# *Escherichia coli* pathotypes involved in swine colibacillosis: PCR detection, fecal prevalence, and antimicrobial susceptibilities

by

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# Abstract

Colibacillosis in swine, which includes neonatal enteritis, postweaning diarrhea, and edema disease, is one of the major economically important diseases. The pathotypes of *E. coli* involved are enterotoxigenic (ETEC), enteropathogenic (EPEC), enteroaggregative (EAEC), and Shigatoxigenic (STEC) *E. coli*. Our objectives were to determine the effects of *in-feed* or *in-water* chlortetracycline (CTC) administration on prevalence of virulence genes and pathotypes implicated in colibacillosis and to compare phenotypic and genotypic susceptibilities to CTC.

A total of 1,296 weaned piglets (21 days age) were used in a 35-d study. Piglets were allocated to 48 pens (27 per pen) and pens were assigned randomly to no CTC, *in-feed* CTC, or *in-water* CTC groups. The CTC was provided from day 0 to 14. Fecal samples were collected from 5 piglets from each pen on days 0, 14 and 28. Samples were enriched in *E. coli* broth and subjected to a 11-plex PCR assay to detect major virulence genes targeting the four pathotypes and to a culture method to isolate and identify the pathotypes. Isolates were subjected to the CTC susceptibility testing by micro-broth dilution and major *tet* genes were determined by PCR.

The fecal prevalence of the virulence genes and the pathotypes were not affected by CTC administration ($P < 0.05$). The predominant enterotoxin genes in pure culture were *astA* (enteroaggregative heat stable, 29%) and *estB* (heat stable enterotoxin B; 21.9%). Shiga toxin genes were detected only in day-28 fecal samples and the *stx*2 gene was more predominant than *stx*1. The pathotypes of *E. coli* isolated were ETEC, atypical EPEC (positive for intimin and enterotoxin genes), STEC and ETEC and STEC hybrids. Although the samples were positive for enteroaggregative enterotoxin gene (*astA*), EAEC pathotype was not detected. All isolates were resistant to CTC and *tet*A gene (91.5%) was the most predominant.

*In-feed* or *in-water* CTC administration had no effect on the fecal prevalence of virulence genes and pathotypes implicated in swine colibacillosis and all the tested isolates showed phenotypic and genotypic resistant to CTC.

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# Dedication

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# Chapter 1 - Swine colibacillosis: Pathogenesis, prevalence, serogroups, and control measures

## Introduction

Swine colibacillosis is a common and economically significant disease in neonatal and weaned piglets. Three forms of enteric disease, neonatal enteritis, post weaning diarrhea, and edema disease together constitute swine colibacillosis. Infection of animals occurs at an early age (neonatal diarrhea) and after weaning (PWD) (Luppi et al., 2016). Generally, the occurrence of disease was noticed within the first 14 days after weaning. The major enteric pathogen of weaned piglets responsible for causing post weaning diarrhea (PWD) and edema disease (ED) is *Escherichia coli*. Enterotoxigenic *E. coli* (ETEC) is the main causative agent for PWD (Fairbrother et al., 2005; Rhouma et al., 2017). The two major virulence factors of ETEC are adhesins and enterotoxins. Adhesins facilitate binding and colonization of pathogens to the host intestinal epithelium, and enterotoxins are responsible for fluid secretion (Nagy et al., 2005; Rhouma et al., 2017). Adhesins are expressed in terms of fimbriae, and the major fimbriae associated with PWD are F4 (K88) and F18 (Luppi et al., 2016; Rhouma et al., 2017). Further colonization of this fimbriae is mediated by F4 and F18, specific receptors located on small intestines. So, the presence of fimbriae and active functioning of these fimbrial receptors play a vital role in determining the swine ETEC infections (Nagy et al., 2005; Rhouma et al., 2017). After initial attachment, ETEC produces enterotoxins, such as heat-labile toxin (LT), heat-stable toxin (STa, STb), and/or enteroaggregative heat-stable toxin 1 (EAST1) as shown in Figure 1.1. The major serogroup of swine associated with PWD of ETEC is O149, which is common with

the combination O149: LT: STa: STb: EAST1:F4ac (Fairbrother et al., 2005; Rhouma et al., 2017).

Among food borne disease causing pathogens, Shiga toxin-producing *Escherichia coli* (STEC) plays a key role and comprises of serologically diverse group of pathogens that causes diseases in humans and animals (Fratamico et al., 2004; Remfry et al., 2021). One of the major subsets of pathogenic intestinal *E. coli* strain is STEC, which can produce Shiga toxins (Stx) (Remfry et al., 2021). There are two types of Shiga toxins, Stx1 and Stx2, which are encoded by *stx*1 and *stx*2 genes respectively, carried on a prophage. These Shiga toxin genes share some amino acid homology; however, they differ in degree of cytotoxicity and antigenicity (Remfry et al., 2021). Among two types of Shiga toxins, Stx2 is more cytotoxic than Stx1. Further, each toxin is categorized into several subtypes. Stx1 toxin has three subtypes (Stx1a, Stx1c, Stx1d), and Stx2 toxin has seven subtypes (Stx2a, Stx2b, Stx2c, Stx2d, Stx2e, Stx2f, Stx2g) (Melton-Celsa et al., 2014). The host intestinal colonization and effacement lesions of STEC is mediated by cluster of genes located on a pathogenicity island, the locus of enterocyte effacement (LEE). One major gene among the cluster of genes is *E. coli* attaching and effacing gene (*eae*) that facilitates the binding of STEC to the host epithelial cells. This *eae* gene encodes for an outer membrane protein called Intimin (Remfry et al., 2021). Among 187 serogroups of *E. coli,* the STEC pathotype is in association with almost 158 serogroups (Valilis et al., 2018; Ludwig et al., 2020). Of which 7 serogroups (O26, O45, O103, 0111, O121, O145 and O157) are considered as top-7 STEC that causes major STEC infections including food borne outbreak (Fratamico et al., 2008; Cha et al., 2018; Remfry et al., 2020). Swine edema disease is caused by the Stx2e subtype of the Stx2 toxin (Fairbrother et al., 2005; Nyholm et al., 2015).

Along with the top-7 STEC serogroups, the majority of non-top-7 (O8, O59, O71, O86, O100, O163, O174 and O184) serogroups have been isolated from swine feces (Tseng et al., 2014; Cha et al., 2018). In a recent study, it was observed that there was an increased prevalence of both the top-7 serogroups (O26, O121, and O157) and non-top-7 serogroups (O8, O86, O91, O100, O174) in finisher pigs (Remfry et al., 2020). The common etiological agents of diarrhea are diarrhoeagenic *E. coli* strains. Among them, one of the pathotype is Enteropathogenic *Escherichia coli* (EPEC). Attachment of EPEC pathotype to intestinal epithelial cells of hosts leads to effacement of enterocyte microvilli, which then leads to attaching and effacing lesions. STEC strains of *E. coli* are also capable of producing attaching and effacing lesions. Based on the presence or absence of EPEC adherence factor, plasmids carrying the *bfp* operon the EPEC strains are further classified in to typical (tEPEC) and atypical (aEPEC) subgroups (Trabulsi et al., 2002). The World Health Organization in 1987 recognized twelve EPEC serogroups: O26, O55, O86, O111, O114, O119, O125, O126, O127, O128, O142, and O158, and that constitutes both typical and atypical EPEC strains (Trabulsi et al., 2002). Among EPEC strains, atypical EPEC (aEPEC) are more closely related to Shigatoxigenic strains and appear to be emerging pathogens (Trabulsi et al., 2002).

## Prevalence of pathotypes associated with swine colibacillosis

The pathogenicity of *E. coli* is attributed to the interplay of several virulence factors, among them O-serogroup is major and mainly associated with PWD and ED (Fair brother and Gyles, 2012). The study conducted in South Korea screened 362 *E. coli* isolates from the feces of weaned piglets over 9 years from 2008 to 2016 and detected 51 O-serogroups. Among them, 5 serogroups (O149, O139, O157, O182, and O14) were typeable isolates. The high prevalence of

O139, O157, and O149 was detected in accordance with other countries (Do et al., 2019). Serogroups O139 and O149 are corelated to ED and PWD, respectively (Gyles et al., 2010). The common primary serogroup detected across 9 years in South Korea was O149. It is the similar virulence factor responsible for causing PWD in other countries like China and Canada (Chen et al., 2004). The predominant frequency of O149 and O139 indicates the risks of PWD and ED of weaned piglets in South Korea, respectively. Studies showed O157 and O8 are the primary serogroups detected in South Korea between 1995 to 1997 (Kwon et al., 1999). The prevalence of O157 gradually decreased, where only 9.1% of O157 isolates are detected from 2008 to 2010, and no single isolate screened positive for O157 from 2015–2016 in South Korea (Do et al., 2019). Studies show the shift in prevalence of primary serogroup from O157 to O139 during 9 years in South Korean swine farms. This indicates that the prevalence of serogroups fluctuates with time (Do et al., 2019).

Fimbriae are the virulence factors that facilitate the adherence of pathogenic *E. coli* to the weaned piglets. Among the fimbrial antigens, F4 and F18 are the most commonly detected fimbriae across countries such as China, Japan, Europe, and USA (Kusumoto et al., 2016; Luppi et al., 2016). The F18 (43.1%) was the predominant fimbriae followed by F4 (20.2%) in another study from South Korea (Do et al., 2019). Regarding the prevalence of enterotoxin genes, 44.9% of the isolates carried STb and it is the primary enterotoxin, followed by STa (33.4%) (Do et al., 2019). This is in accordance with the studies conducted with respect to screening of enterotoxin genes among diarrheic piglets from Japan and the USA, respectively (Zhang et al., 2007; Kusumoto et al., 2016). On the other hand, the predominant increase in prevalence of Stx2e was noticed from 8.0% to 35.1% (Baranzoni et al., 2016). This represents an increased rate of ED in South Korea. The major cause of edema disease in weaned piglets is due to the Stx2e toxin. The

clinical signs of swine colibacillosis varies according to the *E. coli* pathotypes. The enteropathogenic *E. coli* (EPEC) pathotype was dominant in South Korea during 1998, however, a shift in dominance towards ETEC (92.0%) followed by STEC (23.4%) was noticed during a swine study from 2008 to 2016 (Do et al., 2019). Antibacterial growth promoters (AGPs) are used in South Korea to combat *E. coli* infections, but the prohibition of usage of these AGPs (2007) might have aggravated the prevalence of ETEC and STEC types. In general, the pathogenicity of *E. coli* is attributed by combination of its various virulence factors like fimbriae (F4 and F18ac) in association with enterotoxins (LT, STa, STb) and the fimbriae (F18ab) with Stx. The exact reason for the shift in association is not known, but might be due to the usage of antibiotics. In this South Korea swine study, F18 non-typeable isolates (F18nt) were detected, and they are in association with ETEC and STEC pathotypes (Do et al., 2019). Studies showed new variants of F18 have been identified in Germany (Barth et al., 2011) and Korea (Byun et al., 2013).

A study from Europe on the prevalence of *E. coli* pathotypes have screened 339 *E. coli* isolates collected from weaned piglets suffering from PWD from swine farms. They have reported the prevalence of 52.5% *E. coli* isolates that have fimbriae in association with enterotoxin genes, out of which 94.9% are categorized under ETEC pathotype. Further, subtyping of fimbriae revealed the higher prevalence of F4 followed by F18 in cases of PWD. The results agree with the results from other countries, and this study confirms F4 and F18-ETEC are most concerned in causing PWD in piglets (Luppi et al., 2016).

A study conducted in the USA, where a total of 304 *E. coli* strains were isolated from feces of diarrheic piglets between 2004 to 2006 (South Dakota, North Dakota, Minnesota, and Iowa) and screened for *E. coli* pathotypes. These strains were screened for the toxin genes and

virulence factors associated with swine colibacillosis (F4, F18, F41, 987p and K99 fimbria; LT, STa, STb, Stx2, and EAST1 toxins; and AIDA-I, *paa* and *eae* adhesins). Among them, 175 isolates (58%) tested positive for one of the fimbrial genes. Both, F4 and F18 are predominant fimbrial genes and none of the isolates carried 987p. Among the enterotoxin genes, the higher prevalence of enterotoxins (LT, STa, STb) were detected and Stx2 toxin was found only in association with F18. In this study, they noticed the association of the EAST1 gene (34.9%) along with other enterotoxin genes and fimbriae of ETEC. However, non-fimbrial isolates carried at least one of the toxins or one adhesin gene (Zhang et al., 2007). The results of fimbrial prevalence in this study contrast with the previous study conducted in North Carolina where F18 is the predominant fimbria, followed by F4 (Post et al., 2000).

A study was conducted in the USA in 2020 where a total of 598 fecal samples from swine finishers are collected from 8 different states (Iowa, Minnesota, South Dakota, Nebraska, North Carolina, Oklahoma, Kansas, and Ohio). The enriched swine samples are initially screened for Shiga toxin genes (*stx*1, *stx*2) and intimin gene (*eae*) and reported the Prevalence of *stx*2 (65.1%), *stx*1 (25.9%), and *eae* (66.6%) respectively. The samples that were positive for any one of the Shiga toxins are subjected to multiplex PCR for the detection of top-7 serogroups (O26, O45, O103, O111, O121, O145, and O157) and O104 serogroup, as well as non-top-7 serogroups (O8, O20/O137, O59, O86, O91, O100, O120, and O174). Among the top-7 serogroups the higher frequency of O121 (17.6%), O157 (11.5%), and O26 (10.7%) was detected. However, none of the O157 isolates carried any of the Shiga toxin genes. Among non-top-7 serogroups the prevalence's are 88.6% of O8, 35.5% of O86, 24.1% of O174, 20.2% of O100, 15.6% of O91, 4.3% of O59, 4.2% of O120, and 3.2% of O20/O137. The higher prevalence of O8 and O86 was

reported across non-top-7 serogroups. Most of the non-top-7 isolates (95.4%) were Shiga toxin subtype *stx*2e (Remfry et al., 2021).

Enterotoxigenic *E. coli* and Shigatoxigenic *E. coli* are the most common etiological agents of swine and cause huge losses to the swine industry worldwide. The study conducted in the North-western part of peninsular Malaysia in 2013, where they have initially screened a total of 511 isolates, and 345 are *E. coli* isolates. Further, *E. coli* isolates were screened for virulence toxins (LT1, LT2, ST, *eaeA* and ST genes) and only 9 isolates carried the Shiga toxin gene and were categorized as Shigatoxigenic *E. coli.* The remaining isolates were negative for other virulent genes. This study indicates the only prevalence of STEC pathotype in Northern Malaysia (Ho et al., 2013). These study results are similar to a study from Thailand where a higher frequency of STEC (11.2%) is noticed in swine (Prapasarakul et al., 2010). However, previous studies reported ETEC as the most prevalent pathotype in the United States and all over the world (Fairbrother et al., 2005; Zhang et al., 2007). Further, STEC isolates are subjected to subtyping, and they subtype positive for *stx*2e, which is mainly responsible for causing edema disease in piglets. Despite carrying *stx*2e toxin, none of the piglets displayed clinical signs of edema disease. Previous studies reported the association of swine STEC with serogroups of O138, O139, and O141 (Han et al., 2007) but the study conducted in Malaysia has significantly identified O139 serogroup with the respective serotype O139:H1 that is in association with VTEC. This correlates with previous studies where O139:H1 serotype is more prevalent across the world and is responsible for causing PWD and edema disease (Fairbrother et al., 2005). The study also screened two other serotypes, O130:H26 and O168:H21. However, O130:H26 is not the common serotype because very few studies reported this serotype as it has only been isolated once from a diarrheic patient (Muller et al., 2007) and none of the study reported the O168:H21

serotype. In this study, fimbrial antigen F18 was most detected, and these findings are similar to the studies conducted in Slovakia (Vu Khac et al., 2006). They were contrast to the studies conducted in Untied States and Zimbabwe, where they have detected F4 as more prevalent (Zhang et al., 2007 and Madoroba et al., 2009). This indicates the divergence of fimbrial antigen types across various temporal regions. Studies reported that ETEC O139 serogroup in association with F18 caused PWD in Australia and edema disease in Europe (Fairbrother et al., 2005).

A study from South Africa has screened 263 *E. coli* isolates to determine the prevalence of pathogenic *E. coli* and its associated virulence factors. The highest prevalence of Enterotoxigenic *Escherichia coli* (40.3%) followed by Enteroaggregative *Escherichia coli* and Shigatoxigenic *Escherichia coli* was detected (Mohlatole et al., 2013). The study results correspond with the studies conducted in Australia (Do et al. 2005), Korea (Lee et al., 2009) and Czech Republic (Zajacova et al., 2011). This suggests that EAEC is also one of the major pathotype associated with swine colibacillosis (Zajacova et al., 2011). In this study, 17.5 % of EAEC isolates had only EAEC genes, but 10 isolates carried virulence genes of both the EAEC and ETEC pathotypes and two isolates with ETEC and STEC genes. Previous studies also reported that the EAST-1 toxin is not limited to EAEC pathotypes, but can also be found in ETEC and EHEC, which is due to the association of *astA* gene with the toxin genes of ETEC and EHEC isolates (Zajacova et al., 2011). This indicates the extent of threat accounted by EAEC towards swine health. In this study regarding the prevalence of enterotoxin genes, the predominantly detected gene was *estB,* followed by *estA,* and *elt* genes. This result is similar to the study conducted in Spain where they reported high prevalence of *estB* (78.38 %), *estA* (62.16 %), and *elt* (25.68 %) (Blanco et al., 2006). The swine *E. coli* screening study conducted in

China during 2003 to 2008 had a high prevalence of *stx*2*e* (67%), followed by *estA* (32%), and *estB* (13 %) (Wang et al., 2011). In Brazil, 300 diarrheic *E. coli* isolates were screened and found the prevalences of 71%, 44%, 47%, and 3% for *elt, estA, estB,* and *stx*2*e* genes, respectively (Vidotto et al., 2009). Comparative studies across different temporal regions demonstrate the variation in prevalence of virulence genes in different geographical locations, suggesting the vital role of geographical locations. In the South African swine study, none of the detected pathotypes ETEC, STEC, and EAEC are found in association with fimbriae. Similar results are also found in previous porcine studies (Do et al., 2005; Vidotto et al., 2009). It might be achieved due to administration of fimbriae vaccines and to mutations in the genetic elements, which lead the transformation from virulent to avirulent strains (Do et al., 2005). The prevalence of virulence genes and their association with fimbriae and serogroups are listed in a table (Table 1.1).

## Antibiotics used for treatment and prevention of swine colibacillosis

An outbreak of swine colibacillosis needs quick action, often resulting in the utilization of antibiotics for both treatment and prevention. More often, the decision regarding antibiotic selection lies with the swine veterinarian, who frequently relies on retrospective data from the diagnostic laboratories. These retrospective laboratory results provide basic information regarding qualitative susceptibility, whether the bacterial strain is susceptible, intermediate, or resistant to a specific antibiotic and quantitative susceptibility results (minimal inhibitory concentration, MIC) (Luppi, 2017). The categorization of all antibiotics into first, second, and third choice was introduced in a European country, the Netherlands, in 2017. Nonetheless, it is important to note that these classifications are subject to ongoing revisions. Generally, the first

choice of antibiotics is indicated for empirical treatment, whereas second choice of antibiotics are used in cases when first-choice antibiotics are ineffective. The third choice of antibiotics includes third and fourth generation cephalosporins, which are considered last-resort antimicrobials (Burch et al., 2008). The sick animals that exhibit clinical signs need to be administered antibiotics preferably by parental route because of the reduced feed and water intake. But when mortality occurs to all animals, the entire pen needs to be treated by metaphylactic approach. The list of antibiotics commonly employed in swine for the management of swine colibacillosis are shown in Table 1.2. The official journal of the European Union in veterinary medicine (2015/C 299/04) states the indication of metaphylactic antimicrobial approach only when there is need of treatment (http://www.ema.europa.eu/docs/en_GB/document_library/Report/2009/11/WC500008770.pdf).

The antibiotics should be selected in such a way that they need to reach the therapeutic concentrations in the intestinal lumen such as: β-lactam antibiotics (amoxicillin and the combination containing amoxicillin/clavulanic acid), cephalosporins (ceftiofur, cefquinome), aminoglycosides (apramycin, neomycin, gentamycin), aminocyclitols (spectinomycin), sulphonamide combined with trimethoprim (such as trimethoprim/sulphamethoxazole), fluoroquinolones (enrofloxacin, marbofloxacin and danofloxacin), quinolones (flumequine), and polymyxins (colistin sulphate) (Fairbrother et al., 2012).

β-lactam antibiotics (such as penicillins, amoxicillin, cephalosporins, monobactams, and carbapenem) exhibit their mechanism of action by inhibiting peptidoglycan synthesis by binding to penicillin-binding proteins (PBPs). Even though these antibiotics exhibit bactericidal action, their action is limited only to growing cells (Prescott, 2013). Among β-lactam antibiotics, oral administration of amoxicillin has direct impact on the gut flora, especially *E. coli*. Resistance of

gram-negative bacteria towards β-lactam is mediated by efflux mechanisms and modification of porins structures (Prescott, 2013). The combination of clavulanic acid and amoxicillin in the ratio of 2:1 is usually bactericidal in action, and it has potential action in the treatment of neonatal diarrhea and plasmid-mediated β-lactamase-producing bacteria that causes infection in swine. The pharmacokinetic properties of clavulanic acid are similar to amoxicillin (Prescott, 2013). Generally, the mechanisms by which β-lactam acquire resistance are reduced access to the PBPs, decreased affinity of PBP binding, and expression of the β -lactamase enzyme that hydrolyzes β-lactams (Rice, 2012).

Cephalosporins are the safest antimicrobial drugs that have properties similar to β-lactams in terms of drug stability, action against PBPs, and ability to penetrate bacterial cell walls. The broad spectrum of antibacterial activity of second to fourth generation drugs may cause overgrowth of resistant bacteria, including *Clostridium difficile.* As a result, the third and fourth generation of cephalosporins are not considered the first choice of drugs and should be indicated when any other alternative drugs are not effective. Ceftiofur sodium is used in the treatment of swine neonatal colibacillosis in European countries but is not recommended in US (Luppi, 2017). Among cephalosporins, the fourth generation are effective against Enterobacteriaceae and other Gram-negative bacteria that has acquired β-lactam's resistance, as well as resistance to earlier generations of cephalosporins (Prescott, 2013).

The bactericidal activity of aminoglycoside antibiotics like kanamycin, gentamicin, amikacin, and neomycin on aerobic gram-negative bacteria are influenced by pH, where they are significantly active in an alkaline environment but in case of acidic environment, they fail to kill even the susceptible pathogens. Among aminoglycosides, gentamicin is used to treat swine neonatal colibacillosis either by single intramuscular injection or 5 mg oral dose. The absorption

of drug by parental route (intramuscular or subcutaneous) of administration is good and excretion is by glomerular filtration via kidneys. Since excretion of aminoglycosides is mediated only by kidneys, nephrotoxicity is the most common adverse effect of aminoglycoside therapy. Bacteria acquire resistance to aminoglycosides by plasmid-mediated enzymes, such as phosphotransferases, acetyltransferases, and adenyl transferases (Dowling, 2013).

The aminocyclitol antibiotic spectinomycin is a broad-spectrum drug that is usually bacteriostatic, and at concentrations 4 times the MIC it exhibits bactericidal action. The pharmacokinetic properties of spectinomycin are similar to aminoglycosides. However, spectinomycin lacks most of the adverse effects that are caused due to administration of aminoglycosides like nephrotoxicity, but limited usage of spectinomycin is recommended because of development of resistance. An oral solution of spectinomycin is available for treatment of swine colibacillosis (Dowling, 2013).

Diaminopyrimidines (Trimethoprim) are combined with sulfonamides (sulfadiazine, sulfamethoxazole, and sulfadoxine) in a fixed (1:5) ratio. Trimethoprim-sulfonamide is used to control neonatal and post-weaning swine colibacillosis. Where the concentration of diaminopyrimidine is high in tissues and the sulfonamide moves slowly from plasma into tissues (Dowling, 2013).

Fluoroquinolones because of their hydrophilic nature and low (< 50%) protein binding ability, they are rapidly distributed in tissues and found in high concentrations in bile and organs of excretion (liver, intestine, and urinary tract). Bacteria acquire resistance to fluoroquinolones by mechanisms of reduced permeability to drugs, modification of target, and efflux mechanism. These resistance mechanisms can occur simultaneously within the same cells, resulting in higher levels of resistance to drugs (Rice, 2012).

To control intestinal infections caused by Enterobacteriaceae, only oral administration of Polymyxins (colistin sulphate) in pigs is approved in some European countries such as Italy, Spain and Denmark (Rhouma et al., 2016). It is not recommended to administer in USA livestock production systems. One reason for this is because the systemic usage of polymyxins causes nephrotoxic, neurotoxic, and neuromuscular blocking effects, whereas oral or local administrations are tolerable. The usage of colistin varies among countries within Europe. Where usage of below 1 mg/PCU (Denmark and the UK) or much higher, up to 20 to 25 mg/PCU (Italy and Spain) has been noticed. (http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2016/07/WC50211080.pdf). The recommended dose for therapy is 100,000 IU/kg BW (100,000 IU/kg body weight per day or 50,000 IU/kg administered at 12-h intervals), however some non-European countries are authorized to use colistin at a lower dose rate as a feed additive (Kempf et al., 2013).

## Antibiotic alternatives used for treatment and prevention of swine colibacillosis

To prevent bacterial infections in food-producing animals, alternatives to antibiotics have been researched. The nutritional intervention includes antimicrobial minerals (zinc oxide and copper sulfate), organic acids, functional feedstuffs (blood plasma, egg yolk antibodies), direct fed microbials, phytobiotics, and bacteriophages. Other feed additives such as feed enzymes, nucleotides, probiotic oligosaccharides, and clay minerals can enhance intestinal health. Feed additives and supplements have been utilized in newly weaned pigs, including probiotics, direct-fed microbials, phytochemicals, lysozyme, and micro minerals (Sun et al., 2017).

## Bacteriophages

Bacteriophages are a group of viruses with high species-specificity that can kill pathogenic *E. coli*. The study was conducted where animals are categorized in to three groups: Group-1 control, group-2 administered with *E. coli* phage P433/1, and group-3 supplemented with phage P433/2 were utilized. All the piglets in treatment groups were challenged with *E. coli* strain. The fecal examination of group-2 with P433/1 has lower incidence of *E. coli* when compared to group-3. This positive effect of phages was noticed when they were administered prior to onset of diarrhea symptoms. Hence, they are commonly used as therapeutic agents in pigs against PWD (Zhang et al., 2015).

Bacteriophages (PP01) are resistant to *E. coli* O157:H7 strain, and their higher fecal concentration indicates the ability to inhibit ETEC infections in pigs. Supplementation of bacteriophages as feed additives to the PWD infected nursery piglets subsided the attachment of *E. coli* in the ileum and cecum and increased the ratio of villus height to crypt depth in duodenum and jejunum (Lee et al., 2016).

## Blood plasma

Administration of diet supplemented with blood plasma in nursery piglets has inhibited the colonization of ETEC, promoted weight gain and decreased the frequency of ETEC-associated diarrhea that might be achieved due to the existence of specific anti-ETEC antibodies in blood plasma (Owusu-Asiedu et al., 2002). Feeding of a non- mediated diet with blood plasma to pigs improved growth performance, subsided intestinal inflammatory response, and protected F4 fimbriae positive pigs against ETEC infections (Bosi et al., 2004). When F18+ *E. coli* infected nursery pigs were fed non-immune plasma powder added diets, this showed prominent reduction in clinical symptoms, as well as a decrease in fecal *E. coli* count. Previous studies

demonstrated that the presence of higher levels of plasma Ig contributes to maintaining the integrity and barrier function of the gut, thus preventing ETEC infections (Weaver et al., 2014).

## Chicken egg yolk antibodies

The hen egg yolks are a potential source of polyclonal antibodies, which are specific for the virulence factors intimin and the translocated intimin receptor (Tir). The polyclonal antibodies are not specific to the secreted proteins EspA, EspB, and EspD of attaching and effacing *Escherichia coli* (AEEC). As a result, there is a reduced attachment of AEEC strains, and prevented attachment of porcine, bovine, and canine enteropathogenic *E. coli* strains, as well as O157:H7 Shiga toxin-producing *E. coli* strains seen in Figure 1.2. In one study, the weaned piglets are challenged with porcine enteropathogenic *E. coli* strain (ECL1001) and are on an oral supplementation of anti-immunoglobulins, which decreased the severity of attaching and effacing lesions (Girard et al., 2006). Effective usage of capsulated spray-dried egg powder enriched with specific anti-intimin and/or anti-Tir antibodies needs to be supplemented in cattle and swine diets. This passive immunotherapy prevents bacterial colonization and the associated lesions, reduces PWD in pigs (Girard et al., 2006).

## Copper and zinc

Copper is an essential micro mineral that has a vital role in inhibiting bacterial infections in swine (Adewole et al., 2016). Antimicrobial effects of copper is due to its oxidative properties, where it acts by releasing reactive oxygen and subsequent loss of bacterial integrity and cell death. The bactericidal effect of copper is achieved by the administration of copper to nursery piglets, which effectively reduced the fecal *E. coli* count (Mathews et al., 2015). Copper has also ability of contact killing when bacterial cells comes in direct contact with copper, which leads to

the damaging of bacterial membranes, increased cellular copper, and finally degradation of ETEC DNA (Sun et al., 2017).

Zinc is used to control post-weaning diarrhea, which is an essential micro-mineral. It has various roles, including the formation of organs and tissues, maintaining homeostasis, catalytic roles in regulating enzymes and endocrine systems, and regulatory roles in cellular replication and differentiation (Bonetti et al., 2021). It also plays a vital role in maintaining gut epithelial integrity and function (Vallee and Falchuk, 1993). Studies revealed that improvement in growth performance of piglets is facilitated by administration of zinc oxide (ZnO) (Hollis et al., 2005). Zinc also plays a role in the immune system, such as developing immune cells, producing cytokines, and regulating T and B cell signaling (Bonetti et al., 2021). The mechanism of action of ZnO in promoting piglet growth is due to its antibacterial activity, as well as its ability to strengthen the intestinal epithelial tight junctions by modulation of inflammatory cytokines. In addition, administration of ZnO decreased enterocyte damage caused by F4+ ETEC infection by reducing the damage to the cellular membranes of enterocytes (Roselli et al., 2003). Additionally, there are environmental impacts and public concerns with the application of high-dose ZnO in pig feed. As a result, there is a need to reduce the usage of antimicrobial drugs and pharmacological ZnO while also addressing animal health (Kim et al., 2022).

## Direct Fed Microbials / Probiotics

Direct-fed microbials (DFM) are live microorganisms that transfer health benefits to the host. The three main categories of DFMs include *Bacillus* (Gram-positive spore-forming bacteria), lactic acid-producing bacteria, and yeast. The beneficial effects of DFM include the production of antimicrobial products, immunomodulation, regulation of gut microbial profile, and enhancement of epithelial gut barrier function (Kim et al., 2022). *Lactobacillus rhamnosus*

GG (LGG) has promising effects in mitigating diarrhea in post-weaned piglets due to F4+ ETEC, achieved by modification of intestinal microbes, promoting intestinal antibodies, and systemic inflammatory cytokines (Zhang et al., 2010). DFM such as *Sterptococcus faecium*, *Bifidobacterium lactis*, and *Bacillus toyoi* are beneficial in ameliorating the intestinal infection and inflammatory responses due to pathogenic ETEC (Roselli et al., 2005).

Diets formulated with *Bacillus toyoi* or *B. licheniformis* decreased the incidence of diarrhea, and the intestinal ETEC count. Administration of *Enterococcus faecium* inhibited the attachment of F4 fimbriae to its receptors in intestinal mucosa. However, the DFM efficacy is strain dependent. The complex Lactobacilli preparation showed positive performance effect for about 2 weeks, and resistance towards *E. coli* infections (Huang et al., 2004). Comparative studies on efficacy of monostrain DFM (*Lactobacillus acidophilus*) with multi-strain DFM (*L. acidophilus, Lactobacillus casei, Bifidobacterium thermophilum,* and *E. faecium*) revealed that the monostrain did not have any effect on growth performance or on the loin quality, whereas the multi-strain facilitated gain and carcass yield without affecting loin quality. Studies demonstrated that the supplementation of multi-strain DFM to the F18+ ETEC challenged pigs and nursery piglets, promoted growth performance, and reduced the incidence of F18 *E. coli* (Zhang et al., 2010).

Probiotics are one of the potent alternatives to antibiotics administered as feed additives, and they have beneficial effects against intestinal infections in animals (Dubreuil, 2017). Even though they protect against ETEC induced intestinal infections, the exact mechanism is often unknown. However, most of the probiotics inhibit the adherence of pathogens, either by reducing or preventing colonization of pathogens to animal intestines (Dubreuil, 2017). A live culture of *Enterococcus faecium* 18C23 is known to inhibit the attachment of *E. coli* F4ac to the intestinal

mucosa of piglets, and this interaction was found be specific and dose dependent. Inhibition of adherence of *E. coli* F4ac is favored by the molecules from both the *E. faecium* 18C23 cells and the bacterial culture. The steric hindrance among the molecules of *E. faecium* would contribute to the inhibitory effect (Jin et al., 2000). *Pediococcus acidilactici* and *Saccharomyce cerevisiae* are two probiotics currently used in swine since they promote mucosal immunity of pigs and reduce bacterial translocation after ETEC challenge (F4+ O149). The translocation of bacteria to mesenteric lymph node was reduced after administration of either of the strains or a mix of these two strains in swine (Lessard et al., 2009). The combination of prebiotic (raw potato starch) and probiotic administration to piglets had beneficial effect on growth, diarrheal reduction, and increased microbial diversity in the intestines. That was further confirmed due to an inverse relation between probiotic *E. coli* (UM-2 and UM-7) and pathogenic *E. coli* F4 in the ileum, colon, and feces. The efficacy of an *E. coli* probiotic against *E. coli* F4 is due to its ability to produce colicins (a bacteriocin produced by *E. coli*). The *E. coli* strain Nissle 1917 is used as a probiotic against various disorders of intestines since 1920, and this strain was efficient against ETEC. This strain also forms good biofilm and is capable of outcompeting ETEC during biofilm formation. The other probiotic *Lactobacillus zeae* LB1 administration provided 86% protection against inhibiting the ETEC enterotoxin production rather than the colonization of intestines (Zhou et al., 2014).

## Essential oils

Winter savory (*Satureja montana* L. (Sm)) a semi-shrub plant comprises of important compounds of Essential oils (EOs) that have antibacterial activity such as carvacrol, gamma-terpinene, and p-cymene. Another shrub, Manuka (*Leptospermum scoparium* (Ls), constitutes leptospermone, iso-leptospermone and flavesone as the EO compounds (Douglas et al., 2004).

The *in vitro* antibacterial activity of manuka and winter savory EOs both individually or in combination was determined against three bacterial strains isolated from episodes of neonatal diarrhea (ND), post weaning diarrhea (PWD), and edema disease (OD) (Frattini et al., 2020). The three bacterial strains that were tested were previously identified and serotyped as O8, O149, and O139 and carried genes coded for virulence factors; stable toxin a (STa) and labile toxin (LT1) (Blanco et al., 2006). The combined effect of EOs is evaluated by three mixtures: 50% Sm + 50% Ls; 30% Sm + 70% Ls; and 70% Sm + 30% Ls. The main components of Sm were carvacrol (45.4%), p-cymene (10.3%), and thymol (7.0%), and in Ls were cis-calamenene (21.4%), leptospermone (18.3%), and flavesone (6.9%). The MIC and values of each strain against the both individually treated EOs is low when compared to the mixture of two oils; especially 70%-30% Sm/Ls blend had the best values of inhibition against all the tested strains that shows the predominant synergistic action. The effectiveness of the 70%-30% Sm/Ls mixture might be due to the amounts of 29.0% carvacrol and 7.0% leptospermone respectively (Frattini et al., 2020). The predominant effect of mixture of essential oils when compared with single essential oils against *E. coli* strains is in accordance with previous literature (De Medeiros Barbosa et al., 2016). Thus, essential oil blend are potential alternative antibacterial strategies against swine colibacillosis (Frattini et al., 2020).

## Fatty acids

Fatty acids are a category of organic acids with 4-28 carbon chain. These are chemically classified in to short chain (1-5 carbons), medium chain (6-12 carbons), and long chain fatty acids (13-21 carbons). To determine the influence of omega-3 fatty acids (alpha-linolenic acid), sourced by O3 Trial Feed (is a flax seed and algae-derived source of omega-3 fatty acids) on nursery piglet growth performance, a study was conducted where a total of 350 weanling pigs (5

pigs per pen) were assigned for 41-day trial period. Where the pens were randomly assigned to one of the five dietary treatment groups. And the diets are supplemented with increasing omega-3 fatty acids (0, 1, 2, 3, and 4% O3 Trial Feed). On day 25 two pigs per pen were injected with LPS (20 μg *Escherichia coli* LPS per kg BW). However, the increased omega-3 fatty acids did not influence average daily gain, average daily feed intake, growth:feed ratio. The IL-1β and TNF-α concentrations were determined in these challenged pigs and noticed that the concentrations were same across all the treatment groups. Hence increased O3 trail feed did not influence the concentrations of IL-1β and TNF-α and there is no immune response in healthy pigs challenged with LPS (Bromm, 2022).

Short chain fatty acids are the end products of dietary fiber and starch, and when administered play an important role in lipid and glucose metabolism. Butyrate, a short-chain fatty acid reduces the pro-inflammatory response induced by allergic reactions. It also plays a vital role in intestinal development and repair. However, the beneficial effect of butyrate was evident only during the first two weeks of post weaning and later the effect was diminished. So, to enhance the beneficial effect in subsequent growth periods, 0.5% of benzoic acid was added to the increased levels of sodium butyrate concentrations (0%, 0.035%, 0.070%, 0.105%) and fed to swine. Among the various combinations, sodium butyrate added at 0.035% or 0.07% in the diet containing 0.5% benzoic acid improved the overall growth performance of pigs. This indicates the synergistic effect between benzoic acid and butyrate (Wei et al., 2021). The addition of benzoic acid extended the beneficial effects of butyrate in further stages of growth. This might be achieved due to the reduction of pathogenic *E. coli* and increased variety of beneficial bacteria in gut (Wei et al., 2021). The combination of short chain fatty acids such as acetic, propionic, and butyric acids when administered orally at dose of 25 mL/kg to the piglets reduced the incidence

of diarrhea and increased feed intake. The exogenously administered mixture of fatty acids reduced the fat deposition by increasing fat oxidation and improving glucose homeostasis in the liver by downregulating the abundance of glucose to liver. The underlying mechanism of short chain fatty acids on lipid reduction and glucose tolerance might be achieved due to the stimulation of metabolic pathway of unsaturated fatty acids in piglets (Zhou et al., 2021).

## Flavonoids

Flavonoids extracted from green and black tea (*Camellia sinensis*) have bacteriostatic activity against pathogenic *E. coli* strains, and antibacterial activity of these flavonoids is achieved by sequestration of iron from iron-dependent pathogens such as *E. coli* (Friedman, 2007). Experimental studies showed that there is an increased net fluid absorption after perfusion of intestinal segments with black tea extract (BTE), and in the case of ETEC enterotoxin infected nursery piglets. Experimental trials among treatment and control group piglets revealed less prevalence of diarrhea in 0.8% BTE fed group when compared to 0.4% and control BTE diets. This indicates increased levels of intestinal net fluids (Bruins et al., 2010). The ascending growth reversed on supplementation of 10 or 100 µm Fe (II) to the culture medium, which indicates the ability of BTE to limit the availability of iron to *E. coli*. Among 10 and 100 µm Fe (II) dosage administered 10 µm Fe (II) has significant growth rate of ETEC compared to 100 µm Fe (II) (Bruins et al., 2010). The other mechanism of BTE action could be through the binding of BTE phenolic compounds to enterotoxins, and as a result the colonization of enterotoxins is inhibited. These studies indicate that BTE has an inhibitory effect against ETEC pathogens, as well as enterotoxins (Bruins et al., 2010).

## Nucleotides

Nucleotides are bioactive molecules that are vital in maintaining metabolic, structural, and regulatory functions, as well as the maintenance of immune system (Weaver and Kim, 2014a). A study was conducted (Waititu et al., 2016) where ninety weaned piglets were assigned to 3 treatment groups. These treatment groups included Group-1 control; Group-2 supplemented with Nucleotide rich yeast extract (NRYE); and Group-3 supplemented with NRYE and antimicrobial growth promoter. The NRYE is composed of cell wall polysaccharides, carbohydrates, and a mixture of 5 nucleotides (adenosine, cytosine, inosine, uridine, guanosine monophosphates). The antimicrobial growth promoter is a mixture of 55 mg of chlortetracycline and 31.2 mg tiamulin per kilogram of diet. When fecal swabs were screened, group-3 had comparatively reduced fecal shedding of *E. coli,* followed by group-2 (Waititu et al., 2016). In general, diets do not include the required number of nucleotides. Hence, the addition of these nucleotides to the nursery piglet diets showed promising effects on growth performance, decreased intestinal hyperemia, and immunity stimulation (Weaver and Kim, 2014a). Studies revealed feeding of nucleotides to the ETEC challenged pigs promoted growth performance, digestibility, immunity, and reduced the intensity of diarrhea (Li et al., 2015).

## Organic acids

To evaluate the diet acidification on growth performance of piglets the experimental study was conducted (Hutchens, 2021) where a total of 360 weaned piglets were assigned to one of the 8 dietary treatments. The eight treatment diets were Zn (110 mg/kg), diet acidification (with or without 1.2% sodium diformate), and dietary CP with different concentrations (21 or 18%, 1.40 and 1.35% and 1.20% standardized ileal digestible Lys, respectively). Significant increases in average daily gain and gain: feed ratio for pigs fed with pharmacological levels of

Zn, sodium diformate, and 21% CP were recorded. Increased weight gain was noticed for groups fed Zn and sodium diformate. However, the addition of these feed additives doesn't have major effect on fecal dry matter, but independently improved nursery pig performance (Hutchens, 2021).

To evaluate the effect of calcium carbonate (CaCO3) with and without benzoic acid a total of 350 pigs were allocated where CaCO3 was included at 0.45, 0.90, and 1.35% with and without 0.50% inclusion of benzoic acid. There is no evidence of interaction among CaCO3 and benzoic acid, but addition of benzoic acid improved average daily gain, average daily feed intake, and gain: feed ratio and CaCO3 reduced the gain: feed ratio of piglets (Warner, 2022).

To evaluate the effect of organic acids fifty ETEC infected piglets were obtained from a commercial farm, and they were supplemented with 3 different diets, which include group-1 control (regular diet), group-2 regular diet+lactic acid diet, and group-3 regular diet+formic acid (Calinescu et al., 2007). Among the treatment groups, addition of lactic acid to the diets promoted growth, as well as prevented PWD in piglets followed by group-3. Feeding formic acid in supplemented diets resulted in a decreased total number of fecal *E. coli*, which escalated the intestinal morphology, and thus improved the growth performance in piglets. In addition, the *in-vitro* study revealed supplementation of a liquid diet, either with formic acid alone or multiple acids, decreased the Enterobacteriaceae counts (Calinescu et al., 2007). In the case of PWD outbreaks, dietary modulations with organic acids and supplementation of these diets had significantly reduced the intensity of diarrhea and F4+ETEC in the intestine. The natural acidifier hydrochloric acid secreted in stomach also have antibacterial activity and contributes to the integrity of host gut. Hence, supplementation of diets with organic acid could be a potent way to control pH and growth of bacteria in the stomach and intestines (Tsiloyiannis et al., 2001).

The antimicrobial effect of organic acids can be achieved in several ways; hence they are considered one of the most viable swine growth promoters. The addition of organic acids to diets reduces the uptake of pathogenic *E. coli* by feed and water, thus the ingested acids reduce the pH of stomach, and this acidic pH acts as a strong biological barrier function of stomach. The undissociated organic acids can penetrate the bacterial cell wall and thus inhibit the proliferation of bacteria and in turn inhibit the colonization and proliferation of *E. coli* (Mesonero-Escuredo et al., 2016). Benzoic acid is recognized as one of the potential antimicrobial agents and capable of increasing digestive enzyme activity, improves the small intestinal morphology, thus leading to increased nutrient uptake which in turn improves the growth performance of nursery piglets.

## Phytobiotics

Phytobiotics or phytogenic feed additives are plant derivatives that include a wide variety of herbs, spices, and essential oils when administered, which can promote growth performance in pigs (Windisch et al., 2008). The extracts of Macleaya cordata (Wild) at the concentration of 15 to 50 mg/kg when administered to nursery and growing pigs enhanced weight gain and reduced the incidence of diarrhea (Liu et al., 2016). The functional extract of garlic (allicin) promoted weight gain when supplemented to nursery piglets. Supplementation of the phytobiotic additive combination (clove, cinnamon, and fenugreek) to the dietary challenged F4+ ETEC pigs revealed increased weight gain and total tract digestibility of ash (Devi et al., 2015). The administration of extracts of cinnamon, thyme, and oregano inhibited the attachment of *E. coli* in case of nursery piglets (Namkung et al., 2004). Studies revealed that the addition of sanguinarine to the diets facilitated the growth of beneficial bacteria and inhibited the harmful bacteria in pigs (Liu et al., 2016). When nursery pigs are supplemented with diets that are incorporated with various plant extracts like oregano, cinnamon, and Mexican pepper, there was a reduction in total microbial

mass with an increase in *Lactobacilli* to enterobacteria ratio in the ileum (Manzanilla et al., 2004).

## Tannins

Tannins are polyphenolic molecules that can interact and precipitate macromolecules like proteins, alkaloids, and gelatins. Generally, tannins are categorized into three types: condensed tannins (CTs), hydrolysable tannins (HTs), and complex tannins (Sieniawska and Baj, 2017). Cranberry tannins induced structural conformation of *E. coli* that led to the 60% decrease in fimbrial length (from 148 to 53 nm) and reduced the adhesion between bacterial and epithelial cells. Administration of 10 μg of cranberry extract reduced the adhesion of STEC F18. However, 100 μg is needed to strongly inhibit ETEC F4 adhesion *in-vitro*. Further, *in-vivo* experimentation done in pigs demonstrated that the administration of preincubated F4 or F18 fimbriae with cranberry extract eliminated adhesion of fimbriae to intestinal epithelial cells (Coddens et al., 2017). Bacterial biofilm formation facilitates intestinal colonization, exchange of plasmids among bacteria, and concentrates bacterial enzymes that inactivates and decreases antimicrobial penetration, leading to the acquisition of antimicrobial resistance (Bakkiyaraj et al., 2013). Certain tannins inhibit biofilm formation, such as the extracts of *Punica granatum L*. (pomegranate), ellagitannins (mainly punicalagin), and ellagic acid. These tannins inhibit biofilm formation in *Staphylococcus aureus,* methicillin resistant *S. aureus* (MRSA), *E. coli,* and *Candida albicans* (Bakkiyaraj et al., 2013). Some tannins can also inhibit the adhesion of bacterial enterotoxins, like labile toxin (LT), to its GM1 receptor and this inhibition is due to the administration of gallotannins and gallic acid, which are extracts of Galla Chinensis. In a similar way, 750 μg/ml of two HT extracts, one with gallic acid derivative and the other with a

combination of medium to high molecular weight HTs, decreased the binding effect of LT when compared to procyanidin CT extract (Verhelst et al., 2010).

## Challenge study in piglets

One of the primary drivers of mortality and illness in newly weaned pigs is enterotoxigenic *Escherichia coli* (ETEC), making it a significant contributor to the use of antimicrobials in swine production. The process of colonization and infection by ETEC begins with its attachment to the small intestines. ETEC can induce weaning stress, creating conditions conducive for other enteric infectious diseases to manifest, leading to symptoms such as diarrhea or even sudden death. To advance an antibiotic-free production system, efforts to bolster intestinal health and overall performance in weaned pigs are essential practices (Kim et al., 2022). For enterotoxigenic *E. coli* (ETEC) susceptibility, host genotype is important. Enterotoxigenic *E. coli* (ETEC) is the most common pathogen isolated from PWD outbreaks and is characterized by enterotoxin and fimbriae production. The fimbriae of ETEC attach to host receptors that are specific, which enable colonization of the piglet intestine (Rydal et al., 2022). Soon after weaning nursery pigs, there is a significant association with gut health and their growth performance and economic values. Virulence factors of ETEC include adhesins (fimbriae or pili) and enterotoxins. Enterotoxins that are associated with diarrhea in pigs include heat-labile enterotoxins (LT) and heat-stable peptide toxins (ST). The common types of fimbriae on ETEC from PWD pigs are F18+ and F4+. In the past, antibiotics used to be the most effective way to prevent PWD, but with increased bacteria resistance, there is a need to find alternatives to the use of antibiotics. The harmful effects of enterotoxigenic *Escherichia coli* (ETEC) were reduced by effective feeding strategies rather than the usage of therapeutic intervention with antibiotics in

livestock. The development of new feeding strategies plays a crucial role in reduction of postweaning digestive disorders (Heo et al., 2013). Even though the major etiological agent was enterotoxigenic *Escherichia coli* F4ac, the response to feeding strategies varied because of different ETEC genotypes.

One study attempted to induce clinical signs of post-weaning disease in a piglet model using a one-time acute ($1.5 \times 10^{10}$ CFU of a pathogenic heat stable enterotoxin II (STIIB) +, Shiga toxin (Stx2e) +, and heat-labile enterotoxin IIA (LT-IIA) + nalidixic acid-resistant mutant of *Escherichia coli* strain NCDC 62–57 (*E. coli* 62-57nal)) or lower daily chronic dose ($5.4 \times 10^{8}$ CFU ) of the same Shiga toxin-producing and enterotoxigenic *E. coli* 62-57nal strain. Once induced, the disease state was monitored by determining fecal shedding and colonization of the challenge strain, cytokine levels, animal growth performance, fecal calprotectin, fecal metabolomics, histology, and fecal microbiome shifts. In terms of gut health, both the treatment groups (acute and chronic) exhibited similar clinical signs of scours evident on day 2 to day 6 but the control group animals maintained healthy status throughout trail period. When compared to the control group, both chronic and acute groups have significant reduction in average daily gain (ADG) and the acute challenge group has poorest G: F conversion ratio. However, the average daily feed intake (ADFI) of the control group was less when compared to both treatment groups. The lack of blunted villi in these challenged animals is similar to the PWD infected animals, might be the reason for reduced nutrient uptake. Evaluation of *E. coli* infection induced inflammation is by measuring serum interleukins 6 and 8 (IL-6 and IL-8) as they are associated with intestinal inflammation. Among IL-6 and IL-8, a similar increase in concentrations of IL-6 was noticed in both treatment groups. For IL-8, chronic treatment group has significant increase in concentration compared to the acute and control group. Fecal calprotectin acts as a

noninvasive marker of neutrophil intestinal inflammation of swine but fecal calprotectin concentration has no significant treatment effect. In order to notice the changes in gut microbiota due to treatment groups (acute, chronic) targeted 16S qPCR was performed on the fecal samples collected during pre-treatment, treatment and post-treatment phase. A microbiome dominated by *Bacteroidetes* and *Firmicutes* was noticed. Among acute and chronic dose, acute has significant effect on gut microbiome maturation. However, both treatment groups have similar levels of dysbiosis. Further characterization of difference in disease state among treatment groups (acute, chronic) is by performing untargeted metabolomics on fecal samples collected on pre-treatment, treatment and post-treatment phase. The post-treatment fecal samples of both treatment groups have increased levels of amino acid metabolites due to amino acid malabsorption and/or secretion that might be achieved due to inflammation, and microbiome perturbations in gut caused by *E. coli* 62-57nal. The metabolomics study indicated both the treatment groups have similar dysbiosis and differentiated both pre-treatment and post-treatment phase. These interpretations rule out swine that are inoculated with ETEC experience increased crypts depths, sloughing of intestinal villi, and scours. Piglets are likely infected by a chronic exposure of lower doses of *E. coli* as ETEC and STEC can be found in contaminated feed, soil, water, and in the bar environment (Boeckman et al., 2021).

Postweaning diarrhea is characterized by diarrhea, which can lead to severe dehydration, emaciation, and death. Edema disease (ED) in swine is characterized by submucosa edemas of the stomach and mesocolon, which causes swelling of the eyelids, forehead, and in some cases hemorrhagic gastroenteritis, leading to eventual death. Postweaning diarrhea ETEC are primarily associated with *E. coli* producing heat-stable and/or heat-labile enterotoxin, while ED STEC are associated with Shiga toxin, primarily the 2e subtype (*Stx2e*) producing strains, which can be

expressed with or without other enterotoxins. In biomedical research, swine are used as a proxy for humans due to similar physiology, intestinal permeability, immune systems, and intestinal enzymatic profiles. Weaned pigs represent a possible model for human STEC or ETEC infections as porcine gastrointestinal anatomy, ETEC clinical signs, and immune response closely mimic that of humans. An additional study contained two trials with 48 female pigs that have no history of disease. Pigs were weaned at PND 22 in trial 1 (n=15), and PND 23 in trial 2 (n=33). Experimental groups were housed with one pen per room, in separate rooms. The three most middle weight pigs of each of five (trial 1) and eleven (trial 2) litters were randomized into experimental groups to balance the study on sow level and reduce weight variation. Rectal swabs were collected from pigs for ETEC diagnosis at weaning. Swabs were collected from each animal twice daily in trial 1, streaked on BA and incubated at 37 degrees Celsius. One hemolytic *E. coli* colony per pig per day was subcultured on BA and the virulence factors were identified by PCR. In trial 2, rectal swabs were collected once a day and the shedding of hemolytic *E. coli* was evaluated semi-quantitatively as percentage hemolytic *E. coli* out of total bacterial growth. ETEC diarrhea consistency varied between loose (score 3) and watery (score 4). In trial 2, there were single events of diarrhea in four pigs outside the designated sampling in connection with the fourth and fifth inoculation procedure. Across the inoculation groups, five pigs shed hemolytic *E. coli* at baseline, but none of these isolates encoded for ETEC virulence factors. In the study, the diarrhea was observed from mild to severe and lasted 1 to 3 days. There was no association with clinical signs of dehydration, fever, or hypothermia. No spontaneous ETEC F18 diarrhea occurred, but all challenged pigs that were susceptible according to the CHCF1 marker developed ETEC diarrhea with affected secondary disease related outcomes. CHCF1 resistant pigs remained healthy after the challenge with ETEC F4 ab/ac. This study discovered that

CHCF1 genotype was a better marker for F4ab/ac susceptibility in vivo than MUC4. These observations suggest CHCF1 as a promising marker for screening experimental animals and it should be tested in a separate trial (Rydal et al., 2022).

A recent study evaluated the significance of jejunal mucosa-associated microbiota and the impacts on intestinal health in pigs challenged with F18$^{+}$ *Escherichia coli*. In this study, forty-four recently weaned piglets were separated into two treatment groups and were randomized. For 28 days, pigs were fed common diets. At 7 days post-weaning, pigs were inoculated orally with saline solution or F18$^{+}$ *E. coli*. At day 21 post-challenge, blood and feces were collected and pigs were euthanized to collect jejunal tissue to evaluate microbiota and intestinal health. The abundance of Bacteroidetes and Firmicutes were lower in the jejunal mucosa than in the feces. The change of mucosa-associated microbiota by F18$^{+}$ *E. coli* was more prominent. The prominence of *E. coli* correlated with the increased protein carbonyl and reduced villus height in jejunal mucosa impairing the intestinal health of nursery pigs. Pigs challenged with F18$^{+}$ *E. coli* showed a reduced growth performance at the end of experiments. The F18$^{+}$ *E. coli* challenge did not affect the fecal score of pigs from 22 to 28 days post-weaning (Duarte et al., 2022).

The early weaning stress in pigs on clinical disease, intestinal physiology, and immune response to a challenge with enterotoxigenic F18 *E. coli* (ETEC) was evaluated. Pigs were weaned at days 16, 18, and 20, and were given oral challenges of ETEC F18 at day 26. From days 0 and 4 post-infection, pigs were monitored for clinical signs. Early weaned pigs (16 and 18 days weaned) showed more severe diarrhea and decreases in weight gain after the ETEC challenged compared to the late weaned pigs (20 days weaned). The ETEC challenge induced intestinal barrier injury in early weaned pigs, which was shown by the reductions in ileal

transepithelial electrical resistance (TER). The TNF levels increase in ETEC challenged ileal mucosa from early weaned pigs but not in other weaning age groups (McLamb et al., 2013).

Another study investigated the effects of an oral challenge with enterotoxigenic *Escherichia coli* (ETEC) in post-weaning piglets. The treatments did not affect the body weight gain or feed intake. There was a lower feed intake for the pea fiber diet in the first period after the challenge. After the ETEC challenge on day 7, the fecal consistency scores increased in all groups. The addition of peas and pea fractions in weaned piglet diets does not positively or negatively affect performance in gut health in challenged weaned piglets (Jansman et al., 2010).

The study investigated the impact of an F18 enterotoxigenic *Escherichia coli* (ETEC) challenge on growth performance, selected immune responses of piglets, aspects of intestinal function, and to evaluate potential protective effects of direct-fed microbial (DFM) blends. Seventy-two weaned piglets were assigned to one of four treatments, which included nonchallenged (NC), positive challenged control (PC), F18 ETEC-challenged (PC + DFM1), and PC + DFM2 (2 strains of *B. amyloliquefaciens* and one strain of *Bacillus subtilis*). All ETEC-challenged pigs were confirmed to be genetically susceptible to F18. Fecal swabs were collected on dpi 0, 1, 2, 4, 7, and 10 to evaluate ETEC shedding. Blood samples were collected on dpi 0, 1, 2, 4, 7, and 10. Across all challenged treatments, rectal temperature decreased. Removing antibiotics from weaned pig diets has substantial consequences compared to other production stages due to social and environmental stressors, which adversely affect gastrointestinal function and the immune system, leading to increased incidence of disease including diarrhea. The decrease in efficiency of body weight gain combined with increased death loss after ETEC infection results in economic losses. Prior to the challenge, no mortality was observed. After ETEC challenge, 23% mortality was observed among the challenge pigs. There were no

differences among treatments, and it was confirmed via necropsy that mortality was due to severe dehydration. The NC pigs had a higher feed intake than pigs in the other three treatments. There was no difference in fecal scores among challenged treatments over the 10-d challenge period. Pigs in the NC group had no ETEC shedding throughout the experiment, which the PC pigs had higher shedding over the 10-d challenge period. The rate and efficiency of body weight gain in newly weaned pigs is closely associated with intestinal health and function. This study evaluated the effects of two novel *Bacillus*-based DFMs on growth performance, immune response, and intestinal function in weaned pigs challenged with F18 ETEC. All challenged pigs had increased fecal scores and shedding of the F18 ETEC strain compared with NC, which shows that the challenge model was successful. There was no apparent benefit of DFM1 supplements, but DFM2 appeared to partially attenuate the ETEC challenge by decreasing *E. coli* shedding earlier after inoculation (Becker et al., 2020).

The other study evaluated to reduce the incidence of post weaning colibacillosis by oral challenge of F4 enterotoxigenic *Escherichia coli* (ETEC) on animal performance, intestinal microbiota, clinical signs, inflammatory response, intestinal morphology, jejunal gene expression and to evaluate the potential effects of phytogenic blends. A total of 96 of orally challenged F4 ETEC weaned piglets were categorized to one of the four dietary treatments. Control basal diet (T1); T1 supplemented with zinc oxide (T2); T1 supplemented with plant supplements based on essential oils (Colifit I caps) (T3); T3 supplemented with plant supplements based on non-volatile compounds (Phyto Ax cell) (T4). The product Colifit I caps is made up of a blend of essential oils rich in trans-cinnamaldehyde, eugenol, carvacrol, thymol, and diallyl disulfure. Phyto Ax cell is a blend of standardized plants, plant extracts, and green propolis extract that has active compounds such as curcuminoids, carnosic derivatives, naringin flavonoids, salicylic

derivatives, and artepillin-C. There is no significant difference in animal performance in terms of ADG, ADFI, G:F ratio among the treatment groups. To evaluate the intestinal microbiota fecal samples, ileal scrapings, and colon digesta was screened and there is no significant difference in coliforms among treatment groups. The serological concentrations of pig-MAP (major acute phase protein) and cytokine TNF-α concentrations are measured. In swine pig-MAP induces tissue injury and acute inflammatory response. The significant decrease in expression of MAP protein was noticed for T4 treatment group which indicates that phytogenic blends (T4) exhibit anti-inflammatory- property at the site of tissue injury induced due to F4 ETEC. The intestinal morphology in terms of villus/crypt ratio was evaluated and T4 treatment group has significantly improved villus/crypt ratio. The jejunal gene expression of *REG3G* gene was measured. Since, *REG3G* gene acts as a barrier to protect host surface against pathogenic microorganisms by modulating hosts bactericidal activity. The upregulation of *REG3G* gene was noticed for T4 treatment group. The challenged piglets of T4 treatment group have reduced pig MAP protein, increased villus/crypt ratio, and upregulated *REG3G* gene compared to control group. Thus, the phenotypic blends exert positive effect by partially attenuating the ETEC challenge (Montoya et al., 2021).

One study challenged piglets with ETEC F18 to investigate the effect of early inoculation of probiotics to suckling piglets on the ETEC shedding and immune parameters once the piglets were weaned. There was a negative control (non-challenged), positive control (challenged), and a probiotic (challenged and inoculated with a multi-species probiotic) group. On days one and two post-weaning, pigs in the probiotic and positive control groups were challenged with ETEC F18, where pigs in the negative control group were given sodium chloride. The probiotic group had a significantly reduced number of pigs shedding the ETEC F18 and STb toxin in the feces

compared to the positive control group. Providing a multi-species probiotic is helpful in clearing ETEC in newly weaned piglets (Hansen et al., 2022).

The effects of dietary supplementation of bacteriophages against enterotoxigenic *Escherichia coli* (ETEC) K88 was investigated as a therapy against ETEC infection in post-weaning pigs. Two groups of post-weaned pigs were challenged with $3.0 \times 10^{10}$ colony forming units of ETEC K88. The unchallenged and one challenged group were fed a basal nursery diet for 14 days while the other challenged group was fed the basal diet with $1.0 \times 10^{7}$ plaque forming units of the phage per kg. The goblet cell density and villous height: crypt depth (VH:CD) ratio in the intestine and average daily gain (ADG) were less in the challenged group than in the unchallenged group within the animals fed a basal diet. The dietary phage supplementation alleviated the ETEC infection symptoms. This study indicated that the phage therapy is effective in reducing acute ETEC K88 infection in post-weaning pigs (Lee et al., 2016).

Other researchers used metagenomic analysis to investigate the gut microbiota and resistome in piglets that were or were not challenged with enterotoxigenic *Escherichia coli* (ETEC) and had or had not received dietary supplementation with microencapsulated probiotics. The 72 piglets were divided into 6 groups that were either non-ETEC challenged (groups 1-3) or ETEC challenged (groups 4-6). Groups 2 and 5 were supplemented with 10^9 CFU/ml of multi-strain probiotics while group 4 received 10^9 CFU/ml of *P. acidilactici* 72N on days 2, 5, 8, 11, and 14 days of piglet age. Group 3 was given 300mg/kg chlortetracycline in the weaner diet to mimic commercial conditions. The piglets in groups 2 and 5 were enriched with several desirable microbial families, including *Lactobacillaceae, Lachnospiraceae,* and *Ruminococcaceae*. Piglets in group 3 had an increase in members of the *Bacteroidaceae* family and in *tetW* and *tetQ* genes. Metagenomic analysis showed that employing a multi-strain probiotic could transform the gut

microbiota, reduce the resistome, and boost genes associated with food metabolism (Apiwatsiri et al., 2022).

## Vaccines for swine colibacillosis

Swine colibacillosis associated diarrhea leads to morbidity and mortality of neonatal and recently weaned piglets and causes a huge economic loss to swine industry (Chen et al., 2004). Attachment of bacteria to host intestinal enterocytes is mediated by long proteinaceous appendages called fimbriae (Nagy and Fekete, 2005) and later the production of enterotoxins causes imbalances of water electrolyte levels, leading to watery diarrhea. One of the major challenges impairing gut health in nursery pigs is pathogenic infection. The severity of PWD is affected by other factors, such as dietary changes, weaning stress, and deficiency of milk antibodies. In global swine production, *E. coli* infection became a more frequent reason of sudden death or severe diarrhea. Pigs that are infected with ETEC usually show watery diarrhea for about one to five days after infection. Some pigs have shown sudden death without diarrheal symptoms but with intestinal edema. There are three types of vaccines for ETEC that have been applied to pigs in an experiment setting. The first includes intramuscular injectable vaccines, vaccinating sows prevents most neonatal infections either by passive colostrum or lactogenic immunity (Figure 1.3). Neonatal infections are mostly prevented with this method since ETEC infections are non-invasive GIT infections. Lactogenic (systemic) rather than colostrum (passive) immunity plays a key role against the infection. The maternal vaccines available either contain inactivated bacteria with fimbriae or purified fimbriae with or without LT, which are administered by parental route in pregnant sows (Nagy and Fekete, 2005). The lactogenic immunity suddenly stops right after weaning, and the piglet's immune system is highly

susceptible to pathogens that consequently lead to the development of antigen specific secretory IgA, which induces the active intestinal mucosal immune response.

The second vaccine is an oral administration of live wild type or live attenuated enterotoxigenic *E. coli* strains carrying the fimbrial adhesins to pigs. The promising results were noticed with the administration of live F18 ac positive *E. coli* vaccinated to sows, where the suckling piglets received maternal F18 specific colostrum IgA antibodies (Bertschinger et al., 2000). The efficient binding of the vaccine to M cells through the specific IgA antibodies present in the gut lumen of sow might contribute to the efficacy of the vaccine in suckling pigs (Weltzin et al., 1989). Encapsulation strategies were done to enhance the efficacy and protection of oral subunit and live oral vaccines during their transport through GIT and /or against maternal antibodies. The practicality of oral immunization with microencapsulated *E. coli* or fimbriae where they encapsulated F18+ *E. coli* and F18 fimbriae in poly lactide-co-glycoside (PLGA) microspheres by spray drying and oral administration to both newborn and weaned pigs was first tested in 2000 (Felder et al., 2000).

In suckling piglets, oral delivery of live F18 positive *E. coli* strain showed successful results (Bertshinger et al., 2000). However, in both weaned and suckling pig's administration of F18 fimbriae in PLGA particles failed to induce a protective immune response (Felder et al., 2000). Even though the encapsulation technique did not reduce the antigenicity, it might have altered the immune induction mechanism that might prevent the colonization of bacteria in intestines. So, usage of the encapsulated technique that get disintegrated in the small intestines is more appropriate. The comparison of oral administration of F4 fimbriae in enteric-coated pellets to F4 in solution in case of suckling pigs (Snoeck et al., 2003). The administration in the form of encapsulated pellets showed significant reduction in excretion of F4+ETEC when compared to

suckling pigs with oral vaccination of F4 fimbriae in the solution. This demonstrates that the encapsulated pellet provides protection of antigens against degradation by enzymes, as well as neutralization by milk factors. The list of vaccines administered for swine is depicted in Table 1.3.

The third vaccine is the oral administration of purified fimbriae, instead of the whole bacteria. Fimbrial vaccines are routinely administered to pregnant sows in order to control ETEC infections. These vaccines are administered mainly by parental route to pregnant sows in order to increase the level of fimbrial antibody in colostrum and milk. Neonatal piglets receive these lacteal antibodies during suckling and maintain fimbrial antibody levels in the small intestines, which prevents the colonization of ETEC infection during highly susceptible early neonatal period. The first fimbriae based ETEC vaccine (bacterin) was licensed by the United States Department of Agriculture (USDA) in 1980. The vaccines for swine included F4, F5, and F6 fimbrial antigens. Along with fimbrial antigens, one of the swine vaccines included heat labile enterotoxin based on the B subunit ($LT_B$) (Moon et al., 1993). The efficacy of vaccinating sow with fimbriae was first done by using F4 fimbriae in order to protect suckling neonates through passive immunity against ETEC infection. The prevalence of F1 and F41 fimbriae is high in ETEC infections, but their efficacy as vaccines is less defined in swine when compared to F4, F5, and F6 fimbriae types. Most of the animal ETEC strains that produce F1 or F41 are also capable of producing F4, F5, or F6 fimbriae. Vaccination of sows with F5 provides immunity against ETEC strains that has both F5 and F41 fimbriae. Even though maternal vaccines are administered parentally to pregnant sows, lactogenic protection disappears at time of weaning and with age. As a result, the weaned piglets become susceptible to intestinal pathogens. In order to protect these weaned piglets, an active mucosal immunity where local production of F4 and/or

F18 specific sIgA play a vital role in providing immunity against PWD in pigs (Melkebeek et al., 2013). The purified F4ac fimbriae are powerful oral immunogens to weaned piglets when administered at a dose rate of 1mg for two consecutive days. This results in F4 specific antibody secreting cells (ASC) in payer's patches, blood, and mesenteric lymph nodes which was previously demonstrated (Vanden Broeck et al., 1999). When this oral subunit vaccine was administered during three consecutive days and boosted on day 16 post primary vaccination, complete protection was obtained against F4 ETEC in weaned piglets. Induction of protective mucosal response is mediated by the F4 receptor (F4R) located on enterocytes, and the presence or absence of F4R is based on genetic inheritance. Transgenic edible alfalfa was used as a low-cost delivery vehicle for F4 fimbriae adhesive where FaeG is the major subunit. The advantage of large amounts of alfalfa plants is that FaeG remains stable over prolonged storage, which was previously suggested (Joensuu et al., 2006).

The oral administration of purified F18 at a dose rate of 30 mg, along with mucosal adjuvant LT(R192G), did not induce protective immune response against F18 *E. coli* challenged piglets (Verdonck et al., 2007). The variation in immunogenicity among F4 and F18 may be due to the difference in structural aspects of fimbriae. The adhesin of F18 is a minor subunit of FedF, whereas F4 is the major subunit of FaeG, which is present in multiple copies in single fimbriae (Melkebeek et al., 2013). The decreased fecal excretion of F18 *E. coli* in challenged weaned piglets that were immunized with recombinant produce FedF coupled with F4 fimbriae was demonstrated (Tiels et al., 2008). The administration of single live oral vaccine with attenuated *E. coli* strains that express F4ac on day 4 after weaning failed to provide protection 7 days later against the virulent strain was explained (Bozic et al., 2002). However, priming immune system with immunomodulator like levamisole which is administered for three successive days

immediately before the oral vaccination predominantly reduced the clinical signs of PWD (Bozic et al., 2002). Since 2008, coliprotec, a live oral vaccine that is composed of naturally avirulent *E. coli* strain that express F4 fimbriae but do not produce toxins. This vaccine is known to reduce the colonization of F4 ETEC strain, and the duration and severity of diarrhea.

An oral tablet composition of carboxy methyl high amylose starch with live F4+*E. coli* or purified F4 fimbriae (Calinescu et al., 2007) showed better survival rate of bacteria, as well as greater stability of F4 in gastric fluid. Encapsulation of F4 with adjuvant methyl vinyl ether-co-malic anhydride (Gantrez) nanoparticles administration only raised serum antibody response, but did not induce improved protection when compared to soluble F4 fimbriae. However, empty nano particles added to soluble F4 fimbriae are considered as the best adjuvants considering polymeric properties responsible for enhanced immunity rather than protection of antigen against enzymatic degradation, which makes this system less suggestive for vaccination during the suckling period (Vandamme et al., 2011).

Piglets injected with Stx2e- specific antiserum or fed with spray dried sow blood plasma or chicken egg yolks immunized with F18 showed passive immune response against toxin colonization and spread in the body. Oral administration of Stx2e mutant *E. coli* strain in piglets induced strong immune response against wild type Stx2e *E. coli* strain. However, the Stx2e mutant *E. coli* strain needs to be administered daily within a capsule for 4 successive days, which limited the applicability of this approach. Immunization of Stx2e toxoid to suckling piglets induced an increased antibody response levels in serum that strongly prevented the clinical signs in pigs challenged with Stx2e toxin, as well as toxoid immunization had no negative effect on growth performance of piglets, which agrees with previous reports (Bosworth et al., 1996) so administration of Stx2e toxoid provides immunity against edema disease. Immunization of

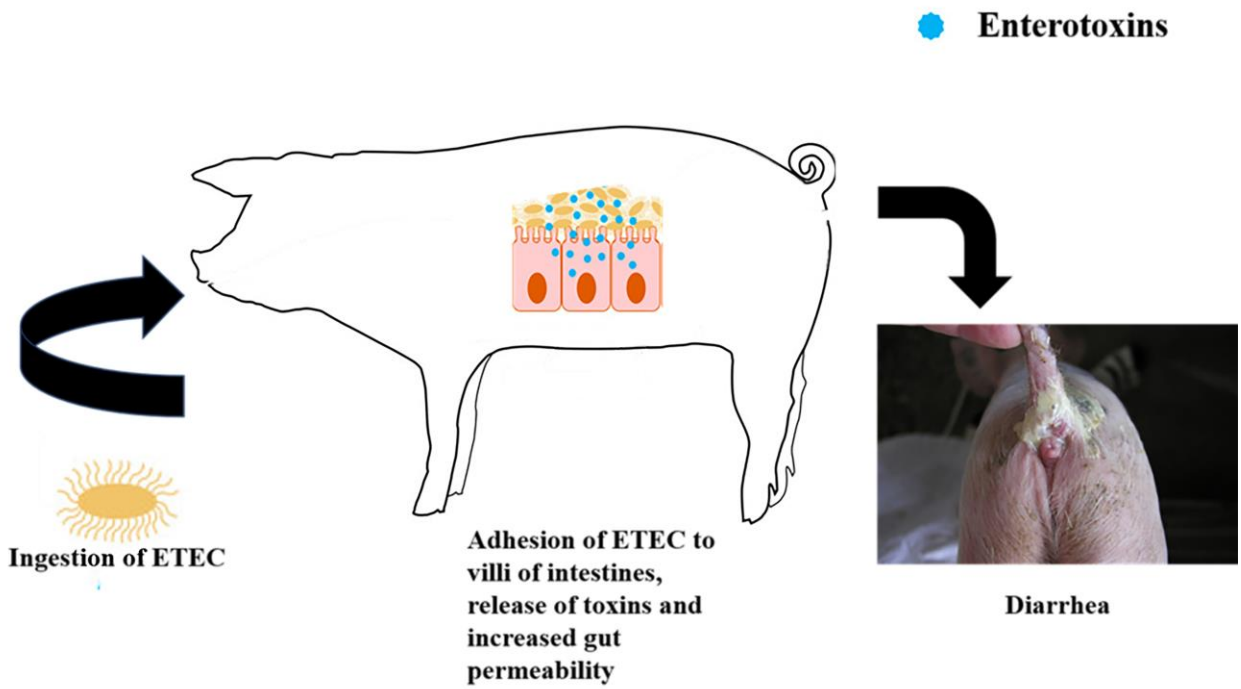
pregnant sows with the Stx2e toxoid led to higher detection levels of Stx2e antibodies in the piglets and persisted for at least one-month of postweaning. This indicates the successful transmission of maternal antibodies to newborn and protection against edema disease (Pravieux et al., 2007). Strong immune response with high levels of passive antibodies in piglets is achieved by active immunization with the Stx2e toxoid (Oanh et al., 2011).

The first effective oral vaccine against edema disease (ED) was developed with Stx2eB-transgenic lettuce with two different strains, such as 2BN and 2BH (Hamabata et al., 2019). The 2BN contains 0.53 mg Stx2eB/g of powdered lettuce dry weight and 2BH contains 2.3 mg of Stx2eB/g of powdered lettuce dry weight. Among the two strains, 2BH strain has a higher immune potential. However, the 2BH strain expressed hemagglutinin (HA)-tag with Stx2eB and carried non-canonical *N*- glycosylation. This led to the development of another two Stx2eB strains, 3 (G+) strain in which HA-tag was removed from 2BH, and the 3 (G-) strain where 73$^{rd}$ Asn was replaced with Ser to prevent non- canonical *N*- glycosylation. Administration of *N*-glycosylated 2BH and 3 (G+) strains in STEC challenged piglets relieved symptoms from ED, which suggests that the presence of HA-tag in the transgene was not related to the vaccine effect. The 3 (G-) strain did not induce protective immune response (Hamabata et al., 2021). That explains *N*- glycosylation at 73 Asn of Stx2eB strains of 2BH and 3 (G+) facilitated for immune response against ED. High molecular stability, $p^H$ stability, and protease-resistance of proteins of non- canonical *N*- glycosylation (Hu et al., 2017) contributed to the immune response. Even glycosylation induces immune reaction by activation of nuclear factor- kB that in turn plays role in innate immunity and inflammatory responses and T helper differentiation of Th1 and Th17 cells (Kingeter et al., 2012). The administration of Stx2eB- lettuce did not induce anti-Stx2e IgA levels despite that the fecal Stx2e-specific antibody levels were below the detection limit, but

Stx2eB in *E. coli* stimulated macrophages to induce inflammatory cytokine tumor necrosis factorα (Hamabata et al., 2019).

In conclusion, the major intestinal pathogenic *E. coli* associated with swine colibacillosis are ETEC, STEC, and EPEC pathotypes, where fimbriae facilitate their attachment to the host intestinal epithelial cells and thus release of toxins leading to infection. However, there are hybrid pathotypes where the isolates carried combinations of various toxin genes from two or more pathotypes. The investigation of hybrid pathotypes should be sought out further to determine the public health importance. The prevalence of these pathotypes both in the US and other countries varies depending on the geographical region. Antibiotics have been used for the treatment and prevention of swine colibacillosis, however, this may lead to increased AMR (antimicrobial resistance). To combat AMR and its spread, the antibiotic alternatives can be employed in the swine production settings. Some of the challenge studies have effectively reduced the frequency of diarrhea, inhibited the colonization of pathogenic *E. coli*, improved growth performance in piglets, facilitated the growth of beneficial bacteria, and maintained the intestinal integrity of swine. Administration of vaccines reduced the prevalence of fimbrial antigens, which reduced the virulence of these pathotypes.

# Figures




Adapted from Barros et al., 2023

**Figure 1.1. The infection model of enterotoxigenic *Escherichia coli* (ETEC) on intestinal epithelial cells**

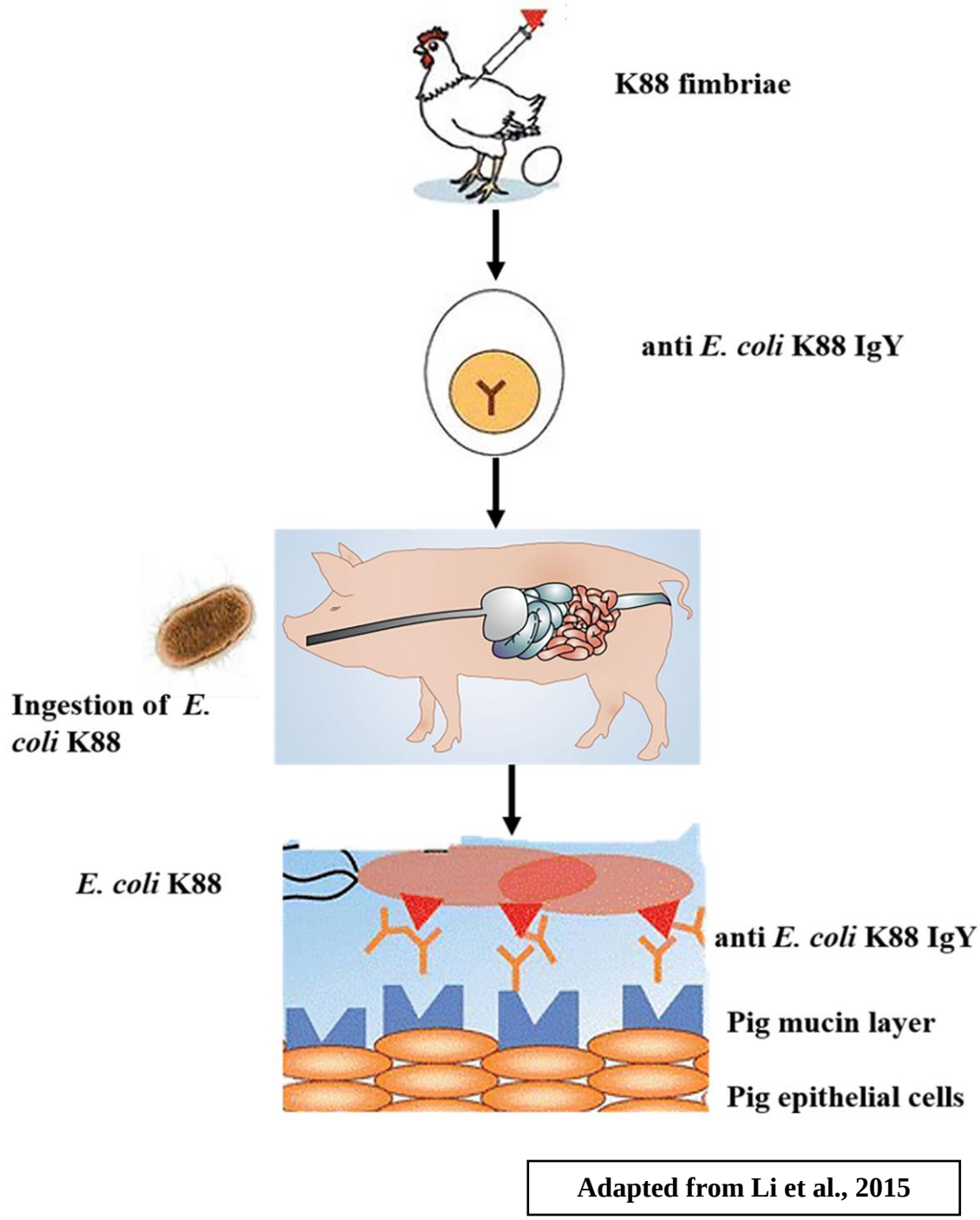



**Figure 1.2. Protective mode of action of IgY antibody against *Escherichia coli* K88**

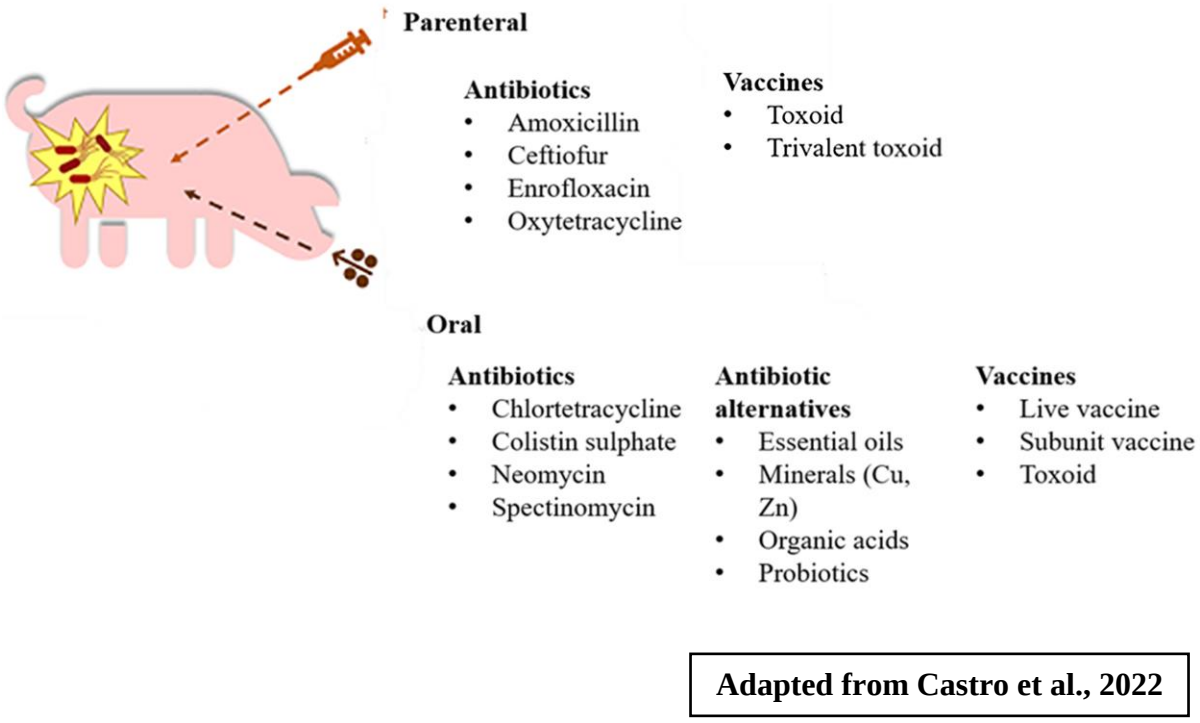



**Figure 1.3. Current treatment strategies used to control post weaning diarrhea (PWD) in piglets**

# Tables

**Table 1.1. Detection and isolation of *Escherichia coli* pathotypes and their serogroups, fimbriae and toxins in swine feces**

| Authors | Country | Study year | Predominant serogroups/ serotypes | Fimbriae | Toxins | Pathotypes | Notes |
|---|---|---|---|---|---|---|---|
| Do et al., 2019 | South Korea | 2008 to 2016 | O139, O157, O149 | F18, F4 | STb, STa | ETEC, STEC | A total of 362 *E. coli* isolates are screened. This study showed the shift in prevalence of primary serogroup from O157 to O139 in 9 years of study period in South Korea swine farms. |
| Luppi et al., 2016 | Europe | 2016 | | F4, F18 | STb, STa, LT | ETEC, STEC | Total of 339 *E. coli* isolates are screened. |
| Ho et al., 2013 | Malaysia | 2013 | O139:H1, O130:H26, O168:H21 | F18 | VT | VTEC | A total of 345 *E. coli* isolates are screened. Among them only 9 isolates carried Shiga toxin genes and all other isolates are negative for enterotoxin genes (LT1, LT2, ST, *eaeA*). |
| Mohlatole et al., 2013 | South Africa | 2013 | | None of the pathotypes carried Fimbriae | STb, STa, LT and *stx*2e | ETEC, EAEC, STEC | A total of 263 *E. coli* isolates are screened. 10 EAEC isolates carried both EAEC and ETEC genes and two isolates carried EAEC and STEC genes. |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Post et al., 2000 | USA (North Carolina) | 2000 | | F18, F4 | LT, STb, STa | ETEC | |
| Zhang et al., 2007 | USA (South Dakota, North Dakota, Minnesota, and Iowa) | 2004 to 2006 | | F4, F18 | STb, LT, EAST1, STa, Stx1 | ETEC, STEC | A total of 304 *E. coli* isolates are screened. This study has noticed the association of EAST1 toxin with the other enterotoxins of ETEC. |
| Remfry et al., 2021 | USA (Iowa, Minnesota, South Dakota, Nebraska, North Carolina, Oklahoma, Kansas, and Ohio) | 2021 | O121 (top-7 STEC) O8, O86 (non-top-7 STEC) | | Stx1, Stx2 | STEC | Total 598 fecal samples from finisher pigs are screened for Shiga toxins and top-7 and non-top-7 STEC associated serogroups. The higher prevalence of *eae* gene was found in association with Shiga toxin genes. |

**Table 1.2. Antimicrobials commonly employed in swine for the management of swine colibacillosis**

| Antimicrobial class | Antimicrobial compounds | Pharmacokinetic properties | Route of administration | Authors |
|---|---|---|---|---|
| Diaminopyrimidines /Sulfonamide | Trimethoprim/sulphamethoxazole | Rapidly absorbed from intestine, well distributed in tissues. | IM and orally | Dowling, 2013 |
| β-lactam antibiotics | Amoxicillin | Poorly absorbed from intestine, relatively poorly distributed in tissues. | IM and orally | Prescott, 2013 |
| β-lactam antibiotics | Amoxicillin + Clavulanic acid | Well distributed in tissues. | IM | Prescott, 2013 |
| Cephalosporins | Ceftiofur | Relatively poor distribution in tissues. | IM | Prescott, 2013 |
| Fluoroquinolones | Enrofloxacin | Well distributed in tissues. | IM | Rice, 2012 |
| Aminoglycosides | Neomycin | Poor absorption rate and relatively poor distribution in tissues. | Orally | Dowling, 2013 |
| Aminoglycosides | Apramycin | Not absorbed from intestine. | Orally | Dowling, 2013 |
| Aminocyclitols | Spectinomycin | | Orally | Dowling, 2013 |

**Table 1.3. Types of vaccines administered for the control and or prevention of swine colibacillosis**

| Vaccines | Composition | Authors |
|---|---|---|
| Oral subunit vaccine (Purified F4ac fimbriae) | Purified F4ac fimbriae | Vanden Broeck et al., 1999 |
| Live oral vaccines (Attenuated *E. coli* strain with F4ac) +Immunomodulator (Levamisole) | Attenuated *E. coli* strain with F4ac) +Immunomodulator (Levamisole) | Bozic et al., 2002 |
| Live oral vaccine (Coliprotec- Natural avirulent *E. coli* strain with F4ac) | Coliprotec- Natural avirulent *E. coli* strain with F4ac | Melkebeek et al., 2013 |
| Live oral vaccine (F18ac- positive *E. coli*) | F18ac- positive *E. coli* | Weltzin et al., 1989 and Bertschinger et al., 2000 |
| Encapsulated subunit vaccine (F4 fimbriae in enteric-coated pellets) | F4 fimbriae in enteric-coated pellets | Snoeck et al., 2003 |
| Encapsulated subunit vaccine (F4 fimbriae + Gantrez) | F4 fimbriae + Gantrez | Calinescu et al., 2007 |
| Stx2e toxoid | Inactivated Stx2e toxin | Oanh et al., 2011 |
| Oral vaccine (Shigatoxin 2eB- transgenic lettuce vaccine) | Shigatoxin 2eB- transgenic lettuce vaccine | Hamabata et al., 2021 |
| Live oral vaccine | Attenuated *E. coli* (expressing $LT_{R192G}$-$STa_{A13Q}$ and $LT_{R192G}$-STb fusion immunogen) | Liu et al., 2015 |

| | | |
|---|---|---|
| Oral toxoid fusion | Chimeric protein (with FaeG-FedF-$LT_{R192G}$A2:5LTB) | Ruan and Zhang, 2013 |
| Live oral vaccine | Attenuated *E. coli* (F4ac-LT $_{R192G-}$ STb) | Ruan et al., 2011 |
| Live oral vaccine | Attenuated *E. coli* (F41-LT $R_{192G-}$ $STa_{A13Q,}$ F41-LT $_{R192G-}$ STb) | Liu et al., 2015 |
| Oral vaccine | *Killed E. coli* F4ac strain and the solubilized fusion protein (F4ac-STa-LTB-STb) | You et al., 2011 |
| Oral vaccine | Fusion protein (LTA-$STa_{A13Q}$-STb-LTA2-LTB-$STa_{A13Q}$-STb) | Feng and Guan, 2019 |
| Parental toxoid vaccine (IM) | STa toxoid ($pLT_{R192G:}STa_{P12F}$: $STaA_{13Q}$) | Zhang et al., 2010 |
| Parental toxoid vaccine (SC) | STa toxoid fusion (BSA-$STa_{A14T}$ or 3x$STa_{N12S}$-$mnLT_{R192G/L211A}$) | Seo et al., 2019 |
| Parental vaccine (IP) | Tripartite chimeric toxoid (FaeG-FedF-$LT_{R192G}$) | Ruan et al., 2011 |
| Parental trivalent vaccine (IP) | Trivalent toxoid protein (STa-LTB-STb) | You et al., 2011 |
| Parental vaccine (IM) | (F4ac: STa-LTB-STb, F5:STa-LTB-STb) | Zhang et al., 2018 |
| Parental toxoid vaccine (SC) | Toxoid protein (F4/LT/STb and F18/STa/STb/Stx2e) | Lu et al., 2020 |

# Chapter 2 - Impact of *In-feed* vs *In-water* Administration of Chlortetracycline on the Prevalence of *Escherichia coli* Pathotypes involved in Swine Colibacillosis


## Abstract

Colibacillosis in swine, which includes neonatal enteritis, postweaning diarrhea, and edema disease, is one of the major economically important diseases. The pathotypes of *E. coli* involved are enterotoxigenic (ETEC), enteropathogenic (EPEC), enteroaggregative (EAEC), and Shigatoxigenic (STEC) *E. coli*. Our objectives were to determine the effects of *in-feed* or *in-water* chlortetracycline (CTC) administration on prevalence of virulence genes and pathotypes implicated in colibacillosis and to compare phenotypic and genotypic susceptibilities to CTC. A total of 1,296 weaned piglets (21 days age) were used in a 35-d study. Piglets were allocated to 48 pens (27 per pen) and pens were assigned randomly to no CTC, *in-feed* CTC, or *in-water* CTC groups. The CTC was provided from day 0 to 14. Fecal samples were collected from 5 piglets from each pen on days 0, 14 and 28. Samples were enriched in *E. coli* broth and subjected to a 11-plex PCR assay to detect major virulence genes targeting the four pathotypes and to a culture method to isolate and identify the pathotypes. Isolates were subjected to the CTC susceptibility testing by micro-broth dilution and major *tet* genes were determined by PCR.

The fecal prevalence of the virulence genes and the pathotypes were not affected by CTC administration ($P < 0.05$). The predominant enterotoxin genes in pure culture were *astA* (enteroaggregative heat stable, 29%) and *estB* (heat stable enterotoxin B; 21.9%). Shiga toxin genes were detected only in day-28 fecal samples and the *stx*2 gene was more predominant than *stx*1. The pathotypes of *E. coli* isolated were ETEC, atypical EPEC (positive for intimin and

enterotoxin genes), STEC and ETEC and STEC hybrids. Although the samples were positive for enteroaggregative enterotoxin gene (*astA*), EAEC pathotype was not detected. All isolates were resistant to CTC, with the *tet*A gene (91.5%) was the most predominant. *In-feed* or *in-water* CTC administration had no effect on the fecal prevalence of virulence genes and pathotypes implicated in swine colibacillosis and all the tested isolates showed phenotypic and genotypic resistant to CTC.

## Introduction

Enteric infections caused by *Escherichia coli*, including subclinical infections, particularly in nursery pigs, are of significant economic importance in the swine industry (Moxley et al., 1999). In addition to mortality, the infections have significant impact on performance, reduced weight gain and feed efficiency (Moxley et al., 1999). Three diseases caused by different pathotypes of *E. coli*, neonatal enteritis, post-weaning diarrhea, and edema disease, collectively called 'colibacillosis', are common in nursery pigs. The pathotypes involved in colibacillosis are enterotoxigenic (ETEC), enteropathogenic (EPEC), enteroaggregative (EAggEC), and Shiga toxigenic (STEC) *E. coli.* The major exotoxins produced by the four pathotypes responsible for the enteric infections include heat labile and heat stable enterotoxins (mainly by ETEC), hemolysin (mainly by EPEC), enteroaggregative heat stable toxin (EAST; mainly by EAggEC), and Shiga toxins and enterohemolysin (mainly by STEC). There is evidence that a single pathotype can produce multiple enterotoxins (Boekman et al., 2021). Additionally, there are certain serogroups and serotypes of *E. coli* within each pathotype that are more prevalent than other serogroups in causing the disease. The pathotype that causes neonatal enteritis is enterotoxigenic *E. coli* (ETEC) (Nordeste et al., 2017). Certain STEC serotypes

produce a subtype of Shiga toxin (Stx), called Stx2e, which causes edema disease in weaned piglets (Garcia-Menino et al., 2018). Some serotypes produce both Shiga toxin and enterotoxins, which could cause edema disease and postweaning diarrhea in the same animal (Garcia-Menino et al., 2018). The EPEC pathotype is primarily responsible for post-weaning diarrhea. The early detection, diagnosis, and treatment of *E. coli* infections are critically important to reduce the economic impact (Wills et al., 2000; Garcia-Menino et al., 2018). The economic impact is because of mortality, decreased animal models, costs for treatment and handling, and vaccinations (Garcia-Menino et al., 2018). Other forms of economic loss include cost of therapy and high morbidity (Holland et al., 1990). Although the major serogroups and serotypes of *E. coli* pathotypes are known, their prevalence and fecal shedding in nursery pigs are not known. Recently, in the commercial swine production settings, *E. coli* infections are on the rise among weaned piglets (Kim et al., 2022). This is mainly attributed to the usage of Duroc sires within the swine industry and their genetic susceptibility to *E. coli* (Ma et al., 2022). Several physical resources at the farm level like floor type, pen divisions, watering, feeding, and feces disposal have a direct association with the onset of diarrhea among pigs at different stages of growth. Other factors like swine genetics, source of replacement stock, biosecurity measures, movement and mingling of pigs also play a role on the onset and occurrence of diarrhea (Pearce, 1999).

Feed and water-based antibiotics are commonly employed with and without the supplementation of minerals like zinc (first 21 days post weaning) for effective mitigation of diarrhea among piglets. Chlortetracycline (CTC), a broad spectrum and a medically important antibiotic, is widely used for treatment of respiratory and enteric disease (Sacristan et al., 2012; Holman et al., 2019). Whether the use of chlortetracycline or route of administration influences antimicrobial resistance of pathogenic *E. coli* is of high interest to the swine industry.

Chlortetracycline can be administered either *in-feed*, *in-water*, or as injectable. The oral route of administration is by far the most common because of ease and convenience. In some cases, vaccines have been used with little success as timing of vaccination can be difficult (https://www.nationalhogfarmer.com/animal-health/resurgence-enterotoxigenic-hemolytic-e-coli). The need for novel methods for combatting infectious diseases has significantly increased, especially because many animal pathogens have developed antimicrobial resistance (Nordeste et al., 2017). With resistance among *E. coli* pathotypes becoming an industry-wide issue, producers and veterinarians are looking for effective alternatives to reduce or mitigate the impact of *E. coli* in pigs (https://www.thepigsite.com/articles/tackle-post-weaning-e-coli-issues-with-aggressive-cleaning-vaccination). The use of antimicrobials for disease treatment and prevention is a critical tool in swine production and management. Because of public health concerns associated with the development of antimicrobial resistance (AMR), there is considerable interest and effort in identifying feeding and management practices to minimize the prevalence of AMR in bacteria.

Our objectives were to develop PCR- and culture-based method to determine prevalence and isolate and identify major virulence genes associated with the pathotypes (ETEC, EPEC, EAggEC, and STEC) and serogroups of *E. coli* involved in enteric infections in nursery pigs, and to compare the phenotypic and genotypic antimicrobial susceptibilities of these isolates to chlortetracycline.

## Materials and methods

### Animals and study design

A total of 1,296 piglets (21 days of age), were split into 48 pens with 27 pigs/pen arranged in a randomized complete block design. The study was conducted in an enclosed

commercial research nursery barn. Each pen was randomly assigned to one of the three treatment groups such as control, *in-feed* chlortetracycline (22 mg/kg BW), and *in-water* chlortetracycline (22 mg/kg BW). Pens were equipped with slatted flooring, six-hole stainless steel self-feeders, and pan waterers to provide *ad libitum* access to feed and water. Fresh fecal samples were collected on days 0 (pre-treatment), 14 (treatment), and 28 (post-treatment) randomly from 5 of 27 piglets from each pen.

### *Escherichia coli* enrichment

Approximately, 1 g of fecal sample was suspended into 9 mL of *Escherichia coli* (EC) broth (Becton Dickinson and Company, Sparks, MD) and vortexed for 1 min and the fecal suspension was incubated at 40°C for 6 h. After incubation, 1 mL of the post enriched fecal suspension was pipetted into a 2 mL centrifuge tube and subjected for DNA isolation as per published procedures (Paddock et al., 2012; Remfry et al., 2020).

### PCR detection of eleven virulence genes

An eleven-plex PCR assay was carried out to detect *aggA, ehxA, estA, estB, bfpA, astA, hlyA, eae, stx*2, *elt,* and *stx*1 genes associated with pathotypes implicated to cause colibacillosis. The information on genes and primer sequences is provided in Table 2.1. The purified DNA from a total of 360 fecal samples was subjected to the 11-plex PCR assay to determine their prevalence.

The assay conditions included a working concentration of primer mix (4.5 pM /µL of each primer). The reaction mix, with a total volume of 20 µL, consisted of 10 µL of GoTaq Green Master Mix (Promega, Madison, USA), 7 µL of sterile PCR grade water, 1 µL of primer mix, and 2 µL of DNA extracted from enriched fecal samples. The PCR running conditions included initial denaturation for 2 min at 94°C, followed by 35 cycles of 15 sec denaturation at

94°C, annealing at 67°C for 80 sec, and extension at 68°C for 2 min with a final hold at 10°C. The *E. coli* strains H10407 and sPRH (ETEC), 2348-69 (EPEC), 17-2 (EAEC) and ATCC 43894 (STEC) served as our reference positive controls for our PCR assay.

## Isolation of *E. coli* by direct plating of fecal samples

Enriched fecal samples positive were streaked onto MacConkey (MAC) agar plates (Becton Dickinson and Company, Sparks, MD) using a sterile loop. Additionally, samples were diluted (1 in 100 dilution) in buffered peptone water, and 100 µL of the diluted fecal suspension is spread-plated onto MAC plates. The plates were incubated at 37°C for 18 to 24 h. After incubation, 10 presumptive *E. coli* colonies (pink lactose fermenting, round, smooth colonies) were selected and streaked onto blood agar plates and incubated at 37°C for 18 to 24 h. The colonies from each pie of blood agar plate were subjected for indole testing Indole positive isolates were subjected for species identification by PCR. The individual colony from each pie is transferred to 100 µL of distilled water and boiled for 5 min at 95°C and centrifuged at 2,200 g for 2 min. After the centrifugation the supernatant was used as the DNA template. The DNA template was tested individually by mPCR assay to detect the 11 genes using afore mentioned assay conditions with a slight modification to annealing step of PCR reaction (67°C for 80 sec). The detection of eleven virulence genes by mPCR was provided in Figure 2.1. If an isolate tested positive for any of the 11 genes, it was stored using a cryo-protect bead (Key Scientific Products, Stamford, Texas).

## Detection of pili or fimbriae genes by PCR

A multiplex PCR assay was carried out to detect F4, F5, F6, F18, F41 that are commonly associated pili with virulence genes implicated to cause colibacillosis. Details regarding the genes and their corresponding primer sequences is given in Table 2.1. The purified DNA from

selected positive virulence genes isolates were subjected to the multiplex PCR assay to determine their prevalence. The assay conditions included a working concentration of primer mix 1 and primer mix 2 (10 pM/uL of each primer respectively) in a 20 µL reaction mix containing 10 µL of GoTaq Green Master Mix (Promega, Madison, USA), 6 µL of sterile PCR grade water, 1 µL of primer mix1 and 1 µL of primer mix 2, and 2 µL of DNA extracted from positive isolates. The PCR running conditions included initial denaturation for 5 min at 94°C, followed by 25 cycles of 30 sec denaturation at 94°C, annealing at 62°C for 30 sec and extension at 68°C for 7 min with a final hold at 8°C.

## PCR detection of serogroups

A multiplex PCR assay was performed to identify serogroups associated with swine *E. coli* pathotypes. The serogroups and primer sequences follow our published procedures (Bai et al., 2012; Shridhar et al., 2016; Remfry et al., 2020; Ludwig et al., 2020). The purified DNA from the selected isolates underwent multiplex PCR assay to determine their prevalence. The assay conditions included a working concentration of primer mix (4-7 pM /µL of each primer) in a 20 µL reaction mix containing 10 µL of GoTaq Green Master Mix (Promega, Madison, USA), 7 µL of sterile PCR grade water, 1 µL of primer mix, and 2 µL of DNA extracted from positive isolates. The PCR running conditions for set no 1-11 include initial denaturation of 5 min at 94°C, followed by 25 or 30 cycles of 30 sec denaturation at 94°C, annealing at 58-68°C for 30 sec and extension at 68°C for 7 min with a final hold at 4°C. The PCR amplification program for set no 12, 13 and 14 were initial denaturation of 1 min at 94°C, followed by 25 cycles of 30 sec denaturation at 94°C, annealing at 58-63°C for 30 sec and extension at 72°C for 60-80 sec with a final hold at 4°C.

## Antimicrobial susceptibility determinations

Isolates positive for either more than one virulence genes were subjected to antimicrobial susceptibility testing of chlortetracycline to determine the minimum inhibitory concentrations (MIC) by micro-broth dilution method according to the Clinical Laboratory and Standards Institute (CLSI, 2018). Stock solution of chlortetracycline antibiotic was prepared by adding sterile distilled water and the final concentration was adjusted to 1,000 µg/mL. Chlortetracycline was tested at the concentrations of 100, 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39, and 0.195 µg/mL. The concentration of bacterial inoculum was adjusted to 0.5 McFarland turbidity standards (Remel Company, Lenexa, KS) by adding individual bacterial colonies from blood agar plate to Mueller-Hinton broth (10 mL) (Becton, Dickinson and Company, Sparks, MD) and vortexed. Then, 100 µL of the Mueller-Hinton broth containing bacterial inoculum were dispensed into 96-well microtiter plates, (Corning Incorporated, Corning, NY) followed by 1:100 dilution of the culture. The microtiter plates were incubated at 37°C for 20 to 24 h, and the results were recorded as either growth or no growth.

## PCR detection of tetracycline resistance genes

Previously published multiplex PCR procedure was employed to detect *tet*A, *tet*B, *tet*C, and *tet*D genes (Ng et al., 2001; Aminov et al., 2002).

## Statistical analysis

The analysis was carried out using SAS (v. 9.4; Cary, NC). Pen was considered as an experimental unit. Bivariate descriptive statistics of all the virulence genes by treatment and sampling day were carried out before building a final model. The PROC MIXED procedure with RFML (residual maximum likelihood) was used to evaluate the prevalence of virulence genes, pathotypes, their association with pili genes, and tetracycline resistance genes. The final model

included fixed effects of treatment and sampling day as a random effect. The outcome variables, modeled using a binary outcome, consisted of the number of positive samples within each pen divided by the total number of samples tested per pen. The least square means were generated using PDIFF option with a Tukey-Kramer adjustment for multiple comparisons by treatment, sampling day and their interaction. The MIC values were log transformed using PROC RANK and analysis was performed on ranked values. Results were considered significant at a *P*-value of $< 0.05$.

# Results

## Prevalence of virulence genes in enriched fecal samples

Of the 360 enriched fecal samples tested by eleven plex PCR assay, 356 (356/360, 98.8%) samples were positive for any one of the 9 virulence genes. None of the fecal samples were found positive for *aggA* and *bfpA* genes. Overall prevalence of *estB* (97%; 349/360) and *astA* (93%; 334/360) genes were higher when compared to other genes across all sampling days and treatment groups with a significant sampling day effect ($P < 0.001$). The prevalence of *ehxA*, *hlyA*, *estA*, *eae*, *elt*, and *stx*2 were 73% (262/360), 62.2% (224/360), 55.5% (200/360), 42.5% (153/360), 15.8% (57/360), and 12% (42/360), respectively. The sampling day had a significant effect ($P < 0.001$) on the prevalence of these genes with neither the treatment groups nor their interaction with the sampling day ($P > 0.05$) for all the virulence genes tested. Only one sample was positive for *stx*1 gene.

The pen prevalence of *estB* and *astA* was 100% across all three sampling days and treatment groups. Similarly, treatment phase (day 14) and post-treatment phase (day 28) had the prevalence of 100% for *ehxA*, *estA*, *hlyA* virulence genes when compared to day 0, with a

significant sampling day $P < 0.001$, across three treatment groups. The pre-treatment phase (day 0) had a prevalence of *ehxA*, and *hlyA* genes ranging from 62.5% to 100% across three treatment groups and 12.5% prevalence of *estA* gene was observed in control and *in-feed* CTC groups. The prevalence of *eae* gene ($P < 0.001$) was 100% in the treatment phase (day 14) and 75% in the post-treatment phase (day 28) across three treatment groups. But, the pre-treatment phase (day 0) had prevalence ranging from 25% to 50% across three treatment groups. The prevalence of *stx*1gene ($P = 0.3765$) was detected only during post-treatment phase (day 28) in *in-feed* CTC group. Similarly, *stx*2 gene ($P < 0.001$) was detected only during day 28 and had prevalence ranging from 37.5% to 62.5% across three treatment groups. The *elt* gene ($P < 0.001$) was detected only during treatment phase (day14) and had the prevalence ranged from 62.5% to 87.5% across three treatment groups. The information on enriched fecal pen prevalence of virulence genes was provided in a table (2.2).

**Prevalence of virulence genes in pure culture**

Fecal samples enriched in *E. coli* broth and positive for any one of the nine virulence genes were inoculated directly on to MAC and incubated and a total of 10 colonies per sample were streaked on to the 10-pie blood agar. Each pie of pure culture was tested by mPCR assay for 11 virulence genes. There were total of 3,600 isolates screened among them 3,263 isolates recovered *E. coli* (3,263/3,560; 91.7%) of which 2,660 isolates are positive for any one gene (2,660/3,263, 81.5%) and 1,923 isolates are positive for more than one gene (1,923/3,263, 58.9%). None of the isolates were found positive for *aggA*, *bfpA* and *stx*1 genes. Overall prevalence of *astA* (29%; 947/3,263) and *estB* (21.9%; 714/3,263) genes were higher when compared to other genes across all sampling days and treatment groups with a significant sampling day effect ($P < 0.001$), treatment and day interaction ($P < 0.001$), respectively. The

prevalence of *ehxA, hlyA, estA, eae*, and *stx*2 were 1.7% (56/3,263), 13.2% (430/3,263), 2.6% (84/3,263), 4.6% (151/3,263), and 7.3% (238/3,263), respectively. The treatment and day interaction were significant on the prevalence of *ehxA* ($P = 0.0019$), *estA* ($P = 0.0079$), *hlyA* ($P = 0.0060$) and *eae* ($P < 0.001$), respectively. Whereas sampling day had a significant effect ($P < 0.001$) on the prevalence of *stx*2. Only two isolates were positive for *elt* gene.

The pen prevalence of *astA* gene ($P < 0.001$) during day 0 (75% to 87.5%) and day 28 (87.5%) was almost similar when compared to day 14 (62.5% to 75%) across all treatment groups. The *estB* gene ($P < 0.001$) on day 0 and day 14 had similar prevalence ranging from 75% to 87.5% across three treatment groups respectively. But day 28 had lower prevalence ranging from 25% to 50% across three treatment groups. The *ehxA* ($P = 0.0690$) and *eae* ($P = 0.0296$) genes had higher range of prevalences on day 14 (50% to 75%; 75%) when compared to day 28 (12.5% to 25%; 25% to 37.5%) and day 0 (0% to 25%; 25% to 50%) across three treatment groups respectively. Similarly, *hlyA* ($P = 0.0156$) and *estA* ($P = 0.0535$) genes also had higher prevalence range on day 14 (50% to 100%; 50% to 75%) in comparison to day 28 (62.5% to 75%; 25% to 37.5%) and day 0 (25% to 37.5%; 0% to 12.5%) across three treatment groups respectively. The *stx*2 ($P = 0.0010$) gene was observed only on day 28 across three treatment groups and had a prevalence of 37.5%. Similarly, *elt* ($P = 0.3765$) gene was detected only on day 14 in control group and had prevalence of 12.5%. The information on pure culture pen prevalence of virulence genes was provided in a table (2.3).

The pen prevalences of ETEC, and STEC, pathotypes of pure culture was provided in tables 2.4 & 2.5 respectively, where either treatment, sampling day or their interaction has no significant effect ($P > 0.05$). However, the pen prevalences of APEC, and hybrid pathotypes of

pure culture has significant sampling day effect ($P = 0.0044$; $P < 0.0001$) respectively and information was provided in tables 2.6 & 2.7.

## Prevalence of fimbriae genes

A total of 1,732 *E. coli* isolated across all sampling day and treatment groups were subjected for the PCR detection of F4, F5, F6, F18, and F41 fimbriae genes by PCR. Overall prevalence of fimbriae genes was very negligible. None of the isolates were positive for F5, F6, and F41 genes. The prevalence of F4 (9/1,732) and F18 (2/1,732) were 0.5% and 0.12%, respectively. The 9 positive F4 isolates also harbored genes for *estA* and *estB* genes. And, 2 of the F18 positive isolates were also positive for *estB* and *elt* genes. Information on prevalence of fimbriae genes was provided in a table (2.8).

## Prevalence of serogroups

A total of 145 *E. coli* isolated from control group across all sampling days were subjected for PCR detection of serogroups (n=123). Among the 145 isolates that were tested 141 (141/145, 97.2%) are from known 21 O serogroups and 4 (4/145, 2.8%) were from unknown serogroups. The O81 was the most predominant serogroup reported among the isolates with the prevalence of 26.8% (33/123). The prevalence of O15 and O35 were 15.4% (19/123), 14.6% (18/123), respectively. The prevalence of O69, O182, O98, O145, O121, O76 were 8.9% (11/123), 5.7% (7/123), 4.9% (6/123), 4% (5/123), 3.3% (4/123), 1.6% (2/123), respectively. The prevalence of O112ab and O123/O186 was 8.1% (10/123) and O8, O23, O26 had a prevalence of 2.4% (3/123) and the lower prevalence of 0.8% (1/123) reported for O7, O11, O24, O88, O141, O150, and O180 serogroups. The information on prevalence of serogroups was provided in a table (2.9).

## Antimicrobial susceptibility testing

*E. coli* isolates (n=165) positive for either two or more virulence genes were selected across all sampling days and treatment groups for antimicrobial susceptibility testing of chlortetracycline by microbroth dilution method. The minimum inhibitory concentrations (MIC) procedure was carried out as per the Clinical Laboratory and Standards Institute (CLSI, 2018). Any isolate with an MIC of ≥16 µg/ml was considered resistant. All the tested isolates were resistant to CTC across three treatment groups control, *in-feed* CTC, *in-water* CTC had MIC values of 50 µg/mL (CI = 37.5 to 62.5), 50 µg/mL (CI = 44.74 to 60.5), 62.5 µg/mL (CI = 50 to 75), respectively. The information on antimicrobial susceptibility testing is provided in table 2.10.

## Prevalence of tetracycline resistance genes

A total of 165 *E. coli* isolates were selected across all three treatment groups and sampling days based on the gene profile. *E. coli* isolates (n=165) used for antimicrobial susceptibility testing were further subjected for genotypic characterization by PCR for the detection of tetracycline resistance genes (*tetA*, *tetB*, *tetC*, and *tetD*). None of the tested isolates were positive for *tetC* gene. The *tetA* was the most predominant gene reported among these isolates. The prevalence of *tetA* gene was 91.5% (151/165) with a significant sampling day effect ($P$ = 0.0074) where the higher prevalence of *tetA* was detected on day 14. The prevalence of *tetB* gene was 16.9% (28/165) and sampling day had a significant effect ($P$ = 0.0091) and the higher prevalence of *tetB* was detected on day 28. The prevalence of *tetD* (12.7%: 21/165) without a sampling day or treatment effect ($P$ = 0.1785). The information on prevalence of tetracycline resistance genes among *E. coli* isolated from piglets was shown in a table (2.11).

## Discussion

In this study ETEC pathotype had higher prevalence ranging from 75% to 87.5% and lower prevalence of STEC (12.5%) pathotype were detected. But the EAEC pathotype was not detected in any sample. Similar results of higher ETEC pathotype prevalence was noticed when isolates are screened for enterotoxin genes in South Africa (Mohlatlole et al., 2013). Among the STEC pathotype, 238 isolates are detected positive for *stx*2 (238 of 3,263) and 18 isolates of 238 also carried *eae* gene in this study. This rare association of *eae* with *stx*2 is also previously reported (Remfry et al., 2021). In this study none of the isolates were positive for *bfpA* gene so the EPEC positive isolates are categorized as atypical EPEC pathotype. This study had the moderate prevalence of atypical EPEC pathotype ranging from 12.5% to 62.5%. The previous study detected 4.9% of typical EPEC and 2.4% of atypical EPEC pathotypes when diarrheic *E. coli* isolates are screened (Dulguer et al., 2003). In this study the EAEC pathotype had 947 isolates positive for *astA* gene (947 of 3,263) and 625 isolates among the 947 are positive only for *astA* and negative for enterotoxin genes. However, none of these *astA* isolates are in association with *aggA* gene. Similar results of higher prevalence of *astA* and absence of *aggA* gene was noticed when the fecal samples of diarrheic children, swine, and companion animals are screened in China (Zhang et al., 2016).

Several laboratory methods are developed for the detection of virulence factors associated with pathogenic *E. coli*. The histopathological method involves immunohistochemical-stained ileal impression smear that reveals the presence of fimbriae of *E. coli* (Francis, 1999). The virulence gene expression of pathogenic *E. coli* can be tested *in-vitro* through cell culture assays and ELISA and *in-vivo* experimentation in lab animals (Isaacson et al 1978; Czirok et al., 1992). Further genetic characterization of virulent *E. coli* by DNA based

molecular detection tests such as PCR and DNA hybridization techniques were developed for the detection of virulence factors (Franck et al., 1998; Tsen and Jian, 1998). Later rapid and sensitive methods for detection of pathogenic *E. coli* by real -time PCR was developed (Fukushima et al., 2003). Many different PCR assays have been reported ranging from single reaction to multiple virulence genes detection. In this study the design and evaluation of multiplex PCR (mPCR) assay was developed for detection of 11 virulence genes (*aggA, ehxA, estA, estB, bfpA, astA, hlyA, eae, stx*2, *elt,* and *stx*1 genes) of pathogenic *E. coli* associated with swine colibacillosis. The mPCR assay is rapid and less time consuming for sample preparation. Since batch preparation and lyophilization of PCR reaction components potentially reduced the chances of contamination, that further strengthened the practicality of mPCR. The eleven virulence genes included in the mPCR detection in the current study were selected because they are well characterized with the pathotypes of *E. coli* concerned with swine colibacillosis and prevalence of these isolates detected from neonatal, PWD, and ED. This mPCR assay is used for rapid characterization of *E. coli* pathotypes of swine colibacillosis (Casey and Bosworth, 2009). The culture method recovered 3,263 pure isolates among them 2,660 isolates (81.5%) are positive for any one gene. Among the pure culture isolates targeted for 11 virulence genes two predominant virulence genes were detected *astA* and *estB* followed by small number of isolates of *hlyA, stx*2, *eae, estA and ehxA* respectively. However, none of the isolates were found positive for *aggA, bfpA* and *stx*1 gene. Similar results of high prevalence of *astA* and *estB* have been reported in feces of piglets of South Korea and South Africa (Lee et al., 2008; Mohlatlole et al., 2013). The *E. coli* isolates from diarrheic and healthy piglets of Spain had higher prevalence of *estB* (78.38%) followed by *estA* (62.16%) and *elt* (25.68%) respectively (Blanco et al., 2006). The isolates collected between 2003 to 2008 from the diseased swine in China had the prevalences of

67% for *stx*2e, 32% for *estA* and 13% for *estB* (Wang et al., 2011). The diarrheic *E. coli* isolates of piglets in Brazil had the prevalences of 71%, 44%, 47% and 3% of *elt, estA, estB* and *stx*2e respectively. This suggests that geographical location plays a key role in prevalence of virulence genes (Mohlatlole et al., 2013).

In this study none of the isolates found positive for *aggA*. Similar results regarding the absence of *aggA* were found in USA when clinical isolates of humans and non-clinical isolates of swine and companion animals are screened (Zhang et al., 2016). However, low prevalence of *aggA* were reported when diarrheic children *E. coli* isolates are screened in Iran (Aslani et al., 2011). This indicates the regional difference in the prevalence of *aggA* gene. The prevalence of *bfpA* gene was not detected in this study. The results were in accordance with the *E. coli* isolates that were screened from feces of healthy piglets in India (Kylla et al., 2020). In this study the high prevalence of *estB* followed by *astA* was noticed across enriched fecal samples whereas in case of pure isolates the prevalence was vice versa. In the case of enriched samples one sample was found to be positive for *stx*1 whereas none of the pure isolates were positive for *stx*1 gene. In a similar way 57 fecal samples are screened positive for *elt* but only one pure isolate positive for *elt* gene. To our knowledge we did not find any articles comparing enriched fecal samples and culture data.

In this study the *E. coli* isolates are screened for five different fimbriae F4, F5, F6, F18, and F41. Among them the prevalence of F4 and F18 were 0.5% and 0.12% respectively. The results are similar to diarrheic *E. coli* isolates of pigs from Europe where they reported high prevalence of F4 (45.1%) and F18 (33.9%) (Luppi et al., 2016). Similarly, a study conducted in USA during 2007 where they have screened 304 *E. coli* strains for enterotoxins and five fimbrial antigens (F4, F18, F41, 987p and K99 fimbria). The 175 isolates tested positive for at least one

of the fimbriae. Among them higher prevalence of F4 and F18 are detected and both are in association with enterotoxin genes. However, Shigatoxin is in association with F18 (Zhang et al., 2007). In contrast, the study conducted in South Korea where they have screened diarrheic piglets and found higher prevalence of F18 (43.1%) when compared to F14 (20.2%) (Do et al., 2019).

Swine *E. coli* has many virulence factors among them O-serogroup is one of the major virulence factors. Several O-serogroups are associated with diarrhea, however reported number of serogroups in association with PWD and ED are less (Blanco et al., 2006; Sato et al., 2016; Do et al., 2019). The previous study conducted in weaned piglets of South Korea from 2008 to 2016 had detected 51 serogroups of which only 5 were constituted as typeable isolates (O149, O139, O157, O182, and O14). Among the identified five serogroups, three serogroups O139, O157, and O149 were frequently detected, which is also similar in other countries such as China and Canada (Noamani et al., 2003; Chen et al., 2004). Among the frequently detected serogroups O149 and O139 are associated with PWD and ED respectively (Do et al., 2019) and O157 serogroup is generally detected worldwide (Paul 2015). Nearly 187 serogroups of *E. coli* were detected among them 158 are associated with STEC pathotype (Ludwig et al., 2020; Remfry et al., 2021). The majority of STEC infections like food borne outbreaks in humans due to consumption of contaminated pork products are mainly due to top- 7 STEC (O26, O45, O103, O111, O121, O145, and O157) serogroups (Scallan et al., 2011; Gould et al., 2013). Even from swine feces, the majority of non-top-7 serogroups O8, O59, O71, O86, O100, O163, O174, and O184 have been isolated (Tseng et al 2014; Remfry et al., 2021). Among the non-top-7 serogroups the highest prevalence of O8, O86, O100 and O36 were reported respectively. The high prevalence O8 serogroup is in accordance with (Kaufmann et al., 2006; Remfry et al., 2021)

previous studies and is the common serogroup associated with PWD in weaned piglets (Frydendahl et al., 2002; Han et al., 2007). The O157 is the most common serogroup of STEC infections in humans. Swine *E. coli* carrying *stx*2e gene does not cause major threat to humans, and they are isolated from healthy as well as mild diarrheic feces of humans (Beutin et al., 2004; Sonntag et al., 2005; Beutin et al., 2008).

In this study the selected isolates positive for virulence genes are tested phenotypically against CTC by microbroth dilution method and all the tested isolates are found to be resistant. The genotypic characterization revealed high prevalence of *tet*A followed by low prevalence of *tet*B and *tet*D. Even in previous studies tetracycline resistance is the most common and prevalent resistant phenotype (Tadesse et al., 2012). The tetracyclines are widely used because of their therapeutic as well as in promoting feed efficiency. So, the persistence of resistance reported in coliforms a decade even after discontinuing either *in-feed* or treatment which indicates that the selection of resistant bacteria due to long-term feeding of antibiotics may not be reversed by withdrawal of antibiotics (Langlois et al., 1983).

In conclusion, we have developed and demonstrated the use of 11-plex PCR for the detection of virulence genes associated with *E. coli* pathotypes. The predominant enterotoxin genes were *astA* and *estB* genes. The occurrence of F4 and F18 fimbriae genes were reported. The *stx*2 was more predominant than *stx*1 gene. The EAEC pathotype was not detected. The predominant serogroup isolated was O81. All the tested *E. coli* isolates were resistant to chlortetracycline with *tetA* being the predominant tetracycline resistant gene. In summary, either *in-feed* or *in-water* CTC administration had no effect on the fecal prevalence of virulence genes and pathotypes implicated in swine colibacillosis.

# Figure

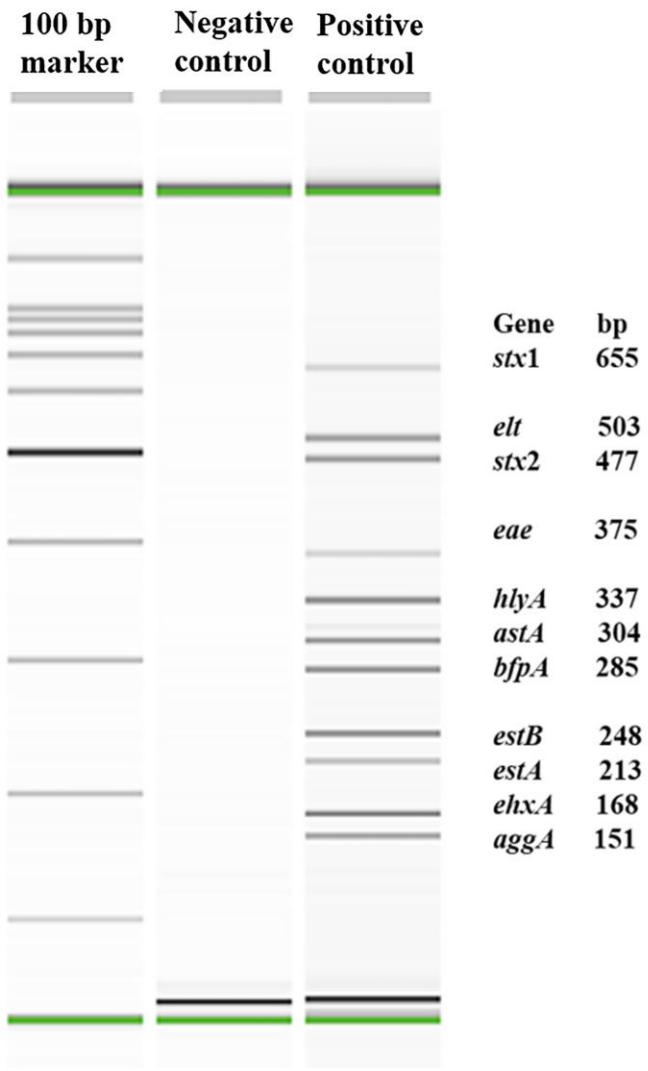



**Figure 2.1. PCR gel electrophoresis image of eleven virulence genes responsible for swine colibacillosis**

# Tables

**Table 2.1. PCR Primers used in the study to detect pathogenic *Escherichia coli* virulence genes and fimbriae genes from fecal samples of piglet diets supplemented with or without *in-feed* and *in-water* chlortetracycline**

| Virulence genes and Pili | Primer | Primer sequences (5' to 3') | Amplicon size (bp) | Source |
|---|---|---|---|---|
| *aggA* | *aggA* -F1 | CGTTACAAATGATTGTCCTGTTACTAT | 151 | Paddock et al., 2013 |
| | *aggA* -R1 | ACCTGTTCCCCATAACCAGAC | | |
| *bfpA* | *bfpA* -F2 | CAGAAGTAATGAGCGCAACG | 285 | Shridhar et al., 2016 |
| | *bfpA* -R2 | CGTAGCCTTTCGCTGAAGTA | | |
| *eae* | *eae* -F2 | TACGCGAAAGATACCGCTCT | 375 | Noll et al., 2015 |
| | *eae* -R2 | CATGCGGAAATAGCCGTTA | | |
| *astA* | *astA* -F | GGCTCAATGTGCTGACTGAA | 304 | This study |
| | *astA* -R | TGCCAGCTTCGGCTTATC | | |
| *ehxA* | *ehxA* -F | GCGAGCTAAGCAGCTTGAAT | 168 | Bai et al., 2010 |
| | *ehxA* -R | CTGGAGGCTGCACTAACTCC | | |
| *hlyA* | *hlyA* -F4 | ACGAAAGTACTGGGTAATGTTGG | 337 | This study |
| | *hlyA* -R4 | ATGTCGTTGCAGCAGCACT | | |
| *elt* | *elt* -F2 | TTATGATCACGCGAGAGGAA | 503 | This study |
| | *elt* -R2 | TTGTGCTCAGATTCTGGGTCT | | |
| *estA* | *estA* -F2 | CATGACGGGAGGTAACATGA | 213 | This study |
| | *estA* -R2 | GGATTACAACAAAGTTCACAGCA | | |
| *estB* | *estB* -F2 | CTTGACTCATATAAAAGCCCACTG | 248 | This study |
| | *estB* -R2 | GCAGTACCATCTCTAACCCCTAAA | | |

| | | | | |
|---|---|---|---|---|
| *stx*1 | *stx*1-F | TGTCGCATAGTGGAACCTCA | 655 | Bai et al., 2010 |
| | *stx*1-R | TGCGCACTGAGAAGAAGAGA | | |
| *stx*2 | *stx*2-F | CCATGACAACGGACAGCAGTT | 477 | Bai et al., 2010 |
| | *stx*2-R | TGTCGCCAGTTATCTGACATTC | | |
| F4 | F4-F | ATTTCAATGGTTCGGTCGAT | 416 | This study |
| | F4-R | CGCAGAAGTAACCCCACCT | | |
| F5 | F5-F | CAGGAAATACTGCTGCTAAAG | 150 | This study |
| | F5-R | GCTGGGCTGAATAGTTAAATGAC | | |
| F6 | F6-F | ACCAGCCAGGCAAATTTAGA | 492 | This study |
| | F6-R | TGTACCTGCTGAACGAATAGTCA | | |
| F18 | F18-F | CAGCAAGGGGATGTTAAATTC | 218 | This study |
| | F18-R | AACTGCCCGCTCCAAGTTA | | |
| F41 | F41-F | TGATTGGACGGAAGGTCAAC | 561 | This study |
| | F41-R | CCTGGCATTAACTTTTCTACATAACC | | |

**Table 2.2. Pen prevalence of the virulence genes of *Escherichia coli* in enriched fecal suspension of piglets' diet supplemented with or without *in-feed* and *in-water* chlortetracycline**

| Virulence genes | Day 0 | | | Day 14 | | | Day 28 | | | *P*- value | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Control | *In-feed* CTC | *In-water* CTC | Control | *In-feed* CTC | *In-water* CTC | Control | *In-feed* CTC | *In-water* CTC | Treatment | Sampling day | Treatment * Sampling day |
| *ehxA* | 5 (62.5) | 8 (100) | 6 (75) | 8 (100) | 8 (100) | 8 (100) | 8 (100) | 8 (100) | 8 (100) | 0.9199 | <.0001 | 0.3365 |
| *estA* | 1 (12.5) | 1 (12.5) | 0 | 8 (100) | 7 (87.5) | 8 (100) | 8 (100) | 8 (100) | 8 (100) | 0.3014 | <.0001 | 0.3110 |
| *estB* | 8 (100) | 8 (100) | 8 (100) | 8 (100) | 8 (100) | 8 (100) | 8 (100) | 8 (100) | 8 (100) | 0.7416 | 0.0082 | 0.8618 |
| *astA* | 8 (100) | 8 (100) | 8 (100) | 8 (100) | 8 (100) | 8 (100) | 8 (100) | 8 (100) | 8 (100) | 0.4699 | 0.0186 | 0.7541 |
| *hlyA* | 6 (75) | 5 (62.5) | 8 (100) | 8 (100) | 8 (100) | 8 (100) | 8 (100) | 7 (87.5) | 8 (100) | 0.7073 | <.0001 | 0.9822 |
| *eae* | 4 (50) | 2 (25) | 2 (25) | 8 (100) | 8 (100) | 8 (100) | 6 (75) | 6 (75) | 6 (75) | 0.6588 | <.0001 | 0.7686 |
| *stx*1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 (12.5) | 0 | 0.3765 | 0.3765 | 0.4183 |
| *stx*2 | 0 | 0 | 0 | 0 | 0 | 0 | 4 (50) | 5 (62.5) | 3 (37.5) | 0.9257 | <.0001 | 0.9888 |
| *elt* | 0 | 0 | 0 | 5 (62.5) | 7 (87.5) | 7 (87.5) | 0 | 0 | 0 | 0.5221 | <.0001 | 0.6232 |

Pen-level prevalence: Calculated as the number of pens that had at least one positive sample for each gene divided by the total number of pens sampled per treatment group (n=8)

**Table 2.3. Pen prevalence of the virulence genes of *Escherichia coli* pure culture isolated from piglets' diet supplemented with or without *in-feed* and *in-water* chlortetracycline**

| Virulence genes | Day 0 | | | Day 14 | | | Day 28 | | | *P*- value | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Control | *In-feed* CTC | *In-water* CTC | Control | *In-feed* CTC | *In-water* CTC | Control | *In-feed* CTC | *In-water* CTC | Treatment | Sampling day | Treatment * Sampling day |
| *ehxA* | 2 (25) | 0 | 0 | 4 (50) | 6 (75) | 4 (50) | 2 (25) | 1 (12.5) | 2 (25) | 0.3874 | 0.0690 | 0.2561 |
| *estA* | 1 (12.5) | 0 | 0 | 5 (62.5) | 4 (50) | 6 (75) | 3 (37.5) | 3 (37.5) | 2 (25) | 0.8812 | 0.0535 | 0.5249 |
| *estB* | 6 (75) | 7 (87.5) | 6 (75) | 6 (75) | 7 (87.5) | 7 (87.5) | 2 (25) | 3 (37.5) | 4 (50) | 0.2676 | <.0001 | 0.8288 |
| *astA* | 6 (75) | 7 (87.5) | 6 (75) | 6 (75) | 6 (75) | 5 (62.5) | 7 (87.5) | 7 (87.5) | 7 (87.5) | 0.7374 | <.0001 | 0.9762 |
| *hlyA* | 2 (25) | 3 (37.5) | 2 (25) | 8 (100) | 4 (50) | 6 (75) | 6 (75) | 5 (62.5) | 6 (75) | 0.5757 | 0.0156 | 0.9633 |
| *eae* | 2 (25) | 3 (37.5) | 4 (50) | 6 (75) | 6 (75) | 6 (75) | 3 (37.5) | 2 (25) | 2 (25) | 0.2570 | 0.0296 | 0.1022 |
| *stx*1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *stx*2 | 0 | 0 | 0 | 0 | 0 | 0 | 3 (37.5) | 3 (37.5) | 3 (37.5) | 0.9636 | 0.0010 | 0.9973 |
| *elt* | 0 | 0 | 0 | 1 (12.5) | 0 | 0 | 0 | 0 | 0 | 0.3765 | 0.3765 | 0.4183 |

Pen-level prevalence: Calculated as the number of pens that had at least one positive sample for each gene divided by the total number of pens sampled per treatment group (n=8)

**Table 2.4. Pen prevalence of major enterotoxigenic genes of *Escherichia coli* isolated from piglets' diet supplemented with or without *in-feed* and *in-water* chlortetracycline based on the culture method**

| Sampling days/ Treatments | Pen prevalence, % | | | *P*- value | | |
|---|---|---|---|---|---|---|
| | ETEC Pathotype* | | | Treatment | Sampling day | Treatment* Sampling day |
| | 0 | 14 | 28 | | | |
| Control | 75 | 87.5 | 87.5 | | | |
| *In-feed* CTC | 87.5 | 87.5 | 87.5 | 0.9291 | 0.6397 | 0.9773 |
| *In-water* CTC | 75 | 87.5 | 87.5 | | | |

*ETEC pathotype: Isolates positive for any one of the four enterotoxin genes (*elt*, *estA*, *estB*, *astA*)

** The total number of pens sampled per treatment group (n=8)

**Table 2.5. Pen prevalence of major Shiga toxigenic genes of *Escherichia coli* isolated from piglets' diet supplemented with or without *in-feed* and *in-water* chlortetracycline based on the culture method**

| Sampling days/ Treatments | Pen prevalence, % | | | *P*- value | | |
|---|---|---|---|---|---|---|
| | STEC Pathotype* | | | Treatment | Sampling day | Treatment* Sampling day |
| | 0 | 14 | 28 | | | |
| Control | 0 | 0 | 12.5 | | | |
| *In-feed* CTC | 0 | 0 | 0 | 0.6101 | 0.1480 | 0.7358 |
| *In-water* CTC | 0 | 0 | 12.5 | | | |

*STEC pathotype: Isolates positive for any one of the Shiga toxin genes (*stx*1, *stx*2) in association with *eae* gene

**The total number of pens sampled per treatment group (n=8)

**Table 2.6. Pen prevalence of major atypical enteropathogenic genes of *Escherichia coli* isolated from piglets' diet supplemented with or without *in-feed* and *in-water* chlortetracycline based on the culture method**

| Sampling days/ Treatments | Pen prevalence, % | | | *P*- value | | |
|---|---|---|---|---|---|---|
| | APEC Pathotype* | | | Treatment | Sampling day | Treatment* Sampling day |
| | 0 | 14 | 28 | | | |
| Control | 25 | 62.5 | 12.5 | | | |
| *In-feed* CTC | 37.5 | 62.5 | 25 | 0.8147 | 0.0044 | 0.8945 |
| *In-water* CTC | 50 | 62.5 | 12.5 | | | |

*APEC pathotype: Isolates positive for *eae* gene, negative for Shiga toxin genes (*stx* 1, *stx* 2) and positive for any one of the four enterotoxin genes (*elt*, *estA*, *estB*, *astA*)

** The total number of pens sampled per treatment group (n=8)

**Table 2.7. Pen prevalence of Hybrid *Escherichia coli* isolated from piglets' diet supplemented with or without *in-feed* and *in-water* chlortetracycline based on the culture method**

| Sampling days/ Treatments | Pen prevalence, % | | | *P*- value | | |
|---|---|---|---|---|---|---|
| | Hybrid Pathotype* | | | Treatment | Sampling day | Treatment* Sampling day |
| | 0 | 14 | 28 | | | |
| Control | 0 | 0 | 37.5 | | | |
| *In-feed* CTC | 0 | 0 | 37.5 | 1.0000 | <.0001 | 1.0000 |
| *In-water* CTC | 0 | 0 | 37.5 | | | |

*****Hybrid Pathotype: Isolates positive for any one of the Shiga toxin genes (*stx* 1, *stx* 2) and positive for any one of the four enterotoxin genes (*elt*, *estA*, *estB*, *astA*)

** The total number of pens sampled per treatment group (n=8)

**Table 2.8. Prevalence of Fimbriae genes detected in pathogenic *Escherichia coli* isolated from piglets' diet supplemented with or without *in-feed* and *in-water* chlortetracycline**

| Fimbriae genes | Control | | | *In-feed* CTC | | | *In-water* CTC | | | Total (n= 1,732) |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0 (n=115) | 14 (n=126) | 28 (n=266) | 0 (n=132) | 14 (n=211) | 28 (n=297) | 0 (n=134) | 14 (n=174) | 28 (n=277) | |
| F4 | 7 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 9* |
| F5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| F6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| F18 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2** |
| F41 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

*Nine F4 positive isolates were also positive for virulence genes *estA, estB*

**Two F18 positive isolates were also positive for virulence genes *estB, elt*

**Table 2.9. *Escherichia coli* serogroups isolated from piglets in association with the pathotypes**

| Pathotypes | Serogroups | Fimbriae |
|---|---|---|
| ETEC | O7 (1), O8 (3), O11 (1), O15 (19), **O23 (3)**, O24 (1), O35 (18), O76 (2), O81 (33), O98 (6), **O141 (1)**, O150 (1), O180 (1), O182 (7) | F4, F18 |
| Atypical EPEC | O15 (19), O26 (3), O112 (10), O123/O186 (10), O145 (5) | |
| STEC | O69 (11) | |
| Untyped | 4 | |

**Table 2.10. Minimum inhibitory concentrations of chlortetracycline for *Escherichia coli* (n=165) isolated from feces of piglets' diet supplemented with or without *in-feed* and *in-water* chlortetracycline**

| Treatment groups | Number of isolates tested | Chlortetracycline, MIC (µg/mL) | |
|---|---|---|---|
| | | MIC | 95% CI* |
| Control | 52 | 50 | [37.5-62.5] |
| *In-feed* CTC | 57 | 50 | [44.74-60.5] |
| *In-water* CTC | 56 | 62.5 | [50-75] |

*Confidence Interval

MIC breakpoint of ≥ 16 µg/ml is considered as resistant for chlortetracycline

**Table 2.11. Prevalence of tetracycline resistance genes among *Escherichia coli* (n=165) isolated from piglets' diet supplemented with or without *in-feed* and *in-water* chlortetracycline**

| Tetracycline genes | Treatments | Isolates positive for tetracycline resistance genes (%, prevalence) | | | | *P*- value | | |
|---|---|---|---|---|---|---|---|---|
| | | Day 0 | Day 14 | Day 28 | Total (n=165) | Treatment | Sampling day | Treatment* Sampling day |
| *tet*A | Control (n=52) | 9 (17.3%) | 23 (44.2%) | 13 (25%) | | | | |
| | *In-feed* (n=57) | 11 (19.3%) | 26 (45.6%) | 15 (26.3%) | 151 (91.5%) | 0.2090 | 0.0074 | 0.2495 |
| | *In-water* (n=56) | 12 (21.4%) | 26 (46.4%) | 16 (28.6%) | | | | |
| *tet*B | Control (n=52) | 1 (1.9%) | 2 (3.8%) | 6 (11.5%) | | | | |
| | *In-feed* (n=57) | 0 | 3 (5.3%) | 6 (10.5%) | 28 (16.9%) | 0.9211 | 0.0091 | 0.7470 |
| | *In-water* (n=56) | 1 (1.8%) | 5 (8.9%) | 4 (7.1%) | | | | |
| *tet*C | Control (n=52) | 0 | 0 | 0 | | | | |
| | *In-feed* (n=57) | 0 | 0 | 0 | - | - | - | - |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *In-water* (n=56) | 0 | 0 | 0 | | | | |
| *tet*D | Control (n=52) | 3 (5.8%) | 3 (5.8%) | 2 (3.8%) | | | | |
| | *In-feed* (n=57) | 1 (1.8%) | 2 (3.5%) | 1 (1.8%) | 21 (12.7%) | 0.2564 | 0.1785 | 0.8111 |
| | *In-water* (n=56) | 3 (5.4%) | 5 (8.9%) | 1 (1.8%) | | | | |

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