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The Effects of Grounded Herbs on the Intestinal Villus Height and Shedding of F18-positive *Escherichia coli* in Weaned Pigs

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ABSTRACT

Antibiotics have been widely used to control and treat infections caused by *Escherichia coli* (*E. coli*) in weaned pigs. The bacteria resistance to antibiotics can occur naturally; however, the misuse of antibiotics can accelerate this resistance. New antibiotics are developed very slowly, and only two new classes of antibiotics have been developed in the past 40 years. This makes herbal medicine a promising method for fighting against antibiotic-resistant bacteria. In the current study, 25 male crossbred (Duroc x Landrace x Large white) weaned piglets with an average weight of 6-8 kg were examined for 24 days. The pigs were randomly assigned to five groups in a completely randomized design with five replicates (1 pig/pen). All treatments included 20% crude protein corn-soybean as the basal diet. The negative control group received no supplementation, while pigs in the second experimental group received a basal diet supplemented with 150 ppm colistin sulfate. Basal diet and herbal mixture (*Andrographis paniculata*, *Zingiber cassumunar*, and *Garcinia mangostana*) were fed to three other experimental groups at 500, 1000, and 2000 ppm. The F18-positive, colistin-resistant *E. coli* were orally inoculated to all pigs for 9 days. The antibacterial and anti-diarrheal effects of this diet and its effect on the inoculated pigs' intestinal villi were evaluated. The results indicated that supplementation of this herbal mixture at levels of 500, 1000, and 2000 ppm had antibacterial effects, with no significant difference between doses. However, the positive effects of this herbal mixture on intestinal villi height and diarrhea were found only in pigs that received 1000 and 2000 ppm of the herbal mixture. From a practical point of view, supplementation of this herbal mixture at 500 and 1000 ppm could be applied for prophylaxis during the weaning period, whereas 2000 ppm of the herbal mixture could be used for the treatment of postweaning *E. coli* diarrhea.

Keywords: *Andrographis paniculate*, *Escherichia coli*, *Garcinia mangostana*, Herbal mixture, *Zingiber cassumunar*

INTRODUCTION

Antibiotic resistance has been among the greatest threats to global health and food security for over four decades (Swann Committee, 1969). One of the accelerating factors of antibiotic resistance is its misuse in humans and animals (WHO, 2020).

Pork is the second most popular kind of meat in terms of global consumption (Shahbandeh, 2022). The most common cause of illness and death in weaners is a gastrointestinal infection resulting from *Escherichia coli* (*E. coli*), which causes diarrhea in neonatal and weaned piglets (Aarestrup et al., 2008). Postweaning *E. coli* diarrhea (PWECD) and edema disease (ED) are common in weaned pigs. The PWECD and ED occur mainly in the first week after weaning (Bertschinger et al., 2000). The death rates of affected pigs may be as high as 25% and 90% in PWECD and ED, respectively (Fairbrother and Nadeau, 2019). The profitability of pig farms affected by PWECD and ED will decrease due to high mortality rates, decreased weight gain, and the high cost of treatments, vaccinations, and feed supplements (Wang et al., 2019). The response to the outbreak of diarrhea caused by *E. coli* needs instant action, such as antibiotics treatment. However, considerable evidence shows that antibiotic therapy in swine induces the selection of resistant bacteria (Österberg et al., 2016; Nguyet et al., 2022). Despite the growing number of antimicrobial-resistant *E. coli* strains found worldwide in pig farms (Peng et al., 2022), the pace of developing new antibiotics has been sluggish. Only two new classes of antibiotics have been developed in the last 40 years. This makes herbal medicine a promising means of fighting antibiotic-resistant bacteria (Adzitey et al., 2019). Nevertheless, the cautionary reminder to use herbs with care is highlighted by the reports of bacterial resistance to herbal antimicrobials reviewed by Vadhana et al. (2015).

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Resistance may develop if only one active principle is involved. Resistance is less likely to occur if multiple active principles are involved (Gupta and Birdi, 2017).

MuPlus® is produced in Thailand from the powder of three grounded herbs, namely *Andrographis paniculata* (*A. paniculata*), *Zingiber cassumunar* (*Z. cassumunar*), and *Garcinia mangostana* (*G. mangostana*). Each powder has shown a wide range of biological activities. For instance, *A. paniculata* has anti-inflammatory (Shen et al., 2002), anti-allergic (Xia et al., 2004), anti-platelet aggregation (Amroyan et al., 1999; Lu et al., 2011), hepatoprotective (Trivedi and Rawal, 2001), anti-HIV (Reddy et al., 2005), antibacterial (Mishra et al., 2013), and anti-diarrheal (Gupta et al., 1990) properties. The *Z. cassumunar* has pharmacological properties, such as antimicrobial, antioxidant, insecticidal, anti-cancer, anti-cholinesterase, and anti-inflammatory activities (Singh et al., 2015.(Finally, the pharmacological activities of *G. mangostana* are antioxidant activities, antitumoral, anti-inflammatory, antiallergy, antibacterial, antifungal, and antiviral properties (Pedraza-Chaverri et al., 2008; Obolskiy et al., 2009). However, these effects have been determined based on single bioactive phytoconstituents of the herbs, and most reports were derived from *in vitro* tests. Studies regarding these herbs in animals mostly involve growth performance (Herawati et al., 2020; Shi et al., 2020) and treatment of some diseases, such as Influenza (Chen et al., 2009) and Mycoplasma gallisepticum (Luo et al., 2022).

In the present study, the antibacterial and anti-diarrheal effects of a combination of these herbs and their effects on intestinal villi height were demonstrated in weaned pigs hosting hemolytic *E. coli* F18.

MATERIALS AND METHODS

Ethical approval

The Institutional Animal Care and Use Committee of Khon Kaen University, Thailand, reviewed and approved the experimental protocol based on the Ethic of Animal Experimentation of the National Research Council of Thailand (IACUC-KKU-60/63).

Escherichia coli strain

Weaned pigs with a sign of convulsion and edema of eyelids from a backyard farm in Khon Kaen province was submitted to the Faculty of Veterinary Medicine, Khon Kaen University, Thailand, for disease diagnosis. Hemolytic *E. coli* was isolated from the intestinal contents of the pigs. At necropsy, edema of mesocolon was also observed. A pure colony of isolated hemolytic *E. coli* strain selected for the experiment was detected for genes encoding F18ac+fimbrial adhesin, heat-stable toxin a (Sta), and verotoxin (STx2e). In addition, the antibiotic-resistant profile was also seen. Gene encoding of the major fimbrial subunit of F18 (fedA) was detected using fedA-specific primers (forward primer FedA 1: 5'-GTGAAAAGACTAGTGTTTATTTC-3' and reward primer FedA 2: 5'-CTTGTAAGTAACCGCGTAAGC-3', size of the amplified product 510 bp) in accordance with Imberecht et al. (1992). The PCR products (fedA) were digested with restriction enzyme *Ngo*MIV (New England Biolabs, USA) following the supplier's instructions, and the size of fedA was observed after electrophoresis on 1.5% agarose gel as described by Imberecht et al. (1994). The presence of Sta was confirmed using Sta primers (forward Sta1: 5'-tctttcccctctttagtcag-3', and reward Sta2: 5'-acaggcaggattacaacaaag-3', size of the amplified product 166 bp) according to Osek et al., (1999) whereas verotoxin-producing gene was confirmed using STx2e primers (forward STx2e -a: 5'-ccttaactaaaaggaatata-3' and reward STx2e -b: 5'-ctggtggtgtatgattaata-3', size of the amplified product 230 bp) according to Johnson et al., (1990). The isolated *E. coli* strain designated as F18ac+StaSTx2e virotype is resistant to colistin sulfate (CS) and was kept in 25% glycerol at 80°C until used.

Herbal mixture

The herbal mixture used (MuPlus®, Thailand) was supplied by Lily FoodAnSci Ltd, based in Thailand. The powder blend consisted of three herbs, namely *A. paniculata*, *Z. cassumunar*, and *G. mangostana*. The *A. paniculata* and *Z. cassumunar* were standardized to include 6% andrographolide and 0.8% volatile oil, while *G. mangostana* contained tannin and xanthones.

Pigs

A total of 25 male crossbred (Duroc × Landrace × Large white) weaned piglets weighing 6-8 kg from the Faculty of Agriculture demonstration farm at Khon Kaen University, Thailand, were used. The piglets arrived at the experimental animal building of the Faculty of Veterinary Medicine at Khon Kean University in Thailand at the beginning of February 2020. The facility was disinfected with a quaternary ammonium compound three days before the start of the experiment to acclimate the animals. Each pig was housed on a concrete floor in an individual stall measuring 1.5 x 1 meters. The piglets were randomly assigned to 5 groups in a completely randomized design with 5 replicates (1 pigs/pen) for 24 days. All treatments used 20% crude protein corn-soybean diet without antimicrobials, zinc oxide, or organic acids as the basal diet. The negative control group received no supplementation, while pigs in the second

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experimental group received a basal diet supplemented with 150 ppm colistin sulfate. Basal diet and herbal mixture (*Andrographis paniculata*, *Zingiber cassumunar*, and *Garcinia mangostana*) were fed to three other experimental groups at 500, 1000, and 2000 ppm. The F18-positive, colistin-resistant *E. coli* were orally inoculated to all pigs for 9 days. Feed mixed with different supplements was fed to the pigs from the beginning of the study, the day pigs were inoculated with hemolytic *E. coli* until day 24.

Inoculum and inoculation

The *E. coli* was prepared following Frydendahl et al. (2003). For each inoculation, *E. coli* was grown at 37°C overnight on blood agar plates. A loopful (10 µl) of colony material was taken from the blood agar and suspended in 50 ml luria-bertani broth. The bottle was incubated overnight at 37°C with shaking. The bacterial culture was centrifuged, and the pellet was suspended in 900 ml of sterile phosphate buffer solution (PBS). This suspension was adjusted to 10^8 CFU/ml adding PBS until the optical density reached 0.1 by the measure at the wavelength of 625 nanometers. The pigs were orally challenged with 1 ml of this suspension daily for up to 9 days.

Clinical sign and fecal scoring

The pigs were checked twice daily for 24 days for clinical signs such as diarrhea, edema, dehydration, anorexia, depression, vomiting, and death. The fecal scoring was performed by the same animal caregiver throughout the experiment. To determine the severity of postweaning diarrhea, the feces were scored by determining the moisture content as hard feces (0), firm feces (1), soft and formed feces (2), diarrhea with unformed and fluid feces (3), and severe diarrhea with watery and frothy feces (4; Siriwathananukul et al., 2010). Piglets with fecal scores higher than 2 were determined to have diarrhea. The pig that died during the experiment was sent to the department of Pathobiology, Faculty of Veterinary Medicine, Khon Kaen University, Thailand, for a postmortem examination. The identification of hemolytic *E. coli* was confirmed using a standard microbiological test (Quinn et al., 2004).

Quantification of *Escherichia coli* in feces

Fecal samples were collected daily from each pig in a clean plastic bag and sealed with rubber rings. The *E. coli* in the feces was measured on the day the feces were collected. One gram of feces was suspended in 9 ml of PBS. A 10-fold serial dilution was prepared. The Miles–Misra technique (for example, the drop count method) was applied to quantify *E. coli* (Miles et al., 1938). Eight drops of 0.02 ml samples were placed on blood agar. The blood agar plates were incubated at 37°C for 24 hours after the drops were dry. Hemolytic bacterial colonies with less than 40 colonies per drop were counted. The CFU number of *E. coli* in each gram of feces was calculated according to Formula 1 (Petersen and McLaughlin, 2016).

$$X = N \times 10^n \times 50 \text{ CFU / gram} \qquad \text{(Formula 1)}$$

Where, X denotes bacteria counted per gram of feces, N is the average bacterial count per 0.02 ml, and n signifies dilution factor of bacteria counted

Sampling of the small intestine

At the end of the experiment, two pigs per group were intramuscularly sedated with 2 mg/kg of azaperone following Hendrikson et al. (1995), and euthanized with an overdose of intravenous barbiturates. Two cross-sectional pieces of 1 cm length from the duodenum (6 inches distal to the pylorus), jejunum (24 inches distal to the pylorus), and ileum (12 inches proximal to the ileocecal valve) were collected from each euthanized pig immediately after death. The first piece was immediately dipped in 10% formaldehyde, whereas the second piece was longitudinally cut and pinned to a piece of styrofoam with the serosal side down. The longitudinally cut piece was then dipped in 10% formaldehyde. The intestine samples were gradually dehydrated, sectioned at 4 µm, and stained with hematoxylin-eosin, according to Nabuurs et al. (1993). One transverse sample from the cross-sectional cut and one from the longitudinal cut from each part of the intestine were mounted per slide. The intestinal morphology was recorded based on a study by Pluske et al. (1996). The height of eight intact villi (10 x objective) was measured, and the results were recorded as mean villous height in µm. The intestinal morphology was captured with the EVOSTM Core Imaging System (Invitrogen) at three megapixels, and the images were analyzed using IMAGEJ software (NIH, USA; Schneider et al., 2012).

Statistical analysis

The parameters in the current study, including the height of the villi, average fecal score, and average colony number of pathogenic *E. coli* score in 6 periods (1-3, 4-9, 10-15, 16-20, 21-24 and 1-24 days) were individually analyzed by one-way analysis of variance (one-way ANOVA) using R version 4.2.0, Vienna, Austria (R Project, 2022) and the R packages of tidyverse version 1.3.1 (Wickham et al., 2019), ggpubr version 0.4.0, ggplot2 version 3.3.6 (Kassambara, 2020) and cowplot version 1.1.1 (Wilke, 2020) were used to manipulate the data and graphic visualization. Tukey HSD

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was used to compare least-squares means between groups when overall significance for that effect was found. A p-value of ≤ 0.05 determined statistical significance.

RESULTS

Clinical results

One pig that received 150 ppm of CS as a supplement died on day 14 of the experiment. The pig was in good condition until being found dead in the morning without any signs of prior sickness. Its eyelids were swollen, its mesocolon was edematous, and its lymph nodes were enlarged. There was a pure culture of a beta-hemolytic colony derived from the mesenteric lymph nodes. Further biochemical tests, such as indole, methyl red citrate, and urease, indicated that this colony was *E. coli*, resistant to oxytetracycline, penicillin, CS, sulfamethoxazole/trimethoprim, and amoxicillin. No fimbria F18 was detected by fedA-specific primer (Imberecht et al., 1992), but a verotoxin gene was found using STx2e primers (Johnson et al., 1990).

Fecal shedding of hemolytic *Escherichia coli*

The number of hemolytic *E. coli* colonies shed was significantly affected by the supplement (antibiotic and herbal mixture) added to the pig feed ($p < 0.05$). The average number of hemolytic *E. coli* colonies in pigs that received no supplementation in their basal diet (negative control) was significantly higher than that of pigs receiving CS, 500 ppm, 1000 ppm, and 2000 ppm of the herbal mixture ($p < 0.05$). No significant difference was observed between pigs that received CS and those that received all three doses of the herbal mixture ($p > 0.05$). Moreover, different doses of the herbal mixture led to no significant difference between the groups ($p > 0.05$; Figure 1a). During the first three days of the experiment, no significant difference was observed in the feces of pigs receiving different treatments regarding hemolytic *E. coli* ($p > 0.05$, Figure 1b). Significant differences in the number of hemolytic *E. coli* shed were observed between pigs receiving no supplementation and those receiving other treatments from days 4-9 of the experiment ($p < 0.05$, Figure 1c). In this period, pigs that received 2000 ppm of the herbal mixture shed significantly higher amounts of hemolytic *E. coli*, compared to pigs that received CS. After hemolytic *E. coli* challenges stopped at day 9, differences in the number of hemolytic *E. coli* shed between treatment groups were significantly observed from days 10-15 ($p < 0.05$), 16-20 ($p < 0.05$), and 21-24 ($p < 0.05$) of the experiment (Figure 1 d, e, f). Pigs without supplements shed the highest number of hemolytic *E. coli* from days 10-15, 16-20, and 21-24. A higher level of hemolytic *E. coli* shedding was observed in pigs that received CS, compared to pigs that received all doses of the herbal mixture from days 10-15. However, differences in shedding resulting from different doses of the herbal mixture were not observed.

The excreted number of colonies in the negative control group increased over time and peaked on day 17 of the experiment (8 days after the challenges stopped). In contrast, when the challenges were stopped, the pigs that received the herbal mixture indicated decreasing hemolytic *E. coli* shedding levels. The excreted number of hemolytic *E. coli* shed by pigs that received CS continued to increase until 3 days after the challenges were stopped (Figure 1g).

Average fecal score

The result showed that pigs fed no supplementation and 500 ppm of the herbal mixture had fecal scores higher than 2, indicating diarrhea (Figure 2a). The overall average fecal scores of pigs in the negative control group were significantly higher than the average fecal scores of pigs that received CS, 500, 1000, and 2000 ppm of the herbal mixture ($p < 0.05$). Pigs administered CS had significantly lower fecal scores (less diarrhea) than pigs receiving 500 ppm of herbal mixture and significantly higher fecal scores than pigs receiving 2000 ppm of the herbal mixture ($p < 0.05$). Looking at pigs that received the herbal mixture at different doses, pigs that received 500 ppm had significantly higher fecal scores than pigs fed 1000 and 2000 ppm ($p < 0.05$). During days 1-3 of the experiment, only pigs that received 2000 ppm of the herbal mixture had significantly lower fecal scores (less than 2), compared to those that received other treatments ($p < 0.05$, Figure 2b). From days 4 to 9 of the experiment, fecal scores higher than 2, which indicated diarrhea, were observed in pigs that received no supplementation and 500 ppm of the herbal mixture (Figure 2c). Pigs that received no supplementation showed significantly higher fecal scores than other treatments. The fecal scores of pigs receiving 500 ppm of the herbal mixture were significantly higher than those receiving 1000 and 2000 ppm ($p < 0.05$). During days 10-15 of the experiment, the fecal scores of pigs that received no supplementation, CS, and 500 ppm of the herbal mixture were higher than 2, and no difference in fecal scores was observed. However, pigs receiving 1000 and 2000 ppm of the herbal mixture had fecal scores equal to or less than 2 and significantly lower fecal scores than other groups ($p < 0.05$, Figure 2d). From days 16 to 20 of the experiment, pigs that received supplements showed fecal scores equal to or less than 2, whereas pigs that received no supplementation had fecal scores higher than 2. Significantly lower fecal scores were observed in pigs that received CS and 1000 or 2000 ppm of the herbal mixture, compared to pigs that received no supplementation ($p < 0.05$, Figure 2e). From days 21 to 24 of the experiment, the pigs in all treatment groups

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indicated fecal scores lower than 2. However, pigs that received no supplementation still had significantly higher fecal scores compared to pigs that received CS and 1000 or 2000 ppm of the herbal mixture ($p < 0.05$, Figure 2f). The daily pattern of fecal score changes in Figure 2f shows that fecal scores decreased over time. The diarrheal periods of pigs that received no supplementation, CS, and 500, 1000, and 2000 ppm of herbal mixture were 21, 14, 17, 7, and 0 days, respectively (Figure 2g).

Intestinal villous height

The average villus heights of the pigs are presented in Figure 3. Pigs that received CS, 1000, or 2000 ppm of the herbal mixture revealed significantly higher villi, compared to pigs that received no feed supplementation or 500 ppm of the herbal mixture ($p < 0.05$; Figure 3a). Regarding the duodenum and jejunum, pigs in the negative control group or those receiving 500 ppm of the herbal mixture showed significantly lower villus heights ($p < 0.05$), compared to pigs that received CS and 1000 or 2000 ppm of the herbal mixture (Figure 3b and c). In the ileum, significantly lower villus heights were found in pigs that received no feed supplementation, compared to pigs that received CS and 1000 or 2000 ppm of the herbal mixture ($p < 0.05$; Figure 3d).

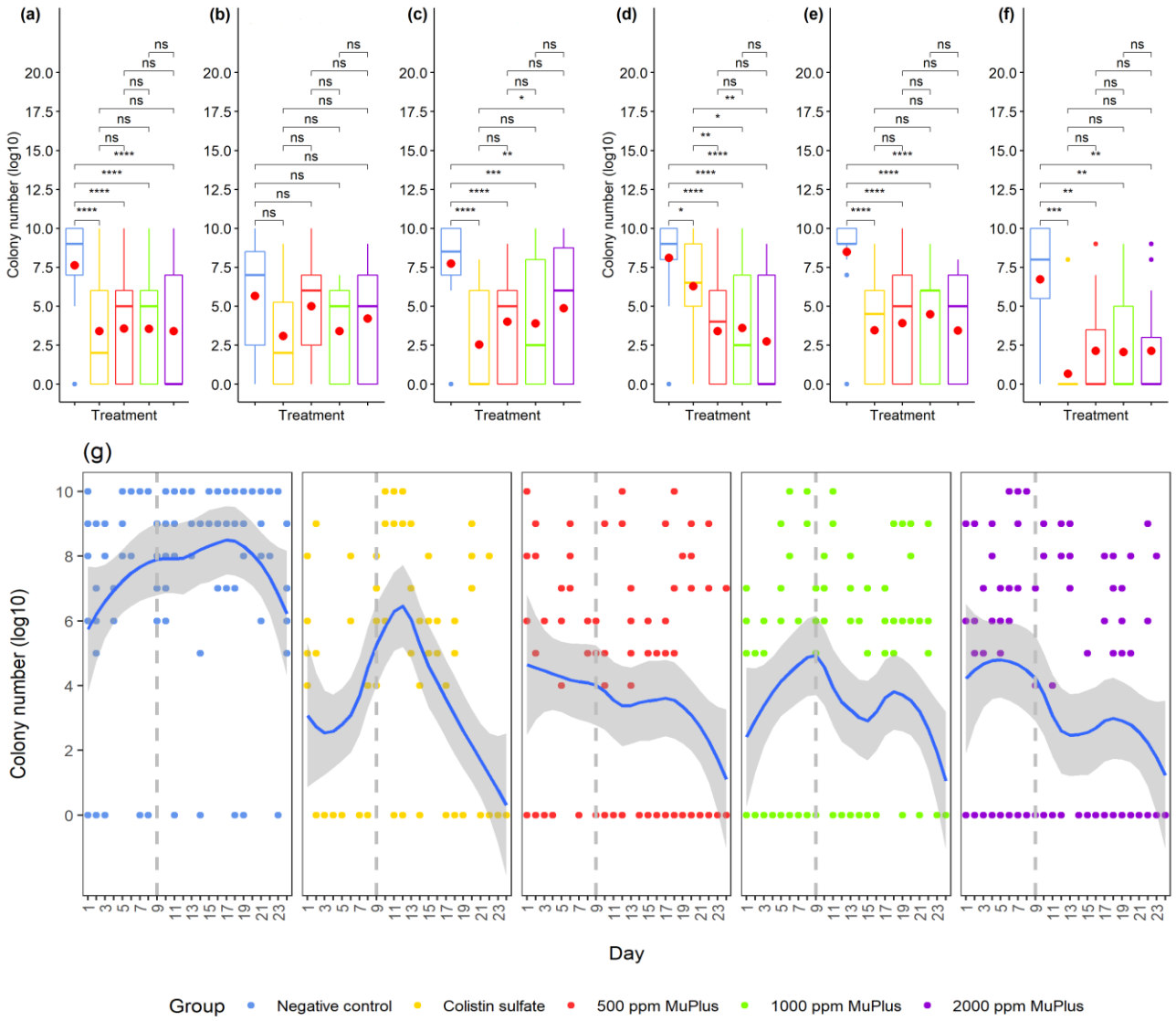

Figure 1. The number of colonies per gram feces of male crossbred (Duroc × Landrace × Large white) pigs challenged with hemolytic *E. coli*. The pigs were randomly assigned to 5 groups which were no supplementation (negative control), 150 ppm CS, 500, 1000 and 2000 ppm of herbal mixture beginning on day one of the experiment. **a:** Overall average colony number in feces of pigs (days 1-24). **b:** Average colony number in feces of pigs from days 1-3. **c:** Average colony number in feces of pigs from days 4-9. **d:** Average colony number in feces of pigs from days 10-15. **e:** Average colony number in feces of pigs from days 16-20. **f:** Average colony number in feces of pigs from days 20-24. The red circles indicate the mean of each group. ****: $p < 0.0001$, ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$, and ns: $p \geq 0.05$. **g:** Log10 colony number change from the beginning to the end of the experiment (day 24). The dots are the data points. The solid curve is the estimated local regression curve. Gray shading indicates the 95% confidence interval. The grey dashed line running vertically illustrates the final day the pigs received hemolytic *E.coli* treatment. Graph were plotted with ggplot2 package and arranged into complex compound figures by cowplot package in R program.

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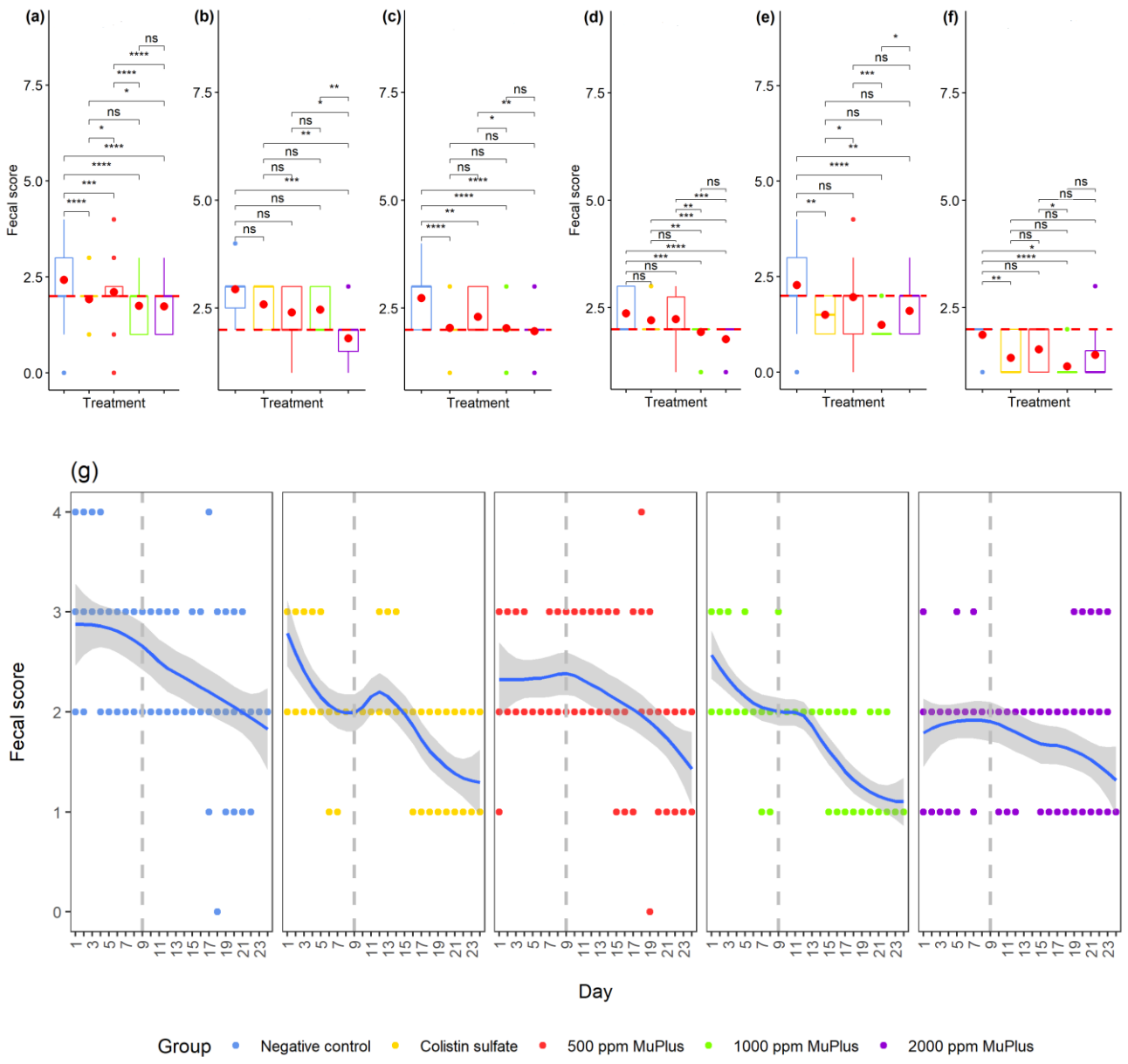

Figure 2. Change in fecal scores of male crossbred (Duroc × Landrace × Large white) pigs challenged with hemolytic *E. coli*. The pigs were randomly assigned to 5 groups which were no supplementation (negative control), 150 ppm CS, 500, 1000 and 2000 ppm of herbal mixture beginning from day one to the end of the experiment (day 24). **a:** Overall average fecal score of pigs. **b:** Fecal score from days 1 - 3. **c:** Fecal score from days 4 - 9. **d:** Fecal score from days 10 - 15. **e:** Fecal score from days 16 - 20. **f:** Fecal score from days 21 - 24. The red circles show the mean of each group. ****: $p < 0.0001$, ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$, and ns: $p \geq 0.05$. **g:** Daily pattern of fecal score changes from the beginning to the end of the experiment (day 24). The dots are data points. The solid curve is the estimated local regression curve. Grey shading indicates the 95% confidence interval. The grey dashed line running vertically illustrates the final day the pigs received hemolytic *E.coli* treatment. Graph were plotted with ggplot2 package and arranged into complex compound figures by cowplot package in R program.

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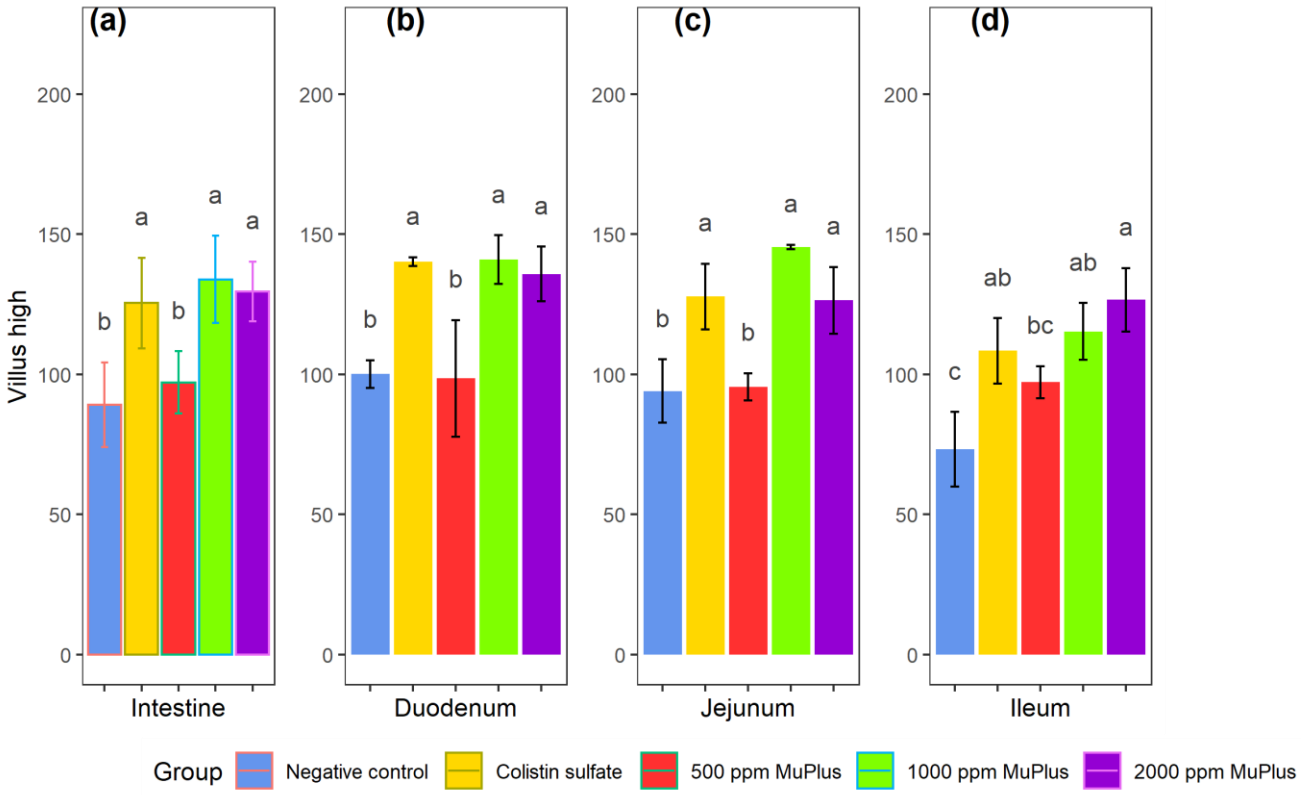

Figure 3. Villus heights at the duodenum, jejunum, and ileum of male crossbred (Duroc × Landrace × Large white) pigs challenged with hemolytic *E. coli*. The pigs were randomly assigned to 5 groups: no supplementation (negative control), 150 ppm CS, 500, 1000, and 2000 ppm of herbal mixture from day one to the end of the experiment (day 24). [abc]Different letters above the error bars indicate significant differences in villus heights ($p < 0.05$)

DISCUSSION

Pigs infected with STx2e-producing *E.coli* may die suddenly without signs of sickness. On the other hand, some affected pigs exhibit symptoms, such as edema of eyelids and forehead, incoordination, and respiratory distress (Fairbrother and Nadeau, 2019). A pig that received 150 mg/kg of CS died 5 days after the last challenge with hemolytic *E. coli*. Postmortem lesions and bacterial identification suggested that the pig died from ED. The hemolytic *E. coli* isolated differed from the one inoculated to weaned pigs because there was no fimbria F18, while STx2e was detected. The detection of other fimbria antigens, such as F4, F5, F6, and F41, was not performed. The study by Baldo et al. (2020) showed that most of the STx2e-producing isolates had the F18 adhesin factor, while only 6.01% had F6 fimbriae, and F4, F5, and F41 were not present. This bacterium could be F6-STx2e virotype, which has become habituated to the swine intestine, where it can survive and persist despite the presence of CS. The bacterium can migrate from the intestinal region to the mesenteric lymph nodes, producing the STx2e toxin that triggers ED (Fairbrother and Nadeau, 2019). Poor absorbance of CS from the gastrointestinal tract to plasma (Rhouma et al. 2016a) and the resistance to CS of this *E. coli* strain could be reinforcing factors that contribute to the development of ED.

This experiment simulated the common real-life situation in which pigs on farms become sick from *E. coli* and shed the bacteria at about 10^8 CFU per gram of feces into the environment (Frydendahl et al., 2003; Boeckman et al., 2022). Pigs in close contact inevitably consume this amount of *E. coli*, colonizing the swine's gastrointestinal tracts and proliferating. The current study revealed that in pigs in the negative control that received no supplementation and were inoculated the same amount of the real-life situation (10^8 CFU), the number of hemolytic *E. coli* shedding increased progressively over time, peaking on day 17. Even after the *E. coli* challenge was stopped, hemolytic *E. coli* shedding continued to grow. This scenario indicated the colonization and proliferation of inoculated *E. coli* in the gastrointestinal tracts of swine (Boeckman et al., 2022). In addition, inoculated *E. coli* induced diarrhea after the first day of inoculation. Despite differences in the strain of the inoculum, the results of this experiment are consistent with those reported by Jensen et al. (2006) and Rhouma et al. (2016b).

In the current study, CS-resistant *E. coli* and 150 mg/kg CS were used as the standard recommended therapeutic dose in pigs (Rhouma et al., 2016a) to simulate a realistic portrait of CS on current pig farms, where pigs might encounter CS-resistant *E. coli*. The supplementation of CS at 150 ppm did not reduce the number of *E. coli* or prevent diarrhea in pigs given CS-resistant hemolytic *E. coli* during the first 3 days or during days 10-15 of the experiment.

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Treatment of secretory diarrhea caused by *E. coli* is based on reducing or eliminating *E. coli*, controlling the motility of the intestines, and controlling secretion of the intestines (Thiagarajah et al., 2015). The Current study revealed that herbal mixture could treat secretory diarrhea caused by *E. coli.* The reduction or elimination of *E. coli* was demonstrated in pigs that received the herbal mixture at 500, 1000, and 2000 ppm. Although there was no direct evidence that motility control was affected by the herbal mixture in the current study, the authors can infer from Nwinyi et al. (2012) that *A. paniculata*, one of the herbal mixture's ingredients, has an anti-motility effect on the gastrointestinal smooth muscle. In addition, secretory diarrhea could be treated with the herbal mixture at 2000 ppm. Gupta et al. (1990) found that *A. paniculata* could control secretory diarrhea caused by the heat-labile and heat-stable enterotoxins of *E. coli*, supporting the result of the present study. The present study indicated that the anti-diarrheal property of *A. paniculata* was dose-dependent. An immediate anti-diarrheal effect was observed at 2000 ppm of herbal mixture. The anti-diarrheal effects at 1000 ppm of the herbal mixture were observed three days after ingestion. In addition to the anti-diarrheal property of the herbal mixture, pigs that received 1000 and 2000 ppm also indicated higher villi in all parts of the small intestine compared to pigs that received no supplementation or lower doses of the herbal mixture.

Regarding the practical applications and economy, each dose of the herbal mixture is appropriate for pig farms with different infection statuses. In farms where diarrhea is not observed, prophylaxis dose of 500 ppm of the herbal mixture can be used. The results from the current study confirmed that this dose of herbal mixture significantly reduced the number of *E. coli* in feces when compared to pigs that received no supplementation. Therefore, a higher dose of the herbal mixture has to be considered in farms where diarrhea is already observed, for example, 1000 ppm for low to moderate diarrhea or 2000 ppm for moderate to high diarrhea.

The herbal mixture used in this study is identical to the antimicrobial synergy concept, which combines two or more antimicrobial agents to achieve a more significant overall effect than the sum of their individual effects (Van Vuuren and Viljoen, 2011). However, combining herbs can result in complex effects due to the potential interactions among their components, leading to undesirable effects (Che et al., 2013). Although the biological activities of the herbs (whether synergistic, additive, or antagonistic) could not be determined in this study, the benefits of the herbal mixture as antibacterial and anti-diarrhea were demonstrated. Furthermore, no antagonistic antibacterial or anti-diarrheal effects or antagonistic effects on intestinal villus height were observed. Regarding the development of bacteria resistance to the herbal mixture, the possibility is slight due to the multiple sources of the compound (Caesar and Cech, 2019).

CONCLUSION

The supplementation of the herbal mixture at 500, 1000, and 2000 ppm had antibacterial effects, with no significant difference between doses. However, the positive effects of this herbal mixture on intestinal villi height and diarrhea were found only in pigs that received 1000 and 2000 ppm of the herbal mixture. From a practical point of view, supplementation of this herbal mixture at 500 to 1000 ppm could be applied for prophylaxis during the weaning period, whereas 2000 ppm of the herbal mixture could be applied for the treatment of postweaning *E. coli* diarrhea.

DECLARATIONS

Data availability

The authors can provide all necessary data to the editor upon request without delay.

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Authors' contribution

Chanthala Laxaphakdy conceptualized the idea and conducted the experimental animal work. Jatedada Jiwakanon and Sirisak Tanpong contribute to data analysis. Sansanee Supankong contributes laboratory work involving *E. coli*. Pitaya Papirom contributed to intestinal morphology work. Sarthorn Porntrakulpipat supervised all the experiments. Chanthala Laxaphakdy and Sarthorn Porntrakulpipat wrote the original manuscript. All authors have read and agreed to publish the final version of the manuscript.

Competing interests

The authors have not declared any conflict of interest.

Ethical consideration

The authors take steps to abide by all ethical standards related to plagiarism, publication approval, inaccuracies in data, multiple submissions, and double publication.

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