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EVALUATION OF BERBERINE AS AN ALTERNATIVE TO ANTIBIOTICS IN
NURSERY PIG DIETS

BY
EMILY SCHOLTZ

A thesis submitted in partial fulfillment of the requirements for the

Master of Science

Major in Animal Science

South Dakota State University

2018

EVALUATION OF BERBERINE AS AN ALTERNATIVE TO ANTIBIOTICS IN
NURSERY PIG DIETS

This thesis is approved as a creditable and independent investigation by a candidate for the Master in Animal Science degree and is acceptable for meeting the dissertation requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to my advisor Dr. Tofuko Woyengo for his support and patience throughout my masters program. I would like to thank other professors Dr. Crystal Levesque and Dr. Robert Thaler for their guidance, suggestions and help along the way as well.

Thank you to all the technical staff, especially Marci Green, with all the help managing Ussing chambers and Dr. Benoit St-Pierre for assistance with my antimicrobial assay. To all the swine graduate students, Jake Koepke, Jung Wook Lee, Jaime Ortman, Casey Zangaro, Ron Navales, Sam Tauer, and Agatha Ampaire for all the assistance and help throughout my trials and graduate degree in countless projects.

Thank you to South Dakota State University for funding the thesis research.

Finally thank you to my friends and family for all the support throughout my schooling I wouldn't be where I am today without having their love and support.

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ABBREVIATIONS

AITC.....	allyl isothiocyanate
EO.....	essential oil
Isc.....	short-circuit current
MIC.....	Minimal Inhibitory Concentration
NC.....	Negative Control
PC.....	Positive Control
TER.....	Transepithelial resistance

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ABSTRACT

EVALUATION OF BERBERINE AS AN ALTERNATIVE TO ANTIBIOTICS IN
NURSERY PIG DIETS

EMILY SCHOLTZ

2018

Pig weaning process results in reduced growth performance and gut health of weaned pigs. Antibiotics can be added in diets for weaned pigs to improve growth performance and gut health, but their use is being discouraged because they can lead to development of antimicrobial resistant microorganisms. Thus, there is need for alternatives to antibiotics in diets for weaned pigs. The overall goal of this thesis research was to determine the effects of plant extracts (berberine, quercetin, and allyl isothiocyanate [AITC]) as alternatives to antibiotics in weaned pig diets.

The first objective of the research was to determine in vitro antimicrobial activities of berberine, quercetin, and AITC against *Escherichia coli* with the goal of identifying the best plant extract for in vivo (animal) studies. Inclusion of berberine in incubation medium at 25, 12.5, 6.25 or 3.125 $\mu\text{g}/100 \mu\text{l}$ reduced ($P < 0.05$) in vitro growth of *E. coli*. However, inclusion of berberine in incubation medium at 1.5625 $\mu\text{g}/100 \mu\text{l}$ did not affect in vitro growth of *E. coli*. Inclusion of AITC and quercetin in the incubation medium at 50.65 $\mu\text{g}/100 \mu\text{l}$ and 11 $\mu\text{g}/100 \mu\text{l}$, respectively, did not affect in vitro growth of *E. coli*. Thus, berberine was selected for animal studies because it was more effective (at a lower concentration [$\sim 3.0\%$]) in inhibiting in vitro growth of *E. coli* than quercetin or AITC.

The second objective was to determine the effects of dietary inclusion of berberine at 3.0% inclusion and antibiotics on growth performance and gut health of weaned pigs. Pigs were fed experimental diets for 7 days after weaning, at end which growth performance and indicators of gut health were measured. Dietary inclusion of berberine decreased ($P < 0.05$) ADFI, ADG, and ileal villous height by 63, 328 and 28%, respectively, and tended to decrease ($P = 0.078$) transepithelial resistance (which is an indicator of gut permeability to toxins) in duodenum by 20%. Dietary antibiotics did not affect any of the response criteria measured in this study.

The last objective was to determine the effects of dietary inclusion of berberine at 0.05% on growth performance and gut health of weaned pigs. Pigs were fed experimental diets (basal diet with or without antibiotics or berberine at 0.05%) for 21 days. Indicators of gut health were determined at day 11 of the study, whereas growth performance was determined at days 11 and 21 of the study. The overall (day 1 to 21) ADG of pigs was increased ($P < 0.05$) by dietary inclusion of antibiotics by 32.4% and tended to increase ($P < 0.06$) by dietary inclusion of berberine by 16.6%. The overall ADFI of pigs was also increased ($P < 0.05$) by dietary inclusion of antibiotics by 21.1%. However, the overall ADFI of pigs was not affected by dietary inclusion of berberine. There was no effect of adding berberine or antibiotics to basal diet on villous height and crypt depth, and villous height to crypt depth ratio in duodenum and jejunum, and on crypt depth in ileum. Also, there was no effect of adding antibiotics to basal diet on villous height and villous height to crypt in ileum. However, villous height to crypt depth ratio in ileum was increased ($P < 0.05$) by dietary inclusion of berberine. There was no effect of dietary treatment on lactulose:mannitol ratio in urine, which is another indicator of gut permeability to toxins.

Inclusion of berberine in basal diet had no effect on TER values in the jejunum and the ileum. However, dietary antibiotics improved ($P < 0.05$) TER in ileum. Dietary berberine increased ($P < 0.05$) short circuit current (Isc, which is an indicator of nutrient absorptive capacity) in jejunum and ileum. However, dietary antibiotics did not affect Isc in jejunum and ileum.

In conclusion, berberine was more effective than quercetin or AITC in inhibiting in vitro growth of *E. coli*. However, berberine did not affect gut health of weaned pigs when it was included in diets at 3% (the lowest concentration at which it inhibited in vitro growth of *E. coli*), which was likely due to the reduced ADFI by dietary berberine because ADFI negatively influence gut health. Dietary berberine at 0.05% improved intestinal nutrient absorptive of and ADG of weaned pigs without affecting ADFI, implying that berberine can improve growth performance of weaned pigs, and that the negative effects of berberine on feed intake of weaned pigs are alleviated when berberine is included in diets at low levels.

GENERAL INTRODUCTION

Piglet weaning is a stressful event that is associated with reduced feed intake, nutrient digestion and absorption, and increased susceptibility to gut infections; hence, growth performance of piglets is reduced (Kiarie, 2008; Wijtten et al., 2011). Piglet stress is due to abrupt changes in their new environment and diet composition, as well as interruption of already established social structure with their littermates and sows. Gut infections characterized with diarrhea are the major cause of reduced growth performance and increased morbidity and mortality of post-weaned pigs and pathogenic *E. coli* are a major cause of the gut infections (Fairbrother et al., 2005). Growth performance of pigs during the weaning phase determines future growth of these pigs and poor performance during this time causes huge economic losses in swine industry (Cutler and Gardner, 1988).

Antibiotics have long been added in diets for the weaned pigs at low levels to improve their performance. The fear of the development of antibiotic resistant microorganisms has limited the addition of antibiotics in the diets for food animals. For this reason, it is important to find effective antibiotic alternatives to manage gut health. Various potential alternatives to antibiotics (feed additives), including prebiotics, probiotics, minerals, plant extracts, and animal-derived antibodies are being investigated; however, majority of these feed additives have been inconsistent in improving gut health and growth performance of pigs (Heo et al., 2013; Thacker, 2013). The use of antibiotic-free diets in the swine industry will continue to be a challenge unless there is an effective alternative.

Weaning process results in increased permeability of intestine of pigs due to increased oxidative stress (Wijtten et al., 2011). The increased intestinal permeability increases translocation of toxins (that are produced by gut microorganisms) into the body, causing inflammatory injuries in the gut wall, which increases the susceptibility of weaned pigs to gut infections. Thus, for an alternative to antibiotics to be effective, it has to reduce growth of pathogenic microorganism in the gut or reduce oxidative stress (that results in increased intestinal permeability), or both.

Quercetin, allyl isothiocyanate and berberine were evaluated as potential antibiotic alternatives. Quercetin is a flavonoid, flavonoids belong to a group of natural substances found in fruit, vegetables, grains, bark, roots, stems, and flowers. They are known for their various beneficial health effects. Quercetin has various properties including; antihypertensive and antiarrhythmic activity; anti-inflammatory and antiallergic properties; and other activities (Formica and Regelson, 1995). Allyl isothiocyanate is a compound formed from hydrolyzed Glucosinolates, in response to plant tissue injury by herbivorous animals or pathogenic microorganisms. AITC has been shown to have strong antimicrobial activity (Lin et al., 2000) and antioxidant activity (Velioglu et al., 1998). Berberine is a plant alkaloid that has already been isolated from various plant species including *Berberis aquifolium* (Oregon grape), *Berberis vulgaris* (barberry), and *Berberis aristata* (tree turmeric) (Mokhber-Dezfuli et al., 2014). Berberine has been shown to reduce diarrhea in humans (Rabbani, 1987) that have been infected with the same diarrhea-causing bacteria in weaned pigs. Berberine has also been shown to be an effective antioxidant by several mechanisms including removal of oxygen, scavenging of reactive oxygen species and nitrogen species or their precursors (Shirwaikar et al., 2006).

Thus, berberine can potentially be used as an alternative to antibiotics in diets for weaned pigs. However, the effects of berberine on growth performance and gut health of weaned pigs haven't been determined.

The main objective of this thesis research was to evaluate berberine, quercetin and allyl isothiocyanate as alternatives to antibiotics in diets for weaned pigs.

CHAPTER ONE

LITERATURE REVIEW

Gut Health at Weaning

The gastrointestinal tract (**GIT**) of a pig is a complex environment. In particular, in newborns and around the time of weaning, the pigs' gut rapidly changes in size, has high protein turnover rates, undergoes rapid changes in microbiota, and quickly alters its digestive and immune functions (Pluske et al., 1997; Vente-Spreuwenberg and Beynen, 2003; Burrin and Stoll, 2003; Lallès et al., 2004). At weaning, pigs are challenged with abrupt changes in their environment, and diet composition, as well as interruption of already established social structure with their littermates and sows. These weaning stressors results in changes in gastrointestinal morphology, microbiology, physiology and immunological challenges (Spreuwenberg et al., 2001; Heo et al., 2012), which in turn, results in a decrease in growth performance, an increase in diarrhea incidences, and an increase in susceptibility to gut infection. The effects of weaning on diarrhea; growth performance; and gastrointestinal morphology, microbiology, physiology and immunology of pigs are discussed below.

Effects of Weaning on Diarrhea. Post-weaning diarrhea is a condition in weaned pigs that is characterized by frequent discharge of watery feces during the first 2 weeks after weaning and represents one of the major economic problems for the pig industry (Cutler and Gardner, 1988). When piglets are abruptly weaned at 3-4 weeks of age, post-weaning diarrhea commonly occurs as a consequence, and the susceptibility to disease increases. Immaturity of the intestinal immune system in combination with lack of dietary supply of IgA and other compounds that are derived from sow milk contributes to

increased susceptibility of the pigs to these diseases (Bailey et al., 1992, 2005; Stokes et al., 2004). Inadequate dietary supply of IgA results in increased bacterial adhesions to epithelial receptors that are normally protected by immune defense systems.

In addition to immature immune system, weaning results in a reduction in absorption of nutrients such as electrolytes from the intestinal lumen of piglets. When fluid and electrolytes influx into the gut lumen exceeds their efflux into the blood, a net secretory condition occurs, which serve as a predisposing factor for secretory diarrhea (Pacha, 2000; Wapnir and Teichberg, 2002). Weaning results in reduction in small intestinal absorptive area and in maturation of enterocytes (Hampson, 1986), which partly explain the reduced nutrient absorptive capacity, and hence increased susceptibility of the pigs to diarrhea and reduced growth rate of pigs in the post-weaning period.

Effects of Weaning on Growth Performance. It has well been established that weaning of pigs is characterized with little or no body weight gain during the first and second week post-weaning, and low feed intake during the first 2 weeks post-weaning seems to be the main reason for the growth stasis after weaning. The changes in diet and environment during weaning have negative effects on feed intake. Wolter and Ellis (2001) determined the effect of feeding liquid-based diet on growth performance of pigs from weaning to slaughter weight. In their study, liquid milk-based diet accelerated growth rate immediately after weaning, but this advantage was not sustained to slaughter weight. Hyum and Ellis (1997) conducted a trial, which looked at the impact of environmental stressors on growth performance. In their study, pigs were subjected to three different stressors (ambient temperature, re-grouping and space allowance) in a factorial arrangement. Of these three stressors, it was found that ambient temperature had

the biggest impact on ADG and ADFI. However, all the 3 stressors were additive, implying that removal of any of these stressors could improve growth performance on pigs.

The weaning process induced-decreased feed intake by pigs can also be due to allergic reaction to dietary soy proteins. Indeed, dietary soybean co-products have negative effects on ADG and ADFI during the first two weeks following weaning (Li et al., 1991). A decrease in villous height and increase in crypt depth as well as an increase in skin-fold thickness were observed when weaned pigs were fed soybean meal-based diets, but not when they were fed skim milk-based diets (Li et al., 1991). Since skin-fold thickness, which is an indicator of antigen sensitivity, is negatively correlated with growth rate, the reduced growth performance of weaned pigs due to dietary soybean products is attributed to the allergic reaction to the soybean proteins (Li et al., 1991). Dreau et al. (2014) conducted a study to determine hypersensitivity of early-weaned pigs to soybean, and observed positive skin tests, and a 25% decrease in villus height in the duodenum due to dietary inclusion of soybean proteins. Friesen et al. (1993) reported that the early-weaned pigs ought to be fed starter diets containing some soybean proteins in order to develop tolerance toward soy protein without causing reduced growth performance because they observed a decrease in ADG, ADFI and F:G of weaned pigs during first 14 days of exposure to soybean meal, but not after the 35-day feeding period.

Effects of Weaning on Intestinal Morphology. The small intestine is lined with finger-like projections called villi and attached to these villi are microvilli. Both these act in increasing surface area and absorptive capacity of the small intestine. There are depressions found between the villi referred to as “crypts”, which are a source of new

cells that will then migrate to the tips of the villi (Kitt et al., 2001). The lining of the intestines has various functions including absorption of nutrients, and secretion of electrolytes, mucins and immunoglobulins, and selective barrier protection against harmful antigens and pathogens (Lalles et al., 2004). Weaning process results in various changes in gut morphology that affect these intestinal functions, leading to a decrease in the digestive and absorptive capacities of the small intestine. These changes include villous atrophy, crypt hyperplasia, and a decline in brush border enzyme activity (GU et al., 2002). Villous atrophy after weaning is caused by either an increased rate of cell loss or a reduced rate of cell renewal (Pluske et al., 1997). Villous shortening that occurs through an increased rate of cell loss, is associated with increased crypt cell production, caused by either a microbial challenge or antigens found in diets (Pluske et al., 1997). Villous atrophy that is due to decreased rate of cell renewal is as a result of reduced cell division in the crypts, and it is caused by a state of anorexia during the wean phase (Pluske et al., 1997). Data from various studies have demonstrated that villous atrophy is correlated with depressed feed consumption and that epithelial morphology improves when normal feed intake patterns resume (McCracken et al., 1999). As a result of the changes in gut morphology, the villous height:crypt depth ratio for weaned pigs is lower than that for unweaned pigs.

Verdonk et al. (2007) determined the effect of feed intake by weaned pigs on structure and permeability of the small intestine, and observed reduced villous height by 17% and reduced crypt depth by 13% due to a decrease in feed intake by 31%. However, in their study, gut wall permeability was not affected by feed intake. Kelly et al. (1991) determined the effect of post-weaning feed intake on digestive capacity in the weaned

pigs using a gastric intubation to control amount of feed consumed, and observed a 19% decrease in villous height and a 13% decrease in crypt depth in pigs that had restricted feed intake (226 g per day) compared with the pigs that had unrestricted feed intake (864 g per day), implying that nutrient intake in the weaned pig affects the morphology of the gut. Pluske et al. (1996) determined the effect of replacing sow milk diet with starter on morphology of small intestine in weaned pigs, and observed that piglets given the starter diet had a 30% decrease in villous height and a 4% increase in crypt depth compared with the pigs fed milk diets ad libitum. There was also a 28% decrease in DM intake in the pigs fed the starter diet versus the milk diet ad libitum, which could be a cause for morphology changes. Results from these 3 studies demonstrates that villous height and crypt depth can be maintained if piglets are maintained on similar sow milk diet, and if the piglets do not suffer from stress of interrupted intake after weaning.

Effects of Weaning on Intestinal Inflammation. Inflammation occurs in weaned pigs in response to physical or chemical damage in GIT or to invasion by an infectious agents or feed toxins. This inflammation impairs the epithelial barrier function and decreases absorption of nutrients.

In addition to the reduction in feed intake, age at weaning, dietary factors, as well as the stressors caused by change in environment and the separations from sow and littermates are all contributors to the changes in intestinal architecture. McCracken et al. (1999) determined the effects of a pelleted milk diet or pelleted soy-based diet on small intestinal inflammatory responses and morphology alterations by examining T cell numbers. They observed a relationship among weaning anorexia, local inflammatory responses, and compromising changes of the piglet's small intestinal morphology. There

was an increase in crypt CD4+ T cell numbers on day 7 of the pigs fed soy-based diet, but not of those fed the milk-based diet, indicating that the dietary soy antigens induced inflammatory and immune responses in the weanling pigs. However, the soy-induced inflammation that was present, likely occurred after the intestinal morphology had already been compromised due to local inflammation caused by anorexia that occurs immediately after weaning.

Effects of Weaning on Intestinal Permeability. Epithelial barrier function in the GIT is reduced in weaned pigs due to stress and dietary alterations. The intestinal barrier consists of a single layer of enterocytes and connecting inter-epithelial tight junctions, which are responsible of regulating the para-cellular flux of solutes and macromolecules (Blikslager et al., 2007). Stress and starvation results in increased intestinal permeability, which can be attributed the effects of stress and diet on IgA production, the main immunologic defense against bacterial adherence to the mucosa (Spitz et al., 1996). The bacterial adherence to intestinal epithelia induces a defect in barrier function that appears to occur at the intercellular tight junctions, which are the rate-limiting seal of the para-cellular pathway between epithelia cells (Spitz et al., 1994). When this rate-limiting seal is dysfunctional, permeability increases allowing toxins, allergenic compounds or bacteria to enter into body, resulting in inflammatory and immunologic responses (Deitch, 1993; Wang, 1995). Heat stress and the stress of transport and shipping can cause a reduction in intestinal blood flow and the destruction of tight junctions. This reduction in intestinal blood flow results in oxidative stress, which can damage cell membranes and open tight junctions (Hall et al., 2001). Moeser et al. (2007) conducted a study to determine the role of weaning on serum corticotrophin-releasing factor and

cortisol, and intestinal permeability of pig, and observed increased intestinal permeability in jejunum (by 59%) and colon (by 34%), serum corticotrophin-releasing factor level (by 144%), and serum cortisol level (by 95%) in weaned pigs due to weaning process. Based on results from these studies, it is apparent that weaning induces activation of the stress pathways, which may be mediating the intestinal barrier dysfunction.

In addition to stress, change in diet composition and reduction in feed intake that occur during weaning can potentially affect intestinal permeability and epithelial barrier function of weaned pigs (Spreeuwenberg et al., 2001). However, Spreeuwenberg et al. (2001) observed no effects weaning diet composition on transcellular transport, but did see a 39% change in paracellular transport on d 2 and 4 compared to d 0 and 1, indicating that the effect of diet composition on mucosal integrity is not as important as the sequential effects of low feed intake during the first 4 d post-weaning. McCracken et al. (1999) also reported that intestinal inflammation subsides and epithelial morphology improve when normal feed intake patterns resume after weaning. Thus, low feed intake and stress seem to be the two main causes of decreases in mucosal integrity.

Dietary Means of Improving Growth Performance and Gut Health of Weaned Pigs

Various feed additives are available to help improve the transition of newly weaned pigs to a new diet and environment, and reduce the incidences of infections. The feed additives include antimicrobial agents, organic acids, prebiotics, probiotics, improved protein sources, and natural plant and herbal extracts. All of these have the potential to improve gut health around weaning, and their effects on growth performance

and gut health of weaned pigs are discussed below. However, it's important to evaluate other feed additives to determine their potential to contribute to gut health.

Effects of Antimicrobial Agents on Growth Performance. Antimicrobial agents promote growth performance mainly through their actions in the GIT. Antibiotics are type of antimicrobial drugs that are used for treatment and prevention of bacterial infections. Antibiotics are included in swine diets as non-nutritive feed additives for their therapeutic potential as well as their ability to promote growth and gut health. Examples of antibiotics that are included in swine diets include carbadox, tylosin, lincomycin, sulfonamides, and tetracyclines. Apart from antibiotics, other anti-microbial agents that are included in swine diets to improve gut health and promote growth include zinc and copper (at pharmacological doses).

When added at low (subtherapeutic) levels in feeds, antibiotics improve growth rate and feed efficiency, reduce mortality and morbidity, and improve reproductive performance (Cromwell, 2001). Some of proposed mechanisms by which antibiotics stimulate growth include the suppression of growth of pathogenic microorganisms, promotion of growth of bacteria that synthesize nutrients that are required by the host animal, and suppression of growth of bacteria that compete with the host animals for dietary nutrients (Cromwell, 2001).

Several studies have been conducted to determine the effects of dietary antibiotics on growth performance of weaned pigs. Teegan et al. (2003) conducted a study in which nursery pigs were fed control diet or a control with Denagard/CTC (35 g/ton Denagard™, 400 g/ton Chlortetracycline), carbadox (g/ton), Neo-Terramycin (140 g/ton Neomycin Sulfate, 140 g/ton Oxytetracycline HCl), or Bio Mos (0.3% mannanoligosaccharide) from

day 0 to 31 post-weaning. Diet that contained Denagard/CTC (35 g/ton Denagard™, 400 g/ton Chlortetracycline) tended to have the greater ADG compared with the control diet or diet with Bio Mos in the first 9 days. From day 9 to 31, pigs fed the diet containing Denagard/CTC had the greater ADG and ADFI than pigs fed all other diets. Overall (d 0 to 31), pigs fed the diet containing Denagard/CTC had the greatest ADG and ADFI compared with pigs fed all other treatment diets. The addition of a mannanoligosaccharides did not improve growth performance compared to antibiotic diet. Thus, data from this study of Teegan et al. (2003) indicate that antibiotics are effective in improving growth performance of weaned pigs, but the effectiveness of antibiotics with regard to improving growth performance is partly depended on type of antibiotics used.

Antibiotics are also used at intermediate levels to prevent disease and at high (therapeutic) levels to treat diseases in animals (Cromwell, 2002). Often times this means treating the entire group of animals with the goal of treating the sick animals and medicating healthy animals to prevent disease. In a study by Van Lunen (2003), pigs fed diets with or without tylosin phosphate supplementation in a biosecure housing system did not differ in growth performance and carcass characteristics, which was attributed to the minimal disease status of the pigs housed in the biosecure area. Studies have shown that disease challenged pigs show a greater response in growth performance when fed diets containing antibiotics at sub-therapeutic levels (CAFA, 1997), indicating that the benefits of adding antibiotics in diets at sub-therapeutic levels are greater when pigs are challenged with diseases.

As previously mentioned, dietary inclusion of micro-minerals (zinc and copper) at pharmacological levels can be an effective tool for preventing diarrhea and promoting growth of weaned pigs. For instance, dietary zinc improved feed efficiency (Hahn and Baker, 1993), ADFI and ADG (Hill et al., 2000) and reduced diarrhea (Broom et al., 2006) in weaned pigs. Stahly et al. (1980) reported that dietary combination of both copper and an antibiotic (CTC or VIR) resulted in a greater feed intake and growth rate and tended to improve efficiency of feed utilization when compared with individual dietary inclusion of these antimicrobial agents, indicating that the effects of the micro-minerals (at pharmacological levels) and antibiotics in weanling pigs are additive in nature.

Probiotics. At weaning, when it occurs early, the transition from milk to a solid diet leads to dramatic changes in the composition of the microbial community during the 7–14 days after weaning (Hillman, 2001). This makes the piglet highly susceptible to enteric diseases. Non-pathogenic gut microflora contributes to intestinal protection against pathogens by competing for binding sites that pathogens would bind, competing for nutrients, as well regulating immune response (Roselli et al., 2005).

Probiotics are orally supplemented living microorganisms used as therapeutic agents for improvement and promotion of health (Bauer et al., 2006). Probiotics have immunological effects because they have high ability to adhere to the intestinal surface, thereby interfering with the adhesion of pathogenic bacteria (Fuller, 1991). Probiotics can also beneficially influence intestinal health by stabilization of the endogenous GIT microbiota (Salmien et al., 1998). This stabilization is partly due to the production of short-chain fatty acids from undigested food materials and endogenous substances, such

as mucus by the probiotic bacteria (Ohashi et al., 2009). Lactobacilli and Bifidobacteria are non-pathogenic and health-promoting bacteria that are mainly used as probiotics.

Several studies have been conducted to determine the effects of probiotics on performance and gut health of pigs. Pollmann et al. (1980) determined the effects of two commercially available lactic acid-producing bacterial feed additives on weight gain and feed conversion ratio of nursery and growing-finishing pigs, and observed a tendency of the probiotics to improve ADG (0.263 vs. 0.270 kg) and F:G (2.28 vs. 2.20) of nursery pigs. However, probiotics did not affect growth performance of the growing-finishing pigs. In a relatively recent study, Shu et al. (2001) determined the effects of supplementing diets for 3-week-old pigs with *Bifidobacterium lactis* HN019 probiotic on severity of post-weaning diarrhea, and observed severe diarrhea scores of 5 and 6 for the control diet on days 1 and 2 and no severe diarrhea for probiotic supplemented diet. The animals that were fed *B. lactis* HN019 also had significantly higher feed conversion efficiency (0.66 vs. 0.25) from day 0 to 9 post-weaning than those that were fed unsupplemented diet. This shows that feeding of the probiotic *B. lactis* HN019 can result in some protection of weaned pigs against post-weaning diarrhea. Van der Peet-Schwering et al. (2014) determined the effects of yeast culture and modified yeast culture as alternatives to antibiotics in diets for piglets on the growth performance, gut integrity, and blood cell composition of weanling pigs. Results from their study showed that piglets fed diets with the antibiotic performed similarly to those fed the diet with the yeast culture. There was no effect of dietary treatment on the concentration of white blood cells and the percentage of lymphocytes or neutrophils within the white blood cell population. Also villous length, crypt depth, and the villous:crypt ratio were unaffected by dietary

treatment. Another study, Chen et al. (2005) determined the effects of adding complex probiotic including *Lactobacillus acidophilus*, *Saccharomyces cerevisiae* and *Bacillus subtilis* in diets at 0.1 and 0.2% on growth performance, nutrients digestibility, blood characteristics and fecal noxious gas content of 40 kg pigs in a 42-day growth trial. Over the 42 day period, pigs fed diet with 0.2% probiotic had greater ADG (by 7.5% and 6%) than those fed the basal diet, those fed diet with 0.1% probiotic. There was no effect of treatment on blood characteristics and nutrient digestibility. Thus, probiotics seem to improve growth performance and gut health in some cases, but not in others. Freitag et al. (1998) performed an evaluation of several different probiotics feed additives on their effects on growth performance and gut health of weaned pigs, and observed that most studies with weaning piglets showed positive effects of probiotics on weight gain and feed conversion, whereas some studies showed no or adverse effects. Significance has rarely been observed between treatments in trials where there was a positive effect of feeding probiotics. Through his evaluation they also observed different doses of *a Bacillus cereus* preparation showed no significant influence on the incidence of diarrhea, while other studies showed significant reductions with supplementation. This indicates strong differences in reactions of the animals toward the individual probiotics.

Prebiotics. The term prebiotics was identified by Gibson and Roberfroid (1995) as indigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of health-promoting bacteria in gastrointestinal tract. For example, prebiotics stimulate growth of Bifidobacterium, which inhibit growth of pathogenic bacteria (Gibson and Wang, 1994). This reduction in growth of pathogenic bacteria, in turn, can help improve the immune system of the animal. Oligosaccharides

are an example of prebiotics that can selectively stimulate the growth of health-promoting bacteria.

The effects of adding prebiotics in diets for pigs on gut health and performance pigs have been determined in several studies. Liu et al., (2015) determined the effect of supplementing diets with chito-oligosaccharide (**COS**) on growth performance, intestinal structure, and fecal shedding of total *E. coli* and *Lactobacillus* in weaned pigs. They observed an increase in ADG (315 vs. 285 g), ADFI (460 vs. 436 g) and G:F (0.69 vs. 0.66 g/g) when COS was supplemented at 200 mg/kg compared with the negative control diet. Dietary supplementation of COS at 200 mg/kg and dietary supplementation of chlortetracycline (**CTC**) increased the villus height and villus:crypt ratio in the ileum and jejunum, indicating that COS can be an effective alternative to dietary antibiotics with regard to improving growth performance and gut health of weaned pigs. Zhao et al., (2011) also conducted a study to determine the effects of mannan oligosaccharides (**MO**) and fructan on growth performance, nutrient digestibility, blood immune variables, and diarrhea score in weaned pigs. During the entire period of the study (d 0 to 28), pigs fed the MO diet had a greater ADG than pigs fed the negative control diet (399 vs. 306 g). Pigs fed the positive control and MO diets had greater ADFI than pigs fed the negative control diet (485 g and 499 vs. 394 g). No differences were observed in diarrhea score among the positive control, negative control plus 0.1% fructan, and negative control plus 0.05% fructan and 0.05% Bio-Mos. However, diarrhea score in pigs fed the MO diet was lower than that of pigs fed the negative control diet. Another study that was conducted by Estrada et al., (2000) determined the effects of the dietary supplementation of fructooligosaccharides (**FOS**) and *bifidobacterium longum*, alone or in combination, on

growth performance and fecal bacterial populations of 18 d-old pigs for 3 weeks in two different experiments. There was a positive effect of FOS and *B. longum* in the first week on ADG (102 vs. 88 g), but not in the second or third week. On day 7, the number of bifidobacteria was higher in the feces for FOS plus *B. longum*-administered pigs than in feces for pigs fed the control diet. However, there were no effects of dietary treatment on bacterial count on days 14 and 21. The *B. longum* supplementation improved BW (13.75 vs. 11.88 kg) and ADG (397 vs 299 g), whereas supplementation of FOS plus *B. longum* negatively affected growth performance. Thus, prebiotics can have a beneficial effect on growth performance and gut health. However, the beneficial effects of prebiotics can vary depending on the type of prebiotic used.

Essential Oils. Essential oils (**EOs**), also called volatile or ethereal oils, are aromatic oily liquids obtained from plant materials (Cho et al., 2006). Most of the EO products that are commercially available are blends of various essential oils; and they include thymol and cinnamaldehyde, oregano, rosemary, and sage to name a few. EO's have been widely reported to have antimicrobial properties, which are their most evident mechanisms by which they improve gut health of animals. However, EOs possess other properties such as antioxidant activity, which oregano is well known for (Economou et al., 1991). Free radicals are continuously produced by the body during oxygen metabolism and exogenous damage, which can cause an inflammatory response or tissue damage (Nijveldt et al., 2001). EO products are quite complex mixtures of several dozens of components, and this complexity makes it often difficult to explain their activities. Some studies have shown that whole EOs (mixture of several EOs) have a greater antibacterial activity than a mixture of a few major components (Gill et al., 2002; Mourey

and Canillac, 2002), which indicates that the minor components also significantly contribute to the antibacterial activity of the EOs products and may have a synergistic effects with major components, or have greater influence on the antibacterial activity of EOs products (Brenes et al., 2010).

EO's can be supplemented to diets in combination with other alternatives to antibiotic to improve gut health and growth performance of weaned pigs. A blend of EO's alone can also be as effective as antibiotics with regard to improving gut health and growth performance of weaned pigs. Li et al., (2012) determined the effects of supplementing diets for weaned pigs (initial body weight of 8.4 kg) with an EOs blend or antibiotics (positive control) for 5 weeks on the growth performance, nutrient digestibility, immune status, intestinal morphology, and intestinal microbiology. The dry matter and crude protein digestibilities were much higher for the pigs fed the positive control (**PC**) and the EOs diets compared to the negative control diet. The IGF-I production and lymphocyte proliferation were higher in the pigs fed PC and EOs diet than those fed NC diet. There was an increase in villus height in the jejunum for the PC diet (509 μm vs. 466 μm) and EOs (535 μm vs. 466 μm) diet compared with the NC diet. They observed an increase in ADG of the pigs by 9 and 14% due to dietary supplementation of EO product and antibiotics, respectively. There was also a decrease in *E.coli* in the cecum, colon, and rectum in pigs fed the PC and EO diet compared with negative control diet. These improvement in immune status, intestinal morphology, and growth performance due to dietary EO diet could be a result of an increase in nutrient digestibility and in the ratio of lactobacilli to enterobacteriaceae in the ileum and cecum in pigs due to dietary inclusion of EO.

Yan et al. (2010) determined the effects of EO supplement (AROMEX®-ME, which is a blend of thyme, rosemary, oreganum extracts and kaolin covered by starch) at dietary inclusion of 0.01%, and low or high dietary nutrient density on growth performance of grower pigs from 23 to 100 kg. Overall, they observed increased ADG due to EO supplementation (0.685 vs. 0.717 kg). However, the magnitude of improvement in ADG due to EOs supplementation was greater in pigs fed the high nutrient density diet (0.795 vs. 0.734 kg) than those fed the low nutrient diets (0.717 vs. 0.685 kg). This indicates that the supplementation of EOs can have a positive effect on growth performance and that the efficacy of EOs products with regard to improving growth performance of weaned pigs is partly dependent dietary nutrient levels. Another study was conducted by Cho et al. (2006) to determine the effects of EO product (Fresta F Conc®), which consisted of fenugreek (40%), clove (12.5%), cinnamon (7.5%) and carrier (40%), alone or in combination with antibiotics [(Chlortetracycline 100 g + Sulfathiazole 100 g + Penicillin 50 g) 0.1%+Fresta F Conc® 0.02%] on growth performance, IgG concentration and fecal noxious gas production of weaned pigs (initial body weight of 5.5 kg) for 49 days. They reported a significant increase in ADG (by 11%) and ADFI (by 8%) due to the inclusion of antibiotics in combination with EOs compared with negative control, but no significant difference between NC and EO diet or diet with antibiotic alone with regard to ADG, ADFI, or F/G.

Based on results from these studies, it is apparent that essential oils can be used to partly replace antibiotic in diets for weaned pigs; however, the combination of EOs with antibiotics results in the better performance than EOs alone, meaning that there is still need to find effective alternatives to antibiotics.

Flavonoids. Flavonoids belong to a group of natural substances found in fruit, vegetables, grains, bark, roots, stems, and flowers. They are known for their various beneficial health effects. Most common benefit being their best-described property to act as antioxidants (Nijveldt et al., 2001). Flavonoids have also been shown to exhibit antimicrobial activity. The antioxidant mechanism of flavonoids can help protect the cells from these damaging effects.

Flavonoids can be added in weaned pig diets to help alleviate the problems caused during weaning. Quercetin, datiscetin, kaempferol, myricetin and isoflavones are some of the most common food flavonoids, and their effects on gut health of animals is discussed below. Quercetin has various properties including; antihypertensive and antiarrhythmic activity; anti-inflammatory and antiallergic properties; and other activities (Formica and Regelson, 1995). Ader et al. (2000) conducted a study to determine bioavailability of quercetin in pigs, and observed absorption of the quercetin from the small intestine mainly in the form of glucuronides. They observed that the amount of quercetin present in plasma does not linearly increase as a function of the oral dose and that plasma levels were lower than levels that were reported to be effective in in vitro assays; however, this does not exclude the fact that some body tissues may accumulate some quercetin or some of its metabolites that are sufficient to exert biological effects. Zou et al. (2016) conducted a study to determine the effects of quercetin supplementation on intestinal integrity, intestinal reactive oxygen species (ROS) levels and intestinal inflammation in finishing pigs under transport stress. Pigs were supplemented with 25 mg of quercetin for 4 weeks and then transported. The quercetin-supplemented pigs showed decreased serum levels of endotoxin, increased height of jejunum villi (442 vs. 320 μm), as well as lower

intestinal levels of ROS (3.5 vs. 5.8 RUL/mgprot) compared with the control diet. This shows that quercetin can improve intestinal injuries in pigs during transport, probably through mechanisms associated with oxidative status and anti-inflammatory activity. The antimicrobial activity of datiscetin, kaempferol, myricetin and quercetin was determined by Xu and Lee (2001) using the disc diffusion method for screening of flavanoids against multi-drug resistant bacteria; the 4 flavonols showed antimicrobial activity against methicillin-resistant *Staphylococcus aureus*.

Isoflavones are a group of flavonoids that are found in some crops such as soybeans and clover (Reinli and Block, 1996). Soy isoflavones have been shown to exhibit antioxidant activity. One of the primary isoflavones in soybean is genistein. Greiner et al. (2001) determined the effects of adding genistein in diets for 10-day old pigs at various concentrations (0, 200, 400 and 800 ppm) on growth performance and virus persistence during a viral challenge. Pre-inoculation (weaning to inoculation) weight gain and feed intake of pigs decreased linearly as dietary genistein concentrations increased. During post-inoculation of PRRS (porcine reproductive and respiratory syndrome) (d 0 to 24 post-inoculation), an increase in dietary level of genistein resulted in a linear reduction in serum concentration of virus and improved ADG by 14% and ADFI by 15% when it was supplemented at 200 ppm treatment; however, genistein had limited effect on weight gain when it was supplemented at 400 ppm and a negative effect on feed intake at both 400 and 800 ppm and a negative effect on weight gain when it was supplemented at 800 ppm.

Another primary soybean isoflavone is daidzein. Soybean daidzein has been reported to enhance phagocytosis rate of macrophages and greater antibody production

and cytotoxic T cell activation (Zhang et al., 1997). Greiner et al. (2001) conducted a study to determine the effects of supplementing 11-day old pigs with different concentrations (0, 200, 400, and 800 ppm) of dietary soy daidzein on pig growth and viral replication during a viral challenge. Prior to inoculation of PRRS pig weight was not altered between dietary groups. Post-inoculation, dietary daidzein supplementation did not alter serum concentrations of virus, but improved ADG by about 5% when it was supplemented at 200 or 400 ppm. The ADG, ADFI and F:G were depressed by dietary supplementation of daidzein at 800 ppm, and limited improvements were seen in ADFI or G:F for all dietary treatments compared to negative control.

Based on results from these studies, it appears that flavonoids can help improve growth of virally challenged animals, exhibit antioxidant properties on stress induced pigs, and reduce growth of specific bacteria.

Alkaloids. Alkaloids are a large group of organic, basic compounds found in plants. They are usually bitter in taste and function in the defense of plants against herbivores and pathogens, and may be toxic in large amounts (Wink, 1999). Alkaloids are found in the leaf, bark, seed, root and other parts of the plant. Berberine is a plant alkaloid with history in Chinese medicinal systems. Berberine has antioxidant (Campisi et al., 2014) and antimicrobial activities (Freile et al., 2003); it has been shown to reduce gut injury in various laboratory animals including rats (Gu et al., 2013), and to reduce diarrhea in humans (Rabbani, 1987) that have been infected with the same diarrhea-causing bacteria in weaned pigs. Beberine has already been isolated from various plant species including *Berberis aquifolium* (Oregon grape), *Berberis vulgaris* (barberry), and

Berberis aristata (tree turmeric) (Mokhber-Dezfuli et al., 2014); and is currently being sold in various pharmacies in North America and in Asia.

Berberine has strong antioxidant activity. It acts as an antioxidant in the body by several mechanisms including removal of oxygen, scavenging of reactive oxygen species and nitrogen species, inhibition of reactive oxygen species and nitrogen species, and by unregulation of endogenous antioxidant defenses (Shirwaikar et al., 2006).

Berberine has been evaluated in many studies for its antidiabetic and antioxidant properties. Bhutada et al. (2011) conducted a study to determine the role of berberine on cognitive dysfunction in streptozotocin-induced diabetic rats. Rats with chronic treatment with berberine (25-100mg/kg twice daily for 30 days) showed improved cognitive performance, lowered hyperglycemia, oxidative stress, and choline esterase activity in diabetic rats. This study demonstrates that berberine treatment can prevent changes in oxidative stress and choline esterase activity.

Berberine has been found to have a wide spectrum of other pharmacological effects. Some of these include antihypertensive, antihyperglycemic, anticancer, and antidepressant, anti-inflammatory (Fan et al., 2015). It has been shown to alleviate diarrhea in humans and has been used as a treatment for cardiovascular and other lifestyle-related diseases. Study on the antimicrobial nature of berberine sulfate on *Vibrio cholerae* and *S. aureus* indicated that berberine exerts a bactericidal action (killing) on *V. cholerae* and a bacteriostatic action (prevents growth) on *S. aureus* (Amin et al., 1968). However, under the same conditions berberine had little effect on *E. coli* strains that are highly resistant to berberine sulfate (Amin et al., 1968). In a clinical trial performed with 400 adults presenting with acute watery diarrhea, the effects of 3 treatments (berberine,

tetracycline, or berberine and tetracycline) on clearance of vibrios from stools in patients with cholera were determined (Khin et al., 1985). Looking at just the patients with the type of cholera diarrhea there was a significant reduction in the number of motions, duration of diarrhea in hospital and volume of intravenous and oral fluid in tetracycline patients. However, there was no significant difference between the patients treated with berberine or group of adults administered placebo. This shows that although berberine is effective against cholera diarrhea and inhibits *V. cholerae* enterotoxin in animals, it does not seem to have antisecretory effect on cholera diarrhea in patients.

Another study conducted by Rabbani et al., (1987) determined the effect of administering berberine sulfate at 400 mg in a single oral dose on the reduction of diarrhea caused by enterotoxigenic *Enterotoxigenic Escherichia coli* (ETEC) and *V. cholerae* in 165 adults administered 400mg of berberine sulfate. After 8 h, there was a significant reduction in diarrhea in patients with ETEC compared to the controls, and at 24 hours after treatment, more patients stopped having diarrhea as compared to the controls (42 vs. 20%). However, in patients with cholera 8 hours after treatment, stool volume was decreased by significantly less margin than for patients that were not treated with berberine sulfate (2.22 L vs. 2.79 L). This indicates that berberine is effective at reducing in ETEC diarrhea patients but antisecretory effect is slightly less effective in cholera patients.

Fan et al. (2015) conducted a study to determine the effects of combination of probiotics, prebiotics and berberine as an alternative to antibiotic in diets for pigs from 60 to 120 days of age, and observed no significant difference between treatments on ADG, ADFI or feed efficiency. They observed a significant reduction of *E. coli* counts in feces

from pigs fed berberine supplemented diet (6.20 vs. 7.35 CFU/g) and an increase in fecal counts of lactic acid bacteria in groups treated with probiotics and oligosaccharides (9.48 vs. 8.46 CFU/g) or with berberine were higher than control (9.47 vs. 8.46 CFU/g). Thus, results from their study show that the addition of probiotics, oligosaccharides, and berberine to pig diets could effectively promote the growth of lactic acid bacteria in the gut and reduce the proliferation of *E. coli*, which would in turn improve pig growth performance and nutrient digestibility and reduce diarrhea rates. Further research looking at the effects of berberine alone in wean pig diets would improve our understanding of the mechanisms by which berberine improves animal performance and health.

Glucosinolates. Glucosinolates are secondary plant metabolites found in various plants, especially plants of *Brassica* family, in which they serve as defence compounds against herbivorous animals and pathogenic microorganisms (Tripathi and Mishra, 2007). Glucosinolates are hydrolyzed by the membrane-bound enzyme known as myrosinase into various compounds (including allyl isothiocyanate; **AITC**) in response to plant tissue injury by herbivorous animals or pathogenic microorganisms. AITC has been shown to have strong antimicrobial activity and antioxidant activity. Lin et al. (2000) determined the antibacterial activity of AITC, streptomycin, penicillin G, and polymyxin B against *Salmonella* Montevideo, *E. coli* O157:H7, and *Listeria monocytogenes*, and observed that AITC and polymyxin B were effective bactericidal agents against the bacteria at all growth stages. *E. coli* O157:H7 and *Salmonella* Montevideo at the exponential growth phase were completely killed following treatment for 2 h with 10 mg/ml polymyxin B or 1,000 mg/ml AITC. Antibacterial activity of AITC was similar to that of polymyxin B. Velioglu et al. (1998) reported that horseradish oil that contains 90-92% AITC had high

antioxidant activity (99.1%), even higher than that of BHA and BHT at 400mg/L (97.2%) and α -tocopherol at 200 mg/L (97.3%), which was used as the standard. However, information is lacking on the effects of dietary glucosinolates on gut health of weaned pigs. Glucosinolates are bitter which may reduce voluntary feed intake when they are included in diets for animals (Tripathi and Mishra, 2007). High dietary levels of glucosinolates can also reduce efficiency of nutrient utilization and even lead to death of pigs, which was observed in dietary glucosinolate levels 1.34 or 2.79 $\mu\text{mol/g}$ resulting in reduced feed intake and growth rate of pigs (Bowland, 1975; McKinnon and Bowland, 1977; Ochetim et al., 1980; Bell et al., 1991). Therefore, it's important to find the level at which glucosinolates can be added in the diets for weaned pigs for improving gut health without having negative effects growth performance.

HYPOTHESIS AND OBJECTIVES

In this thesis, it was hypothesized that dietary berberine, quercetin, and allyl isothiocyanate are suitable alternatives to antibiotics in improving gut health of weaned pigs.

The overall objective of this thesis research was to determine a phytochemical as a suitable alternative to antibiotics.

The specific objectives of the thesis research were:

1. To determine the effect of various levels of berberine, quercetin, and allyl isothiocyanate on in-vitro growth of *E. coli*, with the goal of identifying the most effective one for the use as potential alternative to antibiotics in animal studies.
2. To determine the effect of down selected phytochemical on growth performance, electrophysiological properties of small intestine mounted in Ussing chambers, and small intestinal histomorphology of weaned pigs.

CHAPTER TWO:

Effect of Berberine, Quercetin and Allyl Isothiocyanate on in vitro *E. coli* growth.

ABSTRACT

A study was conducted to determine the effect of 3 phytochemicals (berberine chloride hydrate, quercetin, allyl isothiocyanate) and ampicillin on in-vitro growth of *E. coli*. Inclusion of berberine in incubation medium at 25, 12.5, 6.25 or 3.125 µg/100 µl resulted in reduced growth of *E. coli*. Growth of *E. coli* on incubation medium containing 25, 12.5 or 6.25 µg/100 µl was similar to growth of *E. coli* on incubation medium containing final concentration of ampicillin at 0.04mg/100µl. Inclusion of AITC in the incubation medium at 0.5065 mg/100 µl did not affect in vitro growth of *E. coli*. Also, inclusion of quercetin at 0.011 mg/100 µl in the incubation medium did not affect in vitro growth of *E. coli*. It can be concluded that quercetin and AITC at concentrations used in the current study had limited effects on in vitro growth of *E. coli*. However, berberine exhibited antimicrobial activity similar to that of ampicillin at 25, 12.5 or 6.25 µg/100 µl. Thus, berberine was more effective than quercetin, or allyl isothiocyanate in inhibiting in vitro growth of *E. coli*.

INTRODUCTION

Antibiotics have been added to wean pig diets for many years to help reduce the negatives effects of weaning on growth performance and gut health. With stricter rules pertaining to the use of antibiotics in feed due to the fear of development of antibiotic

resistant bacteria, there has become a need to find alternatives to antibiotics. Plant-based products have been looked at as possible alternatives to antibiotics.

The term phytochemical or phytochemical can be referred to as the plant-derived compounds added into diets to help improve animal production performance and animal health (Steiner, 2009). Phytochemicals can exhibit many modes of action. The 2 modes of action that are important during the weaning stage of pigs are antimicrobial and antioxidant activities because they result in reduced growth of pathogenic bacteria in the gut and reduced oxidative stress in the gut, respectively.

Some of the phytochemicals that can potentially be used as alternatives to antibiotics include: berberine, quercetin, and AITC. Berberine is a nonbasic, plant alkaloid that has been shown to lower oxidative stress in rats (Bhutada et al., 2011) and reduce ETEC diarrhea in humans (Rabbani et al., 1987). Quercetin is a common food flavonoid, and its inclusion in diets has been shown to reduce reactive oxygen species in growing pigs (Zou et al., 2016), as well as reduce to the growth of *Staphylococcus aureus* (Xu and Lee, 2001). Allyl isothiocyanate (AITC) has been shown to have strong antimicrobial activity against *E. coli* (Lin et al., 2000) and exhibit antioxidant activity (Velioglu et al., 1998). However, information is lacking on the effects of these phytochemicals or dosage of these phytochemicals on growth of *E. coli*. The objective of this study was to determine the effect of various levels of these three phytochemicals on in-vitro growth of *E. coli*, with the goal of identifying the most effective one for use as potential alternative to antibiotics in weaned pig studies.

MATERIALS AND METHODS

Three different experiments were conducted at South Dakota State University's Animal Physiology Laboratory; 1 experiment per phytochemical.

Making Pre-culture

A vial of *E. coli* was obtained from the Animal Disease Research and Diagnostic Laboratory at South Dakota State University. It was then streaked on an agar plate and incubated for 24 h at 37°C in an incubator (Imperial III Incubator, Barnstead International). A few colonies were obtained from the plate after the 24 h incubation and immersed in 5 ml LB broth in a 20ml tube. The pre-culture tube was then incubated for 24 hours at 37°C in the fore-mentioned incubator to allow for growth of bacteria in broth.

Determination Antimicrobial Activity of the Phytochemicals

In Exp. 1, ampicillin solution was made by combining 4µl of stock solution ampicillin into 996µl of LB broth. The final concentration of ampicillin was 0.40µg/100µl.

The berberine solution was made by dissolving 0.05 grams of berberine chloride hydrate (Sigma-Aldrich, St. Louis, MO) in 1000 µl of DMSO. The DMSO-dissolved berberine solution was 100-fold diluted using distilled water to create the first working solution of berberine. Four serial 10-fold dilutions were further made from the first working berberine solution to create 5 different working solutions of berberine. The 5 berberine solutions were tested for growth of *E. coli* as described below.

Fifty microliters of each berberine solution and ampicillin solution was pipetted in a 96-well plate in triplicate. Fifty microliters of the pre-culture solution was then pipetted into each well containing berberine or ampicillin solution. Negative control (100 μ l of LB broth) solution and positive control (50 μ l of LB broth plus 50 μ l of pre-culture solution) solution were also pipetted into the wells in triplicate for comparison with berberine and ampicillin solutions. Final concentrations of berberine that were tested were: 25 μ g/100 μ l (Concentration A), 12.5 μ g/100 μ l (Concentration B), 6.25 μ g/100 μ l (Concentration C), 3.125 μ g/100 μ l (Concentration D), and 1.563 μ g/100 μ l (Concentration E). The final concentration that was tested for the ampicillin solution was 0.40 μ g/100 μ l. Each well in peripheral rows of the 96-well plate was filled with 100 μ l of distilled water, and the cover was placed on the 96-well plate to limit evaporation of the contents in the wells. The 96-well plate was then incubated at 37°C for 24 hours in an incubator.

In Exp. 2, 0.01g of quercetin was dissolved in 450 μ l of DMSO to give a final concentration of 0.011 μ g/100 μ l. The dissolved quercetin was diluted and incubated as described above in Exp. 1. Final concentrations of quercetin and ampicillin that were tested included: 0.011 μ g/100 μ l and ampicillin at 0.40 μ g/100 μ l

In Exp. 3, 10 μ l of liquid AITC, with a molecular weight of 1.013g/mL, was added to 990 μ l of distilled water to give a final concentration of 0.5065 μ g/100 μ l. The liquid AITC was diluted and incubated as described above in Exp. 1. Final concentrations of AITC and ampicillin that were tested included: 0.5065 μ g/100 μ l and ampicillin at 0.40 μ g/100 μ l.

Pooling Dilution Samples

After the 96-well plate was incubated for 24 hours, it was taken out from the incubator, and the 3 replicates of each treatment were pooled. They were then diluted once more by taking 10 µl of the pooled samples and combining it with 990 µl of distilled water. This was done in order to be able to count CFU on the plate more accurately.

Making Agar Plates

Agar plates were made by mixing 23.5 g of the agar medium with one liter of distilled water. The mixture was then autoclaved at 121°C for 15 minutes, and cooled for 15 minutes. The cooled agar was agitated in a bottle to achieve a uniform mix, and was poured into plates. The plates were left at room temperature to cool and harden.

Streaking the plate

Plate inoculation was done by spread plate technique. Briefly, 100 µl of the three combined dilution samples was placed in the center of the plate using a sterile pipet. A bent glass rod was sterilized by first dipping it into a 70% alcohol solution and then passing it quickly through the Bunsen burner flame. When all the alcohol had burned off and the rod had air-cooled, the rod was streaked back and forth across the plate working up and down several times. Backtrack was done many times in order to distribute the bacteria as evenly as possible. The plate was turned 90 degrees and repeated with the side to side, up and down streaking. The plate was turned again 45 degrees and streaked a third time. The glass rod was not sterilized between plate turnings. The plate was covered

and sat several minutes before turning it upside down for incubation. Once the plate had been allowed to soak up the sample it was placed in a 37°C incubator for 24 hours.

Counting colonies

Following 24 hour incubation, plates were examined for *E. coli* growth. The number of colonies were counted and expressed in number of colony forming units (CFU) per microliter of sample; the dilution factor was taken into account. The average for the 2 plates in terms of the number of microorganisms per gram or microliter of sample were determined for duplicate plates.

Statistical Analysis

Data were analyzed using mixed procedure of SAS (SAS Inst. Inc., Cary, NC). Plate was used as experimental unit, with eight plates per treatment. To test the hypotheses, the level of significance was set at 5%.

RESULTS

In Exp. 1, inclusion of ampicillin in incubation medium reduced ($P < 0.05$) growth of *E. coli* in terms of CFU per microliter (Table 2.1). Also, inclusion of berberine in incubation medium at 25, 12.5, 6.25 or 3.125 $\mu\text{g}/100 \mu\text{l}$ resulted in reduced ($P < 0.05$) growth of *E. coli*. However, inclusion of berberine in incubation medium at 1.5625 $\mu\text{g}/100 \mu\text{l}$ did not significantly reduce the growth of *E. coli*. A decrease in berberine concentration in incubation medium from 25 $\mu\text{g}/100 \mu\text{l}$ to 12.5 or 6.25 $\mu\text{g}/100 \mu\text{l}$ did not affect the growth of *E. coli*. Also, growth of *E. coli* on incubation medium containing 25,

12.5 or 6.25 $\mu\text{g}/100 \mu\text{l}$ was similar to growth of *E. coli* on incubation medium containing ampicillin.

In Exp. 2, inclusion of ampicillin in incubation medium reduced ($P < 0.0002$) growth of *E. coli* in terms of CFU per microliter (Table 2.2). However, inclusion of AITC in the incubation medium at a final concentration of 0.5065 $\mu\text{g}/100 \mu\text{l}$ did not significantly reduce the growth of *E. coli*. The CFU's per microliter for incubation medium that contained AITC were greater ($P < 0.001$) than those for the negative control or incubation medium that contained ampicillin.

In Exp. 3, inclusion of quercetin at 0.011 $\mu\text{g}/100 \mu\text{l}$ in the incubation medium did not affect CFU's per microliter. However, inclusion of ampicillin in the incubation medium reduced ($P < 0.0004$) growth of *E. coli*. There was no difference in *E. coli* growth between the incubation medium containing ampicillin and that containing no bacteria.

DISCUSSION

Inclusion of berberine in incubation medium at 3.125 $\mu\text{g}/100 \mu\text{l}$ and above reduced growth of *E. coli*. Berberine has been reported to exhibit anti-microbial activity (Yi et al., 2007). Possible mechanisms by which berberine reduces growth of microorganisms include targeting nucleic acids, specifically affecting RNA polymerase and gyrase and topoisomerase IV, both on nucleic acid (Yi et al., 2007). Kosalec et al. (2009) also reported reduced in vitro growth of *Bacillus subtilis* and *Staphylococcus aureus* due to inclusion berberine extracts in incubation medium. In their study, 2 different methods (diffusion method and minimal inhibitory concentration [MIC]

method) were used to determine the effects of berberine extracts (*Berberis croatica* and *Berberis vulgaris*) on growth of *B. subtilis* and *S. aureus*. Berberine exhibited significant antibacterial activity against the Gram-positive bacteria species with inhibition zones ranging between 9.5 mm and 12 mm, and between 9 mm and 16 mm, respectively, when extract was added at 60 μ l. However, berberine-containing extracts of neither barberry species possessed any antimicrobial activity against *E. coli* or *C. albicans* when the diffusion method was used, but the same extracts showed antimicrobial activity when the MIC (≤ 87.5 mg/ml) method was used. Results from this study show that diffusion method and MIC result in different conclusions on the effects to certain bacteria. In this case the different results seen when using the diffusion method was because the berberine extract didn't diffuse into the agar as well as when it was diluted.

The reason for lack of effect of AITC on growth of *E. coli* in the current study is not clear. It could probably have been due to low concentration of the allyl isothiocyanate in the incubation medium. Lin et al. (2000) reported that bactericidal activity of isothiocyanates was dependent on its concentration in incubation medium and exposure time. In their study, inclusion of AITC at 500 μ g/ml in incubation medium did not dramatically affect the in vitro growth of *E. coli* at any time period (1, 2, or 3 hours). However, inclusion of AITC at 2,500 μ g/ml in incubation medium completely inhibited growth of *E. coli* after 30 min treatment. Also, inclusion of AITC in incubation medium at 1,000 μ g/ml, reduced growth of *E. coli* after a 2-h exposure time. Similarly, Delaquis and Sholberg (1997) observed a decrease in colony counts of *Salmonella typhimurium*, *E. coli*, and *Listeria monocytogenes* with increasing times (24, 48, and 72 hours) of AITC exposure and increasing AITC concentration (500, 1000, 1500, or 2000 μ g/L of air).

However, when incubated at 25°C to 35°C and exposed to 500 µg/liter of AITC, *E. coli* was not effected at all, but was strongly inhibited when incubated at both 5 and 40°C. Thus, it appears that the anti-microbial activity of AITC is dependent on dosage, temperature and time of exposure. This research is relevant to our study, because we incubated our plates at 37°C which could have affected the growth of bacteria on the inoculated plates. Higher doses of AITC were not tested in our in vitro assay because AITC is a glucosinolate that has anti-nutritional effects at high of dietary levels. Studies reported that glucosinolates in diets at 1.34 or 2.79 µmol/g reduced feed intake and growth rate of pigs (Bell et al., 1991).

In the current study, inclusion of quercetin at 0.011 mg/100 µl in the incubation medium did not significantly reduce CFU's per microliter. Similarly, Gatto et al. (2002) reported that quercetin at 100 µg/ml of incubation medium did not exhibit antimicrobial activity against *E. coli*. However, Rauha et al (2000) observed slight (1-3 mm) to moderate (3-4 mm) antimicrobial activity of Quercetin at 500µl against *E. coli* using the diffusion method. In an in vivo study that was conducted by Vijaya and Ananthan (1996) with guinea pigs, oral administration of quercetin 142.9 mg/kg body weight resulted in protection of the guinea pigs against an induced *Shigella* infection that killed all shigella-challenged guinea pigs that were not given the quercetin. *Shigella* is a Gram-negative bacteria that is closely related to *E. coli*. Quercetin wasn't tested at higher levels in our in vitro study because it is expensive, and its inclusion in diets for pigs at dosages that are greater than the dosage used in the current study is not cost effective. The cost of Quercetin (#1592409) obtained from Sigma-Aldrich is \$300 per 500 mg.

In conclusion, berberine was the most effective phytochemical at inhibiting the growth of *E. coli*. Berberine exhibited similar antimicrobial activity at 25, 12.5 or 6.25 $\mu\text{g}/100\ \mu\text{l}$ concentrations compared to ampicillin. Quercetin and AITC did not reduce CFU's per microliter compared to the plates incubated with ampicillin. Berberine was tested at multiple levels in order to find the most effective concentration after antimicrobial activity was observed. Further research on effects of different in vitro exposure times and concentrations of quercetin and AITC on growth of *E. coli* needs to be conducted. However, other levels were not tested because of anti-nutritional effects on animals or unfeasible cost of products. The effectiveness of the phytochemicals antimicrobial activity can change with dose and exposure time. Thus, AITC could be tested at longer exposure times than 24 hours to see if that affects antimicrobial activity at that level. Based on results from this study, berberine was identified as potentially most suitable alternative to antibiotics for use in diets for weaned pigs in subsequent studies.

Table 2.1. Effects of including berberine in incubation medium on in vitro growth of *E. coli*

	Negative		Positive		Ampicilin		Concentration		SEM
<i>E. coli</i> growth CFU/microliter	51.66 ^a	640 ^d	3.33 ^a	99.166 ^a	125 ^a	183.33 ^{ab}	308.33 ^{bc}	483.33 ^{cd}	

^{a-d}Within a row, means without a common superscript differ ($P < 0.05$).

¹Negative = LB broth only; Positive =

Table 2.2. Effects of including allyl isothiocyanate (AITC) in incubation medium on in vitro growth of *E. coli*

	AITC ¹	Negative ³	Positive ⁴	Ampicillin ²	SEM
<i>E. coli</i> growth					
CFU/microliter	741.67 ^a	59.16 ^b	642.86 ^a	< 10 ^b	99.50

^{a-b}Within a row, means without a common superscript differ ($P < 0.05$).

¹AITC final concentration was 0.5065 µg/100 µl

²Ampicillin final concentration was 0.40µg/100µl

³Negative = LB broth only

⁴Positive = Preculture only

Table 2.3. Effects of including quercetin in incubation medium on in vitro growth *E. coli*

	Quercetin ¹	Negative ³	Positive ⁴	Ampicillin ²	SEM
<i>E. coli</i> growth CFU/microliter	677.22 ^a	59.16 ^b	642.86 ^a	< 10 ^b	103.25

^{a-b}Within a row, means without a common superscript differ ($P < 0.05$).

¹Quercetin final concentration of 0.011 µg/100 µl

²Ampicillin final concentration of 0.40µg/100µl

³Negative = LB broth only

⁴Positive = Preculture only

CHAPTER THREE:

The effect of dietary berberine at 3.0% on growth performance, electrophysiological properties and histomorphology of small intestine of weaned pigs

ABSTRACT

A study was conducted to determine the effects of berberine on growth performance, electrophysiological properties of small intestine mounted in Ussing chambers, and small intestinal histomorphology of weaned pigs. Twenty-four 3 wk-old weaned pigs (average initial BW = 6.35 kg) were obtained in 2 batches of 12 pigs each, and assigned to 3 experimental diets within batch (4 pigs/diet/batch). The diets included a basal diet without or with antibiotics or 3% berberine. The experiment lasted for 7 days, and at the end, body weight gain and feed consumption were determined. The pigs were then euthanized to determine duodenal, jejunal, and ileal trans-epithelial resistance (TER) and small intestinal histomorphology. Data was analyzed using Mixed procedure of SAS with batch as block and pig as experimental unit. There was no effect of dietary treatment on average daily gain. The average final BW was 5.92 ± 0.34 kg. However, pigs fed antibiotic-containing diet had greater ($P < 0.029$) ADFI than those fed the basal diet (0.147 vs. 0.127 kg). Inclusion of berberine in the basal diet decreased ($P < 0.0001$) ADFI from 0.123 to 0.056 kg. There was no difference in villous height and crypt depth in all sections of small intestine between pigs fed antibiotic-containing diet and basal diet. Inclusion of berberine in the basal diet decreased ($P < 0.05$) crypt depth in duodenum and ileum by an average of 35% and tended to decrease ($P = 0.069$) crypt depth in jejunum by 32%. Ileal villous height was decreased ($P < 0.0001$) by 28% due to inclusion of

berberine in basal diet. However, there was no effect of including berberine in basal diet on villous height in duodenum and jejunum. Pigs fed antibiotic-containing diet and basal diet had similar TER values (which reflects intestinal mucosal barrier function) in all sections of the small intestine. Inclusion of berberine in basal diet tended to decrease ($P = 0.078$) TER in duodenum from 55.53 to 44.31 Ω . It can be concluded that inclusion of antibiotic had minimal impact on intestinal health parameters. Dietary inclusion of berberine appeared to have a negative effect on intestinal health; however, the influence of berberine is confounded by very low feed intake, which can also negatively influence gut health.

Key words: berberine, pig, growth performance, gut health

INTRODUCTION

At weaning, pigs are stressed due to abrupt changes in their new environment and diet composition, as well as interruption of already established social structure with their littermates and sows. The weaning stress results in a decrease in growth performance and an increase in diarrhea incidences. The decrease in growth performance and increase in diarrhea incidences occur mainly during the first 2 weeks after weaning and causes huge economic losses in swine industry (Cutler and Gardner, 1988).

Antibiotics have been added in diets for the weaned pigs at low levels to improve their performance. However, addition of antibiotics in diets for food animals is currently being discouraged because they can lead to development of antibiotic resistant microorganisms. Thus, the effects of various potential alternatives to antibiotics (feed additives), including prebiotics, probiotics, minerals, plant extracts, and animal-derived

antibodies are being investigated; however, majority of these feed additives have been inconsistent in improving gut health and growth performance of pigs (Heo et al., 2013; Thacker, 2013). For this reason, there is still a critical need to develop effective alternative agents to manage gut health. Otherwise, the use of antibiotic-free diets in the swine industry will continue to be a challenge.

Weaning process results in increased permeability of intestine of pigs due to increased oxidative stress (Wijten et al., 2011). The increased intestinal permeability increases translocation of toxins that are produced by gut microorganism into the body, causing inflammatory injuries in the gut wall, thereby increasing the susceptibility of weaned pigs to gut infections. Thus, for an alternative to antibiotics to be effective, it has to reduce growth of pathogenic microorganism in the gut or reduce oxidative stress (that results in increased intestinal permeability), or both.

Berberine is a nonbasic, plant alkaloid that has already been isolated from various plant species including *Berberis aquifolium* (Oregon grape), *Berberis vulgaris* (barberry), and *Berberis aristata* (tree turmeric) (Mokhber-Dezfuli et al., 2014). Berberine has antioxidant (Campisi et al., 2014) and antimicrobial (Freile et al., 2003) activities; it has been shown to reduce gut injury in various laboratory animals including rats (Gu et al., 2013), and to reduce diarrhea in humans (Rabbani, 1987) that have been infected with the same diarrhea-causing bacteria in weaned pigs. In the previous study (see Chapter 2 of this thesis), berberine inhibited in vitro growth of *E. coli* when it added in incubation medium at $\geq 3.125\%$. Thus, berberine can potentially be used as an alternative to antibiotics in diets for weaned pigs. However, the effects of berberine on growth performance and gut health of weaned pigs haven't been determined.

The objective of this study was to determine the effects of berberine on growth performance, electrophysiological properties of small intestine mounted in Ussing chambers, and small intestinal histomorphology of weaned pigs.

MATERIALS AND METHODS

Experimental Animals and Housing

Twenty-four pigs (12 barrows and 12 gilts; large white-Landrace female × Large white-Hampshire male, Pig Improvement Company) weaned at 3 wk of age and with average initial BW of $6.35 \text{ kg} \pm 0.66$ were obtained in 2 batches of 12 pigs (balanced for sex) each from the South Dakota State University's Swine Research Barn and individually housed in metabolism crates with a feeder and nipple drinkers. The mean daily temperature throughout the trial was 28.3°C and they were exposed to lighting all throughout the experiment.

Experimental Diets

The diets fed included a corn-soybean meal-based basal diet (control diet) without or with antibiotics (Tylan 40 at 0.05%) or 3% berberine (Table 1). The antibiotic-containing diet also contained zinc oxide at 0.25%. The basal diet was formulated to meet or exceed NRC (2012) nutrient requirements for weaned pigs. The berberine product used was Berberine Chloride Hydrate, (#4101062757) and was obtained from Sigma-Aldrich (St. Louis, MO). The dietary level of berberine (3%) was chosen based on results from the first study (see Chapter 2) in which inclusion of berberine in incubation medium at $\geq 3.0\%$ reduced in vitro growth of *E. coli*.

Experimental Design and Procedure

The pigs were fed the 3 experimental diets within batch and gender for a total of 4 pigs/diet/batch. The experiment lasted for 10 days. Body weight gain and feed consumption were determined on day 7. On days 7, 8, 9, and 10, 3 pigs balanced for dietary treatment were anesthetized by an intramuscular mixture of telazol-ketamine-xylazine (TKX; telazol and xylazine at 50 mg/mL each; ketamine at 100 mg/mL), and then euthanized by penetrating captive bolt followed by exsanguination daily, and the following procedures took place. Ten centimeter sections of the duodenum (at 70 cm below the pylorus), jejunum (at the middle of small intestine), and ileum (at 70 cm above ileo-cecal junction) were immediately collected and placed in ice-cold Ringer's solution (NaCl; 6.72 g/l, K₂HPO₄; 0.42 g/l, KH₂PO₄; 0.05 g/l, CaCl₂ dihydrate; 0.18 g/l, MgCl₂ hexahydrate; 0.24 g/l, NaHCO₃; 2.1 g/l, glucose 1.80 g/l; pH of 7.3-7.4) for determination of intestinal electrophysiological properties using Ussing chambers technique as described below. Indomethacin (10 μ M) was added in the buffer solution at 3 μ l/L to help minimize the effects of pro-inflammatory eicosanoids on intestinal tissue electrophysiological properties. Also, 5 cm sections of the duodenum, jejunum, and ileum were obtained (from the same locations where sections for Using Chamber were obtained) and placed into 10% buffered formalin solution for later determination of histology as described below.

Histology Analysis

Samples for histology analysis were sent to the Animal Disease Research and Diagnostic Laboratory at South Dakota State University for staining with haematoxylin and eosin. Villous height (from the tip of the villi to the villous-crypt junction) and crypt depth (from the villous-crypt junction to the base) were measured at 20x magnification using a Nikon microscope (Tokyo, Japan) equipped with a DS2MV Nikon camera (Tokyo, Japan) and NIS Elements software (Tokyo, Japan) in 20 well-oriented villi and crypt columns. The villous height-to-crypt depth ratio was calculated.

Determination of Electrophysiological Properties

The electrophysiological properties (potential difference, **PD**; short-circuit current; **I_{sc}** and trans-epithelial electrical resistance, **TER**) were determined using a Ussing chamber (VCC-MC6; Physiologic Instruments Inc., San Diego, CA) containing pairs of current (Ag wire) and voltage (Ag/AgCl pellet) electrodes housed in 3% agar bridges and filled with 3 M KCl. Samples for determining gut permeability were transported (while in ice cold Ringer buffer solution) to the laboratory, where they were opened along the mesenteric border. The opened samples were gently stripped of serosal layer using micro-forceps to remain with the mucosa. The prepared mucosal tissues (4 tissues per intestinal segment) were placed in tissue holders with an aperture of 1 cm². First slider of tissue holder was placed over the serosal side of the tissue. The second slider of tissue holder was placed on mucosal side of the tissue and 2 sliders were gently pressed together. Liquid was dried out from chamber recess and the tissue holders were mounted in the chambers with the mucosal layer facing the left side of the chamber. Ringer buffer solution was added to the serosal and mucosal half chambers (3

mL per each of the half chamber). The chambers were continuously gassed with a mixture of 95% O₂ and 5% CO₂. The temperature of the chambers was maintained at 37°C. After mounting tissues in the chambers, the Ussing chambers system was placed in remote mode to allow for Acquire and Analyze software program to obtain data, and tissues were referenced on the Acquire and Analyze Data acquisition software and hardware system. After referencing, 10 mM glucose was added to the serosal bathing solution, which was balanced on the mucosal side with 10 mM mannitol. The Acquire and Analyze software was turned on (to start collecting data) immediately after adding glucose and mannitol, and tissues were allowed to equilibrate for 10-15 min. After the equilibration, 10 mM glucose was added to the mucosal side and balanced with 10 mM of mannitol of the serosal side. The spontaneous PD was measured using Ringer-agar bridges connected to calomel electrodes, and the PD was short circuited through Ag-AgCl electrodes using voltage clamp that corrected for fluid resistance. The TER (which reflects intestinal permeability) was calculated from the I_{sc} and PD. After 120 minutes, forskolin (10 μM) was added to both the mucosal and serosal sides of the chambers to determine whether or not the tissues were still alive. Significant changes in I_{sc} within 10 min indicated that the tissues were still alive.

Statistical Analysis

Data were analyzed as a randomized complete block design with batch as block using mixed procedure of SAS (SAS Inst. Inc., Cary, NC). Pig was used as experimental unit. Treatment means were compared by probability of difference. To test the hypotheses, the level of significance was set at 5%.

RESULTS

The berberine-containing diet had CP value that was numerically greater and ether extract value that was numerically lower than those for the basal diet or antibiotic-containing diet (Table 3.1). The average final BW of pigs was 5.92 ± 0.34 kg (Table 3.1). The inclusion of berberine in the basal diet decreased ($P < 0.0001$) ADG of pigs. However, there was no difference between basal diet and antibiotic-containing diet with regard to ADG of pigs (Table 3.2). Inclusion of berberine in the basal diet decreased ($P < 0.0001$) ADFI, there was no difference in ADFI between basal diet and antibiotic-containing diet. There was no effect of adding antibiotics to basal diet on villous height and crypt depth in all sections of small intestine (Table 3.3). However, inclusion of berberine in the basal diet decreased ($P < 0.05$) crypt depth in duodenum, jejunum, and ileum. Also, ileal villous height was decreased ($P < 0.0001$) by inclusion of berberine in the basal diet. However, there was no effect of including berberine in basal diet on villous height in duodenum and jejunum. Pigs fed antibiotic-containing diet and basal diet had similar TER values in all sections of the small intestine. Inclusion of berberine in basal diet tended to decrease ($P = 0.078$) TER in duodenum, but had no effect on TER in the jejunum and ileum. There was no difference in Isc values in any sections of the small intestine between diets.

DISCUSSION

Inclusion of berberine in basal diet resulted in increased dietary CP content, which was due to the presence of N in berberine. Berberine is an alkaloid that contain N in its molecular structure (Shirwaikar et al., 2006). The addition of antibiotics to the

basal diet did not affect ADG and ADFI, which as contrary to expectations. The addition of antibiotics in the diets for weaned pigs at sub-therapeutic levels has resulted in improved feed intake and growth rate, and reduced mortality and morbidity (Cromwell, 2001). Stahly et al. (1980) reported increased feed intake and growth rate of 28-day old weaned pigs due to dietary inclusion of both copper and an antibiotic (55 ppm of Chlortetracycline or 27.5 ppm of virginiamycin). Mereu et al. (2016) also observed increased ADFI (507 vs. 480 g), ADG (377 vs. 328 g), and G:F (0.82 vs. 0.78 g/g) of weaned pigs due to inclusion of tiamulin, chlortetracycline and zinc oxide in pre-starter diets at 40, 400 and 2,500 g/metric ton, respectively; and in starter diets at 40, 110, and 2500 g/metric ton, respectively. Several mechanisms by which dietary antibiotics could increase growth performance of animals have been proposed. Some of the proposed mechanisms by which antibiotics promote growth include the suppression of growth of pathogenic microorganisms, promotion of growth of microorganisms that synthesize nutrients such as vitamins and volatile fatty acids that are required by pigs, and suppression of growth of microorganisms that compete with host for nutrients (Cromwell, 2001). The lack of effect of dietary antibiotics and zinc on ADG and ADFI in the current study is not clear. It could probably have been due to the fact that the experimental period of 7 d was not long enough for dietary antibiotics to impact growth performance of weaned. Hong et al. (2004) also observed lack of effect of dietary antibiotics on growth performance of weaned pigs during first 10 after weaning, but observed increased growth performance of the pigs after feeding the antibiotic-containing diets for 20 d after weaning. Keegan et al. (2003) similarly reported increased ADG and ADFI of weaned pigs due feeding of antibiotic-containing diets for 30 d but not for 9 d after weaning.

Studies have shown that disease-challenged pigs show a greater response in growth performance when they are fed diets containing antibiotics at sub-therapeutic levels. For instance, Hsu et al. (1983) determined the effects of adding low levels of tiamulin, a semisynthetic derivative of the antibiotic pleuromulin in diet for *Mycoplasma pneumoniae*-challenged 8 wk-old pigs and observed greater ADG (0.54 vs. 0.44 kg) and feed conversion ratio (3.01 vs. 3.66 kg/kg) for pigs fed antibiotic-containing diets than for those fed nonmedicated diets from d 28 to 42 after medicated feed was withdrawn, but there was no difference in growth parameters for d 0 to 28. Pigs used in the current study were not disease-challenged, which could also partly explain the lack of effect of dietary antibiotics on growth performance of the pigs.

Although antibiotics are effective in improving growth performance of weaned pigs, their inclusion in diets for food animals is being discouraged because they lead to development of antimicrobial resistance. Thus, we conducted the current study to evaluate berberine as possible alternative to antibiotics in diets for weaned pigs. Dietary inclusion of berberine decreased ADG and ADFI of pigs, which was contrary to the expectations. In previous studies, berberine exhibited antimicrobial and antioxidant activities (Campisi et al., 2014; Freile et al., 2003). Also, in the first study of this thesis research, berberine reduced in vitro growth of *E. coli*. Thus, it had been hypothesized that dietary berberine would improve growth performance and gut health of weaned pigs because the reduction in growth performance of pigs after weaning is attributed to increased growth of pathogenic microorganisms in gut, and increased oxidative stress in the gut, which results in increased gut permeability to toxins. However, it should be noted that berberine is an alkaloid with bitter taste, and hence its inclusion in diets at a higher

level can result in reduced palatability of feed. In the current study, berberine was added in diets at 3% because it is at this level that it reduced in vitro growth of *E. coli* in the first study (see Chapter 2). Blaney et al. (2000) observed a decrease in feed intake of sows by 50% due to dietary inclusion of sorghum (that contained 40 mg/kg alkaloids) at 4%. Jugl-Chizzola et al. (2006) and Schone et al. (2006) also observed dose-related reduction in palatability of feed for pigs due to increasing dietary level of essential oils from fennel and caraway, as well as from the herbs thyme and oregano. Similarly, Godfrey et al. (1985) included lupin seed (that contains alkaloids) in diets for growing pigs at concentrations that ranged from 0.05 to 0.52 g/kg, and observed that inclusion of lupin seed in diets at $\geq 0.20\%$ resulted in reduced growth rate (624g vs. 576g), largely a result of reduced feed intake (1.81 vs. 1.69 kg) due to bitterness of the feed. Thus, the reduced feed intake by dietary inclusion of berberine at 3% could have been due to reduced feed palatability. The reduced ADG due to dietary berberine could have been due to the decrease in feed intake by dietary berberine.

Antibiotics have long been used to promote growth, and have been shown to have positive effects on the intestinal health when they are included in diets at sub-therapeutic levels, as stated earlier. Mereu et al. (2016) observed increased growth performance and intestinal TER (163 vs. 115 $\Omega \cdot \text{cm}^2$) of weaned pigs due to inclusion of tiamulin, chlortetracycline and zinc oxide in pre-starter diets at 40, 400 and 2,500 g/metric ton, respectively; and in starter diets at 40, 110, and 2500 g/metric ton, respectively. They also observed decreased Isc (2.2 vs 6.1 $\mu\text{A cm}^2$) of mid-ileum segments of the intestine. Silva et al. (2010) also observed increased height of villus (320 vs. 275 μm) in piglets at 14 days post-weaning due to supplementation of diet with antibiotics alone compared with

supplementation of the diet with antibiotics plus probiotics. However, in the current study, inclusion of antibiotics in diets for weaned pigs did not affect villous height, Isc, and TER, and the reason for this is not clear.

Pigs fed diet with berberine had lower villous height in all sections of the small intestine than pigs fed basal diet, which could have been due to decreased feed intake by dietary berberine. Data from various studies have demonstrated that villous height is positively correlated with feed intake (Cera et al. 1988, Kelly et al. 1991a and 1991b, McCracken et al. 1995, Pluske et al. 1996a and 1996a). Indeed, Verdonk et al. (2007) observed reduced villous height by 17% and reduced crypt depth by 13% due to a decrease in feed intake of weaned pigs by 31%. Also, Kelly et al. (1991b) observed a 19% decrease in villous height and a 13% decrease in crypt depth in pigs due to a decrease in feed intake (by restriction) from 864 to 226 g/day, indicating that nutrient intake in the weaned pigs affects the morphology of the gut.

The addition of berberine in the basal diet tended to decrease TER in the duodenum, and jejunum. Decreased TER reflects increased paracellular permeability of the intestinal mucosa, which in turn, reflects the opening of tight junctions between epithelial cells (Hu et al., 2014), implying that the reduction in TER due to dietary berberine was due to opening of tight junctions. Chang et al. (2005) investigated the functional and morphological changes of the gut barrier of rats after hemorrhagic shock and found that the recovery of gut barrier function was much slower than that of the morphology and there was no direct correlation between them. Hu et al. (2014) observed that mRNA expression of occludin and claudin-1 (which are tight junction proteins) on d 3, 7, and 14 post-weaning and ZO-1 (which is another tight junction protein) mRNA

expression on d 3 and 7 post-weaning was decreased compared with d 0, implying that weaning process resulted in decreased synthesis of tight junction protein. This decrease in synthesis of tight junction proteins results in increased paracellular permeability in the gut, and is mainly attributed to weaning stress. Carey et al. (1994) determined the effects of a 48-hour fast on jejunal ion transport in 23-day-old pigs, and observed reduced mucosal weight, villus height, and crypt depth due to the fasting, which often times is a result of weaning. In their study, fasting increased basal I_{sc} , which reflects active ion transport, and total tissue conductance (G_t) (the opposite of TER) of muscle-stripped jejunal sheets mounted in Ussing chambers. Thus, a sufficient feed intake after weaning prevents the loss of the barrier function that occurs after weaning. Spreeuwenberg et al. (2001) did not observe any effects weaning diet composition on transcellular transport, but did observe a 39% change in paracellular transport on d 2 and 4 compared with day 0 and 1, This trial indicates that the effect of diet composition on mucosal integrity is not as important as the sequential effects of low feed intake during the first 4 days post-weaning as shown by Carey et al (1994). Thus, in the current study, the decrease in TER due to dietary inclusion of berberine could have been due to reduction in feed intake with the dietary inclusion of berberine.

In conclusion, our results show that berberine has negative effects on ADG and gut function when it was included in the diet at 3.0%; however, these results were confounded by reduced voluntary feed intake by pigs due to dietary berberine inclusion.

Table 1. Diet composition

Ingredients (%)	Phase I		
	Basal Diet	Antibiotic Diet	Berberine Diet
Ground Corn	53.58	53.58	53.58
SBM	26.65	26.65	26.65
Whey	10.00	10.00	10.00
Fish Meal 60%	4.50	4.50	4.50
Lysine	0.30	0.30	0.30
Threonine	0.13	0.13	0.13
Methionine	0.15	0.15	0.15
Soy Oil	3.00	3.00	3.00
Monocal 21%	0.50	0.50	0.50
Calcium Carbonate	0.65	0.65	0.65
White salt	0.30	0.30	0.30
Swine Vitamin	0.05	0.05	0.05
Swine Mineral	0.15	0.15	0.15
Zinc Oxide	-	0.25	-
Tylan 40	-	0.05	-
Toxin Binder MMI	0.05	0.05	0.05
Berberine	-	-	3.00
Chemical Composition			
Crude protein (%)	22.55	21.67	23.65
Ether Extract (%)	6.65	6.27	4.05

¹Basal = control diet without antibiotics and berberine; Antibiotic = basal diet plus antibiotics and ZnO; Berberine = basal diet plus berberine at 3%.

²Provided the following per kilogram of diet: 2226 IU vitamin A, 340 IU vitamin D3, 11.3 IU vitamin E, 0.01 mg vitamin B12, 0.91 mg menadione, 2.04 mg riboflavin, 12.5 mg pantothenic acid, 11.3 mg niacin, 0.23 mg folic acid, 0.68 mg pyridoxine, 0.68 mg thiamine, and 0.04 mg biotin.

³Provided the following per kilogram of diet: 75 mg Zn as ZnSO₄, 75 mg Fe as FeSO₄; 7 mg Cu as CuSO₄, and 20 mg Mn as MnSO₄.

Table 3.2. Effects of treatment on growth performance

Item	Antibiotic			SEM	P-value
	Basal Diet	Diet	Berberine Diet		
Initial BW	6.137	6.35	6.475	-	
Final BW	5.925	6.312	5.537	0.223	
ADFI, g	0.127 ^a	0.147 ^a	0.047 ^b	0.010	< .0001
ADG, g	-0.025 ^a	-0.011 ^a	-0.107 ^b	0.014	< .0001
G:F, g/g	-0.236 ^a	0.050 ^a	-4.615 ^b	0.557	< .0001

^{a-b}Within a row, means without a common superscript differ ($P < 0.05$).

¹Basal = control diet without antibiotics and berberine; Antibiotic = basal diet plus antibiotics and ZnO; Berberine = basal diet plus berberine at 3%.

Table 3.3. Effects of treatment on gut health

Item	Basal Diet	Antibiotic Diet	Berberine Diet	SEM	<i>P</i> -value
Duodenum					
Villous Height	161.76	176.89	159.94	10.134	0.560
Crypt Depth	73.91 ^a	74.63 ^a	55.05 ^b	4.259	0.008
Jejunum					
Villous Height	173.71 ^{ab}	203.49 ^a	157.76 ^b	14.357	0.099
Crypt Depth	72.17 ^a	79.30 ^a	54.61 ^b	6.225	0.032
Ileum					
Villous Height	199.88 ^a	183.72 ^a	143.24 ^b	6.592	< .0001
Crypt Depth	78.72 ^a	85.87 ^a	57.90 ^b	5.034	0.004
TER					
Duodenum	61.28 ^a	52.99 ^{ab}	44.31 ^b	6.354	0.202
Jejunum	44.44 ^{ab}	49.15 ^a	34.55 ^b	4.956	0.151
Ileum	67.14	57.45	48.19	7.784	0.279
Isc					
Duodenum	24.54	29.08	36.62	5.135	0.370
Jejunum	26.79	29.82	20.97	7.811	0.785
Ileum	12.08	5.42	9.02	5.090	0.658

^{a-b}Within a row, means without a common superscript differ ($P < 0.05$).

CHAPTER FOUR:

The effect of dietary berberine at 0.05% on growth performance, and electrophysiological properties and histomorphology of small intestine of weaned pigs

ABSTRACT

A study was conducted to determine effects of including berberine in diets for weaned pigs at 0.05% on growth performance, small intestinal permeability, electrophysiological properties of small intestine mounted in Ussing chambers, and small intestinal histomorphology. A total of 216 three-week old pigs with average initial BW of $5.5 \text{ kg} \pm 0.99$ were obtained in 2 blocks of 108 pigs each. Pigs in each batch were housed in 18 pens (6 pigs per pen) and assigned to 3 experimental diets (6 pens/diet/batch). The diets were basal diet without or with berberine or antibiotics. The experimental diets were fed for a period of 21 days and in 2 phases; Phase 1 from day 1 to 11 post-weaning, and Phase 2 from day 12 to 21 post-weaning. During the experimental period, feed intake and BW were determined on days 11 and 21, whereas indicators of intestinal health were determined on day 11 of the experiment. Inclusion of antibiotics or berberine in basal diet increased ($P < 0.05$) ADG of pigs in Batch 1 but not in Batch 2 during the first phase of feeding. There was an interaction ($P = 0.038$) between diet and batch on ADG for during the second phase of feeding such that ADG of pigs in Batch 1 was unaffected by dietary treatment, whereas ADG of pigs in Batch 2 was decreased ($P < 0.05$) by dietary inclusion of berberine but not of antibiotics. There was also an interaction ($P = 0.048$) between diet and batch on ADG for the entire study period (d 1 to 21) such that ADG of pigs in batch 1 was increased ($P < 0.05$) by dietary inclusion of antibiotics by 32% and tended to

increase ($P < 0.06$) due to dietary inclusion of berberine by 16.6%, whereas ADG of pigs in batch 2 was decreased ($P < 0.05$) by dietary inclusion of berberine, but not of antibiotics. There was no effect of adding antibiotics or berberine to the basal diet on ADFI of pigs for day 1 to 11 and for day 12 to 21 regardless of the batch. However, for entire study period (day 1 to 21), pigs fed antibiotic-containing diet had greater ($P < 0.05$) ADFI than those fed the basal diet and those fed the berberine diet. Batch and diet tended to interact ($P = 0.082$) on ileal villous height such that that was no effect of dietary treatment on ileal villous height of pigs in Batch 1, whereas inclusion of berberine (but not antibiotics) in the basal diet increased ($P < 0.05$) the ileal villous height of pigs in Batch 2. There was no effect of dietary treatment on lactulose:mannitol ratio in urine. Inclusion of antibiotics in the basal diet increased ($P < 0.05$) transepithelial resistance (TER) in ileum of pigs in Batch 1 by 35%. Inclusion of berberine in basal diet had no effect on TER values in the jejunum and the ileum. There was no effect of dietary treatment on permeability of the jejunal and ileal mucosa to the FITC-dextran 4 kDa for pigs. Batch and diet interacted on short-circuit current in jejunum and ileum such that dietary inclusion of berberine but not antibiotics increased ($P < 0.05$) Isc in jejunum and ileum of pigs in Batch 1 by at least 60%, whereas dietary inclusion of berberine or antibiotics did not affect Isc in jejunum and ileum of pigs in Batch 2. Some pens of Batch 2 pigs fed diets containing berberine or antibiotics had diarrhea. In conclusion, dietary berberine improved growth performance and Isc intestine of pigs in Batch 1. However, dietary berberine did not ADG and Isc intestine of pigs in Batch 2 likely due to confounding effect of diarrhea of some pigs fed diets containing berberine. The improved ADG and Isc intestine of pigs in Batch 1 by dietary berberine imply that berberine can

improve growth performance of weaned by increasing intestinal nutrient absorptive capacity.

INTRODUCTION

Weaning stressors are associated with changes in gastrointestinal morphology, microbiology, and physiology; and immunological challenges (Spreeuwenberg et al., 2001; Heo et al., 2012). The stress of weaning results in a decrease in growth performance, an increase in diarrhea incidences, and an increase in susceptibility to gut infections. Gut infections characterized with diarrhea are the major cause of reduced growth performance and increased morbidity and mortality of post-weaned pigs and pathogenic *E. coli* are a major cause of the gut infections (Fairbrother et al., 2005). Antibiotics have been added to weaned pig diets for many years to help reduce the effects of weaning stressors on growth performance. However, with stricter rules pertaining to the use of antibiotics in feed for the fear of antibiotic resistant bacteria, there has become a need to find alternatives to antibiotics.

Berberine has been reported to have a wide spectrum of pharmacological effects including antihypertensive, anticancer, and antidepressant, anti-inflammatory, and antioxidant (Fan et al., 2015). It has been shown to alleviate diarrhea in humans and has been used as a treatment for cardiovascular and other lifestyle-related diseases, however there is limited information on effects of including berberine in diets for weaned pigs on growth performance and gut health.

In our previous study (see Chapter 2), inclusion of berberine in incubation medium at 3% reduced growth of *E. coli*. However, in our other previous study (see

Chapter 3), inclusion of berberine in diets for weaned pigs at 3.0% reduced ADFI and ADG. Berberine is a bitter alkaloid (Shirwaikar et al., 2006), and its inclusion in diets at high levels can result in reduced palatability of the diet. Indeed, Godfrey et al. (1985) observed reduced feed intake of growing pigs due to an increase in dietary level of alkaloids to $\geq 0.20\%$ through dietary inclusion of lupin. Hence, the reduction in ADFI and hence ADG of weaned pigs due to dietary inclusion of berberine at 3% in our previous study could have been due to the fact that dietary berberine at 3% was high enough to reduce diet palatability. Berberine at 0.08% of daily feed intake was effective in controlling coccidiosis in chickens (Malik et al., 2014), implying that berberine at dosages less than 1%, may alleviate gut infections.

The objective of this study was to determine the effects of including berberine in diets for weaned pigs at a lower inclusion rate (0.05%) on diet palatability, growth performance, permeability and electrophysiological properties of small intestine mounted in Ussing chambers, and small intestinal histomorphology.

MATERIALS AND METHODS

Two separate experiments were conducted at South Dakota State University.

Exp. 1

The experiment was conducted to determine the effect of dietary inclusion of 0.05% berberine without or with a sweetener (saccharine) on feed intake of weaned pigs. Six three-week old weaned pigs (Large white-Landrace female \times Large white-Hampshire male, Pig Improvement Company) were acquired. Pigs were housed individually in grower pens (2.3 \times 1.8 m) that allowed freedom of movement. Each pen had a metal slate

floor with concrete sides, a 3 space dry feeder, and a nipple drinker. Each pig was given 3 different diets; 1 diet per space of feeder. The diets were a basal diet with antibiotics (Tylan 40), Berberine Chloride Hydrate, (#4101062757) and was obtained from Sigma-Aldrich (St. Louis, MO) at 0.05%, or berberine at 0.05% plus saccharine (an artificial sweetener, obtained from Sigma-Aldrich, St. Louis, MO) at 0.023% (Table 4.1). The experiment lasted for 7 days. During the experimental period, the diets and water were given ad libitum. Diets were placed into feeders in containers that fitted the dimensions of each of the 3 spaces in the feeder. Diets were fed daily to ensure that there was adequate amount of each diet in each feeder for the pig to choose from. On the final day of the study, feed that had remained in the feeders was weighed to determine average daily feed intake of each diet for each pig.

Exp. 2

The experiment was conducted to determine the effects of including berberine in diets for weaned pigs at 0.05% on growth performance, permeability and electrophysiological properties of small intestine mounted in Ussing chambers, and small intestinal histomorphology.

Experimental Animals

A total of 216 three-week old pigs (Large white-Landrace female × Large white-Hampshire male, Pig Improvement Company) with average initial BW of $5.5 \text{ kg} \pm 0.99$ were obtained from a commercial farm in 2 batches of 108 pigs each. Pigs in each batch were weighed upon arrival and housed in 18 pens (6 pigs/pen) balanced for initial BW. Pigs were housed individually in pens ($2.3 \times 1.8 \text{ m}$) that allowed freedom of movement.

Each pen had a metal slate floor with concrete sides, a 3 space dry feeder, and a nipple drinker.

Experimental Diets and Design

Pigs in each batch were fed 3 experimental diets in a completely randomized design (6 pens/diet/batch). The diets included basal diet without or with berberine (berberine chloride hydrate, #4101062757; Sigma-Aldrich, St. Louis, MO) at 0.05%, or control with antibiotic (Table 3 and Table 4). All diets were formulated to meet or exceed NRC (2012) nutrient requirements for weaned pigs. The diets were fed in 2 growth phases based on age; Phase 1 (d 1 to 11 post weaning), and Phase 2 (d 11 to 21 post weaning). The diets were analyzed for CP and ether extract as described in Chapter 3.

Experimental Procedure

During the experimental period, pigs were offered diets and water ad libitum. From d 10 to 12 6 pigs (2 pigs per diet) were euthanized per day for collection jejunal and ileal tissues. Three hours prior to euthanization, a bolus (15 ml/kg) of 5% lactulose and 5% D-mannitol solutions was orally administered to pigs that had been marked for euthanization. Just before euthanization, pigs were anesthetized with 1.5 ml of telazol-ketamine-xylazine (TKX; telazol and xylazine at 50 mg/mL each; ketamine at 100 mg/mL) and the abdomen was numbed with lidocaine. A small incision was made in the lower abdomen, which allowed access to the bladder. Urine collection was collected directly from the bladder using sterile needle and syringe for later determination of gut permeability by lactulose:mannitol ratio in urine as described below. The pigs were then euthanized by captive bolt. Upon the euthanization, the abdominal cavity was exposed by large incision through the abdominal wall. The small intestine was excised and ten

centimeter sections of the jejunum (at the middle of small intestine), and ileum (at 70 cm above ileo-cecal junction) were obtained and placed into Ringer's solution (NaCl; 6.72 g/l, K₂HPO₄; 0.42 g/l, KH₂PO₄; 0.05 g/l, CaCl₂ dihydrate; 0.18 g/l, MgCl₂ hexahydrate; 0.24 g/l, NaHCO₃; 2.1 g/l, Glucose 1.80 g/l; pH of 7.3-7.4) for later measuring intestinal permeability and electrophysiological parameters using Ussing chamber technique as described below. Indomethacin (10 μ M) was added in the buffer solution at 3 μ l/L to minimize the effects of pro-inflammatory eicosanoids on intestinal tissue electrophysiological properties. Also, 5 cm sections were obtained from duodenum (at 70 cm below the pylorus), middle of jejunum and ileum (from the same locations where sections for Using Chamber were obtained), and placed in neutral buffered 10% formalin-containing vials for histomorphology analysis as described below.

Determining Intestinal Permeability by Lactulose:Mannitol Ratio

Permeability was quantified by the ratio of lactulose:mannitol concentrations in the urine as described by Nguyen et al. (2013). Lactulose and mannitol sugars are used due to a consistent increase in the absorption of disaccharide (lactulose) and a reduction in the absorption of monosaccharide (mannitol) after the ingestion of a solution containing the sugar probe molecules. The transport of monosaccharides occurs either through tight junctions between the epithelial cells or through aqueous pores in the cell. Disaccharides are transported through the tight junctions of the crypts. The ratio of the two sugars in the urine provided is used because there are some mucosal factors (gastric emptying, urinary collection) and the ratio is said to not be disturbed by these. An increase in lactulose:mannitol ratio indicates decreased barrier function and vice versa.

Concentrations of lactulose and mannitol in urine were determined using commercial kits from BioAssay Systems (Hayward, CA). Briefly, 4 standards for each kit were prepared and pipetted into 4 wells in a flat bottomed 96-well plate. Then, 20 μ l urine samples were pipetted into the well plate at 2 wells per sample (sample wells). Some wells were left empty to serve as blanks. A working reagent (80 μ l) and blank working reagent (80 μ l) were prepared to be pipetted into each sample well and blank sample well. Well plates for lactulose assay were prepared and incubated in the darkness for 60 min at room temperature before reading optical density at 565 nm. Well plates for mannitol assay were prepared and incubated in light for 30 min at room temperature before reading optical density at 565 nm. Standards were graphed to determine the slope, and concentrations of sample were determined using this equation; *concentration* = $\frac{OD_{sample} - OD_{blank}}{Slope (mM^{-1})} \times n (\mu M)$. If sample wells values were higher than the standard wells, the sample was diluted in water and the assay was repeated. The results were then multiplied by the dilution factor.

Determining Intestinal Permeability and Physiological parameters with Ussing Chambers

The electrophysiological properties I_{sc}, PD and TER were determined using a Ussing chamber (VCC-MC6; Physiologic Instruments Inc., San Diego, CA) containing pairs of current (Ag wire) and voltage (Ag/AgCl pellet) electrodes housed in 3% agar bridges and filled with 3 M KCl. Samples were prepared, mounted in Ussing chambers, and referenced as described in Chapter 3. Also, the PD, I_{sc}, and TER were measured for 120 min as described in Chapter 3.

In addition to PD, Isc, and TER, mucosal permeability in Ussing chambers was assessed by measuring mucosal to serosal fluxes of FITC-dextran 4 kDa (#46944, Sigma-Aldrich, St. Louis, MO). This was because FITC dextran 4 kDa can only pass through larger pores in the gut wall. Thus when it is added to the solution in the chamber at the luminal side, its appearance in the chamber at the serosal side reflects the permeability of the mucosa to the FITC-dextran 4 kDa, and hence permeability to toxins and pathogenic microorganisms. The mucosal to serosal flux of FITC-dextran 4 kDa was measured in darkness as described below. Briefly, the FITC-dextran 4 kDa was added to the mucosal side of the Ussing chambers at a final concentration of 104 nM and 200 μ l samples were taken from the serosal sides at 0 (just before the addition of FITC-dextran 4 kDa on mucosal side), 30, 60, 90 and 120 min for determination of FITC-dextran 4 kDa concentration, and replaced with 200 μ l of buffer solution. After 120 minutes, lights were switched on, and forskolin (10 μ M) was added to both the mucosal and serosal sides of the chambers to determine whether or not the tissues were still alive as described in Chapter 3. The quantity of FITC-dextran 4 kDa was determined by measuring the fluorescence in the collected serosal samples using a fluorescence plate reader (Synergy 2 Multi-detection Microplate Reader, BioTek, Winooski, VT) at 540 nm.

Histomorphology Analysis

Samples for histology analysis were sent to the Animal Disease Research and Diagnostic Laboratory at South Dakota State University for staining with haematoxylin and eosin. Villus height (from the tip of the villi to the villus-crypt junction) and crypt

depth (from the villus-crypt junction to the base) were measured at 20x magnification using a Nikon microscope (Tokyo, Japan) equipped with a DS2MV Nikon camera (Tokyo, Japan) and NIS Elements software (Tokyo, Japan) in 20 well-oriented villi and crypt columns. The VH-to-CD ratio was calculated.

Statistical Analysis

Data from the Experiment 1 was analyzed using mixed procedure of SAS (SAS Inst. Inc., Cary, NC). Pig was used as experimental unit. To test the hypotheses, the level of significance was set at 5%.

Data from Experiment 2 were analyzed as a randomized complete block design with batch as block using mixed procedure of SAS (SAS Inst. Inc., Cary, NC). Pen was used as experimental unit. Main effects of batch and diet and their interactions were determined using specific contrasts. Diet means were compared by probability of difference. To test the hypotheses, the level of significance was set at 5%.

RESULTS

Exp. 1

The ADFI for berberine diet did not differ from that for berberine plus saccharine diet (Table 4.2). The ADFI for the antibiotic diet was greater ($P < 0.05$) than that for the berberine diet or berberine plus saccharine diet.

Exp. 2

Phase 1 basal diet had numerically greater dietary CP content and lower ether extract content than Phase 1 antibiotic diet or berberine diet (Table 4.3). However,

inclusion of berberine in the Phase 2 basal diet resulted in a numerical increase in CP and ether extract contents (Table 4.3).

Some pigs fed antibiotic and berberine diets developed spontaneous diarrhea during the first phase of feeding. For this reason, data is presented by batch to show the effects of batch on response criteria that were measured. Inclusion of antibiotics in basal diet increased ADG of pigs in Batch 1 ($P < 0.05$) for d 1 to 11. Also, inclusion of berberine in basal diet increased ADG of pigs in Batch 1 ($P < 0.05$) for d 1 to 11. However, there was no effect of dietary treatment on ADG of pigs in Batch 2 for d 1 to 11. There was an interaction ($P = 0.038$) between diet and batch on ADG for d 12 to 21 such that ADG of pigs in Batch 1 was unaffected by dietary treatment, whereas ADG of pigs in Batch 2 was decreased ($P < 0.05$) by dietary inclusion of berberine but not of antibiotics. There was also an interaction ($P = 0.048$) between diet and batch on ADG for the entire study period (d 1 to 21) such that ADG of pigs in Batch 1 was increased ($P < 0.05$) by dietary inclusion of antibiotics and tended to increase ($P < 0.06$) by dietary inclusion of berberine, whereas ADG of pigs in Batch 2 was decreased ($P < 0.05$) by dietary inclusion of berberine, but not of antibiotics.

There was no effect of adding antibiotics or berberine to the basal diet on ADFI of pigs for d 1 to 11 and for d 12 to 21 regardless of the batch. However, for entire study period (d 1 to 21), pigs fed antibiotic-containing diet had greater ADFI ($P = 0.03$) than those fed the basal diet and those fed the berberine diet ($P = 0.001$). Inclusion of antibiotics in basal diet increased G:F of pigs in Batch 1 ($P < 0.05$) for d 1 to 11. Also, inclusion of berberine in basal diet increased G:F of pigs in Batch 1 ($P < 0.05$) for d 1 to 11. However, there was no effect of dietary treatment on G:F of pigs in Batch 2 for d 1 to

11. There was an interaction ($P = 0.042$) between diet and batch on G:F for d 12 to 21 such that G:F of pigs in Batch 1 was unaffected by dietary treatment, whereas G:F of pigs in Batch 2 was decreased ($P < 0.05$) by dietary inclusion of berberine but not of antibiotics. There was also an interaction ($P = 0.049$) between diet and batch on G:F for the entire study period (d 1 to 21) such that G:F of pigs in Batch 1 was increased ($P < 0.004$) by dietary inclusion of berberine, whereas G:F of pigs in Batch 2 there was no effect by dietary inclusion of berberine.

There was no effect of adding antibiotics or berberine to basal diet on villous height, crypt depth, and villous height to crypt depth ratio in duodenum and jejunum, and on crypt depth in ileum (Table 4). Batch and diet tended to interact ($P = 0.082$) on villous height in ileum such that that was no effect of dietary treatment on villous height of pigs in Batch 1, whereas inclusion of berberine (but not antibiotics) in the basal diet increased ($P < 0.05$) the villous height of pigs in Batch 2. Also, villous height to crypt depth ratio in the ileum was increased ($P < 0.05$) by dietary inclusion of berberine, but not antibiotics.

There was no effect of dietary treatment on lactulose:mannitol ratio in urine (Table 4.7). Inclusion of antibiotics in the basal diet increased ($P < 0.05$) TER in ileum of pigs in Batch 1. Inclusion of berberine in basal diet had no effect on TER values in the jejunum and the ileum. There was no effect of dietary treatment on permeability of the jejunal and ileal mucosa to the FITC-dextran 4 kDa for pigs in both Batch 1 and Batch 2. There was a tendency ($P < 0.080$) for increased dextran levels in the ileum of the pigs fed the berberine diet compared to the pigs fed the antibiotic diet in Batch 2.

Batch and diet interacted on Isc in jejunum and ileum such that dietary inclusion of berberine but not antibiotics increased ($P < 0.05$) Isc in jejunum and ileum of pigs in

Batch 1, whereas dietary inclusion of berberine or antibiotics did not affect Isc in jejunum and ileum of pigs in Batch 2 (Table 4.7).

DISCUSSION

Exp. 1

The ADFI for diet with antibiotics was greater than that for diet with berberine or diet with berberine plus sweetener, implying that dietary inclusion of berberine at 0.05% reduced diet palatability. There was no difference in ADFI for diet with berberine and diet with berberine plus sweetener, implying that inclusion of the sweetener to the berberine-containing diet did not affect diet palatability. The palatability of a food or feed is dependent on the effect of that food or feed upon several different sensory systems in the mouth. Sweetening agents have been included in feeds for pigs to the palatability of the feeds. However, the effects of sweetening agents on feed palatability has been variable. For instance, Notzold et al. (1955) reported that inclusion of the sweetener saccharin at 0.03 or 0.1% in diets for weaned pigs did not affect feed consumption, growth rate or feed efficiency. However, when the sweetener was included in diets at 0.05% in a 55-day trial, they (Notzold et al., 1955) observed greater feed consumption for sweetened diet than for unsweetened diet during the first half of the study period, implying that there may be specific dietary levels of sweeteners at which there is taste preference for sweetened diets. This was also demonstrated in another study by Goatcher and Church (1970) in which 8 pigs were used to test the preference of water with saccharin at concentrations ranging from 0.25 to 25 g/L, and it was observed that at higher concentrations of saccharin, 6 pigs preferred saccharin solution over water,

whereas 2 pigs preferred water over saccharin solution. Results from the studies of Notzold et al. (1955) and Goatcher and Church (1970) imply that the effect of sweetener on feed palatability can vary among pigs. Thus, the lack of effect of sweetener on palatability of berberine-containing diet in the current study could probably have been due to dosage of the sweetener and pigs used in the study. It should be noted weaned pigs had high preference soybean meal over canola meal (84 vs. 16%) when they were offered both soybean meal-based diet and canola meal-diet (Landro et al., 2012); canola meal contains glucosinolates that are bitter. However, the ADFI for canola-meal-based diet did not differ from that of soybean meal-based diet when the diets the weaned pigs were offered only canola meal-based diet or soybean meal-based diet (Landro et al., 2011). The preference for antibiotic-containing diet over that containing berberine or berberine plus saccharin in the current study was low (43 vs. 33 vs. 24%), implying that weaned pigs can consume sufficient amounts of diets containing ~0.05% when the pigs are only offered berberine-containing diet. Because the ADFI for diet that contained berberine did not differ from that for diet that contained berberine plus saccharin, berberine was included in diets that were fed in Exp. 2 at 0.05% and without a sweetener.

Exp. 2

The addition of antibiotics to the basal diet increased ADG and ADFI of pigs in Batch 1 for d 1 to 11 and for entire period of the study. These results are in agreement with results from several studies (Hays, 1977; CAST, 1981; Zimmerman, 1986; Weber, 2001) that reported improved growth rate and feed efficiency of pigs due to dietary inclusion of antibiotics. Antibiotics are included in swine diets as non-nutritive feed

additives for their therapeutic potential as well as their ability to promote growth. The proposed mechanisms by which antibiotics improve growth performance of weaned pigs include the suppression of bacteria that are responsible for production of toxic compounds such as ammonia and amines in hindgut (Catron, 1953; Henderickx 1981). Reduced production of toxins by these bacteria also result in a decrease in intestinal wall thickness, which results in greater nutrient absorption (Catron, 1953; Henderickx 1981). Dietary antibiotics promote growth of certain microorganisms that synthesize certain nutrients that are required by host animals (Cromwell, 2001). Dietary antibiotics also depress growth of certain microorganisms that compete with host animal for nutrients such as amino acids and vitamins (Cromwell, 2001). The addition of berberine to the basal diet increased ADG of pigs in Batch 1 for d 1 to 11 and tended to increase ADG for entire period of the study, which could partly have been due to increased nutrient absorptive capacity as evidenced by the increased Isc in jejunal and ileal mucosa by dietary berberine. Berberine may improve growth performance by directly affecting the epithelial cells of the gut. Berberine affects pumps and channels involved with chloride secretion and nutrient absorption. A decrease in secretory action plays a role in decreasing electrolyte and water secretion across the intestinal mucosa, which will play a role in improving pig health and performance (Joshi et al., 2011). Berberine is also an effective antioxidant by several mechanisms including removal of oxygen, scavenging of reactive oxygen species and nitrogen species or their precursors, which can occur when the animal is stressed (Shirwaikar et al., 2006), as well as exhibits antimicrobial activities (Freile et al., 2003). Rahimi et al. (2011) observed an increase in feed intake and feed conversion ratio of broiler chicks by the addition of herbal extracts (0.1% thyme or

garlic) to diets fed from hatch to 42 days of age. Nofrarí'as et al. (2014) also observed increased ADFI of weaned pigs by 21% due to supplementation of diets with a plant extract product (5% carvacrol, 3% cinnamaldehyde, and 2% capsicum oleoresin). Inclusion of an extract from *Quillaja saponaria* in diets for 24 day-old weaned pigs at 0, 125, 250, 500 mg/kg did not affect growth performance of the pigs after 28 days of feeding (Turner et al., 2002). In another study, inclusion of *Q. saponaria* in diets for weaned pigs at 65 and 125 mg/kg plus antimicrobials improved growth rate of pigs (Cromwell et al., 1985), which indicated that the *Q. saponaria* extract is beneficial when it is supplemented together with antimicrobials.

The addition of antibiotics or berberine to the basal diet did not increase ADG and ADFI of pigs in Batch 2. It should be noted that some Batch 2 pigs fed antibiotic- and berberine-containing diets developed diarrhea, whereas all pigs fed the basal diet did not develop diarrhea. Thus, the diarrhea could have confounded the effects of dietary antibiotics or berberine in Batch 2 pigs. However, it is not clear why some pigs fed antibiotic- and berberine-containing diets in Batch 2 developed diarrhea.

There was no effect of adding antibiotic to basal diet on villous height, crypt depth or villous height to crypt depth ratio in duodenum and jejunum of pigs. There was, however, an increase in villous height and villous height to crypt depth ratio in ileum of pigs in Batch 2 due to inclusion of berberine in diet. Yazdani et al. (2013) also observed an increase in duodenal and jejunal villous height in 1-day-old broiler chickens due to supplementation of their diets with *Berberis vulgaris* extract in which berberine was the most important active compound. Hanczakowska and Swiatkiewicz (2012) similarly reported an increase in villous height in the ileum of piglets due to supplementation of

diets with extracts from sage (*Salvia officinalis*), lemon balm (*Melissa officinalis*), nettle (*Urtica dioica*), and purple coneflower (*Echinacea purpurea*). The mechanism by which berberine improves gut morphology could potentially be through macrophage apoptosis and inhibition of pro-inflammatory cytokine production, macrophages have been shown to promote repair of damaged mucosal tissue by producing immunosuppressive factors (Pull et al., 2005) which was observed in DSS-induced mice (Yan et al., 2012).

However, the decrease in growth performance of pigs in Batch 2 due to dietary inclusion of berberine we would've been expected to be associated with either a decrease or no change in villous height, since a decrease in villous height is linked to a decrease in absorption in nutrients and barrier protection against harmful pathogens (Lalles et al., 2004). Thus, the reasons for discrepancy between growth performance and ileal histomorphology data are not clear.

Inclusion of antibiotics in the basal diet increased TER values in both the jejunum and ileum of pigs in Batch 1. However, inclusion of berberine in basal diet had no effect on TER values in the jejunum and the ileum. Inclusion of antibiotics in the basal diet had no effect on Isc values of the small intestine in both the jejunum and the ileum. Inclusion of berberine in the basal diet increased Isc in the jejunum and ileum. However, an interaction between diet and batch on Isc was observed such that Isc was increased with the addition of berberine to the basal diet in the ileum in batch 1 but not in batch 2; the same interaction occurred in the jejunum. Stress and restricted energy intake are known to increase basal Isc and increase epithelial conductance (the opposite of TER) (Carey et al., 1994; Santos et al., 2000). Thus, the increase in jejunal and ileal mucosa Isc of pigs in batch 1 by dietary inclusion of berberine could have been due to reduced feed

intake. It is not clear why dietary berberine did not affect Isc in jejunum and ileum of pigs in batch 2. It could have been due to diarrhea by some of the in pigs (in batch 2) fed berberine-containing diet. The lower ADFI for berberine diet than for antibiotic diet could be the reason for the decrease in TER in small intestine of pigs fed the berberine diet because of the decrease in intake. However, an increase in ileal villous height we would be expected to result in an increase in TER in batch two rather than batch one, and the reason for lack of association between TER and villous height is not clear. Contrary to the results in the current study, Robbins et al. (2013) observed a higher mean ileal TER ($62 \Omega/\text{cm}^2$) in *salmonella*-challenged 5-week old pigs fed diet with benzo(c)phenanthridine alkaloid at 1.5 g/1000 kg than in those fed diet with chlortetracycline (59.4 g/1000 kg). Ferraris and Carey (2000) reported that changes in conductance (opposite of TER) largely reflect changes in size or selectivity of the tight junctions that separate the enterocytes, which would allow bacteria or toxins through the tight junctions.

In conclusion, dietary berberine improved ADG, G:F and Isc in jejunum and ileum of pigs in batch 1. However, dietary berberine did not affect small intestinal histomorphology, jejunal and ileal TER, permeability of the jejunal and ileal mucosa to the FITC-dextran 4 kDa, and lactulose:mannitol ratio in urine of pigs in batch 1. Thus, it appears that dietary berberine can improve growth performance of weaned pigs through increasing small intestinal nutrient absorptive capacity because small intestinal Isc is positively correlated with small intestinal nutrient absorptive capacity. However, dietary berberine did not improve growth performance and Isc in small intestine of pigs in batch 2. However, it should be noted that some pigs (in batch 2) fed berberine-containing diets

(also and antibiotic-containing diets) had diarrhea, which could have confounded the effects of dietary berberine on growth performance and small intestinal nutrient absorptive capacity.

Table 4.1. Composition of diets fed in Experiment 1

Item	Diet ¹		
	Antibiotic	Berberine	Saccharine
Ingredient, %			
Corn	53.58	53.58	53.58
Soybean meal	26.65	26.65	26.65
Whey	10.00	10.00	10.00
Fish Meal 60%	4.50	4.50	4.50
L-Lysine.HCl	0.30	0.30	0.30
L-Threonine	0.13	0.13	0.13
DL-Methionine	0.15	0.15	0.15
Soybean oil	3.00	3.00	3.00
Monocalcium phosphate	0.50	0.50	0.50
Calcium carbonate	0.65	0.65	0.65
White salt	0.30	0.30	0.30
Vitamin premix ²	0.05	0.05	0.05
Mineral premix ³	0.15	0.15	0.15
Zinc oxide	0.25	-	-
Tylan 40	0.05	-	-
Toxin binder MMI	0.05	0.05	0.05
Berberine	-	0.05	0.05
Saccharine	-	-	0.23
Analyzed chemical composition, %			
Crude protein	21.67	23.65	22.55
Ether extract	6.27	4.05	6.65

¹Antibiotic = basal diet plus antibiotics and ZnO; Berberine = basal diet plus berberine at 3%. Saccharine diet = Berberine plus 0.23% Saccharine

²Provided the following per kilogram of diet: 2226 IU vitamin A, 340 IU vitamin D3, 11.3 IU vitamin E, 0.01 mg vitamin B12, 0.91 mg menadione, 2.04 mg riboflavin, 12.5 mg pantothenic acid, 11.3 mg niacin, 0.23 mg folic acid, 0.68 mg pyridoxine, 0.68 mg thiamine, and 0.04 mg biotin.

³Provided the following per kilogram of diet: 75 mg Zn as ZnSO₄, 75 mg Fe as FeSO₄; 7 mg Cu as CuSO₄, and 20 mg Mn as MnSO₄.

Table 4.2. Effects of dietary treatment on diet palatability

Items	Antibiotic diet ¹	Berberine diet	Berberine diet + saccharine	SEM	<i>P</i> -value
ADFI	136.23 ^a	103.63 ^b	77.73 ^b	10.95	0.006

^{a-c}Within a row, means without a common superscript differ ($P < 0.05$).

¹Antibiotic = basal diet plus antibiotics and ZnO; Berberine = basal diet plus berberine at 3%. Saccharine diet = Berberine plus 0.23% Saccharine

Table 4.3. Composition of Phase 1 diets (as-fed basis) fed in Experiment 2

Ingredients (%)	Diet ¹		
	Antibiotic	Berberine	Basal
Corn	32.54	33.28	33.35
SBM, 46.5%	15.00	15.00	15.00
Whey, dried	30.00	30.00	30.00
Menhaden Fishmeal	8.00	8.00	8.00
Hamlet Protein 300	8.00	8.00	8.00
L-lysine HCl	0.26	0.26	0.26
L-Threonine	0.06	0.06	0.05
DL-Methionine	0.15	0.15	0.15
L-Tryptophan	0.02	0.02	0.02
Soybean Oil	4.00	4.00	4.00
Monocal	0.48	0.48	0.47
Limestone	0.25	0.25	0.25
Salt	0.25	0.25	0.25
Nursery Vitamin ²	0.05	0.05	0.05
Nursery TM ³	0.15	0.15	0.15
Zinc Oxide	0.42	-	-
Denagard	0.17	-	-
Chlortetracycline	0.20	-	-
Berberine	-	0.05	-
Analyzed chemical Composition			
Crude protein (%)	21.97	22.32	23.37
Ether Extract (%)	9.70	9.00	6.37

¹Basal = control diet without antibiotics and berberine; Antibiotic = basal diet plus antibiotics and ZnO; Berberine = basal diet plus berberine at 0.05%.

²Provided the following per kilogram of diet: 2226 IU vitamin A, 340 IU vitamin D3, 11.3 IU vitamin E, 0.01 mg vitamin B12, 0.91 mg menadione, 2.04 mg riboflavin, 12.5 mg pantothenic acid, 11.3 mg niacin, 0.23 mg folic acid, 0.68 mg pyridoxine, 0.68 mg thiamine, and 0.04 mg biotin.

³Provided the following per kilogram of diet: 75 mg Zn as ZnSO₄, 75 mg Fe as FeSO₄; 7 mg Cu as CuSO₄, and 20 mg Mn as MnSO₄.

Table 4.4. Composition of Phase 2 diets (as-fed basis) in Experiment 2

Ingredients (%)	Diet ¹		
	Antibiotic	Berberine	Basal
Corn	54.84	54.49	55.49
SBM, 46.5%	28.50	28.50	28.50
Whey, dried	10.00	10.00	10.00
Menhaden Fishmeal	3.00	3.00	3.00
L-lysine HCl	0.35	0.35	0.35
L-Threonine	0.09	0.09	0.09
DL-Methionine	0.12	0.12	0.12
L-Tryptophan	0.02	0.02	0.02
Monocal	1.10	1.10	1.10
Limestone	0.83	0.83	0.83
Salt	0.30	0.30	0.30
Nursery Vitamin ²	0.05	0.05	0.05
Nursery TM ³	0.15	0.15	0.15
Zinc Oxide	0.28	-	-
Denagard	0.17	-	-
CTC	0.20	-	-
Berberine	-	0.05	-
Chemical Composition			
Crude protein (%)	21.65	22.68	21.71
Ether Extract (%)	3.24	6.37	2.89

¹Basal = control diet without antibiotics and berberine; Antibiotic = basal diet plus antibiotics and ZnO; Berberine = basal diet plus berberine at 0.05%.

²Provided the following per kilogram of diet: 2226 IU vitamin A, 340 IU vitamin D3, 11.3 IU vitamin E, 0.01 mg vitamin B12, 0.91 mg menadione, 2.04 mg riboflavin, 12.5 mg pantothenic acid, 11.3 mg niacin, 0.23 mg folic acid, 0.68 mg pyridoxine, 0.68 mg thiamine, and 0.04 mg biotin.

³Provided the following per kilogram of diet: 75 mg Zn as ZnSO₄, 75 mg Fe as FeSO₄; 7 mg Cu as CuSO₄, and 20 mg Mn as MnSO₄.

Table 4.5. Effects of treatment on growth performance.

Item	Diet ¹			SEM	P-value		
	Basal	Antibiotic	Berberine		Diet	Batch	Diet*Batch
1-11 days							
ADG (g)							
Batch 1	0.100 ^c	0.151 ^a	0.125 ^b	0.012	0.159	0.005	0.150
Batch 2	0.097	0.096	0.089				
ADFI (g)							
Batch 1	0.160	0.196	0.190	0.014	0.716	0.005	0.207
Batch 2	0.151	0.135	0.109				
G:F							
Batch 1	0.622 ^b	0.770 ^a	0.706 ^{ab}	0.042	0.485	0.319	0.399
Batch 2	0.641	0.618	0.687				
12-21 days							
ADG (g)							
Batch 1	0.307	0.360	0.323	0.014	0.001	0.450	0.038
Batch 2	0.325 ^a	0.378 ^a	0.249 ^b				
ADFI (g)							
Batch 1	0.618	0.699	0.562	0.053	0.093	0.674	0.801
Batch 2	0.639	0.679	0.483				
G/F							
Batch 1	0.498	0.541	0.580	0.041	0.282	0.149	0.042
Batch 2	0.519	0.558	0.330				
1-21 days							
ADG (g)							
Batch 1	0.386 ^b	0.511 ^a	0.450 ^{ab}	0.024	0.007	0.099	0.048
Batch 2	0.422 ^{ab}	0.473 ^a	0.308 ^b				
ADFI (g)							
Batch 1	0.530 ^b	0.642 ^a	0.505 ^b	0.027	0.001	0.493	0.424
Batch 2	0.558 ^{ab}	0.624 ^a	0.431 ^b				
G/F							
Batch 1	0.722 ^b	0.801 ^{ab}	0.898 ^a	0.053	0.804	0.072	0.049
Batch 2	0.758	0.755	0.567				

^{a-c}Within a row, means without a common superscript differ ($P < 0.05$).

¹Basal = control diet without antibiotics and berberine; Antibiotic = basal diet plus antibiotics and ZnO; Berberine = basal diet plus berberine at 0.05%.

Table 4.6. Effects of treatment on intestinal histomorphology

Item	Diet ¹			SEM	P-value		
	Basal	Antibiotic	Berberine		Diet	Batch	Diet*Batch
Duodenum							
Villous height							
Batch 1	148.02	164.97	153.50	10.149	0.704	0.028	0.894
Batch 2	176.64	183.8	184.96				
Crypt depth							
Batch 1	61.53	38.66	61.77	3.016	0.694	0.056	0.570
Batch 2	71.37	70.22	70.62				
VH/CP							
Batch 1	2.41	2.41	2.47	0.134	0.850	0.270	0.933
Batch 2	2.50	2.64	2.65				
Jejunum							
Villous height							
Batch 1	225.56	218.08	200.23	13.131	0.614	0.024	0.226
Batch 2	224.02	266.96	258.50				
Crypt depth							
Batch 1	69.37	70.88	73.32	3.405	0.682	0.003	0.963
Batch 2	80.80	84.68	84.92				
VH/CP							
Batch 1	3.24	3.09	2.73	0.161	0.663	0.996	0.176
Batch 2	2.79	3.15	3.12				
Ileum							
Villous height							
Batch 1	211.49	211.29	210.53	7.214	0.099	0.005	0.082
Batch 2	214.54 ^b	234.51 ^{ab}	260.8 ^a				
Crypt depth							
Batch 1	67.97	71.29	68.25	3.419	0.258	0.001	0.656
Batch 2	79.75	90.72	79.9				
VH/CP							
Batch 1	3.11	2.97	3.09	0.105	0.050	0.196	0.119
Batch 2	2.72 ^b	2.69 ^b	3.29 ^a				

^{a-b}Within a row, means without a common superscript differ ($P < 0.05$).

¹Basal = control diet without antibiotics and berberine; Antibiotic = basal diet plus antibiotics and ZnO; Berberine = basal diet plus berberine at 0.05%.

Table 4.7. Effects of treatment on gut permeability

Item	Diet ¹			SEM	P-value		
	Basal	Antibiotic	Berberine		Diet	Batch	Diet*Batch
TER							
Jejunum							
Batch 1	57.44 ^{ab}	77.59 ^a	41.76 ^b	7.248	0.037	0.9596	0.666
Batch 2	51.48	72.01	52.02				
Ileum							
Batch 1	82.83 ^b	125.58 ^a	77.04 ^b	7.865	0.037	0.179	0.187
Batch 2	81.53	88.08	78.29				
Isc							
Jejunum							
Batch 1	21.94 ^b	19.35 ^b	38.25 ^a	3.616	0.211	0.462	0.039
Batch 2	36.43	26.27	26.28				
Ileum							
Batch 1	16.28 ^b	13.96 ^b	34.56 ^a	2.714	0.011	0.551	0.045
Batch 2	21.17	16.87	21.04				
Dextran							
Jejunum							
Batch 1	2.01	0.85	1.47	0.401	0.521	0.468	0.630
Batch 2	1.05	0.96	1.30				
Ileum							
Batch 1	1.84	0.89	0.86	0.225	0.117	0.484	0.273
Batch 2	1.13	0.69	1.23				
Lactulose/Mannitol							
	0.224	0.465	0.295	0.0827	0.141	0.553	0.527

^{a-b}Within a row, means without a common superscript differ ($P < 0.05$).

¹Basal = control diet without antibiotics and berberine; Antibiotic = basal diet plus antibiotics and ZnO; Berberine = basal diet plus berberine at 0.05%.

CHAPTER FIVE

GENERAL DISCUSSION

The overall objective of this thesis research was to identify a phytochemical that can be as a suitable alternative to antibiotics in diets for weaned pigs. Since the concerns of human risks involved with inclusion of antibiotics in feeds for food animals, there has been a greater need for identification of effective alternatives to antibiotics in diets for food animals.

In the first study, antimicrobial activities of 3 phytochemicals (berberine, quercetin, and AITC) against in vitro growth of *E. coli* were determined with the goal of identifying the best phytochemical for animal studies. As previously mentioned, suitable alternatives to antibiotics should have antimicrobial properties or antioxidant properties, or both. Berberine an alkaloid has exhibited both antimicrobial (Freile et al., 2003) and antioxidant activities (Campisi et al., 2014). Quercetin is a flavonoid, which has various properties including antihypertensive and antiarrhythmic activities, anti-inflammatory and antiallergic properties; and other activities (Formica and Regelson, 1995). The AITC, which is a compound that is derived from glucosinolates, has been shown to have strong antimicrobial (Lin et al., 2000) and antioxidant (Velioglu et al., 1998) activities. Thus, these 3 phytochemicals were hypothesized to be suitable alternatives to antibiotics. Results from the in vitro study indicated that berberine exhibited stronger antimicrobial activity against *E. coli* than quercetin or AITC. The lowest concentration of berberine at which it reduced in vitro growth of *E. coli* was 3.125%. Thus, berberine was selected for weaned pig studies, and it was included in diets for the weaned at 3.0%. However, inclusion of berberine in the diets at 3.0% decreased ADFI, ADG and G:F, and

negatively affected gut health of weaned pigs. The negative effects of berberine on gut health was attributed to reduced voluntary feed intake due to bitterness of berberine.

Berberine at lower dosages (less than 1% of daily food intake) alleviated coccidiosis in chickens (Malik et al., 2014). Thus, it was hypothesized that dietary berberine at less than 1% would be effective in improving gut health of weaned pigs without negative effects on voluntary feed intake, leading to improved growth performance of weaned pigs. A third study was conducted to determine the effects of including berberine in diets for weaned pigs at 0.05% on growth performance and gut health of weaned pigs. Berberine at 0.05% increased in ADG and ISc of weaned pigs, and did not affect ADFI, implying that the negative effects of dietary berberine on gut health in the second study was indeed partly due to reduced voluntary feed intake as result of bitterness of berberine. Because the lowest level of berberine at which it reduced growth of *E. coli* in vitro was 3%, whereas the berberine at 0.05% improved ADG and ISc of weaned pigs, it is apparent that the reduction in growth of *E. coli* is not the major the mechanism by which berberine can improve growth performance and gut health weaned pigs. It appears other mechanisms (such as reduction in oxidative stress) through which berberine can improve can improve growth performance of weaned pigs are more important than reduction in growth of pathogenic microorganisms.

In most studies, the in vivo data match the in vitro ones. Thus, it had been hypothesized that the results from in vitro study will be applicable to in vivo studies. However, this was not the case due to lower palatability of berberine. Thus, in vitro antimicrobial activity assay may be used to evaluate feed additives that are palatable.

The exact mode of action of berberine is not clear from the three studies that were conducted. A decrease in intestinal TER and increase in Isc that were observed in the third study due to dietary berberine has been linked to food deprivation (Carvey et al., 1994). However, intestinal Isc is positively correlated with small intestinal nutrient absorptive capacity, which could be the mechanism by which berberine improved growth performance. However, Robbins et al. (2013) observed a higher mean ileal TER ($62 \Omega/\text{cm}^2$) in salmonella-challenged 5-week old pigs fed diet with benzo(c)phenanthridine alkaloid at 1.5 g/1000 kg than in those fed diet with chlortetracycline (59.4 g/1000 kg). Thus, the alkaloid could have improved TER through reduction in gut permeability toxins. There is need to conduct research to establish mechanisms through which berberine improves gut health and growth performance of weaned pigs.

GENERAL CONCLUSION

Based on results from the antimicrobial activity assay that was conducted, berberine (compared with quercetin and AITC) was the most effective phytochemical at inhibiting the growth of *E. coli*. Berberine had negative effects on ADG and gut function when it was included in the diet at 3.0%; but, these results were confounded by reduced voluntary feed intake by pigs due to dietary berberine inclusion. However, dietary berberine at 0.05% improved ADG, G:F and Isc in jejunum and ileum of pigs without significant effect on ADFI. Thus, it appears that dietary berberine at low dietary concentration (0.05%) has less effect voluntary feed intake of weaned pigs, and can improve growth performance of weaned pigs through increasing small intestinal nutrient absorptive capacity.

FURTHER RESEARCH

Further research is suggested to:

1. Effect of dietary berberine on gut health and growth performance of disease challenged weaned pigs
2. Effect of dietary berberine on gut health and growth performance of other food animals
3. Establishment of mechanisms by dietary berberine improves gut health and growth performance of food animals.
4. Determine the optimal inclusion of berberine at which ADFI isn't negatively affected.
5. Determine the effect berberine and other phytochemicals have on tight junction proteins, because this is an effective tool at determining gut permeability as well.
6. Determine if adding alternatives to antibiotics directly to the Ussing Chambers is an effective tool at determining the effects on gut health prior to inclusion into the diet, because other research has used this as a technique for potential antibiotic alternatives.

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