2007

Colicin E1 addition to the swine diet prevents post weaning diarrhea

Sara Anne Cutler
Iowa State University

Follow this and additional works at: https://lib.dr.iastate.edu/rtd

Part of the Agriculture Commons, and the Animal Sciences Commons

Recommended Citation
Cutler, Sara Anne, "Colicin E1 addition to the swine diet prevents post weaning diarrhea" (2007). Retrospective Theses and Dissertations. 15551. https://lib.dr.iastate.edu/rtd/15551

This Dissertation is brought to you for free and open access by the Iowa State University Capstones, Theses and Dissertations at Iowa State University Digital Repository. It has been accepted for inclusion in Retrospective Theses and Dissertations by an authorized administrator of Iowa State University Digital Repository. For more information, please contact digirep@iastate.edu.
Colicin E1 addition to the swine diet prevents post weaning diarrhea

by

Sara Anne Cutler

A dissertation submitted to the graduate faculty in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Animal Science

Program of Study Committee:
Chad H. Stahl, Major Professor
Ronald W. Griffith
Lloyd L. Anderson
Nancy A. Cornick
Micheal E. Spurlock

Iowa State University
Ames, Iowa
2007

Copyright © Sara Anne Cutler, 2007. All rights reserved.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF FIGURES</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>v</td>
</tr>
<tr>
<td>GENERAL INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>DISSERTATION ORGANIZATION</td>
<td>2</td>
</tr>
<tr>
<td>LITERATURE REVIEW</td>
<td></td>
</tr>
<tr>
<td>Enterotoxigenic <em>E. coli</em></td>
<td>3</td>
</tr>
<tr>
<td>Colicins</td>
<td>14</td>
</tr>
<tr>
<td>Recombinant Yeast Protein Production</td>
<td>20</td>
</tr>
<tr>
<td>Conclusion</td>
<td>21</td>
</tr>
<tr>
<td>CHAPTER 1. DIETARY INCLUSION OF COLICIN E1</td>
<td></td>
</tr>
<tr>
<td>IS EFFECTIVE IN PREVENTING <em>ESCHERICHIA COLI</em> F18 POST-WEANING DIARRHEA IN PIGS</td>
<td></td>
</tr>
<tr>
<td>Abstract</td>
<td>22</td>
</tr>
<tr>
<td>Introduction</td>
<td>24</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>26</td>
</tr>
<tr>
<td>Results</td>
<td>30</td>
</tr>
<tr>
<td>Discussion</td>
<td>33</td>
</tr>
<tr>
<td>CHAPTER 2. DIETARY INCLUSION OF COLICIN E1</td>
<td></td>
</tr>
<tr>
<td>IS EFFECTIVE IN PREVENTING <em>ESCHERICHIA COLI</em> F18 POST-WEANING DIARRHEA IN PIGS - A LONGER DURATION TRIAL</td>
<td></td>
</tr>
<tr>
<td>Abstract</td>
<td>45</td>
</tr>
<tr>
<td>Introduction</td>
<td>46</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>47</td>
</tr>
<tr>
<td>Results</td>
<td>51</td>
</tr>
<tr>
<td>Discussion</td>
<td>52</td>
</tr>
<tr>
<td>CHAPTER 3. EXPRESSION OF AN ACTIVE COLICIN E1 IN <em>PICHIA PASTORIS</em></td>
<td></td>
</tr>
<tr>
<td>Abstract</td>
<td>62</td>
</tr>
<tr>
<td>Introduction</td>
<td>62</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>63</td>
</tr>
<tr>
<td>Results</td>
<td>66</td>
</tr>
<tr>
<td>Discussion</td>
<td>66</td>
</tr>
<tr>
<td>GENERAL CONCLUSION</td>
<td>71</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS 74
WORKS CITED 75
LIST OF TABLES

CHAPTER 1
Primer Sets for Real Time PCR 38
Growth Performance 39
Bacterial Recovery- fecal samples 40
Bacterial Recovery- ileum 41

CHAPTER 2
Primer Sets for Real Time PCR 56
Growth Performance 57
LIST OF FIGURES

CHAPTER 1
  Percentage of Pigs with Diarrhea  42
  Relative Gene Expression of TNFβ  43
  Relative Gene Expression of IL-1β  44

CHAPTER 2
  Percentage of Pigs with Diarrhea  58
  Relative Gene Expression of COX-2  59
  Relative Gene Expression of PGHS  60
  Relative Gene Expression of TNFα  61

CHAPTER 3
  Growth of Recombinant Pichia and Gene  69
  Expression of Colicin E1
  Western Blot of Recombinant Pichia  70
GENERAL INTRODUCTION

Post weaning diarrhea (PWD) caused by enterotoxigenic *Escherichia coli* (ETEC) is a threat to the US swine industry due to losses in production associated with the disease. Early weaned piglets are especially susceptible to disease threats because of an immature immune system, a change in intestinal environment due to the diet transition from the sow's milk to a grain based diet, and exposure to new pathogens in a group housed environment. Colicins represent an alternative to the antibiotics now given in a majority of post-weaning pig diets to combat infections, including PWD. Colicins are proteins produced by *E. coli* that kill *E. coli* and other related bacterial species. We used a highly purified Colicin E1 at 16.5 mg/kg in a five-day study or 20 mg/kg in a two week study to successfully prevent PWD in piglets. In addition to the purified Colicin E1 from the native producing *E. coli* strain we have also expressed the Colicin E1 in *Pichia pastoris* yeast. This technique allows the Colicin E1 to be used as a directly fed antimicrobial that would require no additional purification costs associated with the native strain.
DISSEPTION ORGANIZATION

This dissertation is organized into three chapters. The first chapter is the proof of concept trial which tested two different levels of Colicin E1 inclusion in the diet versus two different ETEC challenge strains. The second chapter is a longer duration trial using a slightly higher level of Colicin E1 in the diet against a seeder pig or natural infection model. In this second trial the Colicin E1 additive was provided in the diet for two weeks, then taken out of the diet for the second two week period to investigate the impact of the diet change on growth and resistance to infection. The third chapter is the expression and characterization of Colicin E1 in recombinant *Pichia pastoris* yeast.

The corresponding author on all research publications is Dr. Chad H. Stahl and Sara Cutler is the primary author. Dr. Steven Lonergan performed all of the protein purification of the Colicin E1. Dr. Anna Butters-Johnson provided animal behavior analysis. Dr. Nancy Cornick provided the ETEC challenge strains as well as input on challenge models for post-weaning diarrhea.

Chapter 1 has been submitted for publication to Antimicrobial Agents and Chemotherapy and is under revision. Chapter 2 is being prepared for submission to the Journal of Animal Science and Chapter 3 is being prepared for submission to Biotechnology Letters.
**LITERATURE REVIEW**

**Introduction**

Post-weaning diarrhea (PWD) in pigs is a multifactorial disease resulting from changes in housing environment after weaning, viral exposure, immune system immaturity, and a transition from a liquid to a solid diet that leaves a vulnerable intestinal environment. Piglets that are weaned at two weeks of age are immunocompromised due to a loss of antibodies in the sow's milk. As a result of this disease threat, prophylactic antibiotics are provided in the post-weaning diet in an effort to combat respiratory and enteric infections. The intestinal environment of the recently weaned pig is particularly susceptible to infection because of new disease threats in a new housing environment and a switch from a milk based diet to one based on grain and protein. Enterotoxigenic *Escherichia coli* (ETEC) are a major cause of opportunistic enteric infections in pigs, in part thought to be due to this diet transition, but also because of rotavirus exposure that further serves to disrupt the intestinal tract of the pig. ETEC-caused PWD in pigs causes production losses due to higher feed to gain ratios and morbidity during the infection, by leaving the animals vulnerable to other pathogens, and by creating a non-uniform set of animals.

**Enterotoxigenic *Escherichia coli***

In swine production ETEC-caused PWD is primarily a disease of just-farrowed neonatal piglets or piglets that have been recently weaned. Enterotoxigenic *E. coli* have multiple traits that are responsible for their virulence and variation in these virulence factors is seen between different strains. The first trait necessary for ETEC to cause PWD is its
ability to attach to the intestinal lining. Bacterial fimbriae mediate the attachment of the ETEC to the intestinal cell. There are two main fimbrial types of *E. coli* associated with PWD in piglets. Frydendahl, et al., reported in 2002 that 92.7% of PWD cases were caused by F4 or F18 and more recently, Zhang, et al. (2007) reported that of the 58% of the suspected ETEC isolates, from cases of PWD submitted for diagnostic evaluation, were PCR positive for fimbriae and of those bacterial isolates 98.9% were F4 or F18. There are three different genetically distinct antigenic variants of *E. coli* F4 fimbriae - F4ab, F4ac, and F4ad and three intestinal receptor phenotypes represented in the swine population (Jin and Zhao, 2000). The intestinal receptors are either glycolipid (ad) or glycoprotein (ab, ac) in structure and they are located on the mucus or the brush border in the intestine, respectively (Jin and Zhou, 2000). Receptors F4ab and F4ac are predominantly found in the jejunum, while F4ad is located in the ileum (Jin and Zhao, 2000).

*Escherichia coli* encode two different F18 subtypes, F18ab and F18ac. Less is known about the intestinal receptor for F18 in swine. However, a polymorphism in the *FUT1* gene has been associated with genetic resistance to F18+ *E. coli* (Meirjink et al., 2000). The phenotypic expression of the F18 receptor has recently been associated with the H-2 histo-blood group antigens expressed on the epithelial cells lining the intestinal tract (Coddens, et al., 2007).

Though the different fimbriae bind only to their corresponding receptor and the brush border receptors (proteins) do not change with the pig’s age, receptors found in the glycolipid or mucus layer of the intestine do change with age and diet (Jin and Zhou, 2000). This observation could further explain why newborn or weaned piglets are more
susceptible to certain types of *E. coli* and why diet potentially has a major influence on intestinal colonization by ETEC.

**ETEC Pathogenesis**

After using their fimbrial adhesins to bind to intestinal receptors, ETEC bacteria, unlike some other intestinal pathogens, do not invade the host cells. Rather, the bacteria remain attached to the enterocyte and when they reach high enough numbers (estimated at \(1 \times 10^6\) CFU), the bacteria produce toxins that utilize the enterocyte’s cellular machinery to generate their pathogenic effects (reviewed, Turner 2006). Enterotoxigenic *E. coli* may produce one or more enterotoxins and the genes encoding these toxins are either located on a plasmid (STa and STb) or on the bacteria’s chromosomes (LT). These toxins are characterized as either heat stable (ST) or heat labile (LT) based on their thermostability. The ST toxins are not inactivated after heat treatment at 100º C for 5 minutes whereas the LT toxin is completely inactivated by treatment at 60º C for 15 min (Fairbrother et al., 2005). The heat stable toxins (ST) all result in fluid loss from the enterocyte although their modes of actions once in the enterocyte vary (Turner, 2006). STa activates guanylate cyclase (GC) in the cell causing an increase of cyclic guanosine monophosphate (cGMP), which results in a disruption of Na and Cl absorption and fluid loss from the enterocyte (Turner, 2006). STb-associated fluid loss in the intestine is thought to be result of prostaglandin secretion (Fairbrother et al., 2005; Gyles, 1992). In addition, another toxin that has structural similarity to STa, EAST1, has been recently associated with ETEC diarrhea in pigs (Fairbrother, et al., 2005; Turner et al., 2006). EAST1 also binds to the GC-C enterocyte receptor and upregulates GC for the production of diarrhea. STa and EAST differ in amino acid sequence and EAST1 has
been associated with human pathogenic *E. coli* disease as well whereas the other ST toxins are primarily associated with animal infections (Fairbrother, et al., 2005; Turner et al., 2006).

Heat labile toxin is an A-B (or AB5) subunit toxin, consisting of an active or A domain and a B or binding domain (Kaper, et al., 2004). The A domain of LT is a type II effector that enters the host cell, and through Gs coupling, activates adenylate cyclase which results in an increase in cyclic adenylate monophosphate (CAMP) and excessive Na, bicarbonate, and Cl secretion, leading to dehydration and fluid loss in the animal (Gyles, 2007; Spangler, 1992). Shiga toxin (STx2e) is associated with edema disease in pigs and also found in association with PWD infections (Fairbrother, et al., 2005). The STx2e toxin (verotoxin) is also an AB subunit toxin but instead of Gs stimulation, STx inhibits protein synthesis in the host, inducing apoptosis (Kaper, et al., 2004). In addition to these toxins, alpha hemolysin has been associated with a majority (82%) of PWD strains when initially isolated (Frydendahl, et al., 2002). The alpha hemolysin, a pore forming cytolysin, can cause damage to host cells and is carried on a plasmid in swine pathogenic ETEC. The alpha hemolysin has been associated with an increase in virulence in PWD causing bacteria, though reports differ on its importance in pathogenesis (Chen, et. al, 2004; Smith and Lingood, 1971; Moxley et al., 1998). The lipopolysaccharide of the ETEC strain recovered served to classify ETEC strains prior to virotyping. Typical O (lipopolysaccharide) antigens in post-weaning diarrhea include O138, O139, O141, and O149), though the specific types vary by region (Fairbrother, et al., 2005).

**Experimental Challenge Models for ETEC**
One of the major challenges of investigating PWD is the development of a reliable, reproducible challenge model that is representative of the disease seen in the field. The use of different challenge strains with varying fimbrial antigens and toxins as well as varying resistance to colonization among the study animals can result in enormous variation among experimentally induced (ETEC caused) PWD studies. Because of these complex factors involved in experimentally replicating PWD, comparing results from one study to the next, let alone between research groups, is a major hindrance to ETEC research. This has impaired the development of intervention strategies, other than changes in management practices (Madec, et al., 2000). Optimizing the efficacy and repeatability of ETEC challenge studies, would allow for dramatic advances in the development of novel interventions for preventing PWD.

Very different challenge protocols have been used to examine PWD. These protocols vary in bacterial load, inoculation frequency, and co-infection or environmental sensitizers (Sarmiento, et al., 1988; Wathes, et al., 1989). The first critical step in a successful infection model is ensuring that the challenge strain is delivered into the intestinal tract in a viable state. Some studies have attempted to increase the amount of their oral challenge strain that survives into the intestine by decreasing the acidity of the stomach with the use of bicarbonate or calcium carbonate (Sarmiento et al., 1988; Madec, et al., 2000; Girard, et al., 2005). Other methods to achieve this same goal include the use of capsules (Tsukahara, et al., 2007) or gastric tubes (Sarmiento, et al., 1988; Girard, et al., 2005). Tsukahara et al. (2007) successfully induced ETEC-caused edema disease by orally administering one capsule containing $4 \times 10^8$ CFU of $0139(F18,STx2e)$ Shiga toxin producing $E. coli$ daily for three days. Sarmiento used $1 \times 10^{10}$CFU of O157:K88ac;
(H43, LT, STb) in a gastric tube with 1.2% sodium bicarbonate and was able to generate clinical signs of disease. Girard, et al., used an intraoesophageal 1.2% calcium carbonate infusion followed by 1x10^{10} CFU of ECL 1001 enteropathogenic *E. coli* (EPEC), and was able to identify histopathologic changes in the intestine but no clinical signs associated with challenge.

Inadvertently or intentionally exposing animals to transmissible gastroenteritis (TGE) or Rota virus predisposes the challenged animals to ETEC infection (Melin and Wallgren, 2002; Benfield, et al., 1988; Cox, et al., 1988; Sarmiento et al., 1988; Geenen et al., 2005). While a viral challenge can lower the resistance of the animal to an ETEC infection (this is a likely scenario in the field), doing so adds a confounding effect in studies examining interventions for ETEC caused PWD.

Other modes of bacterial challenge, such as inclusion of the bacteria in the feed or exposure to a contaminated environment have also been used to induce PWD (Frydendahl, et al., 2003; Melin and Wallgren, 2002). Frydendahl et al. (2003) used a daily dose of 1x10^{8} CFU O138(F18) in the feed and was to achieve diarrhea in 71.4% of the challenged pigs. Repeated inoculation more closely mimics the natural route of fecal-oral infection than does gastric tubing or delivering the bacteria in a capsule. Perhaps more realistic than these methods is exposure to a contaminated environment. When investigating different dietary interventions to prevent PWD, Melin and Wallgren (2002) transported just-weaned piglets in a trailer coated with 2x10^{6} CFU/square meter of ETEC strains 0147:K89,STb and three days after weaning exposed the piglets a second time using the same method same way with different challenge strains [0141:K85(STb,VT2) 0149:K91(K88,STa, STb, LT)]. This challenge method resulted in diarrhea in 34/36
exposed animals, regardless of whether they tested positive for the F4 receptor via a post mortem adhesion assay (Melin and Wallgren, 2002).

It has been speculated that the diet the piglets are fed immediately after weaning may have an effect on the occurrence of PWD as well. A post-weaning starter diet that contains milk products is favored because it eases the transition to grain-based diet (Fairbrother, 2005). However, a post weaning starter diet that contains soybean meal is thought to exacerbate the potential for PWD. A transient inflammatory response to the soy protein adds to the difficult transition from milk to solid feed (Li, et al., 1991; Dreau, et al., 1994).

Combining both nutritional manipulations and ETEC challenge approaches, Madec, et al. (2001) used an inoculum containing four different F4 (LT, STa, STb) ETEC at levels ranging from $10^8$ to $10^{12}$ CFU either once, daily, or at four day intervals. The route of infection (oral or gastric tube), withholding of feed or water prior to inoculation, the use of bicarbonate to reduce gastric acidity, or dietary change to a high protein diet did not seem to influence the development of diarrhea in these pigs. The challenge dose did, however, as did the presence of the F4 receptor (presumptively based on post mortem agglutination testing). Ten out of the sixteen animals challenged at the highest level, $1x10^{12}$CFU, on four challenge days, died. The pigs used in this study were specific pathogen free but only 56% tested positive on the agglutination test for the F4 receptor. In the animals that were the F4 ETEC adhesive type, 56.7% developed diarrhea while 34% of the non-adhesive type showed clinical signs.

With any multifactorial disease, an experimental model that combines several predisposing conditions will increase the chances of reproducing the disease. The
absence of viral infection, the presence of the intestinal receptor for the challenge strain used, and using a post weaning diet that provides adequate nutrition while increasing the amount of crude protein may be the most practical approach to reproducing post-weaning diarrhea in pigs experimentally. The determining factor in the development of PWD in the pig when exposed to sufficient levels of ETEC to cause disease may be genetic background and presence of the fimbrial receptor in the intestine.

Genetic Susceptibility

Despite all of the other factors which can affect the success of an ETEC challenge study in young pigs, the most influential determinant in the development of F18 or F4 caused PWD is the presence of receptors on the ileal or jejunal brush borders or mucosa of the small intestine. Without the receptors for *E. coli* fimbrial adhesion, it is unlikely that colonization will occur. These receptors are highly specific for distinct fimbria. The genetic locus for the F18 receptor is separate from that of the F4 receptor, with the F18 receptor having been mapped to chromosome 6 and the F4 receptor mapped to chromosome 13 (Meijerink, et al., 2000). Both are inherited in Mendelian fashion with the lack of the F4 receptor being on the dominant allele and the lack of the F18 receptor being recessive (Fairbrother, et al., 2005).

The F4 receptor has been the target of selective breeding programs and has been reduced in some swine populations, while the F18 receptor has been mapped to a region of the porcine chromosome near the region of the stress gene and is not of a selective advantage to target this locus (Nagy, et al., 2005). In fact, increases in F18 associated PWD have been noted after the reduction of the stress gene in some swine populations (Nagy, et al., 2005). The DNA polymorphism for susceptibility to F18 colonization on the intestinal
brush border is in the α(1,2) fucosyltransferase gene (Meijerink, et al., 2000) and is detectable by a PCR-RFLP as described by Frydendahl et al. (2003). By selecting test animals that had a polymorphism at this site for susceptibility, the percentage of pigs that developed diarrhea was increased from 5.9% to 71.4%. Although some genetically resistant pigs did shed considerable numbers of the challenge strain and some of the genetically susceptible pigs did not develop diarrhea, this is theoretically due to the transient nature of the expression of the receptor itself. The pigs in this study were challenged for 10 days with $1 \times 10^8$ CFU with the peak shedding occurring around day five.

**Prevention and Control of Post-Weaning Diarrhea**

Prophylactic antibiotics are now given at most large swine facilities (92%) in part to control PWD (USDA NAHMS, 2001). The use of antibiotics in swine feed for growth promotion and/or disease prevention is common practice in the United States. Over 87% of market pigs produced in the U.S. received prophylactic antibiotic treatments in their feed or water (USDA NAHMS, 2001). Despite this practice, ETEC-associated PWD infections still occur. Forty-three percent of large swine producers reported an occurrence of PWD in 2001 (USDA NAHMS, 2001). Bacteria are isolated from the air (Chapin, et al., 2005), water (Anderson and Sobsey, 2006) from pigs (Maynard, et al., 2003; Mathew et al., 1999), and from people who work with the pigs (Campbell et al., 2005) all indicate that a spread of antimicrobial resistant bacteria. In addition, *E. coli* resistant to multiple antimicrobial compounds is on the rise over the past two decades (Maynard, et al., 2003). Alternatives to these antibiotics for use in the animal feed is important as removal of these compounds will add to production costs. The use of
antibiotics in the grower-finisher diet is estimated to save the US producer $3/pig in net return because of increases in efficiency (Cromwell, 2002). If antibiotics are banned from the feed in the US it has been estimated that production costs will rise by $4.50/pig (Hayes and Jensen, 2003).

**Current Alternative Therapies**

Alternatives to antibiotic supplementation in the swine diet include the use of high levels of zinc oxide, pre- and probiotic supplementation, and the inclusion of spray dried plasma. Zinc oxide has been used successfully to reduce the incidence and severity of PWD. A dose of 2.88 mg/kg reduced the duration of infection to four days vs seven for the control animals (n=15) in response to an oral 1x10^{10} CFU/ml O149:K91:F4 challenge (Owusu-Asiedu, 2003). However, this benefit was not seen with a ZnO concentration of 2.50 mg/kg and a more aggressive challenge consisting of three different challenge strains on two occasions [(2x10^{6} CFU/square meter of O147:K89(STb), 0141:K85(STb,VT2), 0149:K91,K88 (STa, STb, LT)] (Melin and Wallgren, 2002). Zinc oxide is known to aid in intestinal development though alteration in bacterial composition and may act as a growth promoter, though results seem to be conflicting for the level needed for the effect (Hojberg, et al., 2005; Hedemann, et al., 2006; Melin and Wallgren, 2002; Davis, et al., 2004). It appears that higher levels of Zinc oxide are more protective from (2500 ppm to 3000 ppm) (Davis, et al., 2004; Smith, et al., 1997). Combining copper sulfate at 200 ppm plus 3000 ppm zinc oxide may provide a synergistic effect (Smith, et al., 1997). This practice of heavy metal supplementation in the swine diet raises environmental concerns however.
Spray dried plasma in the post-weaning diet has been associated with increases in feed intake and a reduction in the severity of PWD as well as bacterial shedding in the feces (Owusu-Asiedu, 2003). The inclusion of spray dried plasma in a weaning pig diet increased intestinal crypt depth and average daily gain in young pigs in response to ETEC challenge (Owusu-Asiedu, 2003; Bosi, 2004). Inclusion of spray dried plasma as 8% of the diet (the limit for economic feasibility), did not improve growth performance or disease resistance in response to an O139:K82 ETEC challenge (Van Dijk, et al., 2002). Pre- and probiotic supplements has been used to promote intestinal health in the poultry industry for some time. Unlike the use of these supplements in the poultry industry, the intestinal tract of the pig is not a "clean slate" like hatchling birds, making it a more difficult task to alter the bacterial flora. At weaning, the pig undergoes a shift in microbiota, and preservation of the lactic acid bacteria present pre-weaning is thought to promote a healthier intestinal environment. This may be accomplished by feeding milk products such as whey protein or the use of lactic acid bacteria as a probiotic. A heat killed, dried *Enterococcus fecalis* (EC-12) preparation at 0.05% of the diet was used as a prebiotic dietary addition and was protective against STEC challenge (Tsuchihara, et al., 2007). At least nine other Enterococcus strains have been shown to have antagonistic effects against K88 ETEC (Garcia-Galaz, et al., 2004). An *Enterococcus fecalis* (EK-13) strain was fed as a probiotic to piglets and increases in lactic acid and propionic acid were noted but no discernable increases in performance were observed (Strompfova, et al., 2006). Liquid feed, allowed to ferment to produce lactic acid, was fed to piglets resulting in increased growth and levels of lactic acid bacteria in the intestinal tract, which is
thought to promote a healthy intestinal environment while excluding potential pathogens (Canibe and Jensen, 2003; Hojberg, et al., 2003).

With most alternative therapies, there are drawbacks such as practicality, questionable efficacy, or concern for the environment. At this time, antibiotic supplementation still represents the best option for the control of PWD and growth promotion in the swine herd. In the search for another solution however, we investigated the addition of Colicin E1 to the swine diet.

**Colicins**

In perhaps the most ancient form of chemical warfare, bacteria produce chemicals to eliminate competing species. Gram-negative bacteria can release a variety of chemicals, including commonly used antibiotics to protect their ecological niche. Colicins are peptides produced by *E. coli* and closely related species that kill other strains of *E. coli*. Colicins may take the form of DNAses, RNAses, or pore formers. Structurally, all colicins consist of three domains: (from the N terminus) a receptor recognition domain, a translocation domain, and an activity domain.

**Mechanism of Action**

Colicins use two basic translocation mechanisms to gain entry into the target cell. Group A colicins (A, E1-E9, K, L, N) use the Tol proteins for uptake into the host cell whereas the Group B colicins (B,D,La,lb, M,V) use the Ton system (Riley and Gordon, 1999). The Ton and Tol translocation mechanisms are associated with nutrient uptake, such as the B vitamin receptor (BtuB) or iron uptake in the target cell (Lazdunski, et al., 1998). Colicins with divergent mechanisms of action such as the pore-formers, endonucleases, or protein or cell wall inhibitors all utilize similar uptake systems (Lazdunski, et al.,
but recognize different receptors and indeed among colicins polymorphisms in the plasmid do exist (Riley, et al., 1994). Colicin E1 and N are both group A pore forming colicins that use the Tol translocation mechanism (Lazdunski, et al., 1998; Cao and Klebba, 2002). Colicin E1 utilizes the BtuB (vitamin $\text{B}_{12}$) receptor for entry into the host cell, while Colicin N uses the OmpF (porin) receptor for entry (Cao and Klebba, 2002). Colicins may use one host cell structure for binding and another for entry and killing as well. Colicin E1 does not require a porin to enter the host cell whereas Colicin N and all other group A colicins do. All receptor binding and entry pathways have one thing in common; however, they all include pathways that are essential to growth or maintenance of the bacterial cell (Cao and Klebba, 2002).

Pore-forming colicins are a useful tool for chemists interested in bacterial membrane transport and images of colicin structure and function have recently become available. Despite their varying binding receptors and translocation techniques, there is one result for the target bacteria. The ionic gradient of the bacterial cell is disrupted by the pore and the cell is lysed.

**Production of Colicins**

*E. coli* produce colicins under conditions of nutrient depletion, overcrowding, osmotic shock, UV light, or DNA-damaging antibiotics (Riley and Gordon, 1999, Spangler, et al., 1985). When faced with these conditions, the bacteria react to the stress by undergoing the SOS response (Konisky, et al., 1982). The LexA protein is the primary transcriptional repressor of colicin synthesis and the SOS response. Under colicinogenic conditions, LexA degradation stimulates cAMP and transcription of the colicin protein (Kuhar and Zgur-Bertok, 1999). Colicin E1 possesses two SOS boxes in its promoter
region (Kuhar and Zgur-Bertok, 1999), which may allow for different environmental conditions to elicit different levels of induction. Kuhar (1999) compared strains containing either Colicin K or E1 plasmids for their colicin production at different growth stages, nutrient densities, and growth phases. While the Colicin E1 gene (*cea*) was induced by an anaerobic environment, Colicin K (*cka*) was not, indicating slightly different genetic control (Kuhar and Zgur-Bertok, 1999). The rate of release of Colicin E1 was also shown to be greater than that of Colicin K in this study (Kuhar and Zgur-Bertok, 1999).

The colicin producing cell is damaged by the production of the colicin and thus has performed a seemingly altruistic act for the preservation of its relatives. If the benefit of colicin production confers an ecological advantage, then it is of benefit for the cell to maintain the plasmid (Riley and Gordon, 1999). When induced, the pore-formers release the colicin through their cell membrane and cause an increase in permeability of their cell membrane, resulting in cell damage (Cao and Klebba, 2002). The colicin producer is not damaged by the action of the colicin itself but rather because of the increase in cellular permeability needed to release the protein into the environment (Cao and Klebba, 2002). Other bacterial cells that possess the plasmid to make the colicin, also have the immunity protein so they survive in the event of colicin secretion by another cell (Alonso, et al., 2000). The immunity protein provides the best defense against secreted colicins and is part of the colicin plasmid which is constitutively produced by the cell in the bacterial lipid bilayer (Espesset, et al., 1994). The immunity protein protects the cell from endogenous colicin by binding to the colicin and preventing the C terminus from entry into the cell membrane; thereby preventing voltage gating and subsequent loss of ionic...
gradient potential in the cell (Lindegerg and Cramer, 2000). Colicin immunity proteins range in size from 85AA to 178AA and are highly specific for an individual colicin. They work by altering the receptor binding motif slightly to stop the colicin encoded on the plasmid from attacking "self" cells (Alonso, et al., 2000). Other bacteria may be resistant or tolerant to colicin invasion as well (Alonso, et al., 2000). Bacteria showing resistance to colicin may have mutated their receptors just enough to evade colicin binding (Alonso, et al., 2000). Colicins show remarkable specificity for their targets, as even a one amino acid mutation in their structure can prevent colicin binding (Alonso, et al., 2000; Cao and Klebba, 2002). Changes in the translocation structure can result in colicin tolerance. Colicin tolerance exists when mutations Tol and Ton transport systems are altered in such a way that the bacteria use a slightly different transportation system to actively bring in nutrients. This tolerance is said to be overcome by simply adding more colicin to the mix, however (Alonso, et al., 2000). The active transport mechanisms needed to bring in nutrients are most likely essential to the target cells existence.

**Colicin Ecology**

How many bacteria have the propensity to produce colicins and what type of colicins are most prevalent is still a matter of debate. Colicin-producing bacteria are often estimated at 30-40% of those cultured (reviewed, Riley and Gordon, 1999) in the natural population in the colon, though ranges are from 9% to 82% (reviewed, Smarda and Obdrzalek, 2001). This amount of variance, and the variety of methods used for the detection of colicinogenic strains, makes it difficult to know how prevalent colicin producing bacteria are in the environment. In a 2001 study, *E. coli* strains were isolated from both healthy persons and those who were suffering from intestinal distress (Smarda and Obdzalek,
In hospital patients infected with *Salmonella*, or afflicted with ulcerative colitis or tumors, there was no apparent trend of colicinogenic strains recovered, but in the case of hemolytic urinary tract infections there were significantly more colicin producers recovered (Smarda and Obdzalek, 2001), though which type were not identified. Among other urinary tract isolates, 44% were colicin producers with Colicin V being the most commonly isolated (Riley and Wertz, 2002). In 266 human fecal isolates, 42% were colicinogenic in a 2006 study (Gordon and O’Brien, 2006). Of those, Colicins Ia and E1 the most prevalent at 9%, followed by M, E7, K, E2, and B all at less than 5%. Of the 102 strains, induced by mitomycin C to make colicin, 41% made two and 16% made 3 different colicins (Gordon and O’Brien, 2006). The authors conclude that *E. coli* strains that carry more than one colicin may benefit from carrying an SOS induced colicin (Ia) along with another that is induced by iron depletion (V).

The relationship between colicogeny and pathogenicity is a curious one as well. Colicin V has been associated with an increase in virulence in urinary tract infection isolates (Waters and Crosa, 1991) and transformation with a Colicin V-containing plasmid can result in an increase in virulence of the strain in chickens (Wooley et al., 1994). Colicinogenic strains were more commonly recovered in cases of pyelonephritis and cystitis than when compared to when urinary shedding of the bacteria was caused no clinical disease (Riley and Wertz, 2002). Colicin-producing strains may indeed be more fit from an ecological perspective.

If a colicinogenic strain can colonize a new niche, such as happens with a systemic, rather than localized infection, the strain is at a selective advantage. On the other hand, colicins are only produced at low levels at “normal” conditions. The colicin production
is only induced in conditions of nutrient scarcity or when the cell senses impending doom such as DNA damage or UV light (Riley and Gordon, 1999). When entering a new environment the cell does not know what other bacteria are resistant to their colicin. From a bacterial ecology standpoint, colicin producing strains always eliminate colicin sensitive strains, but *E. coli* resistant to colicin out compete producers because of the cost of making the colicin puts producers at a disadvantage. Without the presence of colicin producers though in an ecological niche, colicin sensitive strains beat out resistant strains because resistant strains have had to modify their nutrient uptake systems in order to avoid colicin insertion. This then makes resistant strains less efficient and at an ecological disadvantage (Kirkup and Riley, 2004; Riley and Gordon, 1999, Riley and Gordon, 1992).

**Colicins as Antimicrobial Agents**

Due to their efficient bactericidal action colicins make an attractive target for use as an antimicrobial agent (Gillor, et al., 2004). Colicins have been used in at least one human application. Trautner, et al. (2002), used *E. coli* strains which produce Colicin E2 (a DNA endonuclease) to prevent infection causing *E. coli* from colonizing urinary catheters. The food safety applications of Colicin E1 have been investigated as well with the possible application of a carcass spray to reduce the levels of *Listeria monocytogenes* on meat (Patton et al., 2007).

In the search for alternatives to antibiotics in animal production, colicins have been approached as a potential option for control of pathogens of food safety concern in the animal as well. Animal studies exploring the use of direct fed colicins to control a bacterial population are few. In a 2001 study, 20 different colicin-producing strains were
induced to produce colicin and tested against Shiga toxin-producing *E.coli* under aerobic and anaerobic conditions, and in the presence of rumen fluid (Jordi, et al., 2001).

Colicins E1 and K were the most effective under anaerobic conditions and when cultured with rumen fluid (Jordi, et al., 2001). Schamberger, et al., (2004) directly fed eight live Colicin E7 producing strains to cattle, after screening 22 colicin producing strains for their inhibitory level vs *E.coli* O157 (Schamberger et al., 2004; Diez-Gonzalez, et al., 2002). These live bacterial cultures were fed at a level of 1x10⁸CFU/day and significantly reduced fecal *E.coli* O157:H7 shedding. The economic feasibility of using colicins for the prevention of disease in animals has yet to be determined. However, the purification costs associated with feeding a pure protein at this time are prohibitive.

**Yeast Recombinant Protein Production**

*Pichia pastoris*

First recognized by Philips Petroleum Company for its ability to use methanol as its sole carbon source in the 1970’s, *Pichia pastoris* was originally cultured to be an alternative animal protein source (reviewed, Macauley-Patrick, 2005). Subsequently, after the development of expression vectors, *P. pastoris* was exploited for heterologous recombinant protein expression and has since been available from Invitrogen in kit form. As compared to *E. coli* expression systems, the more complex eukaryotic metabolism of *P. pastoris* allows for post-translational modifications such as glycosylation, isomerization, and the addition of functional groups (Daly and Hearn, 2005). Unlike *E. coli* which require maintenance of an antibiotic resistance plasmid, chromosomal integration occurs in yeast transformation and it is not lost after generations.

**Recombinant Yeast Applications**
Recombinant yeast have been used for applications in animal health and nutrition previously. Phytases, such as APPA2, have been included in the swine diet to improve phosphorus utilization (Lee, et al., 2005). Porcine epidermal growth factor (EGF) was expressed in *Pichia pastoris* supernatant and fed to pigs at 1.5mg/kg (Lee, et al., 2006). This addition to an early-weaned pig diet over a 28 day period increased feed efficiency by 30% and stimulated an increase in serum IgA (Lee, et al., 2006). *Pichia pastoris* was also used to express porcine somatotropin into the supernatant (Ouyang, et al., 2003). The recombinant somatotropin showed a cell proliferation effect in Nb2 cells, indicative of somatotropin but injection of the peptide into gilts did not trigger the expected enhancement of growth performance and feed efficiency (Ouyang, et al., 2003). The LTb subunit of the labile toxin has been expressed in *Pichia pastoris* and the secreted, purified protein was shown effective at eliciting an immune response in chickens against cholera toxin and LT involved in ETEC associated diarrhea (Fingerut, et al., 2005).

In addition to the animal applications for the recombinant, yeast produced, colicin, there may be applications for the technology in human medicine and food safety as well.

**Conclusion**

Post-weaning diarrhea remains a threat to swine production. With the emergence of antimicrobial resistant bacteria in the swine population and the perceived threat of the transfer of these bacteria to the human population, alternatives to the antibiotics currently used in swine feed must be developed. Other therapies such as zinc oxide and copper supplementation are not ideal because of environmental concerns of excesses of these minerals in the waste and the recent reports of delay of PWD infections rather than their elimination. Pre- and probiotic supplements are also viable alternatives under
development that may offer some promise. The addition of colicins to the post-weaning 
swine diet, perhaps in combination with probiotic addition or in the recombinant yeast, 
may be a viable option for swine production.
CHAPTER 1
DIETARY INCLUSION OF COLICIN E1 IS EFFECTIVE IN PREVENTING ESCHERICHIA COLI F18 POST-WEANING DIARRHEA IN PIGS


A journal article submitted and under revision to Antimicrobial Agents and Chemotherapy

Abstract

With world-wide concern over the use of antibiotics in animal agriculture and their contribution to the spread of antibiotic resistance, alternatives to conventional antibiotics are needed. Previous research in our lab has shown that Colicin E1 (ColE1) is effective against certain E. coli strains responsible for post weaning diarrhea (PWD) in vitro. In this study we examined the efficacy of dietary inclusion of ColE1 in preventing experimentally induced F18+ enterotoxigenic E. coli (ETEC)- caused PWD. Twenty-four weaned pigs (23 days of age), identified by genotyping to be susceptible to E. coli F18 infections, were individually housed and fed diets containing either 0, 11, or 16.5 mg CoIE1/kg diet. Two days after the start of the trial, all animals were orally inoculated with $1 \times 10^9$ CFU each of 2 F18+ ETEC strains isolated from pigs with PWD. The dietary inclusion of ColE1 decreased the incidence and severity of F18+ ETEC-caused PWD and improved the growth performance of the piglets. Additionally, prevention of PWD by the dietary addition of ColE1 reduced the expression of IL1β and TNFβ mRNA in ileal tissue from these animals. Dietary inclusion of ColE1 may be an effective alternative to
conventional antibiotics in weaning pig diets for the prevention of PWD caused by F18+ 
ETEC.

Introduction

Post-weaning diarrhea (PWD) is a serious threat to the economic success of the swine 
industry, due both to losses as a result of mortalities, as well as reduced growth 
performance of surviving pigs. It is estimated that 50% of piglet mortality worldwide is 
attributable to the causative agent of PWD, enterotoxigenic *Escherichia coli* (ETEC) 
(Fairbrother, et al., 2005). The ETEC strains most commonly associated with PWD in 
pigs possess the F4 or F18 fimbrial type (Fairbrother, et al., 2005, Zhang, et al., 2007). 
As a result of the significant impact that F18+ ETEC infections can have on pig 
production, prophylactic antibiotics are frequently included in the diets of young pigs in 
an attempt to prevent ETEC colonization and the resulting PWD. An estimated 78% of 
large swine farms in the U.S. include subtherapeutic antibiotics in the diets for young 
pigs (USDA, 2001). Despite the use of antibiotic prophylaxis, 40.7% of these farms 
reported an incidence of diarrhea caused by *E. coli* infections (USDA, 2001). The lack of 
effective PWD prevention with the use of prophylactic antibiotics is not surprising, 
because of the frequency and spectrum of antibiotic resistance seen among ETEC strains 
(Choi, et al., 2002; Maynard et al., 2004; Lanz, et al., 2003). It is expected that antibiotic 
resistance will further increase among these strains, based on the overall increase in 
resistance to antibiotics by ETEC over the last 20 years (Maynard et al., 2004).

With worldwide concern over the use of prophylactic antibiotics in animal 
agriculture and its contribution to the spread of antibiotic resistance (FDA, 2003; 
Maynard, et al., 2004), the development of alternatives to conventional antibiotics is
urgently needed to protect swine from these E. coli infections. Public concerns surrounding the antibiotic resistance issue led to the elimination of prophylactic antibiotic use in animal agriculture in Denmark (reviewed, Stein, 2002). This cessation of the use of prophylactic antibiotics in pig production caused a sharp increase in the rate of PWD and a 30% increase in piglet mortality (reviewed, Stein, 2002). These infections led to an increase in veterinarian prescribed antibiotic use in Denmark’s swine industry (Jensen, 2006). The switch from growth promoting or prophylactic antibiotic usage to only veterinary prescribed therapeutic usage resulted in a very modest reduction in total antibiotic usage in Denmark’s swine industry (Jensen, 2006). In order to realize a true reduction in antibiotic use in animal agriculture, effective alternative therapies must be developed.

A potential alternative to conventional antibiotics that holds a great deal of promise are colicins. Colicins are a class of bacteriocins produced by, and effective against, E. coli and closely related bacteria (Fredericq, 1957). These proteins are particularly attractive for use as an alternative to conventional antibiotics for the control of E. coli caused PWD for several reasons. We have previously shown them to be effective against some strains of ETEC responsible for PWD in vitro (Stahl, et al., 2004), and other work has demonstrated that ColE1 is effective against a wide range of E. coli (Yang, et al., 2007; Murinda, et al., 1996; Jordi, et al., 2001; Schamberger and Diez-Gonzalez, 2002). They are not related to any antibiotics that are currently being used in human medicine. Also, colicins would not be absorbed intact by the animals, thereby eliminating concerns over antibiotic residues in meat, and colicins could be effective at low enough concentrations so as not to significantly alter the nutrient density of the diet.
The objective of this study was to determine the efficacy of dietary inclusion of Colicin E1 in preventing PWD due to F18+ ETEC.

**Materials and Methods**

**Colicin Production**

Colicin E1 was produced and purified to homogeneity according to the method of Stahl, et al. (28). Briefly, a Colicin E1 producing strain of *E. coli* was grown in LB and colicin production was induced by the addition of Mitomycin C (EMD Biosciences, San Diego, CA) to the media. The ColE1 was purified from the cell free supernatant by ion exchange chromatography, first utilizing DEAE cellulose (Sigma-Aldrich, St. Louis, MO) and then further purifying the protein utilizing Q sepharose (GE Healthcare, Piscataway, NJ). The purity of the Colicin E1 used in this study is estimated by densitometry at over 95% and the purity of this preparation has been visualized by SDS-PAGE previously (Patton et al., 2007).

**ETEC challenge strains**

*Escherichia coli* F18 strains 2144 (O147:NM) and S1911 (O139) were used as challenge strains. Strain S1191 was isolated from a pig with edema disease and produces heat stable toxins STa, STb, and Shiga toxin 2e and is chloramphenicol resistant. Strain 2144 was a field isolate that was made nalidixic acid resistant and produces the toxins STa and STb. Both strains were grown overnight as pure cultures in LB at 37°C with shaking. They were then individually diluted to an OD$_{600}$ = 0.1 in fresh LB and allowed to grow to an OD$_{600}$ ≈ 1. The cultures were then centrifuged at 4,000 × g for 10 min at 4°C. The bacterial pellets were resuspended in 20% dextrose and 5% non-fat dry milk. The
challenge dose consisted of an equal amount of each strain and was determined by serial
diluting and plating to provide a total of 2×10^9 CFU/0.5 ml oral dose.

**Animals**

All of our protocols involving animals were approved by the Institutional Animal Care
and Use Committee of Iowa State University. The 24 barrows (castrated pigs) utilized
were obtained from the Iowa State University Swine Nutrition Farm and were determined
to be susceptible to *E. coli* F18 based on a restriction fragment length polymorphism test
described by Frydendahl et al. (2003). Briefly, genomic DNA was purified (DNeasy Kit,
Qiagen, Valencia, CA) from pig tail clippings and primers (forward -
TTGGGAACCAGATGGGACAGTATG and reverse -
CCCGCCAAGGAGCGTGCCTGTCTA) were used to amplify a 162 bp section of the 1,
2α fucosyltransferase enzyme by PCR (24). The 162 bp PCR product was then digested
using *HhaI* (New England Biolabs, Ipswich, MD) and the polymorphism was determined
by size comparison on a 3% agarose gel.

Pigs were weaned at 17 days of age and allowed to adjust to solid food (TechStart 17-25,
Kent Feeds, Muscatine, IA). At 21d of age, the pigs were grouped by body weight (n=8)
and transferred to individual pens. Pigs were given 2 d to adjust to individual housing
before the experimental diets were fed. The basal diet for all of the experiments was corn
and soy based and contained no animal products (26% crude protein, 3.51 kcal/kg). This
diet met or exceeded the nutrient requirements of these pigs based on the NRC (1998)
recommendations. Either 0 (control), 11, or 16.5mg of purified ColE1 (supplied at 10
mg/mL in 10 mM Tris, pH 7.6) was added per kg to the basal diet, and the diets were
then pelleted at low temperature (Purina TestDiet, Richmond, IN). The pelleted rations
were provided to the pigs twice daily at a rate which exceeded consumption for each animal (approximately 500 g/d). Unconsumed feed was weighed daily and feed intake was determined.

After receiving the experimental diets for 2 d, all animals were orally inoculated with the two F18+ ETEC strains and their fecal scores were recorded. Fecal scores were determined (0 = dry, hard, and well formed feces; 1 = soft, but formed feces; 2 = pasty and green/brown in color; 3 = viscous and light in color, episodic; 4 = fluid and light in color, episodic; 5 = watery and continuous) twice daily after the bacterial challenge. Fecal samples were obtained 2 d prior to the ETEC challenge and daily after the challenge by inserting a 10 µl fecal loop (Fisher Scientific, Pittsburgh, PA) into the rectum. These samples were immediately diluted in 5 ml of sterile PBS. Serial 10 fold dilutions, (up to 1:100,000) were plated onto MacConkey’s (MAC, Difco, Franklin Lakes, NJ), MAC + chloramphenicol, and MAC + nalidixic acid agar plates for CFU determination. Four days post challenge, all pigs were euthanized by captive bolt and tissue samples were collected. Ileal sections (approximately 10 cm each) were collected from each pig for RNA extraction, and E. coli enumeration. Additionally, rectal and cecal contents were collected for E. coli enumeration.

**Gene expression**

Isolation of RNA from the ileum was performed utilizing a whole ileal homogenate and the RNaseasy kit (Qiagen, Valencia, CA). Genomic DNA was eliminated from the extracted total RNA using the DNA-free kit from Ambion (Austin, TX) according to the manufacturer’s instructions. The RNA was reverse transcribed using Superscript III (Invitrogen Life Technologies, Carlsbad, CA), and then removed from the resulting
cDNA by incubation with *E. coli* RNase H (Invitrogen) according to the manufacturer’s instructions. Concentrations of cDNA were determined utilizing the Quant-it kit (Invitrogen), and the cDNA samples were then stored at -80°C until analysis by real-time PCR. The levels of interferon (*IFN*), interleukin 10 (*IL10*), interleukin 8 (*IL8*), tumor necrosis factor α (*TNFα*), tumor necrosis factor β (*TNFβ*), interleukin1β (*IL1β*), and inducible nitric oxide synthase (*iNOS*) expression were semi-quantitatively determined by real-time PCR using the MyiQ™ Single Color Real-Time PCR Detection System and SYBR Green (Bio-Rad Laboratories; Hercules, CA). Thermocycling conditions included 45 cycles of 30s of melting at 95°C followed by 30s of annealing and extension at 60°C. Following amplification, all samples were subjected to a melt curve analysis to insure that only a single product was produced. Primer oligonucleotides (Table 1) were designed using “PrimerQuest” software available from Integrated DNA Technologies (Coralville, IA). All primer sets were validated to amplify only the sequence of interest and to do so in a linear fashion over a 2 log range of cDNA concentrations. The data from all samples were normalized to cDNA concentration prior to statistical analysis.

**Bacterial enumeration**

Enumeration of *E. coli* from the ileal mucosa, cecal content, and rectal content samples was performed as follows. Samples were weighed, diluted 1:2 with buffered peptone water and placed in a stomacher blender (Seward Stomacher 80, Worthing, UK) for 30 seconds. The samples were then serially diluted over a 4 log range at a 1:200 starting dilution for tissues and 1:500 for fecal samples. Ten µl of each dilution was plated in duplicate on MAC, MAC + chloramphenicol (30 µg/ml), and MAC + nalidixic acid (50 µg/ml) agar plates. Plates were incubated for 16 hours at 37°C and the countable dilution
was then recorded. The limit of detection for our enumeration was $1 \times 10^3$ CFU/g of tissue and $1 \times 10^4$ CFU/g of feces.

**Statistical analysis**

Statistical analysis of the data was performed with Statistical Analysis Software version 9.1 (SAS, Cary, North Carolina) utilizing the GLM procedure. Treatment was considered a fixed effect, and for growth parameters initial body weight was considered a covariate in the analysis. For the bacterial enumeration data, samples that were below the limit of detection were ascribed a value of 1 CFU less than our limit of detection for the calculation of means. All values for bacterial recovery are $\log_{10}$ least squares means.

**Results**

**Fecal scores**

Prior to bacterial challenge, none of the pigs showed any indication of diarrhea or loose stool. There were no significant differences in fecal scores among the treatment groups until 48 h post-challenge (Figure 1). At this point the group fed the control diet had a mean fecal score of 2.38 which was higher ($P < 0.06$) than that of the group fed the 16.5 mg ColE1/kg diet (mean score of 0.5) (Figure 1). Over the entire study, the control group had significantly higher ($P < 0.05$) average fecal score than the high dose (16.5 mg/kg feed) ColE1 group (2.1 vs. 0.59, respectively). The low dose (11mg/kg) ColE1 group also had higher ($P < 0.05$) average fecal scores than the high dose ColE1 group, and these were not significantly different from those of the control pigs. These differences in group fecal scores were caused by an increase in the incidence of diarrhea (fecal scores $\geq 4$) among the control group pigs. In the control group 1, 4, and 5 out of the 8 pigs had diarrhea after 24, 48, and 72h post-ETEC inoculation, respectively. Among the pigs fed
the low dose of ColE1, no animals had diarrhea 24h post-inoculation, however 3 and 4 pigs had diarrhea at 48 and 72h post-challenge, respectively. None of the pigs fed the high dose of ColE1 had any incidence of diarrhea at any time during the experiment.

**Growth performance**

Dietary inclusion of ColE1 had a significant effect (P < 0.05) on the growth performance of the pigs in this study (Table 2). From the time of *E. coli* challenge until the completion of the study, pigs fed the control diet gained an average of 380 g while the pigs receiving either the low or high dose of ColE1 in their diets gained 540 and 940 g, respectively (Table 2). Although the animals fed the control diet gained the least body weight over the course of the study, they consumed significantly (P < 0.05) more feed than either of the groups fed diets containing ColE1. The control group animals averaged a total consumption of 1.54 kg of diet, while the ColE1 treated pigs ate 1.22 kg and 1.44 kg, for the low and high dose ColE1 diets, respectively. Although there were significant differences in both body weight gain and feed consumption, there was not a significant difference in feed conversion efficiency (body weight gain/feed intake, P < 0.19) among any of the treatment groups. In the control group, 2 of the animals lost weight (approximately 400 g each) over the length of the study, causing a negative body weight gain which resulted in a tremendous variation in the feed conversion efficiency for this group.

**Bacterial enumeration**

**Fecal cultures**

There were no colonies isolated on the agar plates containing either chloramphenicol or nalidixic acid from any samples prior to the F18+ ETEC challenge. At 24h post-
inoculation, levels of both total coliforms and the ETEC 2144 challenge strain recovered in the feces were higher (P < 0.05) in the control animals compared with both groups of ColE1 fed pigs (Table 3). There were no significant differences between treatment groups in the recovery of the ETEC S1191 challenge strain at the first day post challenge but in the subsequent day there was a reduction in the high dose ColE1 group as compared to the controls (P < 0.05). By the last day of fecal sampling, the levels of the S1191 strain had dropped below our detection limit for most of the animals regardless of dietary treatment (16/24) and there were no significant differences in the amount of the 2144 strain recovered.

**Tissues**

In the ileum, both treatment groups averaged lower levels of both challenge strains as compared to the controls (Table 4). A trend existed for higher total coliforms in the low group as compared to the controls (P < 0.08). There were no significant differences in the recovery of bacteria from the cecal samples. The S1191 strain was only recovered in cecal samples from 3 of the 8 animals in the high dose ColE1 group compared with 6 and 4 animals in the control and low dose ColE1 treatment, respectively. In the rectum, similar levels of total coliforms and 2144 were recovered for animals in all groups. Significant differences in the levels of the S1191 strain were seen with colicin treatment. Control animals had higher levels of the S1191 strain recovered in their rectal samples compared with either colicin treated group. Interestingly, the low dose ColE1 group had significantly lower (P < 0.05) levels of this bacteria compared to the high dose group.

**Gene expression**
The expression of TNFβ mRNA in ileal tissue was higher (P < 0.05) in the control animals than in either of the ColE1 treated groups (Figure 2). The levels of IL1β mRNA were highest in the low ColE1 supplemented group (6 fold higher than in the controls (P < 0.05), whereas the high ColE1 group had nearly undetectable levels (Figure 3). The levels of expression of both TNFα and iNOS tended (P < 0.1) to be greater in control animals and there were no significant differences in the expression of IFNγ, IL8, or IL10, in the ileum due to colicin treatment.

**Discussion**

We examined the efficacy of ColE1 in preventing F18+ ETEC caused post-weaning diarrhea (PWD) because PWD is a major bacterial threat to the economic sustainability of swine production. This disease has been estimated to be responsible for as much as 50% of the economic losses seen in the production of weaned pigs (Hani, et al., 1971; Fairbrother, et al., 2005). In herds with PWD, up to 2% mortality (USDA, 2001) in weaning pigs can be seen, but of greater economical significance is the morbidity and reduction in growth performance in the pigs that survive these infections. This lost productivity costs the U.S. swine industry several million dollars each year. Although there is a need for alternatives to conventional antibiotics, the development of compounds to combat ETEC-associated PWD is beset with difficulties including having an adequate experimental model of ETEC infection. In the field, PWD is a multifactorial disease and interventions must therefore, be tested against a challenge dose that is high enough to produce diarrhea but not toxic immediately post-challenge. Establishing a good challenge model for this disease has proven to be difficult. Madec et al. utilized four strains of ETEC and several different dosing methods in six trials utilizing a total of 168
specific pathogen free piglets and was only able to generate clinically significant, although transient, diarrhea in 50% of animals (Madec, et al., 2000). Using a viral co-infection such as transmissible gastroenteritis (TGEV) or rotavirus that leads to immunosuppression and intestinal membrane disruption can also lead to an increase in experimental ETEC infection rates (Cox, et al., 1991; Benfield, et al., 1988; Girard, et al., 2005; Nagy, et al., 1992). While a co-challenge model can increase the success rate of experimentally reproducing PWD, it also adds a potential confounding effect particularly if the viral infection alone causes reduced growth performance. An alternative to the viral co-infection model that has also been shown to increase the success of an ETEC bacterial challenge is modification of the post-weaning diet. Alterations of the protein source(s) utilized in the post-weaning diet (shifting away from animal-based protein sources to soy-based) can significantly affect the susceptibility of the pigs to ETEC infection due to a transient intestinal inflammatory response (Bosi, et al., 2004; Dreau, et al., 1994). While both viral co-challenges and dietary alterations can increase infection rates in experimentally induced PWD models, identifying the genetic susceptibility of the animals may offer the most efficient way to increase infection rates in a challenge model. With the use of pre-screening for a F18 receptor polymorphism, the rate of infection can be increased from 5.9% in the F18 resistant genotype pigs to 87% in those that are genetically susceptible (Frydendahl, et al., 2003). In this study, we utilized only genetically susceptible animals, as well as a weaning diet that contained no animal protein and as a result a majority of the protein in the diet was from soybean meal. With this model we achieved over a 60% infection rate in control animals with no mortality after 5 days, no viral co-infection, and an easily-performed one-time oral challenge.
Although our high dose of ColE1 (16.5mg/kg of diet) was able to eliminate all clinical signs of PWD, our low dose of ColE1 was only able to slightly delay the onset of PWD. The level of reduction of the challenge strains of *E. coli* that reached the ileum as a result of feeding the ColE1 may have been the determining factor in the prevention of the development of clinical disease. Colicin fed animals had significantly lower (P < 0.04) fecal shedding of the 2144 challenge bacteria at 1 day post-challenge (Table 3). While this suggests that the addition of colicin to the feed significantly reduced the amount of viable bacteria that reached the distal end of the small intestine, it also demonstrates that our colicin dose was not sufficient to completely eliminate the challenge strains. At the completion of the study there were no significant differences based on colicin treatment on fecal shedding of either of the ETEC challenge strains. We would not anticipate that the feeding of ColE1 would have an effect on reducing the colonization of the ETEC strains if they reached the ileum in a viable state, since ColE1 is sensitive to proteolysis (Cavard, et al., 1989; Bowles, et al., 1981; Brey, et al., 1982). It is likely that not all of the *E. coli* in our large challenge dose would be killed by the ColE1 present in the digesta prior to the inactivation of the ColE1 by proteolysis, but it appears that enough was eliminated as a result of the high dose ColE1 supplementation to prevent disease. The intestinal gene expression data also suggests that fewer of the challenge bacteria reached the intestine in a viable state and were therefore not able to colonize the ileum thus leading to diarrhea in the pigs. This is supported by the lower expression (P < 0.05) of IL1β and TNFβ mRNA in the ileal tissue of pigs fed the high ColE1 diet compared with the control (Figure 3). Interleukin 1β is primarily secreted by macrophages and activates lymphocytes during an immune response. While the concentration of IL1β message was
significantly lower in the ileal tissue of the pigs fed the high dose of ColE1 compared with the control pigs, pigs in the low ColE1 dose group had mRNA levels that were over 6 fold higher than those of the control. At the time of tissue collection there was no longer any significant difference in fecal score between the low dose and the control groups and this elevated expression may be indicative of a delay in the inflammatory response as a result of the ETEC challenge. This would be reasonable since there appeared to be a delay in the onset of PWD with the low dose ColE1 fed pigs compared with the control animals. An increase in IL1β gene expression has been correlated with porcine enteropathogenic E. coli challenge (Girard, et al., 2005), although the associated increase in IL6, IL8, and IL10 seen by Girard et al. were not noted in our study.

Regardless of the dose, reduced levels (P < 0.06) of expression of TNFβ were seen among pigs receiving the ColE1 containing diets (Figure 2). TNFβ, also known as lymphotoxin, is a primary effector of NO production and is associated with inflammatory responses related to T cell recruitment (Ferrante, et al., 1990). The lower levels of expression of TNFβ and IL1β in the ileal tissue of pigs fed the high dose of ColE1 compared with the control animals suggests that ColE1 was able to significantly reduce the bacterial load that initially reached the ileum in these animals; thereby reducing the inflammatory response to this ETEC challenge.

While other researchers have examined the efficacy utilizing colicin producing bacterial cultures as probiotics for cattle in order to reduce E. coli O157 contamination (Schamberger, et al., 2004), we are the first, to our knowledge, to examine a purified colicin as a feed component for the prevention of an ETEC infection. We have demonstrated with growth performance data, clinical indicators of PWD, and intestinal
gene expression that the inclusion of ColE1 can prevent experimentally induced PWD. The efficacy in preventing PWD at a 16.5mg/kg diet level of dietary inclusion, suggests that ColE1 warrants further evaluation as a potential alternative to conventional antibiotics for use in weaning pig diets. This protein may also have significant implications for human food safety as well, since the efficacy of ColE1 against many ETEC of concern for human food safety has been well-documented (Callaway, et al., 2004; Jordi, et al., 2001; Murinda, et al., 1996).
Table 1. Primer sets for semi-quantitative real-time PCR analysis of intestinal gene expression.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer sets</th>
</tr>
</thead>
</table>
| Inducible Nitric Oxide Synthase (iNOS) | F: GGACGTACGAGAAGTGAACCAAACC  
                                      | R: GAACGTACGAGAAGTGAACCAAACC             |
| Interleukin 1 β (IL1β)                 | F: TGAAGAAGAGCCCTGCTCT TGA  
                                      | R: TGCACAAAGCTCATGCAGAACACC             |
| Tumor Growth Factor β (TGFβ)           | F: AGGCCGTACTGTGTTTTACAACA  
                                      | R: TTGGTTCGCTTTCCACCAATTAG             |
| Interleukin 10 (IL10)                  | F: AAGACGTAAATGCGAGGAGGAGAGA  
                                      | R: TGCTAAAGGCACTCTTTACACCTCCT          |
| Interleukin γ (IFN)                    | F: ATGACTTCCAAAACCTGGCTGTGCC  
                                      | R: TATGCACTGCAGATCGAAGTTCTGC           |
| Interleukin 8 (IL8)                    | F: ATGACTTCCAAAACCTGGCTGTGCC  
                                      | R: TATGCACTGCATCGAAGATCTGC             |
| Tumor necrosis factor α (TNFα)         | F: GCCACGTATTGAGGCAATGTCAAA  
                                      | R: GTTGTCATTTTCAGGCTTGACGCTTT         |
| Tumor necrosis factor β (TNFβ)         | F: AGATCAGCTGTCCAGACACACAGA  
                                      | R: TAGAGCGAGGGCTTCCAAAAGAAGAC         |
Table 2. Effect of dietary inclusion of Colicin E1 on growth performance of weaning pigs.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Weight gain, kg</th>
<th>SEM</th>
<th>Feed intake, kg</th>
<th>SEM</th>
<th>Feed Efficiency&lt;sup&gt;1&lt;/sup&gt;</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.38&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.16</td>
<td>1.58&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.062</td>
<td>3.60</td>
<td>0.94</td>
</tr>
<tr>
<td>Low</td>
<td>0.54&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.16</td>
<td>1.22&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.062</td>
<td>2.83</td>
<td>0.74</td>
</tr>
<tr>
<td>High</td>
<td>0.94&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.16</td>
<td>1.40&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.062</td>
<td>1.98</td>
<td>0.74</td>
</tr>
</tbody>
</table>

<sup>a,b</sup> value within a column not sharing a common superscript are different (P < 0.05)

<sup>1</sup> Feed efficiency is defined as feed intake/weight gain.
Table 3. Effect of Colicin E1 on *E. coli* levels in the feces of weaning pigs. Values are Log_{10}.

<table>
<thead>
<tr>
<th>Colicin Dose</th>
<th>Total coliforms</th>
<th>Strain 2144, NAL&lt;sup&gt;R&lt;/sup&gt; SEM</th>
<th>Strain S1191, CAM&lt;sup&gt;R&lt;/sup&gt; SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 day post-challenge</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7.8&lt;sup&gt;a&lt;/sup&gt; 0.4</td>
<td>7.1&lt;sup&gt;a&lt;/sup&gt; 0.4</td>
<td>6.0&lt;sup&gt;a&lt;/sup&gt; 0.3</td>
</tr>
<tr>
<td>Low</td>
<td>6.8&lt;sup&gt;ab&lt;/sup&gt; 0.4</td>
<td>5.7&lt;sup&gt;b&lt;/sup&gt; 0.4</td>
<td>4.8&lt;sup&gt;b&lt;/sup&gt; 0.3</td>
</tr>
<tr>
<td>High</td>
<td>6.5&lt;sup&gt;b&lt;/sup&gt; 0.4</td>
<td>5.6&lt;sup&gt;b&lt;/sup&gt; 0.4</td>
<td>4.7&lt;sup&gt;b&lt;/sup&gt; 0.3</td>
</tr>
<tr>
<td>2 days post-challenge</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>8.0 0.4</td>
<td>6.9 0.6</td>
<td>5.7 0.3</td>
</tr>
<tr>
<td>Low</td>
<td>8.1 0.4</td>
<td>6.2 0.6</td>
<td>5.0 0.3</td>
</tr>
<tr>
<td>High</td>
<td>7.3 0.4</td>
<td>5.4 0.6</td>
<td>4.9 0.3</td>
</tr>
<tr>
<td>3 days post-challenge</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>8.8 0.4</td>
<td>7.5 0.7</td>
<td>4.7 0.2</td>
</tr>
<tr>
<td>Low</td>
<td>8.9 0.4</td>
<td>6.6 0.7</td>
<td>4.9 0.2</td>
</tr>
<tr>
<td>High</td>
<td>7.8 0.4</td>
<td>6.0 0.7</td>
<td>5.2 0.2</td>
</tr>
</tbody>
</table>

<sup>a,b</sup> values within a day and column not sharing a common superscript are significantly different (P < 0.05).
**Table 4.** Dietary inclusion of Colicin E1 affects bacterial recovery in the ileum of weaning pigs. Values are Log_{10}.

<table>
<thead>
<tr>
<th>Colicin Dose</th>
<th>Total coliforms</th>
<th>SEM</th>
<th>Strain 2144, nal^{R}</th>
<th>SEM</th>
<th>Strain S1191, cam^{R}</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.2^{a}</td>
<td>0.4</td>
<td>6.5</td>
<td>0.8</td>
<td>4.4^{a}</td>
<td>0.3</td>
</tr>
<tr>
<td>Low</td>
<td>6.8^{b}</td>
<td>0.4</td>
<td>5.1</td>
<td>0.8</td>
<td>3.1^{b}</td>
<td>0.3</td>
</tr>
<tr>
<td>High</td>
<td>6.8^{b}</td>
<td>0.4</td>
<td>4.8</td>
<td>0.8</td>
<td>3.2^{b}</td>
<td>0.3</td>
</tr>
</tbody>
</table>

^{a,b} Value within a column not sharing a common superscript are significantly different (P < 0.05).
Figure 1. The percentage of pigs with diarrhea indicative of a fecal score higher than three. The low diet had 11 mg/kg ColE1, the high diet 16.5 mg/kg, and the control had no addition.
Figure 2. Relative gene expression, normalized to cDNA concentration (25 ng/reaction), of tumor necrosis factor beta (TNFβ) in intestinal mucosa of pigs fed 16.5 mg/kg Colicin E1 (high), 11 mg/kg Colicin E1 (low) or a basal diet. a,b indicate P < 0.05
Figure 3. Relative gene expression, normalized to cDNA concentration (25 ng/reaction), of Interleukin 1 Beta (IL-1β) in intestinal mucosa of pigs fed 16.5 mg/kg Colicin E1 (high), 11 mg/kg Colicin E1 (low) or a basal diet. a,b indicate P < 0.05.
CHAPTER 2
DIETARY INCLUSION OF COLICIN E1 IS EFFECTIVE AT PREVENTING ESCHERICHIA COLI F18 POST WEANING DIARRHEA IN PIGS-LONGER DURATION TRIAL


A manuscript in preparation for the Journal of Animal Science

Abstract

Post-weaning diarrhea continues to cause significant economic losses in swine production. We have previously shown the efficacy of feeding Colicin E1 to recently weaned pigs as an alternative to sub therapeutic antibiotics to prevent post-weaning diarrhea. A highly purified preparation of Colicin E1 was included at 20 mg/kg for two weeks post-weaning and then removed from the diet for an additional two-week period to determine if withdrawal would have any deleterious side effects. A seeder model was employed for enterotoxigenic Escherichia coli challenge. Eight pigs were orally challenged with 10^9CFU/g of an ETEC strain previously isolated from piglets with PWD. Two animals, paired by body weight, that exhibited severe diarrhea two days post-challenge were placed into pens of piglets that were either fed a diet containing 20mg/kg Colicin E1 or a basal diet with no supplementation. All challenged animals were susceptible to ETEC as determined by PCR/RFLP. In addition, a group of littermates were fed the same diets, in the absence of ETEC challenge to investigate any possible changes in body weight gain between the test diet and control.
In the first week after the seeder pigs were introduced into the ColE1 fed and control fed pens, 6 out of 9 control animals failed to gain over 300 g as compared to 2 of 10 in the ColE1 group. Four of the control animals lost weight and two of those pigs lost more than 10% of their initial body weight. The diarrhea persisted for up to two weeks in the control animals. Gene expression of ileal mucosa showed differences in intestinal development and the persistence of the inflammatory response after ETEC challenge. The challenged control animals had higher levels of TNFα four weeks post ETEC exposure. Expression of COX2 and PGHS were highest in the ColE1 fed ETEC challenged animals, suggesting an upregulation of prostaglandins to preserve membrane integrity in recovery from ETEC challenge.

**Introduction**

Post-weaning diarrhea (PWD) is a serious threat to the economic success of the swine industry, due both to losses as a result of mortality, as well as morbidity as evidenced by reduced growth performance in surviving pigs. It is estimated that 50% of piglet mortality due to diarrhea worldwide is attributable to the causative agent of PWD, enterotoxigenic *Escherichia coli* (ETEC) (Gyles, et al., 2007). The ETEC most commonly associated with PWD in pigs possess the F18 fimbrial type (Fairbrother et al., 2005). As a result of the significant impact that F18 ETEC infections can have on pig production, prophylactic antibiotics are frequently included in the diets of young pigs in an attempt to stave off disease threats and for growth promotion. An estimated 78% of large swine farms in the U.S. include antibiotics in the diets for young pigs (USDA, 2001). Despite the use of antibiotics in the diet, 48% of these farms reported disease caused by *E. coli* infections (USDA, 2001). The lack of effective PWD prevention with
the use of prophylactic antibiotics is not surprising, because of the frequency and spectrum of antibiotic resistance seen among ETEC strains (Maynard et al., 2004; Lanz et al., 2003; Choi et al., 2002). It is expected that antibiotic resistance will further increase among these strains, based on the overall increase in resistance to antibiotics by ETEC over the last 20 years (Maynard et al., 2004).

A potential alternative to conventional antibiotics that holds a great deal of promise are colicins. Colicins are a class of bacteriocins produced by, and effective against, E. coli and closely related bacteria (Fredericq, 1957). These proteins are particularly attractive for use as an alternative to conventional antibiotics for the control of E. coli caused PWD for several reasons. We have previously shown them to be effective against strains of ETEC responsible for PWD in vitro (Stahl et al., 2004) and in vivo (Cutler, et. al., 2007). Colicins are not related to any antibiotics that are currently being used in human medicine. Also, colicins would not be absorbed intact by the animals, thereby eliminating concerns over antibiotic residues in meat, and colicins could be effective at low enough concentrations so as not to significantly alter the nutrient density of the diet. The objectives of this study were to determine if dietary inclusion of Colicin E1 was effective in preventing PWD in young pigs, if withdrawal of the colicin would have any adverse effects on health of the animals, and if there were any growth promotive effects of Colicin E1 feeding in non-challenged animals.

**Materials and Methods**

**Colicin Production**

Colicin E1 was produced and purified to homogeneity according to the method of Stahl, et al. (2004). Briefly, a Colicin E1 producing strain of E. coli was grown in LB and
Colicin production was induced by the addition of Mitomycin C (EMD Biosciences, San Diego, CA) to the media. The ColE1 was purified from the cell free supernatant by ion exchange chromatography, first utilizing DEAE cellulose (Sigma-Aldrich, St. Louis, MO) and then further purifying the protein utilizing Q sepharose (Amersham Biosciences, Piscataway, NJ). The estimated purity of the Colicin E1, measured by densitometry, used in this study was 90% (Patton, et al., 2007).

**Animals**

All of our protocols involving animals were approved by the Institutional Animal Care and Use Committee of Iowa State University. The 28 barrows utilized for the challenge portion of this study were determined to be susceptible to *E. coli* F18 based on a restriction fragment length polymorphism test described by Frydendahl et al. (2003). Briefly, genomic DNA was purified (DNeasy Kit, Qiagen, Valencia, CA) from pig tail clippings and primers (forward -TTGGGAACCAGATGGGACAGTATG and reverse -CCCGCCAAGGAGCGTGCCTGTCTA) were used to amplify a 162 bp section of the 1,2α fucosyltransferase enzyme (*FUT1M307 ECF18R gene*) by PCR (Meijerink et al., 2000). The 162 bp PCR product was then digested using *Hha* (New England Biolabs, Ipswich, MD) and the polymorphism was determined by size comparison on a 3% agarose gel. The remaining 20 animals were not tested for susceptibility to F18 ETEC as these animals were used for feeding and growth comparision only. None of these pigs showed any signs of diarrhea throughout the study. Pigs were weaned at 17 days of age and allowed to adjust to solid food (TechStart 17-25, Kent Feeds, Muscatine, IA). At 21d of age, the pigs were grouped by body weight.
Diets

Pigs were fed with a 57% corn-32% soybean meal starter diet consisting of 2.5% whey, 1% soy protein, and 4% corn oil. Colicin E1 was added to the diet at 20 mg/kg for the treated groups, and no addition was included in the control groups. For the second two week period, the whey and corn oil were removed for a 62.6% corn, 34% soybean meal diet. All animals were fed the same diet for the second two week period.

ETEC challenge strains

*Escherichia coli* F18 strain 2144 (O147:NM) was obtained from the collection of Dr. Nancy Cornick. Strain 2144 is a field isolate that produces the toxins STa and STb. The challenge strain was grown overnight in LB at 37°C with shaking. It was then diluted to an OD$_{600} = 0.1$ in fresh LB and allowed to grow to an OD$_{600} \approx 1$. The culture was then centrifuged at 4,000 x g for 10 min at 4°C in a Sorvall Super T21 centrifuge (Kendro, Newton, CT), and the bacterial pellet was resuspended in 20% dextrose and 5% non-fat dry milk. The challenge dose was determined by serial diluting and plating to provide $2 \times 10^9$ CFU/0.5 ml oral dose.

Challenge method

This study employed a seeder challenge model. Eight animals, genetically susceptible to ETEC, were group housed and orally challenged at 3 weeks of age. After three days, two animals of similar body weight and both showing profuse diarrhea were split one each into the pen provided the control diet (N=9) and the Colicin E1 diet (N=10) for a natural route of infection. Pigs that were used as seeders then became part of each feeding group for a total of 11 pigs in the E1 fed group and 10 in the control diet group.

Fecal Cultures and PCR for the F18+ ETEC
Polymerase chain reaction was performed to confirm shedding of the challenge strain from fecal cultures. A 10µl fecal loop was inserted into the rectum of each pig daily for 14 days after challenge and then placed into 5ml sterile saline, then this sample was serially diluted in saline and plated onto MacConkey’s agar. All recovered bacteria from the countable dilution (less than 50, greater than 10) for each animal from each day were boiled in sterile water for one minute for total DNA extraction, then diluted 1:10 for use as template in the PCR reaction. Primers for F18 (forward: AGC TGA TAT AAC TTG GAG CGG GCA, reverse: TTC TCT TGC TTA GCA GGG AGG CAT) were used to amplify a 342 base pair fragment. Thermal cycling conditions were 95°C for 3 minutes, 30 seconds at 95°C then 65°C for 45 seconds for 30 cycles, then 72°C.

**Analysis of gene expression**

Isolation of RNA from the ileum was performed utilizing ileal mucosal scrapings and the RNeasy Midi kit (Qiagen, Valencia, CA). Genomic DNA was eliminated from the extracted total RNA using the DNA-free kit from Ambion (Austin, TX) according to the manufacturer’s instructions. The RNA was reverse transcribed using Superscript III (Invitrogen, Carlsbad, CA), and the RNA was removed from the resulting cDNA by incubation with *E. coli* RNase H (Invitrogen) according to the manufacturer’s instructions. Concentrations of cDNA were determined utilizing the Quant-it kit (Invitrogen), and the cDNA samples were then stored at -80°C until analysis by real-time PCR. Thermocycling conditions included 45 cycles of 30s of melting at 95°C followed by 30s of annealing and extension at 60°C. Following amplification, all samples were subjected to a melt curve analysis to insure that only a single product was formed. Primer oligonucleotides (Table 1) were designed using “PrimerQuest” software available
from Integrated DNA Technologies (Coralville, IA). All primer sets were validated to amplify only the sequence of interest and to do so in a linear fashion over a 2 log range of cDNA concentrations. The data from all samples was normalized to cDNA concentration prior to statistical analysis.

**Statistical analysis**

Statistical analysis of the data was performed with Statistical Analysis Software version 9.1 (SAS, Cary, North Carolina) utilizing the GLM procedure. Treatment was considered a fixed effect, and for growth parameters initial body weight was used as a covariate.

**Results**

**ETEC Challenge**

The ETEC strain 2144 oral challenge was successful in generating post-weaning diarrhea in 70% of the seeder animals, including the two placed into the treatment pens. The seeder animals had exhibited profuse, watery diarrhea before placement in the treatment group pens and both continued to show clinical signs for at least ten days. Between days five and seven after exposure to the seeder pigs nearly 80% of the control animals exhibited diarrhea symptoms as compared to 30% in the E1 fed group (Figure 1). The duration of the diarrhea in the control animals was an average of eight days. One animal in the E1 fed challenged group showed diarrhea for nine days. PCR for the F18 fimbriae indicated that all of the animals in both groups shed F18+ ETEC for at least two consecutive days.

**Growth Performance**

Four of the 10 control animals lost weight in the first week after exposure to the seeder animal (Table 2). One of the ColE1 fed animals lost weight. In the first week after the
seeder pigs were introduced into the naive animals, 6 out of 9 control animals failed to gain over 300g as compared to 2 of 10 in the ColE1 group. Four of the control animals lost weight and two of the pigs lost more than 10% of their initial body weight. At week one the challenged control group had significantly lower body weights compared to all of the other groups (P < 0.05). At weeks two and three after exposure to the seeder pig, the challenge control animals had lower (P< 0.05) body weights than the animals in the non-challenged ColE1 and control fed groups. A trend existed for lower body weight in the challenged control and ColE1 challenged animals at weeks 2, 3, and 4 (P < 0.1). There were no significant differences in body weight between the nonchallenged animals at any timepoint.

Gene Expression

Expression of TNFα in the ileal mucosa at the end of the four week study was higher in the control, challenged animals than any other group (Figure 2). COX-2 and prostaglandin synthase were both higher in the challenged E1 animals than in the other groups (Figure 3,4). There were no significant differences noted for gene expression of epidermal growth factor or the epidermal growth factor receptor, glucose transporter 2, or interleukin 10 (data not shown).

Discussion

We have previously shown that Colicin E1 in the post-weaning swine diet decreases the duration and severity of PWD in piglets in a short duration, five day trial. That trial used an oral challenge of ETEC strain 2144 in addition to another ETEC strain associated with edema disease (Cutler, et al., 2007). One of the concerns when assessing dietary interventions to reduce PWD is that the intervention merely delays the onset of disease
until the often costly product is removed. To ensure that no deleterious effects would be seen when Colicin E1 was removed from the diet after a two week period, a seeder pig ETEC challenge model was utilized to continuously challenge animals. The ColE1 was removed from the feed and the study was ended after an additional two weeks. There was not a delayed onset of diarrhea in the challenged ColE1 animals two weeks after exposure to a sick animal.

In addition, one of the major challenges of investigating PWD is the development of a reliable, reproducible challenge model. The use of different challenge strains with different fimbrial antigens and toxins expressed as well as varying resistance to colonization among the study animals can result in enormous variation among ETEC caused PWD studies (Madec, et al., 2000). We believe this aggressive challenge model which utilized only genetically susceptible animals, serves to better evaluate the practicality of a Colicin E1 inclusion in the diet. This seeder pig challenge model resulted in a 78% incidence of diarrhea with an average duration of 8 days in the control animals and 30% in the ColE1 fed animals with only one animal with an incidence of diarrhea over two days (Figure 1). Because of the complex factors involved in experimentally replicating PWD, comparing results from one study to the next, is a hindrance to ETEC research and impairs the development of intervention strategies other than changes in management practices (Madec, et al., 2000; Fairbrother, et al., 2005).

The use of genotyping for the F18 receptor in the pig intestine greatly improves the odds of a successful challenge study compared to post mortem analysis of bacterial adhesion to intestinal tissue (Frydendahl, et al., 2003; Madec, et al., 2000). Combining genetic
screening with the use of a natural infection model, may give more clinically relevant results when testing interventions for PWD.

The gene expression data from this study gives an indication of longer term intestinal effects after exposure to ETEC in animals that were actively infected with ETEC challenge as opposed to those who were exposed but did not develop diarrhea. At four weeks post-ETEC exposure and even after their body weight differences had recovered to similar values to that of non-challenged animals and the E1 treated groups, the intestinal development of the control animals may have been lagging behind the other groups. Further, the impact of a prolonged inflammatory response, indicated by higher levels of TNFα in the challenged control animals indicates immune activation four weeks after ETEC exposure (Figure 4). While higher levels of TNFα inhibit intestinal repair, COX2 and prostaglandin synthase increase intestinal epithelial proliferation (Martin and Wallace, 2006). COX 2 enzyme upregulation may also assist in intestinal immunity by increasing Toll like receptor regulation thus making the intestine more responsive to injury and infection (Stenson, 2007; Fukata, et al., 2006). The E1 challenged animals had greater expression of COX2 and PGHS, when compared to all other groups which could indicate a healthy immune environment in the intestine (Figure 3, 4). While the addition of Colicin E1 to the diet of the non-challenged control animals had no effect on COX2 and PGHS, the reduced pathogenesis associated with the ETEC challenge noted in the ETEC challenged Col E1 fed group (shorter duration of diarrhea, better body weight gain). Since Col E1 kills the ETEC challenge strain used in this study, the addition of E1 to the diet most likely decreased the number of challenge bacteria able to cause disease as noted by a reduction in ETEC recovered at day one post challenge (Cutler, et al., 2007).
The pigs that were fed Colicin E1 had lower incidence of PWD and this may be practical for use in the post-weaning swine diet without the environmental concerns of feeding zinc oxide.

In conclusion, we have shown that the addition of 20 mg/kg Colicin E1 to the post-weaning swine diet dramatically reduced the incidence and severity of ETEC caused post-weaning diarrhea in pigs. The inclusion of Colicin E1 in the post weaning diet, especially when used alongside dietary interventions currently in place for PWD could be a practical solution for the reduction of ETEC caused PWD.
**Table 1.** Primer sets for semi-quantitative real-time PCR analysis of intestinal gene expression.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer sets</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epidermal Growth Factor (<strong>EGF</strong>)</td>
<td>F: TGCCATAGACTGGATTTGGCCGTAAG</td>
</tr>
<tr>
<td></td>
<td>R: AGCAATTCCTCGTGGCTGAGAGAT</td>
</tr>
<tr>
<td>Epidermal Growth Factor receptor (<strong>EGFR</strong>)</td>
<td>F: ACCGTGGGAAGATCCCTTGGAGAA</td>
</tr>
<tr>
<td></td>
<td>R: ATAAGACGGCTAAGGCTGAGGCTGAGTA</td>
</tr>
<tr>
<td>Glucose transporter 2 (<strong>GLUT2</strong>)</td>
<td>F: TTCATGTCGGGTGGGACTTTGTGCTA</td>
</tr>
<tr>
<td></td>
<td>R: AATTGCAGGCTCAGTTGCTGAGAA</td>
</tr>
<tr>
<td>Interleukin 10 (<strong>IL10</strong>)</td>
<td>F: AAGACGTAATGCCCAGGACAGAGGA</td>
</tr>
<tr>
<td></td>
<td>R: TGCTAAGGACTCTTCACCTCCT</td>
</tr>
<tr>
<td>Prostaglandin endoperoxide H Synthase (<strong>PGHS</strong>)</td>
<td>F: ATCAGAAGCGAGGACCAGGCTTTCA</td>
</tr>
<tr>
<td></td>
<td>R: ACTTGAGTGTCTTTGGCTGAGGA</td>
</tr>
<tr>
<td>Cyclooxygenase 2 (<strong>COX-2</strong>)</td>
<td>F: ATCAGAAGCGAGGACCAGGCTTTCA</td>
</tr>
<tr>
<td></td>
<td>R: ACTTGAGTGTCTTTGGCTGAGGA</td>
</tr>
<tr>
<td>Tumor necrosis factor <strong>alpha</strong> (<strong>TNFα</strong>)</td>
<td>F: GCCCAGTTGTAGCCAATGCTAAA</td>
</tr>
<tr>
<td></td>
<td>R: GTTGTCTTTTGACTTTACGAGCCGT</td>
</tr>
</tbody>
</table>
Table 2. Effect of dietary inclusion of Colicin E1 on growth performance of weaning pigs. Body weights are least squares means.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Week 1 Body wt (kg)</th>
<th>SEM</th>
<th>Week 2 Body wt (kg)</th>
<th>SEM</th>
<th>Week 3 Body wt (kg)</th>
<th>SEM</th>
<th>Week 4 Body wt (kg)</th>
<th>SEM</th>
<th>Week 5 Body wt (kg)</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.63</td>
<td>0.17</td>
<td>6.52(^b)</td>
<td>0.26</td>
<td>8.6(^b)</td>
<td>0.39</td>
<td>11.94(^ab)</td>
<td>0.59</td>
<td>15.26</td>
<td>0.78</td>
</tr>
<tr>
<td>Challenged</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5.32</td>
<td>0.17</td>
<td>5.50(^a)</td>
<td>0.27</td>
<td>7.5(^a)</td>
<td>0.63</td>
<td>10.32(^b)</td>
<td>0.63</td>
<td>14.18</td>
<td>0.82</td>
</tr>
<tr>
<td>ColE1</td>
<td>5.40</td>
<td>0.17</td>
<td>6.77(^b)</td>
<td>0.28</td>
<td>9.1(^b)</td>
<td>0.63</td>
<td>12.38(^a)</td>
<td>0.63</td>
<td>15.77</td>
<td>0.82</td>
</tr>
<tr>
<td>Challenged</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ColE1</td>
<td>5.68</td>
<td>0.16</td>
<td>6.32(^b)</td>
<td>0.25</td>
<td>8.5(^ab)</td>
<td>0.57</td>
<td>11.99(^ab)</td>
<td>0.57</td>
<td>16.04</td>
<td>0.74</td>
</tr>
</tbody>
</table>

\(^a,b\) value within a column not sharing a common superscript are different (P < 0.05)
Figure 1. Percentage of animals exhibiting diarrhea over a ten day period after exposure to a sick, seeder animal. * indicates difference (P < 0.05) between animals fed 20 mg/kg Colicin E1 and those on a control diet with no addition. 18 days after exposure, no animals exhibited diarrhea.
Figure 2. Relative gene expression, normalized to cDNA concentration (25 ng/reaction), of cycloxyengase 2 (COX-2) in intestinal mucosa of pigs fed 20mg/kg Colicin E1 or a basal diet. Pigs that were challenged were exposed to a seeder pig showing clinical signs of post-weaning diarrhea. \(^{a,b}\) indicate \(P < 0.05\). Tissue was collected 4 weeks after exposure to the seeder animal.

**COX-2**

![Graph showing COX-2 expression levels in challenged and unchallenged pigs fed control or Colicin E1 diets.](image-url)

- \(^{a}\) indicates significant difference from control (untreated) pigs.
- \(^{b}\) indicates significant difference from pigs fed Colicin E1.
- \(^{c}\) indicates additional significant difference compared to other groups.

- **Challenged**
  - Control: Lower expression
  - Colicin E1: Higher expression

- **No Challenge**
  - Control: Normal expression
  - Colicin E1: Decreased expression
**Figure 2.** Relative gene expression of prostaglandin endoperoxide H synthase (PGHS) normalized to cDNA concentration (25 ng/reaction), in intestinal mucosa of pigs fed 20mg/kg Colicin E1 or a basal diet. Pigs that were challenged were exposed to a seeder pig showing clinical signs of post-weaning diarrhea. \(^{ab}\) indicate P < 0.05. Tissue was collected 4 weeks after exposure to the seeder animal.
**Figure 3.** Relative Gene Expression of tumor necrosis factor alpha (TNFα) normalized to cDNA concentration (25 ng/reaction), in intestinal mucosa of pigs fed 20mg/kg Colicin E1 or a basal diet. Pigs that were challenged were exposed to a seeder pig showing clinical signs of post-weaning diarrhea. \(^{a,b}\) indicate \(P < 0.05\). Tissue was collected 4 weeks after exposure to the seeder animal.
CHAPTER 3
EXPRESSION OF AN ACTIVE COLICIN E1 IN PICHIA PASTORIS

Sara A. Cutler and Chad H. Stahl

A Manuscript for submission to Biotechnology Letters

Abstract
Colicins are a class of bacteriocins produced by, and effective against, *Escherichia coli* and closely related species. Previous work in our lab has shown that Colicin E1 is highly effective against the *E. coli* strains of concern for both animal health as well as human food safety. Additionally, Colicin E1 has been shown effective against other food borne pathogens such as *Listeria monocytogenes*. The objective of this study was to produce an active Colicin E1 in a yeast expression system in order to reduce the costs associated with native colicin production and purification.

Introduction
Colicin E1 has shown potential for use both as an alternative to conventional antibiotics in animal feed as well as a novel intervention for the prevention of food borne disease in humans (Trautner, et al., 2002; Patton, et al., 2007, Schamberger, et al., 2004). Colicin E1 is naturally produced by stress induction via the SOS response in *E. coli* containing a plasmid containing the genes for Colicin E1 (*cae*), its resistance protein, and a protein required for its excretion from *E. coli*. In order to utilize this protein in food or feed systems, considerable concentration and purification from native producers is necessary. Other *E. coli* proteins have been successfully expressed in *Pichia pastoris* for use in
animal agriculture (Ouyang, et al., 2003, Lee, et al., 2006, Stahl et al., 2000). The objective of this study was to determine if an active Colicin E1 could be expressed in *P. pastoris*.

**Materials and Methods**

A plasmid containing the Colicin E1 gene (*cae*) was isolated from *E. coli* obtained from the National Collection of Type Cultures (Public Health Laboratory Service London, England) (NC50132 and NC50145). The coding sequence of Colicin E1 was amplified by PCR (forward: CACGTGTATTGGAACCCGCGGTAGCG and reverse: GCGGCCGCTCAAATCCCTAACACCTC), ligated into pGEM-T (Promega Madison, WI) and pGEMT*cae* was transformed into *E. coli* DH5α by electroporation (BTX ECM 630, 1.9kV, 200 ohms). Transformants were then grown for harvesting of this plasmid. The Colicin E1 gene was excised from the pGEM-T*cae* plasmid by digestion with *Pml* I and *Not* I (New England Biolabs Ipswich, MA), ligated into the methanol inducible yeast vector pPICZC (Invitrogen Carlsbad, CA) and transformed into *E. coli* DH5α for maintenance of the plasmid and harvesting of the plasmid for transformation into yeast (Plasmid Maxi kit Qiagen, Valencia, CA). The pPICZaC*cae* plasmid was linearized by digestion with *Sac* I (New England Biolabs), gel purified (Qiagen, QiaQuick Gel Extraction kit), and then 1μg was added to 100μl of electrocompetent *P. pastoris* X33 and these cells were transformed with 1.5kV and 200 Ohms. Electrocompetent *Pichia pastoris* strain X33 cells were prepared according to the method of Cereghino et.al, (2005). One ml of 1M sorbitol was added to a 2mm cuvette after pulsing and this was added to 1ml of yeast peptone dextrose broth (YPD). Transformants were allowed to
recover while shaking at 30°C for one hour and then were plated on YPD/Sorbitol plates with 100µg, 200µg, 500µg, and 1000µg/ml Zeocin (Invitrogen). Colonies were selected from plates with the highest Zeocin concentration and screened for Colicin E1 production by spot testing onto a lawn of DH5α and for killing activity in growing cultures of E. coli.

**Screening for Colicin E1 Activity**

Yeast colonies selected for highest Zeocin resistance were grown in YPD for 48 hours. The cultures were then induced for protein production with 1% methanol daily for two days. Cultures were pelleted in microcentrifuge tubes at 10,000×g and the supernatant was retained. The pellets were resuspended in 100 µl of 10mM Tris-HCL pH 7.6 with approximately 100 µl of sterile glass beads (400-600 µm) and then sonicated (40 Amps, 20 Watts). This mixture was then centrifuged in a microcentrifuge tube at 16,000×g for 10 min. The cell extract was sterile filtered (0.22µm) and spotted onto a lawn of DH5α to determine Colicin E1 activity. The transformant showing the largest zone of inhibition on the spot test was utilized in the expression characterization studies.

**Expression Characterization**

Fermentations with the selected pPICZαCcae X33 yeast clone were performed in YPD (yeast peptone dextrose) media until an OD600 of 15 was achieved, then methanol induction (4%) was performed at 24 hour intervals.

To identify the expression pattern of the selected clones, the cell pellets were sonicated as above and subjected to SDS-Page (4% stacking, 12% resolving, under denaturing conditions) and Western blotting on a nitrocellulose membrane with a commercially
available kit (Supersignal, Pierce). A polyclonal antibody was generated to the Colicin E1 (Antibodies, Inc) in rabbits and was used as the primary antibody. The primary antibody (1.1mg/ml) was diluted 1:15,000 in Superstart™ buffer with 0.01% Tween 20 and was applied to the membrane overnight on a rocker at 4°C. After rinsing the membrane four times with TBS, the secondary horseradish peroxidase antibody was diluted 1:5000 in TBS with 0.01% Tween 20 and allowed to incubated on a rocker for one hour. The membrane was then washed four times with TBS, blotted dry, and chemiluminescent substrate was applied. The blot was exposed using a charged coupled device camera (Fluro-Chem 8800, Alpha Innotech Corporation, San Leandro, CA) for two minutes and the resulting bands were analyzed with AlphaEaseFC software (Alpha Innotech Corporation).

**Expression of Colicin E1 mRNA**

Real time PCR was utilized to show intracellular expression of Colicin E1 mRNA over a time course. Yeast RNA was extracted using the RiboPure Yeast kit (Invitrogen, Carlsbad, CA). RNA was quantified with the Ribogreen kit (Invitrogen) and cDNA was generated with Superscript III and quantified with the OliGreen kit (Invitrogen). Twenty five ng of cDNA was used as template in the reaction and real time PCR was performed with SYBR Green using a BioRad iCycler. Primers were designed to the Colicin E1 sequence (forward, CCA TGC CCG TGA TGC AGA AAT GAA, reverse AAG GAC GGT TCT GAA GCG GAT CAT) with the PrimerQuest software from IDT (Corallville, IA).
Results

Though the construct used for this recombinant *P. pastoris* contained the α factor that is associated with extracellular expression of proteins, the Colicin E1 remained intracellularly expressed under all culture conditions tested.

The growth curve for pPICZαCcae X33 is shown in Figure 1. The Pichia was allowed to grow until to an OD\textsubscript{600} of 15 before methanol induction. After induction the yeast doubled at 48 hours, then stayed at maximal concentration for the duration of the study.

Real time PCR quantitation of mRNA expression of Colicin E1 is also shown on Figure 1. The day before induction there was no detectable measure of Colicin E1 mRNA, but 24 hours after induction with 4% methanol, a sharp increase in relative gene expression was noted with a twenty fold reduction in message at 48 hours after induction, and a drop to nearly undetectable levels in the last 3 days of the 5 day sampling period.

A Western blot showing protein production over the five day time course is Figure 2.

The largest band at 60Kd is at day two post-induction. As shown in figure two after deglycosylation with EndoHF is performed the double band is reduced. The amino acid sequence of Colicin E1 has two possible glycosylation sites at amino acids 26 and 104.

Discussion

After preliminary trials using other media, such as buffered methanol complex media (BMMY), the best Colicin E1 production was accomplished using YPD media at 27°C when the *P. pastoris* reached an OD\textsubscript{600} of 15. We also found that a larger amount of methanol was more effective for protein induction than the recommended 1%. After
preliminary trials, a 4% methanol addition once every 24 hours achieved the greatest protein expression.

Though the alpha factor from *Saccachromyces cerevisiae* was included on the PIC vector used in this expression construct, the *P pastoris* did not secrete the Colicin E1 into the supernatant after methanol induction. Explanations for this may include toxicity to the yeast cell by the colicin, that the colicin is being bound to the yeast cell membrane, or that the glycosylation is changing the structure of the colicin so that it cannot be excreted. Previous trials with the Colicin E1 attempted using a GAP vector with the alpha factor and resulting in very few transformants, possibly indicating that the colicin is toxic to the yeast cell upon excretion.

Intracellular expression allows for feeding the yeast directly to the pigs as a whole cell. Yeast supplementation in the swine diet has been associated with greater feed conversion and immunostimulatory properties (Li, et al., 2006; Eicher, et al., 2006). The use of a recombinant proteins expressed in *P pastoris* in animal feed have been previously used for nutritional, antigenic, or growth promotant applications (Stahl, et al., 2004; Fingerut, et al., 2005; Ouyang, et al., 2003).

The addition of natively produced Colicin E1 to the swine diet has proven to be an effective means to control post-weaning diarrhea (Cutler, et al., 2007), however purification costs of feeding the natively produced *E. coli* protein make this method cost prohibitive at this time. We have expressed a functional Colicin E1 protein in a yeast system. This yeast may be directly fed to pigs, thus lessening the purification costs associated with separating the Colicin E1 from the bacterial cell. Future studies will elucidate the amount of yeast inclusion in the diet to prevent PWD.
In addition to an application in the swine diet as a post-weaning diarrhea preventative, Colicin E1 has recently been shown to be effective against *Listeria monocytogenes* as a carcass spray (Patton, et al., 2007). The use of recombinant, instead of natively induced Colicin E1 could make larger scale production of Colicin E1 possible.
Figure 1. Growth of pPICZaCcae X33 as measured by optical density over a five day induction period is shown on the left axis. The first 4% methanol induction is noted by an arrow, and the yeast was induced daily thereafter. Real time PCR was used to measure mRNA expression of the Colicin E1 within the yeast cell and is shown on the right axis.
Figure 2. Western blot showing pPICZαCcae X33 in the cell extract of the yeast cells over a five day time course study. Molecular weight markers are shown on the right. On the left side of the blot the samples were subjected to EndoHF enzyme treatment to deglycosylate the protein and the Colicin E1 is shown as a single band ~60Kd. On the right side of the blot the samples were denatured.
GENERAL CONCLUSION

Post-weaning diarrhea remains a threat to swine production in the US despite the use of antibiotic feed additives and other alternative therapies utilized in post-weaning diets. With the potential for a reduction or ban on the use of prophylactic antibiotics in the swine diet, alternatives are needed to retain the economic solvency of swine production. Colicins are proteins produced by, and effective against *E. coli*, including the enterotoxigenic *Escherichia coli* (ETEC) responsible for post-weaning diarrhea. The addition of 16.5 of a mg/kg highly purified Colicin E1 (ColE1) to a post-weaning ration reduced the duration and severity of diarrhea caused by a combination of two F18+ ETEC strains over a five day study. Semi-quantitative real time PCR of the ileum revealed lower levels of mRNA expression for inflammatory cytokines IL-1β and TNFβ in the 16.5 mg/kg ColE1 fed group as compared to the control animals. These data, taken together with a lower number of ETEC challenge bacteria recovered from ColE1 fed animals 24 hours after oral challenge, suggests that the dietary ColE1 was able to reduce the numbers of ETEC able to colonize the ileum, thereby rendering it unable to cause diarrhea.

In a longer duration study, utilizing a more natural seeder pig challenge model, ColE1 was also efficacious. This study utilized a post weaning diet containing thought to be protective against PWD such as spray dried plasma and whey protein for the first two weeks. To this diet, 20 mg/kg ColE1 was added. As with commercial practice, these ingredients were removed after two weeks and all
animals were fed a corn/soy diet. Exposure to the seeder animal without dietary ColE1 supplementation resulted in similar rates of PWD as those achieved by direct oral challenge in the previous study. In this 4 week trial, the majority of the diarrhea occurred between days 4 and 10 after exposure to the sick animal, with over 80% of the non-treated animals exhibiting diarrhea during this time period. Much lower rates of diarrhea were noted in the ColE1 fed group (18% during the same period). Body weight gains were significantly higher in the ColE1 fed pigs as a result, compared to those without the dietary addition at weeks 1 and 2. In a group of pigs not exposed to ETEC challenge, no body weight differences were noted when ColE1 was added to the diet. Semi quantitative real time PCR analysis of ileum mucosa showed higher levels of TNF$\alpha$ in the challenged, non-treated animals than those with the ColE1 addition and both diets in the non-challenged room 4 weeks after exposure to the seeder animal. Levels of COX-2 and PGHS, enzymes responsible for maintenance of immune function and barrier function in the intestine, were higher in the ColE1 fed, challenged animals than any other group.

Overall, the addition of the ColE1 purified from a Colicin E1 producing $E.\ coli$ culture reduced the incidence and severity of PWD in both an oral challenge model and a seeder pig challenge model. The expense required to purify the ColE1 used in this study makes it cost prohibitive for use as an additive in swine feed at this time. For this reason, we have utilized recombinant yeast technology to express the ColE1. This method requires no purification cost because intracellular expression of the ColE1 enables the yeast cell to be directly fed to the
pigs. Future studies will include testing of this yeast, the possible use of more than one colicin to offer a broader spectrum against ETEC colonization, and molecular manipulations to the colicin sequence to make it a more effective antimicrobial for other applications, such as food safety.
ACKNOWLEDGEMENTS

The author would like to thank her major professor, Chad Stahl, for his guidance and support as I was both his graduate student and research associate. I learned a tremendous amount not only about research and laboratory work but how much effort it takes to be a good leader and I thank you for the opportunities I was granted. I hope to continue my “witchcraft” at my own expense, in my own lab. I would like to also thank Ronald Griffith for always having faith that I would eventually finish the graduate career I started 8 years ago. Thank you to him for sticking with me that long.

I thank my wonderful husband for listening to me complain on that 40 minute commute home every day. I look forward to being Dr. and Dr. Cutler soon. In my MS thesis, you were my rock and now you are my mountain. Thank you for every day.

I also am in debt to my parents, literally. The importance of education has been emphasized to me my whole life and I think I have taken this as far as it’ll go. Thank you, Dad, for planting the seed of interest in an animal science career. I am fortunate to be among the few scientists who can relate every animal study back to something practical. Thank you, Mom, for your encouragement and support. I couldn’t have done it without you.


FDA, Guidance for Industry #152: Evaluating the safety of antimicrobial new animal drugs with regard to their microbiological effects on bacteria of human health concern. 2003, CVM/FDA/DHHS.


