Title

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Short Communication

Dietary butyrate and valerate glycerides impact diarrhea severity and immune response of weaned piglets under ETEC F4-ETEC F18 coinfection conditions

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Abstract

Enterotoxigenic *Escherichia coli* (ETEC) causes post-weaning diarrhea in piglets, significantly impacting animal welfare and production efficiency. The two primary ETEC pathotypes associated with post-weaning diarrhea are ETEC F4 and ETEC F18. During the post-weaning period, piglets may be exposed to both ETEC F4 and ETEC F18. However, the effects of coinfection by both strains have not been studied. Short chain fatty acid feed additives, such as butyrate and valerate, are being investigated for their potential to improve animal performance and disease resistance. Therefore, this pilot experiment aimed to test the effects of butyrate glycerides or valerate glycerides on growth performance, diarrhea incidence, and immune responses of piglets under ETEC F4-ETEC F18 coinfection conditions. Twenty piglets were individually housed and assigned to one of the three dietary treatments immediately at weaning (21 to 24 d of age). The dietary treatments included control (basal diet formulation), control supplemented with 0.1% butyrate glycerides or 0.1% valerate glycerides. After a 7-d adaptation, all pigs were inoculated with ETEC F4 and ETEC F18 (0.5×10^9 CFU/1.5 mL dose for each strain) on three consecutive days. Pigs and feeders were weighed throughout the trial to measure growth performance. Fecal cultures were monitored for hemolytic coliforms, and blood samples were collected for whole blood and serum analysis. Pigs fed valerate glycerides tended ($P = 0.095$) to have higher final body weight compared with control. The overall severity of diarrhea was significantly ($P < 0.05$) lower in both treatment groups than control. Pigs fed valerate glycerides tended ($P = 0.061$) to have lower neutrophils and had significantly ($P < 0.05$) lower serum TNF-α on day 4 post-inoculation. This pilot experiment established an appropriate experimental dose for an ETEC F4-ETEC F18 coinfection disease model in weaned piglets. Results also suggest that butyrate glycerides and valerate glycerides alleviated diarrhea and regulated immune responses in piglets coinfected with ETEC F4 and ETEC F18.

Lay Summary

Piglets suffer from post-weaning diarrhea associated with Enterotoxigenic *Escherichia coli* (ETEC) F4 and F18, two prevalent strains on swine farms globally. Short chain fatty acids (SCFAs), such as butyrate and valerate, are natural, organic compounds that could potentially promote intestinal health when used as dietary supplements. During the post-weaning period, piglets are vulnerable to simultaneous infection by ETEC F4 and F18. Therefore, this experiment aimed to develop an experimental disease model for coinfection with ETEC F4 and F18, employing a dose of 0.5×10^9 CFU/1.5 mL of each strain, administered over three consecutive days. In addition, the experiment evaluated treatment diets supplemented with 0.1% butyrate or valerate glycerides compared with the control diet. Results from this experiment revealed that the inoculation dose incited infection and diarrhea in piglets, implying its suitability for use in a disease challenge model. Moreover, the results indicated that the inclusion of butyrate and valerate glycerides to pig's diet reduced the severity of diarrhea. Furthermore, pigs fed SCFA glycerides exhibited lowered levels of inflammatory blood markers. In conclusion, the experimental dose induced diarrhea in piglets, and dietary supplementation of butyrate and valerate glycerides alleviated the severity of diarrhea while augmenting inflammatory status.

Key words: diarrhea, enterotoxigenic *escherichia coli*, gut health, immunity, weaned pigs

Abbreviations: BW, body weight; ELISA, Enzyme-linked immunosorbent assay; ETEC, enterotoxigenic *Escherichia coli*; PI, Post-inoculation; SCFA, short chain fatty acid; TNF-α, tumor necrosis factor alpha; UC Davis, University of California, Davis; WBC, white blood cell

Introduction

Newly weaned pigs are highly susceptible to infection with Enterotoxigenic *Escherichia coli* (ETEC), causing severe diarrhea and impaired performance. The administration of antibiotics such as colistin in feed was a common mitigation strategy in the swine industry prior to rising concern regarding increases in antimicrobial resistance (Rhouma et al., 2017). As such, the limitation of antibiotic use in feed is enforced by various government entities (Wierup, 2001; Casewell et al., 2003; Medicine, 2020). Alternative strategies are required to reduce the frequency of morbidity and mortality in piglets associated with post-weaning diarrhea. Dietary

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short chain fatty acids (SCFAs) are a family of weak carboxylic acids, including butyrate (C4) and valerate (C5). Dietary butyrate and its derivatives have demonstrated their capacity to enhance growth performance in pigs at various stages of production (Piva et al., 2002; Lu et al., 2008, 2012). Also, it has been reported that sodium butyrate can reduce the incidence of diarrhea when fed to weaned piglets (Feng et al., 2018). Valerate is present in the intestinal environment as a product of microbial fermentation (McDonald et al., 2018) and is found naturally in the flowering perennial species, *Valeriana officinalis* (Patočka and Jakl, 2010). However, our understanding of its effects on intestinal physiology remains limited. Nonetheless, evidence exists to support the beneficial outcomes associated with endogenous or dietary valerate in the intestinal environment. The production of valerate by certain commensal bacterial species in the gastrointestinal tract has been linked to inflammatory status of the gut in humans (Mondot et al., 2011; Tjellström et al., 2012). Furthermore, two studies have indicated that valerate glycerides enhance resistance to necrotic enteritis in broiler chickens, implying immunomodulation of the intestine during enteric infection (Onrust et al., 2018; Hofacre et al., 2020). The esterification of one or more butyrate or valerate moieties to a glycerol backbone forms glycerides, which have reduced odor compared to free fatty acids, contributing to their palatability. Therefore, the glyceride forms of these SCFAs are of considerable interest for their potential application as feed additives.

Successful ETEC infection and proliferation are partially attributed to antigenic fimbriae, which bind specifically to receptors in the porcine small intestine. ETEC F4 and ETEC F18 are two distinct pathotypes characterized by differential fimbrial structures. Porcine F4 and F18 receptors are particularly abundant at the intestinal lining during adolescence, making weaned piglets highly susceptible to these ETEC strains (Rhouma et al., 2017). Both strains are highly prevalent in swine farm environments worldwide (Amezcua et al., 2002; Luppi et al., 2016; Roy et al., 2018). In addition, the expression of F4 receptors peaks during neonatal and post-weaning stages, while F18 receptors are primarily prevalent in the post-weaning stage (Fairbrother et al., 2005). Consequently, newly weaned piglets are concurrently susceptible to both ETEC F4 and ETEC F18. Despite numerous individual assessments of both strains in published disease challenge studies, as far as our knowledge goes, a coinfection model using both strains has not been developed in weanling pigs. The objectives of this pilot study were to test an inoculation dose of ETEC F4 and ETEC F18, and to evaluate the impacts of dietary butyrate and valerate glycerides on growth performance, diarrhea, and systemic immunity of weaned piglets under ETEC F4-ETEC F18 coinfection conditions.

Materials and Methods

Animals, housing, experimental design, and diet

The current experiment was reviewed and approved by the Institutional Animal Care and Use Committee at the University of California, Davis (UC Davis). Ten barrows and ten gilts (Yorkshire × Landrace; weaned at 21 to 24 d of age) were provided by the Swine Teaching and Research Center at UC Davis. Piglets were selected based on susceptibility trends in litters after testing for F4 and F18 receptor status using methods adapted by Kreuzer et al. (2013) and Garas et al. (2017). Piglets were randomly allotted to one of the three dietary treatments in a randomized complete block design, where animals were blocked by genotyping results and body weight (BW) within sex and litter with pig as the experimental unit. Animals were individually housed (pen size: 0.61 × 1.22 m) at the Cole facility at UC Davis for a total of 14 d. A 7-d adaptation period was allowed, then all animals were inoculated on three consecutive days (days 0, 1, and 2) with ETEC F4 and ETEC F18 (0.5×10^9 CFU/1.5 mL dose for each strain). Inoculums were prepared by the Western Institute for Food Safety and Security at UC Davis. ETEC F18 and F4 was isolated from a field disease outbreak by Dr. John Morris Fairbrother at the University of Montreal (isolate number: ECL22131 and ECL20230). The virotypes for both strains were confirmed positive for production of heat labile toxin, and heat stable toxins A and B. Pigs were not given vaccines or antibiotics prior to weaning and the absence of hemolytic coliforms in fecal cultures were confirmed prior to inoculation. The Swine Teaching and Research Center was free of porcine reproductive and respiratory syndrome virus and rotavirus when this experiment started.

Six replicate pigs were allotted to the control diet and seven replicate pigs were allotted to each experimental treatment. Diets were provided in mash form ad libitum throughout the entire trial period and formulated to meet the nutrient requirements of pigs (NRC, 2012). The three dietary treatments were (1) Control diet: based on corn, soybean meal, and dried whey, (2) Butyrate glycerides diet: control diet supplemented with 0.1% butyrate glycerides, (3) Valerate glycerides diet: control diet supplemented with 0.1% valerate glycerides. SCFA glycerides were generously provided by Perstorp Animal Nutrition (Sweden). The chemical composition of both butyrate and valerate glycerides consisted of 50% monoglycerides, 35% diglycerides, 5% triglycerides, and 10% glycerol. The inclusion dose was determined by consulting our industry advisor.

Clinical observations, and sampling

Throughout the trial period, diarrhea, and alertness were scored based on observations and recorded twice daily. Two independent evaluators assessed diarrhea visually, with the score ranging from 1 to 5 (1 = normal feces, 2 = moist feces, 3 = mild diarrhea, 4 = severe diarrhea, and 5 = watery diarrhea). The frequency of diarrhea (≥3 or 4, %) was calculated using the formula: [(pig days with diarrhea score (≥3 or 4) ÷ (total pig days)] × 100 (Liu et al., 2013b). Alertness was scored from 1 to 3 (1 = normal, 2 = slightly depressed or listless, and 3 = severely depressed or recumbent). Scores for alertness did not exceed two throughout the entire experiment (data not shown). Pigs and feeders were weighed on days −7, 0 before inoculation, and day 7 post-inoculation (PI). Cotton swabs were used to collect fecal samples for culture on days −7, 0, 2, and 4 PI. Swabs were streaked on Columbia Blood Agar with 5% sheep blood to identify lysis of red blood cells surrounding colonies, indicative of hemolytic coliforms. Blood samples were taken from the jugular vein of all pigs on day 0 prior to ETEC coinfection challenge, days 4 and 7 PI in vacutainer tubes with or without EDTA to sample whole blood and isolate serum, respectively. All pigs were euthanized on day 7 PI for tissue sample collection. Prior to euthanasia, pigs were anesthetized with 1 mL mixture of 100 mg telazol, 50 mg ketamine, and 50 mg xylazine (2:1:1) by intramuscular injection. Euthanasia proceeded with intracardiac injection with 78 mg sodium pentobarbital (Vortech Pharmaceuticals, Ltd., Dearborn, MI) per 1 kg of BW.

Complete blood count and serum tumor necrosis factor alpha

Whole blood samples were submitted to the Comparative Pathology Laboratory at UC Davis for total and differential blood cell count. Porcine blood was differentiated using a multiparameter automated programmed hematology analyzer (Drew/ERBA Scientific 950 FS Hematological Analyzer, Drew Scientific Inc., Miami, FL). Serum samples were used to quantify the concentration of pro-inflammatory cytokine, tumor necrosis factor alpha (TNF-α) by Enzyme-linked immunosorbent assay (ELISA; R&D System Inc., Minneapolis, MN). All samples were analyzed in duplicate including standard and control.

Statistical analysis

All data were analyzed in RStudio (RStudio, Boston, MA). Outliers were identified and removed based on the interquartile range method. Normality of data were assessed using the Shapiro–Wilk test or quantile-quantile plots and homogeneity of variance was confirmed using Levene's test. Dietary treatment was the fixed effect and initial BW and sex were random effects. All data were analyzed by ANOVA and pairwise comparisons were made using Dunnett's test to determine significant differences between treatments. The Chi-square test was used for analyzing the frequency of diarrhea. Statistical significance and tendency were considered at $P < 0.05$ and $0.05 \leq P < 0.10$, respectively.

Results

Growth performance, fecal culture, and diarrhea

The initial mean BW of pigs was 8.21 ± 1.25 kg on d −7 PI. Prior to inoculation, the BW of pigs were 10.02, 10.08, and 10.2 kg for control, butyrate glycerides, and valerate glycerides groups, respectively. The final BW of pigs on day 7 PI were 11.03, 11.33, and 12.10 kg in control, butyrate glycerides, and valerate glycerides groups, respectively. Supplementation of valerate glycerides tended ($P = 0.095$) to increase final BW compared with control.

Pigs were free of hemolytic coliforms on day −7 and 0 before ETEC inoculation. After co-inoculation, all pigs exhibited fecal shedding of hemolytic coliforms on day 2 PI, and 95% of pigs exhibited fecal shedding of hemolytic coliforms on day 4 PI. Prior to inoculation, the diarrhea scores of weaned piglets did not exceed a score of two across all groups. The inoculation dose incited diarrhea in piglets, as indicated by the majority of pigs exhibiting diarrhea scores equal or higher than three after day 3 PI. Supplementation of butyrate or valerate glycerides did not impact frequency of diarrhea (score ≥ 3) but reduced ($P < 0.05$) the severity of diarrhea (score ≥ 4) from days 0 and 7 PI (Figure 1).

Systemic immunity

Total white blood cell (WBC) counts, neutrophils, lymphocytes, and serum concentration of TNF-α on days 0, 4 PI, and 7 PI are reported in Table 1. Dietary treatment had no effect on the total number of WBCs or lymphocytes. However, time had a significant ($P < 0.01$) effect, as indicated by increased WBCs, neutrophils, and lymphocytes post-infection. Supplementation of valerate glycerides tended ($P = 0.061$) to reduce neutrophil counts compared with control. The serum concentration of TNF-α was affected ($P < 0.05$) by treatment, where

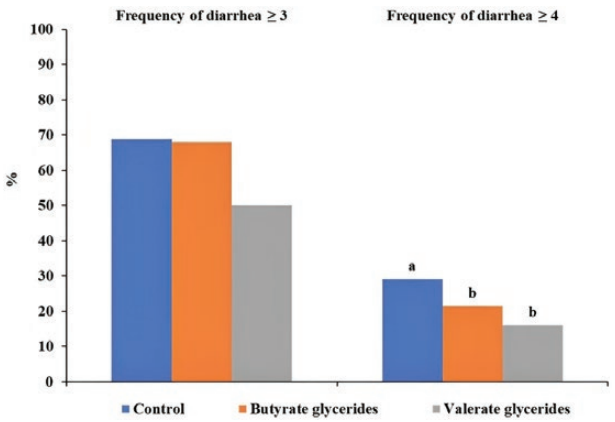

Figure 1. Frequency of diarrhea (days 0 to 7 post-inoculation) of *Escherichia coli*-infected weaned pigs fed diets supplemented with 0.1% butyrate or valerate glycerides. Frequency of diarrhea was calculated as the percentage of pigs with diarrhea score ≥3 or 4 out of total pigs. [a,b]Means without a common superscript are different ($P \leq 0.05$).

both butyrate glycerides and valerate glycerides groups maintained lower serum TNF-α compared with control group at all-time points.

Discussion

While ETEC F18 is more prominent among weaned pigs suffering from diarrhea, ETEC F4 also maintains a high prevalence in commercial swine farm environments. Moreover, both strains may coexist during the post-weaning period (Luppi, 2017). The ETEC challenge model employing a singular strain of F18 has been validated in our prior published research at the dose of 10^{10} CFU/dose used for three consecutive inoculations (Liu et al., 2013a; He et al., 2020; Kim et al., 2022; Wong et al., 2022). The present study marks the first investigation into the coinfection with ETEC F4 and F18 in newly weaned pigs. Results regarding diarrhea and fecal shedding indicate the successful infection using two strains (ETEC F4 and ETEC F18) at 0.5×10^9 CFU/dose/strain, administered orally over three consecutive days. In close agreement with single-strain infections, the severity of diarrhea increased, and hemolytic coliforms were present in feces post-ETEC inoculation (He et al., 2020; Kim et al., 2022). In addition, the counts of total WBCs, neutrophils, and lymphocytes increased over time, indicating the on-going systemic inflammation induced by ETEC infection. However, the current pilot study did not determine the colonization of ETEC F4 and F18 in the intestine, which should be considered in the future study with a longer experimental period and a larger replication numbers. Addtionally, conducting histopathology on the entire intestinal tract is recommended to further investigate the damage caused by ETEC infection.

The beneficial effects of butyrate derivatives on pig performance and health have been demonstrated in numerous studies where sodium butyrate was fed (Piva et al., 2002; Lu et al., 2008). However, a majority of these previously published research studies were conducted with pigs without experimental disease challenge. Additionally, there is limited research on the effects of valerate derivatives in weaned pigs. In this pilot study, it was observed that valerate glycerides tended to enhance growth, and both supplements mitigated the severity of diarrhea in ETEC-infected weaned pigs. These

Table 1. Total and differential white blood cells, and serum TNF-α in weaned pigs under ETEC-F4-ETEC F18 coinfection conditions and fed diets supplemented with 0.1% butyrate or valerate glycerides

Item[1]	Control	Butyrate glycerides	Valerate glycerides	SEM	*P*-value		
					Treatment	Day	Interaction
WBC, 10^3/μL							
day 0	8.33	8.15	10.10	1.22	0.61	<0.01	0.40
day 4 PI	12.78	11.58	11.23	1.13			
day 7 PI	11.39	13.39	13.88	1.13			
Neu, 10^3/μL							
day 0	3.66	3.73	3.60	0.51	0.70	<0.01	0.15
day 4 PI	5.62	4.40	4.05	0.50			
day 7 PI	4.87	5.10	6.02	0.54			
Lym, 10^3/μL							
day 0	3.35	3.19	4.21	0.44	0.32	<0.01	0.87
day 4 PI	4.70	5.10	4.92	0.52			
day 7 PI	4.86	5.48	5.85	0.65			
TNF-α, pg/mL							
day 0	302	175	199	65.42	<0.05	0.53	0.99
day 4 PI	251	147	121	55.48			
day 7 PI	291	159	194	69.45			

[1]WBC, white blood cell; Neu, neutrophil; Lym, lymphocyte; TNF-α, tumor necrosis factor alpha. Each least squares mean represents 6 to 7 observations.

results indicate that both butyrate and valerate glycerides have the potential to serve as feed additives. However, further investigation of both butyrate and valerete glycerides on performance and diarrhea is warranted due to the limitations of the present pilot study, with small animal replication numbers and a short experimental period.

In the present study, systemic immune responses were evaluated by analyzing the total and differential WBC profile and TNF-α concentrations. The results indicate that supplementation of both butyrate and valerate glycerides modulated the systemic inflammatory status of ETEC-infected pigs, as evidenced by lower serum TNF-α levels in both treatment groups than in control. TNF-α is also produced in response to weaning stress (Moeser et al., 2007), which may explain the comparable serum levels observed in control pigs prior to inoculation and post-inoculation. Circulating TNF-α levels are closely associated with the downstream recruitment of neutrophils during instances of inflammation (Colotta et al., 1992). Consequently, the reduced circulatory TNF-α may have contributed to the tendency for fewer neutrophil counts on day 4 PI in pigs that were fed with valerate glycerides. During ETEC infection, diarrhea is exacerbated due to the secretion of bacterial enterotoxins (Loos et al., 2012), which may also stimulate intestinal inflammation and exert further deleterious effects on intestinal barrier function (Feng and Teitelbaum, 2013). Although variations exist in local and systemic immune responses, the two aspects are tightly connected to provide effective protection to animals. Taken all together, the potential benefits of butyrate and valeterate derivatives on growth performance might be related to the modulated systemic immunity in weaned pigs.

In conclusion, the current pilot study indicated that a coinfection of ETEC F4 and F18 could stimulate diarrhea similarly to a single-strain infection. Further research is necessary to optimize the inoculation dose and confirm the presence of the inoculated strains within the intestine of weaned pigs. Additionally, the present study presented preliminary data supporting the potential of dietary butyrate and valerate glycerides to mitigate post-weaning diarrhea in piglets. Future full-scale research should focus on the underlying mechanisms, such as, the modulation of intestinal immunity, the regulation of gene expression through histone deacetylase inhibition (Inan et al., 2000; Yuille et al., 2018) and G-protein coupled receptor agonism (Brown et al., 2003; Poul et al., 2003; Masui et al., 2013).

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Conflict of interest statement

The authors disclose that there was no conflict of interest.

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