowdhury et al., 2009). That report, known as the Swann Report, proposed that antibiotics used for growth promotion should be limited to those that make a significant economic difference in livestock production, have little or no application as therapeutic agents in humans or animals, and/or do not impair efficacy of a prescribed therapeutic drug through development of resistant strains (Swann, 1969). It was almost 30 years after the movement began before there was global

interest or surveillance on the public health impact of antibiotic use in animals (WHO, 1997). Research has shown an increase in antibiotic resistance in humans and the plausibility of transfer of resistant bacteria from animals to humans; however, substantial data are lacking on how much this is a consequence of antibiotic use in animals and if it has a significant effect on human health.

Government Regulations

World Organizations

A 1969 Report of the Joint Committee on the Use of Antibiotics in Animal Husbandry and Veterinary Medicine, commonly known as the Swann Report, reported that antimicrobial use in food-producing animals poses a hazard to human and animal health. In the report, a recommendation was made to only use antibiotics that are not used as therapeutic agents in humans or animals in animal feed (Swann, 1969). In 1970, a FDA task force report recommended antimicrobial drugs used in human medicine should meet certain guidelines to be used for growth promotion (FDA, 1970). This report led to a code of federal regulations that required companies to submit research demonstrating their product does not promote bacterial drug resistance (FDA, 2000). The FDA proposed to withdraw approvals for sub-therapeutic uses of penicillin and tetracycline in animal feed due to safety issues; however, reports from the National Academy of Sciences in 1980 and the Institute of Medicine in 1988 showed there was limited epidemiological research and a lack of direct evidence to either prove or disprove the safety of these antibiotics (FDA, 2012a).

Almost a decade later, the World Health Organization (WHO) issued a report in which they concluded all uses of antimicrobials led to selection of resistant bacteria and stated a low

dose over a longer period of time is more likely to lead to resistance than higher doses over a short period of time (WHO, 1997). The WHO also reported antibiotic resistance has adverse effects on human health and steps should be taken towards a more responsible use of antibiotics in animals (WHO, 1997). The United States government accountability office (GAO) published a report in 1999 stating that the extent to which bacterial resistance, as a result of antibiotic use in animals, has led to human illness or death could not be estimated at that time (GAO, 1999).

The Food and Agriculture Organization (FAO), the World Organization for Animal Health (OIE), and WHO concluded in 2003 there was accumulating evidence of adverse human health effects due to resistant bacteria from antimicrobial use in animals, the food borne route is the major route of transmission, and the consequences are most severe when the antibiotics are critical to humans (FAO, 2003) Also in 2003, the Institute of Medicine (IOM) recommended the FDA ban all antibiotics used for growth promotion that are in the same class as antibiotics used in human medicine (IOM, 2003).

In 2004, FAO, OIE, and WHO released a second joint report recommending the establishment of a list of antibiotics deemed critically important to human health. The report also suggested that good agricultural practices could eliminate the need for in-feed antibiotics and expressed the necessity for resistance surveillance systems (FAO, 2004). A 2004 GAO report was similar to the second joint report by the FAO, OIE, and WHO. GAO recommended the FDA expedite risk assessments of critically important antibiotics to determine if use of those antibiotics should be prohibited in animals and that the Secretary of Agriculture and Health and Human Services develop a plan for collecting data on antibiotic use in animals (GAO, 2004). The American Academy of Microbiology published a report in 2009 stating that “resistance is a natural phenomenon that cannot be eliminated” and recommended an approach to find new ways

to deal with current resistance and try to control future resistance by prudent and responsible use of antibiotics in animals (American Academy of Microbiology, 2009). In 2011, the WHO wrote a report which included recommendations to eliminate the use of antibiotics for growth promotion and require a veterinary prescription for antibiotic use while only using antibiotics deemed critically important for humans when justified (WHO, 2011).

Current Documents in the United States

The FDA has written Guidance for Industry (GFI) documents regarding antimicrobial drug use in food-producing animals. These guidance documents contain non-binding recommendations and are intended to help the industry or sponsors develop products which are considered safe in regards to human health. The FDA currently uses GFI #152 regarding approval of new antimicrobial drugs for use in animals. The FDA must determine that the drug is safe and effective for its intended use in the animal and that it is safe with regard to human health (FDA, 2000). The FDA defines a drug as "safe" if there is reasonable certainty of no harm to human health from the drug's use in food-producing animals (FDA, 2003). Responsibility falls upon the sponsor to provide sufficient evidence of the drug's safety for the FDA to review and either approve or deny its use.

The recommendations from the WHO in 2011 are reflected in the most recent FDA publications. The FDA's Center for Veterinary Medicine published a final guidance document on April 13, 2012 that describes the rising concern for antimicrobial resistance in humans and steps which can be taken to reduce this risk from antibiotic use in animals (FDA, 2012a). GFI #209 recommended two additional principals about the appropriate or judicious use of medically important antimicrobial drugs in food-producing animals which were not included in GFI #152.

These principles include voluntary adoption of the use of these drugs be limited to those uses that are considered necessary for assuring animal health and that antimicrobial use include veterinary oversight or consultation (FDA 2012a).

Antimicrobial drugs for use in feed or water approved prior to 1993 may have been approved for over-the-counter use. Most antimicrobial drugs for use in food-producing animals approved after 1993 must have a veterinary prescription or veterinary feed directive (FDA, 2012b). In the draft of GFI #213, the FDA recommends drug sponsors voluntarily revise the conditions of use for drugs approved prior to 1993. The FDA states their approach in the guidance document is based on the potential of increased bacterial exposure to antimicrobial drugs leads to a greater risk of bacterial resistance (FDA, 2012b).

Government regulation outside the United States

The recommendations are similar from the "Swann Report" in 1969 to the FDA's GFI in 2012 (**Table 1.1**). Several countries outside the United States have eliminated the use of antibiotics for growth promotion. Sweden banned the use of all antibiotics for growth promotion in animals in 1986 (Butaye et al., 2003). Norway prohibited the use of virginiamycin in 1998 and Denmark banned it in 1999 (Butaye et al., 2003). The European Union banned avoparcin in 1997, bacitracin, spiramycin, tylosin, and virginiamycin in 1999, and passed regulation for the phasing out of the use of all antibiotics for growth promotion in farm animals in 2003 (Regulation 1831/2003). The European Union ban on all antibiotics used for growth promotion went into effect on January 1, 2006.

These bans were taken as precautionary steps to the possible risk of antibiotic use in animals leading to adverse human health consequences. Attention has focused mainly on

consequences of using of antibiotic growth promoters, but little attention has been given to the contrary: what are the consequences of discontinuing antibiotic use for growth promotion? Some studies have indicated that bacterial illness rates and resistance levels in humans have increased since the ban in Europe (Hayes & Jensen, 2003). Cox (2005) developed a Rapid Risk Rating Technique (RRRT) which is a multiplicative, top-down approach that starts with data on the number of clinical cases per year and assesses the estimated fraction that would be prevented by interventions. This technique was developed to estimate the impact that antibiotic use in animals has on rates of adverse human health effects; it can be viewed as a risk/benefit analysis of continued use of antibiotics (Cox, 2005). Based on the results from the RRRT, Cox concluded that the expected human health benefits from continued virginiamycin use are much larger than the expected human health risks (Cox, 2005). This risk assessment was based on virginiamycin use in chickens but is estimated to amplify the results if cattle and pigs were included.

Resistance to Streptogramins

Virginiamycin belongs to the antibiotic class Streptogramins. Quinupristin-dalfopristin (QD; trade name: Synercid®) is a semi-synthetic antibiotic which belongs to the same class as virginiamycin. Synercid® was approved for use in the United States in 1999 for treatment of *Enterococcus faecium* infections in humans and *Staphylococcus aureus* and *Streptococcus pyogenes* skin and soft tissue infections (FDA, 2004). Vancomycin is the antibiotic most frequently used to treat *E. faecium* infections in humans (Cox, 2005). In cases where *E. faecium* expresses vancomycin resistance genes, QD may be used to treat vancomycin-resistant *E. faecium* (VREF; Cox, 2005). QD is considered critically important for human medicine by the

WHO (WHO, 2007) and highly important by the FDA (FDA, 2003). Concerns exist that animals given virginiamycin may produce Streptogramin resistant *E. faecium* (SREF) strains that will colonize or transfer genes to enterococci of the human intestine and lead to treatment failures (Bafundo et al., 2003).

Streptogramin resistance was first reported in staphylococci in 1962 (Butaye et al., 2003). The Center of Veterinary Medicine conducted a virginiamycin risk assessment investigating Streptogramin resistance in *E. faecium* attributable to the use of Streptogramins in animals (FDA, 2004). The FDA began the risk assessment in 2000 and released a draft in 2004, but a final document was never published. The FDA's preliminary conclusions in the draft included: (1) SREF has been found in poultry and swine from the United States and Europe and appears to be related to the usage of virginiamycin on farms; (2) resistant bacteria has been found on food animal products from retail sources; (3) resistance occurs at low frequencies in the non-hospitalized population; (4) transfer of resistance from animal to human via food is biologically plausible but the extent cannot be currently estimated; and (5) SREF isolates from animals are associated with high level resistance but not in humans (FDA, 2004). The FDA also calculated risk assessments for the population and reported that if 10% of resistant-*E. faecium* is a result of virginiamycin use in animals, a person in the United States has an estimated 14 in 100 million to 7 in 1 billion chance of having impaired Synercid® therapy. If 100% of resistant-*E. faecium* result from virginiamycin use in animals, the estimated number of impaired Synercid® therapy cases would increase 10-fold (FDA, 2004).

Research investigating the prevalence of virginiamycin-resistant bacteria in swine in the United States is limited. European studies have demonstrated resistance to streptogramins in up to 60% of isolates from poultry, swine, and cattle, whereas, Australian studies have observed

only a 10 to 20% rate of resistant bacteria (Kieke et al., 2006). Some studies have shown a decrease in virginiamycin-resistant *E. faecium* in broilers in Denmark after the banning of antimicrobial growth promoters (Aarestrup et al., 2001; Emborg et al., 2003). Resistance may occur within the animal and in isolates obtained from the animal, but it can also occur in water run-off from production farms. Sapkota and colleagues (2007) observed a higher percentage of virginiamycin resistant enterococci isolated from surface waters down-gradient from a swine facility compared with up-gradient from the facility.

The risks of either banning or continued use of virginiamycin on human health have not been fully evaluated. While there are indications that antibiotic use in animals could potentially have adverse human health effects, there is not a clear, definite connection between antibiotic use in animals causing critical human illness. It is vital to keep investigating the relationship between antibiotic use in animals and human health. An important component is to better understand the mechanisms by which antibiotics promote growth.

Streptogramins Mechanism of Action

Virginiamycin belongs to the class Streptogramins that are naturally occurring compounds produced predominately by members of the genus *Streptomyces* (FDA, 2004). Streptogramins include two types, A and B, and virginiamycin is a mixture of both components termed virginiamycin M and virginiamycin S, respectively. Individually the A and B components only cause bacteriostasis, but together they are bactericidal (Abou-Youssef et al., 1979; Butaye et al., 2003). Virginiamycin binds to bacterial 23S rRNA of the 50S ribosomal subunit, interfering with peptidyltransferase activity and inhibiting protein synthesis, resulting in cell death (Cocito et al., 1997; FDA, 2004). The A component inhibits the elongation phase in

ribosomal assemblage of the protein by interfering with peptidyltransferase function and triggers a conformational change in the ribosome which increases binding affinity for component B (Bouanchaud, 1997; Cocito et al., 1974). The B component prevents extension of polypeptides and induces detachment of incomplete protein chains (Chinali et al., 1988). Streptogramins have a narrow-spectrum including gram positive bacteria (mainly staphylococci, streptococci, and enterococci) and some gram negative cocci. Most gram negative bacteria are naturally resistant due to impermeability of their cell wall (Butaye et al., 2003; Goto et al., 1992).

Antibiotic Modes of Action for Growth Promotion

The proposed mechanisms by which antibiotics work as growth promoters are mainly described under three categories: metabolic, nutritional, and disease-control effects (**Figure 1.2**). Metabolic processes that are directly influenced by antibiotics fall within the metabolic effect category. Research has shown that antibiotics can increase water and nitrogen excretion, liver protein synthesis, and gut alkaline phosphatase and decrease fatty acid oxidation, phosphorylation reactions, and bile degradation products (Braude & Johnson, 1953; Brody et al., 1954; Gaskins et al., 2002; Hash et al., 1964; Moser et al., 1980; Weinberg, 1957).

The nutrition effect includes several proposed mechanisms (**Table 1.2**). One mechanism is that antibiotics can cause a shift in the microbial population of the gut, which may increase the availability of nutrients to the host. A second proposed mechanism is the protection against thickening of the intestinal epithelium secondary to an immune response to intestinal insult, resulting in protection of nutrient absorption capacity (Braude et al., 1955; Henderickx et al., 1981; Niewold, 2007). A third mechanism is a reduction in gut mass resulting in lower energy

expenditure for gut maintenance; therefore, more energy is available for growth (Braude et al., 1955; Koong et al., 1982; Webster, 1981).

The disease-control effect is the most widely accepted theory on how antibiotics promote growth in animals. Early research has focused on this concept. Evidence noted to support this effect is that younger animals, whom are more susceptible to infection, have a greater growth response to antibiotics than older animals and growth response to antibiotics is greater when hygiene is poor (Visek, 1978). The disease control effect includes suppressing disease-causing organisms and production of bacterial toxins, therefore, inhibition of sub-clinical infections (Butaye et al., 2003; Carlson & Fangman, 2000; Cocito, 1979; Feighner & Dashkevicz, 1987; Gaskins et al., 2002; Hays, 1981).

Impact on the Microbiota and Microbial Products

Many of the proposed mechanisms of action relate to changes in the microbiota and, therefore, changes in products produced by intestinal microbiota. Much of the early research has focused on changes associated with the intestinal microbiota. The generally accepted hypothesis is that "antibiotics improve efficiency of animal growth via inhibition of normal microbiota, leading to increased nutrient utilization and reduction in maintenance costs of the gastrointestinal system" (Gaskins et al., 2002).

Virginiamycin decreases the number of lactobacilli in the upper gastrointestinal tract in pigs (Agudelo et al., 2007; Decuypere et al., 1973; Langlois et al., 1978). Song and colleagues (2008) observed a reduction in the total number of bacterial cells in ileal digesta and feces with a reduction in some bacterial species in ileal digesta of growing pigs fed diets supplemented with virginiamycin. *In vitro* data confirm that virginiamycin decreases lactobacilli in gastric and ileal

contents (van Assche et al., 1975; Vervaeke et al., 1979). Lactobacilli are one of the main producers of lactic acid, which is the major end product of microbial carbohydrate metabolism. Virginiamycin has also been shown to reduce lactic acid production to 30% of control pigs and eliminate lactic acid production *in vitro* (Hedde et al., 1981; van Assche et al., 1975). A reduction in lactic acid indicates a reduction in glucose utilization by gut bacteria, which has been demonstrated *in vitro* (Hedde & Lindsey, 1986). Langlois and colleagues (1978) found that the differences in lactobacilli were not sustained when virginiamycin was removed from the diet. These findings indicate that supplementation with virginiamycin has a direct effect on the microbiota and result in more glucose available to the pig for digestion and absorption.

Agudelo and colleagues (2007) observed significant decreases in lactobacilli in ileal contents of pigs fed virginiamycin supplemented diets with normal phosphorus content, but they did not obverse a reduction in pigs fed a phosphorus-deficient diet supplemented with virginiamycin. They also reported a numerical increase in phytate-utilizing bacteria in virginiamycin fed pigs compared with pigs fed diets without virginiamycin in both normal and phosphate-deficient diets (12.45% and 17.2%, respectively). This group is the first to report the effect of virginiamycin on phytate-utilizing bacteria.

Virginiamycin reduced ammonia production by 50% compared with control *in vitro* (van Assche et al., 1975). Dierick and colleagues (1986) demonstrated that virginiamycin-fed pigs had 10% and 15% less ammonia in the small and large intestine, respectively. Ammonia and amines are products of the decarboxylation of amino acids and are toxic, which is not supportive of digestion and absorption. Virginiamycin has been shown to reduce amine production in pigs (Henderickx et al., 1993). *Escherichia coli* is a gram negative bacterium that is a major producer of amines; however, virginiamycin is not active against *E. coli*. Henderickx and colleagues

(1993) proposed the reduction in amine production is a result of increased acidity mediated by lactobacilli. A reduction in ammonia and amines indicate a reduction in the loss of amino acids and, therefore, an increase in amino acids available for the host animal.

Volatile fatty acid production in gastric and ileal contents has been shown to be 50% lower in virginiamycin fed pigs that that of pigs fed diets without virginiamycin (Hedde et al., 1981; Vervaeke et al., 1979). Some researchers have shown a slight increase in coliforms in the gastrointestinal tracts of pigs fed diets supplemented with virginiamycin (Decuypere et al., 1973; Langlois et al., 1978). However, Vervaeke and colleagues (1979) failed to show a difference in coliforms in gastric and ileal contents. Additionally, differences in coliforms and lactobacilli were not sustained when virginiamycin was removed from the diet (Langlois et al., 1978). These findings indicate that antibiotics may have direct, immediate effects on the microbiota that are not sustained once removed from the diet.

Impact on Immune Response

As stated previously, a large portion of the literature has focused on direct antibiotic effects on the microbial population and products. A more recent proposal is a direct effect on the immune response with an indirect effect on the microbiota. Niewold (2007) proposed a concept where antibiotics work to permit growth by inhibiting the production and excretion of catabolic mediators by intestinal inflammatory cells. Niewold (2007) believes the changes observed in the microbiota are a result of altered conditions of the intestinal tissue. Niewold supports this theory by interpreting previous microbiota studies in a different manner. Antibiotics accumulate in inflammatory cells (Labro, 1998; 2000; van den Broek, 1989), which results in lower levels of proinflammatory cytokines and a lower catabolic stimulus; thus, energy is spared in the animal

and can be used for growth. Intestinal inflammation results from the accumulation of inflammatory cells in the mucosa and leads to thicker intestinal epithelium. The thinner intestinal tissue cited for improved nutrient absorption could be reduced inflammation resulting from reduced influx and accumulation of inflammatory cells (Larsson, 2006). Therefore, Niewold proposed that antibiotics directly impact the immune response with secondary effects on the microbiota.

Impact on Rate of Nutrient Passage and Nutrient Digestibility

Researchers have investigated the influence of antibiotics on rate of passage and digestibility of energy, protein, and some minerals. These variables are commonly viewed as secondary effects of antibiotics. Rate of passage has been studied in virginiamycin fed pigs. Fausch (1981) reported the average time of passage was 4 to 11% longer in pigs fed 11 mg/kg of virginiamycin for one week compared with control. This was in agreement with Hedde and colleagues (1981) and Ravindran and colleagues (1984) who demonstrated slower passage rates (2.6 to 29% longer) in grower and finisher pigs fed virginiamycin. A slower rate of passage of feed through the intestine indicates more time for digestion and absorption that could lead to increased uptake and improved feed efficiency.

Virginiamycin improves apparent digestibility of amino acids in the small intestine of pigs by up to 9% compared with controls (Decuypere et al., 1978; Dierick et al., 1986; Stewart et al., 2010). Virginiamycin increased ileal digestibility of nitrogen, lysine, glycine, valine, and methionine by 1.4 to 4.8% compared with control (Dierick et al., 1986). Dierick and colleagues (1986) also observed an inhibition of amino acid breakdown in the small intestine and increased nitrogen retention. Digestibility of dry matter and energy are improved in pigs fed diets

supplemented with 11 mg/kg of virginiamycin (Agudelo et al., 2007; Lindemann et al., 2002; Ravindran et al., 1984). Increased mineral digestibility and retention have also been shown in pigs fed virginiamycin (Agudelo et al., 2007; Lindemann et al., 2002). Virginiamycin supplemented at 11 mg/kg diet has been shown to improve digestibility and retention of magnesium, copper, zinc, and manganese by 17 to 45% (Ravindran et al., 1984). An increase in ileal digestibility by 3.3 to 5% has also been demonstrated for calcium, phosphorus, and zinc with virginiamycin supplementation in pigs (Agudelo et al., 2007; Lindemann et al., 2002). Increased utilization of phosphorus may provide an economic benefit via a reduction in inorganic phosphorus required in feed (Agudelo et al., 2007). Improved digestibility of energy, protein, and minerals indicate that pigs fed virginiamycin are able to utilize feed more efficiently and able to gain weight at a faster rate than pigs fed diets without virginiamycin.

Stewart and colleagues (2010) were the first to investigate amino acid digestibility in corn-soybean meal diets. Grower pigs were fed diets supplemented with 11 or 22 mg/kg of virginiamycin for four weeks. Virginiamycin supplemented at 11 mg/kg diet improved apparent ileal digestibility of all indispensable amino acids, except arginine, histadine, and leucin by 2 to 6.7%. Virginiamycin also improved apparent ileal digestibility of alanine, proline, and tyrosine. Virginiamycin supplemented at 22 mg/kg diet improved apparent ileal digestibility of tryptophan and valine during the four weeks of feeding. These effects were lost in all but lysine and tryptophan when virginiamycin was removed from the diet for two weeks (Stewart et al., 2010). The loss of effect when virginiamycin is removed for the diet indicates a more direct effect of virginiamycin on nutrient digestibility. Increased amino acid digestibility with virginiamycin indicates this may be a mode by which virginiamycin promotes growth in pigs.

Impact on Intestinal Structure

Virginiamycin has been shown to impact the microflora of pigs and influence digestibility and rate of passage. Few studies have investigated the impact of in-feed antibiotics on pig intestinal structure. Only one research project was found in the literature to report virginiamycin and intestinal structure in pigs (van Leeuwen et al., 2002). Pigs weaned at 21 days of age were fed diets containing either 0 or 40 mg/kg of virginiamycin for 32 days, starting at seven days post-weaning. At day 31 post-weaning, pigs were challenged with *E. coli*. The section observed was mid-jejunum taken 5.5 meters distal from the ligament of Treitz. Crypt depth was significantly decreased and the number of crypt goblet cells was significantly increased in the virginiamycin group. In the second trial, pigs were fed for 14 days starting on day fourteen post-weaning. The virginiamycin-fed pigs had longer villi, but crypt depth was unchanged (van Leeuwen et al., 2002).

Impact on Nutrient Transport

Few studies have investigated the impact of in-feed antibiotics on nutrient transport in pigs. No reports were found in the literature on virginiamycin and nutrient transport in pigs. Walsh and colleagues (2012) recently published a study investigating the antibiotic carbadox in newly weaned pigs and nutrient transport before and after a *Salmonella* challenge. Carbadox-fed piglets had less glucose, phosphorus, and glutamine transport at 7 days post weaning compared with control pigs, but transport did not differ at any time post-*Salmonella* challenge (Walsh et al., 2012). Several gut-associated changes occur during weaning so comparability between nutrient transport data in weaning piglets and grower pigs may be limited.

Thesis objective

The effects of in-feed antibiotics on growth and feed conversion have been known for decades. However, the mechanisms by which this occurs are not well defined. In lieu of recent debates on the safety of public health in relation to antibiotic-fed animals, it is important to understand the mechanisms by which these antibiotics work. Beneficial effects of in-feed antibiotics on energy and nitrogen utilization have been shown in several animal species, including swine (Dierick et al., 1986; Vervaeke et al., 1979). Previous studies on the nutritional effect of in-feed antibiotic have focused on digestibility and utilization of protein, energy, and phosphorus. To our knowledge, no study has investigated the effect of virginiamycin on intestinal structure and nutrient and ion transport in grower pigs. Therefore, our study aimed to investigate the structural and functional indices of the intestine in grower pigs feed corn-soybean diets supplemented with virginiamycin. We hypothesized that diets with virginiamycin would increase intestinal surface area and nutrient processing capacity compared with diets without virginiamycin. The mechanism by which in-feed antibiotics promote growth in pigs is most likely multifaceted and intestinal structure and function is an important component in determining the true manner of virginiamycin.

Table 1.1 Summary of agencies' conclusions, recommendations, and actions from 1969 to 2012 regarding antibiotic use in food-producing animals[1]

Year	Organization	Conclusion	Recommendation/Action
1969	Joint Committee[2]	Antimicrobial use in food-producing animals poses a hazard to human and animal health	1. Only use antibiotics in animal feed that are not used as therapeutic agents in humans or animals
1970	FDA		1. Antimicrobial drugs used in human medicine should meet certain guidelines to be used for growth promotion 2. Resulted in code of federal regulations that require companies to submit research demonstrating product does not promote bacterial drug resistance
1980	NAS	Limited epidemiological research available on antimicrobials in animal feed	1. Gain evidence to either prove or disprove the safety of penicillin and tetracycline use in animals
1988	IOM	Unable to find direct evidence that penicillin or tetracycline in animal feed propose a human health hazard	1. Further study on antibiotic use in animals and associated human health effects
1997	WHO	All uses of antimicrobials led to selection of resistant bacteria	1. Do not use same drugs used in humans or with known cross-resistance to antimicrobials used in humans 2. Develop a systematic approach to replacing antimicrobials 3. Risk management needed including prudent use of antibiotics, education and veterinary approval
1999	NRC		1. Establish national databases to support policy development for approval and use of antibiotics in animals 2. FDA establish panel of experts for oversight of development and use of antibiotics
1999	GAO	Extent to which bacterial resistance, as a result of antibiotic use in animals, has led to human illness or death cannot be estimated at this time	1. Departments of Agriculture and Health and Human Services work together to develop a plan to determine use of antibiotics in agriculture

Table 1.1 Continued

Year	Organization	Conclusion	Recommendation/Action
1999	ECR	Actions should be taken promptly to reduce overall use of antimicrobials	1. Antimicrobials used prudently 2. Research new ways to prevent and treat infections
2003	FAO/OIE/ WHO	There is accumulating evidence of adverse human health effects due to resistant bacteria from antimicrobial use in animals	1. WHO appoint a group of experts and develop risk assessments 2. Codex collaborate with OIE for a risk management system
2003	IOM	Determined 13 factors that account for the emergence of new or enhanced microbial threats	1. FDA ban all antibiotics used for growth promotion that are in the same class as antibiotics used in human medicine
2003	FDA (GFI#152)		1. FDA must determine that a drug is safe and effective for its intended use in animals and safe with regard to human health
2004	FAO/OIE/ WHO	Good agricultural practices could eliminate the need for in-feed antibiotics	1. Establish a list of antibiotics deemed critically important to human health by WHO 2. Implement resistance surveillance systems 3. Codex/OIE task force to develop risk management options
2004	GAO	Resistant bacteria can be transferred and may pose a threat to human health	1. FDA expedite risk assessments of critically important antibiotics to see if use of those antibiotics should be prohibited in animals 2. Secretary of Agriculture and Human and Health Services develop a plan for collecting data on antibiotic use in animals
2005	Codex	Provided guidance for the responsible and prudent use of antimicrobials in animals	1. Veterinary prescription for antibiotic use 2. Do not use antibiotics for growth promotion if cross-resistance occurs
2009	AAM	Resistance is natural phenomenon that cannot be eliminated	1. Find new ways to deal with current resistance 2. Try to control future resistance by prudent and responsible use of antibiotics in animals

Table 1.1 Continued

Year	Organization	Conclusion	Recommendation/Action
2011	WHO	Approach a solution to antibiotic resistance in a holistic and multifaceted manner	1. Eliminate the use of antibiotics for growth promotion 2. Require veterinary prescription for antibiotics use and only use antibiotics deemed critically important for humans when justified
2012	FDA (GFI#209)	Antimicrobial resistance in humans is a rising concern	1. Voluntary adoption of limiting use of medically important drugs to times when they are necessary for assuring animal health 2. Veterinary oversight or consultation for antimicrobial use

[1]Abbreviations: NAS, National Academy of Sciences; IOM, Institute of Medicine; WHO, World Health Organization; NRC, National Research Council; GAO, U.S. Government Accountability Office; ECR, European Commission Report; FAO, Food and Agriculture Organization; OIE, World Organization for Animal Health; AAM, American Academy of Microbiology
[2]The Joint Committee on the Use of Antibiotics in Animal Husbandry and Veterinary Medicine, Her Majesty's Stationery Office

Table 1.2 Mechanisms by which in-feed antibiotics may promote growth in animals

Mechanism	Result
Shifts in microbiota	Decrease in microbial use of nutrients resulting in increased nutrients available to the host Decrease in pathogenic microbiota Decrease in toxic products
Immunity	Lower levels of proinflammatory cytokines Reduced influx of inflammatory cells into the mucosa
Transit time	Slower digesta passage rate resulting in increased time for absorption
Nutrient digestibility	Increased digestibility of protein, energy, and minerals
Intestinal structure	Increase in absorptive surface area
Nutrient transport	Increased nutrient uptake via increased surface area or up-regulation of nutrient transporters

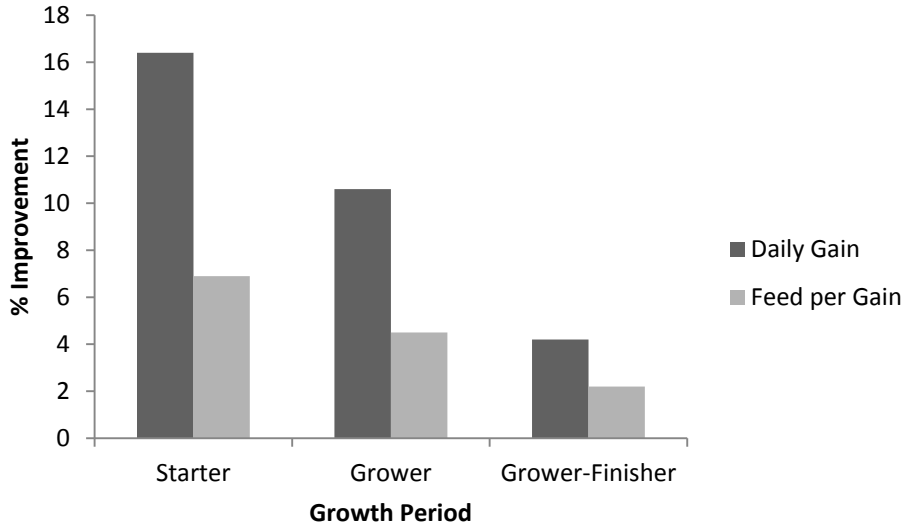


Figure 1.1 Percent improvement in daily gain and feed per unit of gain in swine fed in-feed antibiotics over pigs fed diets without antibiotics. Antibiotics improve daily gain in pigs by 16.4% during the starter period, 10.6% during the grower period, and 4.2% during the entire grower-finisher period. Antibiotics improve feed per unit of gain in pigs by 6.9% during the starter period, 4.5% during the grower period, and 2.2% during the entire grower-finisher period. Adapted from Cromwell, 2002.

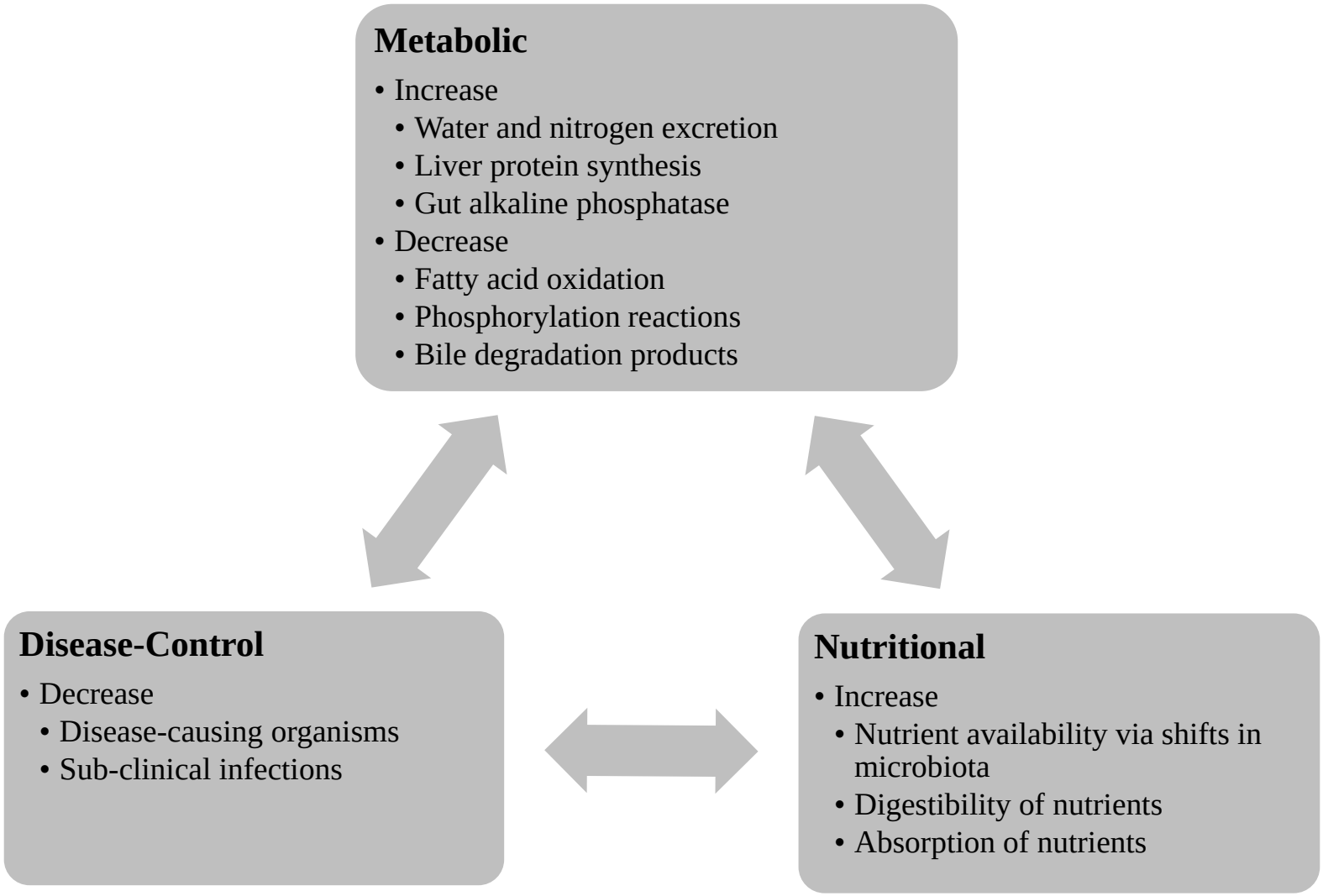


Figure 1.2 The three categories of mechanisms by which subtherapeutic levels of antibiotics may promote growth in animals. (Braude & Johnson, 1953; 1955; Brody et al., 1954; Butaye et al., 2003; Carlson & Fangman, 2000; Cocito, 1979; Feighner & Daskevicz, 1987; Gaskins et al., 2002; Hash et al., 1964; Hays, 1978; Henderickx et al., 1981; Koong et al., 1982; Moser et al., 1980; Webster, 1981; Weinberg, 1957)

Chapter 2: Impact of Virginiamycin on the Development of Intestinal Structure and Functional Capacity in Growing Pigs

Introduction

Growth promoting effects of antibiotics are well documented in several animal species, including swine. However, the mechanisms behind how these antibiotics enhance growth and feed efficiency are not completely understood. Increasing public concern about antibiotic resistance has led to increased political pressure to determine if antibiotic use in animals plays a role in antibiotic resistance and potential treatment failure in humans. Understanding the mechanisms behind antibiotic growth promoters will aid in determining the relationship between in-feed antibiotic use in animals and human health.

The proposed mechanisms by which antibiotics promote growth are described in three main categories including metabolic, nutritional, and disease control effects (Gaskins et al., 2002). Early research mainly focused on how antibiotics suppress the growth of pathogenic antibiotics while more recent research has investigated the nutritional effects of antibiotics. The impacts of in-feed antibiotics on shifts in microbial populations, nutrient availability, rate of passage, and nutrient digestibility have been investigated. However, little research has been done on the impact of antibiotics on intestinal structure and nutrient transport capacity.

Virginiamycin, approved for use in the United States in 1975, is an in-feed antibiotic used for swine (FDA, 2004). Virginiamycin improves average daily gain and feed efficiency in swine (Gaines et al., 2005; Gramm et al., 2006; Hays, 1981; Lewis & Giesemann, 1991; Schinckel, 1993; Zimmerman, 1986). Research has shown that virginiamycin reduces lactic acid bacteria, ammonia production, and volatile fatty acid production (Agudelo et al., 2007; Decuypere et al., 1973; Hedde et al., 1981; Henderickx et al., 1993; Langlois et al., 1978; van Assche et al., 1975;

Vervaeke et al., 1979). Virginiamycin also reduces the rate of passage (Fausch, 1981; Hedde et al., 1981; Ravindran et al., 1984) and improves ileal digestibility of nutrients in pigs (Agudelo et al., 2007; Decuypere et al., 1978; Dierick et al., 1986; Lindemann et al., 2002; Stewart et al., 2010). Research has shown impacts of virginiamycin within the gastrointestinal tract, but the exact mechanisms behind virginiamycin remain largely unknown.

The overall objective of this study was to assess the structural and functional developments induced in the intestine by the consumption of virginiamycin in grower pigs and to determine how these developments may be mechanistically related to the differences in growth observed among treatments. We examined the hypothesis that feeding grower pigs diets with virginiamycin is associated with increased intestinal surface area and nutrient processing capacity compared with pigs consuming diets without virginiamycin.

Materials and Methods

Experimental Design

All animal procedures were approved by the Illinois Institutional Animal Care and Use Committee at the University of Illinois Urbana-Champaign. These piglets were the first generation of pigs housed in clean facilities following a depopulation and extensive cleaning at the Swine Research Center at the University of Illinois at Urbana-Champaign. These piglets were given one injection of Excede® (ceftiofur crystalline free acid) after birth, weaned at 3 weeks of age and raised in a weaning facility consuming a non-medicated swine weaning diet. At allocation, littermate barrows with similar body weight (n=72; 23.0±1.3 kg; pigs nine weeks of age) were randomized to pens in a grower facility. Pens were randomized to one of three treatment groups: 1) a basal corn-soybean meal diet, 2) the basal diet + 11 mg/kg of

virginiamycin, or 3) the basal diet + 27.5 mg/kg of virginiamycin. Within each pen, pigs were further randomized to a time point (7 or 14 days). The control diet was fed to all pigs during their acclimation to the new environment from allocation to experimental day zero (3 to 4 days). The experiments were conducted in three replicates (n=24 pigs/replicate, 4 pigs/pen, 2 pens/treatment/replicate). Feed was a basal corn-soybean meal swine grower diet (**Table 2.1**). Feed was mixed and divided before adding virginiamycin. Before the trial began, feed medication assays were conducted to ensure proper levels of antibiotic in each feed (0, 11, and 27.5 mg/kg of virginiamycin). Virginiamycin at the 11 mg/kg diet level is currently approved for use in pigs up to 120 pounds for growth promotion and feed efficiency. Virginiamycin at the 27.5 mg/kg diet level is currently approved for use in pigs up to 120 pounds for prevention of swine dysentery. These levels of virginiamycin were chosen, in conjunction with our funding agency, to understand the impacts on intestinal function and structure at the level used for improved performance and for prevention of disease.

Body Weight and Feed Intake

Pigs were allowed to consume the feed and water *ad libitum*. Pigs were weighed individually on allocation day, experimental day zero, and every other day throughout the study with final body weight taken on euthanasia day. Feeders were weighed empty, before adding feed, and every other day to record feed consumption of each pen. Feed bags were weighed before being added to the feeder. Feed intake and feed per unit of gain were assessed by pen because individual assessment was not feasible. Total feed intake for each pen was divided by the number of pigs in the pen and then divided by the number of days to determine average daily feed intake per pig. For each individual pig, final weight was subtracted from day zero weight

and divided by days of treatment to determine daily weight gain. Daily weight gain and feed intake was averaged within pens. Average daily feed intake per pig was divided by average daily weight gain to determine feed per unit of gain.

Sample Collection

After the specified time period (7 or 14 days), a telazol, ketamine, and xylazine intramuscular anesthetic was followed by intravenous sodium pentobarbital (Fatal Plus; Vortech Pharmaceuticals, Chicago, IL) for euthanasia. The intestine was quickly removed starting with the duodenum excised proximal to the ligament of Treitz and distal of the stomach. The intestine distal to the ligament of Treitz and proximal to the ileocecal valve was removed and divided equally in half with the proximal half designated as jejunum and distal half designated as ileum. The entire large intestine was removed distal to the ileocecal valve. Segments were flushed with ice-cold saline and weighed. The length of duodenum was measured by suspending it longitudinally with a 10 g weight attached to the distal end while the remaining segments were laid out and measured with a measuring tape. Sample location was consistent among pigs to reduce variation due to sampling location. A 4-cm sample of each intestinal segment was taken and placed in oxygenated Kreb's for electrophysiological analysis. Two 1-cm samples of each segment were immediately snap frozen in liquid nitrogen and stored at -80°C for analysis of DNA, RNA, protein, and disaccharidase activity. A 1-cm section of each segment was scraped free of mucosa and placed on separate slides for mucosa and submucosa weight. An additional 1-cm section of each segment was opened longitudinally, stapled to a square of cardstock at the proximal and distal ends, mucosa side up, and placed in 10% neutral buffered formalin (Sigma-Aldrich, St. Louis, MO) for histomorphology and immunohistochemistry analysis.

Mucosa and Submucosa Mass

Mucosal and submucosal mass was determined gravimetrically by desiccating samples on glass slides and subtracting the mass of the slide from that of slide plus sample. Glass slides were dried in an oven at 120°C for 10 minutes, cooled in a desiccator with a vacuum for 10 minutes, and weighed. Intestinal samples of a specific length were collected as described above. Samples sat at room temperature for at least 24 hours and were then dried for one hour in a drying oven at 95°C. Samples were removed from the oven and immediately placed in a desiccator for one hour. Samples were then removed and weighed immediately.

DNA, Protein, RNA Quantification

Intestinal homogenates for DNA and protein quantification were prepared with 0.25 g intestinal tissue into 1 ml of diethyl pyrocarbonate (DEPC; 2 mL/L; Sigma-Aldrich, St. Louis, MO) homogenized (Tissue Tearor, model 23 985370, BioSpec Products Inc., Bartlesville, OK) individually for 30 seconds. After preparing the appropriate dilution, DNA content of the intestinal samples was determined using the Hoechst microplate method (Latt & Stetten, 1976). Briefly, each well of a black 96-well microplate (Fisher Scientific, Pittsburg, PA) was filled with 8 µl of intesinal homogenate or prepared herring sperm standard and 200 µl of a buffer solution containing 10 mM Tris, 1 mM EDTA, 200 mM NaCl, and 1.6 nmol 33258 bisBenzimide Hoechst dye (Sigma B2883) at pH 7.4. Intestinal DNA concentration was quantified in sample homogenate using fluorescence at an excitation of 360 nm, emission of 450 nm (SpectraMax Gemini XS fluorometer, Molecular Devices, Sunnyvale, CA), and standard curve methodology with a Herring sperm DNA standard (PromegaD1811, Madison, WI).

Intestinal protein concentration was determined using the BSA method (Pierce Biotechnology, Rockford, IL) following the manufacturer's protocol. Briefly, each well of a 96-well plate was filled with 25 µl of each standard or sample replicate and 200 µl of the working reagent. The plate was placed on a plate shaker for 30 seconds and then covered and incubated at 37°C for 30 minutes. The plate was cooled to room temperature and the absorbance was measured at 562 nm using a SpectraMax Plus 384 (Molecular Devices LLC., Sunnyvale, CA). Protein concentration was calculated from a bovine serum albumin (Sigma) standard curve.

RNA concentration was determined spectrophotometrically. RNA was isolated from intestinal tissue samples using TRIzol® reagent (Life Technologies, Inc., Gaithersburg, MD), according to the manufacturer's specifications. An overnight cleaning step was performed by adding 10 µL of 3M sodium acetate at pH 5.2 and 250 µL of 100% ethanol to samples, briefly vortexing and placing samples in a -20°C freezer. The following morning, samples were centrifuged for 15 minutes and the resulting pellet was washed an additional time with 75% ethanol. Total RNA was quantified with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc, Waltham, MA) at OD260 absorbance, and purity was assessed by determining the OD260/OD280 and OD260/OD230 ratios.

Morphometric Analysis of the Mucosal Architecture

Intestinal sections were fixed in formalin and then transferred to 50% ethanol. Samples were infiltrated with paraffin wax and sectioned to approximately 5 µm thickness with a microtome. Slides were cleared of paraffin with xylene and then rehydrated with a series of ethanol washes (100%, 95%, 80%). Slides were rinsed in tap water for 4 minutes. Hematoxylin stain was applied for 3 minutes, acid ethanol for 15 seconds, and Scott's Bluing solution for 4

minutes. Slides were washed with water between each application. Slides were then dehydrated in increasing ethanol baths (80%, 95%, 100%) and Eosin Y (diluted 50% in 95% EtOH) was applied for 30 seconds in the middle of the 95% EtOH bath. Slides were placed in xylene and cover slipped. Sections were visualized with Nanozoomer Slide Scanner Digital Pathology System (Hamamatsu, Bridgewater, NJ) and NDP View imaging software. Villus height, mid-villus width and crypt depth were measured in 8 to 20 vertically well-orientated villi and crypts within each sample. Villus surface area was calculated for each villus by multiplying villus height by villus width.

Epithelial Cell Proliferation

The immunohistochemical detection of proliferating cell nuclear antigen (PCNA) was used as an index of crypt cell proliferation. Formalin-fixed, paraffin-embedded sections sliced to approximately 5 µm thickness were cleared of paraffin with xylene and then rehydrated through graded ethanol washes (100%, 95%, 80%, and 70%). Slides were then rinsed in tap water for 5 minutes. Antigen retrieval was performed by placing slides in a 95°C citrate buffer (10mM citric acid, 0.05% Tween 20, pH 6.0, Sigma-Alrdich, St. Louis, MO) bath for 5 minutes. After 5 minutes, the citrate bath with slides was placed at room temperature for 20 minutes followed by two-5 minute washes in room temperature phosphate buffered saline (PBS; 2 mM $Na_2H_2PO_4 \bullet H_2O$, 8.5 mM Na_2HPO_4, 1.5 mM NaCl, pH 7.4). Endogenous peroxidase activity was quenched with 3% hydrogen peroxide (Fisher Scientific) for 10 minutes followed by two-5 minute washes in PBS. Five percent normal horse serum (NHS) was applied to the slides for 20 minutes to block non-specific binding sites and then slides were incubated for 60 minutes with primary PCNA antibody (Millipore Billerica, MA) diluted 1:325 in 1% NHS PBS. A biotinylated

universal secondary antibody and ABC complex (Vectastain Elite ABC kit, Vector, Burlingame, CA) were prepared and utilized according to manufacturers' instructions. Slides were washed twice for 5 minutes after each application of the primary and secondary antibodies, and ABC complex. Finally, sections were stained for 3 to 4 minutes with VIP (Peroxidase Substrate Kit, Vector), dehydrated in increasing ethanol baths (70-100%), finishing with xylene and cover slipped. Nanozoomer Slide Scanner Digital Pathology System and NDP View imaging software were used to capture images at 20x magnification. PCNA positive cells in 8-10 well-oriented crypts of each sample were counted using ImageJ software (National Institutes of Health, Bethseda, MD).

Disaccharidase Specific Activity

Intestinal homogenates were prepared with 0.25 g intestinal tissue into 1 mL of homogenation buffer containing protease inhibitors (0.45 mol/L sodium chloride, 0.001 mol/L phenylmethylsulfonyfluoride, 0.002 mol/L iodoacetic acid). After preparing the appropriate dilution in homogenation buffer (50 ul homogenate + 50 ul buffer), sucrase and lactase specific activities were determined by the method of Dahlquist (Messer & Dahlqvist, 1966). Briefly, the intestinal homogenates were incubated in either lactose or sucrose buffer for 60 minutes at 37°C. The hydrolysis of the disaccharide by the action of the disaccharidase in the tissue homogenate was stopped by the addition of 2.0% zinc sulfate and 1.8% barium hydroxide. Following centrifugation and transfer of supernatant to a 96-well microplate, the amount of glucose released was detected with a glucose oxidase reagent (Thermo Scientific, Middletown, VA) using a microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). Liberated glucose was quantified using standard curve methodology after subtracting each sample's internal control for

endogenous glucose. Disaccharidase activity was normalized to cellular protein content of the same homogenate as measured by BCA assay and expressed as the amount of glucose release per hour per milligram of protein.

Mucosal Ion and Nutrient Transport

Modified Ussing chambers (Physiologic Instruments, San Diego, CA) were used for electrophysiological analysis of nutrient transport and secretory capacity across intestinal segments as previously described (Kles et al., 2001). Duplicate sections of duodenum, jejunum, ileum and colon were stripped of muscularis (outer serosal layer), cut longitudinally along the mesentery, and mounted in modified Ussing chambers to expose 0.5 cm^2 of tissue. The tissue was bathed in 8 mL of oxygenated (95% O_2/5% CO_2) modified Kreb's buffer maintained at 37°C with a circulating water bath (Fischer Scientific, Itasca, IL). Dual channel voltage current clamps (VCC MC2, Physiologic Insruments Inc.) were utilized to voltage clamp the transepithelial potential of the intestinal segments to zero. Basal transmucosal short-circuit current (I_{sc}; uA/cm^2), resistance (R; $\Omega \bullet cm^2$), and potential difference (Pd; mV) were measured after an equilibration period.

Active transport was determined by measuring the change in short-circuit current induced by the addition of 10 mM glucose, glutamine, or arginine (Sigma-Aldrich, St. Louis, MO) to the medium on the mucosal side. Each addition to the mucosal side was osmotically balanced by the addition of 10 mM mannitol (Sigma-Aldrich) to the serosal reservoir. Neurally mediated secretion was measured by the addition of 0.1 mM serotonin and immune mediated secretion was measured by the addition of 0.1 mM carbachol (Sigma-Aldrich) to the serosal medium. Dual-channel voltage/current clamps (VCC MC2, Physiologic Instruments) with a computer

interface allowed for real time data acquisition and analysis (Acquire & Analyze software, Physiologic Instruments).

Statistical Analysis

Analysis of variance was used to statistically analyze the data using the Mixed Model fitted with a split plot design. Treatment (0, 11, 27.5 mg/kg of virginiamycin) was included in the whole plot, whereas time (7 or 14 days) was included in the subplot. Treatment, time, and the interaction between treatment and time were the main fixed effects. Replicate, the interaction between replicate and treatment, and pen nested within treatment were included as the random effects. Preplanned contrasts between virginiamycin and control groups were completed. When specific intestinal segment data were analyzed, each respective segment was analyzed separately from one another (i.e. duodenal values were not compared with jejunal values). All data were checked for normality by the Shapiro-Wilk statistic. A log 10, square root, or reciprocal transformation was applied to all non-normal data. After p-values were obtained, data were re-transformed to acquire the correct mean estimate and the standard error of retransformed data are presented as an average of standard error (SE) values calculated from both the upper and lower retransformed confidence intervals of group means. If the main effect of time, treatment, or the interaction between time and treatment was significant, means were separated using the least significant difference and considered significant at $p \leq 0.05$.

Principal component analyses (PCA) were completed on data derived from the control and 11 mg/kg virginiamycin groups at day seven (PCA 1; n=23 piglets; 77 variables) and day fourteen (PCA 2; n=24 piglets; 73 variables) as outlined by Jolliffe (2002). Data were not included from the 27.5 mg/kg virginiamycin group because this level of virginiamycin is not

FDA approved for use for growth promotion and feed efficiency. Our primary objective was to obtain an overview of changes in structure and function along the intestinal tract with virginiamycin used for growth promotion and feed efficiency. In both initial PCAs, seventy-five percent of the total variance was accounted for by ten factors; therefore, we retained ten factors in the final analyses. Variables that did not load on any factor retained (correlation coefficient between variables and factors $|r| \leq 0.5$) were excluded from the final analyses. When several variables were significantly correlated ($|r| > 0.6$, $p<0.05$) within a group of similar variables, only the variables with the highest factor loadings were kept for the final analyses. T-tests were conducted on rotated component scores to detect if the principal components discriminated between treatment groups. Statistical analyses were performed using SAS (Version 9.3; SAS Institute, Cary, North Carolina).

Results

A total of 71 pigs completed this study. The one pig not included in the final analysis was eliminated from the study due to illness. Weights from three pens at day seven were not included in final analysis due to equipment malfunction resulting in inaccurate weights for those pigs.

Dietary Intake and Pig Growth

Body weight did not differ among groups at allocation or experimental day zero. Main effect treatment differences were not observed for feed efficiency or feed intake. Average daily gain tended to be higher in the 11 mg/kg virginiamycin group compared with control at day seven ($p=0.056$; **Table 2.2**). Final body weight (7d pooled=31.2±1.1, 14d pooled=36.6±1.0 kg,

p<0.00005), daily feed intake (7d pooled=1.46±0.07, 14d pooled=1.73±0.07 kg, p<0.00005), and feed per unit of gain (7d pooled=1.86±0.13, 14d pooled=2.09±0.12 kg/kg, p=0.015) were higher at day fourteen day compared with day seven (Table 2.2).

Gross Intestinal Morphology

Gross intestinal structure was assessed by intestinal weight, length, mucosal and submucosal mass. Duodenal length per body weight (7d pooled=1.26±0.07, 14d pooled=1.15±0.07 cm/kg) and weight per body weight (7d pooled=1.39±0.08, 14d pooled=1.26±0.08 g/kg) were less at day fourteen compared with day seven (p=0.032, p=0.041, respectively; **Table 2.3**). Duodenal mucosal mass increased over time in the control pigs, but it did not in pigs fed diets supplemented with virginiamycin which resulted in less duodenal mucosa in the 11 mg/kg virginiamycin group at day fourteen compared with control (p=0.038; **Table 2.4**; **Figure 2.1**). Submucosal mass tended to be less in the duodenum with virginiamycin treatment at day fourteen compared with control (p=0.066; Table 2.4).

Jejunal length per body weight decreased over time (7d pooled=22.4±0.89, 14d pooled=18.8±0.88 cm/kg, p<0.00005; Table 2.3). Virginiamycin supplemented at 11 mg/kg diet tended to increase jejunal mass at seven days compared with control (p=0.069; Table 2.3). Submucosal mass decreased over time in the jejunum (7d pooled=0.173±0.021, 14d pooled=0.142±0.021 g/cm, p=0.014; Table 2.4). Virginiamycin increased jejunal submucosal mass compared with control when virginiamycin treatment groups were contrasted with control (p=0.037; Table 2.4; **Figure 2.2**).

Ileal length per body weight (7d pooled=22.4±0.89, 14d pooled=18.8±0.88 cm/kg) and weight per body weight (7d pooled=33.5±0.87, 14d pooled=29.1±0.86 g/kg) were less at day

fourteen compared with day seven ($p<0.00005$, $p=0.0002$, respectively; Table 2.3). Mucosal mass (7d pooled=0.292±0.022, 14d pooled=0.356±0.022 g/cm) and submucosal mass (7d pooled=0.197±0.018, 14d pooled=0.229±0.018 g/cm) increased over time in the ileum ($p=0.002$, $p=0.028$, respectively; Table 2.4).

Colon length per body weight (7d pooled=9.65±0.26, 14d pooled=8.03±0.25 cm/kg) and weight per body weight (7d pooled=45.2±1.2, 14d pooled=41.3±1.2 g/kg) were less at day fourteen compared with day seven ($p<0.00005$, $p=0.005$, respectively; Table 2.3). However, mucosal mass increased over time in the colon (7d pooled=0.398±0.044, 14d pooled=0.464±0.051 g/cm, $p=0.019$; Table 2.4).

Histomorphology

Crypt-villus architecture (villus height, mid-villus width, and crypt depth) was measured by histomorphology to assess the effects of virginiamycin on epithelial architecture. Virginiamycin treatment increased villus surface area in the duodenum compared with control when virginiamycin treatment groups were contrasted with control ($p=0.048$; **Table 2.5; Figure 2.3**). Villus surface area in the duodenum increased with time (7d pooled=78,280±3544, 14d pooled=92,196±3491 μ^2, $p=0.003$) and involved increases in villus height (7d pooled=377±12.6, 14d pooled=416±12.4 µ, $p=0.016$) and width (7d pooled=205±5.0, 14d pooled=221±4.9 µ, $p=0.009$; Table 2.5). Pigs fed the diet containing 27.5 mg/kg of virginiamycin had shallower crypts in the jejunum at day fourteen compared with day seven ($p=0.023$; Table 2.5; **Figure 2.4**). Ileal villus surface area tended to increase with time (7d pooled=61,247±2654, 14d pooled=66,537±2716 μ^2, $p=0.080$; Table 2.5). Colonic crypts were deeper in virginiamycin fed pigs when the 11 mg/kg virginiamycin group was contrasted with control ($p=0.044$; Table 2.5;

Figure 2.5). Colonic crypts were deeper at fourteen days compared with seven days (7d pooled=451±19, 14d pooled=481±19 μ, p=0.013; Table 2.5).

Epithelial Cell Proliferation

Epithelial cell proliferation, measured by immunohistochemistry for PCNA-positive cells, was assessed in pigs fed diets containing 0 mg/kg and 11 mg/kg of virginiamycin for seven days. Pigs consuming the diet containing 11 mg/kg of virginiamycin had more PCNA-positive cells per ileum crypt than control pigs (p=0.003; **Figure 2.6**) with a similar trend in the duodenum (duodenum, p=0.055; **Table 2.6**).

DNA, RNA, and Protein Concentration

The effects of virginiamycin at the cellular level of intestinal tissue were assessed through quantification of intestinal DNA, RNA, and protein concentration. Intestinal DNA, RNA, and protein concentration were not impacted by virginiamycin treatment (**Tables 2.7-2.9**). Cellular RNA concentration tended to be lower at fourteen days compared with seven days in the proximal small intestine (duodenum, 7d pooled=3.93±0.78, 14d pooled=3.07±0.78, p=0.061; jejunum, 7d pooled=0.349±0.013, 14d pooled=0.327±0.016 mg RNA/mg DNA, p=0.083; Table 2.7). DNA concentration was greater at fourteen days compared with seven days in the duodenum (7d pooled=1.20±0.13, 14d pooled=1.55±0.13 mg/mg tissue, p=0.011; Table 2.9).

Intestinal Disaccharidase Activity

Sucrase activity in the duodenum of pigs fed diets containing 11mg/kg of virginiamycin was lower at day seven compared with control but increased over time in the virginiamycin

treatment groups resulting in no differences among groups at day fourteen (p=0.039; **Table 2.10; Figure 2.7**). Lactase activity tended to increase over time with virginiamycin treatment in the duodenum resulting in a trend towards increased lactase activity with virginiamycin treatment compared with control at day fourteen (p=0.086, Table 2.10). Sucrase activity was lower at day fourteen compared with day seven in the ileum (7d pooled=88.6±27, 14d pooled=70.1±22 U/g protein, p=0.041; Table 2.10).

Mucosal Ion and Nutrient Transport

Mucosal resistance, a measure of passive ion transport, was not impacted by virginiamycin treatment. Potential difference, a measure of total ion transport, tended to be lower in the duodenum with virginiamycin when values from pigs fed the diet containing 11 mg/kg of virginiamycin were contrasted with control (p=0.095; **Table 2.11**). Mucosal resistance (7d pooled=103±10, 14d pooled=127±13 $\Omega \cdot cm^2$) and potential difference (7d pooled=1.57±0.40, 14d pooled=2.80±0.62 mV) increased over time in the jejunum (p=0.033, p=0.035, respectively; Table 2.11). Potential difference tended to increase over time in the ileum (7d pooled=1.11±1.1, 14d pooled=1.99±1.1 mV, p=0.066; Table 2.11). Ileal potential difference tended to be higher with virginiamycin treatment when the values from pigs fed the diet containing 11 mg/kg of virginiamycin were contrasted with control (p=0.094; Table 2.11). Potential difference was increased in the colon of pigs fed the diet containing 27.5 mg/kg of virginiamycin compared with control (p=0.048; Table 2.11; **Figure 2.8**). Basal short-circuit current, a measure of active ion transport, tended to be greater in the colon with virginiamycin treatment when values from virginiamycin fed pigs were contrasted with control (p=0.056; Table 2.11).

Sodium-coupled glucose transport was increased with virginiamycin treatment in the duodenum when virginiamycin treatments groups were contrasted with control (p=0.025; **Table 2.12; Figure 2.9**). Virginiamycin tended to increase glutamine transport in the duodenum when the 11 mg/kg virginiamycin group was contrasted with control (p=0.083; Table 2.12). Duodenal glutamine transport was lower at day fourteen compared with day seven (7d pooled=3.12±0.71, 14d pooled=1.63±0.69 $\mu A/cm^2$, p=0.007; Table 2.12). Virginiamycin increased glucose transport in the jejunum when virginiamycin treatments were contrasted with control (p=0.045; Table 2.12; **Figure 2.10**). Jejunal glucose transport was greater at day fourteen compared with day seven (7d pooled=2.33±1.6, 14d pooled=4.68±1.9 $\mu A/cm^2$, p=0.028; Table 2.12). Virginiamycin treatment increased electrogenic glutamine transport in the jejunum compared with control (p=0.010; Table 2.12; **Figure 2.11**). Jejunal glutamine transport tended to decrease over time (7d pooled=2.99±0.65, 14d pooled=2.00±0.44 $\mu A/cm^2$, p=0.088; Table 2.12). Virginiamycin supplemented at 11 mg/kg diet increased arginine transport in the jejunum compared with control (p=0.046; Table 2.12; **Figure 2.12**). Pigs consuming diets supplemented with virginiamycin had less ileal arginine transport compared with pigs consuming the control diet at day seven; however by day fourteen, pigs consuming the diet supplemented with 11 mg/kg of virginiamycin had greater ileal arginine transport compared with control (p=0.042; Table 2.12; **Figure 2.13**).

Serotonin induced chloride secretion tended to increase over time in the duodenum (7d pooled=0.614±0.40, 14d pooled=0.863±0.44 $\mu A/cm^2$, p=0.087; **Table 2.13**). Serotonin induced chloride secretion was decreased in the 27 mg/kg virginiamycin group compared with the 11 mg/kg group in the jejunum (p=0.028; Table 2.13). However, it tended to be higher in the 11 mg/kg virginiamycin group compared with control when the 11 mg/kg virginiamycin group was

contrasted with control (p=0.060; Table 2.13). Carbachol induced chloride (CCH) secretion decreased over time in the jejunum (7d pooled=24.6±11, 14d pooled=13.0±6.0 μA/cm^2, p=0.033; Table 2.13). Ileal carbachol induced chloride secretion decreased in control pigs, but it increased in pigs fed the diet supplemented with 11 mg/kg of virginiamycin over time resulting in increased carbachol induced chloride secretion in the 11 mg/kg virginiamycin group at day fourteen compared with control (p=0.017; Table 2.13; **Figure 2.14**).

Principal Component Analysis

PCA 1

PCA of data from day seven was optimized by removing from the analysis the variables that did not load on any factor retained. Variables that did not load on the retained factors include: mass of the small intestine (SI), mucosal mass of the duodenum, jejunum, and ileum, ileal submucosal mass, duodenal and jejunal villus height, duodenal crypt depth, protein concentration in the duodenum, jejunum, and colon, duodenal DNA, jejunal RNA, duodenal short circuit current (Isc), glucose, glutamine, and arginine transport, jejunal glucose transport, jejunal sucrase and lactase activities, ileal resistance, Isc, potential difference, and CCH, colonic sucrase activity, colonic resistance and glucose transport, and duodenal epithelial cell proliferation (PCNA).

Redundant variables were also removed as described earlier. For example, feed per unit of gain and average daily gain were highly correlated (*r* -.88) and average daily gain was used for the final analysis. Similarly, significant correlations were observed between: colonic mucosal and submucosal mass (*r* 0.63); RNA and DNA within the ileum and colon (*r* -0.91 and *r* -0.74, respectively); duodenal glutamine transport and serotonin-induced chloride secretion

(5HT); jejunal Isc and potential difference (*r* 0.98); jejunal glutamine transport with arginine transport (*r* 0.82), 5HT (*r* 0.89), and CCH (*r* 0.72); ileal glucose transport and 5HT with glutamine transport (*r* 0.92; *r* 0.68, respectively) and arginine transport (*r* 0.85; *r* 0.71, respectively); colonic arginine transport, 5HT and CCH (*r* >0.77).

The final analysis included twenty-three pigs and thirty-seven variables (**Table 2.14**). Eighty-four percent of the variance among pigs was accounted for by the ten retained factors. The first seven factors are shown in Table 2.14. Factor 1 explained 17.8% of total variance. The variables with the highest loadings on factor 1 were: duodenal resistance, colonic arginine transport, ileal 5HT and glucose transport, and jejunal DNA and resistance. Factor 2 explained 14.1% of the total variance. Factor 2 was associated with length of small intestine and colon, average daily gain, duodenal RNA and glutamine transport, and submucosal mass of duodenum and jejunum. Factor 3 explained 12.3% of the total variance. Factor 3 was associated with ileal PCNA and crypt depth, colonic DNA, mass, and glutamine transport, jejunal crypt depth, average daily gain, and duodenal submucosal mass. Factor 4 explained 9.6% of the total variance and was associated with potential difference in the duodenum and colon, jejunal sucrase activity, PCNA, and crypt depth, and submucosal mass in the duodenum. Factor 5 explained 7.6% of the total variance and was associated with colonic crypt depth and PCNA, jejunal Isc, crypt depth, and resistance, and ileal villus height and crypt depth. Factor 6 explained 6.3% of the total variance and was associated with duodenal lactase activity, sucrase activity, and glutamine, lactase activity in the jejunum, and colonic Isc and submucosal mass. The remaining retained factors each explained less than 6% of the total variance.

Proximal gut mucosal resistance and distal gut nutrient transport account for the greatest percent of the total variance compared with all other factors at day seven. The second and third

factors are mainly associated with structural components of the gut. Virginiamycin impacted functional indices to a greater extent than structural components which is reflected in this principal component analysis.

PCA 2

PCA of data from day fourteen was optimized by removing from the analysis the variables that did not load on any factor retained. Variables that did not load on the retained factors include: mucosal and submucosal mass in the jejunum and colon, duodenal and jejunal villus height, colonic crypt depth, duodenal and ileal protein concentration, RNA, and DNA, jejunal RNA and DNA, colonic protein concentration and DNA, duodenal sucrase activity, duodenal and jejunal potential difference and CCH, ileal glucose transport, and colonic resistance and CCH.

Redundant variables were removed as described previously. Significant correlations were observed between: feed per unit of gain and average daily gain (*r* -0.84); length of the small intestine and colon (*r* 0.62); mucosal and submucosal mass within the duodenum and ileum (*r* 0.70 and *r* 0.68, respectively); duodenal arginine transport and 5HT (*r* 0.69); within the jejunum, 5HT was correlated with Isc (*r* 0.72), glucose transport (*r* 0.75), and glutamine transport (*r* 0.74); within the ileum, resistance was negatively correlated with glutamine (*r* -0.64) and 5HT (*r* -0.62) while 5HT was positively correlated with Isc (*r* 0.70), arginine transport (*r* 0.81), and CCH (*r* 0.86); colonic sucrase and lactase activities (*r* 0.79); colonic Isc and potential difference (*r* 0.90).

The final analysis included twenty-four pigs and thirty-four variables (**Table 2.15**). Eighty-five percent of the variance among pigs was accounted for by the ten factors retained. The first seven factors are shown in Table 2.15. Factor 1 explained 16.6% of the total variance.

The variables with the highest loadings were: ileal crypt depth, potential difference, and sucrase and lactase activities, duodenal and jejunal arginine transport, and jejunal protein concentration. Factor 2 explained 12.2% of the total variance. Factor 2 was associated with crypt depth in duodenum and jejunum, glucose transport in duodenum and colon, duodenal lactase activity, and jejunual 5HT. Factor 3 explained 10.6% of the total variance. Factor 3 was associated with duodenal submucosal mass, arginine and glutamine transport in colon, and jejunal resistance. Factor 4 explained 9.8% of the total variance and was associated with SI mass, feed per unit of gain, and SI length. Factor 5 explained 7.9% of the total variance and discriminated between control and 11 mg/kg virginiamycin groups ($p=0.015$). Factor 5 was associated with colonic potential difference, jejunal lactase and sucrase activities and resistance, and duodenal lactase activity. Factor 6 explained 7.2% of the total variance and was associated with mass of the colon, duodenal resistance, glucose transport, and Isc, and jejunal resistance and sucrase activity. Factor 7 explained 6.5% of the total variance and discriminated between control and 11 mg/kg virginiamycin groups ($p=0.050$). Factor 7 was associated with CCH, resistance, submucosal mass, and villus height in the ileum and glucose transport in the colon. The remaining factors each explained less than 6% of the total variance.

Small intestine functional indices account for the greatest percent of total variance compared with all other factors at day fourteen. Proximal gut disaccharidase activity and distal gut ion and nutrient transport dominate the factors that discriminate between control and virginiamycin fed pigs. These variables are indicative of intestinal alteration differences between groups and further support the treatment effects observed for increased intestinal functional capacity in virginiamycin fed pigs.

Discussion

Feed accounts for approximately two-thirds of the cost of producing market-weight swine (Ewan, 2001). Around 75 to 85% of the total feed used per unit of pork marketed is consumed during the growing-finisher period (Cline & Richert, 2001). An improvement in feed efficiency would result in less total feed used and cost-savings for a swine production farm. In addition, higher daily gains would indicate a quicker time to market weight, less time per pig on the farm, and lower costs associated with housing and care. Over 86% of grower-finisher pigs in the United States receive in-feed antibiotics (USDA, 2007). The net economic benefit of using antimicrobials in pig production has been estimated to be $2.64 to $3.93 per market hog (Zimmerman, 1986; Cromwell, 1999). Market hog inventory in the United States as of December 2012 was 60.5 million head (NASS, 2012). Miller and colleagues (2003) analyzed swine data from the National Animal Health Monitoring System and estimated an improvement of 9% in net profits with the use of antibiotics in pigs for growth promotion and feed efficiency in the United States.

The primary outcome of this research was the impact of virginiamycin on intestinal structure and function, but the improvements in growth and feed efficiency observed in this study are comparable to what is reported in the literature. A review by Hays (1981) stated that virginiamycin improved average daily gain by 10.7% and feed per unit of gain by 6.6% in grower pigs. In our study, virginiamycin supplemented at 11 mg/kg improved daily weight gain by 12.2% and feed per unit of gain by 12.9% and virginiamycin supplemented at 27.5 mg/kg improved daily weight gain by 8.0% and feed per unit of gain by 12.1%. Increases in weight gain and feed conversion of this magnitude can have substantial financial benefits for producers and consumers and greatly impact the amount of pork produced each year.

The use of sub-therapeutic levels of antibiotics in food-producing animals has been under increasing scrutiny due to concerns of antibiotic resistance. These concerns resulted in a ban of antibiotic use for growth promotion in the European Union. Impacts of the ban on pig production and economic effects are beginning to be reported in the literature. Aarestrup and colleagues (2010) analyzed productivity data before and after the ban, from 1992 to 2008. They showed a steady increase in the number of pigs per sow per year over the entire study period. Average daily gain actually increased after the ban; however, the increase in finisher pigs was greater prior to the ban. Total antibiotic was highest in 1992, decreased to half that amount in 1998, and remained fairly stable at the level until 2008. No effect on mortality rate was observed for weaned pigs (Aarestrup et al., 2010). The effects of antibiotic growth promoters are well documented, but improvements in performance are observed after the ban indicating that growth promotion is being supported by other factors. Understanding the mechanisms behind how antibiotics improve performance will aid in determining those factors and provide insight into the safety of continued use of antibiotic growth promoters.

We hypothesized that pigs fed diets supplemented with virginiamycin would have increased intestinal surface area compared with pigs fed diets without virginiamycin. Interestingly, few significant changes in absorptive surface area were observed among treatment groups. Others have failed to show increased absorptive surface area in the small intestine of weaned piglets fed antibiotics (Bosi et al., 2011; Shen et al., 2009). Van Leeuwen and colleagues (2002) observed mixed effects on jejunal surface area of weaned pigs fed virginiamycin supplemented diets. Miles and colleagues (2006) actually observed a decrease in ileal surface area of broilers fed virginiamycin. Our results indicate that increased absorptive surface area is not likely a major mechanism by which virginiamycin promotes growth.

Recently, a mechanism was proposed by which antibiotics promote growth via reduced immunologic stress on the animal (Niewold, 2007). In our study, virginiamycin appeared to protect against an increase in thickness of the mucosa and submucosa in the duodenum over time. Additionally, carbachol induced chloride secretion, used as a measure of immune mediated secretion, was increased with virginiamycin in the ileum by day fourteen. These structural and functional changes may support the proposed mechanism by which antibiotics protect the intestine against microbial insult and the subsequent infiltration of immune cells into the intestinal mucosa (Niewold, 2007). We did observe an increase in submucosa in the jejunum but submucosa is the supporting layer of connective tissue directly under the mucosa and less likely directly involved in the immune response. These aspects are only a portion of the immune response mechanism and future studies should investigate immune response parameters, such as pro-inflammatory cytokines, to fully elucidate this mechanism.

Increased digestibility combined with increased growth rate indicates that intestinal functional capacity is greater in pigs fed antibiotics. Virginiamycin has been shown to increase absorption of glucose and arginine in mice (Madge, 1969) and improve digestibility of amino acids and minerals in the small intestine of pigs (Decuypere et al., 1978; Dierick et al., 1981; Dierick et al., 1986; Ravindran et al., 1984; Stewart et al., 2010). In our study, proximal gut nutrient transport was increased by around 2 fold with virginiamycin treatment without an increase in cellular protein abundance. An increase in the number of cells is not responsible for the increase in nutrient transport. This indicates that virginiamycin is not working by creating more mature cells but by enhancing the functional capacity of each, already existing cell. The concentration of virginiamycin would be highest in the proximal gut and that is reflected in the direct impact on structure and functional capacity in the proximal gut.

A unique functional response occurred in the ileum. Increased ileal arginine transport was delayed until day fourteen. Arginine transport may be lower in the ileum at seven days because of increased absorption in the jejunum and, therefore, the nutrients are not reaching the distal small intestine. Additionally, the cells may be rapidly proliferating, immature cells that develop into more mature, functional cells by day fourteen. Virginiamycin has been shown to inhibit amino acid breakdown via bacteria in the small intestine (Dierick et al., 1986). This may result in a secondary effect where more amino acids are available in the ileum for transport by day fourteen. This would support the nutrient sparing effect as a proposed mechanism by which antibiotics promote growth. The nutrient sparing effect is thought to be due to an increase in the amount of nutrients available to the host due to a reduction in nutrient use by bacteria and, therefore, increased absorption. This direct connection would be true if absorption is based on a concentration gradient. Nutrient concentrations are held constant within the Ussing chambers. Increased nutrient transport observed within the Ussing chambers, without increased absorptive surface area, indicate the intestinal tissue has the capability of absorbing more nutrients given the same concentrations.

In this study, pigs fed virginiamycin supplemented diets had greater nutrient transport capacity in the small intestine without increases in absorptive surface area beyond the duodenum. Dierick and colleagues (1981; 1986) suggest that virginiamycin may enhance the activity of alkaline phosphatase, Na^{+}-K^{+} adenosine triphosphatase, and amino peptidases which are all enzymes associated with nutrient absorption. Given the age and diet of grower pigs, maltase activity may provide better insight into discaccharidase activity. Increases in nutrient transport without increases in structural indices may also indicate up-regulation of nutrient transporters or enzymes associated with digestion and absorption. Additionally, virginiamycin increases digesta

passage rate which indicates more time for digestion and absorption. More nutrients available to the pig plus an increase in nutrient transport plus increased time for digestion and absorption may be partly responsible for the increase in pig performance associated with antibiotics.

Principal component analysis was completed to provide an overview of the effects of virginiamycin along the intestinal tract and to derive a smaller set of variables that account for most of the variance to identify a limited number of variables that might indicate mechanisms of virginiamycin. The second PCA identified two factors (factors 5 and 7) that discriminate between control and virginiamycin fed pigs. The factor scores for control were negative which indicates that variables negatively correlated with either factor 5 or 7 are associated with control pigs and may be decreased in control pigs compared with virginiamycin-fed pigs. The factor scores for the 11 mg/kg virginiamycin were positive which indicates that variables positively correlated with either factor 5 or 7 are associated with virginiamycin-fed pigs and may be increased in virginiamycin-fed pigs compared with control. Variables associated with control pigs were confined to the ileum, indicating a decrease in absorptive surface area and a decrease in resistance indicating increased intestinal permeability. Variables associated with virginiamycin-fed pigs involved increases in functional capacity along the entire intestinal tract (**Table 2.16**). Although differences in all of these variables were not statistically significant, this may indicate more subtle differences, that, when applied over the entire intestinal tract can have an impact on growth and feed efficiency.

One limitation to our study was inherently adopted by the study design. Split plot design takes precision away from the main plot to increase precision in the subplot. Virginiamycin treatment effects were our primary interest but treatment had to be the whole plot. Due to limitations in space, pigs were unable to be housed individually and, therefore, dietary treatment

had to be assigned on a pen basis. It was not feasible to feed different diets within the same pen. Another limitation to our study was that it was conducted in a relatively clean facility. Research has shown that growth responses are greater when hygiene is poor (Visek, 1978) and that growth responses are two to three times greater on commercial farms than university settings (Zimmerman, 1986). This may help explain our lack of statistically significant differences in growth response and feed efficiency.

The mechanism behind antibiotic growth promoters is likely multifaceted. With concerns of antibiotic use in animals resulting in adverse human health effects, it is important to understand the mechanism by which antibiotics promote growth. Our study demonstrated that virginiamycin impacted functional capacity to a greater extent than structural indices (**Figure 2.15**). Virginiamycin directly impacted proximal gut nutrient transport with a more delayed response in the distal gut by both increasing nutrient transport and secretion over time and providing protection against loss in nutrient transport and secretory capacity. Increasing our knowledge on the mechanisms of antibiotic growth promotion will help to better understand the safety of in-feed antibiotics and direct government policy on antibiotic use in animals

Table 2.1 Nutrient composition of diets (g/kg)

	Virginiamycin (mg/kg)		
Ingredient	**0**	**11**	**27.5**
Ground corn	694.6	694.6	694.6
Soybean meal	272.1	272.1	272.1
White grease	10.0	10.0	10.0
Lime	9.60	9.60	9.60
Dicalcium phosphate	9.20	9.20	9.20
Trace mineral mix[1]	3.50	3.50	3.50
Vitamin mix[2]	1.00	1.00	1.00
Virginiamycin premix[3]	-	0.10	0.25

[1]The premix supplied the following per kilogram of diet: 125 mg Fe (iron sulfate), 100 mg Zn (zinc oxide), 60 mg Mn (manganese sulfate), 10 mg Cu (copper sulfate), 1.26 mg I (potassium iodate), 0.3 mg Se (sodium selenite)

[2]The premix supplied the following per kilogram of diet: 11,128 IU vitamin A, 2,204 IU vitamin D_3, 66 IU vitamin E, 44 mg niacin, 23.5 mg $_D$-pantothenic acid, 6.58 mg riboflavin, 1.58 mg folic acid, 1.42 mg vitamin K, 0.44 mg biotin, 0.24 mg pyridoxine, 0.24 mg thiamin, 0.03 mg vitamin B_{12}

[3]Contained 110 g of virginiamycin activity per kilogram (V-Max^{TM}50, Phibro Animal Health, Ridgefield Park, NJ)

Table 2.2 Body weight, feed intake, feed efficiency, and daily weight gain[1-2]

	Virginiamycin (mg/kg)			P-value					
				Mixed model			Contrast		
	0	11	27.5	Txt	Time	Interaction	0 vs. 11	0 vs. 27.5	0 vs. txt
Allocation									
Body Weight				0.356	0.445	0.280	0.226	0.256	0.208
7d	23.3 ± 1.3	22.4 ± 1.3	23.3 ± 1.3						
14d	23.4 ± 1.3	23.1 ± 1.3	22.3 ± 1.3						
Day 0									
Body Weight				0.343	0.390	0.188	0.248	0.231	0.210
7d	25.6 ± 1.1	24.1 ± 1.1	25.4 ± 1.1						
14d	25.5 ± 1.1	25.2 ± 1.1	23.8 ± 1.1						
Final									
Body Weight				0.490	**<0.00005**	0.244	0.428	0.460	0.435
7d	30.8 ± 1.4	30.7 ± 1.7	31.9 ± 1.6						
14d	37.3 ± 1.4	36.8 ± 1.4	35.8 ± 1.4						
Daily Gain				0.303	0.336	0.056	0.176	0.262	0.184
7d	0.758 ± 0.09	0.960 ± 0.10	0.857 ± 0.09						
14d	0.834 ± 0.09	0.827 ± 0.09	0.864 ± 0.09						
Daily Feed Intake				0.345	**<0.00005**	0.184	0.245	0.239	0.212
7d	1.50 ± 0.10	1.45 ± 0.10	1.44 ± 0.10						
14d	1.82 ± 0.10	1.68 ± 0.10	1.68 ± 0.10						
Feed per Unit of Gain									
(kg/kg body weight)				0.217	**0.015**	0.114	0.139	0.150	0.117
7d	2.13 ± 0.18	1.64 ± 0.20	1.81 ± 0.19						
14d	2.17 ± 0.19	2.11 ± 0.18	1.98 ± 0.18						

[1]Data are expressed as mean ± SEM

[2]Unit of measure for weight and feed intake is kilograms

Table 2.3 Intestinal weight and length[1]

	Virginiamycin (mg/kg)			P-value					
				Mixed model			Contrast		
	0	**11**	**27.5**	**Txt**	**Time**	**Interaction**	**0 vs. 11**	**0 vs. 27.5**	**0 vs. txt**
Intestinal Weight[2]									
Duodenum				0.351	**0.041**	0.481	0.221	0.378	0.265
7d	1.35 ± 0.11	1.46 ± 0.11	1.38 ± 0.11						
14d	1.23 ± 0.11	1.30 ± 0.11	1.27 ± 0.11						
Jejunum				0.193	0.164	0.069	0.101	0.250	0.131
7d	23.8 ± 1.4	28.7 ± 1.5	27.1 ± 1.4						
14d	25.7 ± 1.4	26.1 ± 1.4	24.8 ± 1.4						
Ileum				0.210	**0.0002**	0.227	0.179	0.355	0.361
7d	32.2 ± 1.5	35.6 ± 1.6	32.8 ± 1.5						
14d	29.8 ± 1.5	29.7 ± 1.5	27.9 ± 1.5						
Colon				0.465	**0.005**	0.476	0.388	0.376	0.365
7d	44.4 ± 2.1	45.5 ± 2.2	45.8 ± 2.1						
14d	41.1 ± 2.1	41.4 ± 2.1	41.4 ± 2.2						
Intestinal Length[3]									
Duodenum				0.498	**0.032**	0.329	0.472	0.492	0.479
7d	1.27 ± 0.12	1.28 ± 0.12	1.22 ± 0.12						
14d	1.12 ± 0.12	1.13 ± 0.12	1.18 ± 0.12						
Jejunum/Ileum				0.431	**0.00005**	0.436	0.430	0.372	0.465
7d	22.6 ± 1.3	22.8 ± 1.4	21.7 ± 1.3						
14d	18.7 ± 1.3	19.1 ± 1.3	18.5 ± 1.3						
Colon				0.416	**0.00005**	0.446	0.398	0.378	0.488
7d	9.68 ± 0.45	9.87 ± 0.45	9.39 ± 0.43						
14d	8.01 ± 0.43	8.11 ± 0.43	7.96 ± 0.43						

[1]Data are expressed as mean ± SEM
[2]Unit of measure is grams per kilogram of body weight
[3]Unit of measure is centimeters per kilogram of body weight

Table 2.4 Mucosa and submucosa mass[1-3]

	Virginiamycin (mg/kg)			P-value					
				Mixed model			Contrast		
	0	11	27.5	Txt	Time	Interaction	0 vs. 11	0 vs. 27.5	0 vs. txt
Mucosa									
Duodenum				0.235	**0.0008**	**0.038**	0.129	0.259	0.154
7d	0.279 ± 0.030[b]	0.280 ± 0.029[b]	0.308 ± 0.029[b]						
14d	0.411 ± 0.029[a]	0.318 ± 0.028[b]	0.334 ± 0.029[ab]						
Jejunum				0.368	0.437	0.315	0.467	0.277	0.381
7d	0.481 ± 0.043	0.451 ± 0.043	0.455 ± 0.041						
14d	0.465 ± 0.041	0.503 ± 0.041	0.436 ± 0.043						
Ileum				0.306	**0.002**	0.350	0.232	0.194	0.183
7d	0.251 ± 0.038	0.311 ± 0.038	0.314 ± 0.037						
14d	0.339 ± 0.037	0.358 ± 0.037	0.370 ± 0.037						
Colon				0.322	**0.019**	0.088	0.224	0.205	0.182
7d	0.401 ± 0.057	0.376 ± 0.061	0.417 ± 0.051						
14d	0.519 ± 0.057	0.460 ± 0.058	0.417 ± 0.041						
Submucosa									
Duodenum				0.366	0.251	0.066	0.346	0.215	0.248
7d	0.155 ± 0.037	0.184 ± 0.037	0.173 ± 0.037						
14d	0.191 ± 0.037	0.147 ± 0.037	0.142 ± 0.037						
Jejunum				0.077	**0.014**	0.347	0.058	**0.044**	**0.037**
7d	0.140 ± 0.026	0.180 ± 0.026	0.198 ± 0.025						
14d	0.118 ± 0.025	0.158 ± 0.025	0.151 ± 0.026						
Ileum				0.391	**0.028**	0.246	0.359	0.260	0.281
7d	0.183 ± 0.031	0.209 ± 0.031	0.198 ± 0.031						
14d	0.213 ± 0.031	0.218 ± 0.031	0.255 ± 0.031						
Colon				0.347	0.099	0.101	0.413	0.206	0.275
7d	0.553 ± 0.049	0.473 ± 0.049	0.433 ± 0.047						
14d	0.504 ± 0.048	0.563 ± 0.047	0.545 ± 0.047						

[1]Data are expressed as mean ± SEM
[2]Unit of measure is grams per centimeter of tissue
[3]Within intestinal segment, different superscripts indicate statistically significant differences

Table 2.5 Intestinal crypt-villus architecture[1-2]

	Virginiamycin (mg/kg)			P-value					
				Mixed model			Contrast		
	0	**11**	**27.5**	**Txt**	**Time**	**Interaction**	**0 vs. 11**	**0 vs. 27.5**	**0 vs. txt**
Villus height[3]									
Duodenum				0.202	**0.016**	0.241	0.314	0.093	0.149
7d	373 ± 22	381 ± 22	378 ± 21						
14d	394 ± 21	407 ± 21	446 ± 21						
Jejunum				0.488	0.134	0.424	0.432	0.491	0.465
7d	456 ± 26	458 ± 27	449 ± 26						
14d	478 ± 27	467 ± 27	487 ± 26						
Ileum				0.317	0.083	0.356	0.208	0.473	0.299
7d	362 ± 27	409 ± 27	376 ± 30						
14d	407 ± 27	419 ± 30	398 ± 27						
Villus width[3]									
Duodenum				0.173	**0.009**	0.236	0.091	0.165	0.097
7d	202 ± 8.6	208 ± 8.6	205 ± 8.2						
14d	207 ± 8.2	231 ± 8.2	224 ± 8.2						
Jejunum				0.274	0.483	0.333	0.174	0.196	0.155
7d	194 ± 7.9	178 ± 8.1	182 ± 7.9						
14d	188 ± 8.1	184 ± 8.1	182 ± 7.9						
Ileum				0.469	0.299	0.181	0.369	0.438	0.388
7d	165 ± 6.7	153 ± 6.9	163 ± 7.7						
14d	161 ± 6.7	167 ± 7.9	161 ± 6.9						

Table 2.5 Continued

	Virginiamycin (mg/kg)			P-value					
				Mixed model			Contrast		
	0	11	27.5	Txt	Time	Interaction	0 vs. 11	0 vs. 27.5	0 vs. txt
Villus surface area[4]									
Duodenum				0.110	**0.003**	0.289	0.112	**0.046**	**0.048**
7d	75,586 ± 6220	79,695 ± 6220	79,559 ± 5955						
14d	83,027 ± 5955	93,889 ± 5955	99,673 ± 5955						
Jejunum				0.350	0.127	0.441	0.223	0.290	0.226
7d	88,123 ± 5840	80,717 ± 6067	81,491 ± 5840						
14d	90,269 ± 6067	86,100 ± 6067	88,771 ± 5840						
Ileum				0.302	0.080	0.420	0.200	0.454	0.332
7d	59,611 ± 4240	62,944 ± 4438	61,186 ± 4885						
14d	65,962 ± 4240	70,284 ± 5198	63,365 ± 4438						
Crypt Depth[3]									
Duodenum				0.304	0.101	0.427	0.167	0.248	0.171
7d	562 ± 25	590 ± 25	575 ± 24						
14d	588 ± 24	597 ± 24	601 ± 24						
Jejunum				0.358	0.086	**0.023**	0.322	0.372	0.467
7d	520 ± 32^{ab}	561 ± 32^{a}	556 ± 32^{a}						
14d	546 ± 32^{ab}	539 ± 32^{ab}	486 ± 32^{b}						
Ileum				0.269	0.310	0.396	0.162	0.219	0.159
7d	310 ± 27	349 ± 27	328 ± 29						
14d	316 ± 27	345 ± 29	349 ± 27						
Colon				0.111	**0.013**	0.086	**0.044**	0.277	0.090
7d	424 ± 23	467 ± 24	463 ± 23						
14d	483 ± 23	496 ± 23	463 ± 23						

[1]Data are expressed as mean ± SEM
[2]Within intestinal segment, different superscripts indicate statistically significant differences
[3]Unit of measure is microns
[4]Unit of measure is microns squared

Table 2.6 Epithelial Cell Proliferation at day 7[1-3]

	Virginiamycin (mg/kg)		P-value
	0	**11**	**Txt**
Duodenum	39.6 ± 2.5	44.3 ± 3.1	0.055
Jejunum	33.2 ± 1.9	36.6 ± 2.0	0.130
Ileum	25.9 ± 1.9[b]	29.9 ± 1.9[a]	**0.003**
Colon	51.8 ± 3.9	59.3 ± 4.0	0.144

[1]Data are expressed as mean ± SEM

[2]Unit of measure is number of proliferating cell nuclear antigen positive cells per crypt

[3]Within intestinal segment, different superscripts indicate statistically significant differences

Table 2.7 Cellular RNA Concentration per Cell[1-2]

	Virginiamycin (mg/kg)			P-value					
				Mixed model			Contrast		
	0	11	27.5	Txt	Time	Interaction	0 vs. 11	0 vs. 27.5	0 vs. txt
Duodenum				0.320	0.061	0.401	0.188	0.305	0.211
7d	3.37 ± 1.0	4.20 ± 1.0	4.20 ± 1.0						
14d	2.66 ± 1.0	3.70 ± 1.0	2.85 ± 1.0						
Jejunum				0.247	0.083	0.233	0.135	0.188	0.130
7d	0.314 ± 0.027	0.365 ± 0.018	0.369 ± 0.022						
14d	0.321 ± 0.026	0.337 ± 0.023	0.323 ± 0.031						
Ileum				0.495	0.110	0.166	0.493	0.461	0.481
7d	0.265 ± 0.037	0.292 ± 0.038	0.270 ± 0.037						
14d	0.262 ± 0.037	0.234 ± 0.037	0.268 ± 0.037						
Colon				0.404	0.266	0.246	0.311	0.281	0.271
7d	1.20 ± 1.5	1.48 ± 1.9	1.41 ± 1.6						
14d	1.46 ± 2.1	1.38 ± 1.8	1.48 ± 1.5						

[1]Data are expressed as mean ± SEM
[2]Unit of measure is milligram of RNA per milligram of DNA

Table 2.8 Cellular Protein Concentration[1-2]

	Virginiamycin (mg/kg)			P-value					
				Mixed model			Contrast		
	0	11	27.5	Txt	Time	Interaction	0 vs. 11	0 vs. 27.5	0 vs. txt
Duodenum				0.337	0.103	0.338	0.279	0.212	0.215
7d	57.0 ± 9.5	64.5 ± 9.5	70.9 ± 9.3						
14d	55.0 ± 9.3	58.1 ± 9.3	55.8 ± 9.3						
Jejunum				0.473	0.111	0.145	0.477	0.383	0.418
7d	8.98 ± 0.70	9.13 ± 0.58	9.83 ± 0.69						
14d	9.05 ± 0.52	9.00 ± 0.68	8.70 ± 0.57						
Ileum				0.385	0.281	0.250	0.437	0.310	0.421
7d	7.63 ± 0.61	8.04 ± 0.63	8.10 ± 0.61						
14d	7.80 ± 0.61	7.14 ± 0.61	8.12 ± 0.61						
Colon				0.404	0.179	0.477	0.289	0.313	0.275
7d	32.8 ± 2.5	31.8 ± 2.6	32.0 ± 2.5						
14d	34.9 ± 2.5	32.9 ± 2.5	33.1 ± 2.5						

[1]Data are expressed as mean ± SEM

[2]Unit of measure is milligram of protein per milligram of DNA

Table 2.9 Tissue DNA Concentration[1-2]

	Virginiamycin (mg/kg)			P-value					
				Mixed model			Contrast		
	0	11	27.5	Txt	Time	Interaction	0 vs. 11	0 vs. 27.5	0 vs. txt
Duodenum				0.435	**0.011**	0.299	0.357	0.323	0.318
7d	1.35 ± 0.22	1.20 ± 0.22	1.05 ± 0.22						
14d	1.55 ± 0.22	1.50 ± 0.22	1.60 ± 0.22						
Jejunum				0.487	0.230	0.061	0.414	0.461	0.428
7d	9.68 ± 0.76	9.99 ± 0.63	9.09 ± 0.66						
14d	9.97 ± 0.62	9.29 ± 0.65	10.4 ± 0.69						
Ileum				0.492	0.413	0.053	0.465	0.433	0.441
7d	11.5 ± 1.1	9.83 ± 1.1	10.0 ± 1.1						
14d	9.53 ± 1.1	10.9 ± 1.1	10.5 ± 1.1						
Colon				0.425	0.214	0.287	0.327	0.298	0.287
7d	1.62 ± 0.15	1.43 ± 0.15	1.46 ± 0.15						
14d	1.46 ± 0.15	1.53 ± 0.15	1.48 ± 0.15						

[1]Data are expressed as mean ± SEM

[2]Unit of measure is milligram of DNA per milligram of tissue

Table 2.10 Intestinal disaccharidase activity[1-3]

	Virginiamycin (mg/kg)			P-value					
				Mixed model			Contrast		
	0	11	27.5	Txt	Time	Interaction	0 vs. 11	0 vs. 27.5	0 vs. txt
Sucrase Activity									
Duodenum				0.269	**0.003**	**0.039**	0.200	0.147	0.138
7d	59.7 ± 49[ab]	33.5 ± 27[c]	38.8 ± 32[bc]						
14d	58.6 ± 49[ab]	76.5 ± 63[a]	61.4 ± 51[a]						
Jejunum				0.442	0.099	0.124	0.342	0.315	0.314
7d	3243 ± 1260	2853 ± 1143	2672 ± 1055						
14d	2804 ± 1100	3622 ± 1419	3974 ± 1656						
Ileum				0.362	**0.041**	0.443	0.452	0.277	0.389
7d	87.3 ± 36	78.0 ± 33	102 ± 42						
14d	63.1 ± 26	64.6 ± 27	84.6 ± 35						
Colon				0.180	0.368	0.092	0.099	0.452	0.190
7d	11.3 ± 50	83.0 ± 40	79.0 ± 40						
14d	10.3 ± 50	64.0 ± 30	13.8 ± 70						
Lactase Activity									
Duodenum				0.462	0.086	0.086	0.380	0.445	0.347
7d	61.0 ± 28	46.0 ± 21	52.0 ± 25						
14d	51.6 ± 24	71.7 ± 34	69.0 ± 33						
Jejunum				0.479	0.227	0.297	0.451	0.391	0.407
7d	2831 ± 813	3399 ± 962	2721 ± 814						
14d	2514 ± 745	2248 ± 127	3033 ± 1147						
Ileum				0.463	0.231	0.130	0.467	0.396	0.457
7d	7.1 ± 1.4	5.2 ± 1.6	6.6 ± 1.3						
14d	4.9 ± 2.3	7.0 ± 1.4	4.6 ± 1.8						
Colon				0.349	0.272	0.233	0.361	0.322	0.475
7d	22.3 ± 7.0	23.1 ± 8.0	17.4 ± 6.0						
14d	18.3 ± 6.0	20.3 ± 7.0	19.7 ± 6.0						

[1]Data are expressed as mean ± SEM

[2]Unit of measure is μmol hydrolyzed substrate per hour (unit (U) of activity) per gram of protein

[3]Within intestinal segment, different superscripts indicate statistically significant differences

Table 2.11 Basal ion transport[1]

	Virginiamycin (mg/kg)			P-value					
				Mixed model			Contrast		
	0	11	27.5	Txt	Time	Interaction	0 vs. 11	0 vs. 27.5	0 vs. txt
Short circuit current[2]									
Duodenum				0.293	0.277	0.202	0.204	0.171	0.151
7d	13.8 ± 5.5	16.0 ± 6.6	13.7 ± 5.1						
14d	27.7 ± 11	9.30 ± 4.7	18.7 ± 9.5						
Jejunum				0.450	0.130	0.436	0.391	0.335	0.342
7d	12.1 ± 5.5	11.8 ± 6.7	14.8 ± 5.5						
14d	14.3 ± 7.4	19.2 ± 8.2	18.5 ± 7.6						
Ileum				0.375	0.424	0.423	0.331	0.233	0.253
7d	7.88 ± 6.1	12.1 ± 7.5	13.0 ± 5.5						
14d	10.9 ± 4.4	11.3 ± 4.4	12.5 ± 4.1						
Colon				0.115	0.453	0.129	0.092	0.061	0.056
7d	21.6 ± 14	22.0 ± 14	28.1 ± 16						
14d	11.6 ± 14	31.1 ± 14	30.8 ± 15						
Transmucosal Resistance[3]									
Duodenum				0.153	0.311	0.185	0.268	0.208	0.462
7d	100 ± 30	71.8 ± 15	114 ± 20						
14d	92.5 ± 21	98.9 ± 21	110 ± 36						
Jejunum				0.423	**0.033**	0.427	0.349	0.296	0.293
7d	95.1 ± 18	99.9 ± 21	113 ± 17						
14d	122 ± 19	132 ± 21	127 ± 32						
Ileum				0.382	0.111	0.126	0.498	0.269	0.359
7d	103 ± 24	128 ± 20	135 ± 25						
14d	148 ± 22	121 ± 25	144 ± 20						
Colon				0.398	0.440	0.311	0.299	0.476	0.397
7d	35.6 ± 17	40.0 ± 19	41.6 ± 20						
14d	41.1 ± 20	44.7 ± 21	34.5 ± 16						

Table 2.11 Continued

	Virginiamycin (mg/kg)			P-value					
				Mixed model			Contrast		
	0	**11**	**27.5**	**Txt**	**Time**	**Interaction**	**0 vs. 11**	**0 vs. 27.5**	**0 vs. txt**
Potential Difference[4]									
Duodenum				0.155	0.394	0.411	0.095	0.468	0.224
7d	1.49 ± 0.64	0.446 ± 0.13	1.40 ± 0.61						
14d	1.27 ± 0.54	0.861 ± 0.37	1.45 ± 0.61						
Jejunum				0.298	**0.035**	0.285	0.238	0.184	0.175
7d	1.40 ± 0.82	1.27 ± 0.61	2.03 ± 0.61						
14d	1.82 ± 0.76	3.51 ± 1.4	3.06 ± 1.0						
Ileum				0.209	0.066	0.180	0.094	0.343	0.116
7d	0.761 ± 1.1	1.30 ± 1.1	1.28 ± 1.1						
14d	1.49 ± 1.1	3.35 ± 1.8	1.27 ± 1.1						
Colon				**0.048**	0.170	0.107	0.265	**0.017**	**0.046**
7d	0.744 ± 0.14	0.583 ± 0.16	1.78 ± 0.63						
14d	0.488 ± 0.26	0.946 ± 0.25	0.771 ± 0.09						
Mean[5-6]	0.603 ± 0.18[b]	0.743 ± 0.13[ab]	1.17 ± 0.32[a]						

[1]Data are expressed as mean ± SEM
[2]Unit of measure is µA/cm^2
[3]Unit of measure is Ω●cm^2
[4]Unit of measure is millivolts
[5]Means pooled within treatment
[6]Different superscripts indicate statistically significant differences

Table 2.12 Nutrient transport[1-3]

	Virginiamycin (mg/kg)			P-value					
				Mixed model			Contrast		
	0	11	27.5	Txt	Time	Interaction	0 vs. 11	0 vs. 27.5	0 vs. txt
Glucose transport									
Duodenum				0.075	0.236	0.310	**0.032**	0.097	**0.025**
7d	0.637 ± 0.49	1.05 ± 0.63	0.713 ± 0.57						
14d	0.549 ± 0.44	1.42 ± 0.81	1.04 ± 0.61						
Jejunum				0.123	**0.028**	0.186	0.094	0.078	**0.045**
7d	2.09 ± 1.4	2.06 ± 1.3	2.87 ± 1.5						
14d	2.41 ± 1.6	5.85 ± 2.5	6.33 ± 4.1						
Ileum				0.312	0.136	0.206	0.174	0.335	0.215
7d	18.8 ± 11	11.0 ± 8.4	19.9 ± 11						
14d	15.6 ± 9.9	11.9 ± 9.1	10.2 ± 8.4						
Colon				0.260	0.443	0.119	0.131	0.335	0.169
7d	1.34 ± 0.85	2.80 ± 1.4	1.00 ± 0.75						
14d	1.30 ± 0.94	1.58 ± 1.1	2.31 ± 1.4						
Glutamine transport									
Duodenum				0.187	**0.007**	0.286	0.083	0.352	0.143
7d	2.36 ± 1.0	4.71 ± 1.8	2.53 ± 0.92						
14d	1.35 ± 0.65	1.89 ± 0.73	1.67 ± 0.82						
Jejunum				**0.010**	0.088	0.356	**0.006**	**0.038**	**0.002**
7d	1.90 ± 0.60	4.61 ± 2.1	2.75 ± 1.0						
14d	0.964 ± 0.25	2.65 ± 0.71	2.68 ± 1.4						
Mean[4]	1.39 ± 0.28[b]	3.56 ± 0.95[a]	2.72 ± 0.81[a]						
Ileum				0.096	0.352	0.327	0.280	0.130	0.425
7d	5.06 ± 2.1	4.97 ± 1.8	4.03 ± 1.4						
14d	4.20 ± 1.5	6.03 ± 2.4	2.93 ± 1.3						
Colon				0.449	**0.048**	0.104	0.459	0.368	0.450
7d	5.66 ± 2.3	9.14 ± 4.5	9.02 ± 2.9						
14d	6.84 ± 2.5	3.47 ± 2.0	5.36 ± 2.2						

Table 2.12 Continued

	Virginiamycin (mg/kg)			P-value					
				Mixed model			Contrast		
	0	**11**	**27.5**	**Txt**	**Time**	**Interaction**	**0 vs. 11**	**0 vs. 27.5**	**0 vs. txt**
Arginine transport									
Duodenum				0.470	0.201	0.347	0.373	0.402	0.364
7d	0.643 ± 0.18	0.833 ± 0.29	0.870 ± 0.32						
14d	1.01 ± 0.34	0.957 ± 0.41	0.873 ± 0.44						
Jejunum				**0.046**	0.338	0.404	**0.025**	0.084	**0.014**
7d	0.768 ± 0.19	1.49 ± 0.52	1.05 ± 0.38						
14d	0.515 ± 0.19	1.17 ± 0.62	1.24 ± 0.82						
Mean[4]	0.630 ± 0.13[b]	1.32 ± 0.38[a]	1.14 ± 0.42[ab]						
Ileum				0.356	0.283	**0.042**	0.326	0.245	0.250
7d	4.06 ± 2.4[a]	1.44 ± 1.2[c]	2.20 ± 1.6[bc]						
14d	1.53 ± 1.1[c]	3.12 ± 2.1[b]	1.77 ± 1.7[bc]						
Colon				0.360	0.409	0.329	0.367	0.335	0.482
7d	1.93 ± 0.79	2.13 ± 0.91	2.61 ± 1.1						
14d	2.61 ± 1.1	1.57 ± 0.65	3.16 ± 1.4						

[1]Data are expressed as mean ± SEM
[2]Unit of measure is μA/cm^2
[3]Within intestinal segment, different superscripts indicate statistically significant differences
[4]Means pooled within treatment

Table 2.13 Serotonin and Carbachol induced-chloride secretion[1-3]

	Virginiamycin (mg/kg)			P-value					
				Mixed model			Contrast		
	0	11	27.5	Txt	Time	Interaction	0 vs. 11	0 vs. 27.5	0 vs. txt
Serotonin									
Duodenum				0.122	0.087	0.318	0.105	0.308	0.279
7d	0.456 ± 0.31	0.917 ± 0.47	0.514 ± 0.34						
14d	0.909 ± 0.42	1.06 ± 0.48	0.643 ± 0.36						
Jejunum				**0.028**	0.088	0.090	0.060	0.208	0.292
7d	1.62 ± 0.87	3.96 ± 1.5	0.828 ± 0.42						
14d	1.15 ± 0.51	1.46 ± 0.64	1.12 ± 0.48						
Mean[4]	1.37 ± 0.46[ab]	2.56 ± 0.68[a]	0.970 ± 0.30[b]						
Ileum				0.381	0.326	0.184	0.238	0.428	0.301
7d	1.28 ± 0.94	0.916 ± 0.48	0.789 ± 0.35						
14d	0.621 ± 0.25	1.86 ± 1.0	1.28 ± 1.0						
Colon				0.490	0.384	0.311	0.454	0.472	0.489
7d	1.50 ± 0.87	1.92 ± 0.89	1.70 ± 0.81						
14d	1.82 ± 0.91	1.24 ± 0.78	1.74 ± 0.81						
Carbachol									
Duodenum				0.200	0.107	0.109	0.258	0.238	0.496
7d	1.56 ± 0.66	2.58 ± 1.3	2.11 ± 5.2						
14d	1.96 ± 0.77	1.78 ± 0.77	0.974 ± 0.42						
Jejunum				0.423	**0.033**	0.427	0.349	0.296	0.293
7d	18.7 ± 8.5	97.6 ± 47.5	8.13 ± 3.6						
14d	11.8 ± 5.3	16.2 ± 7.4	11.5 ± 5.2						
Ileum				0.352	0.406	**0.017**	0.326	0.338	0.485
7d	1.71 ± 0.68[ab]	0.727 ± 0.17[bc]	0.982 ± 0.33[abc]						
14d	0.703 ± 0.14[c]	2.78 ± 1.0[a]	0.780 ± 0.17[abc]						
Colon				0.486	0.382	0.079	0.432	0.482	0.472
7d	7.71 ± 3.4	14.3 ± 6.5	8.44 ± 3.7						
14d	11.6 ± 5.2	5.42 ± 2.4	11.1 ± 5.0						

[1]Data are expressed as mean ± SEM
[2]Unit of measure is μA/cm[2]
[3]Within intestinal segment, different superscripts indicate statistically significant differences
[4]Means pooled within treatment

Table 2.14 Description of the major factors obtained by principal component analysis (PCA) of thirty-seven variables characterizing the gut structure and function of pigs in control and 11 mg/kg virginiamycin groups at day 7[1-2]

Factor	1	2	3	4	5	6	7
Variance explained (%)	17.8	14.1	12.3	9.6	7.6	6.3	5.1
Cumulative (%)	17.8	31.9	44.2	53.8	61.4	67.7	72.8
Loadings of variables[3]							
Duodenum resistance	0.92						
Jejunum DNA	-0.79						
Jejunum resistance	0.60				0.43		
Ileum glucose transport	0.53						
Ileum 5HT	0.87						
Colon arginine transport	0.92						
Average daily gain		-0.74	0.43				
SI length		0.78					
Duodenum RNA		0.72					
Duodenum glutamine transport		0.55				-0.41	
Jejunum submucosal mass		-0.43					
Colon length		0.82					
Duodenum submucosal mass		-0.40	0.41	0.41			
Jejunum crypt depth			0.52	-0.46	0.47		
Ileum crypt depth			0.73		0.41		
Ileum PCNA			0.79				
Colon mass			0.71				
Colon DNA			-0.79				
Colon glutamine transport			0.53				
Duodenum potential difference				0.92			
Jejunum sucrase activity				0.83			
Jejunum PCNA				-0.57			
Colon potential difference				0.89			
Jejunum Isc					0.75		
Ileum villus height					0.53		
Colon crypt depth					0.80		
Colon PCNA					0.75		
Duodenum sucrase activity						0.81	
Duodenum lactase activity						0.83	
Jejunum lactase activity						0.71	
Colon submucosal mass						-0.48	
Colon Isc						0.59	
Duodenum CCH							0.86
Ileum protein							0.75
Ileum RNA							-0.74
Jejunum glutamine transport[4]							
Colon lactase activity[4]							

[1]Abbreviations: 5HT, serotonin induced chloride secretion; SI, small intestine; PCNA, proliferating cell nuclear antigen; Isc, short circuit current; CCH, carbachol induced chloride secretion.

[2]Extraction method: PCA; rotation method: varimax with Kaiser normalization
[3]Only correlations with $|r| \geq 0.40$ are indicated.
[4]Variables included in final analysis but did not load on the first 7 factors

Table 2.15 Description of the major factors obtained by principal component analysis (PCA) of thirty-four variables characterizing the gut structure and function of pigs in control and 11 mg/kg virginiamycin groups at day 14[1-3]

Factor	1	2	3	4	5	6	7
Variance explained (%)	16.6	12.2	10.6	9.8	7.9	7.2	6.5
Cumulative (%)	16.6	28.8	39.4	49.2	57.1	64.3	70.8
Loadings of variables[4]							
Duodenum arginine transport	0.84						
Jejunum protein	-0.55						
Jejunum arginine transport	0.84						
Ileum crypt depth	0.84						
Ileum sucrase activity	0.61						
Ileum lactase activity	0.58						
Ileum potential difference	-0.68						
Duodenum crypt depth		0.73					
Jejunum crypt depth		0.86					
Jejunum 5HT		0.47					
Colon glucose transport		0.77					0.41
Duodenum submucosal mass			0.79				
Jejunum resistance			0.49		0.40	0.49	
Colon glutamine transport			0.75				
Colon arginine transport			0.78				
Feed per unit of gain				0.84			
SI length				0.76			
SI mass				0.88			
Duodenum lactase activity		-0.58			0.62		
Jejunum lactase activity					0.75		
Colon potential difference					0.90		
Duodenum resistance						0.76	
Duodenum Isc						-0.47	
Duodenum glucose transport		0.43				0.53	
Jejunum sucrase activity					0.45	0.51	
Colon mass						-0.79	
Ileum submucosal mass							-0.42
Ileum villus height							-0.42
Ileum resistance							-0.80
Ileum CCH							0.85
Duodenum glutamine transport[5]							
Colon RNA[5]							
Colon sucrase activity[5]							
Colon 5HT[5]							

[1]Abbreviations: 5HT, serotonin induced chloride secretion; SI, small intestine; Isc, short circuit current; CCH, carbachol induced chloride secretion

[2]Extraction method: PCA; rotation method: varimax with Kaiser normalization

[3]Factor scores for control and 11 mg/kg groups are significantly different (factor 5: $p=0.015$; factor 7: $p=0.050$)

[4]Only correlations with $|r| \geq 0.40$ are indicated

[5]Variables included in final analysis but did not load on the first 7 factors

Table 2.16 Summary of factors 5 and 7 of the Principal Component Analysis (PCA) completed with data from control and 11 mg/kg virginiamycin groups at day 14[1-2]

Control (-)[3]	**11 mg/kg virginiamycin (+)**[4]
Ileal submucosal mass Ileal mucosal mass	Duodenal lactase activity
Ileal villus height	Duodenal glucose transport[5]
Ileal resistance	Jejunal disaccharidase activity
	Jejunal resistance
	Ileal carbachol-induced chloride secretion[5] Ileal serotonin-induced chloride secretion
	Colonic potential difference[5] Colonic short-circuit current

[1]Factors 5 and 7 of PCA 2 discriminate between control and 11 mg/kg virginiamycin-fed pigs

[2]Ileal mucosal mass, ileal serotonin-induced chloride secretion, and colonic short-circuit current were not included in the final PCA but were significantly correlated with the variable within the same cell in the table and, therefore, can be used in the interpretation

[3]Factor scores for control were negative

[4]Factor scores for 11 mg/kg virginiamycin were positive

[5]Variable significant by split-plot or contrast analysis

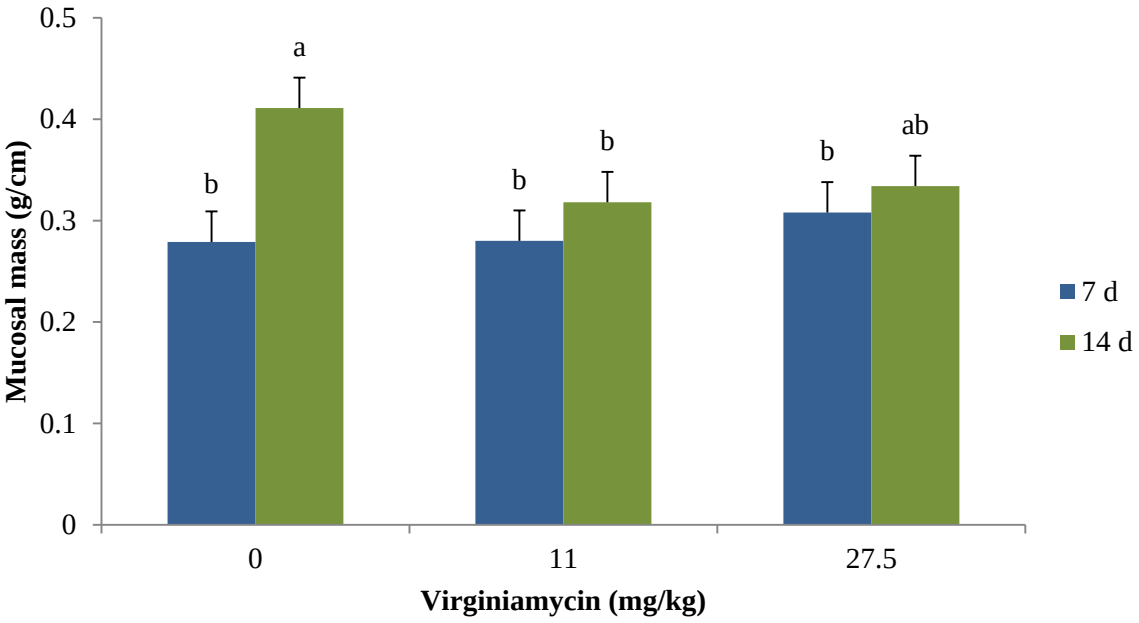


Figure 2.1 Duodenal mucosal mass expressed as grams per centimeter of tissue. Data are expressed as mean ± standard error measurement (SEM). Different letters above bars indicate a statistically significant difference. In the duodenum, mucosal mass increased over time in the control pigs, but it did not in pigs fed diets supplemented with virginiamycin resulting in less mucosal mass in the duodenum in the 11 mg/kg virginiamycin group at day fourteen (p=0.038).

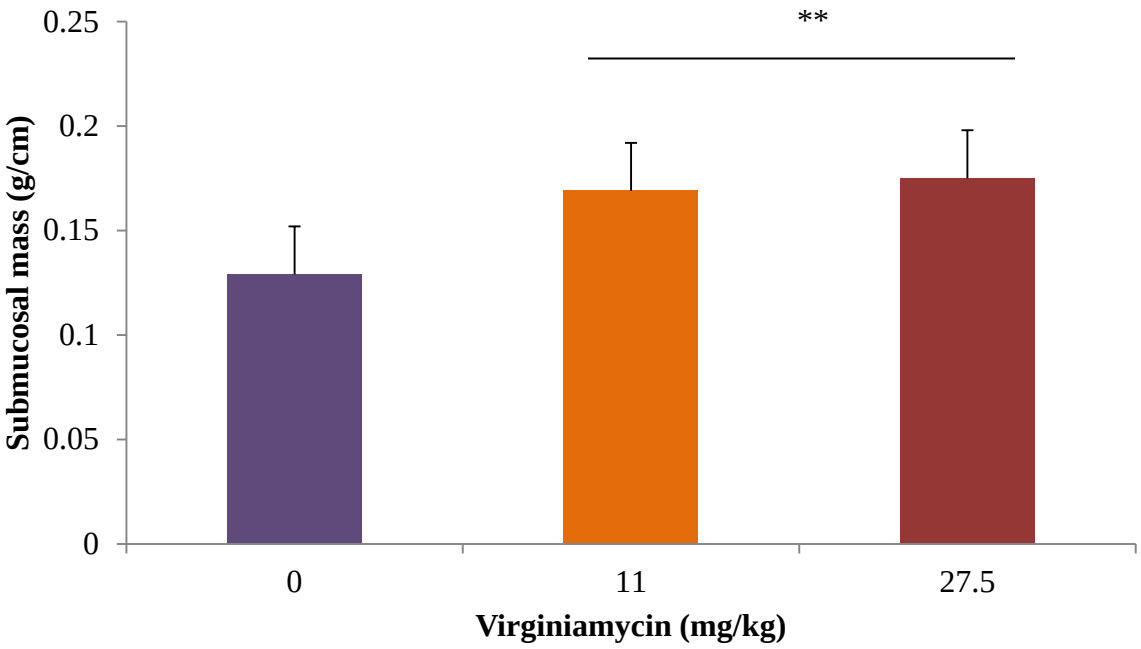
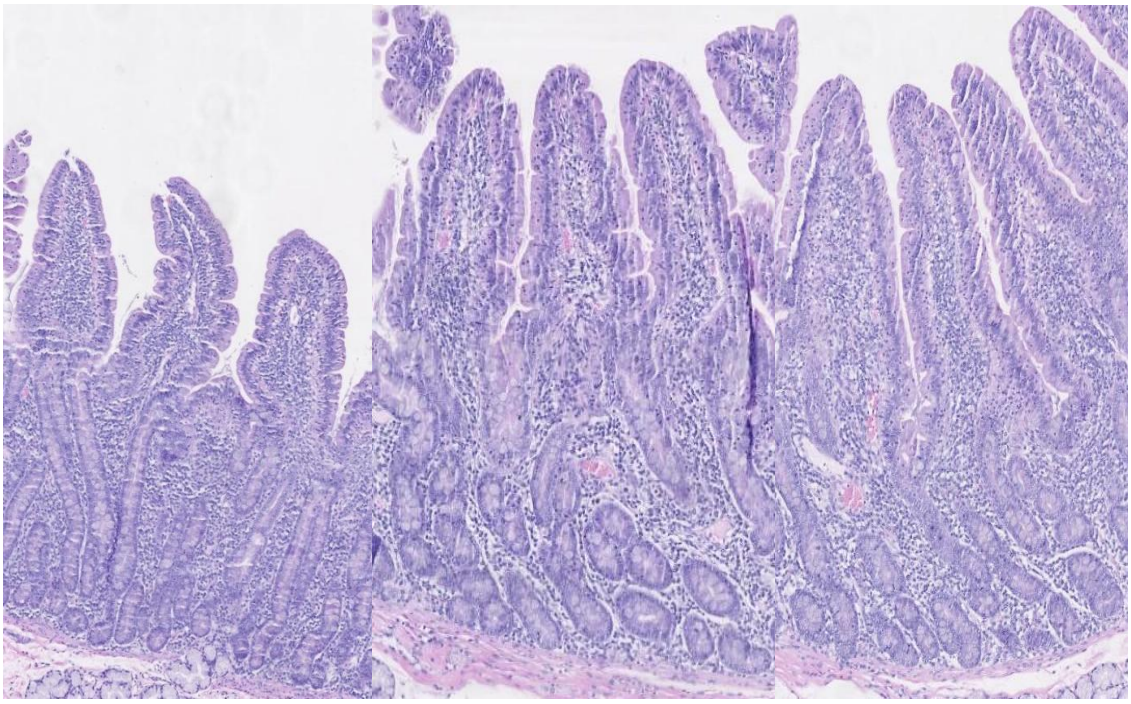


Figure 2.2 Jejunal submucosal mass expressed as grams per centimeter of tissue. Data are expressed as pooled treatment mean ± (SEM). In the jejunum, virginiamycin-fed pigs had more submucosal mass compared with control when virginiamycin groups were contrasted with control (p=0.037).



0 11 27.5

Virginiamycin (mg/kg)

Figure 2.3 Duodenal villus surface area for control, 11 mg/kg, and 27.5 mg/kg virginiamycin. Magnification of 5X with hematoxylin and eosin stain. Villus surface area was greater in the duodenum of virginiamycin-fed pigs compared with control when virginiamycin groups were contrasted with control (p=0.048).

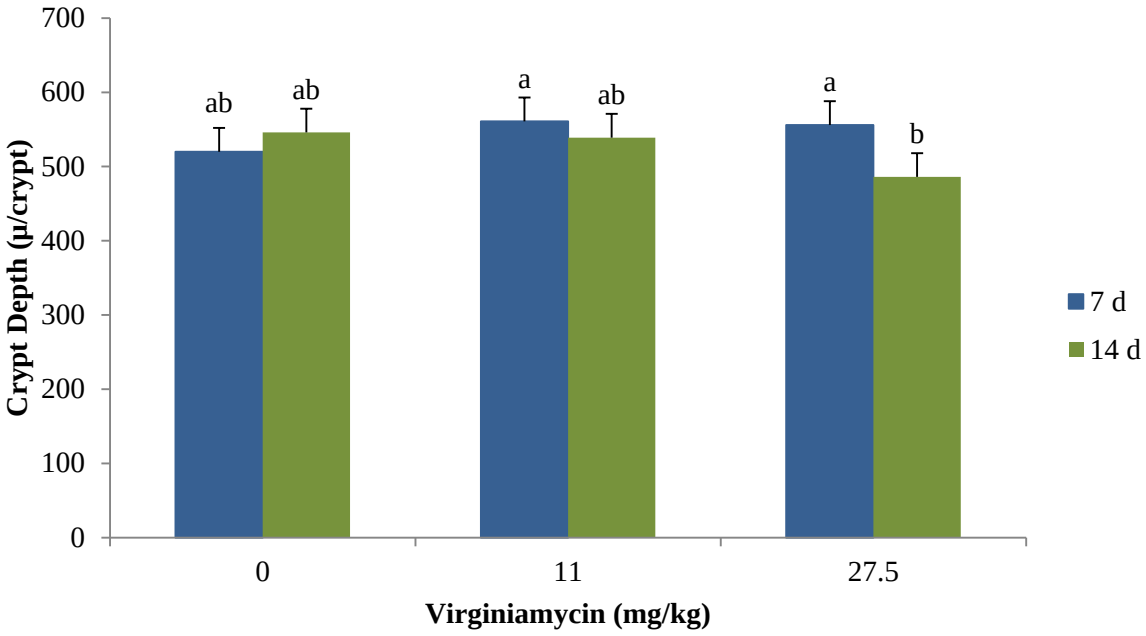


Figure 2.4 Crypt depth in the jejunum. Data are expressed as mean ± SEM. Different letters above bars indicate a statistically significant difference. Crypt depth decreased in pigs fed the diet supplemented with 27 mg/kg of virginiamycin over time in the jejunum (p=0.023)

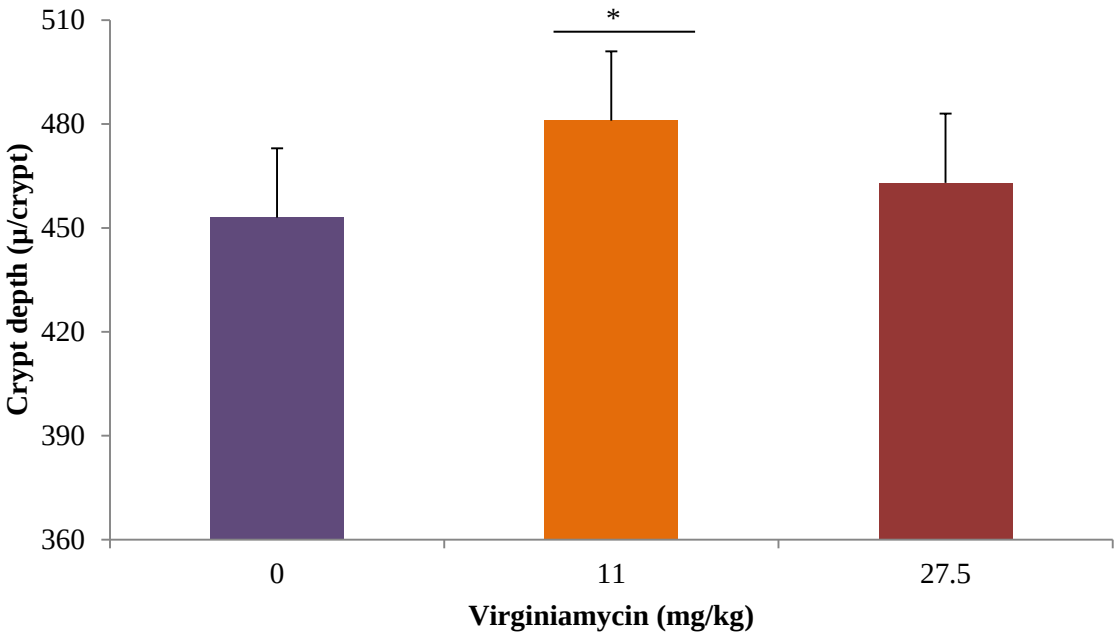
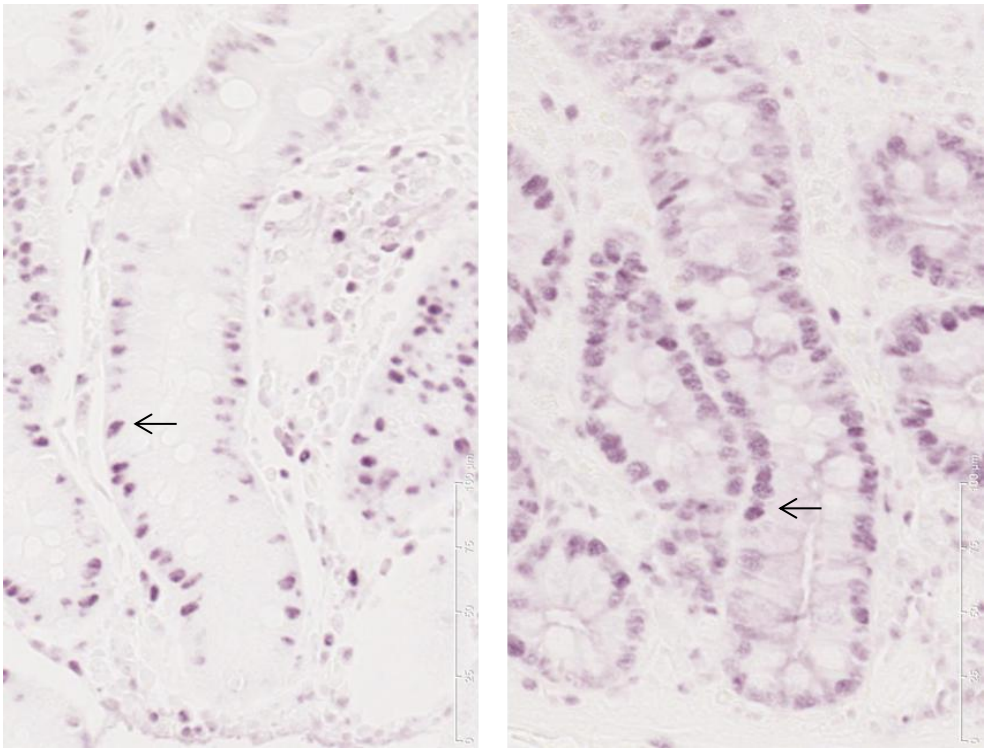


Figure 2.5 Colonic crypt depth. Data are expressed as pooled treatment mean ± (SEM). Pigs fed diets containing 11 mg/kg of virginiamycin had deeper crypts in the colon compared with control when the 11 mg/kg group was contrasted with control (p=0.044).



0 11

Virginiamycin (mg/kg)

Figure 2.6 Ileal epithelial cell proliferation at 7 days in control and 11 mg/kg virginiamycin-fed pigs, as measured by quantifying the number of proliferating cell nuclear antigen (PCNA)-positive cells per crypt. Black arrows indicate PCNA positive cells. Magnification of 5X with VIP stain. Virginiamycin treatment increased the number of PCNA-positive cells per crypt in the ileum at seven days compared with control ($p=0.003$).

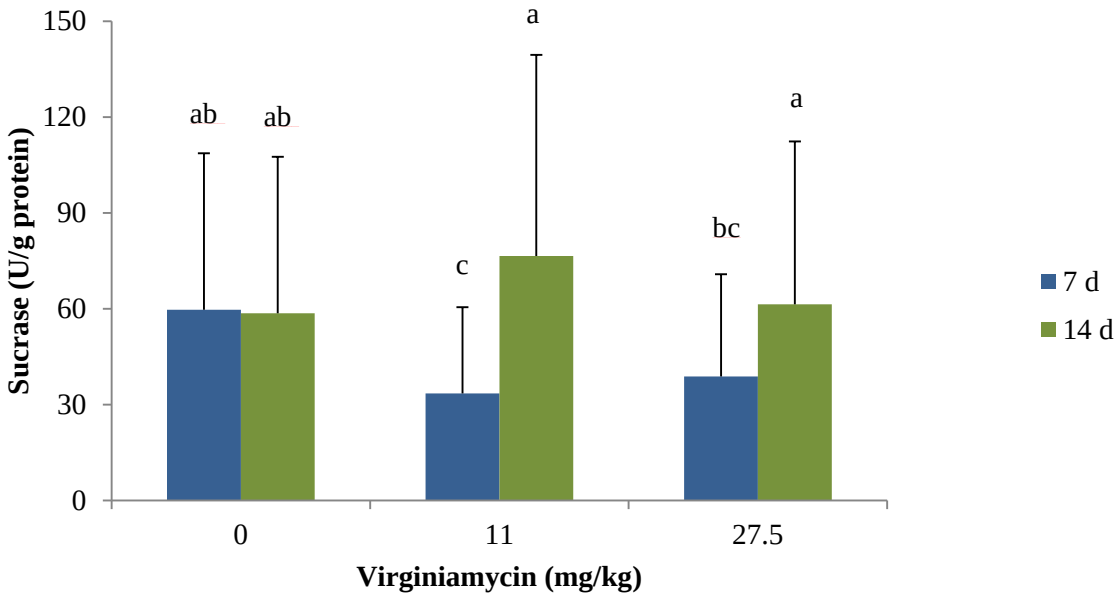


Figure 2.7 Sucrase activity in the duodenum expressed as unit of activity per gram of protein. Data are expressed as mean ± SEM. Different letters above bars indicate a statistically significant difference. Sucrase activity in the duodenum was lower in the 11 mg/kg virginiamycin group compared with control at day seven, but it increased over time in the virginiamycin groups (p=0.039).

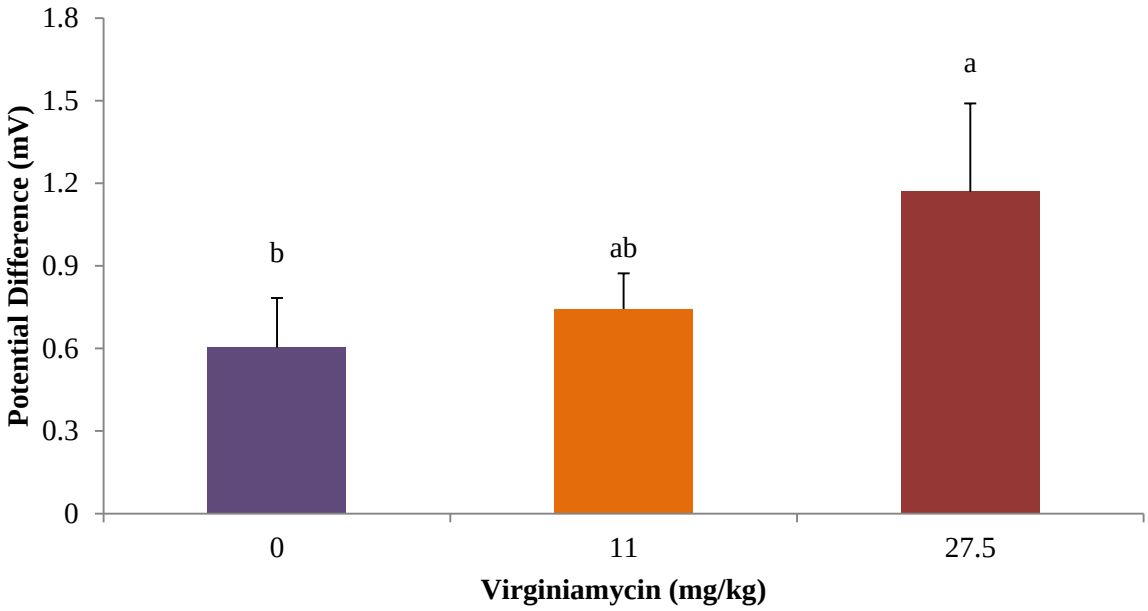


Figure 2.8 Potential difference in the colon. Data are expressed as pooled treatment mean ± SEM. Different letters above bars indicate a statistically significant difference. Potential difference was increased in the colon with the highest virginiamycin treatment compared with control (p=0.048).

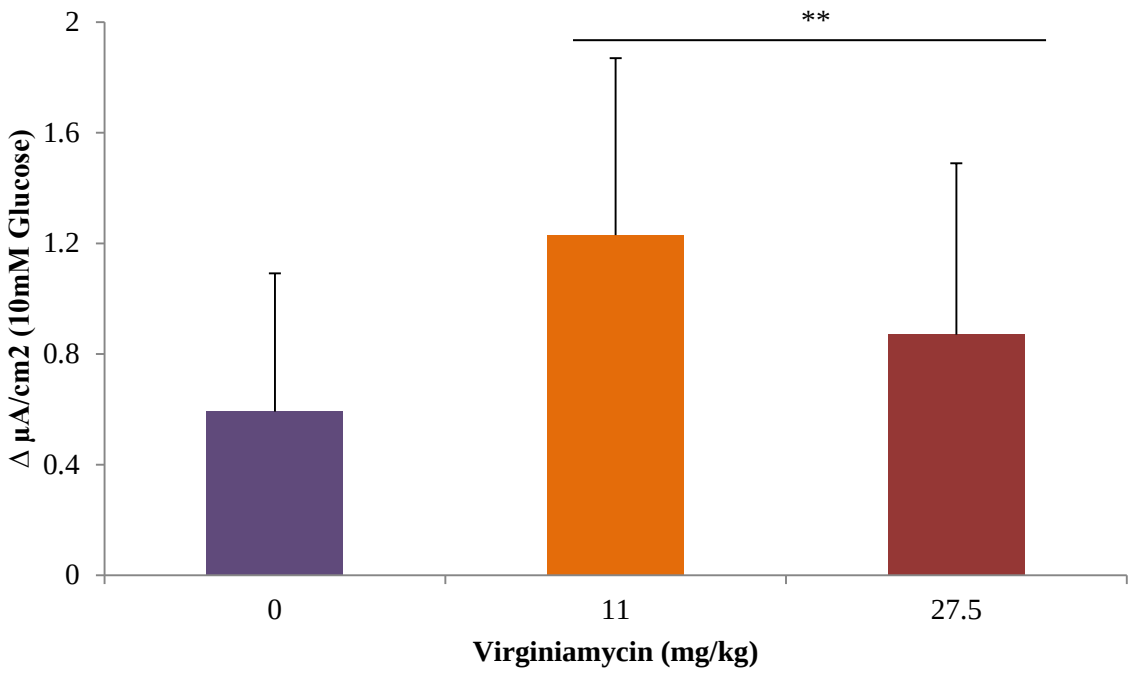


Figure 2.9 Sodium-coupled glucose transport in the duodenum. Data are expressed as pooled treatment mean ± SEM. Virginiamycin treatment increased glucose transport in the duodenal mucosa compared with control when virginiamycin treatments were contrasted with control (p=0.025).

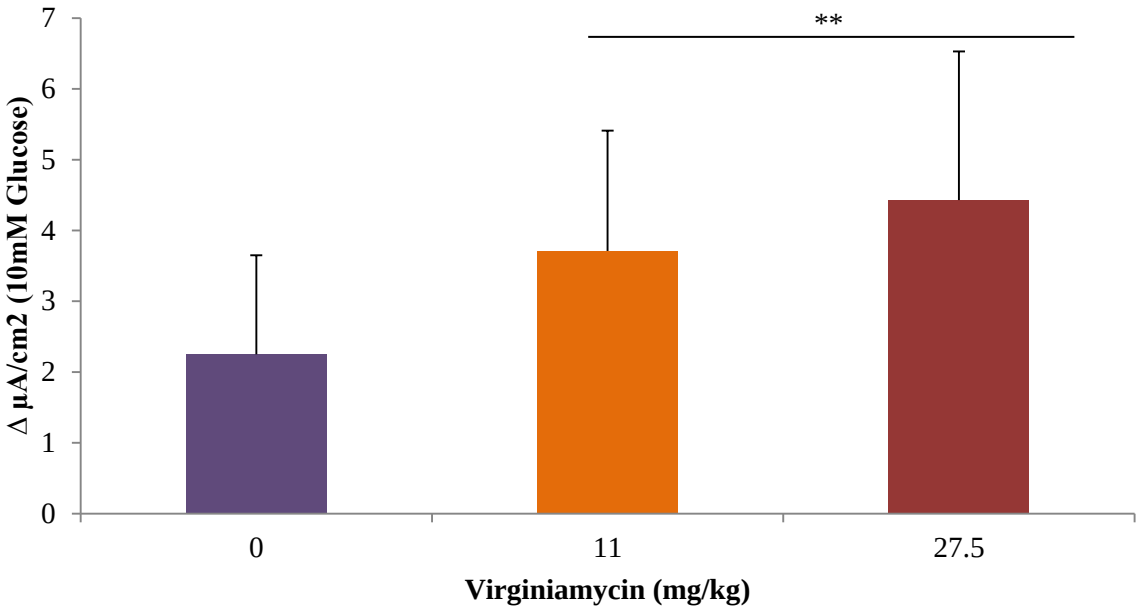


Figure 2.10 Sodium-coupled glucose transport in the jejunum. Data are expressed as pooled treatment mean ± SEM. Virginiamycin treatment increased glucose transport in the jejunum compared with control when virginiamycin treatments were contrasted with control (p=0.045).

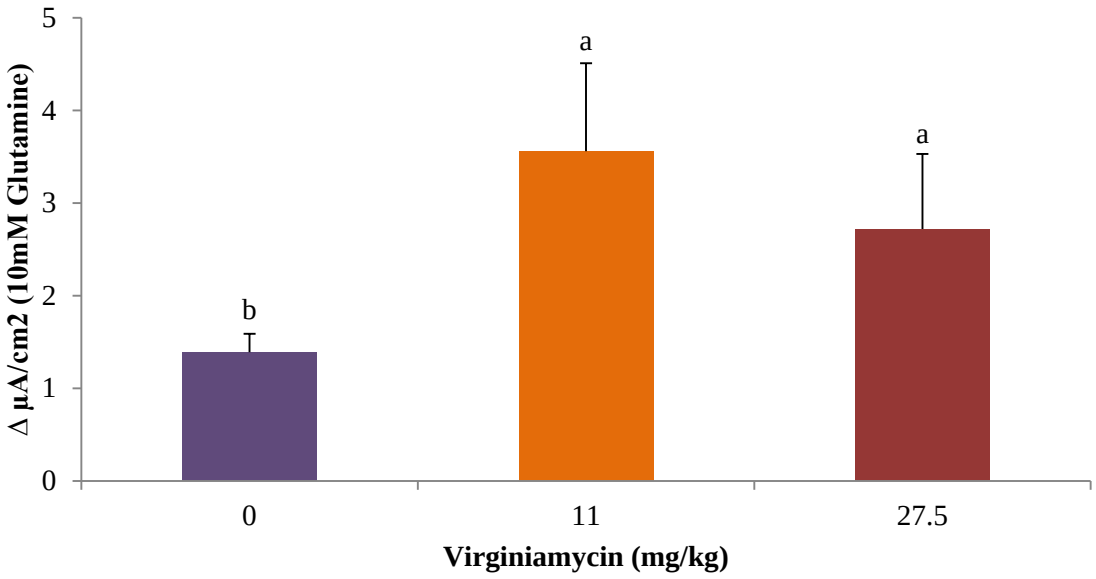


Figure 2.11 Electrogenic glutamine transport in the jejunum. Data are expressed as pooled treatment mean ± SEM. Different letters above bars indicate a statistically significant difference. Virginiamycin treatment increased electrogenic glutamine transport in the jejunual mucosa compared with control (p=0.010).

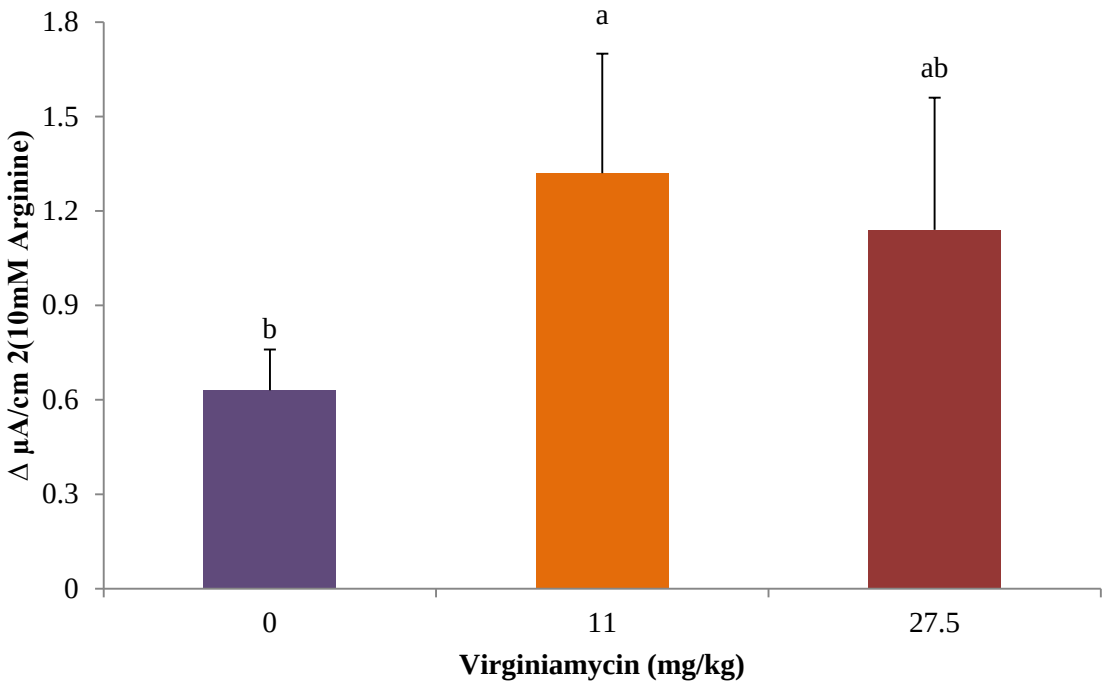


Figure 2.12 Electrogenic arginine transport in the jejunum. Data are expressed as pooled treatment mean ± SEM. Different letters above bars indicate a statistically significant difference. Jejunal arginine transport was significantly greater in the 11 mg/kg virginiamycin group compared with control (p=0.046).

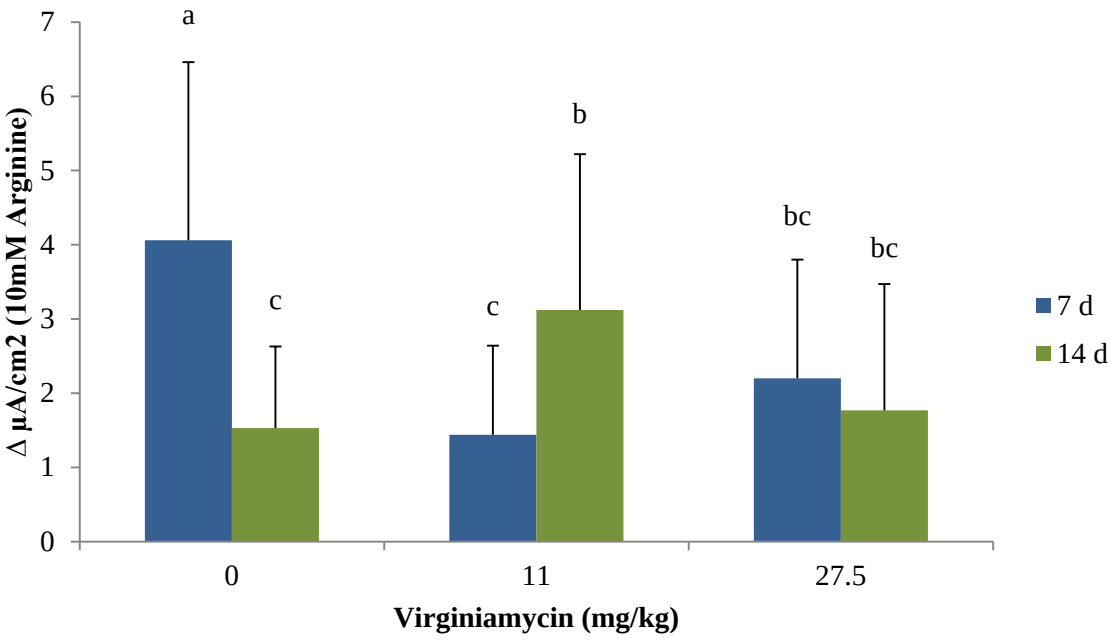


Figure 2.13 Electrogenic arginine transport in the ileum. Data are expressed as mean ± SEM. Different letters above bars indicate a statistically significant difference. Pigs consuming diets supplemented with virginiamycin had less ileal arginine transport compared with control pigs at day seven. Ileal arginine transport decreased over time in control pigs while increasing over time in the pigs fed diets containing 11 mg/kg of virginiamycin resulting in more arginine transport in the ileum of pigs fed diets supplement with 11 mg/kg of virginiamycin diets compared with control at day fourteen (p=0.042).

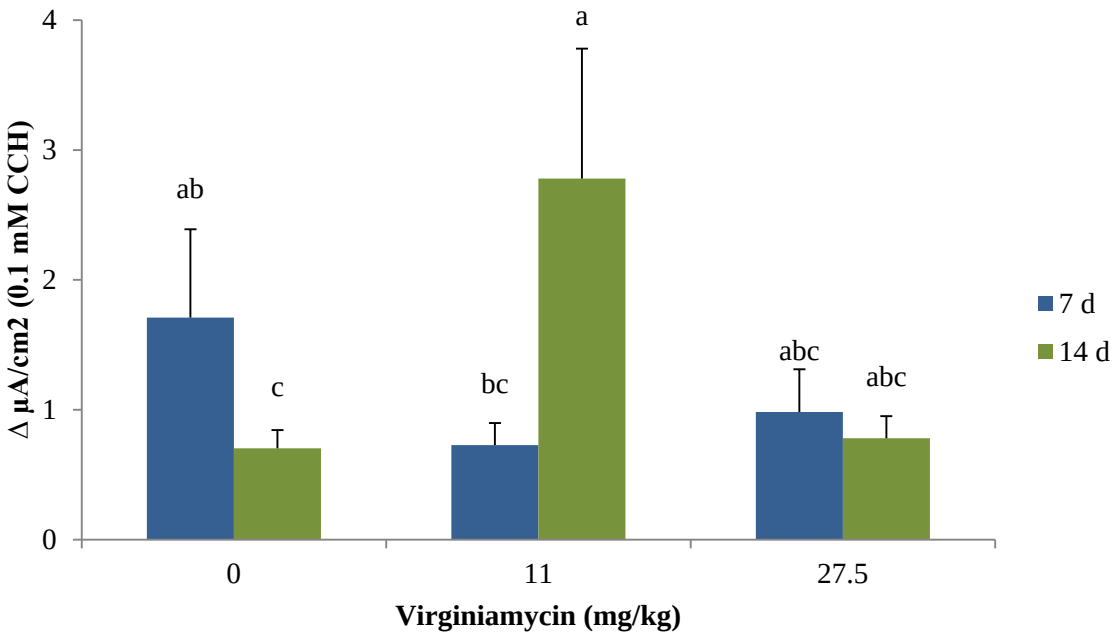


Figure 2.14 Carbachol induced chloride secretion (CCH) in the ileum. Data are expressed as mean ± SEM. Different letters above bars indicate a statistically significant difference. Ileal carbachol induced chloride secretion was decreased in the control pigs, but it increased in pigs consuming the diet supplemented with 11 mg/kg of virginiamycin at day fourteen compared with day seven. This resulted in greater carbachol induced chloride secretion in the 11 mg/kg virginiamycin group at day fourteen compared with control (p=0.017).

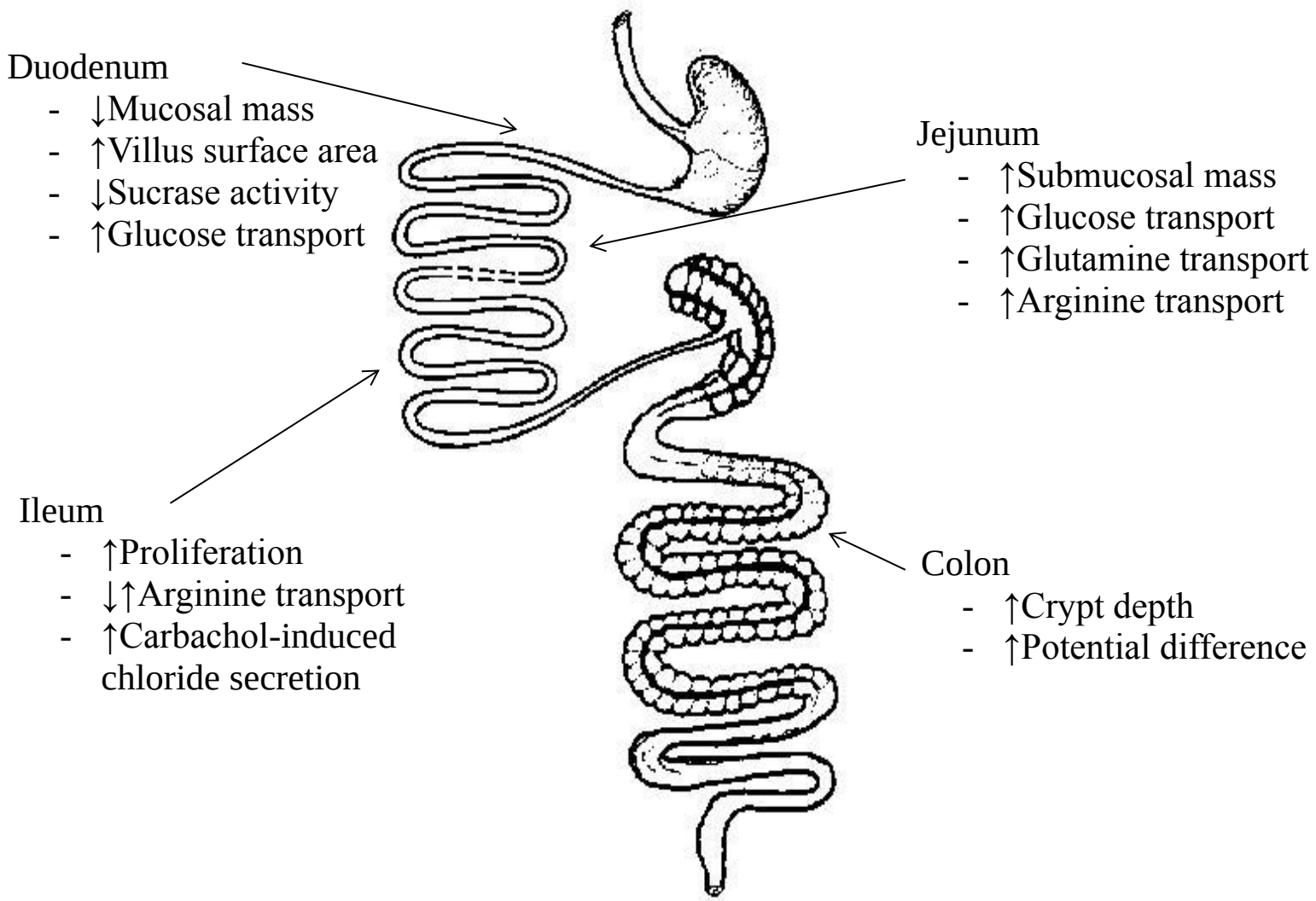


Figure 2.15 Virginiamycin impacts on intestinal structure and function observed in our study.

Chapter 3: Future Directions

In-feed antibiotics play a large role in animal health and production by improving performance and feed efficiency and, thus, increasing affordable pork supply. The safety of in-feed antibiotics has come into question regarding human health. Understanding how antibiotics promote growth will help determine the safety of their use and provide a knowledge basis for alternatives if the use of antibiotics for growth promotion is prohibited. Therefore, the objective of this research was to investigate the structural and functional developments induced in the intestine by the consumption of virginiamycin in grower pigs and to determine how these developments may be mechanistically related to the differences in growth.

Results of this study demonstrate that virginiamycin impacts functional capacity to a greater extent than structural indices. Pigs fed diets supplemented with virginiamycin had increased absorptive surface area in the duodenum but significant increases in nutrient transport along the entire small intestine. Future research is needed to ascertain the mode of action of virginiamycin in grower pigs and investigate the relationship between increased nutrient transport and virginiamycin.

Future direction for this line of research includes investigating:

1) cellular kinetics in the intestinal epithelium

2) nutrient transporters

3) digestive enzyme activity

4) immune response markers

5) microbiota analysis

Future research should focus on the 11 mg/kg level of virginiamycin, because that is the level currently approved for growth promotion and feed efficiency in grower pigs. Grower pigs are good subjects to use for investigating the effects of antibiotics on growth promotion and feed efficiency because growth responses are greater than in finisher pigs without the added dynamics of weaning-associated changes in weaning piglets.

Increased proliferation was observed at seven days in the ileum without increases in the crypt-villus axis. Future work should investigate if there is a subsequent increase in apoptosis at day seven. If apoptosis is not increased at this time point, it may be necessary to investigate time points beyond fourteen days to determine if structural changes occur along the crypt-villus axis at later time points. Additionally, cellular differentiation should be investigated. Immature, rapidly proliferating cells at day seven but more mature, differentiated cells by day fourteen may explain the unique functional response observed in the ileum with virginiamycin.

Nutrient transport was significantly increased along the small intestine with virginiamycin. However, increased absorptive surface area was not observed beyond the duodenum. This suggests that pigs fed diets supplemented with virginiamycin may have up-regulation of nutrient transporters or increased enzyme activity associated with digestion and absorption. Sucrase and lactase activities were not greatly impacted by virginiamycin, but future studies should investigate additional digestive enzymes such as maltase and peptidase activities. Nutrient transporter mRNA expression should be quantified to investigate, in more detail, the molecular basis for treatment differences in functional indices.

Decreased mucosal mass in the duodenum and increased carbachol induced chloride secretion in the ileum may support the immune response theory proposed by Niewold (2007). Future research should investigate immune response markers of innate and acquired immunity.

Assessing intestinal and plasma cytokines and antibody response may provide further support to this mechanism. Pro-inflammatory cytokines are early mediators produced in response to tissue damage and may be impacted by in-feed antibiotics. Virginiamycin has been shown to enhance systemic antibody response to soluble antigens in chickens (Brisbin et al., 2008). Lymphocyte stimulation assays would also provide insight to the effect of virginiamycin on the immune response.

Mucins contribute to the health of the intestinal epithelium by producing a mucus layer to protect the tissue through lubrication and preventing bacterial adhesion. Van Leeuwen and colleagues (2002) observed a significant increase in crypt goblet cells in the jejunum of piglets fed virginiamycin supplemented diets. Studying goblet cells and mucins in the intestinal lumen will provide insight on the effect of virginiamycin on this protection mechanism.

The most widely-accepted mechanism of action for growth promotion via antibiotics is the effect on the microbiota. The intestinal microbial populations greatly impact the intestinal environment and likely play a role in several of the other proposed mechanisms. Previous research has shown that virginiamycin impacts the microbial population in the intestinal tract of pigs. However, those studies have been done with varying amounts of the antibiotic, many of them much higher than the current approved level for growth promotion in swine. Future studies should include analysis of the intestinal microbiota in grower pigs fed diets supplemented with 11 mg/kg of virginiamycin. Microbial analysis should be included with nutrient transport studies and the aforementioned variables to gain a cohesive understanding of the mechanisms behind antibiotic growth promotion.

Overall, the future directions of this research would be aimed at unveiling the mechanisms by which virginiamycin promotes faster weight gain and feed efficiency in pigs.

Understanding how virginiamycin works in grower pigs will provide an evidenced-based working knowledge from which regulatory decisions can be made on the judicious and prudent use of antibiotics in food-producing animals. In the event that the United States decides to ban the use of antibiotics for growth promotion in animals, alternative strategies are needed to maintain the level of uniform performance seen with sub-therapeutic levels of antibiotics. A ban on antibiotic use in pigs for growth promotion, without an adequate alternative or change in practice, may lead to decreased pork production and a decline in animal health.

Antibiotic use for growth promotion has been prohibited in the European Union since 2006; therefore, the European Union provides a valuable reference for evaluating the impact of banning antibiotics and assessing alternative practices. Improving animal husbandry practices can greatly impact animal health and productivity. A reduction in overcrowding and infection control techniques may result in improved animal health and performance. Depopulation and repopulation of farms and commercial feedlots can result in cleaner facilities with lower microbial loads. Increasing the weaning age of piglets may also improve the health and performance of pigs. Early weaned piglets have been associated with inconsistent growth rates, abnormal feed intake, higher cortisol concentrations, and decreased cellular immune reactivity (Johnson et al., 2013). In Europe, as of the beginning of 2013, piglets are not to be weaned at less than 28 days of age. The law also states that "…piglets may be weaned up to seven days earlier if they are moved into specialized housings which are emptied and thoroughly cleaned and disinfected before the introduction of a new group…" (Defra, 2011). This practice was adopted in the European Union to produce healthier piglets, increase survival post-weaning, and increase the number of live pigs per sow per year.

In addition to improve husbandry practices, dietary alternatives to antibiotics have been proposed. In-feed enzymes may be added to feed to help break down certain components of the feed to improve digestion. Probiotics and prebiotics have also been proposed as alternative to antibiotics for growth promotion and there is increasing evidence showing the effects of these on pigs, primarily regarding intestinal adaptation. Proposed mechanisms by which probiotics work are similar to some of the proposed mechanisms of antibiotics, including improving microbial balance in the gut, impacting the immune response, and influencing intestinal metabolic activities. Prebiotics, such as fructooligosaccharides, have been postulated to enhance growth responses in livestock. Zinc and copper have also been investigated as alternatives to in-feed antibiotics. Understanding the mechanisms behind virginiamycin may lead to selection of alternatives that can mimic the growth performance effects of virginiamycin.

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