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Authors

Kim, Kwangwook
Song, Minho
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EDITED BY
Fraser D. Russell,
University of the Sunshine Coast, Australia

REVIEWED BY
Hongbo Yi,
Guangdong Academy of Agricultural Sciences (GDAAS), China
Archana Saxena,
University of South Carolina, United States

*CORRESPONDENCE
Yanhong Liu
yahliu@ucdavis.edu
Peng Ji
penji@ucdavis.edu

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Enterotoxigenic *Escherichia coli* infection of weaned pigs: Intestinal challenges and nutritional intervention to enhance disease resistance

Kwangwook Kim[1], Minho Song[2], Yanhong Liu[1]* and Peng Ji[3]*

[1]Department of Animal Science, University of California, Davis, Davis, CA, United States, [2]Division of Animal and Dairy Science, Chungnam National University, Daejeon, South Korea, [3]Department of Nutrition, University of California, Davis, Davis, CA, United States

Enterotoxigenic *Escherichia coli* (ETEC) infection induced post-weaning diarrhea is one of the leading causes of morbidity and mortality in newly weaned pigs and one of the significant drivers for antimicrobial use in swine production. ETEC attachment to the small intestine initiates ETEC colonization and infection. The secretion of enterotoxins further disrupts intestinal barrier function and induces intestinal inflammation in weaned pigs. ETEC infection can also aggravate the intestinal microbiota dysbiosis due to weaning stress and increase the susceptibility of weaned pigs to other enteric infectious diseases, which may result in diarrhea or sudden death. Therefore, the amount of antimicrobial drugs for medical treatment purposes in major food-producing animal species is still significant. The alternative practices that may help reduce the reliance on such antimicrobial drugs and address animal health requirements are needed. Nutritional intervention in order to enhance intestinal health and the overall performance of weaned pigs is one of the most powerful practices in the antibiotic-free production system. This review summarizes the utilization of several categories of feed additives or supplements, such as direct-fed microbials, prebiotics, phytochemicals, lysozyme, and micro minerals in newly weaned pigs. The current understanding of these candidates on intestinal health and disease resistance of pigs under ETEC infection are particularly discussed, which may inspire more research on the development of alternative practices to support food-producing animals.

KEYWORDS

enterotoxigenic *Escherichia coli*, intestinal health, nutritional intervention, post-weaning diarrhea, weaned pigs

Introduction

Modern swine production becomes highly intensive in order to maximize productivity, however, husbandry-associated stress is also increased. Many physical and/or psychological stress, such as environmental and nutritional changes and increased exposure to infectious diseases, can induce a significant depression in growth performance, alter local or systemic immune responses, and disrupt gastrointestinal homeostasis in different physiological stages of pigs (1). For instance, regrouping, crowding, social isolation, and maternal deprivation may impair the immunity and alter the regulation of the neuroendocrine in pigs, thus inducing gastrointestinal diseases (2). This review is mainly focused on newly weaned pigs and post-weaning stress. The development of the intestinal epithelial barrier, immunity, and enteric nervous system exhibits a high degree of plasticity in the post-weaning period, which can impact the long-term phenotypes and gastrointestinal function (3, 4). Infectious diarrhea disease has long been one of the leading causes of morbidity and mortality in the swine industry (5). Post-weaning diarrhea (PWD) induced by pathogenic Escherichia coli (*E. coli*) infection is one of the most common diseases and is characterized by the discharge of watery feces, dehydration, a thin or unthrifty appearance, and sudden death of piglets. During acute outbreaks of PWD, the pig mortality due to *E. coli* infection may reach 20 to 30% over a 1- to 2-month time span among infected pigs (6). A survey conducted by National Animal Health Monitoring System reported that the mortality rate of nursery pigs ranged from 2.6 to 3.6% in the years 2000, 2006, and 2012, of which diarrhea-caused deaths accounted for 9.4 to 12.6% of the overall mortality (5). Therefore, the prevention of post-weaning *E. coli* infection is extremely important to maintain growth performance and welfare of pigs during the entire lifespan. In-feed antibiotics and a number of feed additives/supplements are discussed in this review to summarize their efficacies on growth promotion and disease resistance in weaned pigs.

ETEC infections in weaned pigs

The commensal *E. coli* strains that colonize the gastrointestinal tract of pigs rarely cause disease. However, *E. coli* expressing specific virulence features confers the ability to cause diarrheal disease (7). The major pathotypes of *E. coli* include enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), diffusely adherent *E. coli* (DAEC), Vero- or Shiga-like toxin-producing *E. coli* (VTEC or STEC) and enterotoxigenic *E. coli* (ETEC) (8). Among these, the most diffuse etiological agents responsible for PWD in pigs are ETEC displaying the fimbriae F4 (K88) and F18 (9).

Clinical signs

The clinical signs observed in *E. coli*-infected animals include diarrhea with watery, light-orange-colored feces, loss of appetite (decreased feed intake), depression, dehydration, rough hair coating, and inflamed perianal regions smeared with feces (10). The watery diarrhea condition typically lasts from 1 to 5 days after infection, but severe cases may result in shock or sudden death without showing obvious symptoms of illness (11). Pigs also change in appearance, as a sallow discoloration of the tip of the nose, the ears, and the abdomen may be observed.

Pathogenesis and toxins

The pathogenesis of ETEC-induced diarrhea is initiated by bacterial attachment to specific receptors expressed on the intestinal epithelium, followed by colonization of ETEC in the small intestine (Figure 1) (18). Fimbriae are hair-like appendages that show characteristic patterns from the outer membrane of the bacterial cells, which facilitate the adhesion of ETEC to the small intestinal mucosa (19). In pigs, F4 and F18 are the fimbrial types that are mostly associated with PWD, and these two fimbrial genes were found in 92.7% of all ETEC-induced PWD (20). Once ETEC successfully adheres to the small intestinal epithelium, colonization is established, and ETEC rapidly proliferates to produce one or more types of enterotoxins.

The secreted enterotoxins, including heat-labile toxins (LTs) and heat-stable toxins (STs), act on stimulating water and electrolyte secretion and reduce fluid absorption in the small intestine (12). Briefly, LTs bind to the receptors on the cell surfaces and activate the adenylate cyclase system to stimulate the secretion of cyclic adenosine monophosphate (cAMP). The up-regulated cAMP induces the activation of an apical chloride channel and a basolateral Na/K/2Cl cotransporter, resulting in chloride secretion from the apical region of enterocytes, reduced sodium absorption, and a concomitant massive water loss into the intestinal lumen (21, 22). STs produced by ETEC are secreted peptides that can be classified as STa and STb based on their solubility and enzyme sensitivity. STa binds to the extracellular domain and accumulates cyclic guanosine monophosphate (cGMP) and consequently opens the ion channel to induce Cl^- and HCO_3^- release into the intestinal lumen (23). STa also enhances the luminal secretion of pro-inflammatory cytokines and chemokines, including interleukin (IL)-6 and IL-8, in the small intestinal mucosa of pigs (24). STb was shown to specifically interact with calcium ion channels on the intestinal epithelial surface to elevate intracellular Ca^{2+} concentration, which may increase paracellular permeability *via* claudin-1 redistribution (25). STb can also concurrently reduce the expression of other tight junction proteins,

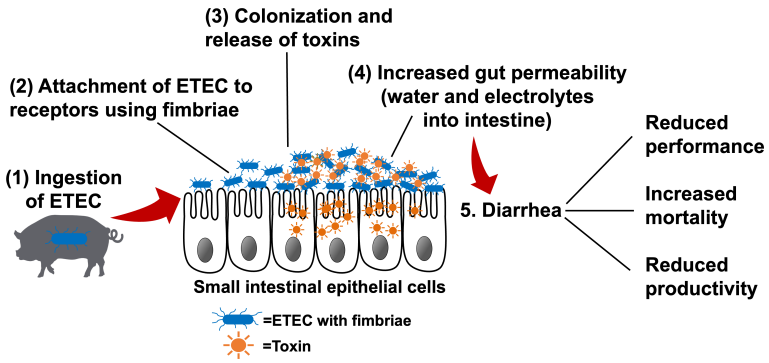

FIGURE 1
The pathogenesis of enterotoxigenic *Escherichia coli* (ETEC) (1) ETEC are ingested by susceptible pigs and enter the gastrointestinal tract. (2) ETEC express fimbrial adhesins, which mediate adherence to specific receptors present on the intestinal epithelial cells. (3) Bacterial colonization occurs in the small intestinal mucosa. Once colonization is established, ETEC rapidly produce toxins (e.g., heat-labile, heat-stable, and/or Shiga toxins). (4) Enterotoxins stimulate water and electrolyte loss into the intestinal lumen, increase gut permeability, and/or transport across the epithelial cells to blood circulation, resulting in edema. (5) Increased gut permeability and massive water loss into the intestinal lumen lead to diarrhea, which results in the poor performance and productivity and increased mortality. Adapted from: Nagy and Feteke (12), Kaper et al. (13), Croxen et al. (14), Fleckenstein et al. (15), and Mirhoseini et al. (16), Rhouma et al. (17).

including zona occludens-1 and occludin, thus accelerating fluid loss into the intestinal lumen (26).

Moreover, Shiga toxins (Stxs) and lipopolysaccharides (LPS) derived from ETEC are also involved in the pathogenicity in the host (13). After binding to the cell surface, Stxs are internalized *via* the Golgi apparatus to the endoplasmic reticulum before being translocated to the cytosol of enterocytes (27). During the translocation, Stxs are able to induce DNA fragmentation and cell apoptosis of infected cells, which further facilitates proteolysis in neighboring cells and toxic effects on the host (28). Additionally, Stxs can stimulate the intestinal epithelial cells to secrete pro-inflammatory cytokines and neutrophil chemoattractant molecules, like IL-8 (29). Bacterial LPS are the major components of the outer membrane of Gram-negative bacteria, including ETEC (30). LPS receptors are mainly located on the cells in the innate immune system, such as macrophages and endothelial cells (31). The activation of immune cells induced by LPS binding can stimulate various immunological signaling pathways, leading to the release of a large amount of cytokines, including tumor necrosis factor-α (TNF-α), IL-6, and IL-1 from target cells (32).

Intestinal barrier disruption during ETEC infection

The intestinal epithelium forms as a single layer lining the gastrointestinal tract and is responsible for the uptake of nutrients and water. Meanwhile, the epithelium also serves as a physical barrier to exclude potential antigens, pathogens, and toxins from the external environment (33). ETEC infection could damage the intestinal epithelial barrier functions, resulting in electrolytes and water imbalance and watery diarrhea, and induce intestinal inflammation in piglets (19, 34, 35).

Mucus

The mucus layer is a gel-like sieve structure covering the luminal surface of the gastrointestinal tract and acts as a physical barrier to bacteria and other antigens present in the lumen (36, 37). Mucus is known to be a highly dynamic matrix, mainly consisting of glycosylated mucin proteins secreted by intestinal goblet cells. In the small and large intestine, mucin 2 (MUC2) is the most abundant mucus protein (38). However, the inner mucus layer also contains antimicrobial peptides, immunoglobulin-A (IgA), and other molecules that are essential in the innate immune defense and the maintenance of intestinal homeostasis (39).

ETEC infection could alter the expression of *MUC2* in the small intestine. An *in vivo* ETEC F18 challenged study observed that ETEC-infected pigs expressed more *MUC2* gene in the jejunal mucosa during the peak of infection (40). The up-regulated *MUC2* gene in the intestinal epithelium was also reported by several *in vitro* studies when LTs or Stxs producing ETEC were used (41, 42). However, a down-regulated *MUC2* gene expression was observed in ETEC F18 infected pigs during the post-peak infection period (40). A growing body of evidence demonstrated that a highly conserved mucin-degrading metalloprotease from ETEC is responsible for mucin reduction, which facilitates the interaction of ETEC with intestinal enterocytes and immune cells and triggers inflammatory responses in the gut (43–45).

Tight junction and epithelial barrier

Intestinal tight junctions are junctional complex in epithelium, consisting of three integral transmembrane proteins, including occludin, claudins, and junctional adhesion molecule (JAM), as well as the cytoplasmatic plaque proteins zonula occludens, cingulin, and 7H6 (46, 47). Occludins and claudins are the major sealing protein. Zonula occludens directly interact with most of the transmembrane proteins localizing at tight junctions and provide the structural basis for the assembly of multiprotein complexes at the cytoplasmic surface of intercellular junctions (48, 49). Tight junctions act as gates or fences to control intestinal permeability and to maintain intestinal integrity (50). Beyond that, growing evidence indicates that tight junctions are also involved in cell-cell signal transduction to guide cell proliferation and differentiation (51).

The alteration of tight junction proteins by bacterial pathogens or enterotoxins can lead to permeability defects in the intestinal epithelium (52). Numerous research articles have reported that ETEC can impair intestinal barrier function by modulating tight junction protein expressions (Table 1), which may induce diarrhea and initiate inflammatory cascades. The common methods to assess *in vivo* intestinal permeability include the mannitol and lactulose test, analyzing the flux of intact FD4, and measuring bacterial translocation. The flux of intact FD4 across the intestinal epithelium occurs primarily through paracellular pathways, thus, increased flux rates of FD4 can reflect the intestinal barrier defects (62). McLamb et al. (34) and Kim et al. (40) reported that ETEC F18 infection elevated FD4 flux rates across the porcine ileum or jejunum, respectively. Bacterial translocation is defined as the passage of viable bacteria or its products from the gastrointestinal tract to normally sterile tissues, including mesenteric lymph nodes and other internal organs (i.e., the spleen) (63, 64). The major mechanisms promoting bacterial translocation are intestinal bacterial overgrowth, deficiencies in host immune defenses by disturbed gut integrity, and increased permeability or mucosal injury (65). It was reported that ETEC F18 clearly increased bacterial translocation from the intestinal lumen to the mesenteric lymph nodes of weaned pigs (66). Collectively, ETEC infection negatively impacts tight junction integrity, and increases paracellular movements of molecules, thus inducing inflammatory responses and diarrhea in pigs.

Immune responses of pigs during ETEC infection

In addition to the physical barrier function of the mucus, the mucosal immune system constitutes an extensive and highly specialized innate and adaptive immune system to protect the host against potential insults from the environment (67). When inflammation occurs in the intestines, the robust innate immune responses are first observed by the marked elevations in the production of inflammatory mediators, including IL-1β, IL-6, and IL-8, which further promote leukocyte accumulation and survival in the inflamed sites (68). The recruited neutrophils and activated macrophages are responsible for the elimination of pathogens and stimulating systemic inflammation and acute-phase reaction (69, 70). ETEC F18 that expressed LT, STa, and Shiga-like toxins remarkably induced the recruitment of neutrophils and macrophages in the ileum of weaned pigs during the peak of infection (71). Consistently, the up-regulated expression of genes encoding inflammatory mediators (e.g., *COX2*, *IL1B*, *IL6*, *IL7*, and *TNF*) were also observed in the ileal mucosa of ETEC F18 infected piglets (40). LPS, the major component of the outer member of ETEC, are highly involved in the activation of innate immunity, as indicated that ETEC F18 increased the mRNA expression of LPS binding protein and *MyD88* in ileal mucosa of

TABLE 1 Enterotoxigenic *Escherichia coli* (ETEC) altered the expression of tight junction proteins in the small intestine of pigs *in vivo* or epithelial cells *in vitro*.

Pathogen	Pig age	Tight junction proteins/outcomes	Reference
ETEC K88	35 d	Reduced protein expression of *occludin* and *claudin* in ileum	Ewaschuk et al. (53)
ETEC K88		Altered the distribution of *ZO-1* and *claudin* in porcine Caco-2 cells (fluorescence microscopy analysis)	Yu et al. (54)
ETEC K88	35 d	Reduced mRNA expression of *occludin* in jejunum; reduced expression of *ZO-1* in jejunum and ileum	Gao et al. (55)
ETEC K88	18 d	Reduced mRNA expression of *occludin* in jejunum	Yang et al. (56)
ETEC K88		Reduced mRNA expression of *ZO-1*, *claudin*, and *occludin* in porcine IPEC-J2 cells; Reduced protein expression of *claudin* and *occluding* in porcine IPEC-J2 cells	Wu et al. (57)
ETEC K88	36 d	Reduced protein expression of *ZO-1* and *occludin* in jejunum	Yang et al. (58)
ETEC K88	36 d	Reduced protein expression of *ZO-1* in jejunum	Li et al. (59)
ETEC F18	39 d	Reduced mRNA expression of *claudin* in jejunum	Kim et al. (40)
ETEC F18	37 d	Reduced mRNA expression of *claudin* in ileum	Li et al. (60)
ETEC F18	38 d	Reduced mRNA expression of *ZO-1* and *occludin* in ileum	Becker et al. (61)

pigs (72). In addition, flagellin, a globular protein in the flagella of ETEC, is also involved in the activation of intestinal immune responses by stimulating IL8 expression in the ileal mucosa (72, 73). Several pathways may be involved in the process of ETEC infection, as the NF-κB and MAPK pathways are particularly important to stimulate downstream inflammatory responses (72, 74–77).

Systemic inflammation could be evoked due to Gram-negative sepsis (78, 79). ETEC F18 infection could induce systemic inflammation, as indicated by the gradually increased total white blood cell counts, neutrophils, and lymphocytes in the blood circulation of infected pigs (71, 80, 81). Systemic levels of pro-inflammatory cytokines (e.g., TNF-α) and acute phase proteins (C-reactive protein and haptoglobin) were also elevated accordingly after ETEC F18 or K88 infection (71, 82–84). The peak of systemic inflammation in weaned pigs appears on day 2 to 7 post-ETEC challenge depending on the severity of infection, while it usually disappears or cannot be detected after day 14 post-infection.

Intestinal microbiota changes during ETEC infection

Intestinal microbiota plays pivotal roles in maintaining the nutritional, physiological, and immunological function of the intestine (85, 86). The pig intestine harbors a very complex and diverse microbial community, which shifts along the intestinal tract and changes by age, diet, and many other factors (87). The colonization of intestinal microbiota in pigs is initiated at birth but still develops at the weaning stage (88). Thus, the intestinal microbial composition in newly weaned pigs could be easily disrupted due to weaning stress and dietary changes, making the pigs more susceptible to pathogenic bacteria (89).

Although *E. coli* is one of the first bacteria to colonize in the intestine of piglets at birth, it is phased out after weaning (90). ETEC infection or increased abundance of *E. coli* during the post-weaning stage could impact intestinal microbiota. Bin et al. (91) reported that ETEC K88 infection reduced microbial diversity and the Bacteroidetes : Firmicutes ratio in jejunum and feces in weaned pigs. Bacteroidetes and Firmicutes are the most dominant intestinal microbial phyla in young pigs, which cooperatively utilize carbohydrates in the gut (92). The reduced fecal Bacteroidetes : Firmicutes ratio is used as a biomarker for intestinal dysbiosis and was also observed in pigs with other types of diarrheal diseases (93, 94). Significant changes in community structure were also reported in many ETEC F18 or K88 infection cases. Pigs challenged with ETEC F18 or K88 were reported to have increased relative abundance of Proteobacteria family in the ileum or colon by increasing *Escherichia-Shigella* or *Helicobacteraceae* (91, 95–97). A reduced relative abundance of *Lactobacillus* was observed in the ileum of weaned pigs when challenged with ETEC F18 (97). The disturbance of intestinal microbiota by ETEC infection further shifts the intestinal ecosystem to be more favorable for the growth of pathogens and reduces the production of volatile fatty acids in the large intestine (97–99). Many of these microbiota changes were reported to be negatively correlated with growth performance and the overall intestinal health of weaned pigs (100, 101).

In-feed antibiotics

In-feed antibiotics were one of the most powerful substances to prevent and treat bacterial infections in food-producing animals. In swine production, the use of antibiotics at intermediate or therapeutic levels served many purposes, including 1) treating sick animals, 2) preventing diseases by mass treatment of the entire population, 3) reducing the negative impacts of stresses, namely weaning stress, and 4) promoting growth. The potential mechanisms of action of antibiotics target different anatomical parts of bacteria. First, antibiotics could induce a lethal malfunctioning of the bacterial cell wall synthesis. The presence of penicillin-binding proteins (PBPs) is critical for proper bacterial cell wall assembly (102). However, PBPs are the main targets of β-lactam and glycopeptide antibiotics in order to inhibit bacterial cell wall synthesis. More specifically, β-lactam agents target PBPs, and their interaction could lead to failure in the synthesis of new peptidoglycan and lysis of bacterium (103). Second, antibiotics could inhibit protein biosynthesis in the ribosomes of bacterial cells. The bacterial ribosome (70S) is composed of two ribonucleoprotein subunits, 30S and 50S subunits, with each performing different functions (104). Some antimicrobial agents, like aminoglycosides and tetracyclines, target the 30S subunit by either preventing the binding of the mRNA to the ribosome or inducing misreading and premature termination of translation of mRNA (105). Chloramphenicol, macrolides, and oxazolidinones antibiotics are the major inhibitors of the 50S subunit. They can prevent the binding of aminoacyl-tRNA to the mRNA-ribosome complex or inhibit the formation of complete peptide chains by targeting the conserved sequences of the peptidyl transferase (106). Third, antibiotics can inhibit bacterial DNA replication. Quinolone antibiotics are the major DNA replication inhibitors. They can inhibit bacterial nucleic acid synthesis by disrupting topoisomerase and DNA gyrase, two critical bacterial enzymes that regulate the chromosomal supercoiling required for DNA synthesis (107). The disturbance of these enzymes can break bacterial chromosomes and cause rapid bacterial death (108). Fourth, antibiotics can inhibit folic acid metabolism. Folate is a cofactor for many enzymes that are required for DNA and RNA biosynthesis and amino acid metabolism in bacteria (109). Sulfonamides and trimethoprim interrupt folic acid synthesis and ultimately disturb the synthesis of purines and thus DNA

biosynthesis (110). A combination of sulfonamides and trimethoprim has shown synergistic antibiotic activities because they target distinct steps in folic acid metabolism (111).

As reviewed by Cromwell (112), in-feed antibiotics improved growth rate by an average of 16.4% and improved the efficiency of feed utilization by 6.9% of young pigs from 7 to 25 kg body weight. Moreover, the inclusion of antibiotics in feed dramatically reduced the mortality of young pigs (3.1%) under high-disease conditions and environmental stress when comparing with non-antibiotic-treated pigs (15.6%) (113). Therefore, approximately 70% of the swine farms in the United States used a wide variety of antibiotics in nursery diets over the past 20 to 30 years before 2015 (5). Several commonly used antibiotics and their effects are summarized in Table 2. However, the potential risks of antibiotic resistance and contamination and the adverse health effects of trace amounts of antibiotics in humans and animals have been increasingly recognized as global health concerns (124). Therefore, effective alternative practices to strengthen the disease resistance of animals are greatly needed.

Nutritional intervention

Research on exploring alternatives to antibiotics is growing and has been reviewed by Pettigrew (125), Lallès et al. (126), Heo et al. (127), and Liu et al. (128). Many nutritional interventions have been widely applied to weanling pigs to enhance their disease resistance and growth performance. Although their exact protective mechanisms may vary and are still not completely understood, one or more following functions may be involved: (1) to favorably affect the characteristics of feed (2), to satisfy the nutritional needs of animals without any adverse effects, or (3) to favorably impact animal production and performance, particularly by regulating gut microbiota, intestinal immunity or digestibility of nutrients.

Zinc oxide

Zinc is an essential micro-mineral required in trace amounts in animal feed. Zinc performs broad types of functional roles, including (1) structural roles in forming components of organs and tissues, (2) physiological roles in maintaining homeostasis, (3) catalytic roles in regulating enzymes and endocrine systems, and (4) regulatory roles in cellular replication and differentiation (129). Zinc also plays a central role in the immune system, as it is crucial for the development and function of immune cells, the production or biological activity of cytokines, and the regulation of T and B cell signaling (130–132). Zinc deficiency affects many aspects of innate and adaptive immunity. Acute zinc deficiency can cause decreased innate and adaptive immune responses, while chronic zinc deficiency is highly associated with many diseases and inflammation (133).

Zinc is commonly added to the nursery diet at pharmacological levels to promote performance and control post-weaning diarrhea (134–136). Numerous studies also reported that supplementation of a high dose of zinc in the form of zinc oxide (ZnO) enhanced disease resistance of weaned pigs against ETEC infection. For instance, the inclusion of 2,880 mg/kg of ZnO reduced the incidence of diarrhea and boosted the recovery of pigs from ETEC F4 infection (119). Kim et al. (118) also reported that 2,400 mg/kg of ZnO administration enhanced average daily gain, reduced diarrhea and fecal shedding of *E. coli*,

TABLE 2 In-feed antibiotics on ETEC infection of weaned pigs.

Pathogen	Antibiotics	Outcome	Reference
ETEC K88	Chlortetracycline+ sulfamethazine+ penicillin	Enhanced immunological responses, improved intestinal morphology	Nyachoti et al. (114)
ETEC K88	Colistin sulfate+ olaquindox	Enhanced feed efficiency, reduced diarrhea	Pan et al. (115)
ETEC K88	Apramycin+ tiamulin+ sulfathiazole+ bacitracin methylene disalicylate	Enhanced growth performance, improved systemic immune responses	Lee et al. (116)
ETEC K88	Colistin	Reduced mortality, reduced diarrhea	Trevisi et al. (117)
ETEC K88	Apramycin	Reduced fecal shedding of ETEC	Kim et al. (118)
ETEC K88	Carbadox	Enhanced growth performance, reduced fecal shedding of ETEC	Owusu-Asiedu et al. (119)
ETEC F18	Chlortetracycline+ tiamulin	Reduced diarrhea, improved systemic immune responses	Hong et al. (120)
ETEC F18	Carbadox	Enhanced growth performance, reduced diarrhea, enhanced intestinal integrity	He et al. (121) Kim et al. (122) Kim et al. (123)

and improved small intestinal morphology in weaned pigs challenged with ETEC F4. Other beneficial effects of pharmacological zinc included the enhancement of intestinal integrity (137), restoration of the injured intestinal mucosa (138), reduction of intestinal permeability by enhancing the expression of tight junction proteins (139), and improvement of intestinal immunity (140) in ETEC-infected pigs. The potential mechanisms of action of high dose ZnO in reducing post-weaning diarrhea include but are not limited to: 1) inhibiting pathogen viability and 2) modulating the intestinal microbial population. Roselli et al. (141) demonstrated that *in vitro* ZnO treatment may protect intestinal epithelial cells from ETEC F4 infection by inhibiting the adhesion and internalization of bacteria. Supplementation of 2,500 mg/kg of ZnO *in vivo* helped to stabilize the microbial community while preventing pathogenic microbes proliferation during the first 2 weeks of post-weaning (142).

Although pharmacological ZnO is very effective in preventing post-weaning diarrhea, its environmental impact is significant and increases public health and safety concerns. Recent research demonstrated that supplementation of pharmacological ZnO may induce the excessive accumulation of zinc in animal tissues, including kidney, liver, and pancreas (143, 144). The overload of ZnO might also contribute to the acquisition and spread of antibiotic resistance genes in pigs (145–147). Therefore, the use of pharmacological ZnO in piglet diets was banned in the European Union from June 2022.

Direct-fed microbials

Direct-fed microbials (DFM) are live microorganisms that confer a health benefit on the host, when administered in adequate amounts (148). There are 3 main categories of DFM, including *Bacillus* (Gram-positive spore-forming bacteria), lactic acid-producing bacteria (e.g., *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, etc.), and yeast (149). The beneficial effects of DFM on the host may be attributed to several mechanisms, including but not limited to: (1) production of antimicrobial products, (2) regulation of gut microbial profile, (3) immunomodulation, and (4) enhancement of epithelial gut barrier function (150, 151).

Bacillus-based DFMs are spore-forming bacteria. They are thermostable for feed storage and processing (e.g., pelleting and extrusion) and are able to survive at low pH in the stomach (152). Some common species of *Bacillus* include *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus pumilus*, *Bacillus amyloliquefaciens*, *Bacillus anthracis*, and *Bacillus cereus*, in which *Bacillus anthracis* and *Bacillus cereus* are known to be pathogenic to humans and animals (153). *Bacillus* spp. can be isolated extensively from plants and their rhizosphere (soil in the vicinity of plant roots) and can also be found in other environments (154). *Bacillus* spp. were characterized as mesophilic and neutrophilic bacteria that can survive and germinate in the gut, form biofilms, and secrete antimicrobials (155, 156). A variety of *Bacillus*-based supplements have been found to promote growth, feed utilization, and intestinal health of pigs (157–159). The potential mechanisms of action of *Bacillus* spp. against ETEC infection include: 1) modulating the host immune responses by regulating the expression of major cytokines that are involved in initiating and regulating immune responses (160), 2) enhancing the expression of tight junction proteins (161), and 3) and promoting the growth of beneficial microbes and overall gut health of the host (162). Our previously published research reported that dietary supplementation of 2.56×10^9 CFU/kg of *Bacillus subtilis* (DSM 25841) enhanced disease resistance and growth performance and reduced diarrhea of weaned pigs infected with ETEC F18 (40, 121). Pigs fed with *Bacillus subtilis* also strengthened intestinal integrity and barrier function, as indicated by reduced transcellular and paracellular permeability and enhanced gene expression of tight junction protein, *ZO1*. In addition, the same *Bacillus subtilis* (DSM 25841) strain was able to reduce the incidence and severity of diarrhea in weaned pigs infected with ETEC F4 (163). Supplementation of *Bacillus subtilis* DSM 25841 was also observed to reduce cecal *Enterobacteriaceae* level, up-regulate the expression of gene sets related to immunity, and improve amino acids metabolism and utilization in jejunal mucosa (163).

Lactic acid-producing bacteria administration can modulate intestinal microbial profiles by competing for the binding sites on the intestinal epithelial cells with pathogens, or by producing microbicidal substances that inhibit or kill pathogens (164–166). *Lactobacillus plantarum* is a widespread strain that can be produced by plant fermentation or directly isolated from the gastrointestinal tract of healthy humans or animals. Lee et al. (116) and Yang et al. (56) demonstrated that supplementation of *Lactobacillus planatrum* (10^{10} cfu/kg of CJLP243 or 5×10^{10} cfu/kg of CGMCC 1258, respectively) enhanced growth performance and reduced diarrhea of weaned pigs challenged with ETEC F4. Pigs fed with *Lactobacillus planatrum* also had enhanced intestinal morphology, reduced fecal shedding of ETEC, or reduced adhesion of ETEC to the small intestinal mucosa. Another study reported that *Lactobacillus plantarum* (CCFM1143 or FGDLZ1M5; 5×10^{10} cfu/kg, respectively) supplementation reduced the relative abundance of *Bacteroidetes* and *Enterobacteriaceae* in feces and increased the concentration of total short-chain fatty acids in the cecum of ETEC infected pigs (99). Consistently, *Lactobacillus rhamnosus* (ACTT 7469; 10^{10} cfu/day or 10^{12} cfu/day) administration ameliorated ETEC F4-induced diarrhea and reduced pathogenic coliform shedding in feces, possibly due to its ability to increase the number of *Lactobacilli* and *Bifidobacteria* in feces (167). Some research also reported that supplementation of lactic acid-producing bacteria could regulate intestinal mucosa immunity and stimulate the immune system

of the host (168, 169). A previous *in vitro* study reported that *Lactobacillus planatrum* (299v) could increase the mRNA expression of *MUC2* and *MUC3* in HT 29 intestinal cells, thus, inhibiting the adherence of enteropathogenic *E. coli* to the intestinal cells (170). Moreover, lactic acid-producing bacteria contribute to an acidic environment in the gastrointestinal tract, which partly alters the growth of pathogenic microorganisms, including *E. coli* (171). Therefore, lactic acid-producing bacteria is another common type of DFMs used in weaned pigs to promote intestinal health.

Yeast consists of a broad range of products, including whole live yeast cells, heat-treated yeast cells, ground yeast cells, purified yeast cell cultures, and yeast extracts. The efficacy of yeast-based products varies depending on their forms (128). The majority of the dry weight of the yeast cell wall is polysaccharides, with α-D-mannan and β-D-glucan as the major components. These polysaccharides have been recognized for their immune-regulatory activities through specific interactions with different immunocompetent cells (172). In particular, α-D-mannan in yeast was reported to bind to mannose-specific receptors that are present in many pathogenic bacteria, including *E. coli* and *Salmonella* spp., thus, inhibiting the adhesion of these pathogens to the mannose-rich glycoproteins lining the intestinal lumen (173). Growing evidence supports the immunostimulatory benefits of β-D-glucans, as it could stimulate the activity of macrophages and neutrophils *via* binding to their receptors (174). *Saccharomyces* spp. are the most studied yeast species for controlling intestinal disorders in young animals due to their remarkable immune-regulatory properties (175, 176). The beneficial effects of live *Saccharomyces cerevisiae* yeast on controlling diarrhea and reducing mortality of weaned pigs infected with ETEC F4 were reported by Trevisi et al. (117). The results of gene expression profiles in jejunal mucosa indicated that supplementation of *Saccharomyces cerevisiae* yeast modified the expression of genes related to mitosis, mitochondria development, metabolic process, and transcription in ETEC-infected pigs (177).

Prebiotics

Prebiotics were originally defined as 'non-digestible food substances that selectively stimulate the growth of favorable species of bacteria in the gut, thereby benefitting the host' by Gibson and Roberfroid (178). This definition has been expanded by including three broad criteria: (1) resistance to gastric acid and hydrolysis by mammalian enzymes and gastrointestinal absorption; (2) ability to be utilized by the gastrointestinal microbiota; and (3) selectively stimulate the growth and/or the activity of intestinal bacteria associated with health-promoting effects (179). The best-characterized prebiotics are non-digestible oligosaccharides, including inulin, lactulose, pyrodextrins, fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), xylo-oligosaccharides, transgalactooligosaccharides, and isomalto-oligosaccharides (180).

The most striking effect of prebiotics is their ability to reshape the composition of gut microbiota in the host. Prebiotics can boost the production of health-promoting bacteria, such as lactic acid-producing bacteria, which can further inhibit the growth of enteric pathogens (e.g., *E. coli*, *Campylobacter*, *Salmonella* spp.) and/or attenuate their virulence (181, 182). The enhancement of the beneficial bacteria population by adding prebiotics could indirectly affect the immunity of the host. In addition to that, prebiotics *per se* can directly interact with intestinal cells, including epithelial cells, goblet cells, or immune cells (183, 184). This interaction may trigger the downstream benefits, as indicated by more mucin production (185), strengthened gut barrier functions (186), or enhanced inflammatory responses (187, 188). Many studies have confirmed the beneficial effects of prebiotic supplementation in weaned pigs challenged with ETEC. For example, supplementation of 2.5 g/kg of FOS extracted from plants can improve growth performance and gut health of pigs infected with ETEC F4 (189). Specifically, pigs supplemented with FOS reduced plasma IL-1β and TNF-α, and improved small intestinal morphology against ETEC F4. Moreover, FOS administration also elevated mRNA expression of duodenal and jejunal *ZO-1* and ileal *occludin*, but down-regulated *TNF-α* and *IL-6* in the small intestine. These results indicated that supplementation of FOS was associated with suppressed inflammatory responses and improved intestinal barrier functions. Luo et al. (190) also observed that dietary supplementation of FOS attenuated the intestinal mucosa disruption in ETEC-infected pigs by increasing their anti-oxidative capacity and intestinal barrier functions. GOS, one of the main bioactive compounds in human milk, was well studied in humans as it supports the colonic health of breast-fed infants (191). GOS exhibited *in vitro* antimicrobial effects on ETEC F4 by inhibiting the adherence of the F4 strains to porcine intestinal mucins (192). This observation suggests that GOS may serve in the prophylaxis of ETEC infection. β-glucans originated from different sources (cereal grains, yeast, or algae) also show prebiotic properties (193). Stuyven et al. (194) reported that β-glucans extracted from yeast reduced the colonization of ETEC F4 to the small intestine, thus alleviating diarrhea of weaned pigs. However, the immune-modulatory activity of β-glucans was more attractive and well-studied in humans and animals. Our previously published research observed that supplementation of algae-derived β-glucans enhanced gut integrity, reduced intestinal paracellular permeability, and boosted intestinal and systemic immune responses in weaned pigs infected with ETEC F18 (81). This study also suggests that dectin, a major β-glucan receptor expressed on many immune cells (e.g., macrophages), is potentially involved in the immune-regulatory effects of β-glucans, thus protecting the host from the ETEC infection (81, 195).

Phytochemicals

Phytochemicals include a large variety of secondary plant metabolites that are naturally derived from plant materials or directly synthesized (e.g., polyphenols, terpenoids, carotenoids, limonoids, flavonoids, catechins, anthocyanidins, indoles, ethnobotanicals, etc.) (196). Phytochemicals exhibited broad biological properties, including antimicrobial, antioxidant, anti-inflammatory, and antiviral effects (197–200). Notably, many phytochemicals display broad-spectrum antibacterial activities against Gram-negative and Gram-positive bacteria (201–203). The antimicrobial mechanism of action varies due to the sources and extraction methods of phytochemicals. Based on the literature view, several potential antimicrobial mechanisms were proposed. First, many plant-derived essential oils could destabilize the phospholipid bilayer, causing the loss of permeability, leakage of intracellular constituents (e.g., ions, proton), and even the coagulation of cytoplasm (204, 205). Second, some phytochemicals contain a high proportion of phenolic compounds that possess strong antibacterial properties by inhibiting the efflux pump (206). Third, phytochemicals could disrupt the enzymes involved in the synthesis, replication, repair, and transcription procedures of virulent bacteria (207). Fourth, certain active components in phytochemicals may prevent the development of adhesion formation (208, 209) and inhibit bacterial adhesion (210, 211).

The anti-inflammatory effects of phytochemicals have also been widely reported with *in vitro* and *in vivo* models. For example, phytogenic compounds (e.g., crude extracts, phenolics, triterpenoids, polysaccharides, saponins, lectins) obtained from fruits, vegetables, and food legumes could suppress the production of inflammatory markers (e.g., C-reactive protein, IL-1, IL-6, TNF-α) or major inflammatory mediators (e.g., NO, iNOS, COX2, PGE_2) in human intervention studies and *in vitro* cell models (212, 213). Essential oils from clove, pine, tea, garlic, cinnamon, and other compounds also possess anti-inflammatory activities that were observed *in vitro* (214, 215) and in livestock, fish, and poultry (216, 217). The anti-inflammatory mechanisms of action have not been completely understood in phytochemicals, and some research indicates that the *in vitro* anti-inflammatory or *in vivo* immune-modulatory effects are partially mediated by blocking the NF-κB activation pathway (218, 219). Other potential modes of action include inhibiting lipoxygenase and cyclooxygenase, two important enzymes in the activation of inflammatory responses (220–222).

The effects of phytochemicals on ETEC F18 and F4 infection have been evaluated in many *in vivo* pig studies. Dietary inclusion of 10 mg/kg of capsicum oleoresin, garlic botanical, or turmeric oleoresin reduced diarrhea and enhanced disease resistance of weaned pigs infected with ETEC F18 (71). Pigs fed with phytochemicals developed better intestinal health, as indicated by higher villi height, lower immune cell accumulation, and milder intestinal inflammation than infected control. The further microarray analysis confirmed that feeding these phytochemicals enhanced the integrity of membranes, especially tight junction-related genes in ileum of weaned pigs (72). In addition, the reduced intestinal inflammation by feeding phytochemicals was also observed at the transcriptional level, as indicated by the down-regulation of genes in the categories of responses to stimulus, antigen processing and presentation, and inflammatory mediators in ileal mucosa (72). Devi et al. (223) reported that supplementation of a 0.05% phytogenic combination, including clove, cinnamon, and fenugreek, improved weight gain and apparent total tract digestibility in pigs under ETEC F4 infection. Likewise, the chestnut extract containing hydrolyzable tannins was reported to reduce diarrhea and enhance the growth performance of pigs challenged with ETEC F4 (224). Cranberry supplementation in feed (10 g/kg) or *via* drinking water (1 g/L) significantly reduced the diarrhea severity of ETEC F18-infected pigs (225).

Lysozyme

Lysozyme is a naturally existing antimicrobial enzyme that can be found in blood, liver, and many bodily secretions. It cleaves 1,4-β-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine in the peptidoglycan layer of the bacterial cell wall, thus inducing cell death (226). Lysozyme is part of innate immunity and plays an important role in limiting bacterial overgrowth at mucosal surfaces. Recent research suggests lysozyme could modulate the host immune responses to infection (227, 228). The lysozyme-mediated degradation and lysis of bacteria enhance the release of bacterial products, such as bacterial peptidoglycans, which further regulate the immune response in the host (229). However, the location of lysozyme activity, the susceptibility of bacterial peptidoglycans to lysozyme digestion, and the amount and composition of bacterial products can all modulate the degree and extent of pro-inflammatory immune responses. Thus, lysozyme could enhance or dampen the innate immune response (229). Other research also reveals that lysozyme may contribute to resolving intestinal inflammation *via* restricting bacterial growth, assisting in intestinal epithelial barrier protection, and reducing phagocyte influx and concomitant cellular inflammatory responses (227, 229, 230).

Lysozyme is one of the suitable alternatives to replace antibiotic growth promoters in swine production. Lysozyme derived from chicken eggs was reported to improve growth performance of weaned pigs, with its efficacy comparable to neomycin/oxytetracycline (101), carbadox/copper sulfate (214), or chlortetracycline/tiamulin hydrogen fumarate (231). Growing evidence also supports that the administration of lysozyme could enhance the disease resistance of weaned pigs against ETEC infection. For instance, Nyachoti et al. (114) reported that supplementation of lysozyme sourced from egg white

improved intestinal development, decreased ETEC counts in the intestinal mucosa, and reduced serum pro-inflammatory cytokines in weaned pigs infected with ETEC F4. Garas et al. (232) observed that feeding lysozyme-rich goat milk reduced the incidence of diarrhea and significantly suppressed total bacteria translocation into the mesenteric lymph nodes in pigs infected with ETEC F4. Supplementing lysozyme-rich milk also reduced the relative abundance of fecal *Enterobacteriaceae* family, in which many prevalent enteric pathogens (e.g., *E. coli* and *Salmonella*) belong to. Similarly, pigs fed with human lysozyme-rich milk had a higher survival rate and reduced diarrhea when they were challenged with ETEC F4 (233). The enriched relative abundance of *Lactobacillus* in feces and enhanced intestinal integrity and mucosa immunity were also observed in these pigs (233).

Conclusions

ETEC is one of the most predominant causes of post-weaning diarrhea in pigs. In-feed antibiotics and pharmacological ZnO were routinely added to the nursery diet to prevent diarrhea and to increase the survival rate of newly weaned pigs. However, the heavy use of medically important antimicrobials in food-producing animals induces the development and spread of antimicrobial resistance. The resistance results in the loss of effectiveness of these drugs as antimicrobial therapies, which poses a serious threat to public and animal health. The significant environmental impacts and public concerns are also highly recognized in the application of high-dose ZnO in pig feed. Thus, the exploration of alternative practices that may help reduce the reliance on antimicrobial drugs and pharmacological ZnO and address animal health needs is warranted. Accumulating evidence has confirmed the importance of nutritional interventions, including modified feeding strategies and nutrient supplements, in the control of diarrheal disease caused by ETEC. Several categories of feed additives are widely applied to nursery pigs to assist in enhancing intestinal barrier function and immunity, balancing intestinal microbiota diversity, and promoting overall health and performance. Although no single substance can fully replace the functions of in-feed antibiotics and high-dose ZnO so far, their beneficial effects on pig health and welfare are promising. Future research should focus on the development of fundamental knowledge on defining healthy gut and robust intestinal function of pigs by adopting novel approaches. Understanding the interaction of host-microbiome-nutrition is also extremely important to exploring the mechanisms of new nutritional interventions.

Author contributions

Conceptualization: KK, YL, PJ. Writing original draft: KK. Reviewing and editing: MS, YL, and PJ. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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