Dietary Inclusion of Colicin E1 Is Effective in Preventing Postweaning Diarrhea Caused by F18-Positive Escherichia coli in Pigs

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Abstract
With worldwide 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 laboratories has shown that colcin E1 is effective against some Escherichia coli strains responsible for postweaning diarrhea (PWD) in vitro. In this study we examined the efficacy of the dietary inclusion of colcin E1 in preventing experimentally induced PWD caused by F18-positive enterotoxigenic E. coli in young pigs. Twenty-four weaned pigs (23 days of age), identified by genotyping to be susceptible to F18-positive E. coli infections, were individually housed and fed diets containing 0, 11, or 16.5 mg colcin E1/kg diet. Two days after the start of the trial, all animals were orally inoculated with $1 \times 10^9$ CFU of each of two F18-positive E. coli strains isolated from pigs with PWD. The dietary inclusion of colcin E1 decreased the incidence and severity of PWD caused by F18-positive enterotoxigenic E. coli and improved the growth performance of the piglets. Additionally, the reduced incidence of PWD due to dietary colcin E1, lowered the levels of expression of the genes for interleukin 1β and tumor necrosis factor beta in ileal tissues from these animals. The dietary inclusion of colcin E1 may be an effective alternative to conventional antibiotics in the diets of weaning pigs for the prevention of PWD caused by F18-positive enterotoxigenic E. coli.

Keywords
Veterinary Microbiology and Preventive Medicine

Disciplines
Agriculture | Meat Science | Nutritional Epidemiology | Parasitology

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Dietary Inclusion of Colicin E1 Is Effective in Preventing Postweaning Diarrhea Caused by F18-Positive Escherichia coli in Pigs\(^*\)

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With worldwide 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 laboratories has shown that colicin E1 is effective against some Escherichia coli strains responsible for postweaning diarrhea (PWD) in vitro. In this study we examined the efficacy of the dietary inclusion of colicin E1 in preventing experimentally induced PWD caused by F18-positive enterotoxigenic E. coli in young pigs. Twenty-four weaned pigs (23 days of age), identified by genotyping to be susceptible to F18-positive E. coli infections, were individually housed and fed diets containing 0, 11, or 16.5 mg colicin E1/kg diet. Two days after the start of the trial, all animals were orally inoculated with \(1 \times 10^7\) CFU of each of two F18-positive \(E.\) coli strains isolated from pigs with PWD. The dietary inclusion of colicin E1 decreased the incidence and severity of PWD caused by F18-positive enterotoxigenic \(E.\) coli and improved the growth performance of the piglets. Additionally, the reduced incidence of PWD due to dietary colicin E1, lowered the levels of expression of the genes for interleukin 1\(\beta\) and tumor necrosis factor beta in ileal tissues from these animals. The dietary inclusion of colicin E1 may be an effective alternative to conventional antibiotics in the diets of weaning pigs for the prevention of PWD caused by F18-positive enterotoxigenic \(E.\) coli.

Postweaning diarrhea (PWD) is a serious threat to the economic success of the swine industry both due to losses as a result of mortalities and due to the reduced growth performance of surviving pigs. It is estimated that 50% of piglet mortality due to diarrhea is attributable to the causative agent of mortalities and due to the reduced growth performance of surviving pigs. It is estimated that 50% of piglet mortality due to diarrhea is attributable to the causative agent of postweaning diarrrhea (PWD) in vitro. In this study we examined the efficacy of the dietary inclusion of colicin E1 in preventing experimentally induced PWD caused by F18-positive enterotoxigenic \(E.\) coli in young pigs. Twenty-four weaned pigs (23 days of age), identified by genotyping to be susceptible to F18-positive \(E.\) coli infections, were individually housed and fed diets containing 0, 11, or 16.5 mg colicin E1/kg diet. Two days after the start of the trial, all animals were orally inoculated with \(1 \times 10^7\) CFU of each of two F18-positive \(E.\) coli strains isolated from pigs with PWD. The dietary inclusion of colicin E1 decreased the incidence and severity of PWD caused by F18-positive enterotoxigenic \(E.\) coli and improved the growth performance of the piglets. Additionally, the reduced incidence of PWD due to dietary colicin E1, lowered the levels of expression of the genes for interleukin 1\(\beta\) and tumor necrosis factor beta in ileal tissues from these animals. The dietary inclusion of colicin E1 may be an effective alternative to conventional antibiotics in the diets of weaning pigs for the prevention of PWD caused by F18-positive enterotoxigenic \(E.\) coli.

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**MATERIALS AND METHODS**

**Colicin production.** Colicin E1 was produced and purified to homogeneity by the method of Stahl et al. (33). Briefly, a colicin E1-producing strain of *E. coli* was grown in LB medium, and colicin production was induced by the addition of mitomycin C (EMD Biosciences, San Diego, CA) to the medium. The colicin E1 was purified from the cell-free supernatant by ion-exchange chromatography, first by using DEAE cellulose (Sigma-Aldrich, St. Louis, MO) and then by further purifying the protein with Q Sepharose (GE Healthcare, Piscataway, NJ). The purity of the colicin E1 used in this study has been visualized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and is estimated to be over 95% pure (30).

**ETEC challenge strains.** *Escherichia coli* F18-producing strains 2144 (O147:NM, where NM indicates nonmotile) and S1191 (O139) were used as challenge strains. Strain S1191 was isolated from a pig with edema disease, produces heat-stable toxins (ST) STa and STb and Shiga toxin 2e, and is chloramphenicol resistant. Strain 2144 was a field isolate that was made nalidixic acid resistant and produces heat-stable toxins STa and STb. Both strains were grown overnight as pure cultures in LB medium at 37°C with shaking. They were then individually diluted to an optical density at 600 nm (OD600) of 0.1 in fresh LB medium and allowed to grow to an OD600 of ∼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% nonfat dry milk. The challenge dose consisted of an equal amount of each strain and was determined by serial dilution and plating to provide a total of 2 × 10⁹ CFU/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) used were obtained from the Iowa State University Swine Nutrition Farm and were determined to be susceptible to F18-positive *E. coli* infections based on the restriction fragment length polymorphism test described by Frynddal et al. (15). Briefly, genomic DNA was purified (DNAeasy kit; Qiagen, Valencia, CA) from pig tail clippings, and primers (forward primer, TTGGGAACCAGATGGGACAGTATG; reverse primer, CCCGCCAGTGAGCTGCGCTGCTTCA) were used to amplify a 162-bp section of the 1.2-kb fusocystethyrase enzyme gene (ECF18R) by PCR (26). The 162-bp PCR product was then digested with HhaI (New England Biolabs, Ipswich, MA), and the resulting product was then digested with HhaI (New England Biolabs, Ipswich, MA), and the RNA was removed from the resulting digest with HhaI (New England Biolabs, Ipswich, MA), and the RNA was removed from the resulting digest with HhaI (New England Biolabs, Ipswich, MA), and the RNA was removed from the resulting digest with HhaI (New England Biolabs, Ipswich, MA), and the RNA was removed from the resulting digest with HhaI (New England Biolabs, Ipswich, MA), and the RNA was removed from the resulting digest with HhaI (New England Biolabs, Ipswich, MA), and the RNA was removed from the resulting digest with HhaI (New England Biolabs, Ipswich, MA), and the RNA was removed from the resulting digest with HhaI (New England Biolabs, Ipswich, MA), and the RNA was removed from the resulting digest with HhaI (New England Biolabs, Ipswich, MA), and the RNA was removed from the resulting digest with 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There were no differences in fecal scores between the treatment groups until 48 h postchallenge. At this point the group fed the control diet had a mean fecal score of 2.38, which tended to be higher ($P < 0.06$) than that for the group fed the 16.5-mg colicin E1/kg diet (mean score, 0.5) (Fig. 1). Over the entire study, the control group had a significantly higher ($P < 0.05$) average fecal score than the high-dose (16.5-mg/kg) colicin E1 group (2.1 and 0.59, respectively). The low-dose (11-mg/kg) colicin E1 group also had higher ($P < 0.05$) average fecal scores than the high-dose group, and these were not significantly different from those for the control pigs. These differences in group fecal scores were caused by the incidence of diarrhea (fecal scores, $\geq 4$) in one, four, and five of the eight control group pigs 24, 48, and 72 h after ETEC inoculation, respectively. Among the pigs fed the low dose of colicin E1, no animals had diarrhea at 24 h postinoculation; however, three and four pigs had diarrhea at 48 and 72 h postchallenge, respectively. None of the pigs fed the high dose of colicin E1 had any incidence of diarrhea at any time during the experiment.

**Growth performance.** The dietary inclusion of colicin E1 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, the pigs fed the control diet gained an average of 380 g, while the pigs receiving the low and high doses of colicin E1 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 colicin E1. The control group of animals averaged a total consumption of 1.54 kg of diet, while the pigs treated with the low and high doses of colicin E1 ate 1.22 kg and 1.44 kg, 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. This was because over the length of the study two of the pigs in the control group lost weight (approximately 400 g each) and one pig gained virtually no weight (less than 10 g) while still consuming feed. This negative body weight gain resulted in a negative feed conversion efficiency, which resulted in tremendous variation in the feed conversion efficiency values for this group.

**Bacterial enumeration. (i) Fecal cultures.** No colonies were isolated on the agar plates containing either chloramphenicol or nalidixic acid from any samples prior to the ETEC challenge. At 24 h postinoculation, the levels of both total coliforms and ETEC challenge strain 2144 recovered in the feces were significantly higher ($P < 0.05$) for the control animals than for both groups of colicin E1-fed pigs (Table 3). There were no significant differences between the groups in the recovery of ETEC strain S1191 at the first day postchallenge, but on the following day there was a reduction in the high-dose colicin E1 group compared to that in the controls ($P < 0.05$). By the last day of fecal sampling, the levels of strain S1191 had dropped below our detection limit for most of the animals, regardless of the dietary treatment (16/24 pigs), and there were no significant differences in the amount of strain 2144 recovered.

(ii) **Tissues.** In the ileum, both colicin E1-fed groups averaged lower levels ($P < 0.05$) of the S1191 challenge strain and coliforms compared to those in the controls (Table 4). There were no significant differences in the recovery of bacteria from the cecal samples among the groups. Strain S1191 was recovered from samples from only three of the eight animals in the high-dose colicin E1 group and was recovered from six and four of the eight animals in the control and the low-dose colicin E1 treatment groups, respectively. In the rectum, similar levels of total coliforms and strains 2144 and S1191 were recovered from the animals in all groups.

**Gene expression.** The concentration of TNF-β mRNA in ileal tissue was higher ($P < 0.06$) in the control animals than in either of the colicin E1-treated groups (Fig. 2). The amount of IL-1β mRNA was the highest in the group supplemented with the low dose of colicin E1 (sixfold higher [$P < 0.01$] than the amount in the controls), whereas the group supplemented with the high dose of colicin E1 had nearly undetectable levels of IL-1β mRNA (Fig. 2). The levels of expression of both TNF-α and iNOS tended ($P < 0.1$) to be greater in the control animals than in the animals receiving the diets with colicin E1 supplementation. There were no significant differences in the concentrations of gamma IFN (IFN-γ), IL-8, or IL-10 mRNA in the ileum among any of the groups.

**DISCUSSION**

We examined the efficacy of colicin E1 in preventing PWD caused by F18-producing *E. coli* because this disease has been estimated to be responsible for as much as 50% of the economic losses seen in the production of weaned pigs (19, 36). In

### Table 2. Effect of dietary inclusion of colicin E1 on growth performance of weaning pigs

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Wt gain (kg)</th>
<th>Feed intake (kg)</th>
<th>Feed efficiency$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.38$^{**}$ (0.13)</td>
<td>1.53$^b$ (0.54)</td>
<td>27.01 (0.55)</td>
</tr>
<tr>
<td>Low dose</td>
<td>0.54$^{***}$ (0.19)</td>
<td>1.21$^b$ (0.42)</td>
<td>2.83 (1.00)</td>
</tr>
<tr>
<td>High dose</td>
<td>0.94$^{***}$ (0.33)</td>
<td>1.44$^b$ (0.51)</td>
<td>1.94 (0.69)</td>
</tr>
</tbody>
</table>

$^a$ The values presented are means (standard errors of the means).

$^b$ The control, low-dose, and high-dose treatments contained 0, 11, and 16.5 mg colicin E1/kg of diet, respectively.

$^c$ Values within a column not sharing a common symbol are significantly different ($P < 0.05$).

$^d$ Feed efficiency is defined as feed intake/weight gain.
herds with PWD, mortality of up to 2% (21, 37) can be seen among weaning pigs, but of greater economic significance is the morbidity and reduction in growth performance of the pigs that survive these infections. Although there is a need for alternatives to conventional antibiotics, the development of compounds that can be used to combat ETEC-associated PWD is beset with difficulties, including the availability of an adequate experimental model of ETEC infection. Establishing a good challenge model for this disease has proven to be difficult. Madec et al. (24) used four strains of ETEC and several different dosing methods in six trials with a total of 168 specific-pathogen-free piglets and was able to generate clinically significant, although transient, diarrhea in only 50% of the animals (24). The use of a viral cochallenge agent, such as transmissible gastroenteritis or rotavirus, that leads to immunosuppression and intestinal membrane disruption can also increase experimental ETEC infection rates (1, 9, 16, 28).

Although our high dose of colicin E1 (16.5 mg/kg of diet) was able to eliminate all clinical signs of PWD, our low dose of pigs to ETEC infection due to a transient intestinal inflammatory response (2, 10). Diets designed to be fed to pigs immediately postweaning typically provide over 40% of the total protein in the diet as protein from animal sources, in part to help prevent this temporary inflammatory response. However, due to the higher cost of animal-based protein sources, there is a constant push to minimize the amount of animal protein included in pig diets. While both viral cochallenges 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 prescreening for an F18 receptor polymorphism, the rate of infection can be increased from 5.9% in pigs with the F18-resistant genotype to 87% in those that are genetically susceptible (15). In our study, we used only genetically susceptible animals, as well as a weaning diet that contained no animal protein. With this model we achieved a 75% infection rate in control animals, with no mortality after 4 days, with no use of viral co-infection, and with the use of an easily performed one-time oral challenge.

Although our high dose of colicin E1 (16.5 mg/kg of diet) was able to eliminate all clinical signs of PWD, our low dose of colicin E1 appeared to be able to slightly delay only the onset of PWD, mortality of up to 2% (21, 37) can be seen among weaning pigs, but of greater economic significance is the morbidity and reduction in growth performance of the pigs that survive these infections. Although there is a need for alternatives to conventional antibiotics, the development of compounds that can be used to combat ETEC-associated PWD is beset with difficulties, including the availability of an adequate experimental model of ETEC infection. Establishing a good challenge model for this disease has proven to be difficult. Madec et al. (24) used four strains of ETEC and several different dosing methods in six trials with a total of 168 specific-pathogen-free piglets and was able to generate clinically significant, although transient, diarrhea in only 50% of the animals (24). The use of a viral cochallenge agent, such as transmissible gastroenteritis or rotavirus, that leads to immunosuppression and intestinal membrane disruption can also increase experimental ETEC infection rates (1, 9, 16, 28).

## Table 3: Effect of dietary inclusion of colicin E1 on E. coli levels recovered from the feces of weaning pigs

<table>
<thead>
<tr>
<th>Postchallenge time and treatment</th>
<th>Total coliforms</th>
<th>E. coli 2144</th>
<th>E. coli S1191</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of CFU/g</td>
<td>E. coli</td>
<td>E. coli</td>
</tr>
<tr>
<td>1 day</td>
<td></td>
<td>2144</td>
<td>S1191</td>
</tr>
<tr>
<td>Control</td>
<td>1.1 × 10⁶*</td>
<td>1.7 × 10⁶</td>
<td>4.9 × 10⁴</td>
</tr>
<tr>
<td>Low dose</td>
<td>1.5 × 10⁷</td>
<td>1.2 × 10⁷</td>
<td>7.4 × 10⁴</td>
</tr>
<tr>
<td>High dose</td>
<td>1.3 × 10⁸</td>
<td>3.7 × 10⁸</td>
<td>4.8 × 10⁴</td>
</tr>
<tr>
<td>2 days</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7.6 × 10⁶</td>
<td>1.6 × 10⁶</td>
<td>4.7 × 10⁵</td>
</tr>
<tr>
<td>Low dose</td>
<td>5.0 × 10⁷</td>
<td>9.1 × 10⁷</td>
<td>3.2 × 10⁵</td>
</tr>
<tr>
<td>High dose</td>
<td>6.0 × 10⁸</td>
<td>1.9 × 10⁸</td>
<td>2.4 × 10⁵</td>
</tr>
<tr>
<td>3 days</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>8.0 × 10⁶</td>
<td>6.3 × 10⁶</td>
<td>6.6 × 10⁵</td>
</tr>
<tr>
<td>Low dose</td>
<td>9.4 × 10⁷</td>
<td>2.9 × 10⁷</td>
<td>3.6 × 10⁵</td>
</tr>
<tr>
<td>High dose</td>
<td>2.3 × 10⁸</td>
<td>3.9 × 10⁸</td>
<td>7.5 × 10⁵</td>
</tr>
</tbody>
</table>

*The control, low-dose, and high-dose treatments contained 0, 11, and 16.5 mg colicin E1/kg of diet, respectively.

## Table 4: Dietary inclusion of colicin E1 affected bacterial recovery in the ileum of weaning pigs

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total coliforms</th>
<th>E. coli 2144</th>
<th>E. coli S1191</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of CFU/g</td>
<td>E. coli</td>
<td>E. coli</td>
</tr>
<tr>
<td>Control</td>
<td>4.7 × 10⁶*</td>
<td>3.2 × 10⁶</td>
<td>2.7 × 10⁵</td>
</tr>
<tr>
<td>Low dose</td>
<td>1.1 × 10⁷</td>
<td>5.6 × 10⁷</td>
<td>1.0 × 10⁹</td>
</tr>
<tr>
<td>High dose</td>
<td>3.8 × 10⁸</td>
<td>2.7 × 10⁹</td>
<td>2.5 × 10⁹</td>
</tr>
</tbody>
</table>

*The control, low-dose, and high-dose treatments contained 0, 11, and 16.5 mg colicin E1/kg of diet, respectively.

*The values presented are least-square means (standard errors of the means).

*Escherichia coli strain 2144 is F18 positive, produces the toxins STa and STb, and was made nalidixic acid resistant.

*Escherichia coli strain S1191 is F18 positive, produces the toxins STa and STb and Shiga toxin 2e, and is chloramphenicol resistant.

*Values within a day and a column not sharing a common symbol are significantly different (P < 0.05).
inclusion may have been the determining factor in the development of clinical disease. Colicin-fed animals had significantly lower levels ($P < 0.04$) of fecal shedding of the strain 2144 challenge bacteria at 1 day postchallenge. 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. By the end of the study there were no significant differences in the fecal shedding of either challenge strain on the basis of colicin treatment. We would not anticipate that the reduced levels of viable bacteria that reached the ileum in a viable state, since colicin E1 is highly sensitive to proteolysis (3, 4, 6). It is likely that not all of the $E. coli$ bacteria in our large challenge dose would be killed by the colicin E1 present in the digesta prior to the inactivation of the colicin E1 by proteolysis, but it appears that enough were eliminated as a result of high-dose colicin E1 supplementation to prevent disease. The gene expression data also suggest that fewer of the challenge bacteria were able to cause the inflammatory response leading to diarrhea in the pigs. This is supported by the lower levels of expression ($P < 0.05$) of IL-1β and TNF-β mRNA in the ileal tissues of pigs fed the high-dose colicin E1 diet compared with those in the control group. IL-1β is primarily secreted by macrophages and activates lymphocytes during an inflammatory response, and increases have been associated with $E. coli$ toxin production (11, 17). While the concentration of IL-1β mRNA was significantly lower in the ileal tissue of the pigs fed the high dose of colicin E1 than in the ileal tissue of the control pigs, pigs in the low-colicin E1-dose group had mRNA levels that were over sixfold higher than those in the control group. At the time of tissue collection there was no longer any significant difference in fecal scores between the low-dose and the control groups, and this elevated expression may indicate a delay in the inflammatory response as a result of the challenge bacteria. This would be reasonable, since there appeared to be a delay in the onset of PWD in the pigs fed the low dose of colicin E1 compared with the time of onset in the control animals. An increase in IL-1β gene expression in the intestinal mucosa has been correlated with enteropathogenic $E. coli$ challenge (16), although the associated increases in the expression of IL-6, IL-8, and IL-10 seen by Girard et al. (16) were not noted in our study. Regardless of the dose, colicin E1 in the diet caused reduced levels ($P < 0.06$) of expression of TNF-β, TNF-β, also known as lymphotoxin, is a primary effector of NO production and is associated with inflammatory responses related to T-cell recruitment (13). The lower levels of TNF-β and IL-1β in the ileal tissues of pigs fed the high dose of colicin E1 compared with those in the control animals suggest that colicin E1 was able to significantly reduce the bacterial load that initially reached the ileum in these animals, thereby reducing the inflammatory response to the ETEC challenge.

While other researchers have examined the efficacy of using colicin-producing bacterial cultures as probiotics for cattle in order to reduce $E. coli$ O157 contamination (32), 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 colicin E1 can prevent experimentally induced PWD. The efficacy in preventing PWD at a level of dietary inclusion of 16.5 mg/kg of diet suggests that colicin E1 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 colicin E1 against many ETEC strains of concern for human food safety has been well documented (5, 8, 31).

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