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Laird, T.J.; Abraham, S.; Jordan, D.; et.al.

Laird, T. J., Abraham, S., Jordan, D., Pluske, J. R., Hampson, D. J., Trott, D. J., & O'Dea, M. (2021). Porcine enterotoxigenic Escherichia coli: Antimicrobial resistance and development of microbial-based alternative control strategies. Veterinary Microbiology, 258. https://doi.org/10.1016/j.vetmic.2021.109117
Document Version: Author's Version

Published Version: https://doi.org/10.1016/j.vetmic.2021.109117

Journal Pre-proof

Porcine enterotoxigenic *Escherichia coli*: antimicrobial resistance and development of microbial-based alternative control strategies<!–<ForCover>Laird TJ, Abraham S, Jordan D, Pluske JR, Hampson DJ, Trott DJ, O'Dea M, Porcine enterotoxigenic *Escherichia coli*: antimicrobial resistance and development of microbial-based alternative control strategies, *Veterinary Microbiology*, doi: 10.1016/j.vetmic.2021.109117</ForCover>–>

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PII: S0378-1135(21)00140-1

DOI: https://doi.org/10.1016/j.vetmic.2021.109117

Reference: VETMIC 109117

To appear in: *Veterinary Microbiology*

Received Date: 21 January 2021

Accepted Date: 13 May 2021

Please cite this article as: { doi: https://doi.org/

Porcine enterotoxigenic *Escherichia coli*: antimicrobial resistance and development of microbial-based alternative control strategies

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Highlights

- Global presence of ETEC strains resistant to critically important antimicrobials
- AMR stewardship and environmental concerns will impact on current strategies
- Future control relies on combination of established and novel strategies
- Bacteriophages, probiotics, posbiotics and vaccines offer potential as alternatives

- Alternatives require additional well-designed experiments and field-based work

Abstract

Strains of enterotoxigenic *Escherichia coli* (ETEC) causing post-weaning diarrhoea (PWD) in piglets have a widespread and detrimental impact on animal health and the economics of pork production. Traditional approaches to control and prevention have placed a strong emphasis on antimicrobial use (AMU) to the extent that current prevalent porcine ETEC strains have developed moderate to severe resistance. This complicates treatment of ETEC infection by limiting therapeutic options, increasing diagnostic costs and increasing mortality rates. Management factors, the use of supra-physiological levels of zinc oxide and selected feed additives have all been documented to lower the incidence of ETEC infection in pigs; however, each intervention has its own limitations and cannot solely be relied upon as an alternative to AMU. Consequently, treatment options for porcine ETEC are moving towards the use of newer antimicrobials of higher public health significance. This review focuses on microorganisms and microbial-derived products that could provide a naturally evolved solution to ETEC infection and disease. This category holds a plethora of yet to be explored possibilities, however studies based around bacteriophage therapy, probiotics and the use of probiotic fermentation products as prebiotics have demonstrated promise. Ultimately, pig producers and veterinarians need these solutions to reduce the reliance on critically important antimicrobials (CIAs), to improve economic and animal welfare outcomes, and to lessen the One Health threat potentiated by the dissemination of AMR through the food chain.

Keywords: Enterotoxigenic *Escherichia coli*; Antimicrobial resistance; Bacteriophages; Probiotics; Vaccines; Pigs

Introduction

Weaning of pigs normally occurs at 18-26 days of age on commercial farms and is an extremely stressful event. The challenges of a new environment and new social order, in addition to dietary changes, a lack of protective antibodies from sows' milk and associated changes in gut structure and

function, all contribute to an increased susceptibility to infections and a greater incidence of diarrhoea in the first two weeks after weaning. The major bacterial aetiological agent of post-weaning diarrhoea (PWD) in swine is enterotoxigenic *E. coli* (ETEC), with other infectious agents causing PWD including coronaviruses and rotaviruses. ETEC-associated disease results in significant costs to the global pig industry due to high morbidity and mortality, substantial veterinary and labour costs, and growth retardation. The main clinical sign of ETEC infection in weaners is watery diarrhoea, with associated depression, inappetence and dehydration. ETEC strains adhere to the host small intestinal epithelial cells using flexible surface fimbriae, which mediate the recognition of and adherence to the corresponding surface receptors (Dubreuil et al., 2016). Fimbrial adhesins F4 and F18 are the most common fimbriae types among ETEC causing PWD, with further divergence seen in the three existing F4 variants, F4ab, F4ac and F4ad, and the two existing F18 variants, F18ab and F18ac (Luppi, 2017; Nadeau et al., 2017). After colonisation, porcine ETEC strains produce one or more heat labile (LT) and/or heat stable (STaP, STb) enterotoxins (Heo et al., 2013; Kusumoto et al., 2016), which activate a flux of electrolytes into the intestinal lumen and the creation of a hypertonic environment. Large volumes of water move from the epithelial cells into the lumen due to this imposed osmotic pressure, with the excessive volumes of water and electrolytes causing hypersecretory diarrhoea. While many experienced veterinarians diagnose ETEC-associated disease based on clinical signs and farm history, definitive diagnosis of ETEC infection is performed through the isolation of a heavy pure or predominately pure growth of β-haemolytic pathogenic *E. coli* from faeces or intestinal samples on blood agar, followed by PCR typing of fimbriae and enterotoxin genes (Heo et al., 2013; Luppi, 2017).

Decades of research into PWD has led to the identification of various genetic, environmental and farm management factors that decrease the susceptibility of weaner pigs to ETEC infection, including for example reduced stocking density, increased feeder space, and the removal of soiled bedding, as reviewed extensively by Rhouma et al. (2017). Another strategy is the selective breeding for pigs that are resistant to infection through selection against gut receptor gene expression using biomarkers including the FUT, MUC13, MUC4, MUC20 and TFRC genes (Sinha et al., 2019). However, this

strategy is limited by the heterogeneity of ETEC fimbriae types, thus breeding out the recessive F4 receptor gene results in pigs being resistant to F4 ETEC infections, but no protection is offered against ETEC strains harbouring other fimbriae types (Rhouma et al., 2017). The impact of all-in-all-out production systems is reported to reduce the incidence of respiratory diseases in swine and may have a similar impact on ETEC infection (Calderón Díaz et al., 2017).

Along with management and dietary factors, antimicrobials have been used extensively for the prevention and treatment of ETEC, with resultant high levels of antimicrobial resistance (AMR) detected in ETEC strains globally (Abraham et al., 2014; Jiang et al., 2019; Luppi et al., 2015; Rosager et al., 2017). Pigs suffering PWD are often placed on empirical therapy whilst awaiting culture and antimicrobial susceptibility results to avoid further morbidity and mortality, and this contributes to the selective pressure exerted on bacteria from antimicrobial usage (AMU) on farms (Heo et al., 2013). The resultant emergence and high prevalence of AMR complicates the control of ETEC infections, and adds further costs to the swine industry through increased mortalities, extended duration of infections and costs associated with extended diagnostics such as antimicrobial susceptibility testing (Luppi, 2017). Furthermore, the development of AMR in porcine ETEC may lead to the use of critically important antimicrobials (CIAs) including extended spectrum cephalosporins (ESCs) (ceftiofur, cefquinome), colistin and fluoroquinolones (enrofloxacin) (Marshall and Levy, 2011). This usage is dependent on the legislation concerning the use of CIAs in swine and differs between countries; for example, Vietnam, France and Germany commonly use colistin for the control of PWD whilst colistin is not registered for use in pigs in Australia (Cutler et al., 2020; Rhouma et al., 2017). This use indirectly poses a potential threat to human health, by transfer of antimicrobial resistance genes (ARGs) towards CIAs from porcine to human commensal *E. coli* (Marshall and Levy, 2011). These clones which are of heightened concern for One Health are termed high risk, and recently have been defined by de Lagarde et al. (2021) as being emergent clones that carry multiple resistance genes, have a high capacity for dissemination and have high pathogenicity. Identification of high risk ETEC clones allows focused investigation into the spread of these ETEC strains and the intensive development of methods for controlling them. Currently

established antimicrobial alternatives for ETEC infections in swine include supplementation with high levels of zinc oxide, the addition of organic acids in feed and the reduction of protein in diets (Bednorz et al., 2013; Ciesinski et al., 2018; Heo et al., 2015; Tugnoli et al., 2020; Wang et al., 2018). Whilst these three alternatives have been reported to aid in reducing the impact of ETEC infection, the use of these have not completely removed the need for antimicrobial treatment, and therefore other possible alternatives need to be pursued. Investigations into microbial-based ETEC control methods, such as the use of microbial-derived feed additives, vaccines and bacteriophage therapy have shown some promise, however results are variable and require further investigation.

Antimicrobial usage

Amoxicillin/clavulanic acid, apramycin, gentamicin, neomycin and trimethoprim-sulphonamide are all antimicrobials, defined in this review as registered antibacterial drugs, commonly selected for the treatment, metaphylaxis and prevention of ETEC infection (Cutler et al., 2020; Luppi, 2017). The more extensive list in Table 1 includes a wider range of antimicrobials, some of which are less commonly used, and includes the highest priority critically important antimicrobials (CIA) ceftiofur, colistin and enrofloxacin, as well as the high priority CIAs apramycin, gentamicin and neomycin. This refers to the classification system designed by the WHO to reserve CIAs for the treatment of life-threatening infections in humans (WHO, 2019). Whilst this list aids countries in restricting the use of specific antimicrobials, the legislation and label constraints differ significantly between countries. This is evident in Australia where the antimicrobials enrofloxacin, colistin and gentamicin are not registered for use in food-producing animals despite many other countries using these antimicrobials for control of ETEC infection (Cutler et al., 2020). Historically subtherapeutic levels of certain antimicrobials have also been used in livestock as growth promoters (AGP), and although this use is now regulated in many countries, large amounts of antimicrobials continue to be used for the metaphylactic and therapeutic treatment of PWD, exerting selective pressure on all bacteria present in the gut (Holman and Chenier, 2015). Upon emergence of resistance, treatment shifts to antimicrobials with lower levels of resistance, but arguably of more importance for maintaining human health

(Rhouma et al., 2017). Continued increases in resistance against these antimicrobials is inevitable until viable alternatives become readily available and used in livestock.

Table 1. List of antimicrobials used for treatment of ETEC infection in swine categorised according to the World Health Organisation classification of antimicrobials for human health.

Antimicrobial class	Antimicrobial agent
Highest priority critically important antimicrobials	
Cephalosporins (3rd and higher generation)	Ceftiofur
Polymyxins	Colistin
Quinolones	Enrofloxacin
High priority critically important antimicrobials	
Aminoglycosides	Apramycin, gentamicin, neomycin
Penicillins	Ampicillin, amoxicillin-clavulanic acid
Highly important antimicrobials	
Amphenicols	Florfenicol
Sulphonamides	Sulphamethoxazole, trimethoprim
Important antimicrobials	
Aminocyclitols	Spectinomycin

Antimicrobial resistance in enterotoxigenic *E. coli*

Despite differences in study design making international comparisons difficult, high levels of resistance are evident globally in porcine ETEC. A study conducted across 15 states in the USA between 2013 and 2014 identified 89.1%, 49.1%, 32.7% and 30.9% of the 55 ETEC isolates as being resistant to ampicillin, neomycin, gentamicin and trimethoprim-sulphamethoxazole, respectively. Resistance towards the highest priority CIAs enrofloxacin and ceftiofur also was detected, with 58.5%, and 25.5% of isolates being resistant, respectively (Jiang et al., 2019). By contrast all 70 Australian porcine ETEC isolated between 1999 and 2005 were susceptible to the highest priority

CIAs cefotaxime and ciprofloxacin, with a lower prevalence of resistance to gentamicin (34.3%) detected. In comparison to the above study, a similar high prevalence of resistance to tetracycline and ampicillin was detected in 67.1% and 50% of isolates respectively (Abraham et al., 2014). The threat and complications arising from AMR are intensified through the accumulation of various AMR mechanisms resulting in single strains conferring resistance to three or more antimicrobial classes, termed multidrug resistant (MDR) (Abraham et al., 2014). In some cases, the ARGs encoding resistance determinants are located adjacent to each other within mobile genetic elements. Consequently, selecting for resistance to one drug will select for another; this is termed co-selection (Abraham et al., 2017a). Multidrug resistance enhances selection pressures, and clinically limits the number of antimicrobials that can be used to successfully treat infections, leading to increased infection durations and associated costs, higher mortality and increased use of CIA classes. Global studies conducted on ETEC isolates from Australia between 1999 and 2005, China between 2010 and 2013, Denmark in 2014 and the USA in 2013 and 2014, have demonstrated a concerningly high prevalence of MDR ETEC in swine with 64.3%, 69.6%, 86.2% and 96.1% of isolates being MDR, respectively (Abraham et al., 2014; Jiang et al., 2019; Rosager et al., 2017; Xu et al., 2015).

Despite the strict control of CIA usage in some countries, resistance to these antimicrobial classes has emerged in swine-origin ETEC. The discovery of various plasmid-mediated CIA resistance mechanisms has amplified concerns regarding dissemination of this resistance due to the ease of horizontal transmission of mobile genetic elements (Abraham et al., 2017b; Liu et al., 2016). Whilst these CIA resistance mechanisms are widely documented in commensal *E. coli*, the prevalence and mechanisms of CIA resistance in ETEC strains in numerous countries are lacking. Studies that have examined CIA resistance in porcine ETEC have varied in study designs and measurement systems, demonstrated by the restrictions such as analysis of only MDR ETEC isolates (Abraham et al., 2014; Smith et al., 2014), and analysing only F4 ETEC strains (Luppi et al., 2015). This needs to be considered when viewing Figure 1 and through the following discussion of CIA resistance rates. Despite the difference, these studies have demonstrated high levels of phenotypic resistance across many countries, with over 85% of ETEC isolates being resistant to ampicillin in Vietnam and the

USA and over 50% of ETEC being resistant to enrofloxacin in the US, Brazil, China and Italy (Figure 1) (Abraham et al., 2014; EcL; Jiang et al., 2019; Luppi et al., 2015; Rosager et al., 2017; Sato et al., 2015; Smith et al., 2014; Xu et al., 2015).

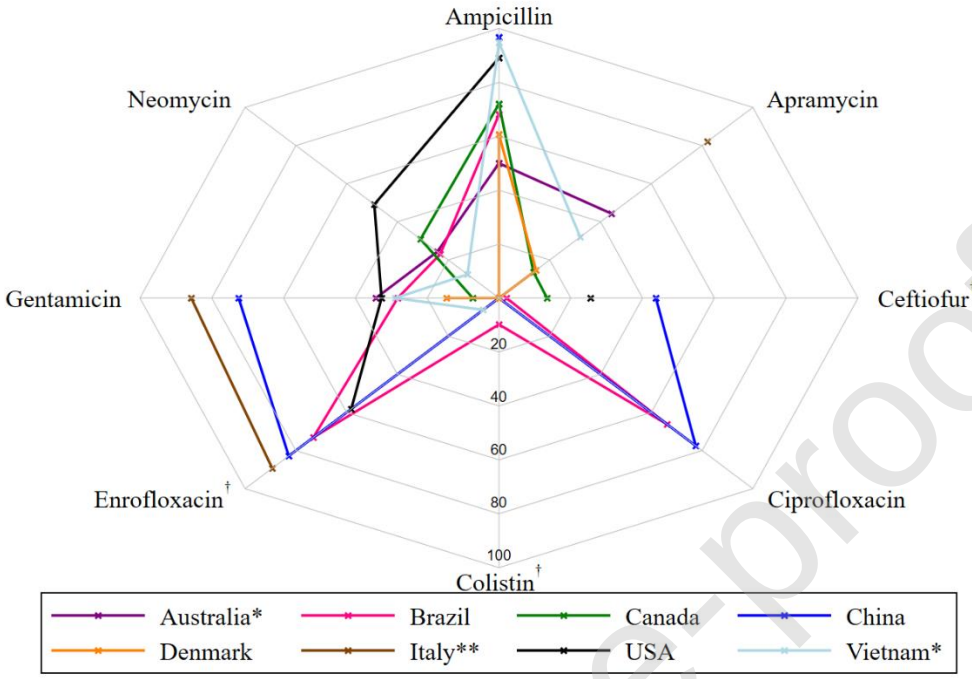

Figure 1. Percent of porcine ETEC demonstrating phenotypic resistance against CIAs as published in different countries.

Country/drug combinations without data points were unavailable. Caution is advised regarding the variation in assertation in breakpoints of published data. † Antimicrobials categorised as highest priority critically important antimicrobials. * Countries with data only on MDR ETEC isolates available. ** Countries with data only on F4 ETEC isolates included in the study.

Globally, there is a wide range in the prevalence of fluoroquinolone (FQ) resistance amongst porcine ETEC strains (Figure 1), with resistance conferred via the accumulation of specific point mutations in the quinolone resistance-determining regions of target chromosomal genes and/or from acquiring

plasmid-mediated FQ resistance genes via horizontal gene transfer. In separate studies conducted in Denmark and Australia, both of which are countries that strictly control the use of FQs in swine, all ETEC strains were susceptible to ciprofloxacin (Abraham et al., 2014; Rosager et al., 2017). In comparison, 3.2% of ETEC isolates from Vietnam from 2001 were found to be phenotypically resistant to ciprofloxacin, with the plasmid-mediated quinolone resistance gene *qnrB* detected in 52.3% of these isolates (Smith et al., 2014). This low level of phenotypic FQ resistance and high carriage of quinolone resistance genes is of concern, and further research is needed to determine current CIA resistance levels among ETEC in many countries. Studies conducted in Brazil and China demonstrated higher phenotypic prevalence at 66.2% and 77.5% of ETEC strains, respectively (Sato et al., 2015; Xu et al., 2015). The resistance mechanism was not assessed in the Brazilian isolates whilst isolates from China underwent screening for plasmid-mediated resistance genes, with *oqxAB, qnrS, qnrB, qepA* and *aac(6')-Ib-cr* being detected (Xu et al., 2015).

Extended-spectrum cephalosporins (ESC) are another CIA class that are administered for the treatment of porcine ETEC infections in some countries (Luppi, 2017; Rhouma et al., 2017), whilst other countries have restricted the use of third generation cephalosporins (3GC) in pig production. This is demonstrated in Australia where ceftiofur is not registered for use in pigs (Cutler et al., 2020). Despite this, 4.8% of ETEC isolated from Australian swine between 2013 and 2014 were resistant to ceftiofur (van Breda et al., 2018). In contrast, ETEC isolated from Denmark were all susceptible to ceftiofur, whilst 13.0% from Canada in 2003 and 25.5% from Italy between 2002-2011 were resistant (Boerlin et al., 2005; Luppi et al., 2015; Rosager et al., 2017). Extended-spectrum cephalosporin resistance also was common in China, with 43.7% and 41.7% of isolates being resistant to the 3GCs ceftiofur and cefotaxime, respectively. Additionally, some of these isolates also carried quinolone resistance genes, with an association found between $bla_{\text{CTX-M-14}}$ and *oqxAB* indicating these genes were likely to be linked on the same plasmid (Xu et al., 2015).

Countries including China, Vietnam, Belgium, Germany and France also use colistin in swine for the control of gastrointestinal pathogens including ETEC (García et al., 2018; Rhouma et al., 2016). A study in Spain identified 76.9% of ETEC and shiga-toxin producing *E. coli* (STEC) isolates from

2006 to 2017 as being resistant to colistin, with plasmid-mediated colistin resistance genes *mcr-1*, *mcr-4* and *mcr-5* found in 26.4%, 72.8% and 3.6% of the isolates, respectively (García et al., 2018). This contrasts with 9.8% of ETEC isolates from Brazil being resistant to colistin, and all ETEC isolates tested from Denmark and China being susceptible to colistin (Rosager et al., 2017; Sato et al., 2015; Xu et al., 2015). Despite this low prevalence of colistin resistance in ETEC strains, these ARGs also have been detected in commensal *E. coli* from swine, and therefore concern about resistance transfer through the food chain is warranted (Abraham et al., 2017b). The discovery and detection of plasmid-mediated colistin resistance in swine across the globe has led to calls for an urgent limit on colistin use in animals, with many countries now banning its use (Rhouma et al., 2016). The emergence and dissemination of these CIA resistance mechanisms in both pathogenic and commensal bacteria, coupled with the lack of current AMR prevalence of ETEC strains across various countries, highlights the need for ongoing studies that monitor CIA resistance carriage levels in ETEC.

Alternative treatment options

Zinc oxide

Zinc oxide (ZnO) is well-established as a feed additive, with multiple studies having demonstrated its positive effects when included at supra-physiological levels in the diet in alleviating ETEC-induced PWD, and having shown its potential as an agent that could be used in place of antimicrobials in feed (Heo et al., 2013). Despite this, there are two major issues concerning the use of feed containing supra-physiological levels of ZnO. The first is the high quantity of unabsorbed zinc (Zn) that is excreted in faeces, resulting in environmental soil contamination from piggery effluent (Ciesinski et al., 2018). The second issue preventing the long-term usage of ZnO is the finding that high doses of ZnO select for antimicrobial-resistant bacteria. The mechanisms behind this effect are theorised to include: cross-resistance due to the heavy metal impact on bacterial efflux pumps, with co-selection due to the gene conferring heavy metal resistance being located on a mobile genetic element carrying ARG; the heavy metal effecting the bacterial conjugation system; and/or the direct effect of heavy metals on antimicrobials themselves (Bednorz et al., 2013). The concern surrounding these issues has resulted in the European Union banning veterinary medicinal products containing supra-physiological

levels of ZnO from 2022; therefore, the commercialisation of other alternative strategies for effective treatment and control of PWD is a priority (EC, 2017).

Lower protein diets

Diets containing high levels of crude protein (CP) result in a greater proportion of protein remaining undigested in the small intestine, with this undigested protein, as well as excess endogenous nitrogen, passing through to the large intestine. The unabsorbed protein is fermented in the large intestine by nitrogen utilising bacterial species, including *E. coli*, encouraging the growth of this potentially pathogenic species. The fermentation process results in increased levels of toxic end products that damage the gut mucosa, with these compounds also being implicated in the aetiology of PWD (Heo et al., 2015; Wang et al., 2018). Furthermore, the decreased pH levels associated with high protein diets favour the proliferation of many pathogenic bacterial species in the large intestine (Wang et al., 2018), including ETEC. As a result, a reduction of dietary CP has been proposed as a method to reduce PWD in swine by limiting fermentation of protein in the distal GIT, and therefore, reducing proliferation of bacterial species and levels of detrimental compounds. This has been demonstrated numerous times in pigs fed lower protein diets supplemented with essential amino acids, with a measurable decrease in protein fermentation and reduction in the incidence of PWD (Heo et al., 2015; Wang et al., 2018). Heo et al. (2015) analysed four datasets from experiments comparing low and high protein weaners diets, demonstrating a positive linear relationship between daily dietary protein intake and incidence of PWD in diets containing more than 60 g of daily protein per pig. Nevertheless, some studies have shown inferior performance with feeding lower protein diets after weaning, which is most likely attributable to an imbalance in the ideal protein ratio associated with types and concentrations of amino acids (Heo et al., 2013; Wang et al., 2018).

Organic acids

The addition of various organic acids to feed has been studied for their effects on reducing symptoms caused by ETEC infection in weaner pigs. Organic acids reduce stomach pH values, subsequently increasing feed digestion, inhibiting the growth of pathogenic bacteria and favouring the growth of many beneficial bacteria (Ren et al., 2019; Tugnoli et al., 2020). The acidic conditions also boost the

physiological functions of the GI tract, activating pepsin, chelating minerals and enhancing secretion of enzymes (Tugnoli et al., 2020). Ren et al. (2019) studied the impact of organic acids in pigs challenged with ETEC. Formic and propionic acid added to the feed (1% mixture of 64% formic acid and 25% propionic acid) led to a reduced faecal consistency score (FCS) and rectal temperature compared to control pigs at 9- and 24-hours post-challenge. Despite this difference, the faecal pH values, faecal total coliform counts and faecal *Lactobacillus* counts were not significantly different between the groups at 24- and 48-hours post challenge (Ren et al., 2019). However, this may be due to these data representing the effect on faeces, with the effect in the small intestine being unmeasured. Further studies identifying the effects of organic acids against ETEC infection in all sections of the GI tract are required to optimise organic acid parameters in weaners diets.

Prebiotics

Prebiotics, non-living products which are indigestible by the host and instead fermented by the host microbiota, can also be supplemented into swine diets, promoting the development of a healthy microbiota and conferring host health benefits. Substrates that have potential as prebiotics must selectively favour the growth of beneficial bacterial genera, such as *Lactobacillus* and *Bifidobacterium*. This manipulation of the gut microbiota alters its composition and improves its functionality, subsequently heightening protection against enteric pathogens (Angelakis, 2017). A prebiotic effect was demonstrated *in vitro* with casein glycomacropeptide and mannan-oligosaccharides reducing ETEC adherence to porcine intestinal epithelial cells (Hermes et al., 2011); however, the underlying mechanisms of prebiotics remain poorly characterised and limited studies on ETEC-challenged weaners have been completed (Angelakis, 2017).

Microbial-based control methods

Vaccines

Commercially, only a single live oral vaccine is available against ETEC-associated PWD in swine although there have been countless studies investigating the use of subunit, encapsulated and parenteral vaccines (Hedegaard and Heegaard, 2016; Melkebeek et al., 2013). These sub-optimal success rates of vaccines against ETEC are due to the challenges surrounding the timing of

vaccination and the diversity of ETEC strains in swine (Melkebeek et al., 2013). Many of these vaccines target the specific fimbrial antigens due to the strong immune response elicited, with the antibodies generated preventing ETEC attachment, yet they provide no cross-protection against ETEC strains expressing other fimbriae types (Fairbrother et al., 2017; Melkebeek et al., 2013; Rhouma et al., 2017). Enterotoxins have also been researched as possible ETEC vaccine targets; however, the presence and production levels of enterotoxins also varies significantly between strain and serogroups (Wang et al., 2020). The difficulty in importing live vaccines into countries with high biosecurity further limits the capability of vaccination programs, as many vaccines undergoing development against ETEC infection use live *E. coli* strains. Autogenous vaccines are another vaccination strategy commonly developed as a targeted approach against highly virulent bacterial or viral strains affecting an individual herd (Hoelzer et al., 2018). The advent of whole genome sequencing technology has enabled the full characterisation of ETEC strains responsible for the disease syndrome and may help determine if an autogenous vaccine is warranted whilst also identifying virulent ETEC strains. However, the efficacy of autogenous vaccines suffers a similar lack of broad-spectrum activity when swine herds are infected by more than a single ETEC strain, whilst also being limited in their administration to only the infected farm generating the strain (van Breda, 2017).

The two approaches to ETEC vaccination are vaccination of sows and vaccination of piglets. Sow vaccination capitalises on the transfer of maternal antibodies to neonatal piglets via colostrum and milk, therefore protecting piglets against infection. Whilst this approach has been demonstrated to provide effective protection against ETEC infection in neonatal pigs, the loss of milk at weaning is followed by a decay of maternal antibodies received from vaccinated sows, with pigs being most vulnerable to ETEC infection in this post-weaning period. One method to extend the protective duration of antibodies is the use of passive immunisation (Hedegaard and Heegaard, 2016). Virdi et al. (2013) incorporated anti-ETEC antibodies in the feed of piglets using variable domains of llama heavy chain-only antibodies (VHH) against ETEC fused with the Fc portion of a porcine IgA immunoglobulin. ETEC challenged pigs receiving VHH-IgA based antibodies demonstrated superior clearance of ETEC shedding in faeces, with ETEC shed in faeces for three days after challenge

compared to the control pigs in which ETEC was detected in faeces up until day 8. An increased weight gain and lower immune response to the ETEC challenge were additionally reported in pigs receiving VHH-IgA based antibodies (Virdi et al., 2013). Whilst administration of antibodies specific to F4 and F18 fimbria have demonstrated potential in controlling ETEC-associated PWD, the dosage and timing of immunisation requires optimisation with a need for the spectrum of protection to be expanded (Hedegaard and Heegaard, 2016). Rausch et al. (2017) designed a vaccine which elicited broad spectrum protection against ETEC infection using two toxoid multi-epitope fusion antigens (MEFA). The vaccine was first tested in a murine model, with mice administered the vaccine demonstrating increased levels of serum anti-LT, anti-STb and anti-Stx2e IgG titers measured *in vitro* using ELISA. An ETEC challenge model in swine was then completed, with sows receiving vaccination at 8 weeks prior to farrowing, and piglets orally challenged with ETEC 24 hours after birth. All piglets in the control group (n=6) developed severe diarrhoea and no LT, STa, STb or Stx2e specific antibodies were detected in serum samples. In contrast, antibodies specific to LT, STa, STb and Stx2e were detected in serum of pigs belonging to the vaccination group (n=7) and mild diarrhoea was only reported in a single piglet (Rausch et al., 2017). This vaccine requires further testing to confirm broad spectrum protection against ETEC-associated PWD.

Vaccination of piglets against ETEC infection has similar obstacles as sow vaccination, with the timing of vaccination and multiple ETEC strains restricting the success of vaccine development. The vaccine must be given so that protection exists over the expected period of peak incidence of ETEC in the production cycle, with the neutralisation of vaccines caused by maternal antibodies and the time taken to produce an immune response in piglets limiting the time-frame for vaccination. Fairbrother et al. (2017) tested the efficacy of the live oral vaccine, Coliprotec F4 (Prevtec Microbia GmbH, Germany), which consists of a $F4^+$ tame *E. coli* strain, when administered at various time intervals. The $F4^+$ ETEC challenge strain was administered at 3, 7 or 21 days post vaccination (dpv). A significant level of protection was induced when the challenge strain was administered at 7 and 21 dpv, while pigs challenged at 3 dpv showed less protection in terms of diarrhoea prevalence and reduction in ETEC colonisation and shedding. This study emphasised the importance of the timing of

vaccine administration against ETEC infection (Fairbrother et al., 2017). Another study also investigated the use of avirulent strains for live vaccines, focusing on widening the narrow spectrum protection provided by vaccination with a single *E. coli* strain through the design of a bivalent *E. coli* vaccine composed of live F4ac and F18ac *E. coli* strains. Challenge strains and vaccines were administered to pigs via drinking water with challenge occurring either 7 or 21 dpv in two separate trials; pigs in one trial were challenged with a F4ac ETEC strain whilst the other trial used a F18ab ETEC-challenge strain. In the F18-ETEC model, vaccinated pigs demonstrated a significant reduction in severity and duration of diarrhoea and an increase in average daily gain (ADG). This trend also was observed in the F4-ETEC model, although the effect was not statistically significant. This may have been attributed to the low sample number of pigs used per group (n=10 or 20) and complications in establishment of ETEC infection, with only 50% and 22% of control pigs demonstrating moderate to severe diarrhoea from the 7 and 21 dpv challenges, respectively. Despite this, a significant reduction in faecal shedding of F4- and F18 ETEC was measured in vaccinated pigs from corresponding studies, as well as increased serum levels of anti-F4 and anti-F18 IgM and IgA antibodies, overall indicating protection against F4-ETEC and F18-ETEC induced by a single vaccine (Nadeau et al., 2017). The last decade of research has greatly progressed vaccination against ETEC infection in weaners, delivering methods that are beginning to overcome the narrow spectrum and administration issues of vaccination against ETEC. However, until a multivalent vaccine or a vaccine utilising sufficiently conserved toxoids is commercialised, other control methods for ETEC infection in swine need to be explored and implemented.

Probiotics, postbiotics and synbiotics

Probiotics are live microorganisms which confer health benefits to the host when administered at sufficient levels, whilst postbiotics (a relatively new term) are bioactive compounds from food-grade microorganisms resulting from a fermentation process (Wegh et al., 2019). Furthermore, specific probiotics and prebiotics have demonstrated a superior health benefit when applied in combination, termed synbiotics, compared to the administration of a single modality (Wang et al., 2019). A very large range of probiotics are commercially available for supplementation into pig diets with the

primary purpose to reduce the impact of pathogenic bacterial infections directly or via positive effects on host immune function. The immature microbiota of weaner pigs leaves them susceptible to colonisation by enteric pathogens, with this production stage being an ideal target to test the effects of probiotic, postbiotic, prebiotic and synbiotic use in aiding the establishment of a diverse, healthy, and ultimately protective, gut microbiota (Angelakis, 2017).

Probiotics are designed to provide protection against enteric pathogens through interference with the adhesion of pathogens, reduction in nutrient availability through competitive exclusion, modification of the pH of the GI tract, and modulation of the host immune response (Angelakis, 2017). Strains typically developed for probiotic use are commonly isolated from the target species, as these strains have already demonstrated the capability to adhere to and colonise the host. Lactobacilli, lactic acid-producing Gram-positive bacteria which inhabit the GI tract of swine, have been demonstrated to alleviate both symptoms and the reduced growth performance induced by ETEC infection in *in vivo* studies (Table 2) (Lee et al., 2012; Yang et al., 2014). In one study, supplementation with *Lactobacillus plantarum* (*L. plantarum*) increased ADG from 199 grams in control pigs to 394 grams whilst villous height and villous height: crypt depth (VCR) were also greater in pigs receiving probiotics compared to control pigs (Yang et al., 2014). Another study also reported an increased ADG in pigs receiving *L. plantarum* compared to control pigs, additionally measuring reduced duration of elevated rectal temperature after ETEC-challenge, reduced FCS and modulation of cytokine response (Lee et al., 2012). Another microorganism historically used as a probiotic is the fungal species *Saccharomyces cerevisiae* (*S. cerevisiae*). ETEC-challenged swine receiving *S. cerevisiae* var. *boulardii* showed a significant reduction in FCS, duration and severity of diarrhoea, and shedding of ETEC in faeces, as well as an increase in growth performance compared to control pigs (Trckova et al., 2014). Supplementation with both *S. cerevisiae* and *Bacillus licheniformis* gave similar results, with increased ADG, reduced diarrhoea incidence and reduced *E. coli* concentration in the caecum - whilst additionally reporting increased *Lactobacillus* concentration in the caecum (Pan et al., 2017). In contrast, Luise et al. (2019a) described no impact on growth performance measures or microbiota alpha and beta diversity indexes as a result of feeding the probiotics *Bacillus subtilis* (*B.*

subtilis) or *Bacillus amyloliquefaciens*. An increased abundance of Enterobacteriaceae was demonstrated in pigs receiving either supplement, with only *B. subtilis* tending to reduce FCS, hence showing that the benefits of probiotics are species dependant (Luise et al., 2019a). Whilst these studies demonstrate the beneficial impact of probiotics on ETEC infections, the specific mechanism of action of many probiotic strains remains uncertain. Recent studies have begun to shed light on the matter, including those of Tian et al. (2016) demonstrating *E. faecium* acts by inhibiting adhesion of ETEC to enterocytes and reducing the proinflammatory response induced by ETEC infection; however, more studies that increase our understanding of the action of probiotics are necessary to optimise probiotic use.

Table 2. Impact of probiotic, postbiotics and synbiotics on ETEC-challenged weaners

Study	Type of additive	Product	Results
Yang et al. (2014)	Probiotic	*Lactobacillus plantarum*	Increased bodyweight, average daily gain and average daily feed intake, villous height, villous height: crypt depth, levels of *occludin* mRNA in jejunum Decreased crypt depth
Lee et al. (2012)	Probiotic	*Lactobacillus plantarum*	Increased average daily gain Decreased heightened rectal temperature duration, faecal consistency scores, number of ETEC-positive pigs, serum IL-6 concentrations and serum TNF-α concentrations, duration of heightened serum INF-γ concentrations
Trckova et al. (2014)	Probiotic	*Saccharomyces cerevisiae*	Decreased faecal consistency scores, duration of diarrhoea and ETEC faecal shedding Increased growth performance, serum IgA concentrations
Pan et al. (2017)	Probiotic	*Bacillus licheniformis* and *Saccharomyces cerevisiae*	Increased average daily gain, average daily feed intake, mucosal sIgA concentrations in jejunum and ileum, quantity of occluding protein in jejunal mucosa, villous height, villous height: crypt depth, caecum *Lactobacillus* concentration Decreased diarrhoea incidence, serum diamine oxidase, endotoxin levels, caecum *E. coli* concentration
Luise et al. (2019)	Probiotic	*Bacillus subtilis*	Tendency ($P = 0.06$) to decrease faecal consistency scores

			Decreased concentrations of metabolites in plasma (lysine, glycine, serine and P.aa.C30.0), abundance of Enterobacteriaceae in caecum microbiota No impact on growth performance, microbiota alpha and beta diversity Increased serum IgA concentration
		Bacillus amyloliquefaciens	No impact on faecal consistency scores, growth performance, microbiota alpha and beta diversity Decreased concentrations of metabolites in plasma (arginine, lysine, ornithine), abundance of Enterobacteriaceae in caecum microbiota Increased concentrations of metabolites in plasma (glycine, glutamine), serum IgM concentration
Kiarie et al. (2011)	Postbiotic	*Saccharomyces cerevisiae* fermentation products	Reduced ETEC adherence to ileal mucosa, levels of ammonia in colon, prevalence of Enterobacteriaceae in ileal digesta, faecal consistency scores Increased bacterial diversity in ileal digesta
Nordeste et al. (2017)	Postbiotic	*Lactobacillus acidophilus* fermentation products	Reduced faecal consistency score, demeanour scores, abundance of ileum and colonic *E. coli* Increased abundance of *Lactobacillis* spp., Firmicutes, Bacteroides/Prevotella and Clostridial cluster XIVa in faeces
Wang et al. (2019)*	Synbiotic	*Lactobacillus plantarum* and fructo-oligosaccharide	Increased average daily gain, apparent digestibility of DM and CP, faecal abundance of *Lactobacillus* spp., serum IFN-γ concentration, serum IgG concentration Decreased feed-to-gain ratio, faecal abundance of Enterobacteriaceae No impact on serum haptoglobin, IgA or IgE

*Tested on healthy weaners without ETEC challenge

Postbiotics, the fermentation products of probiotic strains, are often a poorly defined mixture of sugars, amino acids and proteins, but these are receiving increased attention as potential modulators of gut health and are being investigated for advantageous effects on the gut microbiota and overall host health. A beneficial health impact was detected in ETEC-challenged pigs receiving *S. cerevisiae* fermentation products (SFP), demonstrated as a greater bacterial diversity and a reduced prevalence of Enterobacteriaceae in the ileal digesta (Kiarie et al., 2011). Furthermore, a reduction in the number of ETEC adhering to the ileal mucosa and reduced levels of ammonia in the colon were also detected when compared to the control group. *L. acidophilus* fermentation products also led to a reduction in

FCS in pigs receiving varying concentrations of the feed additive. Pigs receiving the highest concentration had an average FCS of 0.56 compared to 1.16 in the control group on day 2 post challenge, whilst pigs receiving the lowest concentration had an average FCS of 0.17 compared to the 0.83 in the control group on day 5 post challenge. In another study a healthier microbiota was detected in pigs receiving postbiotics compared to control pigs, with an increased abundance of several beneficial bacteria in faeces, including *Lactobacillus* spp. and Firmicutes; however, no significant differences in weight gain were reported (Nordeste et al., 2017).

Probiotics and prebiotics also have been studied for their synergistic potential. Wang et al. (2019) reported the effects of seven oligosaccharides on the probiotic *L. plantarum* ZLP001. Fructo-oligosaccharide (FOS) were demonstrated to improve the temperature stress tolerance and increase the growth performance of the probiotic. When supplemented into weaner diets of healthy pigs, the synbiotic was shown to significantly increase ADG to 439 grams, compared to the 398 grams ADG of the control group. In comparison, groups receiving either only the probiotic or the prebiotic were reported to have an ADG of 422 and 423 grams, respectively, which was not statistically significant from either the control group or the synbiotic group. Furthermore, the synbiotic group demonstrated a significant decrease in faecal shedding of Enterobacteriaceae compared to treatment with single modalities, with all three treatment groups demonstrating a significant decrease when compared to the control group. These Enterobacteriaceae faecal shedding concentrations were 4.14, 3.41, 3.78 and 3.01 $\log_{10}$ CFU/g for the control group, probiotic group, prebiotic group and synbiotic group, respectively (Wang et al., 2019). Despite the potential value of synbiotics, there are countless combinations of probiotics and prebiotics that need *in vitro* and *in vivo* testing, with their preventative impact on ETEC infection in weaners needing additional, rigorous assessment. Furthermore, the financial aspects of these benefits in comparison to the increased costs associated with incorporation of multiple products in feed need to be evaluated to determine if it is financially viable.

The main limitation in commercial use of probiotics, postbiotics and synbiotics is the uncertainty surrounding their effects due to the possibility of reporting bias, contradictory effects between studies and the difficulties surrounding ETEC challenge models. A search of the PubMed database of trials

reported from 2011 using the search string "probiotics" and "swine" returned 35 publications, with 91%, 6% and 3% of studies reporting positive, moderate/little and no effect, respectively. Despite the overwhelming positive results, there is no clear determinant that can be drawn on dosages and effect of these additives. A large proportion of studies also report positive outcomes against a measured variable but no significant effect on other variables, followed by studies with contradictory results and measurement of different variables. The uncertainty of effects, for 'biotics' as a collective as well as other alternative strategies, on ETEC infections specifically are heightened due to issues surrounding infection models. The lack of an ability to induce a consistent and reproducible ETEC infection across pigs leads to issues in comparability within and between trials, reduces confidence in results obtained and may explain the contradictory results (Luise et al., 2019b). Urgent progression of ETEC challenge models are required to strengthen *in vivo* ETEC studies investigating the potential of 'biotics' and other control strategies. Currently used methods, and recommendations for standardisation, are reviewed in detail by Luise et al. (2019b).

Phage therapy

Bacteriophages (phages) are viruses that target bacterial cells, with virulent phages following a strictly lytic cycle of infecting the cell, hijacking the host's biochemical machinery to produce new phage progeny, and causing a bactericidal effect upon release of the phage progeny due to the breakdown of the bacterium cell wall (Cha et al., 2012). The high specificity of phages is ideal for therapeutic use due to the reduced risk of treatment affecting non-targeted bacteria, with the high natural abundance of phages also being an ideal characteristic (Yu et al., 2018). Phages are commonly isolated from environments in which the target bacterium is present, with this phenomenon being beneficial from a biosecurity standpoint as it minimises movement of biologicals between farms (Cha et al., 2012; Jamalludeen et al., 2009).

Phage therapy has been reported to alleviate the severity of clinical symptoms induced by ETEC infection in swine (Cha et al., 2012; Jamalludeen et al., 2009; Lee et al., 2017). This was measured as a reduction in the mean FCS of 1.25 to 0.79 accompanied by an increase in ADG from 98 g to 186 g in F4-ETEC challenged pigs receiving phage therapy compared to the positive control group. Phage

L86 demonstrated lytic activity against F4-ETEC *in vitro*, and following spray-drying, was supplemented in swine feed at a rate of 10^7 plaque-forming units (PFU)/kg. Another beneficial effect of phage supplementation in this study was the measured reduction in faecal shedding of ETEC (Lee et al., 2017). This reduction of faecal ETEC load due to phage supplementation was also detected in a study by Cha et al. (2012) where pig feed was supplemented with freeze-dried phages. The faecal ETEC load in pigs was reduced by 64% and 61% compared to the positive control group in pigs receiving 10^6 and10^8 plaque-forming units (PFU)/kg, respectively (Cha et al., 2012). Isolation and storage of these and other ETEC-lysing phages will allow for selective host range testing, decided from previous specificity testing of stored phages, hence shortening the time for identification of a phage for treatment of the outbreak ETEC strain on farm (Figure 2). The promising effects of phage therapy in reducing the severity of ETEC infection in swine, as well as reducing the risk of spread of ETEC by reducing faecal ETEC load, has already been documented. Upon optimisation of phage selection and cocktail design, phage therapy offers the pig industry a highly targeted therapeutic strategy.

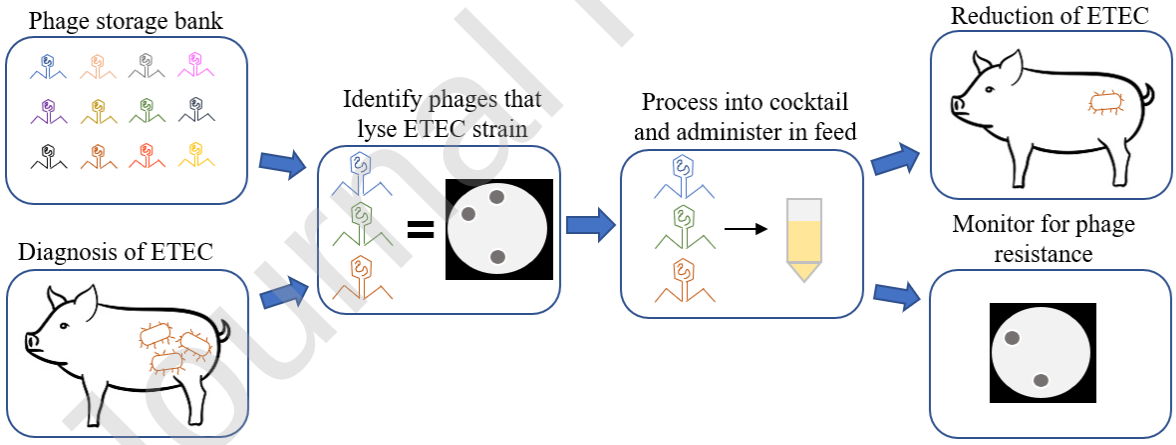

Figure 2. Phage therapy for treatment of ETEC infection in swine.

Phage therapy has progressed to utilise multiple phages in a single cocktail with the aims of overcoming the development of phage resistant bacteria and/or broadening the range of bacterial

strains that are lysed. The mechanisms by which bacteria have developed phage resistance include prevention of phage adsorption and blocking of phage DNA entry (Yu et al., 2018). However, unlike antimicrobials, phages also can evolve, regaining lytic activity against the mutated bacteria by switching the host receptor or through the breakdown of extracellular matrixes produced by the bacteria that prevent phage attachment (Labrie et al., 2010). Furthermore, a study by Yu et al. (2018) anticipated the emergence of phage JDP1 resistant ATCC25922 *E. coli* strains. To overcome this the authors used a phage cocktail that included phages RBP and RSP, which have lytic activity against the phage-resistant variants of ATCC25922, termed Rb and Rs, respectively. Inclusion of the latter phages allowed for the lysis of JDP1-resistant bacterial strains as they developed, resulting in a decreased generation and mutation frequency of phage-resistant strains (Yu et al., 2018). The co-evolution of bacteria and phages diminishes the potential of bacterial cells to become permanently resistant to phage therapy; however, monitoring of phage resistance needs to be conducted following treatment (Figure 3).

A decrease in AMR in bacterial strains due to evolutionary trade-offs to gain phage resistance also has been detected. This was demonstrated when *Pseudomonas aeruginosa* isolates were found to alter their binding site for phage OMKO1, the outer membrane protein OprM of the multi-drug efflux (Mex) systems MexAB and MexXY, to evolve resistance to the phage. The Mex system transports antimicrobials out of the cell, with the system increasing resistance to multiple classes of antimicrobials including quinolones, macrolides and tetracyclines. The mutation reducing the expression of OprM resulted in a reduced efficiency of the Mex systems coupled to an increased susceptibility to the antimicrobials ciprofloxacin, ceftazidime, erythromycin and tetracycline (Chan et al., 2016).

Prior to the exploitation of these types of evolutionary trade-offs in phage therapy, the selection of phages for treatment, as well as the phage processing and administration method, need further advances. Creation of phage storage banks, containing phages capable of lysing a range of ETEC strains, will allow for directed screening of previously isolated and studied phages upon diagnosis and typing of the ETEC strain. After selection of phages, it is essential to optimise processing of the phage

to reduce phage titre loss, with studies demonstrating a superior effect on the reduction of bacteria using highly concentrated phage treatments. Firstly, the processing method needs to ensure stability of phage titre, with phages being susceptible to high temperatures, neutralisation by organic compounds and mechanical stresses due to their protein structure (Malik et al., 2017). Secondly, whilst some phages naturally have high acidic resistance, protection of pH-susceptible phages against the low pH of the GI tract is required to prevent inactivation of phages before they reach their target site of action in the distal small intestine (Jamalludeen et al., 2009). Phage encapsulation and the administration of antacids prior to phage administration are methods developed to protect phages against these harsh conditions (Malik et al., 2017). Finally, the prime administration route of phages for treatment in pigs has not been well established, with initial studies demonstrating faster bacterial clearance from incorporation of phages in feed, suggesting that food protects against the low pH within the animal's GI tract (Carvalho et al., 2010). The incorporation of phages in feed also is beneficial when treating large numbers of animals due to the simplicity and reduced labour requirements for administration, particularly for repeated dosage. Phage processing, administration dosage and route of administration need to be examined further to ensure optimal use of phage therapy.

Advanced phage modifications

Advancements in biotechnology have sparked research into widening the potential of phages for pathogen control. Engineered phages have been demonstrated to enhance pathogen control by increasing the antibacterial activity and broadening the host range of the natural phage itself, with phages also developed to deliver bactericidal genes upon recognition of specific DNA sequences and reduce AMR levels of target bacterial strains: this has been reviewed in detail by Pires et al. (2016). A study by Yosef et al. (2015) explored the use of phages as a delivery system to target AMR bacteria, and to select for susceptible strains by sensitising the AMR bacterial strains whilst concurrently conferring the susceptible bacterial strains with resistance to lytic phages. This strategy was implemented by using lysogenic phages for the delivery of clustered regularly interspaced short palindromic repeats (CRISPR) and the CRISPR-associated system (cas), termed phage-transferable CRISPR-Cas system. In this study, the *E. coli* type I-E CRISPR system was modified to contain

spacers that target the ARGs *bla*$_{\text{NDM-1}}$ and *bla*$_{\text{CTX-M-15}}$. Furthermore, T7 phages were engineered to contain spacers identical to the above CRISPR array triggering degradation of the T7 phage by the CRISPR-Cas system. Upon inoculation of bacterial culture with these lytic phages, the bacterial strains which had reverted to being susceptible to the antimicrobials and therefore carried the CRIPSR array, were able to mount a defence against the phages whilst the bacterial strains remaining resistant to antimicrobials contained no CRISPR-Cas system to target the T7 phage and were lysed by the phages. In summary, the phage-transferable CRISPR-Cas system not only reverted AMR bacterial strains, it selected for antimicrobial susceptible strains by providing protection against the engineered lytic phage (Yosef et al., 2015). While CRISPR techniques have not been applied directly to ETEC toxin genes, studies using the phage-transferable CRISPR-Cas system to specifically target and eliminate virulent bacterial strains have been reported (Bikard et al., 2014), demonstrating future potential of targeting ETEC strains whilst not affecting commensal *E. coli* strains. Overall, these studies begin to demonstrate the potential of engineered phages in targeting specific bacterial strains. Future investigation into their use against ETEC infections, including ETEC strains harbouring AMR, in swine is warranted.

Limitations

The main aspect hindering upscaling phage therapy in pigs is the high specificity of phages, despite this being advantageous when considering the impact of treatment on the gut microbiota. The narrow spectrum nature of phages means a phage capable of lysing an ETEC strain on one farm is unlikely to target an ETEC strain from another farm. This means that upon each new outbreak on each farm, a new phage will need to be isolated. A high level of communication between farm workers, veterinarians and laboratory staff is required to achieve this, a requirement that is attainable. There is also a reliance on the isolation of phages capable of lysing the ETEC strain, with the isolation and testing of phage properties being required. The second issue with phage therapy relates to the fact that most phages are easily isolated. This trait has resulted in most studies isolating new phages and analysing their potential instead of analysing a common phage in various studies. Consequently, the current literature presents a low level of knowledge of numerous phages instead of an in-depth

knowledge of a few strong phage candidates. Finally, phage therapy in swine remains novel, with the approval and regulation of phage-based products needing to be developed before products are commercially available. Currently, phage-based products have been approved for use in food safety and processing in many countries, with the US recently approving PLSV-1™ and INT-401™ for use in poultry against *Salmonella* and *Clostridium perfringens*, respectively (Fernández et al., 2018). Despite this, no commercial phage-based product is available against ETEC infection in swine.

Future directions – Control and treatment combinations

Research has highlighted potential antimicrobial alternatives; however, there is not enough consistent evidence to promote the long-term use of any one strategy. Currently no single suitable approach that encompasses environmental health and animal welfare, has been found to be as effective as antimicrobials for the control of ETEC. As such, the expectation that cessation of AMU for the prevention and treatment of ETEC infection will occur in order to manage emerging public health concerns remains uncertain. Currently, it is at the producer's discretion to administer alternative strategies, with decisions centred around the cost of the product versus the potential benefits. The future management of ETEC infection in weaner pigs points towards reliance on a combination of alternative control strategies, collectively aiming to reduce the incidence of ETEC infection and provide targeted therapeutic approaches for established infections - as outlined in Figure 3.

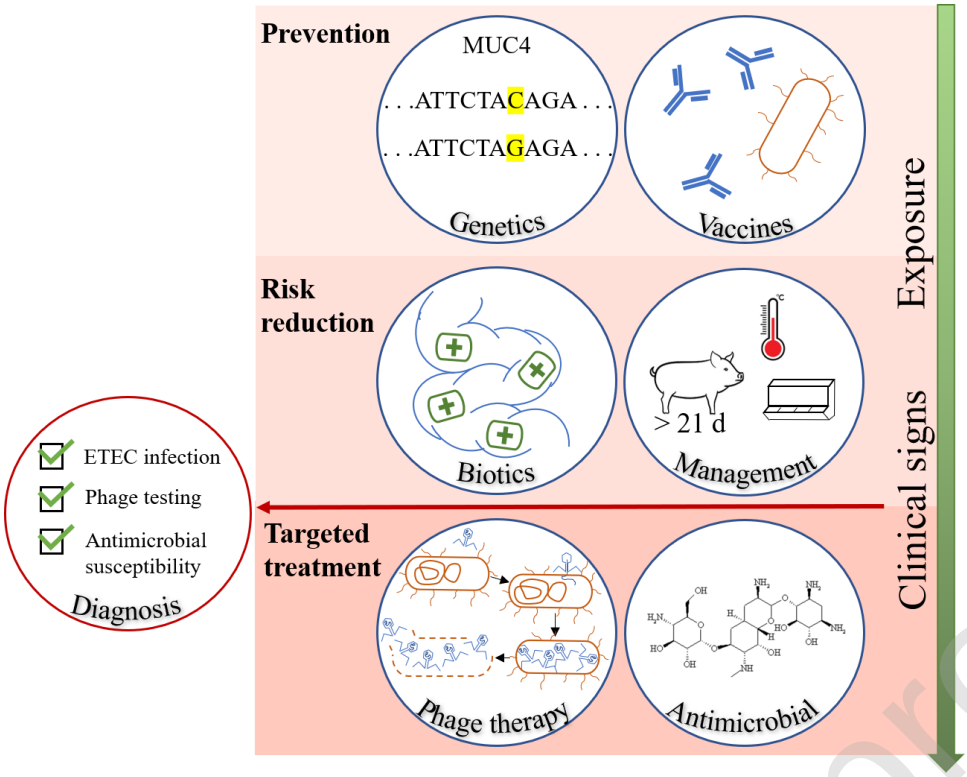

Figure 3. Future ETEC control scheme encompassing a combination of current, alternative and novel strategies.

Note: MUC4 shown as an example of genetic susceptibility testing.

These strategies include current, alternative and novel approaches which collectively focus on preventing colonisation of ETEC by reducing the risk of infection whilst providing targeted treatment for control of outbreaks. The continued development of broad-spectrum vaccines, as well as the identification and selection of genes effecting the host's resistance to ETEC infection, may help control the specific ETEC fimbrial type present on farms. The global implementation of improved farm management, which encompasses heightened hygiene and biosecurity approaches such as through the all-in-all-out stocking approach and optimisation of feeding regimens, can continue to reduce the rate of infection. Meanwhile, supplementation of diets with probiotics, prebiotics or synbiotics to modulate the immune response and support the development of a healthy gut microbiome is limited. Further investigation into these additives is required, and upon a deeper understanding of the mechanisms at play, this strategy may potentially lead to a reduction of pathogen adhesion and aid in pathogen control upon colonisation. After presentation of clinical signs, it is

essential to diagnose the causative pathogen, with further testing including antimicrobial susceptibility testing, whole genome sequencing and phage host testing being needed to guide the best treatment options. The identification of phages capable of lysing the involved ETEC strain in a stored phage bank offers a highly targeted therapeutic method and may become more desirable as industries attempt to transition from high to low AMU. The use of broad-spectrum antimicrobials will remain as a last resort for strains in which phage therapy is not available or not successful. The integration of a combination of alternative strategies offers the swine industry an approach to managing the production and financial burden of ETEC infection in the face of the One Health threat imposed by AMR.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

N/A

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