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Impacts of Monoglycerides on Intestinal Integrity and Disease Resistance of Weaned Pigs

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Impacts of Monoglycerides on Intestinal Integrity and Disease Resistance of Weaned Pigs

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ABSTRACT

Monoglycerides, glycerol monoesters of fatty acids, offer several advantages over organic acids by increasing stability, reducing unpleasant odors, and enabling the gradual release of bioactive substances (i.e., organic fatty acids) throughout the intestine, where they can positively affect intestinal integrity. These properties make monoglycerides attractive nutritional interventions for improving animal health and growth. In this context, three studies were performed to investigate the potential beneficial effects of monoglycerides as a practical strategy to improve disease resistance and overall health in pigs.

The first study aimed to evaluate the potential of a monoglyceride blend and zinc glycinate as sustainable methods for improving intestinal mucosal integrity in weaned pigs. *In vitro* cell culture models (porcine enterocyte cell line [IPEC-J2] and porcine alveolar macrophages [PAM]) were used to assess intestinal barrier and immunomodulatory functions. IPEC-J2 cells treated with 250 and 1,000 μg/mL of monoglycerides had increased ($P < 0.05$) transepithelial electrical resistance (TEER) values at 48 and 72 h after treatment, while 5 μg/mL of zinc glycinate showed increased ($P < 0.0001$) TEER values only at 72 h after treatment, compared with the control group. Lipopolysaccharide (LPS) challenge increased ($P < 0.05$) the production of tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β) from PAM. In the non-challenge group, 50 or 100 μg/mL of monoglycerides stimulated ($P < 0.05$) TNF-α and IL-1β production from PAM. Treatment with 25 or 100 μg/mL of zinc glycinate also enhanced ($P < 0.05$) TNF-α production from PAM. In LPS-treated PAM, 1,000 μg/mL of monoglycerides increased ($P < 0.05$) IL-1β production, while zinc glycinate suppressed ($P < 0.0001$) the secretion of TNF-α and IL-1β at the doses of 100, 250, and 500 μg/mL. The results from *in vitro* culture assays indicate that

monoglycerides have a beneficial effect on strengthening epithelial barrier function, while zinc glycinate may have strong anti-inflammatory benefits.

The second study evaluated the effects of dietary monoglycerides (blend of short- and medium-chain fatty acids; BalanGut™ LS L; BASF SE) on the gut health and immunity of weaned pigs experimentally infected with an enterotoxigenic *Escherichia coli* (ETEC) F18. Pigs in high-dose zinc oxide (ZNO; 3,000 mg/kg) and antibiotic groups had lower ($P < 0.05$) severity of diarrhea than control, but the severity of diarrhea was not different between antibiotic and monoglycerides groups. Pigs fed with monoglycerides or ZNO had lower ($P < 0.05$) serum haptoglobin on d 2 or 5 post-inoculation (PI) than control. Pigs in ZNO had greater ($P < 0.05$) goblet cell numbers per villus, villus area and height, and villus height-to-crypt depth ratio (VH:CD) in duodenum on d 5 PI than pigs in control, monoglycerides, and antibiotic groups. Pigs supplemented with monoglycerides, ZNO, or antibiotic had reduced ($P < 0.05$) ileal crypt depth compared with control on d 5 PI, contributing to the increase ($P = 0.06$) in VH:CD. Consistently, pigs in ZNO expressed the lowest ($P < 0.05$) *TNFa*, *IL6*, *IL10*, *IL12*, *IL1A*, *IL1B*, and *PTGS2* in ileal mucosa on d 5 PI, while no difference was observed in the expression of those genes between ZNO and monoglycerides. Supplementation of ZNO or antibiotic had significant impacts on metabolic pathways in the serum compared with control, particularly on carbohydrate and amino acids metabolism, while limited impacts on serum metabolites were observed in monoglycerides group when compared with control. Pigs supplemented with ZNO had greater ($P < 0.05$) growth performance than other treatments, but no difference was observed in average daily feed intake between ZNO and monoglycerides groups during the post-challenge period. The second study indicates that supplementation of monoglyceride blend may enhance disease resistance of weaned

pigs by alleviating the severity of diarrhea and mitigating intestinal and systemic inflammation, although the effectiveness may not be comparable to high-dose zinc oxide.

The third study was conducted to explore the effects of dietary supplementation of organic acids (blend of formic acid, lactic acid, and sodium formate; Acitra G20C, Eastman), monoglycerides (blend of short- and medium-chain fatty acids; Entero-Nova 410C, Eastman), and combination of both acid-based additives on diarrhea, immune responses, and growth performance of ETEC F18-challenged weaned pigs. Supplementation of organic acids, monoglycerides, or their combination significantly reduced ($P < 0.05$) the frequency of diarrhea compared with control group. ETEC F18 infection increased ($P < 0.05$) counts of total white blood cells, neutrophils, and lymphocytes on d 5 and 14 PI, compared with d 0. Supplementation of the combination of organic acids and monoglycerides reduced ($P < 0.05$) the counts of total white blood cells, lymphocytes, and the ratio of neutrophils to lymphocytes, and tended ($P < 0.10$) to reduce neutrophil count on d 5 PI, compared with control. Pigs fed with monoglycerides had higher ($P < 0.05$) neutrophil counts than pigs in the control group on d 14 PI. Pigs fed with monoglycerides tended ($P < 0.10$) to have lower serum granulocyte-macrophage colony-stimulating factor, interleukin-1 alpha, and TNF-α concentrations on d 0, 5, or 14 PI compared with control. However, supplementation of organic acids, monoglycerides, or the combination of both had limited impacts on growth performance throughout the experiment. The third study suggests that dietary supplementation of organic acid blend, monoglyceride blend, and or their combination may alleviate diarrhea, intestinal damage, or inflammation caused by ETEC infection in pigs. Moreover, the potential for synergistic effects related to immune-modulatory effect observed in the combination group requires further investigation. In summary, monoglycerides investigated in the present studies have positive effects

on disease resistance and the overall health of weaned pigs under ETEC F18 infection, by improving gut integrity and modulating local and systemic inflammation.

KEY WORDS: enterotoxigenic *Escherichia coli*, gut integrity, monoglycerides, post-weaning diarrhea, systemic immunity, weaned pigs

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CHAPTER 1

LITERATURE REVIEW

1.1 Gut health of weaning pigs

1.1.1. Gastrointestinal function development and early life stress

The gastrointestinal tract is responsible not only for the breakdown and transport of nutrients but also for maintaining fluid balance and acting as a barrier (physical, biochemical, and immunological) to protect the host from the external environment (Vancamelbeke and Vermeire, 2017). More than two-thirds of the body's immune cells are located in the gut, and this key immune organ plays a critical role in maintaining balance among the complex interactions of nutrients, microbes, and the epithelium (Blikslager et al., 2007; Pluske et al., 2018; Szabó et al., 2023). Therefore, maintaining good gut health is crucial for the efficient utilization of ingested nutrients and proper epithelial barrier and immune response, which are essential for the growth and health of pigs (Heo et al., 2013). The intestinal health of pigs can be threatened throughout their lives by various factors, including anti-nutritional factors, antibiotic misuse, and environmental stress. However, early life stages are particularly vulnerable due to physiological immaturity (Campbell et al., 2013).

Weaning is the most stressful event in the early life of pigs, typically occurring at 3 to 4 weeks of age under commercial conditions. During the weaning stage, piglets experience an immunity gap characterized by a decline in passive immunity and insufficient active immunity (Moeser et al., 2017). Additionally, stress induced by weaning during the critical period of postnatal intestinal maturation can compromise the development and function of the gut, disrupting

its ability to achieve optimal developmental trajectories (Figure 1.1) (Moeser et al., 2017; Tang et al., 2022). This disruption may also affect gut health and susceptibility to diseases in subsequent phases (Medland et al., 2016; Pohl et al., 2017; Pluske et al., 2018). Although many studies have explored practical strategies to manage the transition (Smith et al., 2010; Lancheros et al., 2020; Park et al., 2020b), the complex interplay of physiological, nutritional, and environmental challenges makes it difficult to manage weaning stress.

1.1.2. Post-weaning diarrhea

The combination of stressors during the weaning period impairs the efficient use of resources in weanling pigs by causing physiological changes such as decreased appetite, intestinal dysfunction, and immune dysregulation (Lallès et al., 2007). At the same time, compromised gut integrity increases the host's susceptibility to external factors, such as antigens, pathogens, and toxins, leading to post-weaning diarrhea (PWD) and undesirable inflammation (McLamb et al., 2013; Jayaraman and Nyachoti, 2017). PWD typically occurs within a few days to weeks after weaning and is a common gastrointestinal disease in piglets. It not only causes growth retardation but also has a high mortality, resulting in significant economic losses and animal welfare concerns (Liu et al., 2013). Although a variety of pathogens can cause diarrhea in weanling piglets, pathogenic *Escherichia coli* (*E. coli*) infection is the most common cause of PWD (Figure 1.2) (Almeida et al., 2022; Paiva et al., 2023). Theoretically, acute diarrhea is associated with altered intestinal motility, decreased nutrient assimilation, and/or increased water and electrolyte secretion (Hornbuckle et al., 2008). These symptoms closely align with those observed in *E. coli*-associated PWD in pigs, such as loose stools, dehydration, metabolic acidosis, and poor appearance.

1.1.3. Diarrheagenic *Escherichia coli*–enterotoxigenic *Escherichia coli*

E. coli, a member of the *Enterobacteriaceae* family, is a facultatively anaerobic, Gram-negative bacterium commonly found to be commensal in the intestine of healthy animals (Kaper et al., 2004). *E. coli* maintains biological activity by regulating cytoplasmic pH within an optimal range (i.e., pH 7.2-7.8) to survive and colonize across a wide range of gastrointestinal pH (i.e., pH 4.5-9) (Gonzales et al., 2013). The optimal conditions for the growth of *E. coli* are 37℃ and a pH close to neutral or slightly alkaline (Tuttle et al., 2021; Xu et al., 2023). In contrast to commensal *E. coli* strains, which typically do not induce disease under normal conditions, some *E. coli* strains with certain specific virulence factors have been classified as enteropathogenic based on their pathogenesis and clinical symptoms (Kaper et al., 2004; Mare et al., 2021; Yu et al., 2021). These include: 1) enteropathogenic *E. coli* (EPEC); 2) enterohaemorrhagic *E. coli* (EHEC); 3) enteroinvasive *E. coli* (EIEC); 4) diffusely adherent *E. coli* (DAEC); 5) enteroaggregative *E. coli* (EAEC); and 6) enterotoxigenic *E. coli* (ETEC). Among the diarrheagenic *E. coli* strains, ETEC is the predominant pathotype of *E. coli* associated with PWD in pigs (Dubreuil, 2021). Although surface structures of *E. coli*, including O (lipopolysaccharide; LPS) and H (flagellar) antigens, can also be used for serological classification, serotyping is considered inefficient and inaccurate due to cross-reactivity resulting from high diversity (Fratamico et al., 2016; Debroy et al., 2018; Mare et al., 2021).

1.1.3.1. Pathogenesis of *Escherichia coli*

In the pathogenesis of pathogenic bacteria, including ETEC, several virulence factors enable the bacteria to attach to host cells, proliferate, evade the defense system, and cause adverse effects (Turner et al., 2006; Nash et al., 2015; Kim et al., 2022). The primary virulence factors essential to the pathogenic process of ETEC are colonization factors (CF; i.e., adhesions) and

enterotoxins (Dubreuil, 2021). Upon ingestion through contaminated sources (e.g., fecal-oral route), ETEC reaches the small intestine, where infection begins as ETEC colonizes the small intestine by attaching to specific receptors on the intestinal epithelium via fimbrial adhesins (Zhang et al., 2022). This colonization of ETEC facilitates the stable and intense production and secretion of enterotoxins. These toxins then bind to specific receptors on the intestinal epithelium, causing local lesions and impairing gut integrity (Kim et al., 2022; Svennerholm and Lundgren, 2023).

Adhesion and invasion–adhesins. The major adhesive surface antigens in ETEC are fimbriae (Sun and Kim, 2017). These fimbrial structures recognize and bind to specific receptors on the surface of small intestinal enterocytes (Heo et al., 2015). There are five groups of porcine ETEC fimbrial adhesions, including F4 (K88), F5 (K99), F6 (987P), F7 (F41), and F18. There is also an age-related correlation based on fimbrial types. F5 (K99), F6 (987P), and F7 (F41) fimbriae are primarily associated with diarrhea in newborn piglets. F4 (K88) fimbriae are observed in both neonatal/suckling and weaned pigs, while F18 fimbriae are mainly associated with PWD (Frydendahl, 2002; Hartadi et al., 2020). This relationship is not surprising given that intestinal receptors are expressed differently depending on the age of the pig (Nagy et al., 1992), and successful attachment is a prerequisite for ETEC infection. Differences in the relationship between the adhesin and the host receptor may also contribute to the host specificity of ETEC (Fleckenstein et al., 2010). According to a recent report from the Iowa State University Veterinary Diagnostic Laboratory (Paiva et al., 2023), ETEC F18 fimbriae were observed to be predominant when genotyping was performed on cases submitted for diagnosis of pig PWD (Figure 1.3).

F18ab and F18ac, variants of ETEC F18, are known to induce different diseases in pigs. F18ab is associated with edema disease, whereas F18ac is known to cause PWD in pigs (Duarte et al., 2023a). The operon encoding F18 fimbriae includes five genes (*fed*A, *fed*B, *fed*C, *fed*E and

*fed*F). The sequence of FedF, which is associated with adhesin, has been reported to be conserved across different regions and variants, suggesting that both variants share the same receptor (F18 receptor; F18R) (Cox et al., 2012). Polymerase chain reaction (PCR) test can be used to detect the presence of the F18 receptor in pigs, assessing their susceptibility to ETEC F18 infection. It has been reported that a point mutation at nucleotide 307 of the *FUT1* gene, which encodes alpha(1,2)-fucosyltransferase, confers resistance to ETEC F18 in pigs with the A/A homozygous genotype. In contrast, pigs with the G/G or G/A genotypes are susceptible (Meijerink et al., 2000; Kreuzer et al., 2013). The *FUT1* gene is involved in the expression of H blood group antigens (HBGA), which are related to the receptor for ETEC F18 fimbriae (Duarte et al., 2023a). Previous studies have reported that the *FUT1M307* genotype in pigs is highly associated with ETEC F18 susceptibility (87%), although a resistant genotype does not completely exclude the risk of ETEC F18 infection (6%) (Frydendahl et al., 2003; Sun and Kim, 2017).

Cytotoxic effects–enterotoxins. Enterotoxins produced by ETEC can compromise intestinal integrity and lead to infection and disease (Boeckman et al., 2022). Based on their thermal stability, these toxins are divided into two groups: heat-labile (LT) and heat-stable (ST) toxins. Enterotoxins primarily induce secretory diarrhea due to fluid imbalance.

LT are classified into LT-I (LT-Ih and LT-Ip) and LT-II (LT-IIa, LT-IIb, and LT-IIc) based on their antigen properties (Wang et al., 2019). LT is a high-molecular-weight (MW = ~88 kDa) complex that remains stable at 60℃ for up to 15 minutes. Upon binding to enterocyte receptors, LT activates adenylate cyclase (AC) (Read et al., 2014). The activation of AC induces an excessive increase in cyclic adenosine monophosphate (cAMP), which affects the cystic fibrosis transmembrane regulator (CFTR) chloride ion channel through protein kinase A (PKA) activation, resulting in electrolyte and water loss (Read et al., 2014). In addition to the loss of intestinal

permeability associated with ion channels, LT has been reported to support adhesion for colonization (Berberov et al., 2004; Horstman et al., 2004; Sun and Kim, 2017) and to increase levels of pro-inflammatory cytokines (Soriani et al., 2002; Read et al., 2014).

ST are low-molecular-weight monomeric peptides that remain stable at 100℃ for up to 15 minutes. They exist in two variants: STa and STb (MW = 2 and 5 kDa, respectively) (Cox et al., 2012). STa binds to the intestinal receptor, guanylyl cyclase-C (GC-C), leading to the activation of guanylate cyclase and a subsequent increase in cyclic guanosine monophosphate (cGMP) synthesis (Read et al., 2014). Increased cGMP, similar to the effect of LT, leads to electrolyte and water loss through the alteration of the CFTR channels via protein kinase G II activation (Vaandrager et al., 1997; Vaandrager et al., 2000). Additionally, cGMP indirectly affects CFTR by inhibiting phosphodiesterase 3 (PDE3), which further regulates cAMP and PKA (Dubreuil, 2017). STa also has adverse effects on intestinal epithelial barrier integrity, leading to intestinal mucosal disorders (Whipp et al., 1985) and increased levels of pro-inflammatory cytokines (Loos et al., 2013). In contrast to STa, STb is susceptible to proteolytic enzymes, and its detrimental effects on intestinal barrier function begin with binding to acidic glycosphingolipid (i.e., sulfatide) on the intestinal epithelium (Butt et al., 2020). STb increases intracellular calcium influx and activates chloride channels by increasing calcium-dependent enzymes, thereby inducing chloride efflux. Elevated intracellular calcium levels can also cause fluid loss through several mechanisms, including alterations in ion channels (i.e., CFTR and NHE3) and the synthesis of intestinal secretagogues (i.e., prostaglandin E2 and 5-hydroxytryptamine) (Cox et al., 2012). STb has also been reported to have adverse effects on gut integrity, including damaging intestinal mucosa (Whipp et al., 1985; Whipp et al., 1986; Whipp et al., 1987) and altering tight junction protein

expression (Dubreuil, 1997; Mukiza and Dubreuil, 2013) or redistribution (Mukiza and Dubreuil, 2013; Nassour and Dubreuil, 2014; Dubreuil, 2017).

Lipopolysaccharides. LPS is a heat-stable endotoxin that is an essential and specific surface structure of bacteria, especially Gram-negative bacteria such as *E. coli* (Alexander and Rietschel, 2001; Miyamoto et al., 2009). It has been reported that the outer membrane of *Salmonella* and *E. coli* consists primarily (> 75%) of LPS (Klein and Raina, 2019; Avila-Calderón et al., 2021). LPS is structurally composed of three parts from the outside: polysaccharide (O-antigen), core oligosaccharide, and lipid A (glycolipid) (Neidhardt et al., 1987; Amor et al., 2000). Generally, the external structure (O-antigen) is variable, which allows for specific discrimination (serotyping) among bacteria strains, while the internal structure (lipid A) is more conservative (DebRoy et al., 2011; Debroy et al., 2018; Farhana and Khan, 2023). Lipid A is the primary component responsible for toxicity (i.e., immune activation) and can vary in structure depending on the bacterial species (Caroff and Novikov, 2020; Farhana and Khan, 2023).

LPS has been widely utilized in numerous *in vitro* and *in vivo* studies of physiology, immunology, endocrinology, metabolism, and toxicity due to its immune-activating properties (Molteni et al., 2016; Farhana and Khan, 2023). LPS is recognized by pattern recognition receptors (PRR) of the host as a specific pathogen-associated molecular pattern (PAMP) (Rallabhandi et al., 2006; Yu et al., 2010). Toll-like receptor 4 (TLR4), an LPS-specific PRR, is primarily expressed on the surface of innate immune cells (e.g., macrophages, neutrophils, and endothelial cells) and induces signaling cascades associated with inflammatory responses (Akira and Takeda, 2004; Akira et al., 2006). In the interaction between LPS and its receptor, LPS binding protein (LBP), cluster of differentiation 14 (CD14), and myeloid differentiation factor 2 (MD2) act as cofactors to trigger downstream signaling (Khan et al., 2018). This signaling leads to the regulation of

transcription factors (e.g., mitogen-activated protein kinases [MAPK] and nuclear factor-kappa B [NF-κB] activation) and the activation of innate immune cells (Alexander and Rietschel, 2001; Pålsson-McDermott and O'Neill, 2004; Hayden et al., 2006). Consequently, this process results in the secretion of inflammatory mediators (e.g., cytokines/chemokines) and the inflammatory response (Figure 1.4). While this response is essential for maintaining homeostasis, it is also crucial to manage properly to prevent host cell damage from excessive exposure (Renz et al., 2012; Page et al., 2022; Javed et al., 2023).

1.1.3.2. Clinical signs

Pigs infected with ETEC are characterized by acute diarrhea (watery and yellowish stools) due to fluid loss caused by the toxic effects of virulence factors that disrupt electrolyte regulation in the intestinal wall (Liu et al., 2014; Kim et al., 2022). This condition is often accompanied by a dirty appearance, loss of appetite, poor physical condition, dehydration, and depression. In severe cases, it can lead to electrolyte imbalance, nutritional deficiencies, metabolic acidosis, or sudden death without obvious symptoms (Fairbrother et al., 2005; Hornbuckle et al., 2008; Luppi, 2017). This impact of ETEC infection is significant not only due to the economic losses associated with stunted growth, mortality, morbidity, and treatment costs (e.g., preventive/therapeutic medications or additives), but also with regard to animal welfare (Zhang et al., 2007; Nadeau et al., 2017; Cremonesi et al., 2022).

Farm history and clinical signs in piglets can be used to diagnose PWD. Additionally, specific culture media containing lactose (such as MacConkey agar) or mammalian blood (such as blood agar) can help identify some ETEC associated with PWD (i.e., ETEC F4 and F18), which are characterized by their ability to utilize lactose and their hemolytic properties (Luppi, 2017; He et al., 2020a; Mueller and Tainter, 2023). Most ETEC isolates from pigs with PWD on farms in

Europe have been identified as hemolytic (Luppi et al., 2016). However, a definitive diagnosis requires strain isolation and the identification of enterotoxin and fimbriae by PCR (Navez et al., 2023). Necropsy can reveal characteristic ETEC lesions, such as enlargement or hyperemia of the stomach and mesenteric lymph nodes, which aid in guiding further laboratory investigations (Luppi, 2017). Acute diagnosis is crucial for the effective and efficient management of PWD caused by ETEC.

ETEC-infectious diarrhea in weaned pigs, which requires a minimum dose of 10^9-10^{10} ETEC, typically has an average incubation period of two days. Acute diarrhea may last up to 10 days (Sun and Kim, 2017; Khalil et al., 2021; Duarte et al., 2023a). The intensity and/or duration of PWD and the associated symptoms can vary depending on several factors, including the condition of the animal and management strategies used during the period. The detrimental effects of ETEC infection can persist even after the symptoms disappear (Fairbrother et al., 2005; Gebhardt et al., 2020; Duarte and Kim, 2022). A recent review paper summarized several studies on the effects of experimental challenge with ETEC F18 on the health of weaned pigs (Duarte et al., 2023a), including:

1) Reduced average daily gain (by 28%), average daily feed intake (by 10%), and gain-to-feed ratio (by 20%) (Liu et al., 2013; Andersen et al., 2017; Kim et al., 2019; Li et al., 2019; Becker et al., 2020; Duarte et al., 2020; He et al., 2020b; Sun et al., 2021; Wong et al., 2022; Caprarulo et al., 2023; Chang et al., 2023; Duarte et al., 2023b; Jang et al., 2023; Jerez-Bogota et al., 2023).

2) Increased levels of interleukin 6 (IL-6; by 60%) (Duarte et al., 2020; Wong et al., 2022), IL-8 (by 43%) (Becker et al., 2020; Duarte et al., 2020; Xu et al., 2022), tumor necrosis factor-alpha (by 28%) (Duarte et al., 2020; Duarte and Kim, 2022; Xu et al., 2022; Duarte et al., 2023b; Jang et al., 2023).

3) Altered villus height (VH; reduced by 14%), crypt depth (CD; increased by 6%), and villus height-to-crypt depth ratio (reduced by 18%) (Liu et al., 2013; Kim et al., 2019; Li et al., 2019; Duarte et al., 2020; Sun et al., 2021; Duarte and Kim, 2022; Xu et al., 2022; Chang et al., 2023; Duarte et al., 2023b; Jang et al., 2023).

1.1.3.3. Conventional practices and other strategies

Antibiotics (e.g., chlortetracycline, tiamulin, tylosin, and carbadox) and pharmacological doses (2,000–3,000 mg/kg) of zinc oxide had/have been widely used in nursery diets to control PWD and to promote animal health and growth (Pluske et al., 2002; López-Gálvez et al., 2021; Xie et al., 2021). They have antimicrobial properties and increase nutrient availability by enhancing gut health and resource utilization, reducing gut infections, and modifying gut microbial composition and metabolism (Hung et al., 2020; Kim et al., 2022). Despite their effectiveness, public concerns about potential risks to human health, antibiotic resistance, and environmental impact have led to restrictions on the use of antimicrobial growth promoters (Table 1.1) and a reduction in the use of high-dose zinc oxide in feed (Bonetti et al., 2021; Monger et al., 2021; Rahman et al., 2022). These concerns align with the One Health approach, recognizing the interactions of humans, animals, and the environment, and may result in further restrictions or bans on conventional practices (Aslam et al., 2021; Stoica and Cox, 2021). Therefore, there is an urgent need for alternative practices, including health, management, and nutritional interventions, to promote animal health and welfare while further reducing the use of antibiotics in pig production and reducing reliance on high-dose of zinc oxide in animal feed. Advances in conventional practices are not limited to agents that modulate gut microbiota with antimicrobial activity, but also include strategies that improve growth performance through enhanced gut health (Rhouma et al., 2017; Canibe et al., 2022). Optimal alternative practices should meet the following criteria

(Cheng et al., 2014): 1) no toxicity or side effects to animals, and minimal short-term residues; 2) ability to enhance disease resistance and improve growth and feed efficiency; 3) stability with ease of handling and no impact on palatability; 4) no negative environmental impact.

1.2. Organic acids

1.2.1. Acidification function

Organic acids have been employed as additives in animal feed and water primarily to inhibit the growth of undesirable microorganisms and to lower the pH of the feed (Tugnoli et al., 2020). Inorganic acids, such as hydrochloric acid (HCl), sulfuric acid (H_2SO_4), and phosphoric acid (H_3PO_4), are also used as acidifiers due to their low cost. However, they present safety concerns due to their strong corrosivity and have shown limited research and inconsistent results compared to organic acids (Kim et al., 2005; Che et al., 2012; Liu et al., 2018). Organic acidifiers are chemical compounds with carboxyl group (–COOH). Examples include benzoic acid, citric acid, fumaric acid, propionic acid, lactic acid, and formic acid (Papatsiros and Billinis, 2012). The effectiveness of each organic acid as an acidifier depends on its pKa (acid dissociation constant; Table 1.2), which varies due to differences in carbon chain length and saturation. The pKa influences how the acid interacts with the pH of gastrointestinal environment, affecting its antimicrobial and acidifying activity (Suiryanrayna and Ramana, 2015; Nguyen et al., 2020; Pearlin et al., 2020). In general, organic acids used as additives have a pKa in the range of 3-5 and can be selected based on their intended purpose (Dibner and Buttin, 2002; Tugnoli et al., 2020). Organic acids with relatively low pKa are more effective for lowering stomach pH, while organic acids with relatively high pKa are generally used for feed preservation.

In the early stages of a pig's life, especially during weaning, the gastrointestinal system is immature, making it difficult to maintain proper stomach acidity, and the pH can be further

compromised (up to pH 5) by external factors such as feed (Heo et al., 2013; Park et al., 2020a; Song et al., 2022). It may have an adverse effect on the nutrient digestion and absorption as well as on the growth of pigs. Maintaining appropriate pH in the stomach not only acts as a chemical barrier, but also improves pepsin activity (most active around pH 3), which is related to protein digestibility (Kim et al., 2005; Suiryanrayna and Ramana, 2015). The increased pH level may also accelerate the gastric emptying rate, which may not allow feed to stay in the gastrointestinal tract long enough to be digested (Johnson, 2006). In this regard, lowering the gastric pH by supplementing organic acids during the weaning period can inhibit the invasion of external factors (e.g., pathogens) and increase pepsin activity, thus improving digestibility and performance.

Dietary inclusion of fumaric acid (0.7%) or citric acid (1%) has been reported to reduce the stomach pH of weanling pigs from 4.6 to 4.2 and 3.5, respectively (Scipioni et al., 1979; Suiryanrayna and Ramana, 2015). A meta-analysis indicated that dietary acid supplementation can significantly reduce dietary pH by more than 1 unit; however, the impact on gastric pH was less pronounced, changing from pH 3.73 to pH 3.66 (Tung and Pettigrew, 2006). In addition to lowering pH, several studies have demonstrated that adding organic acids enhances protein and mineral digestibility/utilization (Mroz et al., 2000; Sauer et al., 2009; Liu et al., 2018; Xu et al., 2018) as well as improves growth performance (Partanen and Mroz, 1999; Pettigrew, 2006; Wang et al., 2022) in pigs. This is supported by reports indicating that organic acid supplementation enhances enzyme secretion from the pancreas (Dibner and Buttin, 2002; Adil et al., 2010; Khan and Iqbal, 2016), and may reduce the gastric emptying rate (Van der Sluis, 2002; Nguyen et al., 2020). Comparative research has reported lactic acid to be more effective than other organic acids in improving growth performance in weaned pigs (Tsiloyiannis et al., 2001). However, improved feed efficiency and intestinal morphology have been observed in previous research without

changes in intestinal pH (Lee et al., 2022). Several studies have also shown that supplementation with organic acids (e.g., formic acid and lactic acid) has antibacterial effects against pathogens, like ETEC, which helps alleviate PWD (Tsiloyiannis et al., 2001). The reduction of intestinal digesta pH by organic acids is correlated with reduced growth of coliform bacteria (Knarreborg et al., 2002), suggesting that their effects may be due to an antimicrobial activity in addition to stomach acidification (Cherrington et al., 1991; Risley et al., 1992).

Dissociated organic acids lower the pH in the gut, creating an unfavorable environment for the growth and survival of pathogens, especially acid-intolerant bacteria, thereby reducing their opportunity to proliferate (Canibe et al., 2001). It has been reported that lactic acid supplementation effectively lowers gastric pH and inhibits *E. coli* proliferation in the intestine (Thomlinson and Lawrence, 1981; Ø verland et al., 2007). Formic acid has been reported to have antimicrobial properties against a wide range of pathogens, including bacteria, fungi, and yeast (Partanen and Mroz, 1999; Suiryanrayna and Ramana, 2015). On the other hand, undissociated organic acids can penetrate the cell wall and dissociate, lowering the intracellular pH and disrupting the metabolic functions of the microorganism, such as proton pump activation and increased osmotic pressure, which are essential for homeostasis and survival of bacteria (Holyoak et al., 1996; Lambert and Stratford, 1999; Warnecke and Gill, 2005). Gram-negative bacteria are often more resistant to various hydrophobic and hydrophilic antibiotics than Gram-positive bacteria due to their distinct outer membrane barrier (Delcour, 2009; Zeinab et al., 2023; Kim et al., 2024). However, undissociated organic acids with lipophilicity may be more effective against Gram-negative bacteria through the mechanisms mentioned above (Suiryanrayna and Ramana, 2015). These internal and external pH stresses burden acid-vulnerable bacteria, while creating an

environment favorable for bacteria adapted to relatively low pH (Gauthier, 2002; Mani-López et al., 2012).

The expected and possible effects of organic acids that result in improved animal productivity include: 1) increased pepsin activity, reduced digesta passage rate, and improved digestibility/utilization of nutrients through lowering gastrointestinal pH; 2) utilization as an energy source; and 3) reduced pathogenic bacteria growth and prevention of PWD (de Lange et al., 2010). However, these effects have been reported to vary depending on the buffering capacity of the diet (Blank et al., 1999), and inconsistent responses in animals have also been reported (Risley et al., 1991; Risley et al., 1993; Gottlob et al., 2006; Weber and Kerr, 2008). Meta-analysis of previous studies also found that the effect of organic acid supplementation on growth rate improvement decreases with animal age (Tung and Pettigrew, 2006; Wang et al., 2022). While researchers have explored various approaches to optimize the use of organic acids, including different forms and/or combinations (Pearlin et al., 2020; Wang et al., 2022), interactions with several factors (e.g., age and health of the animal, feed palatability, solubility/stability, buffering capacity from external factors, and safety) still remain critical considerations in the application (Ravindran and Kornegay, 1993; Heo et al., 2013; Tugnoli et al., 2020).

1.2.2. Biological activity on intestinal health

1.2.2.1. Short-chain fatty acids

In general, fatty acids with saturated short carbon chains (< C6) are classified as short-chain fatty acids (SCFA) and are produced primarily by microbial breakdown of carbohydrates, especially dietary fiber (i.e., indigestible polysaccharides), in the large intestine (Scheppach and Weiler, 2004; Zhang et al., 2022). The average production of SCFA in monogastric animals, particularly in humans, is 550 mmol per day (Bergman, 1990; Dalile et al., 2019) and their

concentrations in the weaned pigs' colon are reported to range from a minimum of 58 to a maximum of 98 mmol per kg of dry matter, depending on dietary composition (Hedemann and Bach Knudsen, 2007; Rossi et al., 2010). Previous research observed increased SCFA levels in the colonic digesta of piglets fed a high-fiber diet supplemented with 4% wheat bran and 2% sugar beet pulp (Hermes et al., 2009). In the large intestine, particularly the colon, acetate, propionate, and butyrate are the predominant SCFA produced, although their presence has also been observed in the proximal and distal parts of the small intestine (Williams et al., 1997; Cong et al., 2022). The hindgut of weaned pigs can absorb SCFA, which provide a significant portion of the energy required by colonocytes, with the majority of the produced butyrate used for colonocyte metabolism (Jacobi and Odle, 2012; Liu et al., 2021). Butyric acid, one of the most studied among SCFA, has been reported to improve intestinal health by affecting the proliferation and apoptosis of enterocytes in both the large and small intestines (Sakata, 1987; Bartholome et al., 2004; Kien et al., 2007; Liu, 2015).

In addition to serving as an energy source, SCFA may also have beneficial effects on animal health by alleviating inflammation and enhancing intestinal integrity. SCFA have been shown to improve gut integrity, as indicated by improved intestinal barrier formation and protection. This has been demonstrated through analyses of intestinal histology and morphology, permeability (e.g., transepithelial electrical resistance, marker molecules, and fluorescein isothiocyanate-dextran), tight junction protein expression, reactive oxygen species, and immunofluorescence assays (Willemsen et al., 2003; Liu, 2015; Yan and Ajuwon, 2017; Bedford and Gong, 2018; Feng et al., 2018). These changes have been observed alongside anti-inflammatory effects and improved immunity, as indicated by inflammatory and immune

biomarkers (e.g., cytokines and immunoglobulins), which also contribute to improving intestinal integrity (Vinolo et al., 2011; Wen et al., 2012; Liu et al., 2021).

Improvements in the intestinal barrier and immune function contribute to enhanced intestinal integrity and are associated with the role of SCFA in regulating gene expression at the molecular level (Canani et al., 2011; Feng et al., 2018; Mann et al., 2024). For example, SCFA directly affect gene expression in epithelial or immune cells by inhibiting histone deacetylase or increasing histone acetylation (Figure 1.5) after entering the cells via passive diffusion and monocarboxylate transporters (Cuff et al., 2005; Xiong et al., 2019; Zhang et al., 2022). This causes changes in chromatin structure (i.e., relaxation), leading to alterations in transcription and variable responses depending on cell type, dosage, and exposure time (Yu et al., 2022). Moreover, it has been reported that SCFA affect cellular processes by acting as ligands for various receptors found in different cells, including G-protein-coupled receptors (GPCR), TLR, and peroxisome proliferator-activated receptors (PPAR) (Dalile et al., 2019; Nogal et al., 2021; Yu et al., 2022). Activation of these receptors triggers downstream signaling pathways, leading to cellular changes such as regulation of cytokine secretion, cell differentiation and recruitment, and alleviation of oxidative stress (Zhao et al., 2018; Siddiqui and Cresci, 2021; Strosznajder et al., 2021). GPR43, GPR41, and GPR109A are the most widely studied receptors activated by SCFA, also known as free fatty acid receptor (FFA/FFAR) 2, FFA/FFAR3, and hydroxycarboxylic acid receptor 2/nicotinic acid receptor 1, respectively (Tan et al., 2014; Siddiqui and Cresci, 2021). These receptors are found in diverse regions, including adipocytes, enterocytes, and immune cells, where GPR109A primarily binds to butyric acid, while both GPR43 and GPR41 are activated by various SCFA (Tan et al., 2014; Mann et al., 2024). The biological effects of organic acids discussed in the previous section, such as pH reduction and antimicrobial effects, were not covered here, but it

has been reported that antibacterial ability is mainly exerted in the upper part of the gastrointestinal tract (Knarreborg et al., 2002).

1.2.2.2. Medium-chain fatty acids

Saturated fatty acids with medium-length carbon chains (C6-12) are classified as medium-chain fatty acids (MCFA) and are commonly found in dietary fat sources (e.g., coconut oil and milk) (Borrelli et al., 2021; Luo et al., 2022). MCFA have pKa values around 5 (i.e., they are weak acids), which means they dissociate at neutral pH (Bach and Babayan, 1982; Zentek et al., 2011). One study reported that a significant portion (around 80%) of MCFA in the upper part of the small intestine may have antibacterial activity (Dierick et al., 2002). However, the dissociation rate may vary depending on animal factors (e.g., age, health status, and feed intake) or external factors (e.g., buffering capacity) (Rossi et al., 2010; Zentek et al., 2011; Nhara et al., 2024). Previous research has observed that feeding MCFA increases nutrient (i.e., protein, fat, or fiber) digestibility, leading to improved growth performance (Hanczakowska et al., 2011; Hanczakowska et al., 2013). This section does not describe the details of biological activities, following the decrease in pH and antimicrobial activity mentioned in the previous section.

Natural MCFA include C6 (caproic/hexanoic acid), C8 (caprylic/octanoic acid), C10 (capric/decanoic acid), and C12 (lauric/dodecanoic acid), as fatty acids found in nature are made up of C2 units (acetyl-CoA) (Zentek et al., 2011; Hanczakowska, 2017). The common type of fat, triglyceride form, has also been investigated to verify the effects of MCFA as MCFA have unpleasant odor and are released from triglycerides in the digestive tract during digestion by lipase (Dierick et al., 2002; Decuypere and Dierick, 2003). MCFA are primarily absorbed by enterocytes, transported via the portal vein to the liver, and metabolized through processes such as beta-oxidation, lipogenesis, and ketogenesis. This transport process is more similar to that of SCFA

than to long-chain fatty acids, which are incorporated into chylomicrons (water-soluble lipoproteins) (Papamandjaris et al., 1998; Schönfeld and Wojtczak, 2016; Huang et al., 2021). This rapid absorption in the upper part of the intestine makes it difficult to expect the effects of MCFA in the lower part of the intestine (Zentek et al., 2011; Yang et al., 2024).

MCFA have been reported to have favorable effects on gut health, including serving as an energy source (Guillot et al., 1993; Liu, 2015), improving intestinal structure and barrier function, and enhancing immune response (Hanczakowska et al., 2011; Martínez-Vallespín et al., 2016; Lee and Kang, 2017), as well as reducing pathogen growth rate and diarrhea (Boyen et al., 2008; Lei et al., 2017). Positive changes in these health parameters may lead to improved performance in weaned pigs (Marounek et al., 2004; Hanczakowska et al., 2016; Zhang et al., 2019). The improved gut integrity induced by MCFA may result from modulation of transcription factors and metabolic pathways similar to SCFA, although this has not been studied in pigs as extensively as SCFA (Zentek et al., 2011). GPR84, expressed on the surface of various cells and primarily studied in immune cells, has been reported to be activated by MCFA, with C10-12 being the most effective ligands (Wang et al., 2006). Activation of GPR84 under inflammatory conditions induces an inflammatory response in macrophages, including increased phagocytosis and secretion of inflammatory mediators (Suzuki et al., 2013; Recio et al., 2018). GPR84 is associated with the M1 polarization of macrophages, implying that its activation by MCFA may promote cell-mediated immunity rather than anti-inflammatory effects by influencing the T helper 1 and T helper 2 balance (Wang et al., 2006; Wynn and Barron, 2010; Zhang et al., 2022). The expression of GPR84 can be increased by LPS (e.g., endotoxemia) and pro-inflammatory molecules (Bouchard et al., 2007; Nagasaki et al., 2012; Recio et al., 2018). However, contradictory reports suggest that C10 may have anti-inflammatory effects (Park et al., 2011; Huang et al., 2014), indicating that further

research is needed to clarify tissue-specific effects and the underlying mechanisms. Another receptor activated by MCFA is GPR40 (FFAR1), which is predominantly expressed in the brain and pancreatic β-cells. Activation of GPR40 leads to an increase in the secretion of pancreatic-derived hormones (i.e., insulin) (Briscoe et al., 2003; Itoh et al., 2003). Moreover, intracellular MCFA may modulate nutrient metabolism (e.g., glucose and lipid) by enhancing mitochondrial oxidative capacity and activating PPAR-γ (Ahmadian et al., 2013; Montgomery et al., 2019; Huang et al., 2021).

1.2.3. Application of organic fatty acid derivatives–monoglycerides

Despite the versatile biological activities of organic fatty acids, which affect gut health through intestinal structure/barrier, immunity, and metabolism, these compounds and their related products have limitations such as instability, unpleasant flavor, and corrosiveness (Lauridsen, 2020; Tugnoli et al., 2020; Yang et al., 2024). These issues lead to inconsistent results when those products are supplemented in animal feed (Walsh et al., 2007; Kil et al., 2011). In this context, monoglycerides have emerged as a promising and practical alternative for improving the health of weaned pigs. Monoglycerides offer several advantages, including enhanced stability and palatability compared to organic fatty acids, as well as antibacterial and antiviral effects (Thormar and Hilmarsson, 2007; Jackman et al., 2020).

Monoglycerides (MG; i.e., monoacylglycerols) are ester forms of organic fatty acids, consisting of one molecule of glycerol and one fatty acid, and their strong covalent bond contributes to their stability (Jackman et al., 2022). Considering production efficiency and sustainability, MG are typically synthesized rather than extracted from natural sources (Luo et al., 2022; Yang et al., 2024). The properties and names of MG are determined by the attached fatty acids (Damstrup et al., 2006; Rarokar et al., 2017). For example, different MG have different

melting points, which affect their primary form (e.g., monocaprin is a liquid, while monolaurin is powder) and their application (e.g., a mixture may be a liquid) (Jackman et al., 2020; Jackman et al., 2022). In addition to addressing the limitations of organic acids, such as unpleasant flavor and oxidation, a significant benefit of MG is that they protect the active substances (i.e., organic fatty acids) from early losses in the upper part of the gastrointestinal tract. These substances are then slowly released throughout the intestine by lipolytic enzymes (Sampugna et al., 1967; Namkung et al., 2011).

The beneficial effects of organic fatty acids on the immune system and gut health have already been discussed earlier (Hamer et al., 2008; Rossi et al., 2010; Liu, 2015; Lauridsen, 2020). In poultry, various biological activities of MG on gut health and productivity have been studied, including antimicrobial activities, intestinal integrity, and performance (Appleton et al., 2024). However, limited research has been conducted in pigs, especially weanling pigs, which are vulnerable to pathogen exposure. Interestingly, in some regions, MG may not require registration for inclusion in animal feed if they are categorized as feed materials (Jackman et al., 2020). In the U.S. food industry, MG are widely used in food processing and production and are classified as GRAS (Generally Recognized as Safe) due to their recognized safety (Zhao et al., 2020; Kong et al., 2021).

MG are amphipathic molecules with both hydrophilic (carboxyl group) and hydrophobic (carbon chain) regions. Their amphiphilic nature allows MG to form micelles that disrupt bacterial cell membranes, leading to increased permeability (Hyldgaard et al., 2012; Churchward et al., 2018). Moreover, MG have a higher pKa (around 14) compared to organic acids. The pKa value above the normal physiological pH range indicates that MG can act pH-independently (Churchward et al., 2018; Appleton et al., 2024; Yang et al., 2024). Effective antibacterial activity

is observed at concentrations above the critical micelle concentration (CMC). MG require lower concentrations to form micelles compared with MCFA, and the molecular length may also affect their activity (Yoon et al., 2015; Yoon et al., 2017; Jackman et al., 2020). Numerous *in vitro* studies have demonstrated antimicrobial activity against a wide range of bacteria (Hyldgaard et al., 2012; Churchward et al., 2018; Kovanda et al., 2019), and developing resistance to MG has been reported as difficult (Schlievert and Peterson, 2012; Borrelli et al., 2021). Generally, MG are more effective against Gram-positive bacteria than Gram-negative bacteria due to differences in cell membrane structure (Churchward et al., 2018). However, external factors, such as acidic pH and temperature, can increase the susceptibility of Gram-negative bacteria (e.g., *E. coli*) to MG (Bergsson et al., 2002; Thormar et al., 2006; Thormar and Hilmarsson, 2007). This is likely because these external factors attenuate the interference of LPS, allowing MG to induce membrane instability more efficiently (Bergsson et al., 2002; Thormar and Hilmarsson, 2007). In this context, combining MG with OA may have synergistic effects due to their distinct properties and modes of action.

SUMMARY

During the weaning period, piglets face significant challenges in maintaining intestinal health and homeostasis due to multiple stressors, making them particularly vulnerable to external factors. Effective management of pathogenic infections and overall health in weanling pigs is crucial not only for short-term and long-term productivity but also for animal welfare. Diarrhea caused by ETEC in post-weaning piglets is a common gastrointestinal disease that results in considerable economic losses in swine production. Antibiotics and high-dose zinc oxide have traditionally been used in swine feed to control diseases and enhance productivity. They have proven effective in promoting growth and mitigating economic losses from ETEC-induced diarrhea during the critical weaning period. However, public concerns regarding potential risks to

human health, antibiotic resistance, and environmental impact have led to restrictions on antimicrobial growth promoters and a reduction in the use of high-dose zinc oxide in feed. These concerns align with the One Health approach and may lead to further restrictions or bans on these conventional practices. The ongoing challenge has highlighted the need for innovative alternatives to support and sustain pig production. In this regard, MG have emerged as one of the promising options due to their various biological activities, including broad-spectrum antimicrobial effects. MG are relatively easy to use as feed additives since they allow active substances to be gradually released throughout the intestine compared with their acid form and salt form. However, there is limited research on the effects of MG on the growth and health of pigs, particularly in disease models such as ETEC F18 infection. Therefore, comprehensive investigations were conducted to explore the impact of MG supplementation as a promising dietary intervention on disease resistance and the overall health of weaned piglets using both *in vitro* cell culture models and *in vivo* ETEC challenge models.

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TABLES AND FIGURES

Table 1.1. Regulations regarding the use of antibiotics as growth promoters in different countries (adapted from Rahman et al., 2022)

Country	Year	Action
Australia	2017	Antibiotics used in human medicine are not licensed as growth promoters. However, five antibiotics (olaquindox, avilamycin, bambermycin, monensin, and salinomycin) not currently used in human medicine are used as growth promoters in poultry, pigs, cattle, and sheep [23].
Canada	2020	Growth promotion claims on medically important antimicrobials (MIAs) (Category I, II, and III antimicrobials) will no longer be permitted. Ionophore and coccidiostat products will be unaffected, as they are not considered MIAs [24,25].
China	2020	All antibiotic growth promoters except herbal medicine have been banned [26].
European Union	2006 2022	Illegal across the EU The EU will ban the importation of meat and dairy produced using antibiotic growth promoters. However, fluoroquinolones continue to be licensed in the UK and in many EU countries [27].
New Zealand	2017	No banning claim found. The Ministry of Health and the Ministry for Primary Industries (MPI) stated in 2017: "*If antibiotics used in food-producing animals, MPI must also be satisfied that the antibiotic will not leave residues above the maximum residue level in food from the treated animals*". Compared to EU countries, New Zealand uses more cephalosporins and macrolides, but less quinolones [28].
Sweden	1986	First country to ban the use of antibiotics as growth promoters [29].
USA	2017	Medically important antimicrobials are banned. However, bacitracin and carbadox, which are classified as medically important by the World Health Organization, are still used as growth promoters in pigs [30].

Table 1.2. List of the most common acids and their properties (adapted from Tugnoli et al., 2020)

Category	Name	Molecular Formula	pKa	Physical Form	Odor and Taste	Solubility	Chemical Safety
Short chain	Formic	CH_2O_2	3.75	Colorless liquid	Pungent odor	Miscible in water, ether, acetone, ethyl acetate, methanol, ethanol	Severe skin burns and eye damage
	Acetic	$C_2H_4O_2$	4.74	Colorless liquid	Pungent vinegar-like odor Sour and burning taste	Miscible in water, alcohol, glycerol, ether, carbon tetrachloride	Flammable liquid and vapor, severe skin burns and eye damage
	Propionic	$C_3H_6O_2$	4.88	Colorless liquid oily	Very pungent rancid odor	Miscible in water, soluble in alcohol, ether, chloroform	Severe skin burns and eye damage
	Butyric	$C_4H_8O_2$	4.82	Colorless liquid oily	Rancid unpleasant odor, acrid taste, with a sweetish after taste	Miscible in water, alcohol, ether	Severe skin burns and eye damage
Medium chain	Caproic (or hexanoic)	$C_6H_{12}O_2$	5.09	Colorless to light-yellow liquid oily	Characteristic goat-like odor	Soluble in ethanol and ether	Toxic in contact with skin, severe skin burns and eye damage
	Caprylic (or octanoic)	$C_8H_{16}O_2$	4.89	Colorless to light-yellow liquid oily	Characteristic goat-like odor	Miscible in ethanol, chloroform, acetonitrile Soluble in alcohol, chloroform, ether, carbon disulfide petroleum ether and glacial acetic acid	Severe skin burns and eye damage; harmful to aquatic life with long lasting effects

Category	Name	Molecular Formula	pKa	Physical Form	Odor and Taste	Solubility	Chemical Safety
	Capric (or decanoic)	$C_{10}H_{20}O_2$	4.90	White crystalline powder	Characteristic goat-like odor	Soluble in ethanol, alcohol, ether, chloroform, benzene and carbon disulfide	Skin and eye irritation; harmful to aquatic life with long lasting effects
	Lauric (or dodecanoic)	$C_{12}H_{24}O_2$	5.30	White flakes	Bay-like odor	Miscible with benzene Very soluble in methanol and ethanol Soluble in acetone	Skin irritation, serious eye damage and irritation
Others	Sorbic	$C_6H_8O_2$	4.76	White crystalline powder or granules	Odorless Acrid and sour taste	Very soluble in ether. Soluble in ethanol	Skin and eye irritation. May cause respiratory irritation
	Benzoic	$C_7H_6O_2$	4.19	Colorless crystalline powder	Pungent odor Bitter taste	Soluble in alcohol, ether, and benzene	Skin irritation, eye damage; damage to organs through prolonged or repeated exposure
	Lactic	$C_3H_6O_3$	3.86	Colorless to yellow viscous liquid or crystals	Odorless Acrid taste	Soluble in water, ethanol and diethyl ether	Skin irritation, serious eye damage

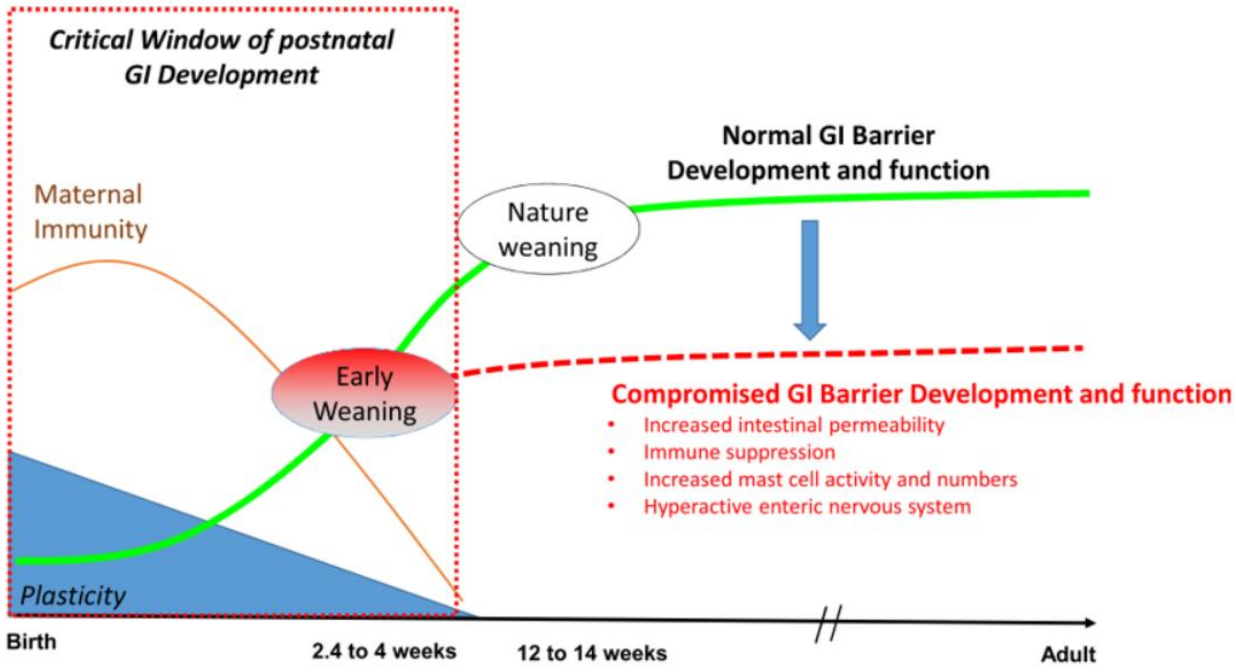


Figure 1.1. Impact of early weaning on the developmental trajectory of gastrointestinal (GI) barrier function (adapted from Moeser et al., 2017)

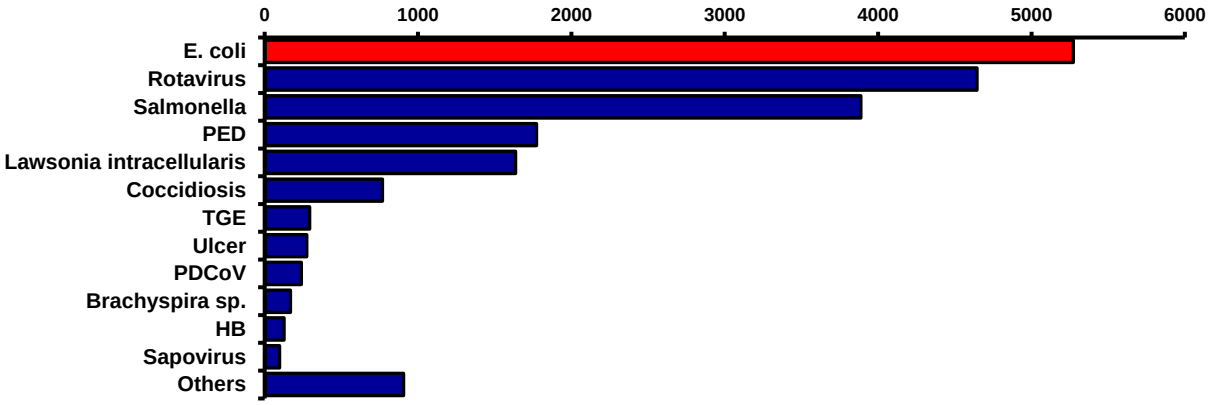
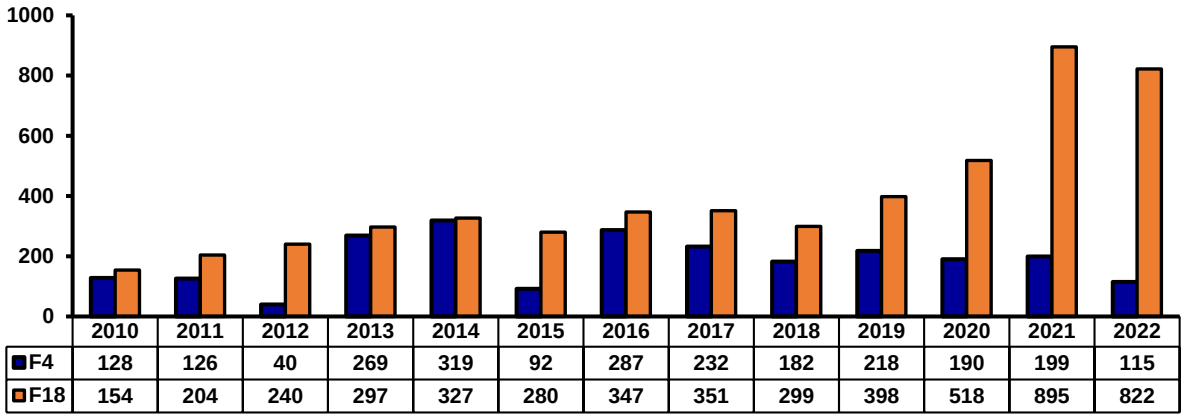


Figure 1.2. Number of diagnostic accessions at the Iowa State University Veterinary Diagnostic Laboratory resulting in each specific disease diagnosis from samples collected from pigs with post-weaning diarrhea from 2010 to 2022 (adapted from Paiva et al., 2023)



	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021	2022
F4	128	126	40	269	319	92	287	232	182	218	190	199	115
F18	154	204	240	297	327	280	347	351	299	398	518	895	822

Figure 1.3. Frequency of *Escherichia coli* F18 and F4 fimbrial gene detection in cases where genotyping was performed at the Iowa State University Veterinary Diagnostic Laboratory from 2010 to 2022 (adapted from Paiva et al., 2023)

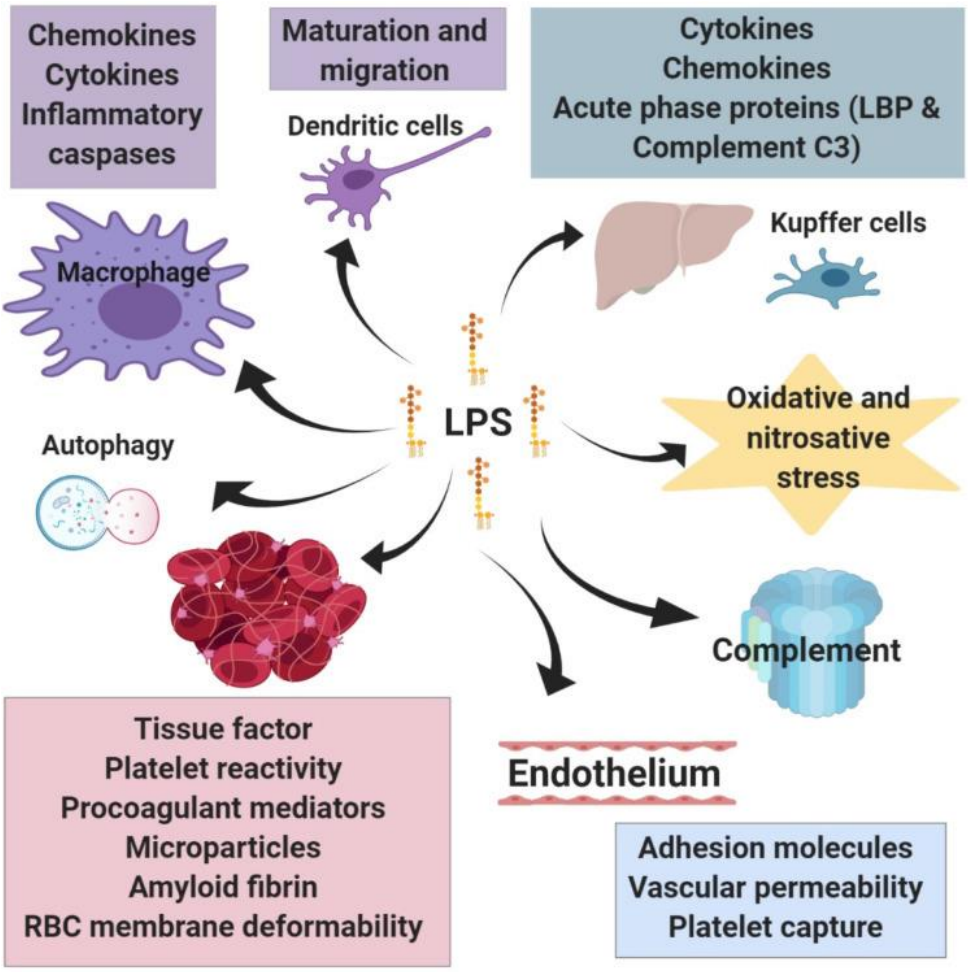


Figure 1.4. Summarised downstream effects of LPS signalling. LPS influences a range of cell types and physiological processes (adapted from Page et al., 2022)

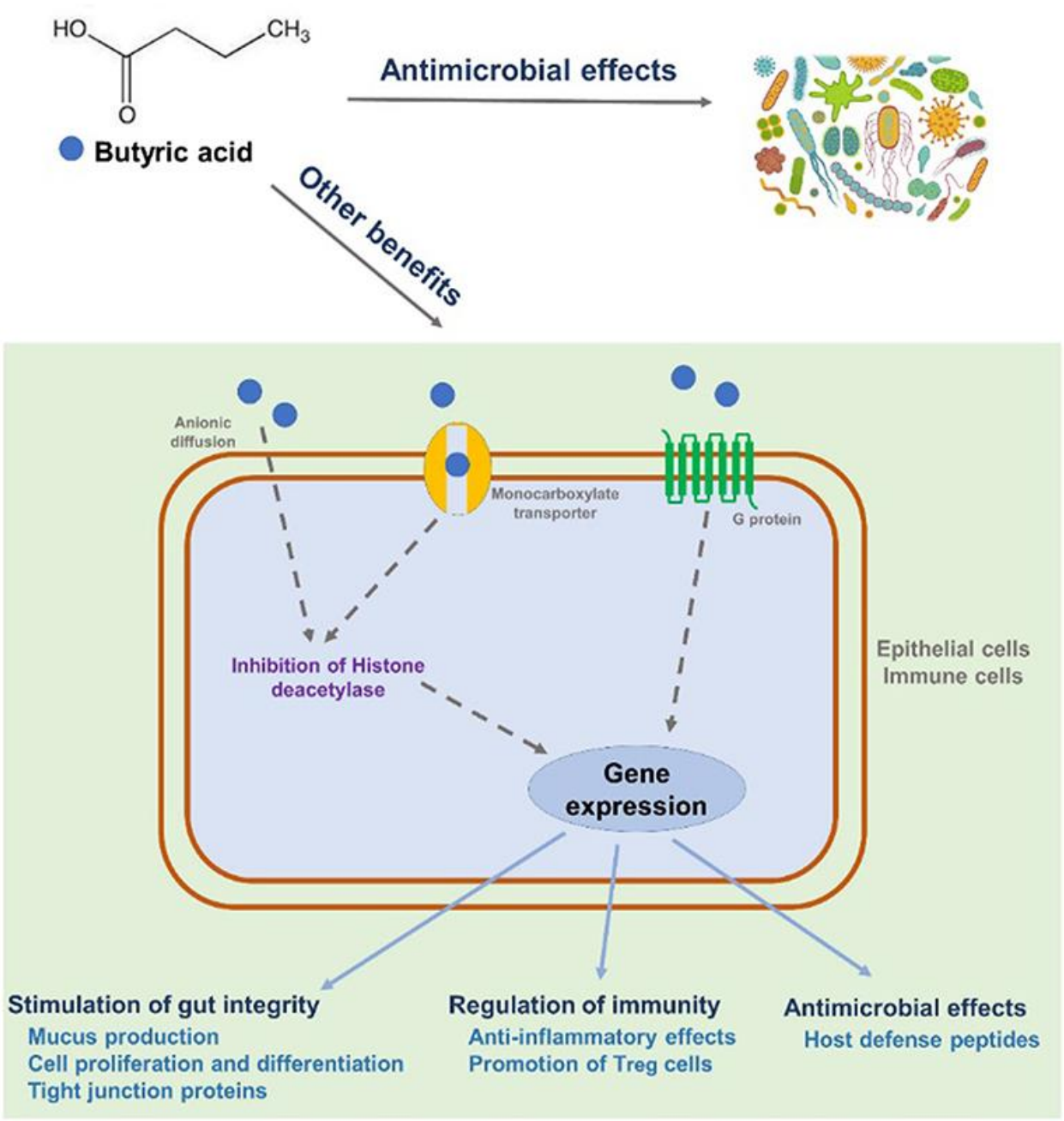


Figure 1.5. Proposed mechanisms of action on the beneficial effects of butyric acid (adapted from Xiong et al., 2019)

CHAPTER 2

IN VITRO INVESTIGATION OF MONOGLYCERIDES AND ZINC GLYCINATE: ANTI-INFLAMMATORY AND EPITHELIAL BARRIER FUNCTION

ABSTRACT

The objectives of this study were to investigate the *in vitro* immune-modulatory effects of monoglycerides and zinc glycinate with porcine alveolar macrophages (PAM) and their impact on epithelial barrier integrity using the intestinal porcine enterocyte cell line (IPEC-J2). A cell viability assay was performed for each compound to determine the appropriate dose range for each cell. In Exp. 1, IPEC-J2 cells (5×10^5 cells/mL) were seeded and treated with each compound (monoglycerides: 0, 25, 100, 250, 500, and 1,000 µg/mL; zinc glycinate: 0, 2, 5, 12.5, 25, and 50 µg/mL). Transepithelial electrical resistance (TEER) was measured by Ohm's law method at 0 h (before treatment) and at 24, 48, and 72 h post-treatment. In Exp. 2, PAM were collected from six clinically healthy piglets and seeded at 10^6 cells/mL. After incubation, the cells were treated with each compound and/or lipopolysaccharide (LPS). The experimental design was a 2 × 6 factorial arrangement with 2 doses of LPS (0 or 1 µg/mL) and 6 doses of each compound (monoglycerides: 0, 50, 100, 250, 500, and 1,000 µg/mL; zinc glycinate: 0, 25, 50, 100, 250, and 500 µg/mL). Cell supernatants were collected to analyze the concentrations of tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β) by enzyme-linked immunosorbent assay kits. Data were analyzed by ANOVA using PROC MIXED of SAS with a randomized complete block design. IPEC-J2 cells treated with 250 or 1,000 µg/mL of monoglycerides had increased ($P < 0.05$) TEER values at 48 and 72 h post-treatment, while 5 µg/mL of zinc glycinate showed increased ($P < 0.0001$)

TEER values at 72 h post-treatment, compared with control. LPS challenge increased ($P < 0.05$) the production of TNF-α and IL-1β from PAM. In the non-challenge group, 50 or 100 μg/mL of monoglycerides stimulated ($P < 0.05$) TNF-α and IL-1β production from PAMs. Treatment with 25 or 100 μg/mL of zinc glycinate also enhanced ($P < 0.05$) TNF-α production from PAM. In LPS-treated PAM, 1,000 μg/mL of monoglycerides increased ($P < 0.05$) IL-1β production, while zinc glycinate suppressed ($P < 0.0001$) the secretion of TNF-α and IL-1β at the doses of 100, 250, and 500 μg/mL. In conclusion, the results of this *in vitro* study indicate that monoglycerides positively affect the barrier function of the epithelium, while zinc glycinate may have strong immune regulatory benefits. Future *in vivo* animal studies will be required to verify their impacts on animal gut health, systemic immunity, and growth performance.

Key words: alveolar macrophage, immunomodulation, IPEC-J2 cells, monoglycerides, transepithelial electrical resistance, zinc glycinate

INTRODUCTION

Gastrointestinal barrier dysfunction is considered a primary factor in the development of various gastrointestinal diseases, causing inflammatory responses that disrupt host homeostasis and affect animal health and productivity (Pluske et al., 2018a; Pluske et al., 2018b). In this regard, weanling piglets become more vulnerable to environmental factors such as pathogens and toxins due to multifactorial stressors associated with weaning, combined with the immaturity of intestinal barrier elements (Moeser et al., 2017; Xiong et al., 2019; Tang et al., 2022). Pathogenic *Escherichia coli* infection is one of the major causes of diarrhea post-weaning, further compromising the intestinal system (He et al., 2020; Almeida et al., 2022). Restrictions on conventional practices and public concerns aligned with One Health have spurred research to identify alternatives that improve intestinal health and immunity in weaned pigs, aiming to prevent losses caused by enteric disease and maximize productivity (Liu et al., 2018; Patience and Ramirez, 2022; Rahman et al., 2022).

Recent studies have highlighted the potential and utilization of nutritional interventions that have demonstrated beneficial effects on modulating immune function and intestinal barrier function. Monoglycerides, fatty acid derivatives, are considered practical applications that overcome the limitations of fatty acids, such as unpleasant odors, and enhance their effectiveness by improving stability (Bedford and Gong, 2018; Jackman et al., 2020). It is well known that the length of fatty acids affects their biological activities, including immunomodulation and maintenance of intestinal integrity (Tugnoli et al., 2020; Zhang et al., 2021; Yan et al., 2023; Cundra et al., 2024). Similarly, zinc glycinate represents a sustainable approach that can reduce reliance on inorganic forms of zinc, such as zinc oxide, without compromising biological efficacy. As an organic form of zinc, it increases bioavailability (Nitrayova et al., 2012; Pearce et al., 2015).

Improved absorption and utilization of zinc not only reduces the amount needed to achieve the desired effects on animal health, but also mitigates potential risks associated with excessive zinc oxide use (Liu et al., 2016; Behjatian Esfahani et al., 2021). In addition, unlike other forms of zinc, glycine and zinc form stable chelates, enabling efficient binding and minimizing unnecessary reactions of zinc glycinate with other molecules (Kulkarni et al., 2011; Sun et al., 2020).

However, despite the potential of monoglyceride blends and zinc glycinate as practical practices, there is limited research regarding their effects on intestinal integrity and immune responses, which are crucial for protecting the host from external factors. Understanding the interactions between these practical practices and the intestinal barrier is essential for effective application, and a foundation can be established through *in vitro* experiments before proceeding to *in vivo* studies. Therefore, the objectives of this study were 1) to determine the effects of monoglycerides and zinc glycinate on epithelial barrier function by measuring transepithelial electrical resistance (TEER) with intestinal porcine enterocyte cell line (IPEC-J2); and 2) to investigate the *in vitro* anti-inflammatory effects of monoglycerides and zinc glycinate on lipopolysaccharide (LPS) treated porcine alveolar macrophages (PAM).

MATERIALS AND METHODS

Two experiments were conducted to achieve the objectives of this study. The monoglyceride blend of butyric, caprylic, and capric acids (BalanGut LS L) and zinc glycinate used in this study were obtained from BASF SE (Ludwigshafen, Germany). To prepare cell culture treatment, monoglyceride blend was first dissolved in dimethyl sulfoxide (DMSO) and was further diluted with sterile Dulbecco's Modified Eagle Medium (DMEM/F12) or Roswell Park Memorial Institute (RPMI)-1640 medium for culturing IPEC-J2 cells or PAM, respectively. Culture media contained heat-inactivated fetal bovine serum (HyClone Laboratories, Inc., Logan, UT) and

antibiotics (Penicillin-Streptomycin; Mediatech, Inc., Manassas, VA). The final concentration of DMSO in monoglyceride blend treatment did not exceed 0.05%. Zinc glycinate is in solid form and was dissolved directly into cell culture medium in each experiment. The procedures of this study were adapted from previous research (Liu et al., 2012; Hejna et al., 2021).

Cell viability assay

Cell viability assays were performed in two experiments to determine the impacts of monoglycerides and zinc glycinate on the cell viability of IPEC-J2 cells and PAM by a Vybrant MTT Cell Proliferation Assay Kit (Invitrogen Corporation, Carlsbad, CA). MTT, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, measures the metabolic activity of cells via mitochondrial enzymes which catalyze a color change reaction (Mosmann, 1983). Briefly, cells in 96-well plates were treated with different treatments as described in each experiment, then incubated at 37℃ and 5% CO_2 for 24 h. After the incubation, all media was removed and fresh culture medium and MTT solution were added to each well. After 4 h of incubation at 37℃, all cell medium was removed from each well, and then DMSO was added and thoroughly mixed. The color change reaction was quantified by optical density (OD) measured at 540 nm with a reference wavelength of 630 nm (Synergy HTX Multi-Mode Microplate Reader, BioTek, Winooski, VT) after 10 min of incubation. The average OD of the untreated control group was calculated and set to 100%. The relative viability was calculated using the following Eq. [1]:

$$\text{Cell viability, \%} = (OD_{\text{treated cells}}/\text{mean } OD_{\text{untreated control}}) \times 100 \qquad [1]$$

The percentage of live cells obtained for each treatment represents both viability and proliferation.

Experiment 1. Epithelial Barrier Function with IPEC-J2 Cells

IPEC-J2 cells were purchased from Leibniz Institute DSMZ-German Collection of Micro-organisms and Cell Cultures GmbH (Braunschweig, Germany) and cultured with DMEM/F12 growth media. The final cell concentration was adjusted to 5×10^5 cells/mL (Geens and Niewold, 2011). Then 0.5 mL of IPEC-J2 cell suspension was seeded in each well of 12-well plates on 1.12 cm^2 Corning polycarbonate transwell tissue culture treated inserts (0.4 µm pores; Corning Inc., Corning, NY). Plates were then incubated at 37˚C in a humidified 5% CO_2 incubator for adherence. Culture media was changed every other day and cells were cultured for a total of 4 to 5 days until confluence was reached and the TEER values were close to 1,000 Ωcm^2. After that, all cells were treated with 0.6 mL new media containing different doses of monoglycerides and zinc glycinate in the apical chamber of the transwell inserts. The experimental design for each tested compound was 4 (different time points) × 6 (6 different levels of tested compound) factorial arrangement in a randomized complete block design with 6 replicates in duplicate wells. The tested doses for monoglycerides were: 0, 25, 100, 250, 500, and 1,000 µg/mL. The tested doses for zinc glycinate were: 0, 2, 5, 12.5, 25, and 50 µg/mL. These doses were selected for cell culture treatments based on preliminary cytotoxicity assays using IPEC-J2 cells. TEER of IPEC-J2 cells were measured (Ωcm^2) at 0 h (before treatment) and at 24, 48, and 72 h post-treatment using a Millicell ERS-2 voltohmmeter (MilliporeSigma, St. Louis, MO). Wells in duplicate containing transwell inserts and culture medium with no cells were used as blank measurements at each time point. Culture plates were allowed to reach room temperature before measurements in order to attain stable TEER readings. Electrodes were rinsed with 70% ethanol, 0.1 M NaCl solution, and finally pre-warmed medium between readings of wells containing different treatments. The resistance of each monolayer was calculated (Eq. 2) and was inversely proportional to the area of the membrane (Eq. 3), based on methodology by Srinivasan et al. (2015):

$$R_{monolayer}, \Omega = R_{sample} - R_{blank} \quad [2]$$

$$TEER_{reported}, \Omega cm^2 = R_{monolayer}, \Omega \times \text{monolayer area, } cm^2 \quad [3]$$

Experiment 2. Anti-inflammatory Effects with Porcine Alveolar Macrophages

The protocol for this study was reviewed and approved by the Institutional Animal Care and Use Committee at the University of California, Davis (IACUC# 21875). PAM were collected from six clinically healthy piglets at 7 wk of age. Before euthanization, pigs were anesthetized with 1 mL mixture of 100 mg telazol, 50 mg ketamine, and 50 mg xylazine (2:1:1) by intramuscular injection. After anesthesia, pigs were euthanized by intracardiac injection with 78 mg Fatal-Plus solution (sodium pentobarbital, MWI Animal Health, Visalia, CA) per 1 kg of body weight. PAM were collected by bronchoalveolar lavage according to the procedures as described in previous research (Liu et al., 2012). Nonviable cells were distinguished by trypan blue (Sigma-Aldrich Co., St. Louis, MO) staining method, while live cells that excluded the dye were counted using a hemocytometer (Fisher Scientific, Inc., Pittsburgh, PA). The final cell concentration was adjusted to 10^6 cells/mL. The viability of the cells was greater than 97%. In this paper, we use the term “porcine alveolar macrophages” because the majority of bronchoalveolar lavage fluid cells are macrophages (Dickie et al., 2009).

PAM were cultured in 24- or 96-well cluster plates at a density of 10^5 cells/mL. All plates were incubated overnight at 37℃ in a humidified 5% CO_2 incubator to allow macrophages to adhere to the bottom. The nonadherent cells were washed away with pre-warmed RPMI-1640 medium. Adhered macrophages were treated in duplicate with fresh pre-warmed RPMI-1640 containing different treatments. Monoglycerides and zinc glycinate were tested with the same experimental design as a 2 (without or with 1 μg of LPS/mL) × 6 (6 different levels of tested compound) factorial arrangement in a randomized complete block design. The tested doses for

monoglycerides were: 0, 50, 100, 250, 500, and 1,000 μg/mL. The tested doses for zinc glycinate were: 0, 25, 50, 100, 250, and 500 μg/mL. These doses were selected for cell culture treatments based on preliminary cytotoxicity assays using PAM. After 24 h more of incubation, the supernatants in duplicates were collected, pooled, and stored at −80°C. Supernatants were analyzed for the concentrations of tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β) by commercial enzyme-linked immunosorbent assay kits (R&D Systems, Inc., Minneapolis, MN).

Statistical Analysis

All data generated from different assays were analyzed by ANOVA using the MIXED procedure (SAS 9.4, SAS Institute Inc., Cary, NC) with different statistical models. The statistical model of the PAM assays included LPS challenge, doses of treatment, and their interaction as fixed effects and block as a random effect. In the TEER assays, a pool of duplicate wells was considered an experimental unit and the statistical model included the effects of dose as a fixed effect and plate as a random effect. Data are presented as least-squares means and standard error of the means. Probability values of < 0.05 were considered to be significant.

RESULTS

Transepithelial Electrical Resistance of IPEC-J2 Cells

Cell viability was not affected by monoglycerides with the dose up to 1,000 μg/mL. Zinc glycinate exhibited ($P < 0.05$) cytotoxic effects on IPEC-J2 cells when the dose was increased to 50 μg/mL. Compared with the control, 250 and 1,000 μg/mL monoglycerides increased ($P < 0.05$) TEER values in IPEC-J2 cells at 48 and 72 h post-treatment (Figure 2.1a). Cells treated with 5 μg/mL zinc glycinate had increased ($P < 0.0001$) TEER values at 72 h post-treatment

compared with control (Figure 2.1b). However, cells treated with the highest dose (50 μg/mL) of zinc glycinate had the lowest ($P < 0.05$) TEER values at 24, 48, and 72 h post-treatment.

Pro-inflammatory Cytokines Secretion in Porcine Alveolar Macrophages

The lowest cell viability of PAM for both treatments was around 86%, indicating no cytotoxic effects from supplementing monoglycerides up to 1,000 μg/mL and zinc glycinate up to 500 μg/mL. The LPS challenge increased ($P < 0.05$) cell viability of PAM compared with the non-challenged group in both tested compounds. The levels of pro-inflammatory cytokines secreted by PAM in the absence or presence of LPS are shown in Figure 2.2. Treatment with LPS stimulated ($P < 0.05$) TNF-α and IL-1β production from PAM. In the non-challenge groups, supplementing with 50 or 100 μg/mL of monoglycerides increased ($P < 0.05$) TNF-α and IL-1β production from PAM. Treatment with 25 or 100 μg/mL of zinc glycinate also enhanced ($P < 0.05$) the production of TNF-α from PAM, but not for IL-1β. In the LPS challenge groups, no difference was observed in TNF-α production when PAM were treated with monoglycerides at any dose, however, 1,000 μg/mL of monoglycerides increased ($P < 0.05$) IL-1β production. Zinc glycinate suppressed ($P < 0.0001$) the secretion of TNF-α and IL-1β at the doses of 100, 250, and 500 μg/mL from LPS-treated PAM.

DISCUSSION

The gastrointestinal barrier, comprising both physical and immunological factors, plays an important role in protecting the host from external pro-inflammatory agents (Wong et al., 2022; Wang et al., 2023). To develop effective management strategies for weaned piglets, which are susceptible to enteric diseases such as enterotoxigenic *Escherichia coli* due to intestinal barrier dysfunction, it is essential to understand the interactions among the components of the intestinal barrier. The present *in vitro* study investigated the potential of a monoglyceride blend and zinc

glycinate to enhance epithelial barrier integrity and regulate inflammatory responses, which are critical for preventing inflammatory diseases and improving intestinal health and productivity in animals. The results of this study provide a foundational understanding that can be extended to *in vivo* research.

IPEC-J2 cells, derived from the jejunum of a neonatal piglet, have been used to assess the influence of test compounds on parameters related to epithelial homeostasis, as they effectively mimic the barrier function of the porcine intestinal epithelium (Vergauwen, 2015; Marks et al., 2022). To evaluate the effect of monoglycerides and zinc glycinate on the barrier integrity of IPEC-J2 cells, the TEER value of the polarized IPEC-J2 cell monolayer was measured. TEER measurements have been widely used to monitor changes in the integrity of epithelial monolayer at various time points without undesirable interference with the cells (Srinivasan et al., 2015). Epithelial cells treated with monoglycerides up to 1,000 µg/mL developed stronger barrier function *in vitro*. This observation may be partially explained by the beneficial effects of fatty acids on cell metabolism, including serving as an energy source for enterocytes, which helps maintain intestinal integrity (Xiong et al., 2019; Jia et al., 2020; Lauridsen, 2020). Similar to our results, previous studies have reported improved intestinal development, as indicated by intestinal morphology and tight junction protein expression in starter phase chickens supplemented with monoglycerides (Kumar et al., 2021; Liu et al., 2023). Zinc glycinate also shows potential for improving epithelial barrier function over time, as indicated by the improved TEER when cells were treated with 5 µg/mL of zinc glycinate. However, at higher concentration, zinc glycinate may begin to exert detrimental effects, such as stress, potentially leading to decreased permeability. This aligns with the decreased cell viability observed in the MTT results at the highest concentration of zinc glycinate. Zinc is known to play a crucial role in various biological processes

related to the immune system, enzyme activity, and intestinal integrity (Bonetti et al., 2021). However, it may have toxic effects at excessively high concentrations (Brink et al., 1956; Burrough et al., 2019). Our observations may be attributed to the high bioavailability of zinc glycinate and suggest the possibility of different thresholds for adverse effects across various cell types.

Appropriate immune responses to external factors are important to maintain intestinal homeostasis, but excessive inflammatory responses may lead to leaky gut and additional exposure to pro-inflammatory factors (Lambert, 2009; Liu, 2015). Macrophages play an important role in mediating inflammatory responses, and PAM cell model has been used to investigate cytokine secretion by macrophages induced by LPS (Liu et al., 2012; Hejna et al., 2021). Low doses of monoglycerides and zinc glycinate increased pro-inflammatory cytokine production from non-challenged macrophages, which indicates that both products may stimulate the activation of innate immune responses under normal conditions. While monoglycerides had limited anti-inflammatory effects, zinc glycinate exhibited strong anti-inflammatory benefits when macrophages were challenged with LPS, consistent with the beneficial effects of zinc on intestinal homeostasis, including immune-modulatory effects mentioned above (Bonetti et al., 2021). Consistent with our findings, Kloubert et al. (2018) observed an improvement in innate immune cell function, as indicated by phagocytosis and oxidative burst, with zinc supplementation. In addition, Roselli et al. (2003) also reported zinc supplementation reduced pro-inflammatory cytokine secretion induced by enterotoxigenic *Escherichia coli*. Meanwhile, results obtained with monoglyceride blend in the current study are in line with the report of Sivinski et al. (2020), who reported that monoglyceride increased nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation in macrophages in the absence of LPS challenge, but decreased NF-κB activation in a

dose-dependent manner under LPS. Further studies are needed to elucidate the immunomodulatory effects of monoglycerides and their complex dose-response relationship in a weaned pig model.

In conclusion, the current *in vitro* cell culture assays suggest that monoglycerides target epithelial cells and enhance barrier function, while confirming the anti-inflammatory benefits of zinc glycinate. Future research is needed to explore the potential of monoglycerides and zinc glycinate as practical practices for improving intestinal homeostasis in animal studies and to focus on their modes of action. Additionally, the present study also indicates that epithelial cells are more sensitive to zinc glycinate than immune cells. Further investigation into the cytotoxic tolerance difference between these cell types would be valuable.

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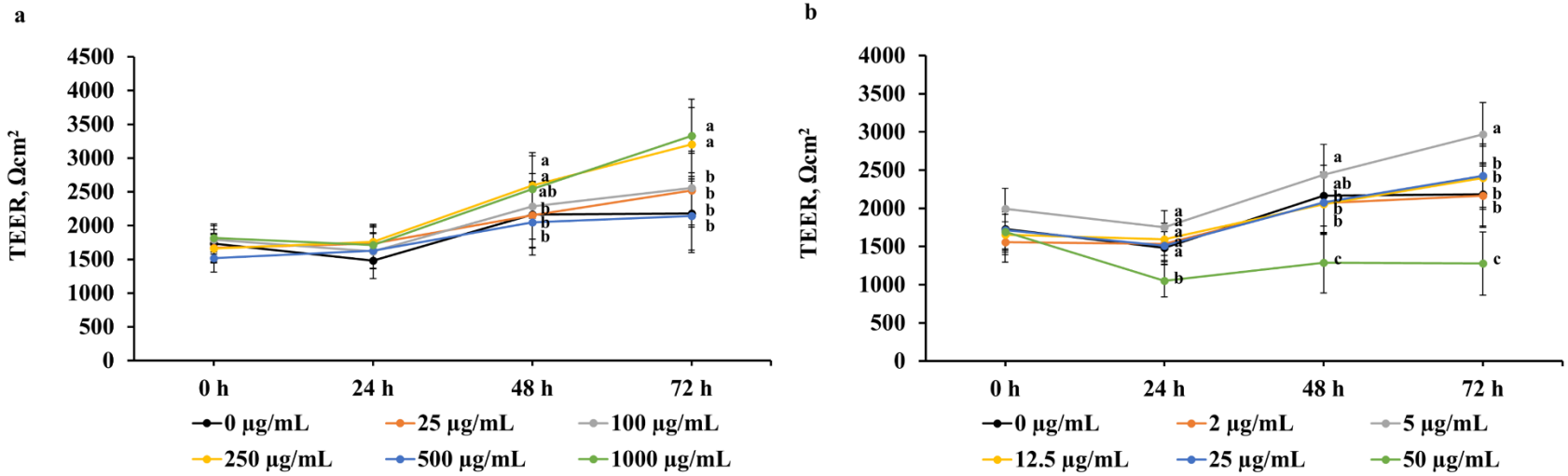

Figure 2.1. Effects of monoglycerides (**a**) and zinc-glycinate (**b**) on the transepithelial electrical resistance (TEER) of IPEC-J2 cells (0 to 72 h). The tested doses were 0, 25, 100, 250, 500, and 1,000 µg/mL for monoglycerides and 0, 2, 5, 12.5, 25, and 50 µg/mL for zinc-glycinate. The results were means of values from 6 observations. [a,b,c]Means without a common superscript are different ($P < 0.05$).

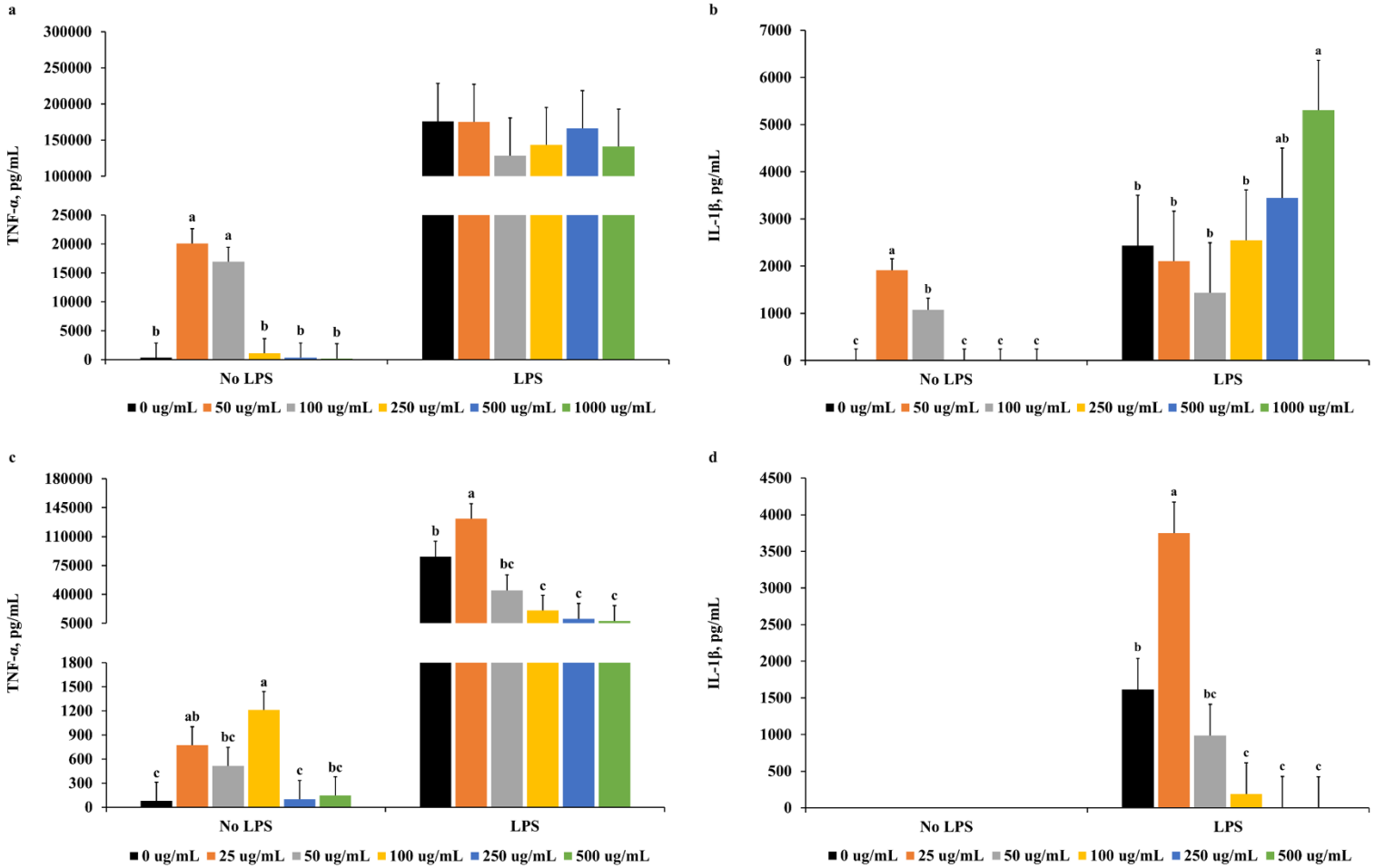

Figure 2.2. Monoglycerides (**a** and **b**) and zinc-glycinate (**c** and **d**) influenced the production of tumor necrosis factor-alpha (TNF-α; **a** and **c**) and Interleukin-1 beta (IL-1β; **b** and **d**) from porcine alveolar macrophages (PAM) in the absence or presence of lipopolysaccharide (LPS). PAM were incubated with various concentrations (monoglycerides = 0, 50, 100, 250, 500, and 1,000

µg/mL; zinc-glycinate = 0, 25, 50, 100, 250, and 500 µg/mL) of each compound in the presence or absence of LPS (0 or 1 µg/mL) for 24 h. The results were means of values from 6 observations. [a,b,c]Means without a common superscript are different ($P < 0.05$).

CHAPTER 3

EFFECTS OF MONOGLYCERIDE BLEND ON SYSTEMIC AND INTESTINAL IMMUNE RESPONSES, AND GUT HEALTH OF WEANED PIGS EXPERIMENTALLY INFECTED WITH A PATHOGENIC *ESCHERICHIA COLI*

ABSTRACT

Monoglycerides have emerged as a promising alternative to conventional practices due to their biological activities, including antimicrobial properties. However, few studies have assessed the efficacy of monoglyceride blend on weaned pigs and their impacts on performance, immune response, and gut health using a disease challenge model. Therefore, this study aimed to investigate the effects of dietary monoglycerides of short- and medium-chain fatty acids on the immunity and gut health of weaned pigs experimentally infected with an enterotoxigenic *Escherichia coli* F18. Pigs supplemented with high-dose zinc oxide (ZNO; 3,000 mg/kg) had greater ($P < 0.05$) growth performance than control, monoglycerides, and antibiotic supplemented groups; however, no difference was observed in average daily feed intake between ZNO and monoglycerides groups during the post-challenge period. Pigs in ZNO and antibiotic groups had lower ($P < 0.05$) severity of diarrhea than control, but the severity of diarrhea was not different between antibiotic and monoglycerides groups. Pigs fed with monoglycerides or ZNO had lower ($P < 0.05$) serum haptoglobin on d 2 or 5 post-inoculation than control. Pigs in ZNO had greater ($P < 0.05$) goblet cell numbers per villus, villus area and height, and villus height:crypt depth ratio (VH:CD) in duodenum on d 5 post-inoculation than pigs in other treatments. Pigs supplemented with monoglycerides, ZNO, or antibiotic had reduced ($P < 0.05$) ileal crypt depth compared with control

on d 5 post-inoculation, contributing to the increase (P = 0.06) in VH:CD. Consistently, pigs in ZNO expressed the lowest (P < 0.05) *TNFa*, *IL6*, *IL10*, *IL12*, *IL1A*, *IL1B*, and *PTGS2* in ileal mucosa on d 5 post-inoculation, and no difference was observed in the expression of those genes between ZNO and monoglycerides. Supplementation of ZNO and antibiotic had significant impacts on metabolic pathways in the serum compared with control, particularly on carbohydrate and amino acids metabolism, while limited impacts on serum metabolites were observed in monoglycerides group when compared with control. The results suggest that supplementation of monoglyceride blend may enhance disease resistance of weaned pigs by alleviating the severity of diarrhea and mitigating intestinal and systemic inflammation, although the effectiveness may not be comparable to high-dose zinc oxide.

Keywords: Diarrhea, Enterotoxigenic *Escherichia coli*, Gut health, Monoglycerides, Systemic immunity, Weaned pigs

INTRODUCTION

Weaning piglets, the process of separating them from their mother, exposes them to nutritional, physiological, and environmental challenges (Weary et al., 2008; Heo et al., 2013; Pluske, 2016). These weaning stressors impair intestinal barrier function and induce intestinal and systemic inflammation, in addition to the typically occurring decrease in feed intake (Campbell et al., 2013; Xiong et al., 2019). The compromised intestinal barrier increases the risk of external factors (e.g., toxins, antigens, and pathogens) entering the body, making piglets vulnerable to enteric diseases (Smith et al., 2010; Tang et al., 2022). Post-weaning diarrhea, caused by the infection of enterotoxigenic *Escherichia coli* (ETEC) F18, is one of the common problems in young pigs (Nagy and Fekete, 1999; Nagy and Fekete, 2005). This disease is characterized by watery diarrhea and deterioration of intestinal health, causing tremendous economic losses in swine production due to growth lag, morbidity, cost of medication, and mortality (Zhang et al., 2007; Nadeau et al., 2017; Cremonesi et al., 2022; Kim et al., 2022b). In-feed antibiotics or pharmacological doses of zinc oxide (2,000–3,000 mg/kg) have been widely applied to nursery diets for controlling post-weaning diarrhea and promoting animal health and growth (De Briyne et al., 2014; López-Gálvez et al., 2021; Xie et al., 2021). However, along with the increased public health concern regarding antimicrobial resistance (McEwen, 2006; EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP), 2014; Kraemer et al., 2019; Aslam et al., 2021; Lallès and Montoya, 2021; Monger et al., 2021), the use of antibiotics for growth promoting purposes in animal production has been restricted since 2017 in the United States (Food and Drug Administration, 2013). Furthermore, considering sustainable animal agriculture, it is noteworthy that Europe not only banned the use of pharmacological doses of zinc oxide but also limited dietary zinc oxide supplementation to 150 mg/kg (European Commission, 2003; European Commission,

2016; Bonetti et al., 2021). Hence, alternative practices, including animal management and nutrition interventions, are needed to promote animal health and welfare, as increased morbidity and economic losses due to the constraints of conventional practices are inevitable.

Numerous nutritional interventions (e.g., exogenous enzymes, bioactive compounds derived from animals or plants, microbiome modulators) have been investigated and adopted in the swine industry to address the emergence of the post-antibiotic era (Kil and Stein, 2010; Liu et al., 2018). One promising alternative is a group of products based on organic acids, specifically short-chain fatty acids (SCFA; less than 6 carbons) or medium-chain fatty acids (MCFA; 6–12 carbons). Research has shown that SCFA and MCFA have strong antibacterial activity (Van Immerseel et al., 2004; Mathis et al., 2005; Kovanda et al., 2019). In addition, they also exhibit various biological activities in pigs (Decuypere and Dierick, 2003; Nguyen et al., 2020; Tugnoli et al., 2020), including beneficial effects on growth performance, intestinal physiology, and immunity, making them more than just an energy source. However, the effectiveness of supplementing organic fatty acids is often hindered by limiting factors such as unpalatable flavor and losses prior to reaching the lower gastrointestinal tract (Oprean et al., 2011; Pituch et al., 2013). In this respect, monoglycerides, composed of fatty acid esterified to glycerol, may address the limitations due to the two criteria: (1) they are relatively easy to handle; and (2) they allow active substances to be gradually released throughout the intestine (Bedford and Gong, 2018). Moreover, *in vitro* antimicrobial activity against a wide range of pathogenic bacteria was observed in glycerol esters derived from SCFA and MCFA (Namkung et al., 2011; Hyldgaard et al., 2012; Churchward et al., 2018; Kovanda et al., 2019; Wang et al., 2020). There is growing interest in monoglycerides as antibacterial lipids in nutrition and health. Their physiological activities have been extensively studied in poultry (Jahanian and Golshadi, 2015; Arabshahi et al., 2021; Kumar et al., 2021),

however, limited research has been reported on the efficacy of monoglycerides in weaned pigs using disease models. Therefore, the objective of this study was to investigate the influence of dietary supplementation of a monoglyceride blend on growth performance, intestinal health, and systemic immunity of weaned pigs experimentally infected with ETEC F18.

MATERIALS AND METHODS

Animals, housing, experimental design, and diet

Sixty weaned pigs with 28 barrows and 32 gilts (average body weight [BW] = 6.49 ± 0.74 kg; around 21 to 24 d old) were obtained from the Swine Teaching and Research Center at the University of California, Davis. The sows and piglets used in this experiment did not receive *Escherichia coli* vaccines, antibiotic injections, or antibiotics in creep feed. Before weaning, feces were collected from sows and all their piglets destined for this study to verify the absence of β-hemolytic *Escherichia coli*. The ETEC F18 receptor status was also tested by polymerase chain reaction (PCR)-restriction fragment length polymorphism (Kreuzer et al., 2013), and piglets susceptible to ETEC F18 were selected for this study. After weaning, all pigs were randomly assigned to one of the four dietary treatments (15 replicates/treatment) in a randomized complete block design with BW within sex and litter as the block and pig as the experimental unit. Pigs were housed in individual pens (0.61 m × 1.22 m) for 28 days, including 7 days before and 21 days after the first ETEC challenge. All piglets had free access to feed and water. The light was on at 07:30 h and off at 19:30 h daily in the environmental control unit.

The four dietary treatments included: 1) a corn-soybean meal-based basal diet (control); 2) the basal diet with 0.3% monoglyceride blend (BalanGut™ LS L; BASF SE, Ludwigshafen, Germany) of butyric, caprylic, and capric acids; 3) the basal diet with 3,000 mg/kg of zinc oxide (ZNO); 4) the basal diet with 50 mg/kg of carbadox (antibiotic). A 2-phase feeding program was

used with the first two weeks as phase 1 and the last two weeks as phase 2 (Table 3.1). Spray-dried plasma, antibiotics, and high levels of zinc oxide exceeding recommendation and normal practice were not included in basal diet. All diets were formulated to meet pig nutritional requirements (NRC, 2012) and provided as mash form throughout the experiment.

After 7 days of adaptation, all pigs were orally inoculated with 3 mL of ETEC F18 for three consecutive days from d 0 post-inoculation (PI). The ETEC F18 was originally isolated from a field disease outbreak by the University of Montreal (isolate number: ECL22131). The ETEC F18 expresses heat-labile toxin and heat-stable toxins a and b. The inoculums were prepared at 10^{10} colony-forming units per 3 mL dose in phosphate buffered saline. This dose caused mild diarrhea in the current study, consistent with our previously published research (Liu et al., 2013; Kim et al., 2019a; He et al., 2022).

Clinical observations and sample collections

The procedures of this experiment were adapted from previous research (Liu et al., 2013; Kim et al., 2019b; He et al., 2020; Wong et al., 2022). Clinical observations (fecal score and alertness score) were recorded twice daily throughout the study. The fecal score of each pig was assessed each day visually by two independent evaluators, 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 was calculated as the percentage of the pig days with fecal score of 3 or greater, as well as calculated as the percentage of the pig days with fecal score of 4 or greater. 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 experiment (data not shown).

Pigs were weighed on weaning day (d -7; initial BW), d 0 (before the first inoculation), 5, 14, and 21 PI. Feed intake was recorded throughout the study. Average daily gain (ADG), average daily feed intake (ADFI), and feed efficiency (gain:feed ratio) were calculated for each period. Fecal samples were collected from the rectum of all pigs throughout the experiment using a cotton swab on d -7, 2, 5, 7, 10, 14, and 21 PI to test β-hemolytic coliforms and the percentage of β-hemolytic coliforms to total coliforms (Liu et al., 2013; Kim et al., 2019b; He et al., 2020; Wong et al., 2022). Blood samples were collected from the jugular vein of all pigs before ETEC challenge (d 0), and on d 2, 5, 14, and 21 PI to collect serum samples, which were stored at −80 °C until further analysis.

Twenty-four pigs (6 pigs/treatment, 3 barrows and 3 gilts) were euthanized on d 5 PI near the peak of ETEC infection, and the remaining pigs were euthanized at the end of the experiment (d 21 PI). Before euthanization, pigs were anesthetized with 1 mL mixture of 100 mg telazol, 50 mg ketamine, and 50 mg xylazine (2:1:1) by intramuscular injection. After anesthesia, intracardiac injection with 78 mg Fatal-Plus solution (sodium pentobarbital, MWI Animal Health, Visalia, CA, USA) per 1 kg of BW was used to euthanize each pig. Intestinal mucosa samples were collected from jejunum and ileum, snap-frozen in liquid nitrogen, and then stored at −80 °C for gene expression analysis. Three 4-cm segments from the duodenum, the middle of the jejunum, and the ileum (10 cm close to the ileocecal junction) were collected and fixed in 10% neutral buffered formalin for intestinal morphology analysis.

Detection of β-hemolytic coliforms

Briefly, fecal samples were plated on Columbia Blood Agar with 5% sheep blood (Biological Media Service, School of Veterinary Medicine, University of California, Davis) to identify hemolytic coliforms, which can lyse red blood cells surrounding the colony. Fecal samples

were also plated on MacConkey agar to enumerate total coliforms. Hemolytic colonies from the blood agar were sub-cultured on MacConkey agar (Biological Media Service, School of Veterinary Medicine, University of California, Davis) to confirm that they were lactose-fermenting bacteria and flat pink colonies. All plates were incubated at 37 ℃ for 24 h in an air incubator. Populations of both total coliforms and β-hemolytic coliforms on blood agar were visually scored from 0 to 8 (0 = no bacterial growth, 8 = very heavy bacterial growth). The ratio of scores of β-hemolytic coliforms to total coliforms was calculated.

Measurements of serum cytokine and acute phase proteins

Serum samples were analyzed for tumor necrosis factor-α (TNF-α; R&D Systems Inc., Minneapolis, MN, USA), C-reactive protein (CRP; R&D Systems Inc., Minneapolis, MN, USA), and haptoglobin (Aviva Systems Biology, San Diego, CA, USA) using porcine-specific enzyme-linked immunosorbent assay kits following the manufacturer's procedures. All samples, including standards, were analyzed in duplicate. The intensity of the color was measured at 450 nm with the correction wavelength set at 530 nm using a plate reader (BioTek Instruments, Inc., Winooski, VT, USA). The intra-assay coefficients of variation for TNF-α, CRP, and haptoglobin were less than 7%. The inter-assay coefficients of variation for TNF-α, CRP, and haptoglobin were less than 10%. The concentrations of each analyte in the tested samples were calculated based on a standard curve.

Intestinal morphology

Fixed intestinal tissues were embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin. The slides were photographed by an Olympus BX51 microscope at 10× magnification, and all measurements were conducted in the image processing and analysis software (Image J, NIH). Ten straight and integrated villi and their associated crypts and surrounding areas were selected to analyze villus height (VH), area, and width; crypt depth (CD)

and width; and goblet cell number per villus as described in previous studies (Park et al., 2020b; Wong et al., 2022).

Immunohistochemistry

The immunohistochemistry procedures were based on previous research (Janjatović et al., 2008; Liu et al., 2013). Briefly, the embedded ileal tissues were sectioned at 5 μm and placed on the microslides. The slides were incubated with 5 μg/mL porcine neutrophil-specific antibody PM1 (BMA Biomedicals, Augst, Switzerland) or 0.4 μg/mL porcine macrophage-specific antibody MAC387 (Thermo Scientific, MA, USA). Antibody binding was visualized by using the avidin-biotin complex, and the diaminobenzidine chromogen (Vector Laboratories, Inc., CA, USA). Hematoxylin was applied as a counter stain. Slides incubated without the primary antibodies but with PBS were used as negative controls. Images were captured by an Olympus BX51 microscope at 10× magnification, and all measurements were analyzed by Image J software. Eight straight and integrated ileal villi were selected for measurement. The unit was the number of cells per square millimeter.

Intestinal barrier and innate immunity

Jejunal and ileal mucosa samples were analyzed for gene expression by quantitative real-time PCR (qRT-PCR). Briefly, approximately 100 mg of mucosa sample was homogenized using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Then, total ribonucleic acid (RNA) was extracted following RNA extraction procedural guidelines provided by the reagent manufacturer. The quality and quantity of RNA were evaluated using a Thermo Scientific NanoDrop 2000 Spectrophotometer (Thermo Scientific, Inc., Waltham, MA, USA). The complementary DNA (cDNA) was produced from 1 μg of total RNA per sample using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems; Thermo Fisher Scientific, Inc.,

Waltham, MA, USA) in a total volume of 20 μL. The mRNA expression of Mucin 2 (*MUC2*), Claudin-1 (*CLDN1*), Zonula occludens-1 (*ZO-1*), and Occludin (*OCLN*) in jejunal mucosa and the mRNA expression of Tumor necrosis factor-alpha (*TNFa*), Interleukin 6 (*IL6*), Interleukin 7 (*IL7*), Interleukin 10 (*IL10*), Interleukin 12 (*IL12*), Interleukin-1 alpha (*IL1A*), Interleukin-1 beta (*IL1B*), *MUC2*, and Prostaglandin-endoperoxide synthase 2 (*PTGS2*) in ileal mucosa was analyzed. Data normalization was accomplished using 18S ribosomal RNA as a housekeeping gene. Primers were designed based on published literature and commercially synthesized by Integrated DNA Technologies, Coralville, IA, USA. All primers were verified prior to qRT-PCR (Table 3.2). The qRT-PCR reaction conditions followed the published research (Liu et al., 2014). The $2^{-\Delta\Delta CT}$ method was used to analyze the relative expression of genes compared to control (Livak and Schmittgen, 2001).

Untargeted metabolomics analysis

The untargeted metabolomics analysis was performed by the NIH West Coast Metabolomics Center at the University of California, Davis, using gas chromatography (Agilent 6890 gas chromatograph controlled using Leco ChromaTOF software version 2.32, Agilent, Santa Clara, CA, USA) coupled with time-of-flight mass spectrometry (GC/TOF-MS) (Leco Pegasus IV time-of-flight mass spectrometer controlled using Leco ChromaTOF software version 2.32, Leco, St. Joseph, MI, USA). Metabolite extraction was performed following procedures previously described by Fiehn et al. (2008). Briefly, frozen serum samples (approximately 30 μL) were homogenized using a Retsch ball mill (Retsch, Newtown, PA, USA) for 30 s at 25 times/s. After homogenization, a prechilled (−20 °C) extraction solution (isopropanol/acetonitrile/water at the volume ratio 3:3:2, degassed with liquid nitrogen) was added at a volume of 1 mL/20 mg of sample. Samples were then vortexed and shaken for metabolite extraction. After centrifugation at

12,800 × *g* for 2 min, the supernatant was collected and divided into two equal aliquots and concentrated at room temperature for 4 h in a cold-trap vacuum concentrator (Labconco Centrivap, Kansas City, MO, USA). To separate complex lipids and waxes, the residue was re-suspended in 500 µL of 50% aqueous acetonitrile and centrifuged at 12,800 × *g* for 2 min. The resultant supernatant was collected and concentrated in the vacuum concentrator. Dried sample extracts were derivatized and mixed with internal retention index markers (fatty acid methyl esters with the chain length of C8 to C30). The samples were injected for GC/TOF analysis, and all samples were analyzed in a single batch. Data acquisition by mass spectrometry and mass calibration using FC43 (perfluorotributylamine) before starting analysis sequences. Metabolite identifications were performed based on the two parameters: 1) Retention index window ± 2,000 U (around ± 2 sec retention time deviation), and 2) Mass spectral similarity plus additional confidence criteria as detailed below. A detailed methodology for data acquisition and metabolite identification was described in a previously published article by Fiehn et al. (2008).

Statistical analysis

The normality of data was verified and outliers were identified using the UNIVARIATE procedure (SAS Institute Inc., Cary, NC, USA). Outliers were identified and removed as values that deviated from the treatment mean by more than 3 times the interquartile range. All data except frequency of diarrhea and metabolomics were analyzed by ANOVA using the PROC MIXED of SAS (SAS Institute Inc., Cary, NC, USA) in a randomized complete block design with the pig as the experimental unit. The statistical model included diet as the main effect and block as a random effect. Treatment means were separated by using the LSMEANS statement and the PDIFF option of PROC MIXED. 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.

The metabolomics data were analyzed using different modules of a web-based platform, MetaboAnalyst 5.0 (https://www.metaboanalyst.ca) (Pang et al., 2021). Data were filtered for peaks with detection rates less than 30% of missing abundances and normalized using logarithmic transformation and auto-scaling. Mass univariate analysis was performed using one-way ANOVA followed by Fisher's least significant difference test (adjusted $P \leq 0.05$). Fold change analysis and *t*-tests were also conducted to determine the fold change and significance of each identified metabolite. Statistical significance was declared at a false discovery rate (FDR, Benjamini and Hochberg correction; q) < 0.2 and fold change > 2.0. Partial least squares discriminant analysis (PLS-DA) was carried out to further identify discriminative variables (metabolites) among the treatment groups. Pathway analysis and metabolite set enrichment analysis were performed on identified metabolites that had a Variable Importance in Projection (VIP) score > 1. The pathway with a *P*-value less than 0.05, as well as an impact value greater than 0.1, was defined as a significant impact pathway.

RESULTS

Growth performance, diarrhea, β-hemolytic coliforms

There were no significant differences in the initial (d -7) and d 0 BW of pigs among dietary treatments (Table 3.3). In comparison to control and antibiotic groups, supplementation of monoglycerides did not affect BW, ADG, and ADFI throughout the experiment. Pigs supplemented with ZNO had greater ($P < 0.05$) BW on d 5, 14, and 21 PI, increased ($P < 0.05$) ADG from d 0 to 5 PI, d 0 to 14 PI, and d 0 to 21 PI, and enhanced ($P < 0.05$) ADFI from d 0 to 14 PI and d 0 to 21 PI than the other treatments. However, the ADFI from d 0 to 21 PI was not different between ZNO and monoglycerides groups. Pigs supplemented with ZNO had greater ($P < 0.01$) gain:feed ratio from d 0 to 5 PI compared with the other treatments, but the difference did

not persist throughout the post-challenge period. The gain:feed ratio on d 0 to 21 PI was lower ($P < 0.05$) in monoglycerides than in control and antibiotic groups, but did not differ from ZNO group.

Pigs in the ZNO group had the lowest ($P < 0.05$) fecal score from d 1 to 10 PI among dietary treatments (Figure 3.1). The incidence of diarrhea was 32.09% in control, 30.41% in monoglycerides, 4.01% in ZNO, and 22.53% in antibiotic, while the severity of diarrhea was 19.26% in control, 16.22% in monoglycerides, 0.31% in ZNO, and 12.35% in antibiotic, respectively (Figure 3.2). The incidence of diarrhea (fecal score ≥ 3) was lower ($P < 0.05$) in ZNO and antibiotic groups compared to control and monoglycerides groups. The severity of diarrhea (fecal score ≥ 4) in ZNO and antibiotic groups was also lower ($P < 0.05$) than that in control, but there was no difference observed in the severity of diarrhea between monoglycerides and antibiotic groups. The ZNO group had the lowest incidence and severity of diarrhea throughout the experimental period.

No β-hemolytic coliforms were identified in fecal samples of pigs in all groups prior to ETEC inoculation. Beta-hemolytic coliforms were identified in all pigs' feces on d 2 PI. Pigs in ZNO group had lower ($P < 0.05$) percentage of β-hemolytic coliforms in feces on d 5 PI than pigs in control, while no difference was observed among monoglycerides, ZNO, and antibiotic groups (Figure 3.3). No difference was observed in the percentage of β-hemolytic coliforms in feces among all dietary treatments on d 7, 10, 14, and 21 PI.

Systemic immunity

No difference was observed in serum TNF-α concentrations among all treatments at d 0 before ETEC inoculation, and at d 2, 5, and 21 PI (Table 3.4). Dietary supplements tended ($P = 0.07$) to impact serum TNF-α on d 14 PI, pigs fed with ZNO had the lowest TNF-α and pigs fed with control had the highest level of TNF-α among all treatments. Pigs in monoglycerides group

had lower ($P < 0.05$) serum CRP than pigs in the antibiotic group on d 0 before ETEC inoculation. Supplementation of ZNO reduced ($P < 0.10$ and $P < 0.05$) serum CRP on d 14 and 21 PI, tended ($P = 0.06$) to reduce serum haptoglobin on d 0, and reduced ($P < 0.05$) serum haptoglobin on d 2 and 5 PI. Pigs fed with monoglycerides also had lower ($P < 0.05$) serum haptoglobin on d 5 PI, compared with control pigs.

Intestinal morphology

On d 5 PI, pigs in ZNO had more ($P < 0.05$) goblet cell numbers per villus, greater ($P < 0.05$) villus area and VH, and higher ($P < 0.05$) VH:CD in duodenum than pigs in other treatments (Table 3.5; Figure 3.4). Supplementation of monoglycerides, ZNO, or antibiotic reduced ($P < 0.05$) ileal CD compared with control. Consistently, pigs in ZNO group tended ($P = 0.06$) to have the biggest VH:CD in the ileum, followed by pigs in monoglycerides and antibiotic groups. On d 21 PI, pigs supplemented with ZNO tended ($P = 0.07$) to have more goblet cells per villus, and had the largest ($P < 0.05$) villus area and highest ($P < 0.05$) VH in the duodenum, when compared with other treatments.

Immunohistochemistry

Supplementation of ZNO or antibiotic reduced ($P < 0.05$) neutrophil counts in ileal villi on d 5 PI compared with control (Table 3.6). However, no significant differences in neutrophil counts were observed among monoglycerides, ZNO, and antibiotic groups. Pigs supplemented with ZNO had the lowest ($P < 0.05$) number of macrophages in ileal villi among all treatments on d 5 PI. Pigs fed with antibiotic also had significantly lower ($P < 0.05$) recruitment of macrophages in ileal villi than control group, but comparable to that in pigs fed with monoglycerides.

Intestinal barrier and innate immunity

No differences were observed in the mRNA expression of *MUC2*, *CLDN1*, *ZO-1*, and *OCLN* in jejunal mucosa of weaned pigs among different treatments on d 5 and 21 PI (Figure 3.5). On d 5 PI, pigs fed with ZNO had lower ($P < 0.05$) mRNA expression of *TNFa*, *IL6*, *IL10*, *IL12*, *IL1A*, *IL1B*, and *PTGS2* in ileal mucosa, compared with other treatments (Figure 3.6). However, no difference in the expression of listed genes was observed between pigs supplemented with monoglycerides or ZNO. Pigs supplemented with monoglycerides expressed the lowest ($P < 0.05$) *PTGS2* in ileal mucosa compared with other treatments on d 21 PI.

Metabolite profiles in serum

A total of 483 (165 identified and 318 unidentified) metabolites were detected in serum samples. Based on statistical threshold and VIP scores, pantothenic acid and fructose were up-regulated by ZNO, compared with the pigs in control group on d 5 PI (Table 3.7). Supplementation of monoglycerides changed the relative abundances of 14 metabolites (7 up-regulated and 7 down-regulated) compared with ZNO, and upregulated lactose and cellobiose compared with antibiotic on d 5 PI. On d 14 PI, supplementation of ZNO changed abundances of 10 metabolites (7 up-regulated and 3 down-regulated) compared with control. Supplementation of monoglycerides up-regulated 2 metabolites (hippuric acid and indole-3-propionic acid) and down-regulated 8 metabolites (including glutaric acid, serotonin, mannose, etc.) compared with pigs in the ZNO. Pigs fed with antibiotic had greater abundances of hippuric acid and indole-3-propionic acid, but had lower thymine, pantothenic acid, glycerol, and piperidone compared with the pigs in the ZNO group. Limited differential metabolites were identified when comparing control vs. monoglycerides, and control vs. antibiotic throughout the experiment (data not shown).

Based on the identified metabolites and VIP scores, a PLS-DA score with 95% confidence ranges (shaded areas) showed a clear separation between control and ZNO, between

monoglycerides and ZNO, between monoglycerides and antibiotic, and between ZNO and antibiotic groups on d 5 PI (Figure 3.7A) and/or d 14 PI (Figure 3.7B). To further explore the metabolic profile differences among dietary treatments, PLS-DA was performed for the following comparisons: (1) control vs. ZNO, (2) monoglycerides vs. ZNO, (3) monoglycerides vs. antibiotic, and (4) ZNO vs. antibiotic on d 5 and 14 PI. The score plot again distinguished control from ZNO (Figure 3.8A and B), monoglycerides from ZNO (Figure 3.8C and D), monoglycerides from antibiotic (Figure 3.9A and B), and ZNO from antibiotic (Figure 3.9C and D).

Pathway analysis and metabolite set enrichment analysis were performed on the identified metabolites in serum with VIP > 1 (Table 3.8). On d 5 PI, taurine and hypotaurine metabolism and phenylalanine metabolism were the most affected metabolic pathways in a comparison of control vs. monoglycerides (Figure 3.10A and B). Arginine biosynthesis, beta-alanine metabolism, arginine and proline metabolism, pyruvate metabolism, citrate cycle (TCA cycle), glyoxylate and dicarboxylate metabolism, and glycolysis/gluconeogenesis were the most affected metabolic pathways when comparing control with ZNO (Figure 3.11A and B). Citrate cycle, taurine and hypotaurine metabolism, and beta-alanine metabolism were the most affected metabolic pathways when monoglyceride blend was compared with ZNO (Figure 3.12A and B). Taurine and hypotaurine metabolism, nicotinate and nicotinamide metabolism, and beta-alanine metabolism were the most affected metabolic pathways in a comparison of monoglycerides vs. antibiotic (Figure 3.13A and B). Beta-alanine metabolism and citrate cycle were the most affected metabolic pathways when comparing ZNO with antibiotic (Figure 3.14A and B). On d 14 PI, glyoxylate and dicarboxylate metabolism and taurine and hypotaurine metabolism were the most affected metabolic pathways in a comparison of control vs. monoglycerides (Figure 3.10C and D). Alanine, aspartate and glutamate metabolism, citrate cycle, glyoxylate and dicarboxylate metabolism, and

pyrimidine metabolism were the most affected metabolic pathways when comparing control with ZNO (Figure 3.11C and D). Citrate cycle, glyoxylate and dicarboxylate metabolism, alanine, aspartate and glutamate metabolism, and pyrimidine metabolism were the most affected metabolic pathways when monoglyceride blend was compared with ZNO (Figure 3.12C and D), while citrate cycle was the most affected metabolic pathway in comparison of monoglycerides vs. antibiotic (Figure 3.13C and D). Alanine, aspartate and glutamate metabolism, glyoxylate and dicarboxylate metabolism, citrate cycle, D-glutamine and D-glutamate metabolism, pyrimidine metabolism, arginine biosynthesis, and beta-alanine metabolism were the most affected metabolic pathways when comparing ZNO with antibiotic (Figure 3.14C and D).

DISCUSSION

The present study investigated the potential of a monoglyceride blend containing butyric, caprylic, and capric acids in mitigating the adverse effects of ETEC F18 infection on systemic and intestinal immune responses, as well as intestinal health in weaning pigs. Additionally, the study identified metabolic changes resulting from monoglycerides supplementation, shedding light on potential mechanisms underlying the observed physiological responses.

Post-weaning diarrhea, a prevalent gastrointestinal disease occurring shortly after weaning, is often attributed to the adhesion and proliferation of ETEC F18 or F4 in the small intestine. Clinical signs typically include watery diarrhea, dirty appearance, stunted growth, dehydration, and lethargy (Fairbrother et al., 2005; He et al., 2020). In this study, successful ETEC F18 infection was confirmed through fecal shedding of β-hemolytic coliforms and the manifestation of typical infection symptoms, including growth retardation and severe diarrhea. These observations are consistent with our previous research (Kim et al., 2019b; Wong et al., 2022). The observed pattern of gradual recovery after the peak of infection (d 3 to 5 PI) also aligns with our previous studies

using the same ETEC F18 strain (Liu et al., 2013; Kim et al., 2022a; Wong et al., 2022). The results of fecal score and the frequency of diarrhea indicated that supplementation of high-dose zinc oxide or antibiotics significantly reduces both the incidence and severity of diarrhea in weaned pigs infected with ETEC F18. However, the impact of dietary monoglycerides on diarrhea was limited.

ETEC toxins can disrupt the regulation of intestinal ion transporters, leading to fluid and electrolyte imbalances (Kaper et al., 2004; Mirhoseini et al., 2018). Although the percentage of β-hemolytic coliforms in feces was similar across treatments post-infection, supplementation of high-dose zinc oxide notably reduced the β-hemolytic coliforms on d 5 PI, which may be attributed to zinc oxide's antimicrobial properties and its ability to support intestinal barrier function and epithelial tissue regeneration (Pearce et al., 2015; Liu et al., 2018; Bonetti et al., 2021). Similarly, both monoglycerides and antibiotics showed comparable reductions in ETEC shedding, likely due to their antibacterial activity (Bedford and Gong, 2018; Rahman et al., 2022). This reduction corresponded with a decreased incidence of diarrhea across all supplemented groups.

It is well known that ETEC infection can disrupt essential intestinal functions, such as nutrient transport, epithelial barrier integrity, and immune function (Kim et al., 2022b; Duarte et al., 2023). All of these result in reduced digestive and absorptive capacity, and increased resource expenditure for maintaining intestinal homeostasis, ultimately leading to compromised performance in infected animals (He et al., 2020; Sheikh et al., 2022; Daneshmand et al., 2023). The beneficial effects of high-dose zinc oxide on intestinal morphology were significant, and supplementation with monoglycerides improved CD and VH:CD in the ileum of ETEC-infected pigs on d 5 PI, comparable to high-dose zinc oxide. However, there were limited changes in intestinal morphology on d 21 PI, likely due to the pigs' recovery from ETEC infection. Consistent

with our observations, previous studies have reported the positive effects of pharmacological doses of zinc oxide in managing post-weaning diarrhea caused by ETEC and have summarized its beneficial effects on growth performance, gastrointestinal tract health, and immunity (Bonetti et al., 2021). Although the exact modes of action of carbadox are unclear, the observed changes in serum inflammatory markers and ileal morphology may be due to their ability to compete for sites important for nutrient absorption and ETEC colonization, thereby reducing resource costs and improving nutrient availability. Intestinal morphology results are also consistent with findings reported by Hung et al. (2020), who observed that carbadox in the diet decreased CD and increased VH:CD in the small intestine of weaned pigs.

In addition to changes in intestinal morphology, high-dose zinc oxide and carbadox supplementation showed a mitigating effect on the recruitment of neutrophils and macrophages in the ileal villi. Supplementation with high-dose zinc oxide also reduced the relative gene expression of inflammatory cytokines (*TNFa*, *IL6*, *IL10*, *IL12*, *IL1A*, *IL1B*, and *PTGS2*) in ileal mucosa, indicating a moderating effect on the intestinal immune response. Although monoglycerides supplementation partially attenuated intestinal inflammation, its efficacy was not comparable to that of high-dose zinc oxide. The observed changes in the supplementation of monoglycerides suggest reduced intestinal epithelial cell renewal and attenuated inflammatory responses, indicating reduced energy and nutritional costs similar to conventional practices (Hung et al., 2020). These findings also suggest that supplementing monoglycerides may overcome primary obstacles associated with the use of organic acids as feed additives, including undesirable losses in the upper intestine and unfavorable taste and aroma. The antibacterial effects of organic acids and their monoglycerides against *Escherichia coli* have been verified through numerous *in vitro* studies (Thormar et al., 2006; Hyldgaard et al., 2012; Kovanda et al., 2019; Wang et al., 2020).

The biological activity of butyric acid, which constitutes a major portion of our glyceride blend (~60%), has been well documented, including its modulation of various cellular responses via histone deacetylase inhibition and G protein-coupled receptor activation in various cell types (Hamer et al., 2008; Pituch et al., 2013; Bedford and Gong, 2018; Salvi and Cowles, 2021), further supporting our findings.

Moreover, local inflammation can influence systemic immunity, and immune activation by external factors can exacerbate the performance status during the weaning period due to metabolic changes (Klasing, 1988; Spurlock, 1997; Park et al., 2020a). For instance, ETEC infection activates immune cells and increases the secretion of pro-inflammatory cytokines (Schilling et al., 2001; Liu et al., 2013; Wong et al., 2022), leading to alterations in the absorption and utilization of nutrients or energy, including anorexia, decreased gut motility, and increased hepatic acute-phase protein synthesis (Klasing, 1988; Elsasser et al., 2008; Davis, 2022). Supplementation with high-dose zinc oxide was associated with a significant reduction in inflammatory biomarkers throughout the experiment, and an anti-inflammatory effect of monoglycerides was also observed during peak infection. This finding is supported by observations reported by Tian et al., (2022), where inclusion of glycerol butyrate in pig diet reduced pro-inflammatory factors (*TNFa*, *IL6*, and, *IL1B)* in jejunum and ileum to ETEC infection by inhibiting the NF-κB/MAPK pathway.

Given the biological effects of high-dose zinc oxide discussed earlier and the observed changes in diarrhea, intestinal morphology, and intestinal and serum inflammatory markers, it is not surprising that the pigs fed with high-dose zinc oxide had the greatest growth performance throughout the experimental period among all treatments. On the other hand, carbadox supplementation reduced feed intake compared to high-dose zinc oxide, but feed efficiency was higher than that of monoglycerides throughout the post-challenge period. These results reflect the

multifactorial nature of animal growth and suggest that high-dose zinc oxide and antibiotics are likely to exert their beneficial effects through different mechanisms (Hung et al., 2020). In the present study, the monoglyceride blend had limited effects on the growth performance of weaned pigs infected with ETEC F18. This finding aligns with other research showing that dietary supplementation of SCFA or MCFA monoglycerides did not affect the performance of weaned pigs (Cui et al., 2020; Thomas et al., 2020; Li et al., 2022; Li et al., 2023). Recent studies in poultry also confirmed that dietary supplementation of monoglyceride blend (butyric, caprylic, and capric acids) did not affect the growth performance of early growth stage in broilers infected with necrotic enteritis (Gharib-Naseri et al., 2021; Kumar et al., 2021). In this study, supplementation of monoglyceride blend reduced gain:feed ratio of ETEC-infected pigs. However, it is noteworthy that this change was the result of increased feed intake. The observed improvement in feed intake in pigs fed with monoglycerides is further supported by the previously discussed anti-inflammatory effects of monoglycerides. Weaning stress is associated with reduced nutrient and energy intake, which may not recover even two weeks after weaning (Bruininx et al., 2001; Dong and Pluske, 2007). Thus, the potential impacts of the monoglyceride blend on the feed intake of newly weaned pigs need to be further investigated in a performance trial with a larger number of animals.

The physiological changes caused by external factors, such as nutritional interventions or disease, can be comprehensively evaluated through a metabolomics analysis, providing valuable insights into the underlying mechanisms (Martínez-Reyes and Chandel, 2020; Kim et al., 2024). In this study, pigs supplemented with high-dose zinc oxide exhibited significant alterations in serum metabolites primarily associated with carbohydrate and amino acid metabolism, compared to pigs in the control and monoglycerides groups. These changes are consistent with the

mechanistic measurement results discussed earlier, and are also in line with the inferred effects suggested by other research related to nutrient and energy availability (Hung et al., 2020). For example, the citrate cycle is a major metabolic pathway regulated to meet diverse cellular metabolic needs, including playing an important role in energy production and providing intermediates required for biosynthesis (Fernie et al., 2004). Recent studies have shown that these intermediates are also involved in cell signaling and have diverse functions, such as the regulation of chromatin modification and DNA methylation, as well as immunomodulation (Williams and O'Neill, 2018; Martínez-Reyes and Chandel, 2020).

Interestingly, monoglycerides supplementation had limited effects on serum metabolites compared to the control; however, significant pathway alterations were observed in serum metabolites when pigs were supplemented with monoglycerides. Specifically, taurine and hypotaurine metabolism was one of the metabolic pathways significantly affected by the supplementation of monoglycerides during the peak of ETEC infection in pigs. Taurine and hypotaurine are known to play crucial roles in cellular homeostasis and antioxidant responses (Aruoma et al., 1988; Mizota et al., 2021). Similar to high-dose zinc oxide, carbadox supplementation had impacts on carbohydrate and amino acid metabolism in serum metabolites compared to control or monoglycerides. These changes include alterations in the citrate cycle and beta-alanine metabolism. Beta-alanine is a naturally occurring amino acid involved in the synthesis of carnosine, which exhibits beneficial biological activity, including antioxidant and anti-inflammatory properties (Culbertson et al., 2010; Jukić et al., 2021; Chen et al., 2023). Additionally, it has been reported that Mas-related G protein-coupled receptors, specifically responsive to beta-alanine, may have beneficial effects on immune stress and homeostasis (Shinohara et al., 2004; Zhang et al., 2021).

In conclusion, the findings of this study suggest that supplementation of monoglyceride blend including C4, C8, and C10 saturated fatty acids may enhance disease resistance by mitigating intestinal and systemic inflammation in weaned pigs challenged with enterotoxigenic *Escherichia coli* F18. Although the effects on performance and disease resistance were not comparable to that of high-dose zinc oxide, the efficacy was similar to the supplementation of carbadox. Additional research is needed to further evaluate the effects of monoglycerides supplementation on growth performance of weaned pigs under various external challenges in commercial conditions. Another area of research may be to explore combinations of monoglycerides with other acids, such as formic acid, as a potential alternative to conventional practices.

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TABLES AND FIGURES

Table 3.1. Ingredient compositions of experimental diets[a]

Ingredient, %	Control, phase 1	Control, phase 2
Corn	44.41	57.27
Dried whey	15.00	10.00
Soybean meal	18.00	22.00
Fish meal	10.00	7.00
Lactose	6.00	-
Soy protein concentrate	3.00	-
Soybean oil	2.00	2.00
Limestone	0.56	0.70
L-Lysine·HCl	0.21	0.23
DL-Methionine	0.08	0.05
L-Threonine	0.04	0.05
Salt	0.40	0.40
Vit-mineral, Sow 6[b]	0.30	0.30
Total	100.00	100.00
Calculated energy and nutrient		
Metabolizable energy, kcal/kg	3463	3429
Net energy, kcal/kg	2601	2575
Crude protein, %	22.27	20.80
Arg,[c] %	1.23	1.15
His,[c] %	0.49	0.47
Ile,[c] %	0.83	0.76
Leu,[c] %	1.62	1.55
Lys,[c] %	1.35	1.23
Met,[c] %	0.45	0.39
Thr,[c] %	0.79	0.73
Trp,[c] %	0.23	0.21
Val,[c] %	0.91	0.84
Met + Cys,[c] %	0.74	0.68
Phe + Tyr,[c] %	1.45	1.38
Ca, %	0.80	0.70
Total P, %	0.68	0.59
Digestible P, %	0.47	0.37

[a]In each phase, three additional diets were formulated by adding 0.3% monoglyceride blend, 3,000 mg/kg zinc oxide, or 50 mg/kg carbadox to the control diet, respectively

[b]Provided the following quantities of vitamins and micro minerals per kilogram of complete diet: vitamin A as retinyl acetate, 11,136 IU; vitamin D_3 as cholecalciferol, 2,208 IU;

vitamin E as *DL*-alpha-tocopheryl acetate, 66 IU; vitamin K as menadione dimethylpyrimidinol bisulfite, 1.42 mg; thiamin as thiamine mononitrate, 0.24 mg; riboflavin, 6.59 mg; pyridoxine as pyridoxine hydrochloride, 0.24 mg; vitamin B_{12}, 0.03 mg; *D*-pantothenic acid as *D*-calcium pantothenate, 23.5 mg; niacin, 44.1 mg; folic acid, 1.59 mg; biotin, 0.44 mg; Cu, 20 mg as copper sulfate and copper chloride; Fe, 126 mg as ferrous sulfate; I, 1.26 mg as ethylenediamine dihydriodide; Mn, 60.2 mg as manganese sulfate; Se, 0.3 mg as sodium selenite and selenium yeast; and Zn, 125.1 mg as zinc sulfate

[c]Amino acids were indicated as standardized ileal digestible amino acids

Table 3.2. Gene-specific primer sequences and polymerase chain reaction conditions[1]

Gene[2]	Acc. No[3]	Forward primer (5'-3')	Reverse primer (5'-3')
MUC2	AK231524	CAACGGCCTCTCCTTCTCTGT	GCCACACTGGCCCTTTGT
CLDN1	NM_001244539	TCTTAGTTGCCACAGCATGG	CCAGTGAAGAGAGCCTGACC
ZO-1	AJ318101	CCGCCTCCTGAGTTTGATAG	CAGCTTTAGGCACTGTGCTG
OCLN	NM_001163647	TTCATTGCTGCATTGGTGAT	ACCATCACACCCAGGATAGC
IL1A	NM_214029	CAGCCAACGGGAAGATTCTG	ATGGCTTCCAGGTCGTCAT
IL1B	NM_214055	CCTTGAAACGTGCAATGATG	TTCAAGTCCCCTGTGAGGAG
IL6	NM_214399.1	TAAGGGAAATGTCGAGGCCG	TTGTGTTCTTCATCCACTCGT
IL7	NM_214135	CAACTGCACCAGCAAGGTTAAAG	AAGTCCCCCTGTCTTTTCTGTTC
IL10	NM_214041.1	TCGGCCCAGTGAAGAGTTTC	GGAGTTCACGTGCTCCTTGA
IL12	NM_213993	CGTGCCTCGGGCAATTATA	CGCAGGTGAGGTCGCTAGTT
PTGS2	AF207824	ATAAGTGTGACTGCACCCGAAC	GGTGGGCTATCAATCAGATGTG
TNFa	NM_214022.1	CGTGAAGCTGAAAGACAACCAG	GATGGTGTGAGTGAGGAAAAC
18S rRNA	NM_213940.1	AGGAAAGCAGACATCGACCT	ACCTGGCTGTACTTCCCATC

[1]Thermal cycling conditions were 95℃ for 20 sec and 95℃ for 1 sec, followed by 40 cycles with 20 sec at 60℃.

[2]*MUC2* = Mucin 2; *CLDN1* = Claudin-1; *ZO-1* = Zonula occludens-1; *OCLN* = Occludin; *IL1A* = Interleukin-1 alpha; *IL1B* = Interleukin-1 beta; *IL6* = Interleukin 6; *IL7* = Interleukin 7; *IL10* = Interleukin 10; *IL12* = Interleukin 12, *PTGS2* = Prostaglandin-endoperoxide synthase 2; *TNFa* = Tumor necrosis factor-alpha; *18S* rRNA = 18S ribosomal ribonucleic acid.

[3]Accession number in GenBank database.

Table 3.3. Growth performance of enterotoxigenic *Escherichia coli* F18-challenged weaned pigs fed experimental diets

Item[c]	Control	Monoglycerides	ZNO[d]	Antibiotic	SEM	*P*-value
BW, kg						
d -7	6.50	6.47	6.51	6.48	0.19	0.95
d 0	7.44	7.46	7.81	7.43	0.23	0.12
d 5 PI	7.56[b]	7.38[b]	9.25[a]	7.95[b]	0.34	<0.01
d 14 PI[e]	12.88[b]	12.56[b]	14.71[a]	12.77[b]	0.43	<0.05
d 21 PI[e]	17.43[b]	17.00[b]	19.14[a]	17.33[b]	0.52	<0.05
ADG, g						
d -7 to 0	154	145	186	135	20.97	0.30
d 0 to 5 PI	38[b]	44[b]	287[a]	104[b]	34.14	<0.01
d 0 to 14 PI[e]	346[b]	330[b]	452[a]	362[b]	26.98	<0.05
d 0 to 21 PI[e]	470[b]	431[b]	526[a]	457[b]	15.29	<0.01
ADFI, g						
d -7 to 0	271	267	278	273	28.71	0.98
d 0 to 5 PI	353	376	451	405	28.64	0.14
d 0 to 14 PI[e]	553[b]	607[b]	718[a]	577[b]	25.93	<0.01
d 0 to 21 PI[e]	680[b]	741[ab]	826[a]	719[b]	32.32	<0.05
Gain:Feed						
d -7 to 0	0.55	0.54	0.67	0.48	0.054	0.10
d 0 to 5 PI	0.10[b]	0.11[b]	0.60[a]	0.23[b]	0.069	<0.01
d 0 to 14 PI[e]	0.62	0.55	0.63	0.61	0.029	0.25
d 0 to 21 PI[e]	0.66[a]	0.58[b]	0.62[ab]	0.64[a]	0.014	<0.05

[a,b]Means without a common superscript are different ($P < 0.05$)

[c]*BW* Body weight, *ADG* Average daily gain, *ADFI* Average daily feed intake, *PI* Post-inoculation. Each least squares mean represents 14–15 observations

[d]*ZNO* High-dose zinc oxide

[e]Each least squares mean represents 8–9 observations

Table 3.4. Serum tumor necrosis factor-alpha and acute-phase proteins in enterotoxigenic *Escherichia coli* F18-challenged weaned pigs fed experimental diets

Item[d]	Control	Monoglyceride	ZNO[e]	Antibiotic	SEM	*P*-value
d 0 before inoculation						
TNF-α, pg/mL	187.51	197.13	208.46	226.26	38.15	0.90
CRP, µg/mL	7.57[ab]	5.56[b]	8.26[ab]	11.88[a]	2.61	<0.05
Haptoglobin, µg/mL	1531.22[ab]	1236.94[ab]	756.45[b]	2217.13[a]	402.98	0.06
d 2 post-inoculation						
TNF-α, pg/mL	236.35	277.77	235.19	232.65	42.62	0.86
CRP, µg/mL	9.15	5.95	8.96	10.59	2.06	0.18
Haptoglobin, µg/mL	1637.06[a]	1783.59[a]	818.70[b]	1724.84[a]	284.28	<0.05
d 5 post-inoculation						
TNF-α, pg/mL	271.96	334.52	291.19	271.28	51.42	0.81
CRP, µg/mL	6.49	6.98	4.19	8.76	1.62	0.21
Haptoglobin, µg/mL	1800.29[a]	1118.21[b]	365.53[c]	1300.01[ab]	224.95	<0.01
d 14 post-inoculation[f]						
TNF-α, pg/mL	462.00[a]	254.71[ab]	213.75[b]	400.18[ab]	69.97	0.07
CRP, µg/mL	7.95[ab]	8.42[a]	6.92[b]	8.21[ab]	1.56	0.09
Haptoglobin, µg/mL	1280.88	1250.54	551.28	1002.63	332.56	0.42
d 21 post-inoculation[f]						
TNF-α, pg/mL	421.64	275.89	273.94	290.19	79.20	0.49
CRP, µg/mL	9.10[a]	9.12[a]	4.18[b]	8.23[a]	1.33	<0.01
Haptoglobin, µg/mL	626.76	770.43	174.50	130.36	201.65	0.11

[a,b,c]Means without a common superscript are different ($P < 0.05$)

[d]*TNF-α* Tumor necrosis factor-alpha, *CRP* C-reactive protein. Each least squares mean represents 14–15 observations

[e]*ZNO* High-dose zinc oxide

[f]Each least squares mean represents 8–9 observations

Table 3.5. Intestinal morphology of enterotoxigenic *Escherichia coli* F18-challenged weaned pigs fed experimental diets

Item[c]	Control	Monoglycerid	ZNO[d]	Antibiotic	SEM	*P*-value
d 5 PI						
Duodenum						
Goblet cells, per villus	10.97[b]	8.61[b]	18.58[a]	12.86[ab]	2.40	<0.05
Villus area, µm^2	12473[b]	14342[b]	20838[a]	16097[b]	1549	<0.01
Villus height, µm	183.56[b]	190.73[b]	274.42[a]	216.75[b]	13.11	<0.01
Villus width, µm	70.92	81.33	81.22	79.10	4.62	0.13
Crypt depth, µm	245.58	236.32	237.64	251.08	21.00	0.88
Crypt width, µm	25.02	24.88	24.99	24.24	1.12	0.91
VH:CD	0.77[b]	0.83[b]	1.21[a]	0.91[b]	0.072	<0.01
Jejunum						
Goblet cells, per villus	4.87	6.95	4.73	4.08	1.53	0.60
Villus area, µm^2	12434	12002	16387	13376	1817	0.30
Villus height, µm	200.14	205.14	236.62	213.79	17.42	0.42
Villus width, µm	64.45	61.02	69.64	63.84	3.86	0.28
Crypt depth, µm	147.26	135.38	138.27	145.15	13.03	0.81
Crypt width, µm	22.73	23.42	24.23	25.18	1.049	0.23
VH:CD	1.41	1.68	1.79	1.56	0.16	0.35
Ileum						
Goblet cells, per villus	15.38	16.40	17.00	17.28	2.90	0.97
Villus area, µm^2	12826	9723	11251	11128	1101	0.11
Villus height, µm	184.86	174.00	193.78	173.11	12.18	0.58
Villus width, µm	67.95	64.39	60.84	63.62	2.92	0.27
Crypt depth, µm	170.20[a]	123.54[b]	136.34[b]	137.90[b]	12.29	<0.05

Crypt width, µm	22.34	22.85	23.64	24.13	0.64	0.50
VH:CD	1.20[b]	1.36[ab]	1.57[a]	1.34[ab]	0.110	0.06
d 21 PI[e]						
Duodenum						
Goblet cells, per villus	28.80[ab]	25.04[ab]	37.07[a]	21.44[b]	3.98	0.07
Villus area, µm^2	30642[ab]	24823[b]	34452[a]	25941[b]	2336	<0.05
Villus height, µm	309.02[b]	278.81[b]	366.04[a]	271.89[b]	18.64	<0.05
Villus width, µm	98.56	88.51	96.46	97.05	3.17	0.18
Crypt depth, µm	275.04	272.96	282.99	250.34	14.52	0.41
Crypt width, µm	27.29	28.85	27.28	27.05	0.90	0.52
VH:CD	1.21	1.15	1.37	1.18	0.101	0.42
Jejunum						
Goblet cells, per villus	8.89	10.08	11.57	9.08	1.41	0.45
Villus area, µm^2	18156	18460	18590	19026	1343	0.97
Villus height, µm	257.12	276.95	282.69	279.23	15.19	0.66
Villus width, µm	73.33	69.45	71.08	73.11	2.71	0.71
Crypt depth, µm	165.89	170.21	180.80	165.23	11.99	0.56
Crypt width, µm	26.48	26.67	26.13	26.44	0.80	0.97
VH:CD	1.69	1.70	1.71	1.79	0.15	0.95
Ileum						
Goblet cells, per villus	18.40	19.74	22.26	18.18	3.45	0.73
Villus area, µm^2	14508	15979	16654	15200	2130	0.57
Villus height, µm	220.74	239.54	248.57	236.31	17.99	0.42
Villus width, µm	70.96	75.91	73.84	71.49	5.13	0.66
Crypt depth, µm	166.96	154.41	161.84	148.58	14.19	0.59

Crypt width, µm	27.50	28.50	25.85	26.24	1.038	0.21
VH:CD	1.40	1.70	1.90	1.70	0.12	0.22

[a,b]Means without a common superscript are different ($P < 0.05$)

[c]*PI* Post-inoculation, *VH:CD* Villus height-to-crypt depth ratio. Each least squares mean represents 6 observations

[d]*ZNO* High-dose zinc oxide

[e]Each least squares mean represents 8–9 observations

Table 3.6. Number of neutrophils and macrophages in the ileum of enterotoxigenic *Escherichia coli* F18-challenged weaned pigs fed experimental diets

Item	Control	Monoglycerides	ZNO[d]	Antibiotic	SEM	*P*-value
d 5 post-inoculation[e]						
Neutrophils	2596[a]	1759[ab]	1382[b]	1406[b]	382	<0.05
Macrophages	2236[a]	1715[ab]	676[c]	1085[bc]	369	<0.05

[a,b,c]Means without a common superscript are different ($P < 0.05$)

[d]*ZNO* High-dose zinc oxide

[e]Each least squares mean represents 6 observations

Table 3.7. Serum metabolites that differed among the dietary treatment groups

Metabolite	Fold change[a]	VIP[b]	FDR[c]
Control vs. ZNO[d], d 5 post-inoculation			
pantothenic acid	0.32	1.97	0.156
Fructose	0.43	2.08	0.137
Monoglycerides vs. ZNO, d 5 post-inoculation			
piperidone	0.12	2.05	0.093
kynurenine	0.34	1.87	0.099
beta-alanine	0.36	1.78	0.099
xylonic acid	0.41	1.82	0.099
nicotinamide	0.44	1.83	0.099
pantothenic acid	0.46	1.77	0.099
glutaric acid	0.48	1.60	0.141
hippuric acid	2.08	1.56	0.157
6-Oxopiperidine-2-carboxylic acid	2.28	1.75	0.099
histidine	2.29	1.50	0.177
sucrose	2.43	1.72	0.110
indoxyl sulfate	2.45	1.59	0.141
glycyl tyrosine	2.69	1.59	0.141
alpha-aminoadipic acid	4.48	1.51	0.177
Monoglycerides vs. Antibiotic, d 5 post-inoculation			
lactose	2.38	2.49	0.034
cellobiose	2.41	2.52	0.034
Control vs. ZNO, d 14 post-inoculation			
piperidone	0.09	1.79	0.014
glutaric acid	0.30	1.21	0.146
glycerol-3-galactoside	0.33	1.47	0.067
oleic acid	0.37	1.25	0.129
5-methoxytryptamine	0.38	1.47	0.067
mannose	0.42	1.81	0.014
thymine	0.43	1.71	0.024
conduritol-beta-epoxide	2.18	1.62	0.037
hippuric acid	3.36	1.51	0.062
indole-3-propionic acid	3.48	1.48	0.067
Monoglycerides vs. ZNO, d 14 post-inoculation			
piperidone	0.10	1.70	0.031
glycerol	0.22	1.75	0.031
taurine	0.22	1.14	0.194
glutaric acid	0.25	1.35	0.118
serotonin	0.30	1.62	0.032
oleic acid	0.37	1.28	0.137
glycerol-3-galactoside	0.40	1.31	0.129
mannose	0.47	1.75	0.031
hippuric acid	3.31	1.43	0.078
indole-3-propionic acid	5.25	1.63	0.032
ZNO vs. Antibiotic, d 14 post-inoculation			
hippuric acid	0.34	1.72	0.158
indole-3-propionic acid	0.43	1.70	0.158
thymine	2.09	2.05	0.070
pantothenic acid	2.60	1.70	0.158
glycerol	2.73	1.79	0.150
piperidone	4.84	1.83	0.150

[a]Fold change values less than one indicate that the differential metabolites were reduced in the Control compared to ZNO or Monoglycerides compared to ZNO or Monoglycerides compared to Antibiotic or ZNO compared to Antibiotic, respectively

[b]*VIP* Variable importance in projection

[c]*FDR* False discovery rate

[d]*ZNO* High-dose zinc oxide

Table 3.8. Significant impact pathways in serum that affected by the dietary treatment groups

Pathway name	Impact[a]	*P*-value[b]
Control vs. Monoglycerides, d 5 post-inoculation		
Taurine and hypotaurine metabolism	0.71	0.017
Phenylalanine metabolism	0.36	0.027
Control vs. ZNO[c], d 5 post-inoculation		
Arginine biosynthesis	0.41	< 0.001
beta-Alanine metabolism	0.40	0.026
Arginine and proline metabolism	0.34	0.006
Pyruvate metabolism	0.21	0.030
Citrate cycle (TCA cycle)	0.13	0.023
Glyoxylate and dicarboxylate metabolism	0.10	0.017
Glycolysis / Gluconeogenesis	0.10	0.046
Control vs. Antibiotic, d 5 post-inoculation		
Citrate cycle (TCA cycle)	0.21	0.002
Glyoxylate and dicarboxylate metabolism	0.11	0.012
Phenylalanine metabolism	0.36	0.032
Monoglycerides vs. ZNO, d 5 post-inoculation		
Citrate cycle (TCA cycle)	0.13	0.024
Taurine and hypotaurine metabolism	0.71	0.025
beta-Alanine metabolism	0.40	0.028
Monoglycerides vs. Antibiotic, d 5 post-inoculation		
Taurine and hypotaurine metabolism	0.71	0.001
Nicotinate and nicotinamide metabolism	0.19	0.010
beta-Alanine metabolism	0.40	0.026
ZNO vs. Antibiotic, d5 post-inoculation		
beta-Alanine metabolism	0.40	0.004
Citrate cycle (TCA cycle)	0.19	0.023
Control vs. Monoglycerides, d 14 post-inoculation		
Glyoxylate and dicarboxylate metabolism	0.21	0.014
Taurine and hypotaurine metabolism	0.29	0.022
Control vs. ZNO, d 14 post-inoculation		
Alanine, aspartate and glutamate metabolism	0.16	< 0.001
Citrate cycle (TCA cycle)	0.31	< 0.001
Glyoxylate and dicarboxylate metabolism	0.24	< 0.001
Pyrimidine metabolism	0.12	0.003
Control vs. Antibiotic, d 14 post-inoculation		
Citrate cycle (TCA cycle)	0.21	0.003
Glyoxylate and dicarboxylate metabolism	0.11	0.015
Taurine and hypotaurine metabolism	0.71	0.023
Monoglycerides vs. ZNO, d 14 post-inoculation		
Citrate cycle (TCA cycle)	0.28	< 0.001
Glyoxylate and dicarboxylate metabolism	0.24	< 0.001
Alanine, aspartate and glutamate metabolism	0.16	0.003
Pyrimidine metabolism	0.10	0.012
Monoglycerides vs. Antibiotic, d 14 post-inoculation		
Citrate cycle (TCA cycle)	0.19	0.017
ZNO vs. Antibiotic, d14 post-inoculation		
Alanine, aspartate and glutamate metabolism	0.36	< 0.001
Glyoxylate and dicarboxylate metabolism	0.22	< 0.001
Citrate cycle (TCA cycle)	0.27	< 0.001
D-Glutamine and D-glutamate metabolism	0.50	< 0.001
Pyrimidine metabolism	0.18	0.001
Arginine biosynthesis	0.12	0.010
beta-Alanine metabolism	0.40	0.031

[a]Pathway impact value; cumulative percentage from the matched metabolite nodes that calculated from pathway topology analysis

[b]Original *P*-value calculated from the enrichment analysis

[c]*ZNO* High-dose zinc oxide

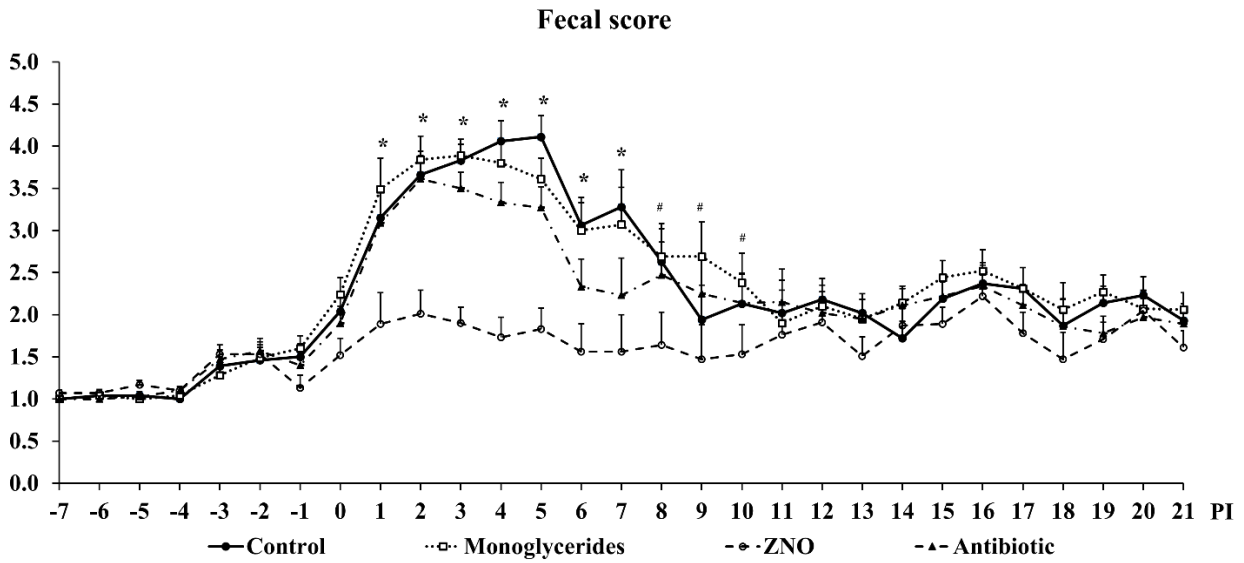

Figure 3.1. Daily fecal score of enterotoxigenic *Escherichia coli* F18-challenged weaned pigs fed diets supplemented with monoglycerides, high-dose zinc oxide (ZNO), or antibiotic. Fecal score = 1, normal feces, 2, moist feces, 3, mild diarrhea, 4, severe diarrhea, 5, watery diarrhea. $^{*}P < 0.05$, indicating fecal scores were significantly different among treatments. $^{\#}P < 0.10$, indicating fecal scores tended to different among treatments. Each least squares mean represents 14–15 observations before d 5 post-inoculation (PI) and each least squares mean represents 8–9 observations after d 5 PI.

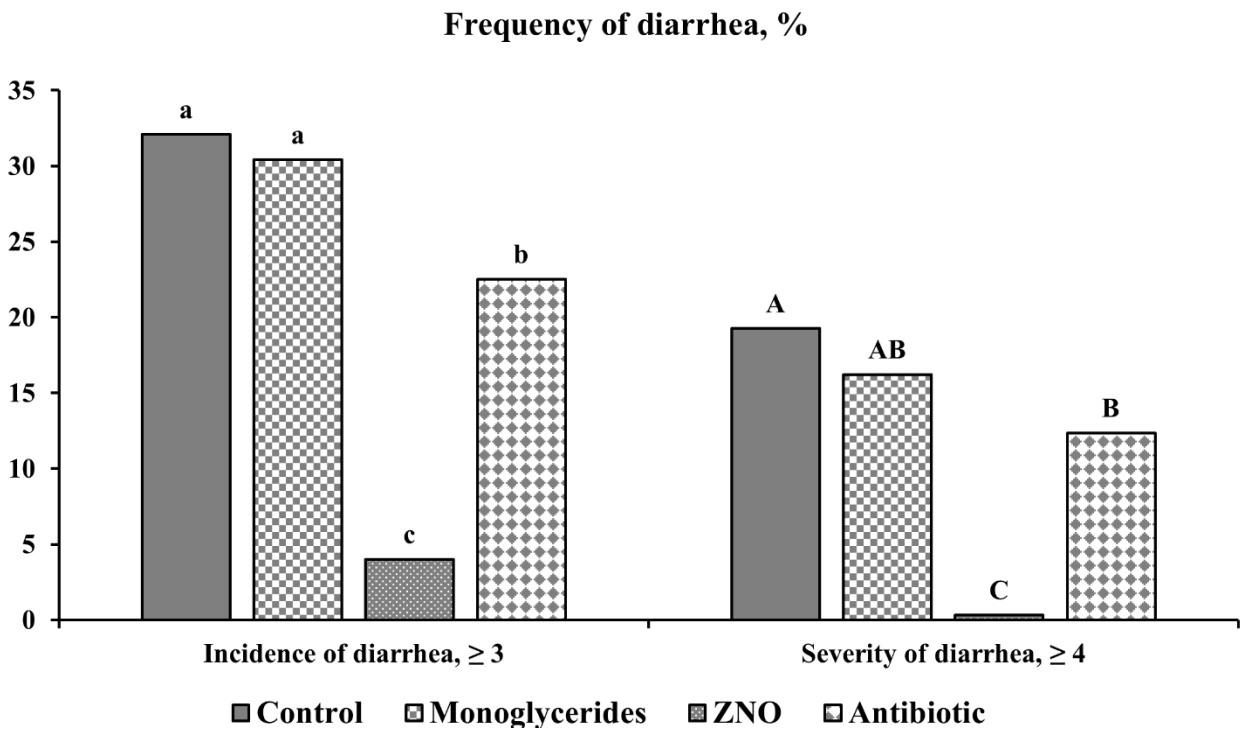

Figure 3.2. Frequency of diarrhea (overall period) of enterotoxigenic *Escherichia coli* F18-challenged weaned pigs fed diets supplemented with monoglycerides, high-dose zinc oxide (ZNO), or antibiotic. Frequency of diarrhea was calculated as the percentage of pig days with fecal score ≥ 3 or 4 in the total of pig days. ^{a,b,c}Means without a common superscript are different ($P < 0.05$) in frequency of diarrhea ≥ 3. ^{A,B,C}Means without a common superscript are different ($P < 0.05$) in frequency of diarrhea ≥ 4.

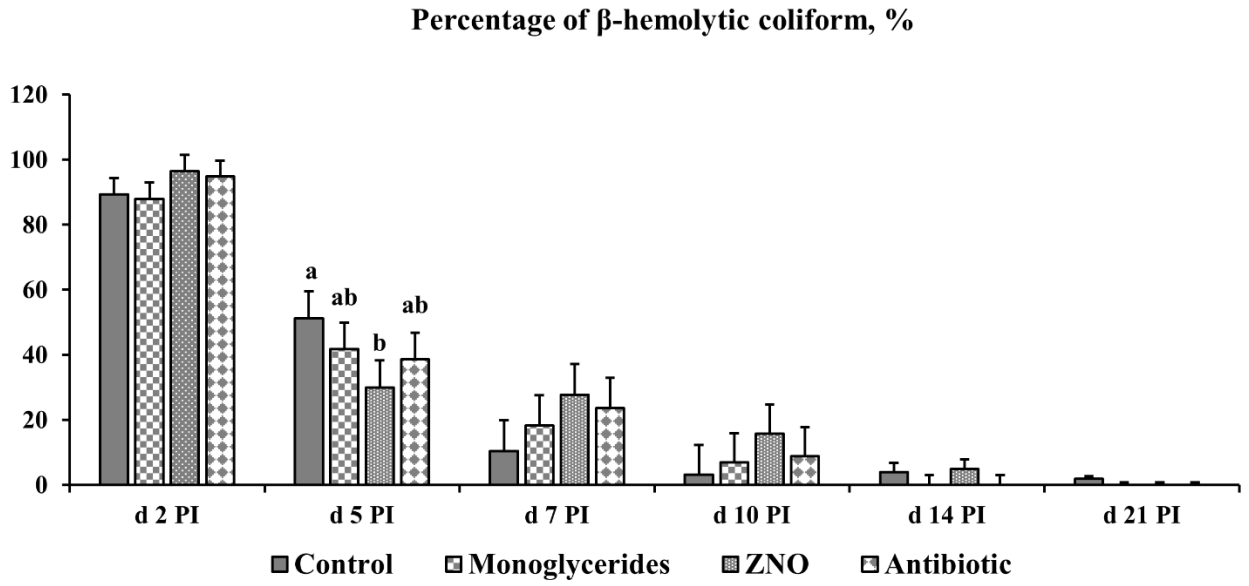

Figure 3.3. The percentage (%) of β-hemolytic coliforms in fecal samples of enterotoxigenic *Escherichia coli* F18-challenged pigs fed diets supplemented with monoglycerides, high-dose zinc oxide (ZNO), or antibiotic. No β-hemolytic coliforms were observed in the fecal samples of pigs before *Escherichia. coli* challenge. β-hemolytic coliforms were only observed in control pigs on d 21 post-inoculation (PI). Each least squares mean represents 14–15 observations on d 2 and 5 PI and each least squares mean represents 8–9 observations on d 7, 10, 14, and 21 PI. [a,b]Means without a common superscript are different ($P < 0.05$).

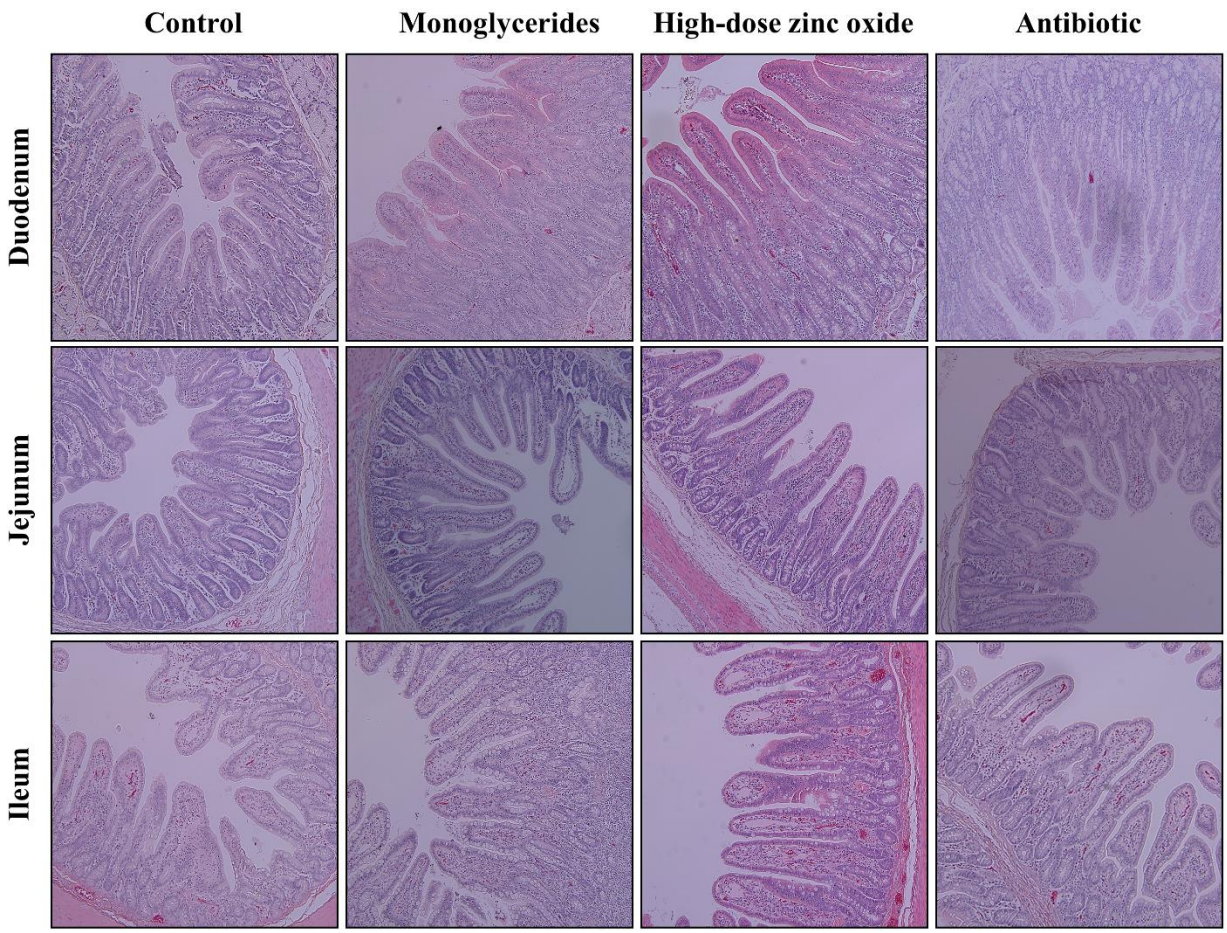

Figure 3.4. Intestinal morphology of enterotoxigenic *Escherichia coli* F18-challenged weaned pigs fed experimental diets on d 5 post-inoculation.

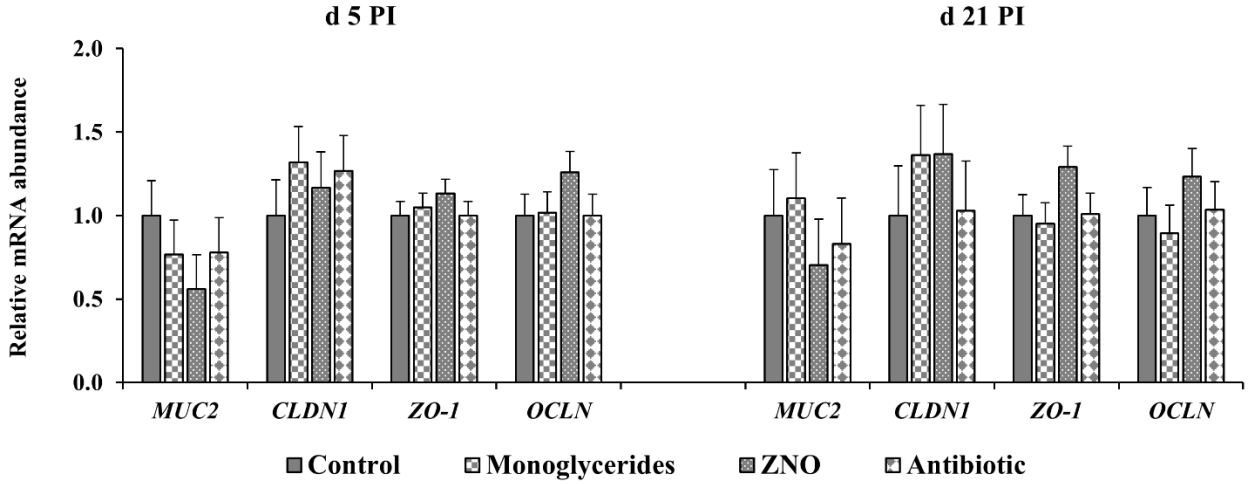

Figure 3.5. Relative mRNA abundance of genes in jejunal mucosa of enterotoxigenic *Escherichia coli* F18-challenged weaned pigs fed diets supplemented with monoglycerides, high-dose zinc oxide, or antibiotic. Each least squares mean represents 6–9 observations. PI = Post-inoculation; *MUC2* = Mucin 2; *CLDN1* = Claudin-1; *ZO-1* = Zonula occludens-1; *OCLN* = Occludin

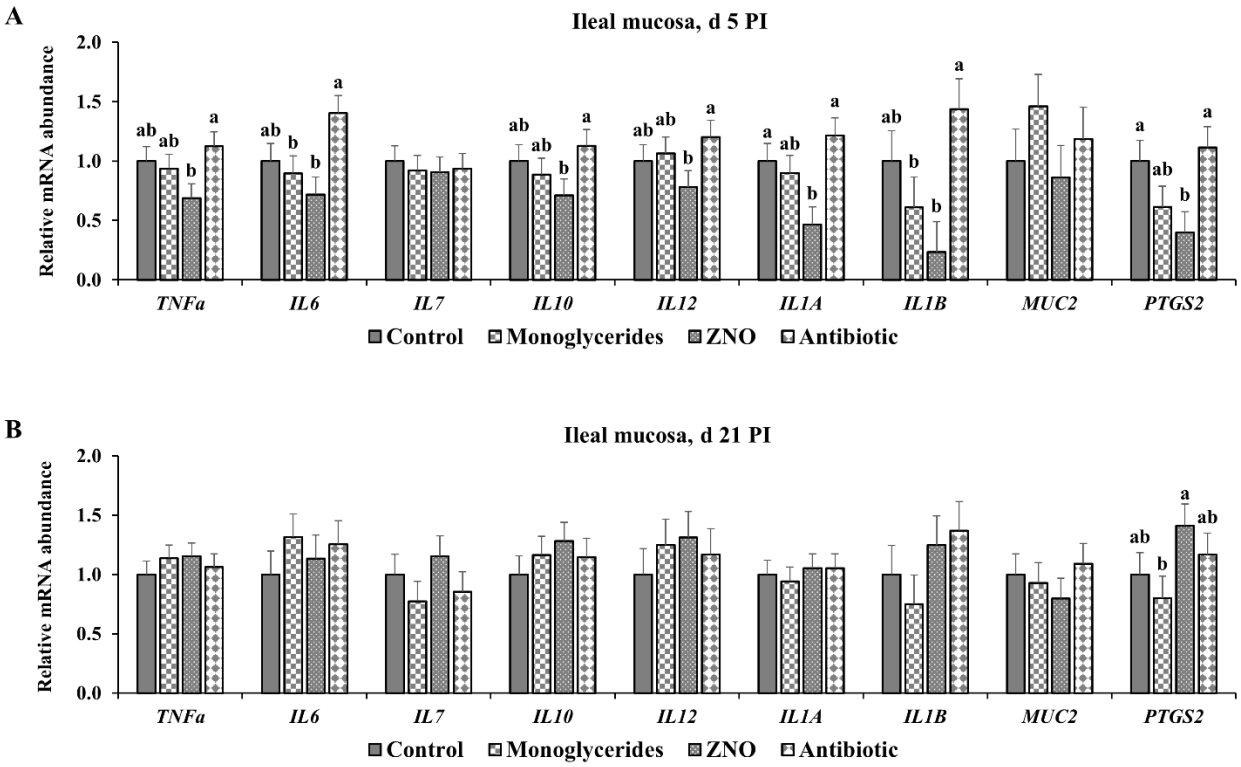

Figure 3.6. Relative mRNA abundance of genes in ileal mucosa of enterotoxigenic *Escherichia coli* F18-challenged pigs supplemented with monoglycerides, high-dose zinc oxide, or antibiotic on d 5 (**A**) and 21 PI (**B**). [a,b]Means without a common superscript are different ($P < 0.05$). Each least squares mean represents 6–9 observations. PI = Post-inoculation; *TNFa* = Tumor necrosis factor-alpha; *IL6* = Interleukin 6; *IL7* = Interleukin 7; *IL10* = Interleukin 10; *IL12* = Interleukin 12; *IL1A* = Interleukin-1 alpha, *IL1B* = Interleukin-1 beta; *MUC2* = Mucin 2, and *PTGS2* = Prostaglandin-endoperoxide synthase 2

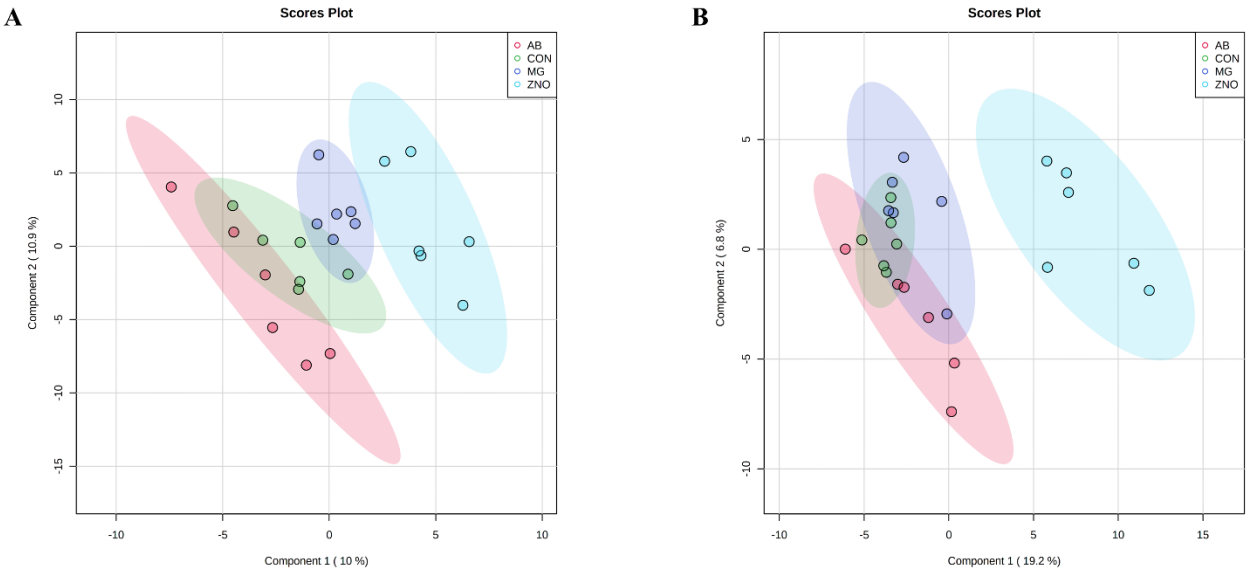

Figure 3.7. Partial Least Squares Discriminant Analysis (PLS-DA) 2D score plot of the metabolites in serum showed separated clusters between the CON and ZNO, MG and ZNO, MG and AB, and ZNO and AB groups on d 5 (**A**) and/or d 14 (**B**) post-inoculation, respectively. CON = Control; MG = Monoglycerides; ZNO = High-dose zinc oxide; AB = Antibiotic. Shaded areas in different colors represent in 95% confidence interval.

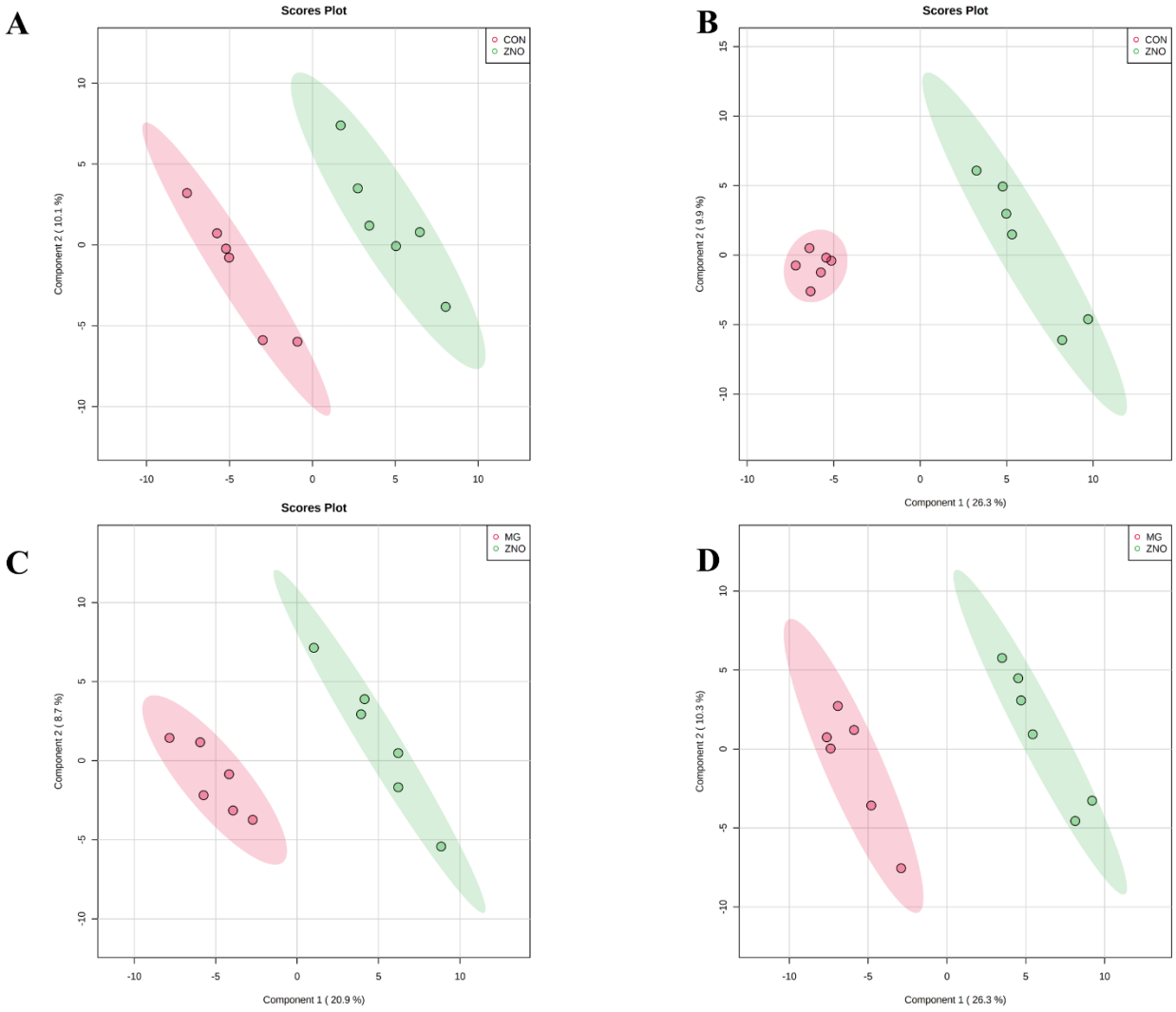

Figure 3.8. Partial Least Squares Discriminant Analysis (PLS-DA) 2D score plot of the metabolites in serum showed separated clusters between the CON and ZNO (**A** and **B**), MG and ZNO (**C** and **D**) on d 5 (**A** and **C**) and d 14 (**B** and **D**) post-inoculation, respectively. CON = Control; MG = Monoglycerides; ZNO = High-dose zinc oxide. Shaded areas in different colors represent in 95% confidence interval.

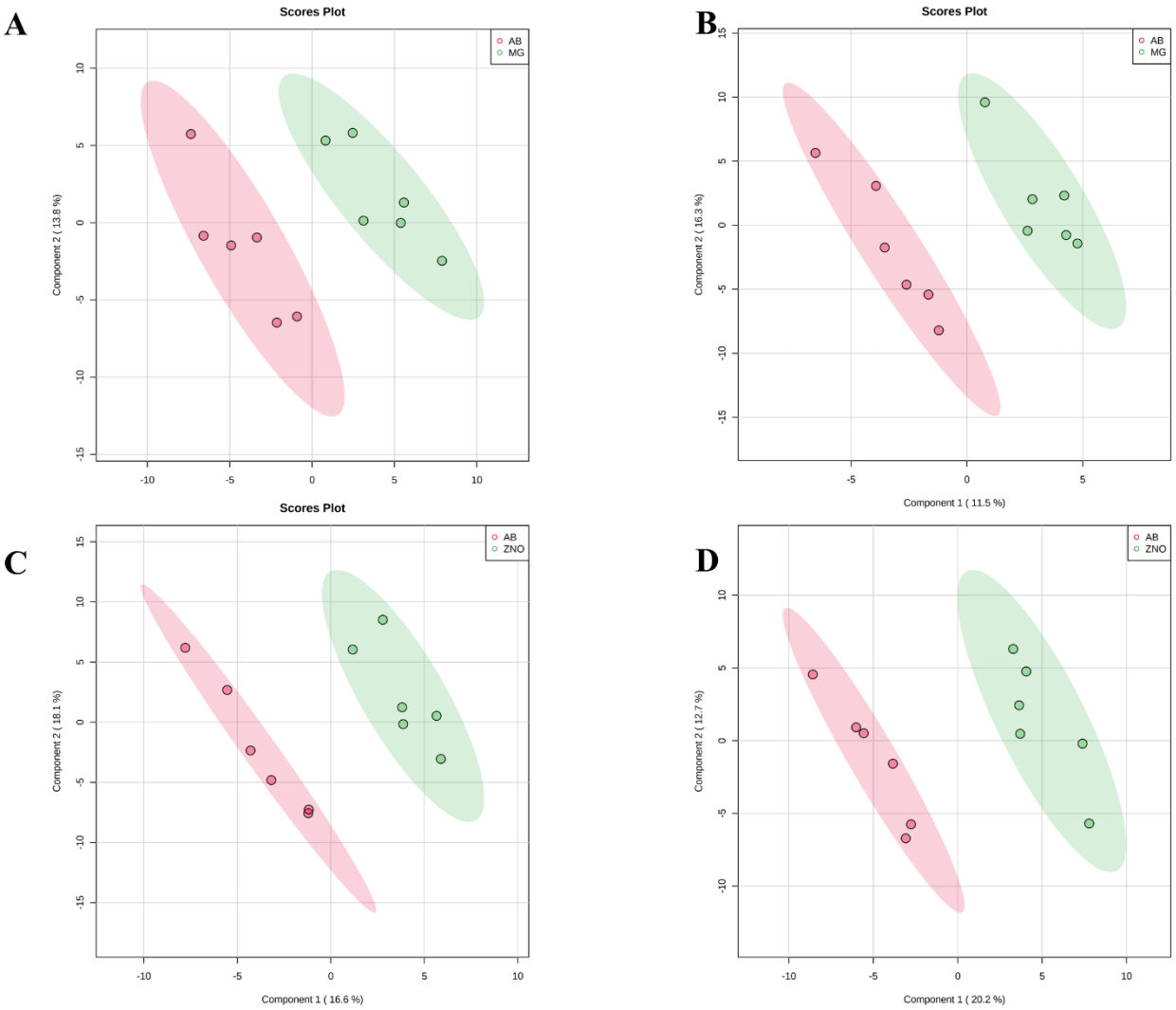

Figure 3.9. Partial Least Squares Discriminant Analysis (PLS-DA) 2D score plot of the metabolites in serum showed separated clusters between the MG and AB (**A** and **B**), ZNO and AB (**C** and **D**) on d 5 (**A** and **C**) and d 14 (**B** and **D**) post-inoculation, respectively. MG = Monoglycerides; ZNO = High-dose zinc oxide; AB = Antibiotic. Shaded areas in different colors represent in 95% confidence interval.

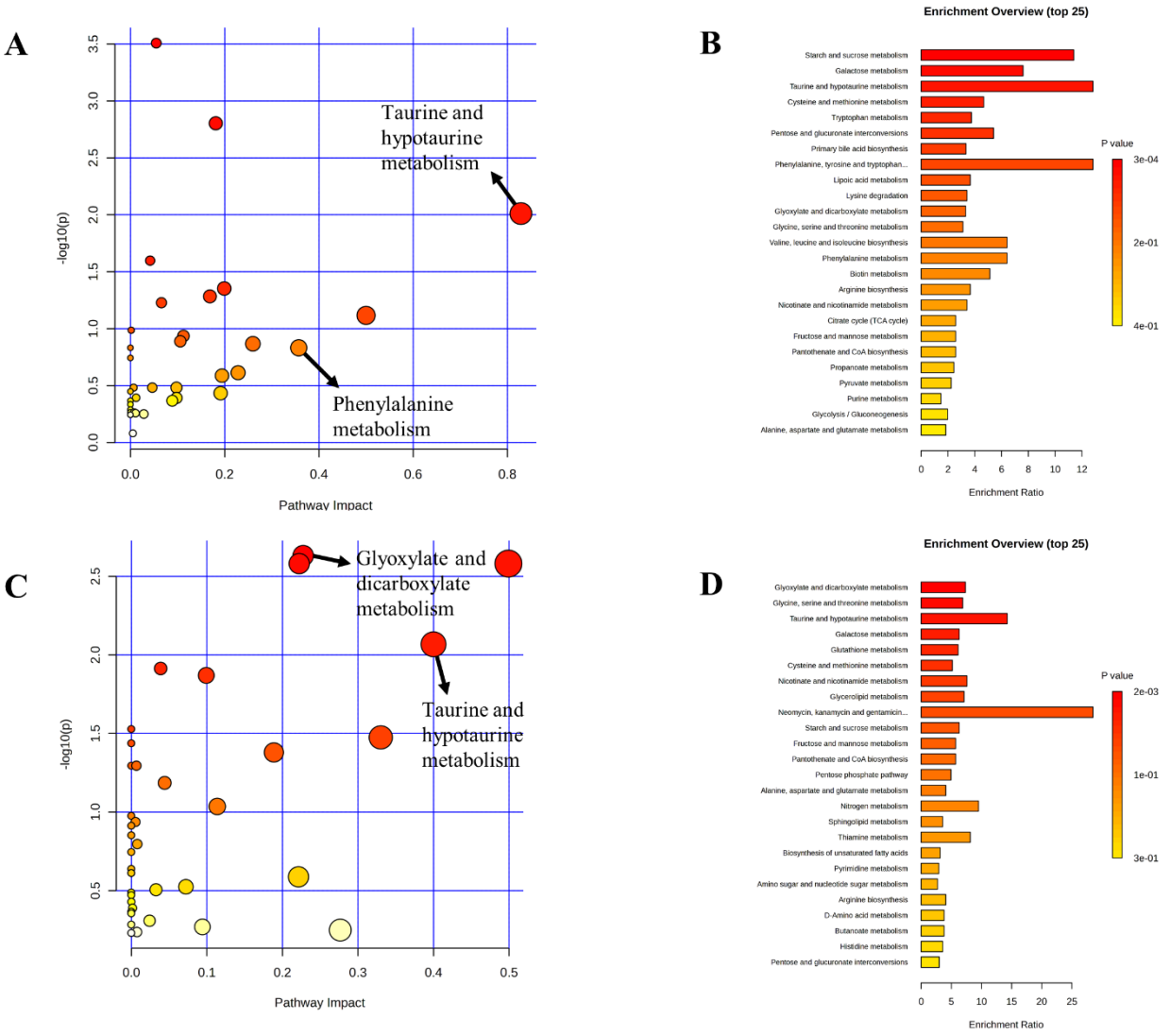

Figure 3.10. Significantly changed pathways in serum between the control and monoglycerides groups on d 5 (**A**) and d 14 (**C**) post-inoculation, respectively. The x-axis represents the pathway impact values and the y-axis represents the –log(P) values from the pathway enrichment analysis. Metabolite set enrichment analysis shows the metabolic pathways were enriched in control compared with monoglycerides on d 5 (**B**) and d 14 (**D**) post-inoculation, respectively. Both pathway analysis and metabolite set enrichment analysis were performed using identified metabolites with VIP > 1.

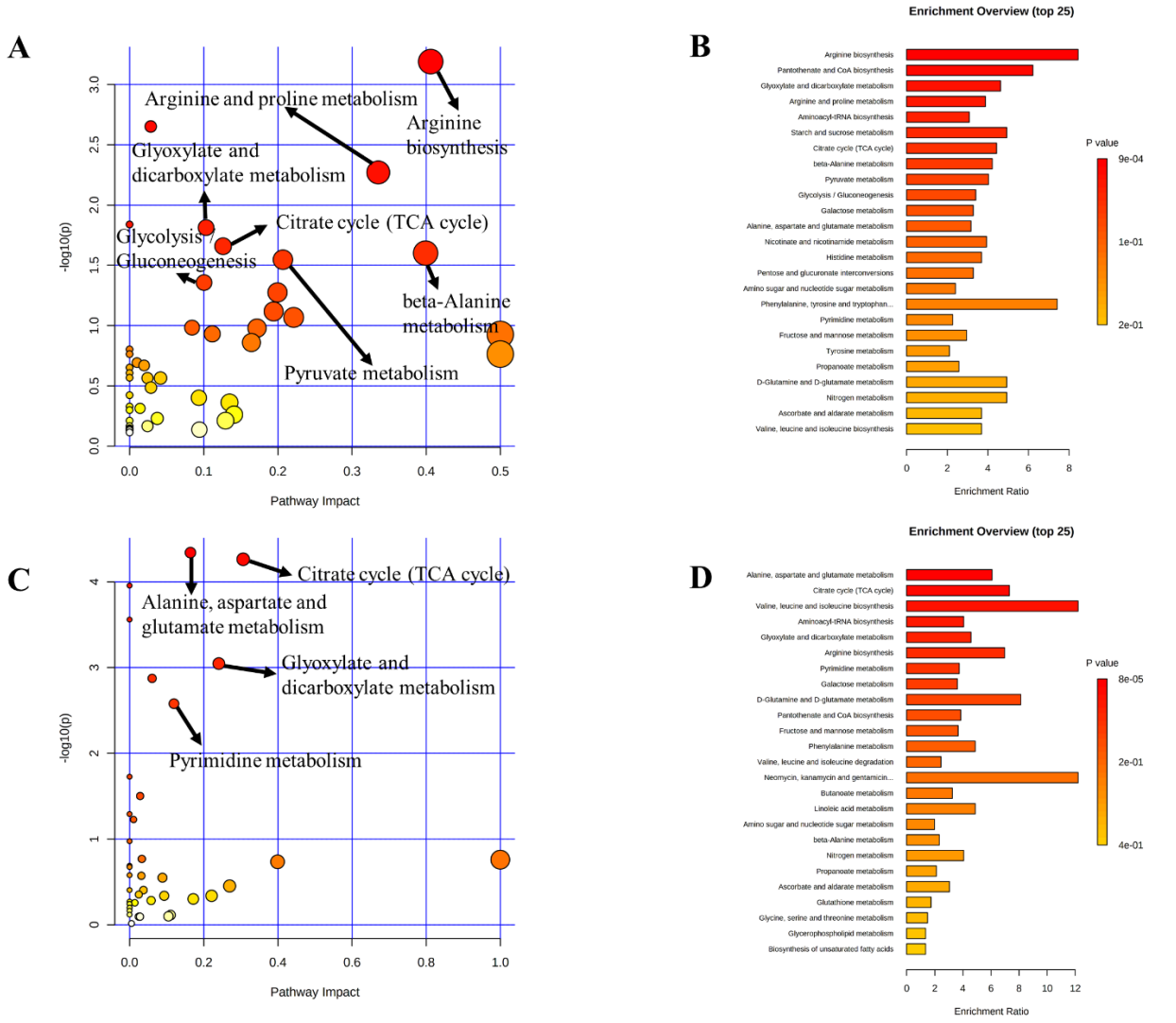

Figure 3.11. Significantly changed pathways in serum between the control and high-dose zinc oxide (ZNO) groups on d 5 (**A**) and d 14 (**C**) post-inoculation, respectively. The x-axis represents the pathway impact values and the y-axis represents the –log(P) values from the pathway enrichment analysis. Metabolite set enrichment analysis shows the metabolic pathways were enriched in control compared with ZNO on d 5 (**B**) and d 14 (**D**) post-inoculation, respectively. Both pathway analysis and metabolite set enrichment analysis were performed using identified metabolites with VIP > 1.

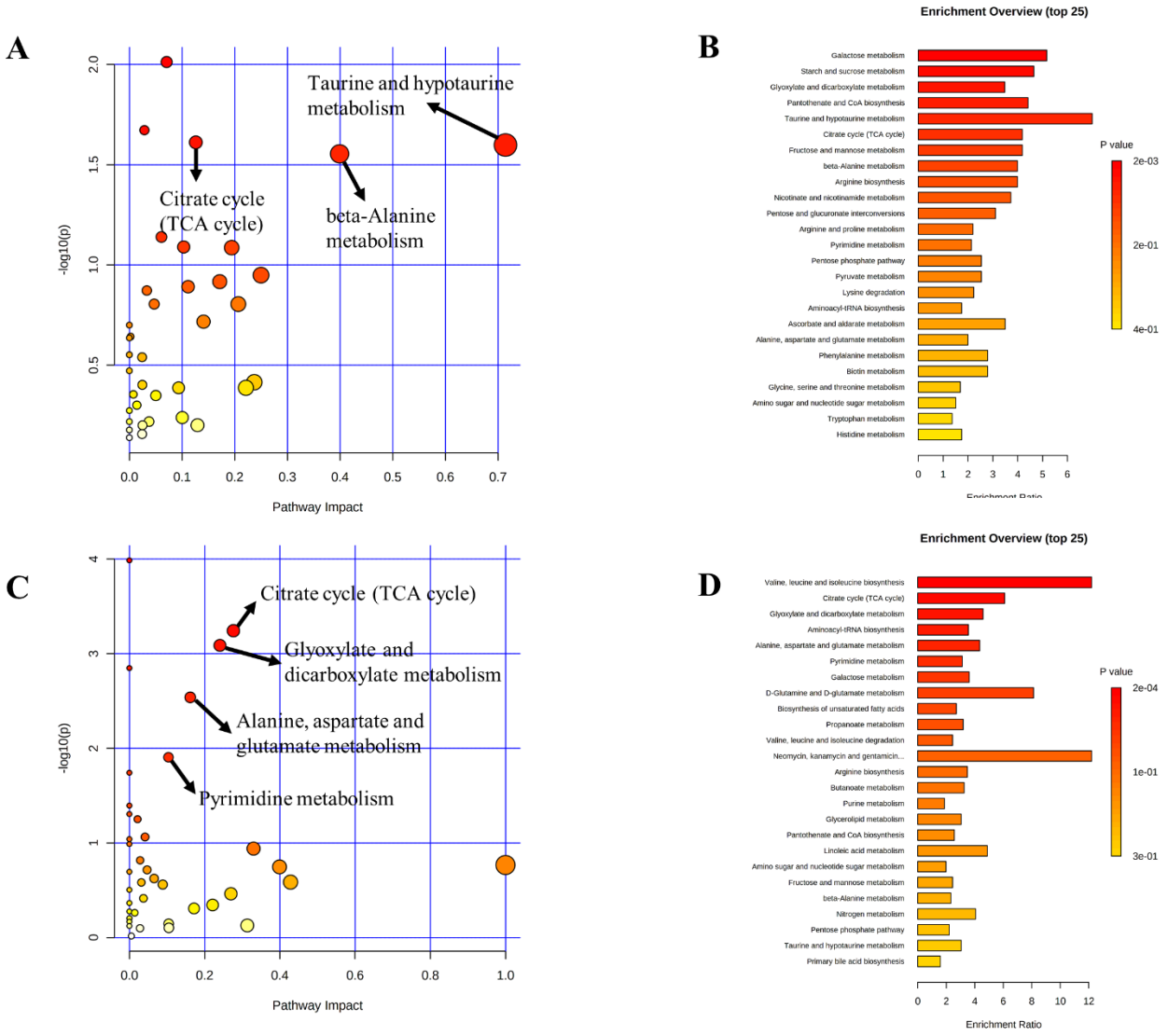

Figure 3.12. Significantly changed pathways in serum between the monoglycerides and high-dose zinc oxide (ZNO) groups on d 5 (**A**) and d 14 (**C**) post-inoculation, respectively. The x-axis represents the pathway impact values and the y-axis represents the –log(P) values from the pathway enrichment analysis. Metabolite set enrichment analysis shows the metabolic pathways were enriched in monoglycerides compared with ZNO on d 5 (**B**) and d 14 (**D**) post-inoculation, respectively. Both pathway analysis and metabolite set enrichment analysis were performed using identified metabolites with VIP > 1.

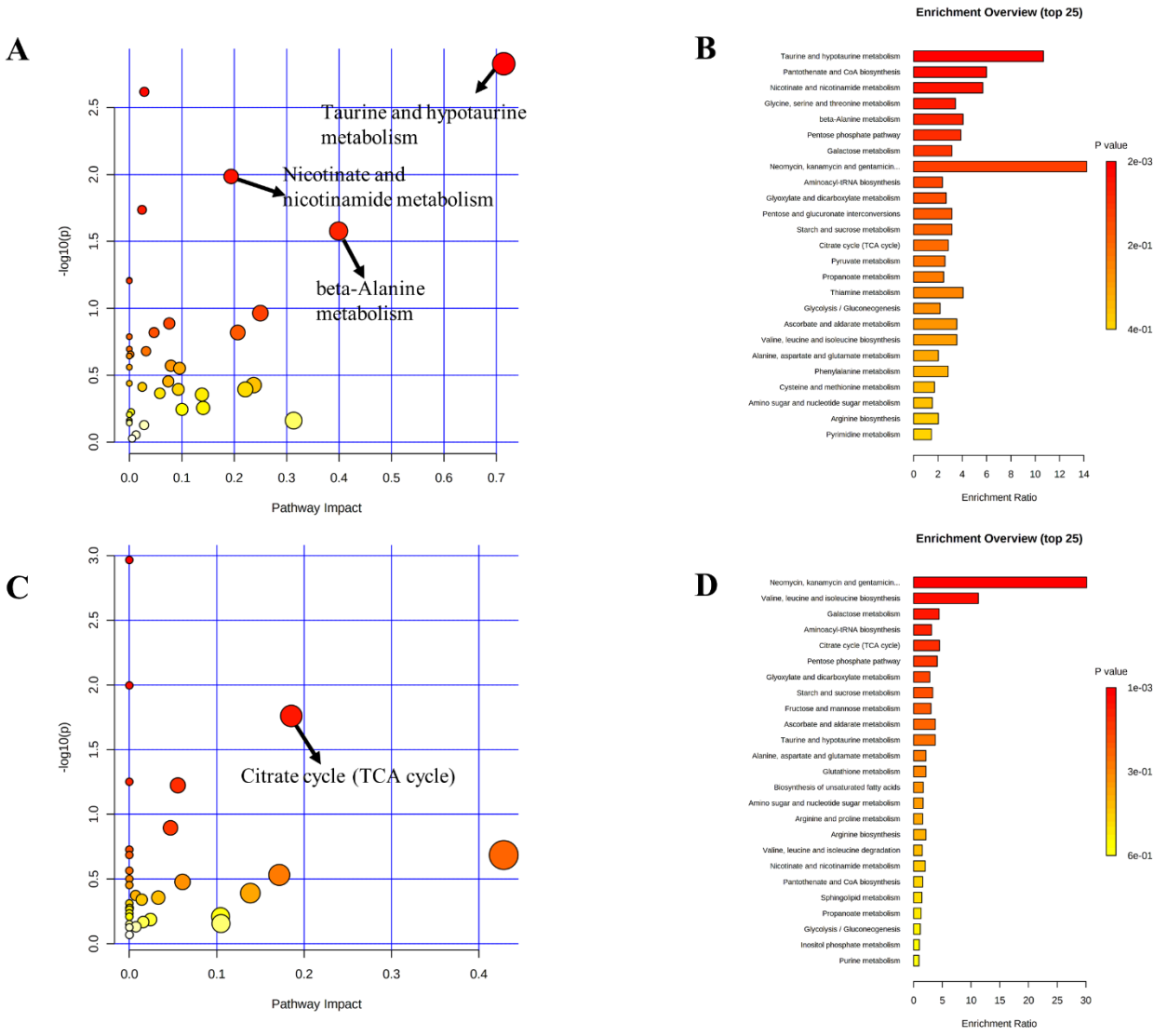

Figure 3.13. Significantly changed pathways in serum between the monoglycerides and antibiotic groups on d 5 (**A**) and d 14 (**C**) post-inoculation, respectively. The x-axis represents the pathway impact values and the y-axis represents the –log(P) values from the pathway enrichment analysis. Metabolite set enrichment analysis shows the metabolic pathways were enriched in monoglycerides compared with antibiotic on d 5 (**B**) and d 14 (**D**) post-inoculation, respectively. Both pathway analysis and metabolite set enrichment analysis were performed using identified metabolites with VIP > 1.

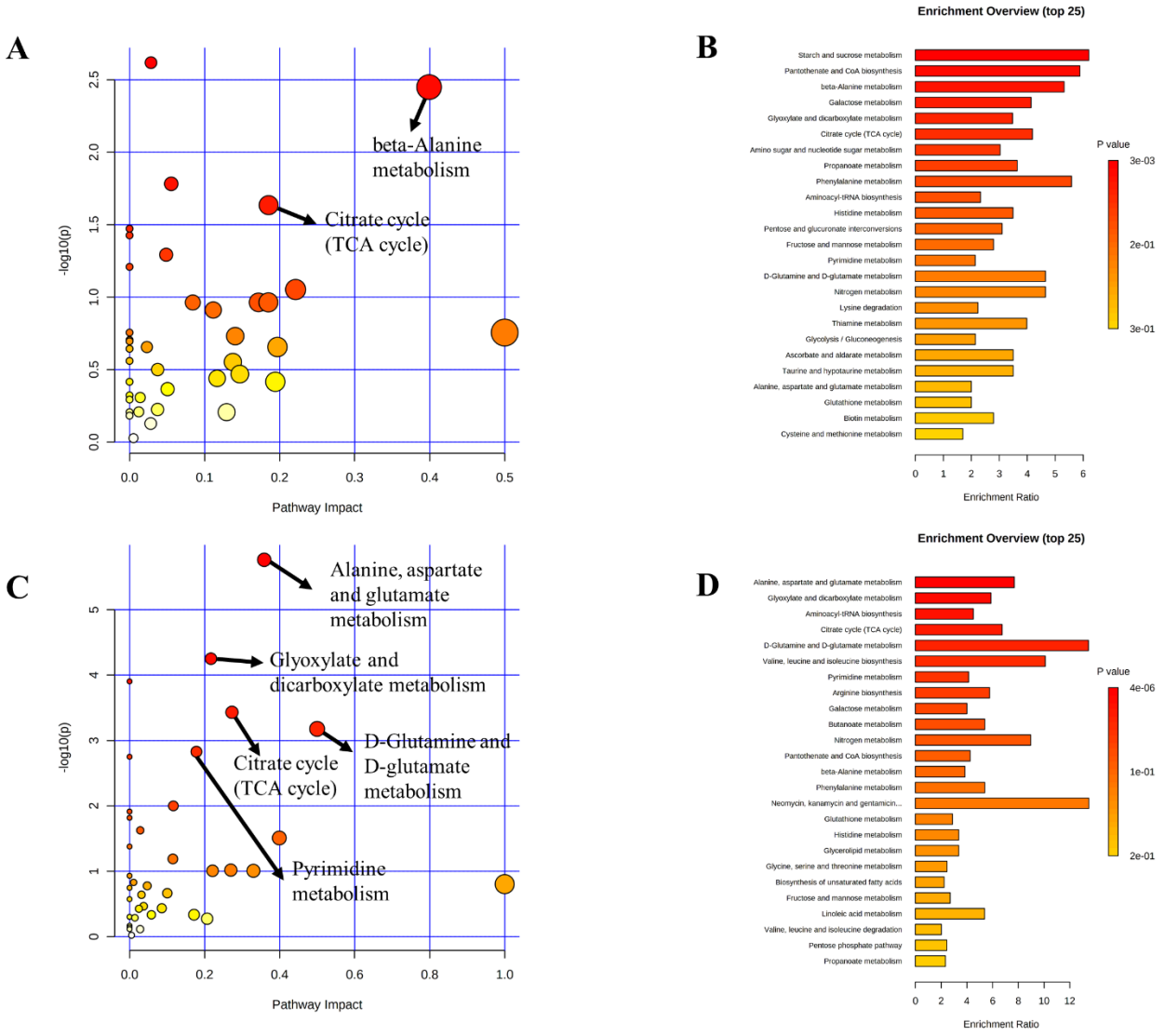

Figure 3.14. Significantly changed pathways in serum between the high-dose zinc oxide (ZNO) and antibiotic groups on d 5 (**A**) and d 14 (**C**) post-inoculation, respectively. The x-axis represents the pathway impact values and the y-axis represents the –log(P) values from the pathway enrichment analysis. Metabolite set enrichment analysis shows the metabolic pathways were enriched in ZNO compared with antibiotic on d 5 (**B**) and d 14 (**D**) post-inoculation, respectively. Both pathway analysis and metabolite set enrichment analysis were performed using identified metabolites with VIP > 1.

CHAPTER 4

DIETARY SUPPLEMENTATION OF BLEND OF ORGANIC ACIDS AND MONOGLYCERIDES ALLEVIATED DIARRHEA AND SYSTEMIC INFLAMMATION OF WEANED PIGS EXPERIMENTALLY INFECTED WITH ENTEROTOXIGENIC *ESCHERICHIA COLI* F18

ABSTRACT

The emergence of antibiotic resistant microorganisms associated with conventional swine production practices has increased interest in acid-based compounds having antimicrobial properties and other biological functions as nutritional interventions. Despite the interest in organic acids and monoglycerides, few studies have examined the effects of the combination of these acid-based additives in weaned pigs under disease challenge conditions. Therefore, this study aimed to investigate the effects of dietary supplementation with blends of organic acids or medium-chain fatty acid monoglycerides on intestinal health and systemic immunity of weaned pigs experimentally infected with an enterotoxigenic *Escherichia coli* (ETEC) F18. Dietary supplementation of organic acids, monoglycerides, or both organic acids and monoglycerides (combination) reduced ($P < 0.05$) frequency of diarrhea of ETEC F18-infected pigs throughout the experimental period. This is consistent with the reduced ($P < 0.05$) proportion of β-hemolytic coliforms in feces observed in organic acids and combination treatments on d 10 post-inoculation. Supplementation of organic acids, monoglycerides, or combination also reduced ($P < 0.05$) bacterial translocation in mesenteric lymph nodes on d 21 post-inoculation. ETEC infection increased ($P < 0.05$) total white blood cells, neutrophils, lymphocytes, monocytes, eosinophils,

and basophils. ETEC infection also decreased ($P < 0.05$) red cell distribution width and increased ($P < 0.05$) percentage of packed cell volume and levels of hemoglobin and total protein. Pigs fed with monoglycerides or combination had lower ($P < 0.05$) white blood cells on d 5 post-inoculation, and pigs in combination group also had lower ($P < 0.05$) lymphocytes than pigs in control group. Monoglyceride supplementation increased ($P < 0.05$) white blood cells and neutrophils compared with control group on d 14 post-inoculation. However, supplementation with organic acid blend, monoglyceride blend, or combination did not affect growth performance in this experiment. Supplementation with monoglycerides or organic acids alone or in combination ameliorates the detrimental effects of ETEC F18 infection in weaned pigs, as indicated by reduced diarrhea, fecal shedding, and bacterial translocation, and thus enhancing disease resistance. Monoglycerides reduce the inflammatory response during peak infection, but their immunomodulatory and possible synergistic effects with organic acids need to be further investigated.

Keywords: Acidifiers, Antimicrobial agents, Diarrhea, Enterotoxigenic *Escherichia coli*, Monoglycerides, Systemic immunity, Weaned pigs

INTRODUCTION

Weaning, a critical and inevitable stage in pig production, presents a variety of challenges, including rapid dietary transitions, immature physiological and immune systems, and environmental changes (Pluske, 2016; Park et al., 2020). The multifactorial stressor decreases appetite, induces intestinal dysfunction, and increases susceptibility to pathogens in weaned pigs, resulting not only in post-weaning diarrhea but also in inflammatory response (Xiong et al., 2019). Colonization of enterotoxigenic *Escherichia coli* (ETEC) in the small intestine, especially strains expressing the F18 fimbriae, is the most common cause of post-weaning diarrhea in pigs (Rhouma et al., 2017). The changes in the host resulting from interaction with ETEC F18 are not fully understood, but disturbances in fluid secretion and electrolyte imbalances caused by virulence factors have been reported (Fleckenstein et al., 2010; Kim et al., 2022b). ETEC F18 infection in weaned pigs leads to diarrhea, dehydration, stunted growth, and even significant mortality (Sun and Kim, 2017; Navez et al., 2023). Moreover, stress in the early life stage can exacerbate the severity and duration of this enteric disease and vice versa (McLamb et al., 2013).

Nutritional strategies to manage the adverse effects of weaning stress and post-weaning diarrhea have become even more important, particularly as usage of antibiotic growth promoters has been restricted and reduced due to increasing concerns over public health risks from antimicrobial resistance and environmental footprint (Liu et al., 2018; Pluske et al., 2018; Helm et al., 2019). Organic acids, organic compounds containing a carboxyl group, have been proposed as a viable alternative to antibiotic growth promoters due to their benefits on animal health and growth (Tugnoli et al., 2020). During the weaning period, pigs have immature digestive systems and produce insufficient hydrochloric acid, making supplementation with organic acids particularly significant (Ferronato and Prandini, 2020). For example, organic acid supplementation

can lower the stomach pH, thereby increasing pepsin activity and reducing pathogen load by creating a hostile environment for pathogens (Kim et al., 2005). The benefits of organic acid supplementation extend beyond these effects, encompassing a variety of bioactivities that ultimately enhance nutrient utilization and growth performance in pigs (Suiryanrayna and Ramana, 2015; Ferronato and Prandini, 2020). Although organic acids have been utilized in swine feed for a while, inconsistent responses in animals have prompted further research to determine the optimal application methods and efficacy across various factors (e.g., types and combinations of organic acids, animal conditions, etc.) (Liu et al., 2018; Nguyen et al., 2020; Tugnoli et al., 2020).

Monoglycerides, glycerol monoesters of organic fatty acids, have attracted interest as an alternative to corresponding fatty acids. Esterification of organic fatty acids with glycerol increases stability, reduces unpleasant odors, and allows for a gradual release of the active substance throughout the intestine, facilitating their use as additives (Jackman et al., 2020). Monoglycerides of medium-chain fatty acids (MCFA; C6–12) have demonstrated antibacterial activities against a broad spectrum of pathogens, including *Escherichia coli* (Hyldgaard et al., 2012; Churchward et al., 2018; Kovanda et al., 2019), and are classified as GRAS (generally recognized as safe) in the United States and widely utilized in the food industry (Moonen and Bas, 2014). MCFA monoglycerides have been reported to play various biological roles, such as immunomodulation (Wang et al., 2006; Zhang et al., 2016) and improvement of intestinal health (Amer et al., 2021; Kong et al., 2021), based on *in vitro* and *in vivo* experiments. In addition, synergistic antibacterial effects have been observed when MCFA or their esters were combined with organic acids (Thormar et al., 2006; Kim and Rhee, 2013). Despite the interest in organic acids and monoglycerides and their potential synergy, few studies have explored their use as blends to enhance disease resistance and resilience in pigs. Therefore, the objectives of the current study

were 1) to evaluate the influence of dietary supplementation of organic acids or monoglycerides on the growth performance, intestinal health, and immune responses of weaned pigs experimentally infected with ETEC F18; and 2) to investigate whether combination of these acid-based additives has synergistic effects on animal health in *in vivo* challenge model.

MATERIALS AND METHODS

Animals, housing, experimental design, and diet

The protocol for this study was reviewed and approved by the Institutional Animal Care and Use Committee at the University of California, Davis (IACUC #21875). A total of 40 piglets (initial body weight [BW] = 7.81 ± 0.84 kg) weaned around 21 d of age were obtained from the Swine Teaching and Research Center at the University of California, Davis. The sows and piglets used in this experiment did not receive *Escherichia coli* vaccines, antibiotic injections, or antibiotics in creep feed. Before weaning, fecal samples were collected from sows and all their piglets destined for this study to verify the absence of β-hemolytic *Escherichia coli*. The *Escherichia coli* F18 receptor status was also tested based on the methods of Kreuzer et al. (2013), and only piglets susceptible to *Escherichia coli* F18 were selected for this study. After weaning, all pigs were randomly assigned to one of the four dietary treatments (10 replicates/treatment) in a randomized complete block design with BW as the block and pig as the experimental unit. Pigs were housed in individual pens (0.61 m × 1.22 m) for 28 days, including 7 days before and 21 days after the first *Escherichia coli* challenge. All piglets had free access to feed and water. The light was on at 07:30 h and off at 19:30 h daily in the environmental control unit.

The four dietary treatments included: 1) a corn-soybean meal-based nursery basal diet (control); 2) the basal diet with 0.3% organic acid blend (Acitra G20C, Eastman, blend of formic acid, lactic acid, and sodium formate); 3) the basal diet with 0.3% monoglyceride blend (Entero-

Nova 410C, Eastman, blend of short- and medium-chain fatty acids); 4) the basal diet with Acitra G20C at 0.2% and Entero-Nova 410C at 0.2% (combination). A 2-phase feeding program was used, with the first two weeks as phase 1 and the last two weeks as phase 2 (Table 1). Spray-dried plasma, antibiotics, and high levels of zinc oxide exceeding recommendation and normal practice were not included in the basal diet. All diets were formulated to meet pig nutritional requirements (NRC, 2012) and provided as mash form throughout the experiment.

After 7 days of adaptation, all pigs were orally inoculated with 3 mL of ETEC F18 for three consecutive days from d 0 post-inoculation (PI). The ETEC F18 was originally isolated from a field disease outbreak by the University of Montreal (isolate number: ECL22131). The ETEC F18 expresses heat-labile toxin and heat-stable toxins a and b. The inoculums were prepared at 10^{10} colony-forming units (CFU) per 3 mL dose in phosphate-buffered saline. This dose caused mild diarrhea in the current study, consistent with our previously published research (Liu et al., 2013; Kim et al., 2019a; He et al., 2022).

Clinical observations and sample collections

The procedures of this experiment were adapted from previous research (Liu et al., 2013; Kim et al., 2019b; He et al., 2020; Wong et al., 2022). Clinical observations (fecal score and alertness score) were recorded twice daily throughout the study. The fecal score of each pig was assessed each day visually by two independent evaluators, 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 was calculated as the percentage of the pig days with fecal score of 3 or greater. 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 experiment (data not shown).

Pigs were weighed on weaning day (d -7; initial BW), d 0 (before first inoculation), 7, 14, and 21 PI. Feed intake was recorded throughout the study. Average daily gain (ADG), average daily feed intake (ADFI), and feed efficiency (gain:feed ratio) were calculated for each period. Fecal samples were collected from the rectum of all pigs on d -7, 0, 2, 5, 7, 10, 14, and 21 PI using a cotton swab to test β-hemolytic coliforms and the percentage of β-hemolytic coliforms to total coliforms (Liu et al., 2013; Kim et al., 2019b; He et al., 2020; Wong et al., 2022). Blood samples were collected from the jugular vein of all pigs with or without ethylenediaminetetraacetic acid to yield whole blood and serum, respectively, before ETEC challenge (d 0), and on d 2, 5, or 14 PI. Serum samples were collected and immediately stored at −80 °C before further analysis.

All pigs were euthanized at the end of the experiment. Before euthanization, pigs were anesthetized with 1 mL mixture of 100 mg telazol, 50 mg ketamine, and 50 mg xylazine (2:1:1) by intramuscular injection. After anesthesia, intracardiac injection with 78 mg Fatal-Plus solution (sodium pentobarbital, MWI Animal Health, Visalia, CA, USA) per 1 kg of BW was used to euthanize each pig. Mesenteric lymph nodes were aseptically collected and then pooled within the pig, grounded, diluted, and plated on brain heart infusion agar for measurement of total bacteria, and the results were expressed as CFU per g of mesenteric lymph nodes (Almeida et al., 2013; Garas et al., 2016). Spleen samples were analyzed in the same manner as mesenteric lymph nodes for bacterial translocation.

Detection of β-hemolytic coliforms

Briefly, fecal samples were plated on Columbia blood agar with 5% sheep blood (Biological Media Service, School of Veterinary Medicine, University of California, Davis) to identify hemolytic coliforms, which can lyse red blood cells (RBC) surrounding the colony. Fecal samples were also plated on MacConkey agar (Biological Media Service, School of Veterinary

Medicine, University of California, Davis) to enumerate total coliforms. Hemolytic colonies from the blood agar were sub-cultured on MacConkey agar to confirm that they were lactose-fermenting bacteria and flat pink colonies. All plates were incubated at 37 ℃ for 24 h in an air incubator. Populations of both total coliforms and β-hemolytic coliforms on blood agar were visually scored from 0 to 8 (0 = no bacterial growth, 8 = very heavy bacterial growth). The ratio of scores of β-hemolytic coliforms to total coliforms was calculated.

Measurement of immune response biomarkers

Whole blood samples collected on d 0, 5, and 14 PI were used for measuring total and differential blood cell counts by Comparative Pathology Laboratory at the University of California, Davis. A multiparameter, automated programmed hematology analyzer (Drew/ERBA Scientific 950 FS Hematological Analyzer, Drew Scientific Inc., Miami, FL, USA) was used for the assay to differentiate porcine blood optimally. Serum samples collected from d 0, 2, 5, and 14 PI were analyzed for C-reactive protein (CRP; R&D Systems Inc., Minneapolis, MN, USA) and haptoglobin (Aviva Systems Biology, San Diego, CA, USA) using porcine-specific enzyme-linked immunosorbent assay kits following the manufacturer's procedures. All samples, including standards, were analyzed in duplicate. The intensity of the color was measured at 450 nm with the correction wavelength set at 530 nm using a plate reader (BioTek Instruments, Inc., Winooski, VT, USA). The concentrations of each analyte in the tested samples were calculated based on a standard curve. The intra-assay coefficients of variation for CRP and haptoglobin were 3.8% and 8.5% respectively. The inter-assay coefficients of variation for CRP and haptoglobin were 5.6% and 8.2%, respectively. Serum samples collected from d 0, 5, and 14 PI were also analyzed for inflammatory cytokines (granulocyte-macrophage colony-stimulating factor, interleukin [IL]-1

alpha, IL-6, IL-8, and tumor necrosis factor-alpha) using a Porcine Immunology Multiplex Discovery Assay (PD13; Eve Technologies Corp, Calgary, AB, Canada).

Statistical analysis

The normality of data was verified and outliers were identified using the UNIVARIATE procedure (SAS Institute Inc., Cary, NC, USA). Outliers were identified and removed as values that deviated from the treatment mean by more than 3 times the interquartile range. All data except frequency of diarrhea were analyzed by ANOVA using the PROC MIXED of SAS (SAS Institute Inc., Cary, NC, USA) in a randomized complete block design with the pig as the experimental unit. The statistical model included diet as the main effect and block as a random effect. Treatment means were separated by using the LSMEANS statement and the PDIFF option of PROC MIXED. 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, diarrhea incidence, β-hemolytic coliforms

There was no significant difference in the initial BW of pigs among dietary treatments (Table 2). In comparison to control, supplementation of organic acids, monoglycerides, or combination did not affect BW, ADG, ADFI, and gain:feed ratio of pigs throughout the experiment. Supplementation with organic acids, monoglycerides, or combination of both did not affect average daily fecal scores throughout the experiment (Figure 4.1). However, the frequency of diarrhea (fecal score ≥ 3) was lower ($P < 0.05$) in organic acids (29.77%), monoglycerides (25.49%), and combination groups (28.57%) than control group (39.29%) during the overall experimental period (Figure 4.2).

No β-hemolytic coliforms were identified in fecal samples of pigs before ETEC F18 inoculation (Figure 4.3), but the β-hemolytic coliforms were detected in feces from all pigs on d 2 PI. No significant differences were observed in the percentage of fecal β-hemolytic coliforms at each time point, except that organic acid blend and combination reduced ($P < 0.05$) the percentage of fecal β-hemolytic coliforms on d 10 PI compared to control. However, comparable percentages of β-hemolytic coliforms in feces were observed in the three supplemented groups on d 10 PI.

Bacterial translocation

Pigs supplemented with organic acids, monoglycerides, or combination had lower ($P < 0.05$) counts of total coliforms (CFU/g) in mesenteric lymph nodes on d 21 PI compared with control group (Figure 4.4). The total coliforms (CFU/g) in mesenteric lymph nodes did not differ between the three supplemented groups. There was no significant difference in total coliforms (CFU/g) in spleen among the dietary treatments.

Systemic immunity and red blood cell profile

ETEC F18 inoculation increased ($P < 0.05$) the total white blood cell (WBC) number, and the counts of neutrophils, lymphocytes, monocytes, eosinophils, and basophils as the pig age increased (Table 3). The proportion of eosinophils and basophils was increased ($P < 0.05$) on d 14 PI compared with d 0 or 5 PI. The ETEC F18 infection also affected the RBC profile by decreasing ($P < 0.05$) red cell distribution width on d 5 and 14 PI, but increasing ($P < 0.05$) the percentage of packed cell volume and levels of hemoglobin and total protein on d 14 PI.

No difference was observed in the WBC profile among dietary treatments on d 0 before ETEC F18 inoculation (Table 4). Supplementation of organic acids did not affect the WBC profiles on d 5 and 14 PI. Pigs fed with monoglycerides or combination had lower ($P < 0.05$) WBC counts on d 5 PI, compared with control. Supplementation of combination also reduced ($P < 0.05$) number

of lymphocytes and neutrophil-to-lymphocyte ratio (NLR) on d 5 PI compared with control. However, pigs supplemented with monoglycerides had greater ($P < 0.05$) numbers of WBC and neutrophils and higher ($P < 0.05$) NLR on d 14 PI compared to control.

The effects of dietary supplementation of organic acids, monoglycerides, or combination on the RBC profiles of weaned pigs were limited on d 0, with the exception that pigs in organic acid blend group had increased ($P < 0.05$) mean platelet volume (Table 5), which continued into the subsequent period. Pigs in the combination group had the greatest ($P < 0.05$) RBC on d 5 PI. On d 14 PI, pigs in the combination group had greatest ($P < 0.05$) RBC and hemoglobin counts, but the lowest ($P < 0.05$) platelet counts among dietary treatments.

Pigs in the monoglycerides group tended ($P < 0.10$) to have the lowest IL-1 alpha level on d 0 and 14 PI, followed by pigs in the combination group (Table 6). However, dietary treatments did not affect IL-6 and IL-8 levels. Supplementation of monoglycerides also tended ($P < 0.10$) to reduce serum granulocyte-macrophage colony-stimulating factor and tumor necrosis factor-alpha on d 0 and 5 PI. No difference was observed in C-reactive protein and haptoglobin levels among dietary treatments throughout the experiment.

DISCUSSION

In the swine industry, post-weaning diarrhea caused by ETEC F18 is a significant disease in terms of the negative impact on productivity, profitability, and sustainability, particularly due to issues related to antibiotic resistance and environmental impacts (Bonetti et al., 2021; Duarte et al., 2023). Despite growing interest in the bioactivity and effects of nutritional interventions such as organic acids and monoglycerides, there has been limited research on disease resistance and resilience in weaned pigs under disease challenge conditions. The present study aims to contribute to our understanding of practical nutritional intervention by investigating the impacts of various

mixtures of acid-based feed additives on performance, diarrhea, fecal culture, bacterial translocation, and systemic inflammation of weaned pigs challenged with ETEC F18.

ETEC with the F18 fimbrial adhesin, the primary cause of post-weaning diarrhea, adheres to and proliferates in the small intestine, producing enterotoxins that disrupt fluid homeostasis (Almeida et al., 2022; Kim et al., 2022b; Almeida et al., 2023). Diarrhea, bacterial fecal shedding, and associated signs are commonly used to assess challenge efficacy (Luise et al., 2019). Following ETEC F18 inoculation, increased diarrhea and β-hemolytic coliform fecal shedding were observed, indicating successful infection in our challenge model. These observations are consistent with our previous studies using the same ETEC strain, which also showed peak infection on d 3 to 5 PI followed by gradual recovery (Liu et al., 2013; Kim et al., 2022a; Wong et al., 2022). The current study demonstrates that supplementation with organic acid blend, monoglyceride blend, or their combination effectively reduced the frequency of diarrhea in ETEC F18-infected pigs. This is supported by changes in the percentage of β-hemolytic coliform in feces. While there was no difference in β-hemolytic coliform fecal shedding among the treatment groups during the early infection stage, pigs in the organic acids and combination groups exhibited the lowest fecal shedding on d 10 PI, followed by pigs in the monoglycerides group. These findings are consistent with previous research suggesting that the reduction in diarrhea of pigs may be due to the inhibition of ETEC proliferation by supplementing organic acids (Tsiloyiannis et al., 2001), monoglycerides (Kovanda et al., 2023), or combination (Ren et al., 2020).

Organic acids have unique pKa values and exhibit different degrees of dissociation depending on the pH of their environment. The undissociated form can penetrate bacterial cell membranes, lowering intracellular pH and disrupting bacterial homeostasis (Tugnoli et al., 2020). Combinations of acids with different pKa values can exert broad effects throughout the

gastrointestinal tract (Ravindran and Kornegay, 1993; Nguyen et al., 2020). Monoglycerides, on the other hand, act pH-independently (Wang and Johnson, 1992; Yoon et al., 2018) and primarily disrupt bacterial phospholipid membranes, altering membrane permeability and exerting antibacterial effects (Yoon et al., 2018; Jackman et al., 2022). Synergistic antibacterial effect of the combination of acid and monoglycerides has been reported in *in vitro* microbiology studies. In the current *in vivo* challenge study, both organic acid and monoglycerides reduced frequency of diarrhea and fecal β-hemolytic coliforms, but no additional reduction was observed in the combination group. The results of diarrhea frequency and fecal shedding suggest that continuous supplementation with mixtures of acid-based additives has the potential to enhance intestinal barrier function and resilience to pathogenic challenges over time.

To investigate the effects of organic acids, monoglycerides, and their combination on intestinal barrier function and immune responses in ETEC-infected pigs, bacterial translocation and systemic immunity were examined. Regardless of the harmfulness of bacteria in the gut, bacterial invasion into the other organs due to bacterial overgrowth, intestinal barrier dysfunction, or compromised immune system can lead to systemic inflammation and damage (Nagpal and Yadav, 2017). This invasion is often observed in disease conditions and can serve as an indicator of bacterial infection (Berg, 1995; Nagpal and Yadav, 2017). Different bacteria may vary in their efficiency of translocating from the gastrointestinal tract to organs, such as mesenteric lymph nodes, spleen, and liver. Mesenteric lymph nodes are particularly efficient site for translocating *Enterobacteriaceae* family (Gram-negative, facultatively anaerobic; e.g., *Escherichia coli*) (Berg, 1995; Yadav and Kumar, 2022). The results of bacterial translocation indicate that organic acid blend, monoglyceride blend, and their combination improved the integrity of the intestinal barrier during ETEC infection, potentially reducing systemic spread of pathogens.

The activation of innate immunity by lipopolysaccharides (LPS) in the outer membrane of ETEC leads to the release of inflammatory cytokines, immune cell recruitment, and disruption of intestinal integrity, resulting in systemic inflammation (Kim et al., 2022b; Duarte et al., 2023). The increase in WBC counts observed in this study aligns with previous research (Song et al., 2012; Liu et al., 2013; Kovanda et al., 2023), indicating that innate immunity plays a significant role in both the early stages as well as throughout the progression of ETEC infection, reflecting a systemic inflammatory response. Supplementation with monoglycerides or the combination reduced WBC counts on d 5 PI, suggesting modulation of the inflammatory response. Moreover, although the blend of organic acid or monoglyceride groups showed intermediate levels, the combination group exhibited lower lymphocytes and NLR than the control group, indicating enhanced immune-modulatory effects in ETEC F18-challenged pigs near the peak of infection. These findings are in close agreement with our previous research involving other types of acid-based products (i.e., monobutyrin or monovalerin) (Kovanda et al., 2023), suggesting that supplementation with the combination may synergistically reduce systemic inflammation induced by ETEC infection. Our observations of reduced inflammatory cytokines further support the immunomodulatory effects of monoglycerides.

Interestingly, monoglycerides supplementation further increased WBC, neutrophils, and NLR on d 14 PI, potentially linked to the activation of G protein-coupled receptors (GPCR) by monoglyceride-derived MCFA. It has been reported that MCFA act as ligands for various GPCR present in the plasma membrane of various cells, influencing cellular processes (Ikeda et al., 2022). Specifically, GPR84, predominantly expressed on immune cells, enhances the inflammatory response upon activation (Recio et al., 2018; Huang et al., 2021; Yang et al., 2024) and has been reported to be upregulated by LPS (Huang et al., 2021; Yang et al., 2024). Therefore, the relatively

high level of neutrophils in the monoglycerides group on d 14 PI may be associated with sustained innate immune response (Marwick et al., 2018; Loh and Vermeren, 2022; Silva et al., 2023), contributing to pathogen clearance.

Despite the aforementioned symptomatic and physiological improvements, there were no differences across treatments in the growth performance of newly weaned pigs challenged with ETEC F18 in this study. These findings are also consistent with previous studies where the addition of acid-based additives improved intestinal and immune status but did not significantly impact growth performance (Zentek et al., 2013; Amer et al., 2021). It is important to note that enhancing overall disease resistance in pigs, particularly during the post-weaning period, may reduce the risk of other enteric diseases and the necessity for antibiotic use.

In summary, the present study thoroughly investigated the effects of several mixtures of acid-based feed additives on the performance, diarrhea, fecal shedding, bacterial translocation, and immunity of newly weaned pigs challenged with ETEC F18. Supplementation with organic acid blend (formic acid, lactic acid, and sodium formate), a monoglyceride blend of SCFA and MCFA, or a combination of both acid-based additives benefited the control of post-weaning diarrhea and may alleviate intestinal damage caused by ETEC infection as indicated by reduced fecal shedding and bacterial translocation. The monoglyceride blend of SCFA and MCFA exhibited immunomodulatory effects on the host and shows promise for synergistic effects with organic acids, warranting further investigation.

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TABLES AND FIGURES

Table 4.1. Ingredient compositions of experimental diets[a]

Ingredient, %	Control, phase 1	Control, phase 2
Corn	44.70	54.29
Dried whey	15.00	10.00
Soybean meal	21.50	30.50
Fish meal	3.00	-
Lactose	6.00	-
Soy protein concentrate	5.00	-
Soybean oil	2.00	2.00
Limestone	0.98	1.00
Dicalcium phosphate	0.55	0.90
L-Lysine·HCl	0.34	0.39
DL-Methionine	0.14	0.12
L-Threonine	0.09	0.10
Salt	0.40	0.40
Vit-mineral, Sow 6[b]	0.30	0.30
Total	100.00	100.00
Calculated energy and nutrient		
Metabolizable energy, kcal/kg	3418	3375
Net energy, kcal/kg	2564	2508
Crude protein, %	20.84	20.19
Arg,[c] %	1.20	1.19
His,[c] %	0.48	0.48
Ile,[c] %	0.80	0.77
Leu,[c] %	1.57	1.53
Lys,[c] %	1.35	1.29
Met,[c] %	0.44	0.40
Thr,[c] %	0.79	0.76
Trp,[c] %	0.23	0.23
Val,[c] %	0.86	0.82
Met + Cys,[c] %	0.74	0.71
Phe + Tyr,[c] %	1.44	1.42
Ca, %	0.80	0.75
Total P, %	0.62	0.61
Digestible P, %	0.40	0.37
Analyzed nutrients, %		
Dry matter	92.00	90.40
Crude protein	23.00	22.70
Acid detergent fiber	2.80	3.60
Neutral detergent fiber	6.40	8.40

Total Ca	1.39	1.48
Total P	0.64	0.65

[a]In each phase, three additional diets were formulated by adding 0.3% organic acids, 0.3% monoglycerides, or blend of 0.2% organic acids and 0.2% monoglycerides to the control diet, respectively

[b]Provided the following quantities of vitamins and micro minerals per kilogram of complete diet: Vitamin A as retinyl acetate, 11136 IU; vitamin D_3 as cholecalciferol, 2208 IU; vitamin E as *DL*-alpha tocopheryl acetate, 66 IU; vitamin K as menadione dimethylprimidinol bisulfite, 1.42 mg; thiamin as thiamine mononitrate, 0.24 mg; riboflavin, 6.59 mg; pyridoxine as pyridoxine hydrochloride, 0.24 mg; vitamin B_{12}, 0.03 mg; *D*-pantothenic acid as *D*-calcium pantothenate, 23.5 mg; niacin, 44.1 mg; folic acid, 1.59 mg; biotin, 0.44 mg; Cu, 20 mg as copper sulfate and copper chloride; Fe, 126 mg as ferrous sulfate; I, 1.26 mg as ethylenediamine dihydriodide; Mn, 60.2 mg as manganese sulfate; Se, 0.3 mg as sodium selenite and selenium yeast; and Zn, 125.1 mg as zinc sulfate

[c]Amino acids were indicated as standardized ileal digestible amino acids

Table 4.2. Growth performance of enterotoxigenic *Escherichia coli* F18-challenged weaned pigs fed experimental diets

Item[a]	Control	OA[b]	MG[c]	OA+MG[d]	SEM	*P*-value
BW, kg						
d -7	7.72	7.77	7.77	7.76	0.43	0.96
d 0	9.09	8.90	9.10	8.96	0.26	0.87
d 7 PI	11.61	10.96	11.53	11.59	0.40	0.55
d 14 PI	14.83	14.23	15.07	15.02	0.60	0.73
d 21 PI	19.38	18.70	19.18	19.89	0.73	0.69
ADG, g						
d -7 to 0	196	154	196	167	49.34	0.48
d 0 to 7 PI	367	306	360	381	60.31	0.44
d 7 to 14 PI	459	470	509	491	47.04	0.80
d 14 to 21 PI	650	640	583	696	31.24	0.098
d 0 to 21 PI	554	554	546	593	33.54	0.71
d -7 to 21 PI	417	390	410	432	30.51	0.68
ADFI, g						
d -7 to 0	326	302	333	315	34.36	0.80
d 0 to 7 PI	580	553	545	569	70.91	0.95
d 7 to 14 PI	793	784	839	797	55.43	0.89
d 14 to 21 PI	1059	1117	1131	1177	69.24	0.62
d 0 to 21 PI	925	949	986	986	60.24	0.83
d -7 to 21 PI	690	691	712	715	52.03	0.95
Gain:Feed						
d -7 to 0	0.59	0.50	0.58	0.48	0.101	0.31
d 0 to 7 PI	0.61	0.56	0.66	0.66	0.050	0.29
d 7 to 14 PI	0.55	0.60	0.61	0.62	0.034	0.46
d 14 to 21 PI	0.62	0.58	0.52	0.59	0.035	0.08
d 0 to 21 PI	0.60	0.58	0.56	0.60	0.028	0.40
d -7 to 21 PI	0.60	0.57	0.58	0.60	0.020	0.57

[a]*BW* Body weight, *ADG* Average daily gain, *ADFI* Average daily feed intake, *PI* Post-inoculation. Each least squares mean represents 9-10 observations

[b]*OA* Organic acid blend

[c]*MG* Monoglyceride blend

[d]OA+MG = combination of organic acids and monoglycerides

Table 4.3. Total and differential white blood cells and red blood profile in weaned pigs throughout the experiment

Item[d]	d 0	d 5 PI	d 14 PI	SEM	*P*-value
WBC, 10^3/μL	7.81[c]	10.09[b]	11.95[a]	0.44	< 0.01
Neu, 10^3/μL	3.69[b]	5.09[a]	5.26[a]	0.31	< 0.01
Lym, 10^3/μL	3.34[c]	4.08[b]	4.96[a]	0.22	< 0.01
Mono, 10^3/μL	0.53[c]	0.78[b]	0.94[a]	0.050	< 0.01
Eos, 10^3/μL	0.15[b]	0.19[b]	0.55[a]	0.051	< 0.01
Baso, 10^3/μL	0.011[b]	0.019[a]	0.021[a]	0.002	< 0.01
Neu, %	46.07	48.96	44.99	1.55	0.20
Lym, %	43.06	40.72	41.73	1.53	0.54
Mono, %	7.13	7.47	8.09	0.47	0.29
Eos, %	1.89[b]	1.54[b]	4.89[a]	0.49	< 0.01
Baso, %	0.13[b]	0.15[ab]	0.18[a]	0.017	< 0.05
Neu:Lym	1.03	1.19	1.06	0.079	0.18
RBC, 10^6/μL	7.38	7.34	7.55	0.14	0.39
HGB, g/dL	8.16[b]	8.11[b]	8.68[a]	0.28	< 0.05
HCT, %	30.39[b]	29.72[b]	32.57[a]	0.55	< 0.01
MCV, fL[e]	41.58[ab]	40.66[b]	42.70[a]	0.81	< 0.01
MCH, pg	11.35	11.16	11.78	0.51	0.22
MCHC, g/dL	27.16	27.01	27.43	0.72	0.88
RDW, %	29.23[a]	27.29[b]	26.31[b]	0.68	< 0.01
Platelets, 10^3/μL	461	441	421	57.24	0.42
MPV, fL[e]	7.74	7.52	7.61	0.21	0.21
Total protein, g/dL	4.98[b]	4.94[b]	5.15[a]	0.078	< 0.05

[a,b,c]Means without a common superscript are different ($P < 0.05$)

[d]*WBC* White blood cell, *Neu* Neutrophil, *Lym* Lymphocyte, *Mono* Monocyte, *Eos* Eosinophil, *Baso* Basophil, *RBC* Red blood cell, *HGB* Hemoglobin, *HCT* Hematocrit (Packed cell volume); *MCV* Mean corpuscular volume, *MCH* Mean corpuscular hemoglobin, *MCHC*

Mean corpuscular hemoglobin concentration, *RDW* Red cell distribution width, *MPV* Mean platelet volume. Each least squares mean represents 9-10 observations

[e]*fL* Femtolitre (10^{-15} L)

Table 4.4. Total and differential white blood cells in enterotoxigenic *Escherichia coli* F18-challenged weaned pigs fed experimental diets

Item[c]	Control	OA[d]	MG[e]	OA+MG[f]	SEM	*P*-value
d 0 before inoculation						
WBC, $10^3/\mu L$	8.16	7.68	7.68	7.74	0.83	0.91
Neu, $10^3/\mu L$	3.91	3.51	3.56	3.77	0.60	0.92
Lym, $10^3/\mu L$	3.47	3.42	3.24	3.21	0.36	0.78
Mono, $10^3/\mu L$	0.63	0.46	0.54	0.48	0.095	0.21
Eos, $10^3/\mu L$	0.113	0.135	0.187	0.175	0.095	0.67
Baso, $10^3/\mu L$	0.010	0.012	0.012	0.010	0.005	0.95
Neu, %	46.30	44.97	46.26	46.77	3.15	0.97
Lym, %	43.72	43.05	41.90	43.56	3.00	0.97
Mono, %	8.03	6.14	8.00	6.37	0.90	0.28
Eos, %	1.38	1.65	2.39	2.14	0.91	0.63
Baso, %	0.112	0.144	0.145	0.123	0.033	0.89
Neu:Lym	1.11	0.95	1.07	0.97	0.137	0.70
d 5 post-inoculation						
WBC, $10^3/\mu L$	11.59[a]	10.19[ab]	9.86[b]	8.72[b]	0.83	< 0.05
Neu, $10^3/\mu L$	5.83	5.32	4.79	4.41	0.60	0.33
Lym, $10^3/\mu L$	4.66[a]	3.87[ab]	4.11[ab]	3.68[b]	0.36	< 0.05
Mono, $10^3/\mu L$	0.89	0.69	0.83	0.71	0.095	0.36
Eos, $10^3/\mu L$	0.193	0.257	0.170	0.154	0.095	0.63
Baso, $10^3/\mu L$	0.018	0.018	0.019	0.020	0.005	0.98
Neu, %	50.08	51.48	48.84	45.46	3.15	0.30
Lym, %	40.40	39.75	39.82	42.89	3.00	0.66
Mono, %	7.63	6.28	8.30	7.66	0.90	0.33
Eos, %	1.28	1.51	1.59	1.77	0.91	0.89
Baso, %	0.119	0.153	0.180	0.130	0.033	0.41
Neu:Lym	1.32[a]	1.20[ab]	1.26[a]	0.99[b]	0.137	< 0.05
d 14 post-inoculation						
WBC, $10^3/\mu L$	10.29[b]	12.20[ab]	12.74[a]	12.57[ab]	0.83	< 0.05
Neu, $10^3/\mu L$	4.09[b]	5.48[ab]	6.33[a]	5.15[ab]	0.60	< 0.05
Lym, $10^3/\mu L$	4.86	5.32	4.67	4.97	0.36	0.77
Mono, $10^3/\mu L$	0.94	0.86	1.05	0.93	0.095	0.72

Eos, $10^3/\mu L$	0.418	0.529	0.547	0.716	0.095	0.50
Baso, $10^3/\mu L$	0.021	0.017	0.026	0.019	0.005	0.67
Neu, %	42.91	42.49	51.12	43.45	3.15	0.33
Lym, %	44.05	41.94	37.74	43.18	3.00	0.62
Mono, %	8.71	8.15	7.98	7.51	0.90	0.85
Eos, %	4.18	4.35	5.34	5.68	0.91	0.79
Baso, %	0.161	0.148	0.226	0.189	0.033	0.34
Neu:Lym	0.89[b]	0.94[ab]	1.36[a]	1.04[ab]	0.137	< 0.05

[a,b]Means without a common superscript are different ($P < 0.05$)

[c]*WBC* White blood cell, *Neu* Neutrophil, *Lym* Lymphocyte, *Mono* Monocyte, *Eos* Eosinophil, *Baso* Basophil. Each least squares mean represents 9-10 observations

[d]*OA* Organic acid blend

[e]*MG* Monoglyceride blend

[f]OA+MG = combination of organic acids and monoglycerides

Table 4.5. Red blood cell profile in enterotoxigenic *Escherichia coli* F18-challenged weaned pigs fed experimental diets

Item[d]	Control	OA[e]	MG[f]	OA+MG[g]	SEM	*P*-value
d 0 before inoculation						
RBC, 10^6/μL	7.22	7.36	7.38	7.54	0.25	0.78
HGB, g/dL	8.22	7.89	8.29	8.24	0.47	0.80
HCT, %	29.19	30.70	30.68	31.00	1.05	0.56
MCV, fL[h]	41.51	42.18	41.46	41.18	1.13	0.78
MCH, pg	11.42	11.76	11.26	10.96	0.68	0.66
MCHC, g/dL	27.38	27.68	27.09	26.50	1.03	0.77
RDW, %	29.11	29.32	29.27	29.23	1.08	0.98
Platelets, 10^3/μL	482	522	396	443	69.08	0.32
MPV, fL[h]	7.50[b]	8.24[a]	7.53[b]	7.70[ab]	0.26	< 0.05
Total protein, g/dL	5.01	5.08	4.89	4.96	0.12	0.46
d 5 post-inoculation						
RBC, 10^6/μL	7.26[ab]	7.04[b]	7.33[ab]	7.76[a]	0.25	< 0.05
HGB, g/dL	8.14	7.66	8.23	8.43	0.47	0.65
HCT, %	29.61	28.50	29.44	31.36	1.05	0.37
MCV, fL[h]	40.98	40.80	40.16	40.72	1.13	0.86
MCH, pg	11.23	11.16	11.22	11.02	0.68	0.99
MCHC, g/dL	27.77	27.13	27.85	26.89	1.03	0.74
RDW, %	27.06	27.84	26.64	27.62	1.08	0.85
Platelets, 10^3/μL	384	494	441	443	69.08	0.37
MPV, fL[h]	7.47[ab]	7.91[a]	7.26[b]	7.45[ab]	0.26	< 0.05
Total protein, g/dL	4.83	5.07	4.99	4.86	0.12	0.46
d 14 post-inoculation						
RBC, 10^6/μL	7.23[b]	7.20[b]	7.62[ab]	8.16[a]	0.25	< 0.05
HGB, g/dL	8.68[ab]	8.01[b]	9.05[ab]	9.69[a]	0.47	< 0.05
HCT, %	31.74[bc]	29.53[c]	33.24[ab]	35.75[a]	1.05	< 0.05
MCV, fL[h]	42.73	41.23	42.86	44.00	1.13	0.54
MCH, pg	12.05	11.28	11.84	11.95	0.68	0.86
MCHC, g/dL	27.97	26.85	27.89	27.03	1.03	0.81
RDW, %	26.74	27.04	25.24	26.20	1.08	0.88
Platelets, 10^3/μL	509[a]	434[ab]	409[ab]	331[b]	69.08	< 0.05

MPV, fL[h]	7.72[ab]	8.05[a]	7.26[b]	7.41[b]	0.26	< 0.05
Total protein, g/dL	5.14	5.05	5.30	5.08	0.12	0.29

[a,b,c]Means without a common superscript are different ($P < 0.05$)

[d]*RBC* Red blood cell, *HGB* Hemoglobin, *HCT* Hematocrit (Packed cell volume); *MCV* Mean corpuscular volume, *MCH* Mean corpuscular hemoglobin, *MCHC* Mean corpuscular hemoglobin concentration, *RDW* Red cell distribution width, *MPV* Mean platelet volume. Each least squares mean represents 9-10 observations

[e]*OA* Organic acid blend

[f]*MG* Monoglyceride blend

[g]OA+MG = combination of organic acids and monoglycerides

[h]*fL* Femtolitre (10^{-15} L)

Table 4.6. Serum inflammatory cytokines and acute phase proteins in enterotoxigenic *Escherichia coli* F18-challenged weaned pigs fed experimental diets

Item[c]	Control	OA[d]	MG[e]	OA+MG[f]	SEM	*P*-value
d 0 before inoculation						
GM-CSF, pg/mL	97.63[a]	66.40[ab]	19.88[b]	106.11[a]	41.78	0.06
IL-1α, pg/mL	136.68[a]	142.19[a]	17.35[b]	65.23[ab]	63.87	0.07
IL-6, pg/mL	120.02	183.34	83.06	207.92	83.49	0.52
IL-8, pg/mL	140.32	125.81	136.91	177.77	25.04	0.29
TNF-α, pg/mL	228.58[ab]	136.31[ab]	49.05[b]	302.93[a]	88.09	0.056
C-reactive protein, µg/mL	6.77	4.32	4.62	6.13	1.54	0.63
Haptoglobin, mg/mL	1.70	1.40	0.99	1.42	0.47	0.24
d 2 post-inoculation						
C-reactive protein, µg/mL	17.85	19.55	18.13	16.99	4.95	0.93
Haptoglobin, mg/mL	2.16	2.82	2.14	2.23	0.66	0.60
d 5 post-inoculation						
GM-CSF, pg/mL	51.06[ab]	53.02[ab]	27.57[b]	88.94[a]	22.39	0.055
IL-1α, pg/mL	138.70	152.51	31.53	62.66	66.99	0.12
IL-6, pg/mL	148.57	209.48	117.35	213.25	90.72	0.76
IL-8, pg/mL	106.33	150.79	110.00	211.33	39.63	0.12
TNF-α, pg/mL	129.86	141.61	31.45	194.62	67.52	0.18
C-reactive protein, µg/mL	7.81	9.01	10.24	8.27	2.06	0.79
Haptoglobin, mg/mL	1.43	1.67	1.39	0.97	0.68	0.57
d 14 post-inoculation						
GM-CSF, pg/mL	48.18	47.16	32.45	50.28	6.58	0.22
IL-1α, pg/mL	103.34[ab]	148.50[a]	27.40[b]	47.13[b]	67.27	0.07
IL-6, pg/mL	83.62	169.12	101.47	145.67	60.16	0.68
IL-8, pg/mL	96.90	108.91	93.83	171.07	49.02	0.10
TNF-α, pg/mL	81.19	54.00	29.66	76.27	22.41	0.35
C-reactive protein, µg/mL	7.59	8.17	11.28	9.01	2.33	0.69
Haptoglobin, mg/mL	0.83	0.94	0.88	0.54	0.39	0.74

[a,b]Means without a common superscript are different ($P < 0.05$)

[c]*GM-CSF* Granulocyte-macrophage colony-stimulating factor, *IL-1α* Interleukin-1 alpha, *IL-6* Interleukin 6, *IL-8* Interleukin 8, *TNF-α* Tumor necrosis factor-alpha. Each least squares mean represents 9-10 observations

[d]*OA* Organic acid blend

[e]*MG* Monoglyceride blend

[f]OA+MG = combination of organic acids and monoglycerides

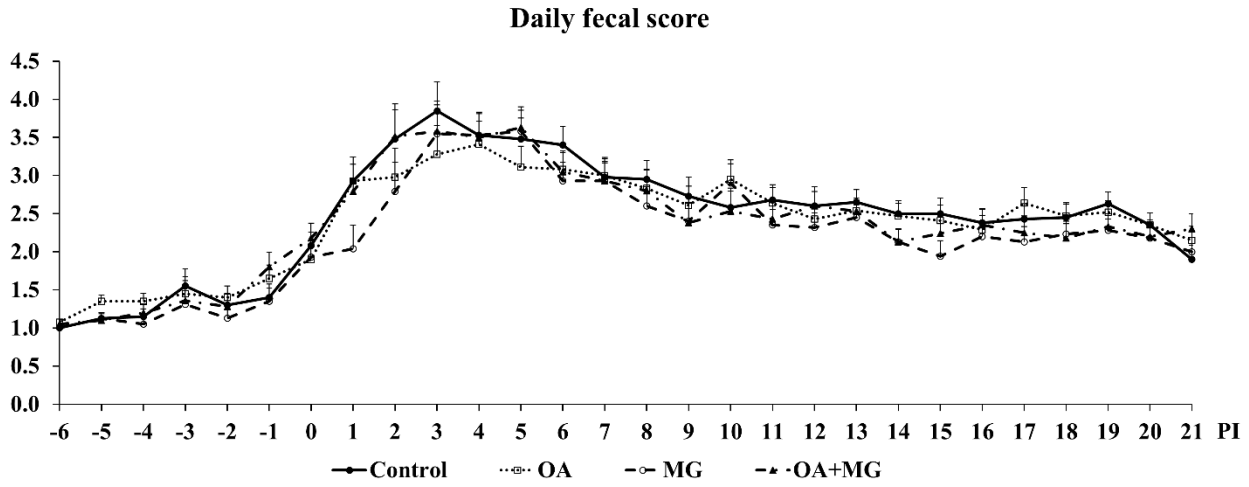

Figure 4.1. Daily fecal score of enterotoxigenic *Escherichia coli* F18-challenged weaned pigs fed diets supplemented with organic acid or monoglyceride blends. Fecal score = 1, normal feces, 2, moist feces, 3, mild diarrhea, 4, severe diarrhea, 5, watery diarrhea. PI = post-inoculation. Each least squares mean represents 9-10 observations. OA = organic acid blend; MG = monoglyceride blend; OA+MG = combination of organic acids and monoglycerides.

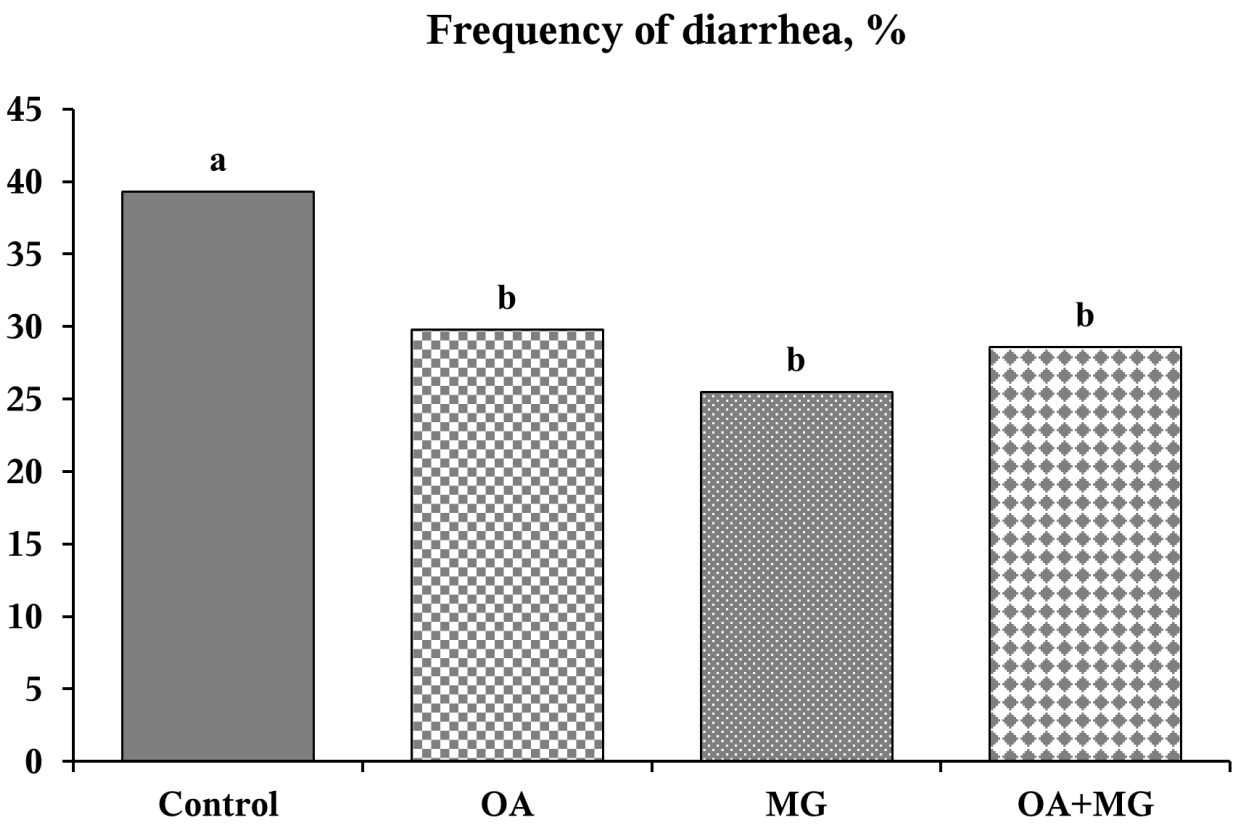

Figure 4.2. Frequency of diarrhea of enterotoxigenic *Escherichia coli* F18-challenged weaned pigs fed diets supplemented with organic acid or monoglyceride blends during the entire experimental period. Frequency of diarrhea was calculated as the percentage of pig days with diarrhea score ≥ 3 in the total of pig days. [a,b]Means without a common superscript are different ($P < 0.05$). OA = organic acid blend; MG = monoglyceride blend; OA+MG = combination of organic acids and monoglycerides.

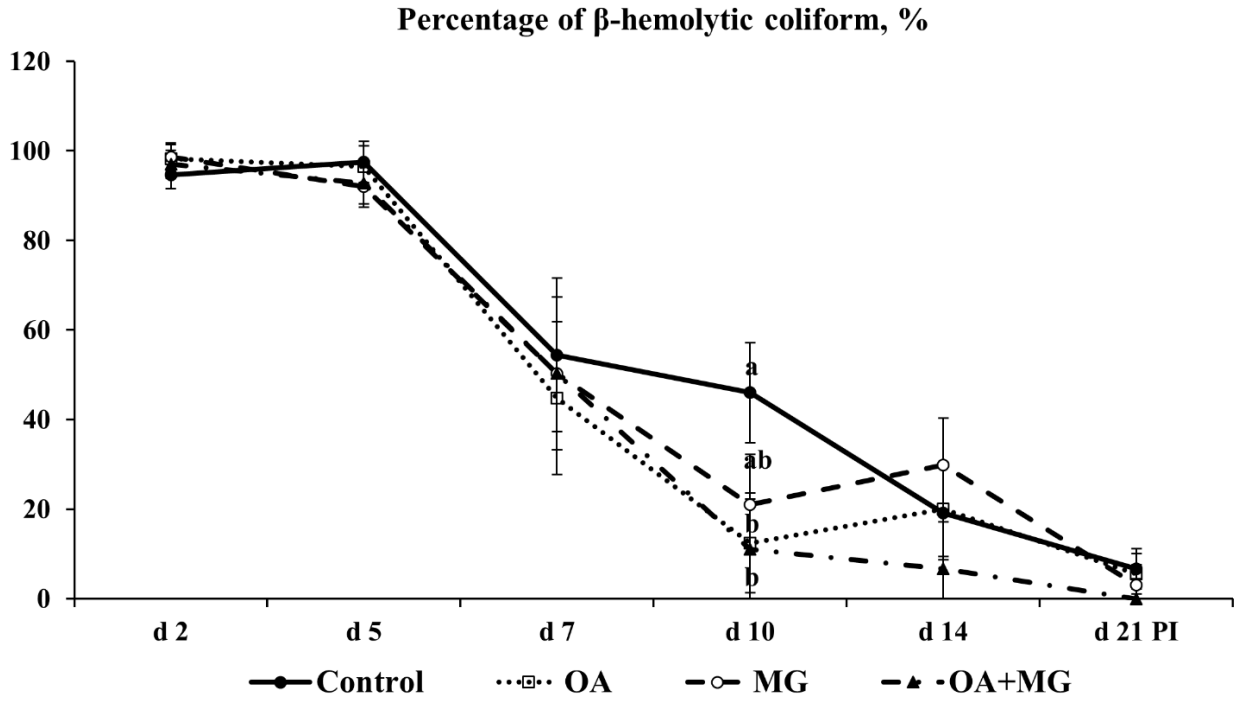

Figure 4.3. The percentage (%) of β-hemolytic coliform in fecal samples of enterotoxigenic *Escherichia coli* F18-challenged pigs fed diets supplemented with organic acid or monoglyceride blends. Each least squares mean represents 9-10 observations. [a,b]Means without a common superscript are different ($P < 0.05$). Control pigs had more ($P < 0.05$) β-hemolytic coliform in feces, compared with pigs in the combination of organic acids and monoglycerides, or pigs only fed with organic acid blend on d 10 post-inoculation (PI). OA = organic acid blend; MG = monoglyceride blend; OA+MG = combination of organic acids and monoglycerides.

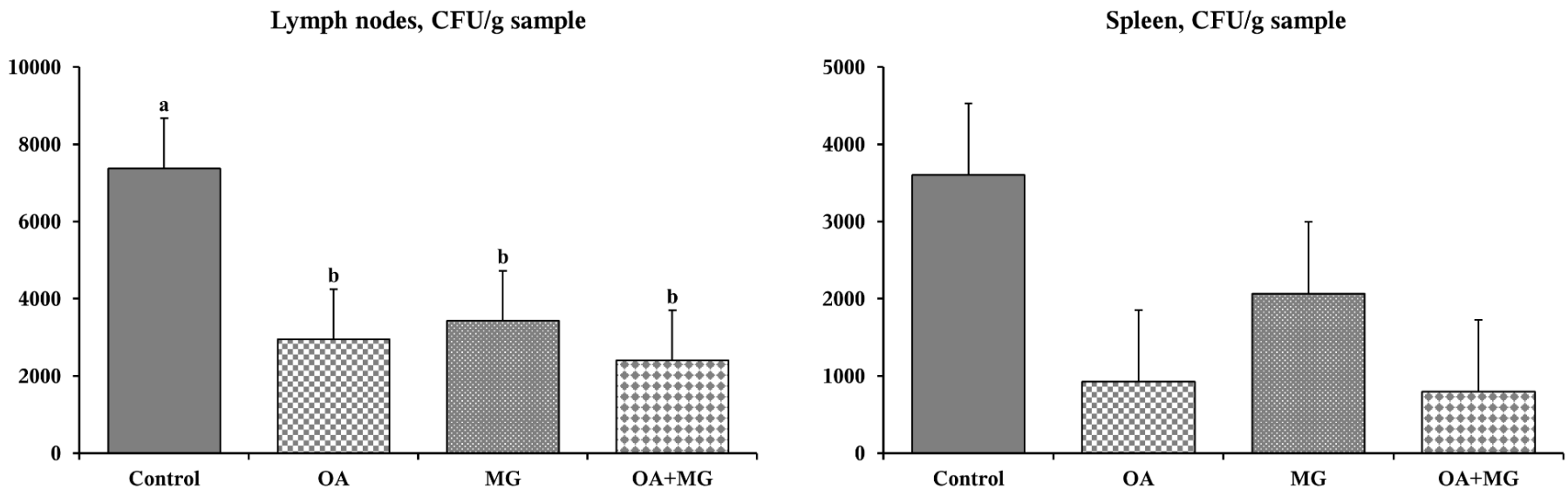

Figure 4.4. Total coliforms in mesenteric lymph nodes and spleen of enterotoxigenic *Escherichia coli* F18-challenged weaned pigs fed experimental diets. [a,b]Means without a common superscript are different ($P < 0.05$). Each least squares mean represents 9-10 observations. OA = organic acid blend; MG = monoglyceride blend; OA+MG = combination of organic acids and monoglycerides.

CHAPTER 5

GENERAL SUMMARY, DISCUSSION, AND CONCLUSION

Gut integrity is associated not only with the efficient transport of nutrients through digestion and absorption, but also with proper intestinal barrier and immune function, which includes the ability to isolate and protect the host from external factors detrimental to health and homeostasis. Multifactorial weaning stressors, combined with the abrupt transition of diet from easily digestible liquid to a complex plant-based diet, can result in intestinal dysfunction as well as decreased appetite in weaned pigs, making them more susceptible to pathogen exposure and infection. Diarrhea caused by enterotoxigenic *Escherichia coli* (ETEC) infection within a few weeks following weaning is common in piglets, compromising gut integrity and negatively affecting both short-term recovery/survival and long-term development of gastrointestinal function, ultimately impacting productivity and animal welfare in swine production. Antibiotics and pharmacological levels of zinc oxide (2,000–3,000 mg/kg) have been widely used during the critical period of swine production to improve productivity and reduce losses by managing post-weaning diarrhea and promoting growth. Despite their effectiveness as antimicrobial growth promoters, the use of antibiotics and high-dose zinc oxide in feed has been restricted or reduced due to public concerns about human health, antibiotic resistance, and environmental damage. These challenges, along with the importance of sustainable agriculture, make it essential to reduce reliance on conventional practices and find nutritional interventions for pig growth and health.

Monoglycerides, formed by attaching a fatty acid to glycerol, exhibit broad-spectrum antibacterial effects as well as antiviral activity against enveloped viruses due to their amphiphilic nature. Moreover, the molecular structure of monoglycerides improves handling characteristics

and minimizes the loss of unwanted bioactive substances (i.e., organic fatty acids) before reaching the intended site in the intestine. This benefits the intestinal barrier and metabolism of the host. These properties make monoglycerides a valuable and practical nutritional intervention for enhancing disease resistance and/or resilience in weanling pigs by improving intestinal integrity and modulating inflammatory responses. Despite the potential advantages of monoglycerides for growth and health of pigs, few studies have investigated their biological activity and potential use in the transitional period of piglets, particularly in cases of ETEC infection, which commonly occurs after weaning. Therefore, a comprehensive investigation was conducted to explore the benefits and potential use of monoglycerides as a sustainable strategy to improve disease resistance and overall health in weaned pigs through *in vitro* cell culture and *in vivo* ETEC challenge models.

The first *in vitro* study evaluated the biological activities of monoglycerides by measuring the integrity of epithelial monolayer with an intestinal porcine epithelial cell line (IPEC-J2), and assessing immunomodulatory responses with porcine alveolar macrophages (PAM). In terms of epithelial barrier integrity, epithelial cells treated with up to 1,000 μg/mL of monoglycerides exhibited enhanced barrier function, as indicated by increased transepithelial electrical resistance. Low doses (50 or 100 μg/mL) of monoglycerides increased pro-inflammatory cytokine production from non-challenged macrophages, while the highest dose (1,000 μg/mL) increased inflammatory marker in macrophages activated by lipopolysaccharide (LPS). The results from this study suggest that monoglycerides help improve epithelial cell lining and integrity, potentially due to metabolic changes such as providing energy or regulating tight junction protein expression. The study also indicated that monoglycerides have the potential to act as inflammatory modulators, contributing to early immune responses and immune enhancement, which may be related to the regulation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) in immune cells.

The second study evaluated the effectiveness of dietary monoglycerides (a blend of short- and medium-chain fatty acids; BalanGut™ LS L; BASF SE) on several parameters related to disease resistance and productivity in pigs, including growth performance, diarrhea, local and systemic immune responses, intestinal health, and metabolism. Antimicrobial growth promoter (carbadox) improved growth performance, reduced diarrhea, and decreased fecal shedding of ETEC-infected pigs, while monoglyceride supplementation provided comparable but somewhat limited benefits. Pigs fed monoglycerides had lower serum haptoglobin levels at the peak of infection compared to control pigs. Supplementation of monoglyceride reduced ileal crypt depth and induced changes similar to those observed with carbadox, including reduced neutrophil and macrophage numbers and decreased mRNA expression of *TNFA*, *IL* (Interleukin) *6*, *IL10*, *IL1A*, *IL1B*, and Prostaglandin-endoperoxide synthase 2 (*PTGS2*) in the ileal mucosa. Moreover, monoglyceride supplementation influenced serum metabolites associated with cellular homeostasis and antioxidant responses during the peak of ETEC infection in pigs. These results suggest that supplementation of monoglycerides may enhance disease resistance by mitigating intestinal and systemic inflammation and promoting intestinal health in weaned pigs challenged with ETEC. Potential modes of action include modulating various cellular responses through HDAC inhibition and G-protein coupled receptor activation, as well as reducing the inflammatory response to ETEC infection by modulating the NF-κB/MAPK pathway. The second study indicates that supplementation of monoglycerides can alleviate the severity of diarrhea and mitigate intestinal and systemic inflammation in weaned pigs challenged with ETEC.

The third study aimed to evaluate the efficacy of monoglycerides (a blend of short- and medium-chain fatty acids; Entero-Nova 410C, Eastman) in modulating immune response and maintaining gut integrity of ETEC-infected pigs, and their compatibility with organic acids (a blend of formic acid, lactic acid, and sodium formate; Acitra G20C, Eastman). Supplementation

with organic acids, monoglycerides, or their combination alleviated the diarrheal condition compared to the control group. These results are supported by reduced fecal shedding and bacterial translocation to immune organs observed in ETEC-infected pigs supplemented with either organic acids, monoglycerides, or the combination, suggesting mitigation of ETEC colonization or its adverse effects. Supplementation of monoglycerides and the combination of both acid-based additives reduced inflammation markers (white blood cells, neutrophils, lymphocytes, and the ratio of neutrophils to lymphocytes) at the peak of ETEC infection compared to the control group. Additionally, pigs supplemented with monoglycerides had higher levels of white blood cells and neutrophils than the control group after the peak of the infection, which may be associated with a sustained innate immune response or surveillance contributing to recovery and pathogen clearance. The third study demonstrated that monoglycerides or organic acids alone or in combination can ameliorate the detrimental effects of ETEC infection in weaned pigs by alleviating intestinal damage and modulating both intestinal and systemic inflammation.

In summary, monoglycerides have demonstrated enhanced disease resistance and overall health in weaned pigs by mitigating excessive diarrhea and inflammatory responses induced by ETEC infection. These effects are associated with improved intestinal structure and barrier function, as well as modulated local and systemic immunity, and altered metabolites related to gut integrity. As the push toward more sustainable livestock production and a One Health approach, nutritional interventions that reduce reliance on antimicrobial use are increasingly important. Monoglycerides are a promising dietary approach because they meet several key criteria for effective practices, including safety, stability, antibacterial activity with minimal resistance development, improved disease resistance and performance, ease of handling, and compatibility. However, more research is needed to assess the impact of monoglycerides supplementation on growth performance of weaned pigs in commercial settings under various external challenges.

Future studies should also explore the synergistic effects of monoglycerides combined with other acid-based additives to further validate their potential combined benefits in *in vivo* models. Additionally, targeted metabolomics and other omics approaches should be considered to provide valuable insights into the biological activities and interactions of different types of monoglycerides.