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Effect of Fermented Feeds on Weaned Piglets Experimentally Infected with *Escherichia coli*

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ABSTRACT

Sixty large white breed piglets aged between 28 and 35 days were randomly assigned to five replicate groups (Bu1, Bu2, Bd1, Bd2, Bs1, Bs2, Lc1, Lc2, C1, and C2) with six pigs per group. The Bu1 and Bu2 groups were fed with wet and dry basal diets, respectively, supplemented with 0.8 mL (6 x 10^6 colony-forming units (CFU)) of *Lactobacillus* isolated from burukutu (beverage made from sorghum grains). The Pg1 and Pg2 groups were fed with wet and dry basal diets, respectively, supplemented with 0.8 mL (6 x 10^6 CFU/mL) of *Lactobacillus* isolated from pig hindguts. The Bs1 and Bs2 groups were fed with wet and dry basal diets supplemented with 0.8 mL (6 x 10^6 CFU/mL) of *Bacillus subtilis* and *Bacillus pumilus*, respectively. The Lc1 and Lc2 groups were fed with wet and dry basal diets supplemented with 0.8 mL (6 x 10^6 CFU/mL) of *Lactobacillus acidophilus*, respectively. Meanwhile, the C1 and C2 groups as the control groups were fed with wet and dry basal diets, respectively. The treatment and control animals were infected with *Escherichia coli* at 6 mL (1 x 10^{10} CFU/mL) orally. Aseptically collected fecal samples from the piglets in each group showed significant bacteriological and pathological differences. This study suggested that *Lactobacillus* species isolated from burukutu, pig hindguts, and industrial probiotics could inhibit colibacillosis.

Keyword: colibacillosis, *Escherichia coli*, fermented feed, *Lactobacillus* sp., probiotic

INTRODUCTION

In industrial pig farming, piglets are weaned at increasingly earlier ages although they are psychologically immature and more susceptible to common post-weaning problems (Valentim et al., 2021). This process is considered stressful and can lead to changes in the immune system of the piglets (Kick et al., 2012). Studies on the effect of stress on porcine immunity have shown that early weaning between days 14 and 21

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decreases lymphocyte proliferation (Blecha et al., 1983) and increases steroid production by activating the neurohumoral system, which affects the immune status of the piglets and lowers their resistance to infection (Kanitz et al., 2008).

The main causes of diarrhea in weaned piglets are weaning stress, decreased lactogenic immunity, nutrition, and environmental changes. Early weaning stress continually impairs the intestinal barrier function in pigs (Kick et al., 2012). Post-weaning diarrhea (PWD) in piglets is typically associated with the proliferation of enterotoxigenic *E. coli* (ETEc) (Fairbrother et al., 2005). It is also caused by β-hemolytic enterotoxigenic strains of *Escherichia coli* (ETEc). Weaning can result in significant economic losses due to growth retardation, morbidity, mortality, sudden death, diarrhea, and dehydration within two weeks of weaning (Fairbrother et al., 2005; Rhouma et al., 2017).

The use of antibiotics as growth promoters has been shown to be effective in reducing post-weaning diarrhea and promoting immunomodulatory and anti-inflammatory responses, thereby improving the growth of weaned piglets (Rhouma et al., 2017). However, increasing bacterial resistance (multi-drug-resistant pathogens) leading to therapeutic failures on farms and antibiotic residues in animal products, as well as increasing public awareness of antimicrobial residues, now becomes a global health concern (Rhouma et al., 2017). Therefore, intensive research and numerous clinical trials are essential for the development of alternatives to antimicrobials in preventing post-weaning diarrhea in piglets. Several alternatives to antibiotics have been developed in recent years, including probiotics which have gained prominence (Abudabos et al., 2013; Khan & Naz, 2013). Studies have shown that probiotic supplementation in diets for weaned piglets can have a similar effect to antibiotics (Kritas & Morrison, 2005).

Increasing demand for animal protein has led to an increase in pig population for pork production in Nigeria. Disease outbreaks are the primary obstacle to profitable pig farming in areas where pork production and consumption are not restricted by religious beliefs (Igbokwe & Maduka, 2018). A study conducted in Lagos, Nigeria revealed that food-producing animals in Nigeria are a reservoir of multidrug-resistant *Escherichia coli* that can be transmitted to humans through the food chain (Adenipekun et al., 2014).

To the best of our knowledge, no study has been conducted to evaluate the effect of fermented feed on weaned piglets infected with *E. coli*. Therefore, this study aims to evaluate the effect of fermented feed on weaned pigs experimentally infected with *E. coli*.

MATERIALS AND METHODS

Research location

This study was conducted between July and December 2021 at the Samaru Campus of Ahmadu Bello University (A.B.U) Zaria, Sabon Gari Local Government Area of Kaduna State, Nigeria. Zaria is located at a latitude of 11° 11´N, 07°, 38´E and 686 m above sea level in the Northern Guinea Savannah, with a temperature range of 13.8° to 36.7°C and an annual rainfall level of 1092.8 mm (Aliyu et al., 2013).

Probiotic bacteria

Probiotic bacteria were isolated and characterized from the gastrointestinal tracts (GIT) of five large white breed piglets. A combination of laboratory culture, isolation, and sequencing (16S ribosomal RNA) techniques as described by Lo Verso et al. (2018) was used to identify the probiotics. The probiotics were prepared in MRS agar slants and stored at -4°C. *Lactobacillus subtilis,*

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Lactobacillus pumilus, Lactobacillus acidophilus, and *Lactobacillus* from burukutu (BKT) and pig hindguts were also revived by inoculation in Man, Rogosa, and Sharpe broth (MRS; Oxoid, England). The probiotics were then grown in anaerobic jars with CO_2-generating kits (Anaerogen; Thermofisher, UK) at 37°C for 24 hours. The revived probiotic strains were inoculated at 1% (v/v) on the corresponding culture and grown anaerobically in MRS broth overnight following the procedures described by Mulaw et al. (2019).

Animals

Weaned piglets (n = 60) aged between four to five weeks with an average body weight of 5.5 kg were sourced from breeders in Hayin Danyaro, a small village in Samaru that is popularly known as pig city, Sabon Gari Local Government Area of Kaduna State, Nigeria. The weaned piglets were transported and housed separately in the piggery pens at the Department of Veterinary Medicine, Faculty of Veterinary Medicine, A.B.U. Zaria, Kaduna State. The piglets were kept in a temperature-controlled building with concrete flooring and provided unrestricted access to water throughout the trial period.

The piglets were randomly assigned to five replicate groups (A1, A2; B1, B2; C1, C2; D1, D2; and E1, E2) with six piglets per pen. The A1 and A2 groups were fed with wet and dry basal diets supplemented with 0.8 mL (6×10^6 CFU/mL) of *Lactobacillus* isolated from burukutu, respectively. The B1 and B2 groups were fed with wet and dry basal diets supplemented with 0.8 mL (6×10^6 CFU/mL) of *Lactobacillus* bacteria isolated from pig hindguts, respectively. The C1 and C2 groups were fed with wet and dry basal diets supplemented with a 0.8 mL (6×10^6 CFU/mL) mixture of *Bacillus subtilis* and *Bacillus pumilis,* respectively. The D1 and D2 groups were fed with wet and dry basal diets supplemented with 0.8 mL (6×10^6 CFU/mL) of *Lactobacillus acidiphilus,* respectively. Finally, the E1 and E2 groups as the control groups were fed with wet and dry basal diets only, respectively.

Source of pathogenic *E. coli* for experimental infection

Fecal samples were collected from weaned piglets with a diarrhea in the piggery of a pig farmer in Hayin Mallam, Samaru, Sabon Gari Local Government of Kaduna State. The feces were collected in a sterile polythene bag from piglets exhibiting typical signs of *E. coli* infection, such as lethargy and yellowish stool. The samples were transported to the bacteria zoonoses laboratory of the Department of Veterinary Public Health and Preventive Medicine, Ahmadu Bello University, Zaria. A total of 90 mL of Tryptone soy broth was inoculated with 10 g of feces and incubated at 37°C for 24 hours. A loopful of the broth culture was streaked onto EMB agar medium and incubated at 37°C for 24 hours. Colonies exhibiting the characteristics of *E. coli* (green metallic sheen) were subjected to biochemical tests as shown in Table 1.

Table 1. Biochemical tests of the colonies exhibiting the characteristics of E.*coli*.

Biochemical test	Results	Description
Triple sugar iron	Acid	Positive for *E. coli*
Citrate	Negative (light blue)	Positive for *E. coli*
Urea	Negative (pink)	Positive for *E. coli*
SIM (indole)	Positive (red/motility)	Positive for *E. coli*
Methyl red	Pink/red	Positive for *E. coli*

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Serotyping of the isolated pathogenic *E. coli*

A homogenous mixture was formed by emulsifying a colony from the plate in a loopful of sterile normal saline on a clean glass slide. A drop of phase 3 polyvalent sera (Oxoid, U.K.) was added and gently vortexed for approximately five seconds until *E. coli* antigens were detected by agglutination reactions.

Diets and feeding regimen

A diet for growers was formulated as shown in Table 2. Two dietary treatments were prepared with or without bacterial inclusions. Dry feed (DF) was provided as a dry meal, while wet feed (WF) was provided as a wet meal. The WF was prepared by mixing meal and water in a ratio of 1:4 in a 20-liter tank that was clearly labeled. The tank was agitated at room temperature (27°C) before all treatment groups were inoculated with *Lactobacillus* from different sources, including BKT, pig hindgut, and PSTAB and PTASB patented bacteria. The control group was fed only with the basal diet without LAB. The same procedure above was repeated for the dry feed preparation, except that no water was added to the feed.

Table 2. Composition of compound feed used to feed the experimental pigs

Feed ingredient	Weight (kg)
Bag of maize	50
Bag of bran (wheat and guinea corn husk)	40
Groundnut cake	23
Methionine	0.1
Lysine	0.1
Bone meal	3
Cray fish	0.1
Salt	0.1
Premix	0.25
Limestone	1.0

The piglets in all the groups were initially fed for a period of three weeks prior to inoculation. Subsequently, the different bacterial isolates were added to their respective rations, as previously described by Gracia et al. (2004) and Ahmed et al. (2014). The piglets were fed with appropriate amounts of feed according to their body weights, ensuring that the entire rations were consumed within approximately 30 minutes once a day at 8:30 a.m. Fresh water was provided ad libitum. After the treatment period, fecal samples were collected from the rectum of all the piglets in each treatment group to determine the level of *Lactobacillus* present in the feces.

Infection of weaned piglets

The piglets in both the treatment and control groups were exposed to *E. coli*. Each piglet was administered with 6 mL (1×10^{10} CFU/ml) of the *E. coli* strain using a 10 mL syringe injected at the back of their oral cavities at 5:00 p.m., following the method described by Owusu-Asiedu et al. (2003).

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Fecal sampling and bacteriological analysis of infected piglets

Fecal samples were collected from each piglet 12 hours after the administration at 8:00 a.m. the following day. The samples were serially diluted (0.1 in 9.9 mL) and two tubes at 10^{-4} and 10^{-6} were selected and spread-plated on EMB-agar (Oxoid, U.K.). These tubes were incubated at 37°C for 24-48 hours, following the method described by Foo et al. (2001). The numbers of colony-forming units (CFU) were expressed as log10 CFU per gram to obtain the number of colonies grown daily over a period of two weeks. Subsequently, the results were collated. Similarly, the control groups were infected at the same time and observed for clinical signs of colibacillosis, such as diarrhea, rough coat, and lethargy.

Fecal sampling and bacteriological analysis of infected piglet

Enterotoxigenic *E. coli* strains were collected from piglets with diarrhea in Samaru village, that exhibited typical signs of *E. coli* infection, such as lethargy and yellowish stool. The isolates were previously identified by molecular typing as ETEc 089, ETEc 0114, ETEc 0125, ETEc 0127, and ETEc 0128 (Karaye et al., 2020). After four weeks of probiotic administration, the piglets in each treatment group were infected orally with 6 mL of a solution containing 1×10^6 CFU (Coliform Forming Unit) of the *E. coli* strains. After twelve hours of administration, fecal samples were collected aseptically from the rectum of each piglet in each group (A-D) using a swab. *E. coli* counts on the plates were determined for seven days and the presence or absence of diarrhea was also noted.

Microbiological analysis of piglet's fecal samples

Fecal samples were collected to calculate bacterial shedding. All piglets were clinically scored for diarrhea on days zero to seven after the ETEc administration. The presence of ETEc in fecal samples was confirmed by molecular typing. The severity of diarrhea was determined using the fecal consistency (FC) scoring system as described by Marquardt et al. (1999). Fecal consistency was scored on a scale of 0 to 3 (0 = normal; 1 = loose stool; 2 = mild diarrhea; 3 = severe diarrhea). The scoring was performed by two trained personnel from the microbiology laboratory at the Faculty of Veterinary Medicine, A.B.U. Zaria, without prior knowledge of the dietary treatment assignments.

Gross and histopathology

At the end of the experiment (day 28), two piglets per replicate were randomly selected from the infected and treated groups based on the average weight of each replicate. The piglets were euthanized by electrical stunning followed by exsanguination. Postmortem examination of intestinal morphology was conducted on three replicates with six pigs per treatment (n = 3). Segments of intestinal tissues, each measuring two centimeters, were collected from various parts of the weaned pigs, including the duodenum, jejunum, ileum, caecum, colon, rectum, as well as tissues from other organs, such as spleen, lymph nodes, liver, and pancreas. These samples were immediately fixed in 10% neutral buffered formalin (Origin Pure Bio Sci and Tech, Taipei City, Taiwan). The tissues were processed and sectioned on a clean, grease-free microscope glass slide at a thickness of five µm (3 cross-sections from each piglet) using a rotary microtome (Thermo Scientific, Waltham, MA, USA). Subsequently, the slides were stained with hematoxylin and eosin. The morphometry of the intestinal villi, crypts, and other organ and tissue sections in the histological and pathological specimens was examined using a light microscope equipped with an ocular micrometer, according to the method described by Crouch and Woode (1978). The severity of lesions in each tissue and organ was evaluated by randomly selecting well-

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oriented villi and their associated crypts at each location. Desquamation and necrosis in both infected and probiotic-fed piglets were also evaluated.

Data analysis

The results of fecal shedding from the in vitro trial and the different treatments of the two feeds with different bacterial inoculations were subjected to analysis of variance (ANOVA) using the SAS software package (version 9.0). Mean differences among treatments were separated using the least significant difference (LSD) at 5% significance level.

Ethical approval

Procedures carried out in this study complied with all relevant legislation regarding the protection of animal welfare and were approved by the Ethical Clearance Committee for Animal Use and Care of Ahmadu Bello University, Zaria with the approval number ABUCAUC/2016/005.

RESULTS

Clinical signs

All piglets infected with ETEc in the control group developed diarrhea after 72 hours of infection. Severe diarrhea was observed in four pigs infected with ETEc 089, ETEc 0114, ETEc 0125, ETEc 0127, and ETEc 0128. Four piglets from the infected and non-treated groups, namely two from the control wet-fed group and two from the control dry-fed group, with diarrhea were euthanized after 96 hours of infection. In all infected groups, severe diarrhea was observed in 40% of the piglets in the control groups. Two pigs died on day 5 after the administration of ETEc 089, ETEc 0114, ETEc 0125, ETEc 0127, and ETEc 0128.

Gross pathology

The distal one-half to two thirds of the large intestine was dilated with a significant amount of greenish yellow to gray watery materials. The intestinal wall appeared thin and flaccid, while the stomachs were distended. The mesenteric lymph nodes showed slight congestion. Intestinal changes were more severe than those in other tissues and organs. Signs of diarrhea associated with mild dehydration were also present. The intestines were filled with watery material. However, no signs of hyperemia or inflammation were apparent.

Intestinal bacterial load

Figure 1 shows the computed data on bacterial counts for fecal *E. coli* shedding in piglets fed with wet and dry probiotics in four treatment groups during the seven-day period or one week following oral administration with enterotoxigenic *Escherichia coli* (ETEc). The results showed a significant linear increase ($p < 0.05$) from 0.42 (10^{10} CFU/mL) at 12 hours after administration to 2 (10^{10} CFU/mL) on day two. The bacterial count significantly decreased from 1.5 (10^{10} CFU/mL) on day three after administration to 0.7 (10^{10} CFU/mL) and 0.33 (10^{10} CFU/mL) on days four (10^{10} CFU/mL) and six (10^{10} CFU/mL). On day seven, the count further decreased to 0.16 (10^{10} CFU/mL) and remained at this level until no colonies were visible on the plate (Figure 1A). For piglets fed with dry feed containing *Lactobacillus* species, the values were 0.41 (10^{10} CFU/mL) at 12 hours after administration, increasing significantly with a linear curve to 1.5 on day two, followed by a slight decrease to 1.41 (10^{10} CFU/mL) on day two, a further decrease to 0.66 (10^{10} CFU/mL) on day three, a slight increase to 1.1 (10^{10} CFU/mL) on day four, and a consistent decrease to 0.23 (10^{10} CFU/mL) at the end (Figure 1B).

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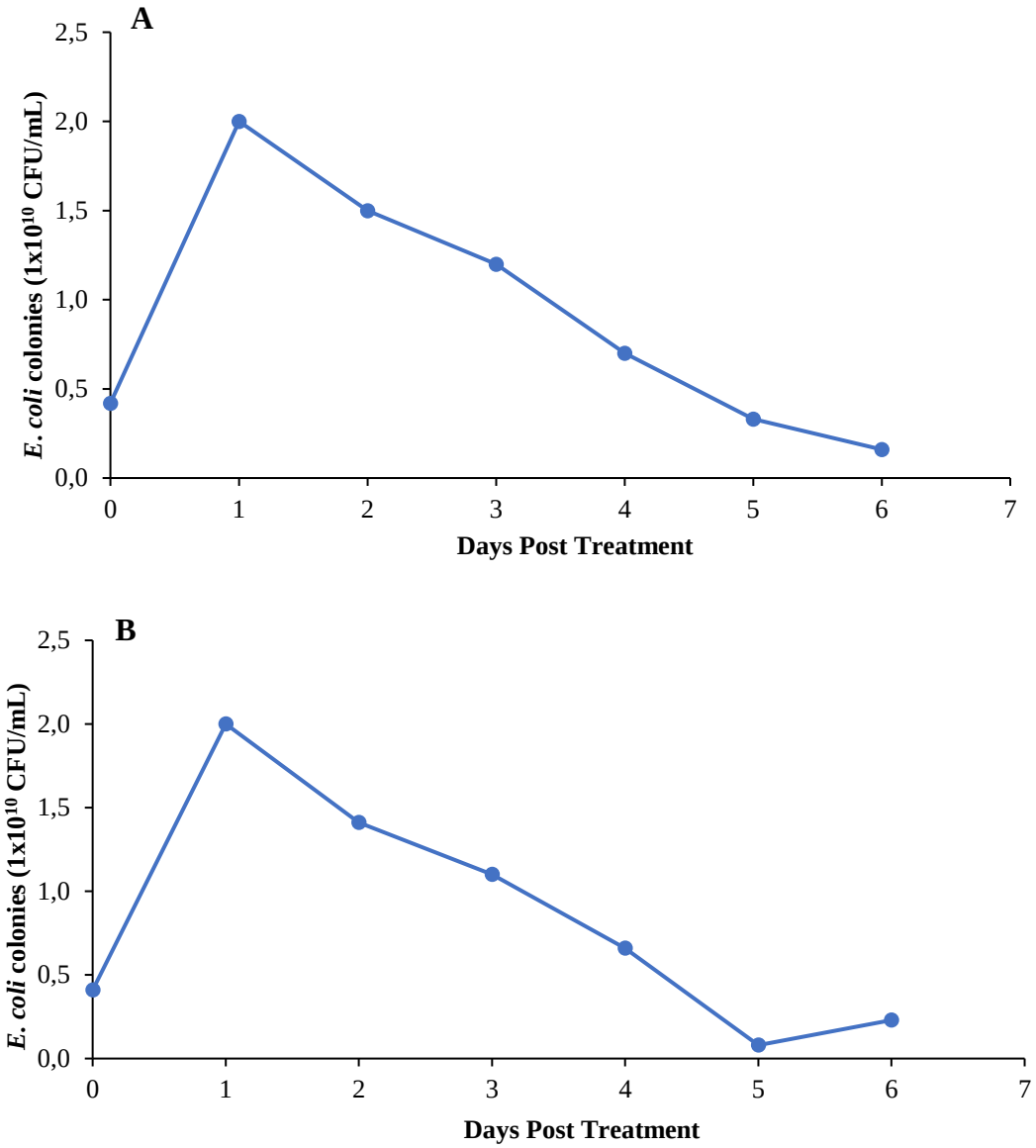

Figure 1. Scoring of fecal *E. coli* shedding in pigs fed with wet (A) and dry (B) probiotics with *Lactobacillus* isolated from burukutu and measured during seven days of oral administration with enterotoxigenic *Escherichia coli* (ETEc; 6 mL (1 x 10^{10} CFU/mL)).

At 12 hours after the ETEc administration, the fecal ETEc value of piglets fed with wet feed inoculated with *Lactobacillus* from pig hindguts (Figure 2A) was 0.24 (10^{10} CFU/mL). It increased to 1.1 on day one and peaked at 3.69 (10^{10} CFU/mL) on day two. It later decreased to 0.73 (10^{10} CFU/mL), 0.6, and 0.34 (10^{10} CFU/mL) on days three, four, and five, respectively. On days six and seven, it decreased to 0.1 (10^{10} CFU/mL) and remained at this level until no colonies were observed. For piglets fed with dry feed but inoculated with *Lactobacillus* from pig

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hindguts, the fecal value at five hours after the ETEc administration as shown in Figure 2B was 0.58 (10^{10} CFU/mL), which decreased to 0.5 (10^{10} CFU/mL) on day one, peaked at 1.6 on day two, and decreased to 0.65 (10^{10} CFU/mL) on day three. The values continued to decrease from 0.3 (10^{10} CFU/mL) on day four to 0.04 (10^{10} CFU/mL) on day five, and finally to 0.29 (10^{10} CFU/mL) on days six and seven.

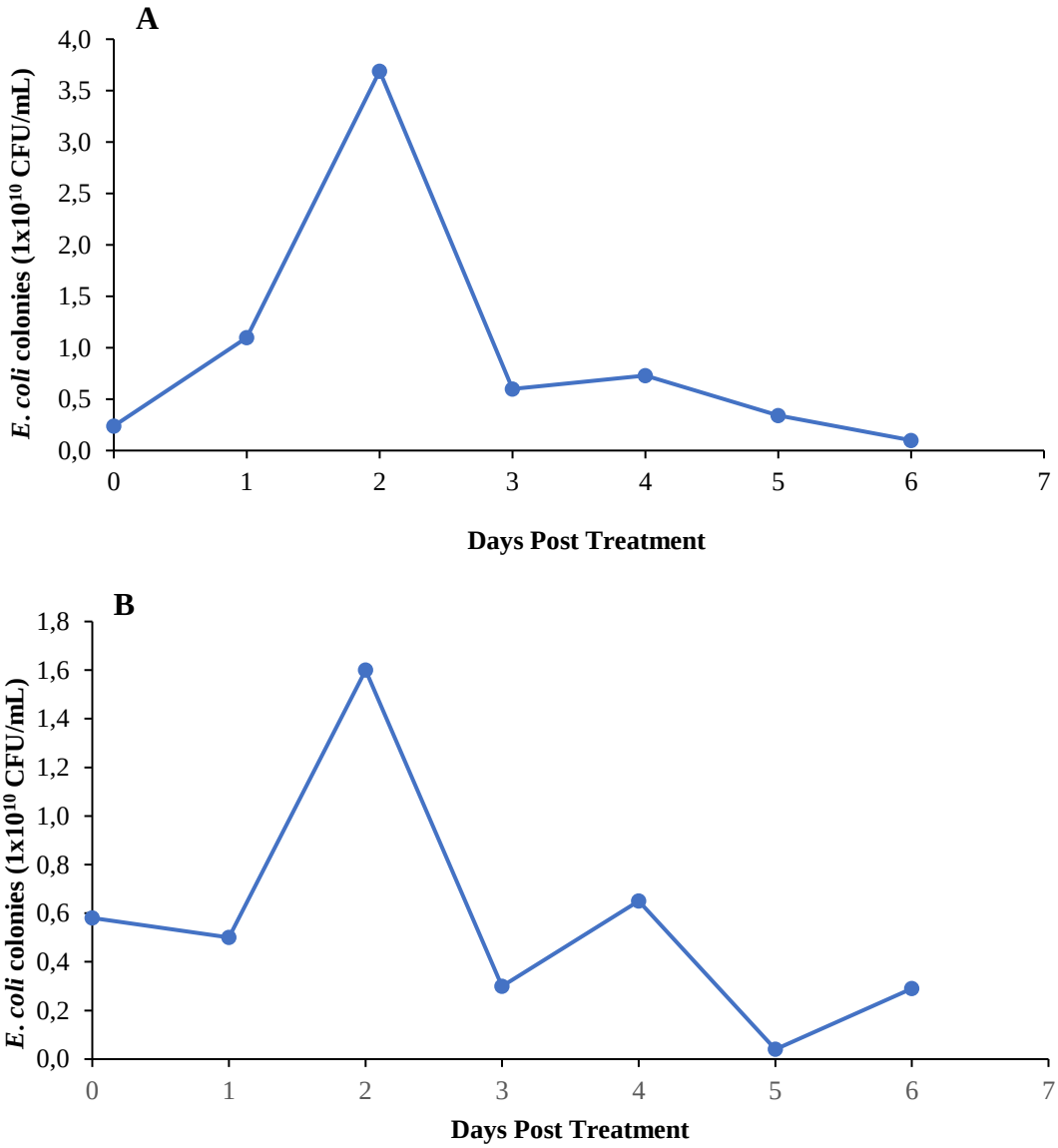

Figure 2. Scoring of fecal *E. coli* shedding in pigs fed with wet (A) and dry (B) probiotics with *Lactobacillus* isolated from pig hindguts and measured during seven days of oral administration with enterotoxigenic *Escherichia coli* (ETEc; 6 mL (1 x 10^{10} CFU/mL)).

Fecal samples collected five hours after the ETEc administration in piglets fed with wet feed inoculated with patented *Lactobacillus* (PSTAB) (Figure 3A) showed a

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value of 0.11 (10^{10} CFU/mL), which increased to 0.76 (10^{10} CFU/mL) on day one and remained at the same level on day two. The bacterial count peaked on day three at 1.3 (10^{10} CFU/mL) before decreasing to 0.4 (10^{10} CFU/mL) on day four. Subsequently, it slightly increased to 0.5 (10^{10} CFU/mL) on day five before decreasing again on days six and seven, with a value of 0.4 (10^{10} CFU/mL).

Piglets fed with dry feed inoculated with patented probiotic (PSTAB) (Figure 3B) showed a value of 0.03 at 12 hours after the ETEc administration. It increased to 3.3 on day one and slightly decreased to 2.44 (10^{10} CFU/mL) on day two. On day three, the value decreased to 1.1 (10^{10} CFU/mL), which decreased further to 0.72 (10^{10} CFU/mL), 0.2 (10^{10} CFU/mL), and 0.2 (10^{10} CFU/mL) on days four, five, and six, respectively, until no colonies were observed on the plate.

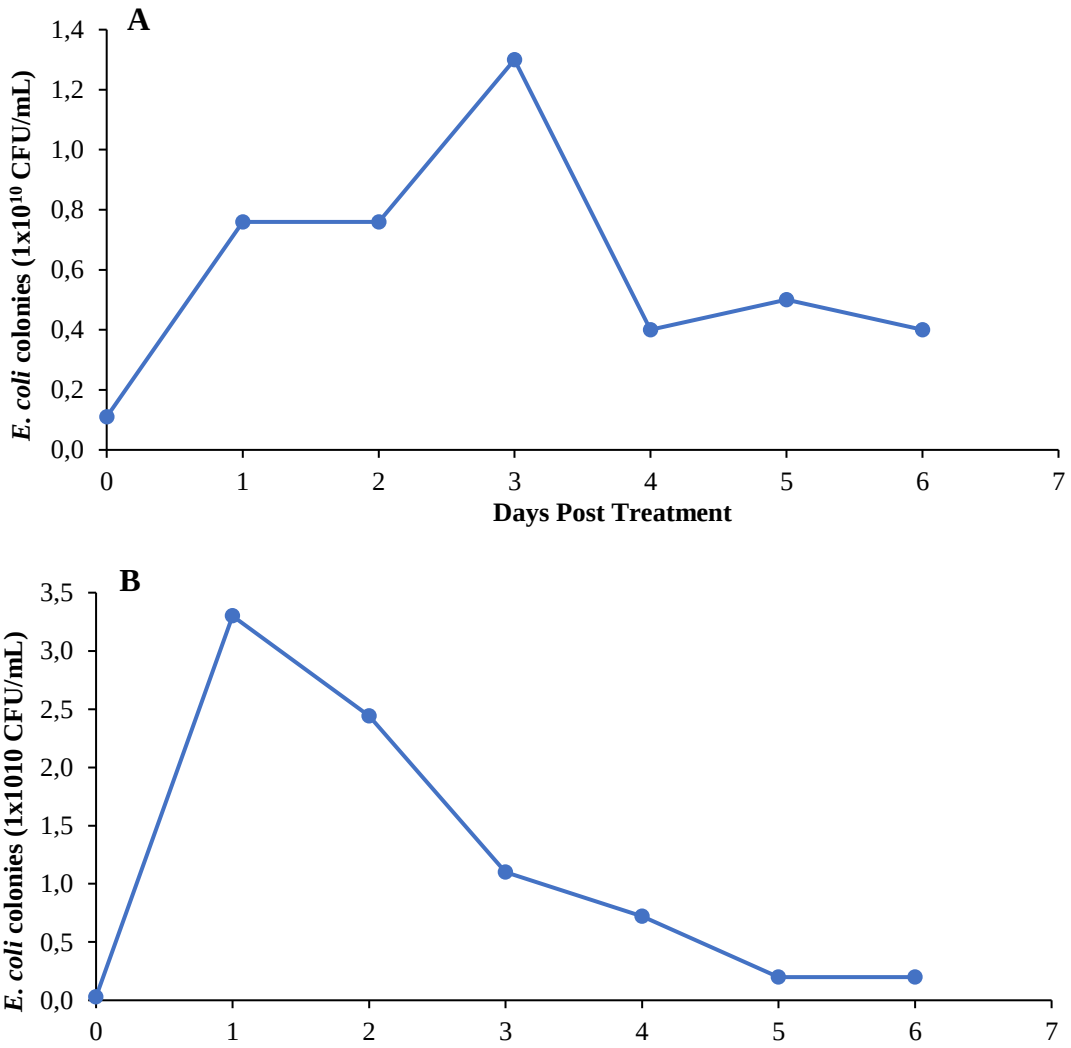

Figure 3. Scoring of fecal *E. coli* shedding in pigs fed with wet (A) and dry (b) probiotics with *Lactobacillus* isolated from *Bacillus pumulis* and *Bacillus subtilis* and measured during seven days of oral administration with enterotoxigenic *Escherichia coli* (ETEc; 6 mL ($1x10^{10}$ CFU/mL)).

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Figure 4A shows the findings from fecal samples collected from piglets fed with wet feed, inoculated with patented bacteria (PTAB), and administered with ETEc from five hours to day seven. The value was 0.03 (10^{10} CFU/mL) at 12 hours, and peaked on day one at 6. It decreased significantly to 1.7 (10^{10} CFU/mL) on day two and further decreased to 0.4 (10^{10} CFU/mL) on day three. Subsequently, it increased slightly to 0.50 (10^{10} CFU/mL) on day four and before decreasing to 3.3 and 0.1 on days five and six. Piglets that were fed with dry feed, inoculated with patented bacteria, (PTAB) and administered with ETEc showed bacterial counts of 0.41 (10^{10} CFU/mL), 1.7 (10^{10} CFU/mL), 6 (10^{10} CFU/mL), 0.15 (10^{10} CFU/mL), 0.73 (10^{10} CFU/mL), 0.7 (10^{10} CFU/mL), and 0.4 (10^{10} CFU/mL), as shown in Figure 4B.

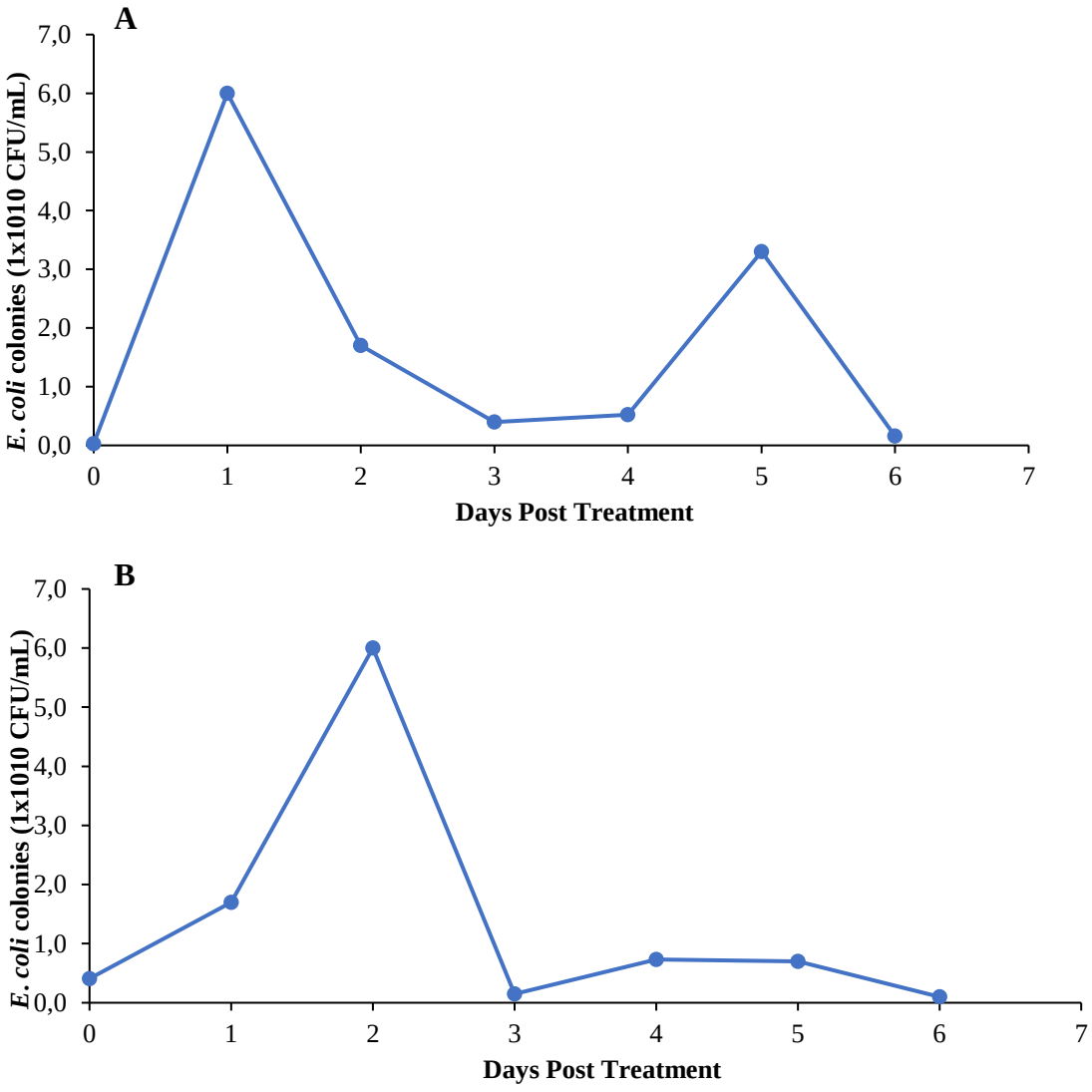

Figure 4. Scoring of fecal *E. coli* shedding in pigs fed with wet (A) and dry (B) probiotics with *Lactobacillus acidiphilus* and measured during seven days of oral administration with enterotoxigenic *Escherichia coli* (ETEc; 6 mL (1 x 10^{10} CFU/mL)).

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In conclusion, the total scoring of fecal *E. coli* shedding in pigs fed with wet and dry probiotics showed different patterns for the four different *Lactobacillus* isolates on day seven after oral administration with enterotoxigenic *Escherichia coli* (Figure 5).

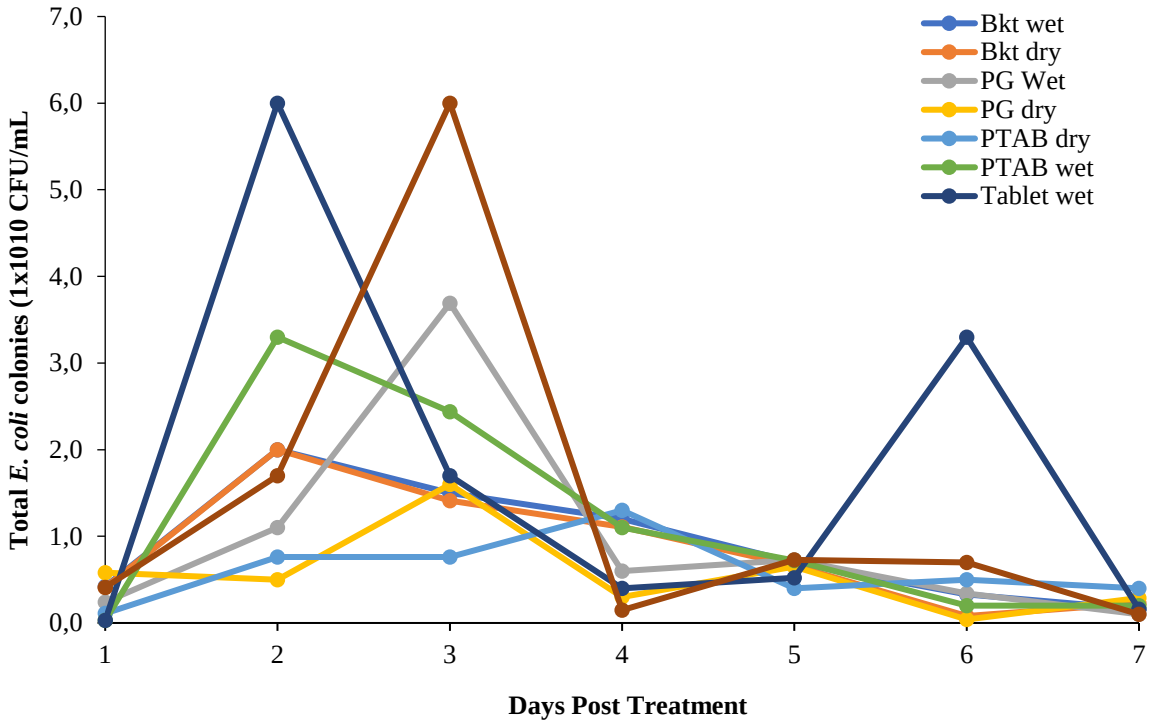

Figure 5. A graph comparing the various scorings of fecal *E. coli* shedding in pigs fed with wet and dry probiotics with four different *Lactobacillus* isolates measured during seven days of oral administration with enterotoxigenic *Escherichia coli* (ETEc; 6 mL (1×10^{10} CFU/mL)).

DISCUSSION

This study found that fecal shedding of *Escherichia coli* (*E. coli*) strains increased in the treatment groups receiving dry and wet fermented feed. Notably, the trend of *E. coli* shedding was similar and decreased by day seven in all groups. These findings suggested that the administration of feed inoculated with *Lactobacillus* over a period of time can protect the gut wall of animals. The protective ability is influenced by the duration of administration and the amount consumed. Similarly, Mourand et al. (2021) observed a decrease in the level of fecal excretion of CTX^R- *E. coli* in *E. coli* ED1a-treated pigs compared to that of non-treated pigs, which was usually less than 1 $\log_{10}$ CFU and was mainly observed during the probiotic administration period. Amezcua et al. (2002) used fermented liquid whey inoculated with

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specific lactic acid bacteria of pig origin to reduce the severity and progression of postweaning enterotoxigenic *E. coli* bacteria infected with *E. coli* 0149:k91:F4. It was found that the LAB counts of 9 Log_{10} CFU/g were reached on day three after infection, which was associated with a decrease in pH to less than 4. These parameters are considered the optimum values for fermentation of liquid feed and have been associated with the production of lactic acid, hydrogen peroxide, and bacteriocin (Van Winsen et al., 2001).

These LAB metabolites have the potential to reduce Enterobacteriaceae counts in the feed by no less than 3.2 Log_{10} CFU/g. When fed to pigs, thy can reduce the number of coliform bacteria in the gastrointestinal tract. Hojberg et al. (2003) found that Enterobacteriaceae counts were similar between dry-feed groups and fermented-feed groups. This suggested that feed supplemented with *Lactobacillus* can reduce in Enterobacteriaceae in the stomach, which is reflected in the amount shed in feces. This effect was also observed in pigs of all ages in an earlier study (Jensen et al., 2014). The amount of Enterobacteriaceae shed in feces usually reflects Enterobacteriaceae counts in the stomach contents of pigs. The use of fermented feed can reduce Enterobacteriaceae in the stomach, which in turn reduces Enterobacteriaceae shedding (Van Winsen et al., 2001). Gardiner et al. (2002) reported lower counts of Enterobacteriaceae in pigs on days 15, 22, and 26 post probiotic consumption and during the first week of *E. coli* inoculation. Lactic acid bacteria and yeasts that occur naturally in fermented feed have been reported to proliferate and produce lactic acid, acetic acid, and ethanol. This fermentation process reduces the pH of the fermented feed, which inhibits the growth of pathogenic organisms. Eventually, feeding pigs with fermented feed can lead to a decrease in stomach pH, which can prevent the growth of pathogens such as coliforms and *Salmonella* in the gastrointestinal tract of piglets (Missotten et al., 2015).

In an experiment comparing dry and wet fermented feed, piglets fed with fermented wet feed had a significantly higher concentration of yeasts ($p < 0.05$) in their gut compared to those fed with dry feed. The high concentration of yeasts in the fermented wet feed may have potential benefits, depending on the strains that are present (Missotten et al., 2015). Additional benefits of wet feed include improved nutrient digestibility, enhanced intestinal morphology, reduced antinutritional contents in feeds, and lower dust levels in swine barns (Missotten et al., 2015). This study also found that feed containing multiple strains of LAB was found to increase the shedding of enterotoxigenic *E. coli* (ETEc) in pig feces by preventing adhesion compared to the control group. Similar previous studies (Tuohy et al., 2007; Tsai et al., 2008) also found that piglets fed with feed containing LAB shed more ETEc.

It has been suggested that the reduction of Enterobacteriaceae and *Salmonella* species is influenced by the undissociated form of lactic acid and volatile fatty acids (VFA) (Russell & Diez-Gonzalez, 1998). The undissociated acid can freely cross the bacterial membrane, while the dissociated form cannot. Inside the bacterial cell, the acid dissociates, causing a decrease in the intracellular pH. This can lead to the cessation of enzymatic processes and the collapse of proton motive force, ultimately resulting in cellular damage or death (Russell & Gonzales, 1998). In addition, Dell'Anno et al. (2021) observed that the in-feed supplementation of *L. plantarum* and *L. reuteri* positively contributed to eubiosis in the intestinal environment and that single strains or a combination of these lactic acid bacteria, in either dry or wet feed, could help prevent diarrhea. Similar observations were

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reported by Park et al. (2015) in lactating pigs.

A comparison of the colonization rate of bacteria in the group that received wet formulated feed and the group that received *Lactobacillus* in dried feed formulation showed that the administration and shedding of ETEc in the former group was significantly higher than in the latter group. This suggested that long-term use of probiotics in feed, especially in the wet form, can effectively prevent pathogen colonization in the gastrointestinal tract of piglets. Similarly, Van Winsen et al. (2001) suggested that other mechanisms, such as nutrient availability, competition for receptor sites, and immunological responses, may also contribute to the reduction of Enterobacteriaceae. In addition, there is a significant positive correlation between pH and *Lactobacillus* in the stomach contents of pigs fed with dry feed as well as in the stomach contents of pigs fed with wet fermented feed. This suggested that fermented feed have an effect on the bacterial ecology of the gastrointestinal tract, resulting in a reduction in the *Enterobacteriaceae counts* in various parts of the gastrointestinal tract.

Fermented feed contains high concentrations of lactic acid and several volatile fatty acids, including acetic acid, butyric acid, and propionic acid, as well as large numbers of lactobacilli and and a low pH (Dell'Anno et al., 2021). These four parameters, either individually or in combination, can affect the bacterial ecology of the gastrointestinal tract (Van Winsen et al., 2001). Lactic acid and VFA are also produced by the indigenous microflora in the gastrointestinal tract (Van Winsen et al., 2001; Ishii & Sadowsky, 2008). *Lactobacillus* species may inhibit pathogenic bacteria from adhering to host cells through competitive inhibition. This inhibition may be due to the production of various metabolic and anti-bacterial substances, such as organic acids, bacteriocin, proteinase, and hydrogen peroxide. Ultimately, *Lactobacillus* species may regulate the immune system activity of host cells, either individually or in combination with the above functions. In conclusion, this study and other similar studies support the use probiotics and biotherapy over antibiotics for the prevention and treatment of ETEc infections.

CONCLUSION

The available data from this study showed that dietary inoculation of *Lactobacillus*-based probiotic bacteria in wet or dry forms positively affected the pigs in each treatment group by altering the microbial environment, which increased the shedding of ETEc in all groups. It was also found that the numbers of *Enterobacteriaceae* in the different treatment groups were not significantly different, particularly on days 1 and 2 post-treatment. The findings of this study suggested that the reduced shedding of Enterobacteriaceae in the fermented feed groups could have significant implications for the infection pressure of entero-pathogens belonging to this group of bacteria. However, further case-controlled studies are needed to determine the efficacy of LAB preparations, as well as the optimal supplementation levels and doses.

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