Novel approaches for managing and controlling antimicrobial resistance in pigs

by

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Thesis Declaration

I, Tanya Jane Laird, declare that:

This thesis is my account of my research and has been substantially accomplished during enrolment in this degree, except where other sources are fully acknowledged. All co-authors, where stated and certified by my Principal Supervisor or Executive Author, have agreed that the works presented in this thesis represent substantial contributions from myself. The thesis contains as its main content, work that has not previously been submitted for a degree at any other university. In the future, no part of this thesis will be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of Murdoch University and where applicable, any partner institution responsible for the joint-award of this degree.

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This thesis contains published work and/or work prepared for publication, some of which has been co-authored.

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David Jordan assisted in conceptualising the experiment, data analysis and reviewed and edited the manuscript.

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Tanya Laird, Sam Abraham, and Mark O'Dea

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Tanya Laird, Josie Mansfield, Shafi Sahibzada, Diana Turpin, John Pluske, Sam Abraham, and Mark O'Dea

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Thesis Abstract

Antimicrobial resistance (AMR) continues to threaten One Health with a projected 10 million deaths per year by 2050 if left uncontrolled. While the human medical field has historically demonstrated poor control in the use of antimicrobials, usage in livestock, both historic and current, is also of concern. Resistance to critically important antimicrobials (CIA's), those reserved for use in life-threatening human infections, has been detected in swine globally. This resistance has the potential to transfer between animals and into humans, and with the accelerated growth of AMR, investigation into novel control strategies for AMR and bacterial infections is now equally as important as surveillance.

This thesis determined the diversity of AMR in commensal *Escherichia coli* (*E. coli*) within and between pigs and farms, hypothesising that this diversity results in underrepresentation of the true prevalence of AMR by current national AMR surveillance approaches. Secondly, this thesis investigated novel approaches for the control of AMR *E. coli* and a ubiquitous pathogen, enterotoxigenic *E. coli* (ETEC), in swine. Postbiotics in the form of *Lactobacillus acidophilus* fermentation products (LFP) and *Saccharomyces cerevisiae* fermentation products (SFP) have demonstrated potential effects in alleviating symptoms induced by ETEC infection and in reducing AMR. The thesis evaluated LFP and SFP, as well as their combined effects, on weaner pigs challenged with F4-ETEC, and in a second experimental trial, on weaner pigs challenged with extended-spectrum cephalosporin (ESC) resistant *E. coli*. Following these trials, a large-scale field-based trial was completed analysing the effects of these postbiotics on AMR *E. coli*. Finally, the combination of bacteriophages and competitive exclusion clones (CECs) were evaluated *in vitro* as a novel and targeted approach for the control of ESC-resistant *E. coli*. The high throughput robotics platform, Robotic Antimicrobial Susceptibility Platform (RASP), was used throughout the project to determine its future application in AMR surveillance and evaluation of control strategies.

High levels of phenotypic and genotypic diversity were detected in commensal *E coli* at both the host and farm level. This was evident in 89% of pigs harbouring more than a single AMR index in the eight *E. coli* colonies subjected to antimicrobial susceptibility testing (AST). Furthermore, 58 different multi-locus sequence types (MSLTs) were identified from the 151 isolates subjected to whole genome sequencing. This diversity highlights the low reliability of current national surveillance methods that use 1 isolate per farm and less than 200 isolates per animal species.

Supplementation with LFP and SFP demonstrated indirect benefits in ETEC-challenged weaner pigs. This was detected as increased growth performance and modulation of the faecal microbiome through increased alpha diversity and abundance in the beneficial bacterial family *Lactobacillaceae*. However, the postbiotics demonstrated no direct impact on ETEC infection measured through bacterial quantification and faecal consistency scores. Furthermore, the effect of these postbiotics on growth performance and faecal shedding of resistant *E. coli* were also studied in healthy weaner pigs. The postbiotics demonstrated no impact on growth performance of healthy weaner pigs. Although the postbiotics demonstrated a reduction in ESC-resistant *E. coli* in pigs challenged with ESC-resistant *E. coli*, no effects on ciprofloxacin, tetracycline and ESC-resistant *E. coli* were detected in the farm-based trial. These conflicting results highlight the importance of evaluating strategies in field trials, however, both studies provided a detailed examination of the natural reduction in AMR-carriage over time, whether in experimental models or on farm. Finally, a targeted approach for controlling ESC-resistant *E. coli* was analysed. The combination of bacteriophages and CECs demonstrated a complimentary relationship, significantly reducing and possibly eliminating ESC-resistant *E. coli in vitro.* Overall, the high-through put robotics platform, RASP, provided cost-effective bacterial quantification and testing of selected bacterial strains across the project. The sample number in experiments is often restricted due to labour constraints especially during these

already labour-intensive trials, however implementation of RASP allowed a high number of samples to be processed and tested, demonstrating future use in AMR research.

In conclusion, a more in-depth sampling model for AMR surveillance is necessary to account for the heterogeneity of AMR. Application of robotic platforms, such as the RASP, in AMR surveillance offers a highly economic and low laborious approach for processing this increased sample size. The RASP additionally demonstrated use in investigation of AMR and ETEC control methods, demonstrated by its high resolution into the dynamics of AMR *E. coli* and ETEC in faecal contents of pigs. Lastly, an *in vitro* combination approach, using both targeted and preventative therapies, demonstrated superiority in reduction of ESC-resistant *E. coli*. This novel combined approach requires future analysis to determine if these results are replicated on farm.

Note on Thesis Layout

This thesis consists of a series of chapters prepared as individual manuscripts for publishing. Chapter 1 has been peer-reviewed and published as a review article. Chapter 2 and 6 have been peer-reviewed and published as journal articles. Chapters 3, 4 and 5 have been prepared as fully formed scientific manuscripts for publishing.

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Chapter 1 - Porcine enterotoxigenic *Escherichia coli*: antimicrobial resistance and development of microbial-based alternative control strategies

1.1 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.

1.2 Keywords

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

1.3 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.

1.4 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

1.4.1 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).

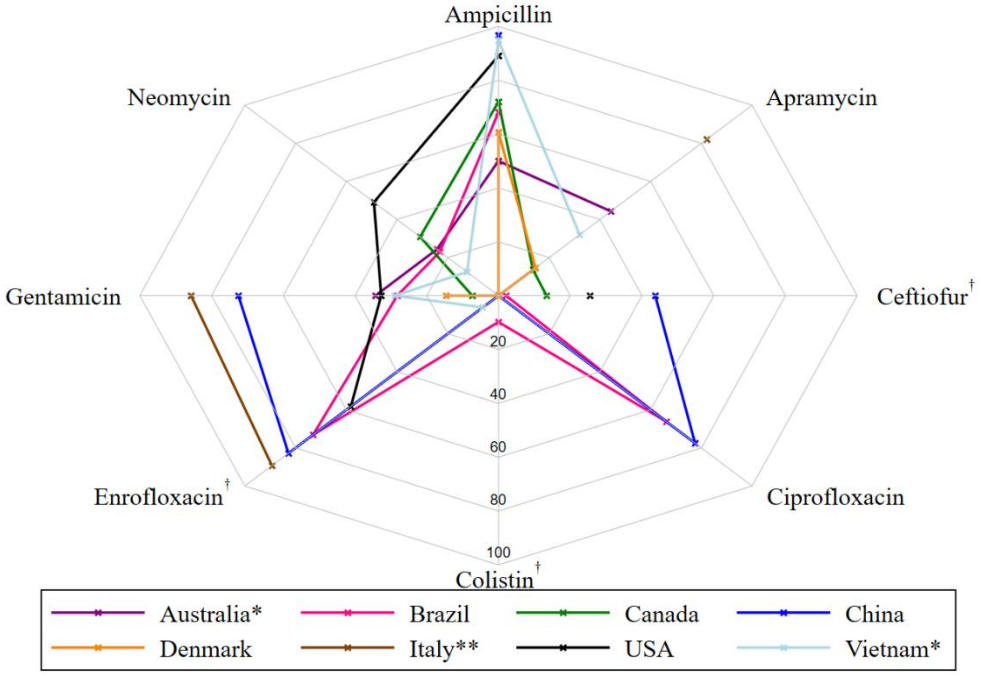

Figure 1. Percent of porcine ETEC demonstrating phenotypic resistance against CIAs as published in different countries.

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.

1.5 Alternative treatment options

1.5.1 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).

1.5.2 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).

1.5.3 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.

1.5.4 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).

1.6 Microbial-based control methods

1.6.1 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., 2020a). 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.

1.6.2 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).

1.6.3 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.

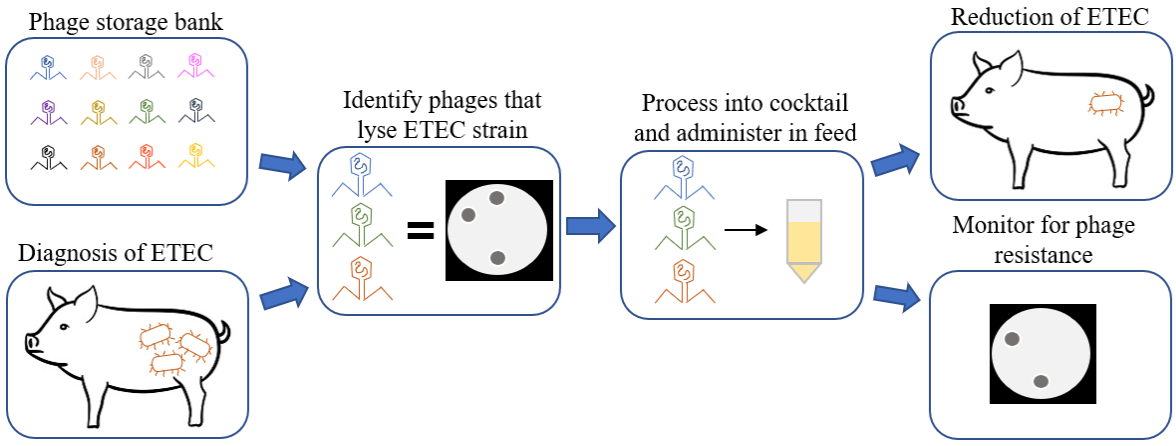

Figure 2. Phage therapy for treatment of ETEC infection in swine.

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 2).

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.

1.6.3.1 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.

1.6.3.2 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.

1.7 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*.

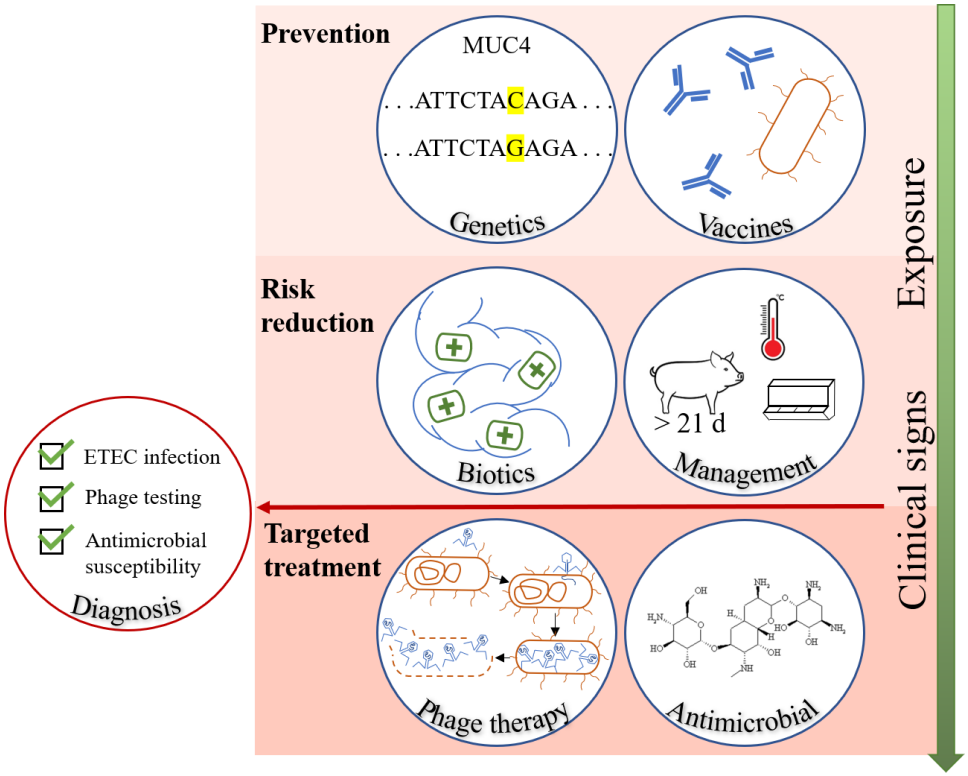

Figure 3. Future ETEC control scheme encompassing a combination of current, alternative and novel strategies.

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.

Chapter 2 - Diversity detected in commensals at host and farm level reveals implications for national antimicrobial resistance surveillance programs

2.1 Synopsis

2.1.1 Background

A key component to control antimicrobial resistance (AMR) is the surveillance of food animals. Currently, national programs test only limited isolates per animal species per year - an approach tacitly assuming that heterogenicity of AMR across animal populations is negligible. If the latter assumption is incorrect then the risk to humans from AMR in the food chain is underestimated.

2.1.2 Objectives

Demonstrate the extent of phenotypic and genetic heterogeneity of *Escherichia coli (E. coli)* in swine to assess the need for improved protocols for AMR surveillance in food animals.

2.1.3 Methods

Eight *E. coli* isolates were obtained from ten pigs on each of ten farms. For these 800 isolates AMR profiles (MIC estimates for 6 drugs) and PCR-based fingerprinting analysis were performed and used to select a subset (n=151) for whole genome sequencing.

2.1.4 Results

Heterogeneity in the phenotypic AMR traits of *E. coli* was observed in 89% of pigs with 58% of pigs harbouring three or more distinct phenotypes. Similarly, 94% of pigs harboured two or

more distinct PCR-fingerprinting profiles. Farm level heterogeneity was detected with ciprofloxacin resistance detected in only 60% of pigs from a single farm. Furthermore, 58 MLST were identified with the dominant sequence types being ST10, ST101, ST542 and ST641.

2.1.5 Conclusion

Phenotypic and genotypic heterogenicity of AMR traits in bacteria from animal populations are real phenomena posing a barrier to correct interpretation of data from AMR surveillance. Evolution towards a more in-depth sampling model is needed to account for heterogeneity and increase the reliability of inferences.

2.2 Introduction

Bacteria harbouring resistance to antimicrobials have a profound impact on the health and welfare of humans and animals. The global action plan to control antimicrobial resistance (AMR) in food animals, and its national derivatives, all strive for a reduction in antimicrobial usage guided in large by the surveillance of resistance (DANMAP, 2019; WHO, 2015). Owing to the ubiquitous occurrence of commensal *E. coli* in terrestrial animals and humans, the dynamic nature with which this organism responds to selection for resistance, and the species' pathogenic potential, commensal *E. coli* are heavily relied upon as an indicator organism in surveillance for AMR in livestock (DANMAP, 2019).

Current approaches to performing national surveillance emphasise use of AST by broth microdilution.(WHO, 2015) However, logistics of sampling coupled with laboratory expenses restricts the number of *E. coli* isolates that are analysed with national programs to a few hundred commensals. This typically comprises a single *E. coli* isolate per epidemiological unit, with no more herds assessed across a country in a single year than the number of isolates evaluated (Authority et al., 2019; DANMAP, 2019). Two issues arising from these sampling limitations stand out: first is the lack of

sensitivity for detecting emerging resistances, especially involving critically important antimicrobials (CIAs), due to their low prevalence and possible non-uniform distribution within herds; second is the paucity of information that is provided on individual herds for the purpose of informing the farmer and veterinarian on improving their antimicrobial stewardship. To address these matters there is the need to understand the heterogeneity of AMR in *E. coli* carried by livestock within and across farms.

Studies examining this heterogeneity have largely neglected AMR diversity, instead focusing on genomic analysis using PCR-based fingerprinting techniques, with few studies performing WGS due to the high associated costs (Abraham et al., 2012; Ahmed et al., 2017; Herrero-Fresno et al., 2017; Herrero-Fresno et al., 2015). By harnessing the advancements of robotic systems for microbiological testing, including automated WGS preparation and AST (Truswell et al., 2021), per isolate costs can be reduced with the potential to affordably increase resolution of AMR phenotypic and genetic diversity. Complementary use of AMR indexing schemes (Truswell et al., 2021), which weight antimicrobials based on public health significance, offer an analytic summary of resistance from these large datasets, whilst additionally providing translatable outcomes for end-users and stakeholders. Because indexing delivers data occurring on a continuum, this facilitates in-depth examination of statistical relationships between AMR datasets of varying geographical regions and time frames (Smith et al., 2014; Smith et al., 2010), allowing real-time review of antimicrobial stewardship efforts.

This study provides a detailed examination of the diversity of *E. coli* in Australian swine, using an AMR index to provide a summary-measure of harboured resistance. We here show the heterogeneity of *E. coli* phenotypes and genotypes at the pig and farm level, providing critical data highlighting the importance of adequate subsampling to estimate AMR levels in livestock production systems, and the need to revaluate the methodology and interpretation of conventional AMR surveillance in livestock.

2.3 Materials and methods

2.3.1 Isolate collection

Freshly defecated faecal samples from ten finisher pigs were collected from each of ten randomly selected Australian commercial extensive swine farms, between November 2018 and February 2019. Samples were transported on ice to Murdoch University, Australia. Samples were stored overnight at 4°C and processed the day after collection. Faecal samples were plated on CHROMagar ECC agar (Edwards Group, Australia) to isolate and select eight individual *E. coli* colonies per pig using the high throughput Robotic Antimicrobial Susceptibility Platform (RASP) as outlined by Truswell et al. (2021) Species identification of all the 800 *E. coli* isolates were confirmed by MALDI TOF (Bruker).

2.3.2 Antimicrobial susceptibility testing

Broth microdilution was performed on all isolates as per ISO 20776-1:2019 and CLSI guidelines (CLSI, 2020). The six antimicrobials tested were tetracycline, ampicillin, ciprofloxacin, ceftriaxone, gentamicin, and trimethoprim-sulfamethoxazole. MIC testing was completed using the RASP (Truswell et al., 2021) with imaging of plates completed via VISION software, reading conducted manually and results analysed using ECOFF breakpoints (Testing, 2021) with CLSI breakpoints (CLSI, 2020) applied to obtain AMR prevalence data based on clinical resistance.

2.3.3 Random amplified polymorphic DNA (RAPD) analysis

DNA was extracted from all isolates using 6% Chelex 100 (Bio-Rad, Australia) as outlined by Abraham et al. (2018) RAPD analysis was performed on all isolates using RAPD primer 1254 as previously described with minor variations and manual identification of RAPD profiles (Abraham et al., 2012; Abraham et al., 2014).

2.3.4 Whole-genome sequencing

Isolates were selected for WGS based on RAPD results with 151 isolates selected representing each unique RAPD profile from each farm. DNA extraction and sequencing was performed as outlined by O'Dea et al. (2019) with the exception of the library preparation performed using a Celero DNA-Seq library preparation kit (Nugen).

2.3.5 Genomic analysis

Sequencing files were assembled using SPAdes (v3.14.0). MLSTs were identified using MLST (Seemann T) (v2.15) with new STs assigned through EnteroBase. Antimicrobial resistance genes (ARGs) were detected using ABRIcate (v0.8.7) with default settings. ABRIcate was also used for the detection of virulence factors using the *E. coli* virulence finder database and a manually curated database with the cut-offs of ≥ 95% coverage and ≥ 99% identity applied. Minimum spanning trees were created using GrapeTree (v0.1.8) and based on allelic profiles from MLST.

Mutations within the quinolone resistance determining region (QRDR) were identified using Snippy (v4.1.0). Snippy-core (v4.1.0) was used for determination of core genome SNPs for phylogenetic analysis with the phylogenetic tree created using RAxML (v8.2.11) on 130 isolates after exclusion of low-sequencing depth samples (n=21). Regions of recombination were removed using clonalframeML (v1.11) with iTOL (v5.7) used to annotate the final tree.

All sequenced data was deposited at NCBI under BioProject number PRJNA720242.

2.3.6 Statistical analysis

Statistical analysis was completed using STATA (v15.1 and v16.1).

2.3.7 AMR index scheme

The AMR index is a summary measure of the public health significance of resistance present in an isolate with resistance towards CIAs given a higher weighting than that to antimicrobials

of less concern. The weighting of antimicrobials was based on the Australian Strategic and Technical Advisory Group rating of antimicrobials with the exception of the weighting for ciprofloxacin being increased from three to four due to fluoroquinolones not being registered for use in Australian food animals (ASTAG, 2018) (Table 3). The weighting for each single phenotypic resistance (defined by ECOFF's) carried by an isolate were added, giving a summary score referred to as the AMR index. An isolate that was fully susceptible would receive an AMR index score of zero whilst an isolate resistant to all six antimicrobials, and therefore of the highest public health concern, would receive an AMR index score of 13 - the cumulative sum of all antimicrobial weightings.

Table 3. Weighting of each antimicrobial used for the AMR index score and based on the antimicrobial's public health significance.

Antimicrobial	Weighting
Ampicillin	1
Ceftriaxone	3
Ciprofloxacin	4
Gentamicin	2
Trimethoprim-sulfamethoxazole	2
Tetracycline	1

2.4 Results

2.4.1 Bacterial isolates and resistance

An AMR index score of 0 was assigned to 24.0% of isolates whilst the average AMR index score across all 800 *E. coli* isolates was 1.7 (Figure *4*). The highest AMR index score detected was 10 out of a potential maximum score of 13, with these three isolates displaying a multidrug-resistant (MDR) profile (Figure 5) defined as isolates resistant to three or more antimicrobial

classes. Overall, 10.5% (n=84) of isolates were classified as MDR with a total of three different MDR profiles identified. All isolates were susceptible to ceftriaxone with low prevalence of resistance against gentamicin (0.8%), ciprofloxacin (3.8%) and trimethoprim-sulfamethoxazole (20.4%) and higher levels of resistance detected against tetracycline (50.1%) and ampicillin (62.0%) (Table 4). Meanwhile, 1.8% of isolates were clinically resistant to ciprofloxacin.

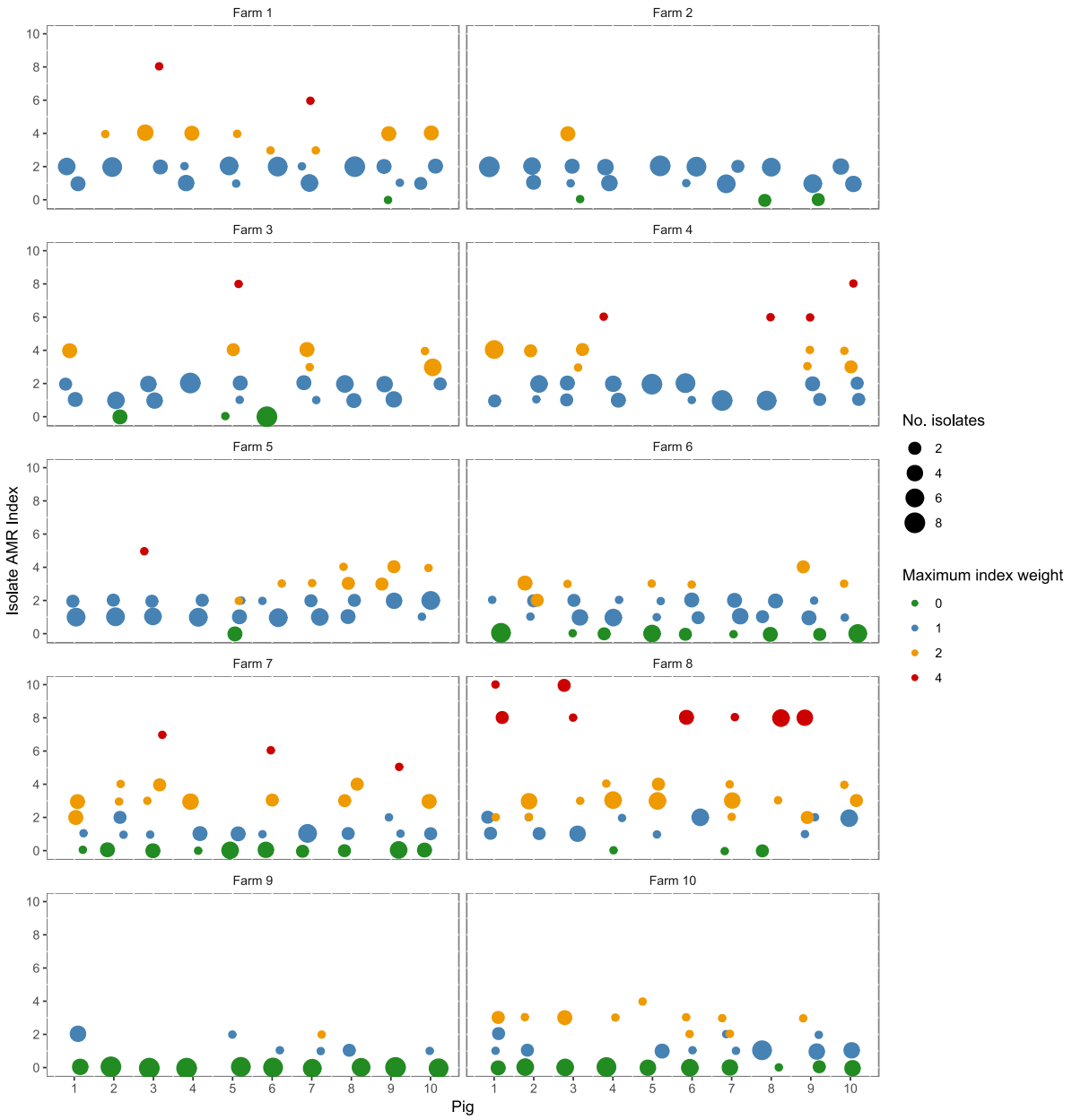

Figure 4. Variation in the antimicrobial index for 800 *E. coli* isolates distributed amongst 10 farms and 10 pigs per farm. A total of eight *E. coli* were analysed per pig. Pig ID is across x-axis and isolate AMR index on y-axis. Displayed is also the number of isolates, and the index weighting of the highest antimicrobial resistance detected in each isolate.

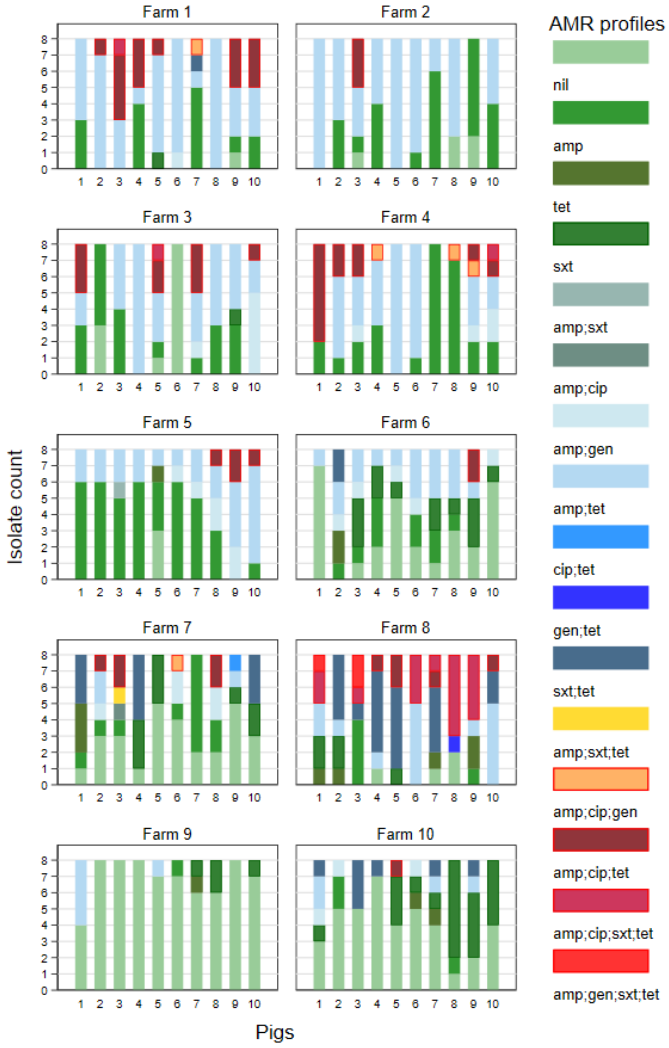

Figure 5. Antimicrobial resistance profile diversity among *E. coli* in individual pigs across Australian pig farms. Pig ID is across x-axis. Abbreviation of antimicrobials: amp = ampicillin, cro = ceftriaxone, cip = ciprofloxacin, gen = gentamicin, sxt = trimethoprim/sulfamethoxazole and tet = tetracycline.

2.4.2 Intra-host diversity

Heterogeneity of AMR indexes at the host level was demonstrated with only 11 pigs sharing a single AMR index (Figure 4) and AMR profile (Figure 5) across all eight isolates. A combination of two indexes was most common and detected in 33% of pigs while 29%, 21% and 6% harboured three, four and five different AMR index scores, respectively. The range of

AMR index scores within pigs varied highly with pigs harbouring three different AMR indexes having a range of two to nine. DNA fingerprinting techniques supported intra-host variation with one to six unique RAPD profiles detected per pig with an average of 3.46 +/- 0.12 (SE) (Figure 9). Only six pigs, belonging to four farms, had the same RAPD profile attributed to all isolates. Overall, 56 unique RAPD profiles were detected from the 800 *E. coli* isolates with the most dominant being P1 (30.3%), P25 (11.4%) and P9 (7.5%).

2.4.3 Intra-farm diversity

Intra-farm variation was detected, evident in Farm 8 with Pig 7 having five unique AMR indexes with a range of eight whilst only three different AMR index scores with a range of 2 were detected in Pig 10 (Figure 4). In comparison, Farm 9 showed less variation between pigs with an AMR index range of zero, one and two detected in four, three and three pigs, respectively. Figure 5 illustrates the intra-farm variation with only three of the farms containing two pigs with the same AMR profiles. Furthermore, ciprofloxacin-resistant *E. coli* was detected in only 60% of pigs on Farm 8.

A variety of RAPD profiles were also observed between pigs on the same farm (Figure 10). Farm 1 showed the least variation of the number of unique RAPD profiles between pigs with Farm 9 showing high variation, despite this farm having a low variation of AMR indexes.

2.4.4 Inter-farm diversity

Overall, a large diversity of AMR indexes was observed between farms with the mean AMR index score at the farm level being between 0.2 and 3.7 (Figure 4). The range of index scores, AMR profiles and AMR prevalence also varied highly between farms. The highest average range of AMR indexes detected within pigs was highest on Farm 8 at 5.8. In comparison, Farm 9 had an average range of AMR indexes within pigs of 0.9. In this farm, 86.3% of isolates were fully susceptible. Overall, 16 different phenotypes were identified with large variations in the

prevalence of each phenotype between farms (Figure 5) as illustrated in isolates that harboured resistance to both ampicillin and tetracycline being present in 58.8% of isolates from Farm 2 but only 3.8% of the isolates from Farm 7. The prevalence of AMR differed between farms as demonstrated in Figure 6 with the largest divergence, ranging from 7.5% to 100% of isolates, detected for ampicillin. Similar variation in farm level prevalence was observed for tetracycline and trimethoprim-sulfamethoxazole with lower variation against ciprofloxacin and gentamicin. Lastly, the percentage of MDR isolates per farm varied between 0% of isolates on Farm 9 and 30.0% of isolates on Farm 8 (Table *4* - Supplementary. Percent of the 800 E. coli isolates resistant to antimicrobials across farms using ECOFF breakpoints.Table 4).

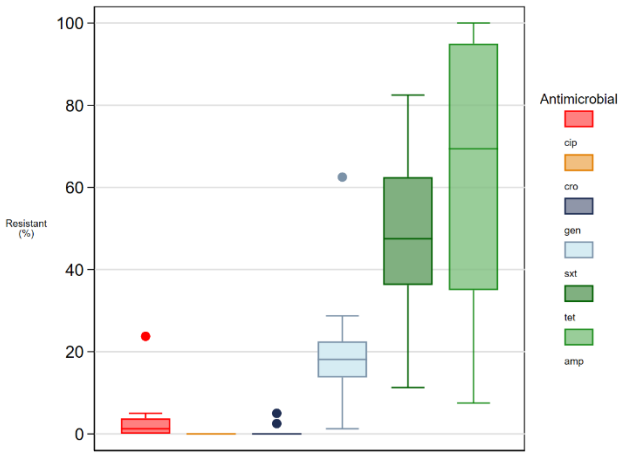

Figure 6. Prevalence of AMR in commensal *E. coli* across 10 Australian pig farms. Variation in the percent resistant to antimicrobials across farms is displayed with the middle line of each box representing the median prevalence, the boxes representing the inter-quartile range and the whiskers displaying the highest and lowest prevalence detected. The public health significance of each antimicrobial is represented by colour with green, blue, orange and red representing a weighting of 1, 2, 3 and 4, respectively. Abbreviation of antimicrobials: cip = ciprofloxacin, cro = ceftriaxone, gen = gentamicin, sxt = trimethoprim/sulfamethoxazole, tet = tetracycline and amp = ampicillin.

Heterogeneity between farms was further supported by RAPD profiles with farms having between 8 and 19 unique RAPD profiles each with an average of 14.9 +/- 1.2 (SE) RAPD profiles per farm.

2.4.5 Genomic analysis

WGS was applied to evaluate the clonal distribution of strains demonstrating different RAPD profiles. Among the representative isolates (n=151) 58 STs were identified including 5 newly assigned STs (ST11,165, 11,171-11,173 and 11,230) (Figure 7). The most dominant STs were ST10 (n=18), ST101 (n=17), ST542 (n=9) and ST641 (n=7) with 31 STs occurring only in single isolates. Variation in STs was detected within the 42 pigs which had more than a single isolate subjected to WGS with an average of 2.2 ± 0.1 (SE) different STs detected. A single pig had four different STs detected among the four isolates sequenced with 11 pigs having three different STs. At the farm level an average of 9.8 ± 0.70 (SE) STs were detected per farm with between eight and 20 isolates sequenced from each farm (Figure 7). Phylogenetic analysis showed clustering of STs, however, STs and clonal complexes were not clustered by farms, further supporting the hypothesised diversity of *E. coli* within and between farms (Figure 8). No correlation was found between STs and RAPDs, most apparent in ST10 which harboured 15 different RAPD profiles with these profiles also identified in other STs belonging to different clonal complexes (Figure 10).

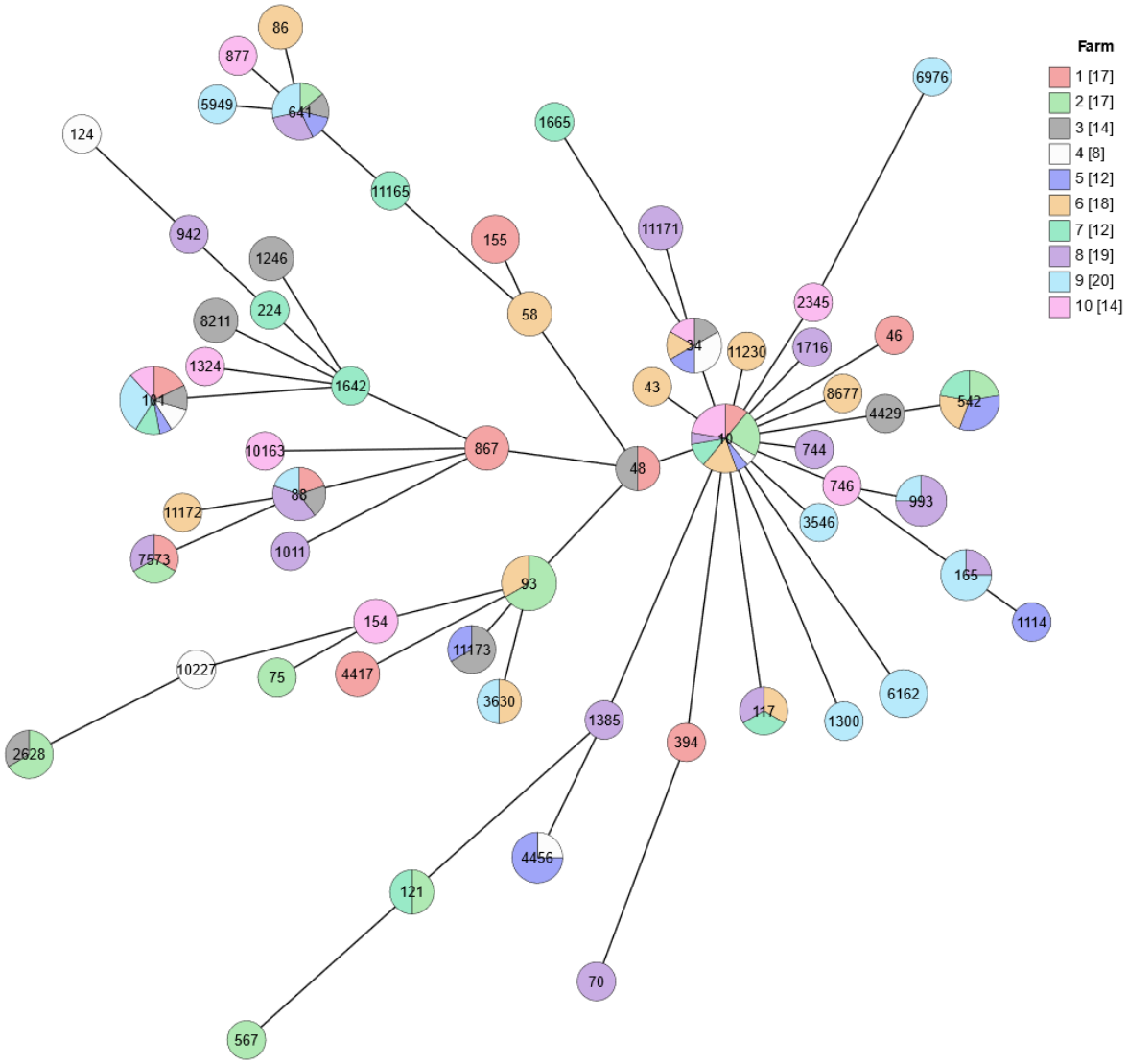

Figure 7. Minimum spanning tree based on multilocus sequence types of 151 *E. coli* isolates from Australian swine coloured by farm. Numbers in brackets of legend indicate number of isolates typed per farm.

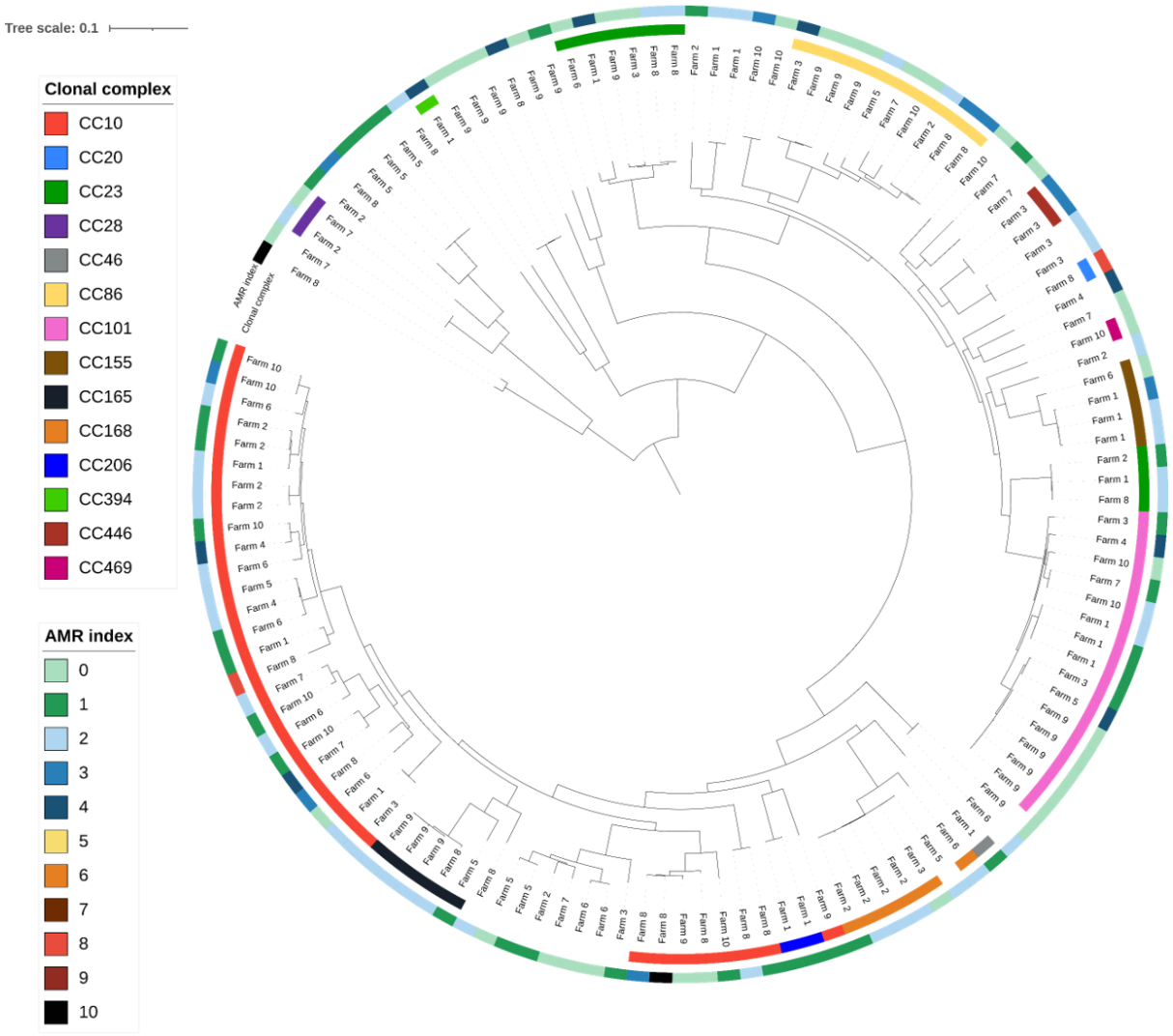

Figure 8. Midpoint-rooted maximum likelihood core genome SNP tree of 130 *E. coli* isolated from Australian swine. Labels indicate farm. Clonal complexes are represented by inner colour strip with AMR index represented by coloured squares on outmost layer, ranging from 0 to 10.

2.4.6 Antimicrobial resistance genes

Overall, 44 variants of ARGs conferring resistance to nine antimicrobial classes were detected with variance in the combinations of ARGs and the number of ARGs detected within pigs and farms. No extended-spectrum cephalosporin (ESC) resistance genes were detected in agreement with phenotypic susceptibility observed for ceftriaxone. The plasmid-mediated quinolone resistance (PMQR) gene *qnrS* was detected in a single isolate belonging to ST942

which was phenotypically wild type and clinically susceptible to ciprofloxacin. The ciprofloxacin-resistant isolate subjected to WGS belonged to ST744 with no PMQRs detected. However, a combination of SNP mutations was detected in the QRDR, *gyrA* (S83L and D87N) and in *parC* (S80I). Only single QRDR mutations were detected in four other isolates with one of these isolates being ciprofloxacin resistant (defined by ECOFF) but not clinically resistant. The detection of ARGs agreed with phenotypic classification based on ECOFF's for ampicillin, tetracycline, gentamicin, and trimethoprim-sulfamethoxazole in 88.1%, 74.8%, 100.0%, and 78.2% of isolates, respectively. In comparison, agreement between "clinical resistance" phenotype and ARGs was present in 91.4% and 96.0% of isolates for ampicillin and trimethoprim-sulfamethoxazole, respectively. For tetracycline, 14 isolates were phenotypically resistant whilst lacking any *tet* gene variants, however all these isolates harboured the MDR gene *mdfA*, which has been demonstrated to confer tetracycline resistance (Edgar and Bibi, 1997). This gene was detected in 98.0% (n=148) of isolates. Additional ARGs were detected amongst isolates known to confer resistance to chloramphenicol, florfenicol, fosfomycin and macrolides, however phenotypic data for these antimicrobials were not obtained.

Investigating ARG variations identified a total of 62 combinations. The dominant profiles were *mdfA* alone (n=33), and *mdfA* and bla_{TEM} in combination (n=11). Profiles carrying resistance towards a single antimicrobial class was most common (22.5% of isolates), whereas ARGs for two and four antimicrobial classes were each seen in 17.2% of the isolates. A single isolate carried ARGs conferring resistance to eight different antimicrobial classes, supporting phenotypic data with this isolate classed as MDR.

Variation in the number of ARGs was detected at both the farm and pig level with between zero and 14 ARGs detected per isolate. The average number of ARGs detected in isolates across farms differed with Farm 9 having the lowest average at 1.4 ARGs compared to Farm 3 and Farm 8 having averages of 5.1 and 7.1 ARGs detected per isolate, respectively. Variation was

also detected between pigs on the same farm, evident in pigs belonging to Farm 2 having an average of between one and seven ARGs detected per isolate. This variation was lower in Farm 9 with pigs having an average of between one and 2.2 ARGs per isolate. Pig level variation was additionally observed with a single pig having isolates with either two, three, five or nine ARGs detected. This was further evident in pigs in which more than a single isolate was sequenced, having an average of two ARGs detected per isolate.

2.5 Discussion

National and international efforts to control AMR rely heavily on active surveillance, and whilst the current methodologies of AMR surveillance programmes have aided in addressing many fundamental issues of antimicrobial usage, the extent of the bacterial diversity demonstrated in this study within and between farms and animals, and the potential underestimation of the prevalence of CIA and multi-drug resistance, highlights a challenge modern AMR surveillance must overcome. This diversity was detected using in-depth sampling with variation in AMR index within hosts evident in 89% of pigs carrying multiple AMR index scores (Figure 4). Meanwhile the significance of the variance between pigs belonging to the same farm was highlighted with 60% of pigs on Farm 8 harbouring ciprofloxacin-resistant *E. coli* (Figure 5). These levels of variation risk CIA resistance being undetected by single isolate sampling as only 23.8% of all isolates from Farm 8 were resistant to this CIA. Whilst national surveillance programmes early on acknowledged the impact of diversity between farms and therefore sampled multiple farms (Authority et al., 2019; DANMAP, 2019), the level of diversity within farms and hosts has not previously been demonstrated. The factors that may affect this diversity include antimicrobial use, biosecurity, pig source and feed type, and upon comparison between farms, may enable the identification and modification of practices that account for high AMR indexes. Delivery of evidence that can positively impact on antimicrobial stewardship at farm-level is a vital goal for future

surveillance since farms are the fundamental unit of decision-making for antimicrobial use in food animals. For this to occur, surveillance must embrace the demonstrated variation inherent at the host level, intra-farm level and inter-farm level and raises the question about what sampling designs are needed in the future.

The variation in AMR composition was supported by genetic analysis with an average of 2.2 ± 0.11 (SE) unique STs detected within each pig which had more than a single isolate subjected to WGS, supporting previous findings of genetic variation in REP (repetitive element sequence based) PCR profiles of commensal *E. coli* (Ahmed et al., 2017; Herrero-Fresno et al., 2015). An average of 5.87 different REP profiles were detected per pig when analysing 50 *E. coli* from each of four nursery pigs from across five farms in Denmark. Despite only four pigs representing each farm, variation between farms was detected with farms carrying between eight and 21 unique REP profiles (Herrero-Fresno et al., 2015). In support, the current study detected an average of 9.8 ± 0.70 (SE) and 14.9 ± 1.2 (SE) unique STs and RAPD profiles within farms. Overall, 58 MLSTs were identified among the 151 sequenced isolates with 31 STs only found in a single isolate. The four main sequence types detected were ST10, 101, 542 and 641 which have all previously been detected in Australian swine (Abraham et al., 2015; Zingali et al., 2020). WGS provided further valuable resolution into the genetic variation of *E. coli* with observed variation in ARGs evident in Farm 2 having an average of between one and seven ARGs detected within each pig. This variation supports the heterogeneity detected in phenotypic data, highlighting the necessity for adapting current sampling methods in AMR surveillance. Genotypic resistance was more consistently associated with phenotypic resistance when CLSI breakpoints were applied compared to ECOFF breakpoints. Tetracycline demonstrated the lowest consistency which may be attributed to the presence of the MDR gene *mdfA*, which encodes for a transport protein with phenotypic resistance dependant on its expression levels (Bibi et al., 2001). Overall, genetic analysis supported the high levels of

diversity in commensal *E. coli* and whilst STs correlated with core genome analysis, no trend was observed between these and AMR indexes, highlighting the necessity in determining diversity of *E. coli* in terms of both AMR phenotypic data and genetic diversity.

The statistically robust sampling method in this study supports the finding that CIA resistance previously detected in Australian pig herds continues to occur at low levels despite the geographic isolation of the Australian continent, the virtual ban on importation of livestock, and the strict regulation of CIA use in Australian livestock (Abraham et al., 2017b). This regulation prevents the use of fluroquinolones in food animals and restricts the use of ceftiofur to off-label use in pigs (Abraham et al., 2017b; Mukerji et al., 2017). Despite these measures, ESC-resistant *E. coli* have been detected in Australian swine (Abraham et al., 2015; Abraham et al., 2018). More recently, Kidsley et al. (2018) detected no ESC-resistant *E. coli* upon analysis of 201 isolates from healthy pigs from across 19 Australian farms. The absence of ESC-resistance was supported in the current study however, ESC-resistance was not selected for and therefore may have been present at a very low proportion. Similar prevalence of ciprofloxacin clinical resistance was also seen between these two recent studies with 2% detected by Kidsley et al. (2018) compared to the 1.8% detected in this study. The PMQR gene *qnrS1*, demonstrated to confer low level resistance (Garoff et al., 2017), was detected in a clinically susceptible isolate, with this gene previously detected in *E. coli* from Australian swine (Kidsley et al., 2018). The ciprofloxacin-resistant *E. coli* subjected to WGS belonged to ST744, a globally disseminated ST commonly harbouring CIA resistance and detected in Australian silver gulls and swine (Abraham et al., 2015; Haenni et al., 2017; Hasan et al., 2012; Mukerji et al., 2019), with resistance attributed to the detection of three QRDR mutations. Detection of CIA resistance in livestock in the absence of drug use, has previously been postulated as originating from incursion of human or bird derived isolates as evidenced by the emergence of human-associated MRSA in Australian pigs (Sahibzada et al., 2017) and

fluoroquinolone-resistant *Campylobacter* in chickens (Abraham et al., 2020). The dissemination of these CIA resistant strains despite regulation of drug use, coupled with their low prevalence and the diverse nature of AMR in *E. coli* observed in this study, underlines the need for further evolution of AMR surveillance methods. Whilst the current sample method lessens this risk, a bias towards highly abundant *E. coli* strains may occur when isolating from *E. coli* specific agar. Consequently, if CIA-resistant *E. coli* occurs at lower abundance, it may remain undetected in samples with the true prevalence underrepresented. Investigation into the absolute abundance of different resistant *E. coli* types in faeces, by serial dilution onto selective agar plates supplemented with antimicrobials, may allow this to be evaluated and further aid in describing AMR in commensal *E. coli*. Nonetheless, even taking the limitations of this study into account, the diversity in both genomic and AMR composition is vast and a critical shortcoming of traditional AMR surveillance. In-depth surveillance can allow countries similar to Australia with respect to low antimicrobial usage and AMR, to monitor the emergence of resistance towards CIAs whilst alternatively providing a benchmark for countries with higher resistance levels for feedback on the efficacy of antimicrobial stewardship efforts. These findings support the necessity for further examination into the diversity of AMR in livestock focusing on heightening our understanding of AMR and strengthening surveillance for AMR in animals.

2.6 Acknowledgements

This project was supported by funding from the Australian Government Department of Agriculture, Water and the Environment as part of its Rural R&D for Profit programme and Australian Pork Limited. Authors would like to acknowledge the Veterinarians who collected the sample and farms who participated in the study.

2.7 Funding

This project was supported by funding from the Australian Government Department of Agriculture, Water, and the Environment as part of its Rural R&D for Profit programme.

2.8 Transparency declarations

None to declare.

2.9 Supplementary

Table 4 - Supplementary. Percent of the 800 *E. coli* isolates resistant to antimicrobials across farms using ECOFF breakpoints.

Farm	amp (n)	cft (n)	cip (n)	gen (n)	sxt (n)	tet (n)	mdr
1	96.5 (77)	0	2.5 (2)	0	22.5 (18)	78.8 (63)	21.3 (17)
2	93.8 (75)	0	0	0	3.8 (3)	62.5 (50)	3.8 (3)
3	83.8 (67)	0	1.3 (1)	0	20.0 (16)	52.5 (42)	12.5 (10)
4	100.0 (80)	0	5.0 (4)	0	21.3 (17)	60.0 (48)	20.0 (16)
5	95.0 (76)	0	1.3 (1)	0	13.8 (11)	35.0 (28)	5.0 (4)
6	42.5 (34)	0	0	0	12.5 (10)	42.5 (34)	2.5 (2)
7	35.0 (28)	0	3.8 (3)	2.5 (2)	28.8 (23)	36.3 (29)	8.8 (7)
8	55.0 (44)	0	23.8 (19)	5.0 (4)	62.5 (50)	82.3 (66)	30.0 (24)
9	7.5 (6)	0	0	0	1.3 (1)	11.3 (9)	0
10	13.8 (11)	0	0	0	16.3 (13)	40.0 (32)	1.3 (1)
Total	62.3 (498)	0	3.8 (30)	0.8 (6)	20.4 (163)	50.1 (401)	10.5 (84)

Abbreviation of antimicrobials: amp = ampicillin, cro = ceftriaxone, cip = ciprofloxacin, gen = gentamicin, sxt = trimethoprim/sulfamethoxazole, tet = tetracycline and mdr = multi-drug resistant.

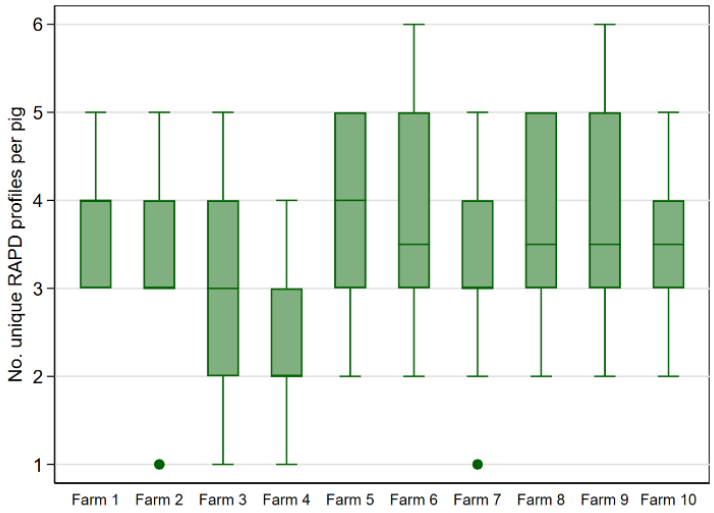

Figure 9 - Supplementary. Variation of number of unique RAPD profiles (n=800) of the eight *E. coli* representing each pig and distributed amongst 10 farms.

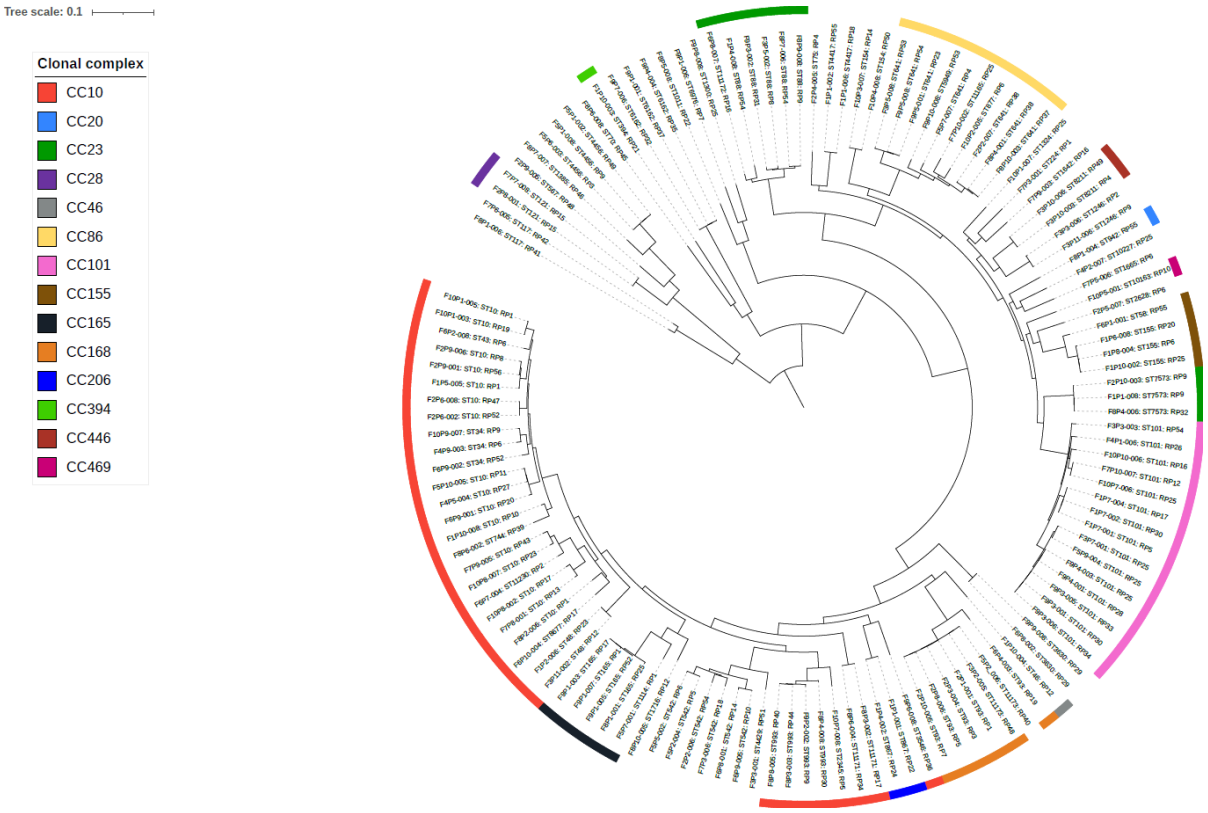

Figure 10 - Supplementary. Midpoint-rooted maximum likelihood core genome SNP tree of 130 *E. coli* isolated from Australian swine. Isolates are named using the following formula: FarmPig_Isolate number: ST: RAPD profile i.e. Farm 1 Pig 2 Isolate 3 of ST45 and RAPD profile i.e. Farm 1 Pig 2 Isolate 3 of ST45 and RAPD profile 6 = F1P2_003: ST45: RP6.

Chapter 3 – Effects of *Lactobacillus* and *Saccharomyces* fermentation products on growth performance and faecal microbiome in ETEC challenged weaners

3.1 Abstract

3.1.1 Background

Enterotoxigenic *E. coli* (F4-ETEC) pose an economic and animal welfare threat to the swine industry through reduced growth, increased mortality and treatment costs. Prevention and treatment of ETEC often relies on antimicrobials; however, due to the threat of antimicrobial resistance, usage must be minimised and, alternative control methods are needed. Medicinal levels of zinc oxide can alleviate ETEC infection, although this also has implications for resistance and environmental contamination with heavy metals. Postbiotics, the fermentation products of probiotic strains, have been reported to increase host health and defence against pathogens and in contrast to zinc oxide, has no impact on AMR or the environment. In a randomised, controlled-trial this study investigated the effects of postbiotics in the form of *Lactobacillus acidophilus* fermentation products (LFP) and *Saccharomyces cerevisiae* fermentation products (SFP), on weaner pigs challenged with an F4 ETEC strain.

3.1.2 Results

Automation of ETEC quantification provided high resolution into the measurement of ETEC faecal shedding; however the fermentation products, either individually or combined, had no impact on ETEC concentration in faeces. Analysis of the faecal microbiota supported these findings with no variance in the abundance of Enterobactericeae between treatment groups. Furthermore, no differences in the faecal consistency scores were detected between treatment

groups. A difference in mean liveweight (± 95% CI) was detected at the final timepoint, being 16.9 (16.7, 17.0), 17.0 (16.8, 17.2) and 17.9 (17.4, 18.3) kg for pigs supplemented with LFP, SFP, and the combination, respectively compared to 16.2 (15.9, 16.5) and 16.1 (15.8, 16.6) kg in pigs left untreated and those supplemented with zinc oxide, respectively. Analysis of the faecal microbiome detected an increased abundance of Lactobacillaceae in pigs supplemented with LFP whilst supplementation with SFP, and its combination with LFP, increased alpha diversity.

3.1.3 Conclusion

Dietary supplementation with LFP and SFP marginally increased growth performance of pigs challenged with ETEC and beneficially modulated the faecal microbiota, suggesting these postbiotics may have a role in management of porcine ETEC.

3.2 Background

Newly-weaned pigs are vulnerable to a multitude of infectious agents, with serotypes of enterotoxigenic *Escherichia coli* (ETEC) being amongst the most globally ubiquitous of these pathogens (Zimmerman et al., 2019). Infection with F4-ETEC results in the induction of post-weaning diarrhoea (PWD), not only impacting the overall health of animals and potentially resulting in mortality, but also incurring financial losses due to poor growth performance and inflated treatment and management costs (Fairbrother et al., 2005). Historically, antimicrobials have been used to control bacterial infections including ETEC. However, due to the emergence and dissemination of ETEC strains resistant to commonly used antimicrobials, coupled with industry directions to minimise antimicrobial usage, antimicrobials can no longer solely be relied upon for control of ETEC (Jahanbakhsh et al., 2016; Laird et al., 2021a). Medicinal zinc oxide is a well-established feed additive and recognized as a therapeutic option for ETEC in

the nursery. However, concerns with zinc pollution in the environment and heavy metal resistance co-selecting for antimicrobial resistance (Bednorz et al., 2013; Ciesinski et al., 2018; Heo et al., 2013) has resulted in the European Union banning veterinary products containing zinc oxide from June 2022(EC, 2017). Numerous alternative in-feed and management-related strategies to ZnO including probiotics, postbiotics, bacteriophages and vaccination programs are all being investigated for their efficacy and environmental impact in comparison to ZnO (Laird et al., 2021a).

Postbiotics, the fermentation products of probiotic strains, potentially offer an alternate strategy for minimising antimicrobial use after weaning. The mixture of sugars, proteins and amino acids in fermentation products is being investigated for advantageous effects on the gastrointestinal tract (GIT) microbiota and host health in protecting against pathogens and their associated diseases. *Lactobacilli* and the yeast species *Saccharomyces cerevisiae*, are two researched probiotics, with studies describing alleviation in clinical signs of ETEC infection in pigs (Lee et al., 2012; Trckova et al., 2014). The postbiotic potential of these species is being explored with studies reporting contrasting effects of *Saccharomyces cerevisiae* fermentation products (SFP) and *Lactobacillus acidophilus* fermentation products (LFP) against enteric pathogens in pigs (Bass and Frank, 2017; Brewer et al., 2014; Harris et al., 2017; Kiarie et al., 2011; Kiarie et al., 2012; Knapp et al., 2018). A reduction in ileal mucosa ETEC count and a reduction in the abundance of Enterobacteriales in the ileal microbiota has been reported in F4-ETEC-challenged pigs supplemented with SFP (Kiarie et al., 2011; Kiarie et al., 2012). No studies have investigated the effects of LFP on ETEC-challenged pigs, with only two studies reporting effects in healthy weaner pigs, described as increased average daily gain (ADG), increased abundance of *Lactobacillus* and decreased abundance of *E. coli* (Bass and Frank, 2017; Lan et al., 2016). These reported results suggest this postbiotic may potentially modulate the microbiome of ETEC-infected pigs and minimise effects of ETEC infection on growth

performance. With weaning being the ideal target period for postbiotic treatment, due to the susceptibility to ETEC infection and the potential to influence the rapid diversification of the GIT microbiota that occurs at this time (Guevarra et al., 2019), further investigation into these fermentation products is warranted.

Using a controlled clinical trial with random allocation of piglets to treatment and blind assessment of outcomes, this study attempts an unbiased examination of the impact of LFP and SFP, when administered alone or in combination. This was analysed during and following an F4-ETEC infection in weaner pigs and compared to the effects of medicinal ZnO. It was hypothesised that these postbiotics, would reduce the duration and severity of ETEC infection, measured through the enumeration of faecal ETEC shedding and the presence of diarrhoea. It was additionally hypothesised that LFP and SFP will improve growth performance and promote diversification of the microbiome.

3.3 Materials and methods

This trial was approved by the Animal Ethics Committee of Murdoch University (R3101/19).

3.3.1 Pre-screening for F4-ETEC susceptibility

Bristles with attached follicles were collected from 225 pigs one week before weaning. Bristles were placed in a labelled sterile 1.5 mL microcentrifuge tube and placed on ice with sanitisation of equipment implemented between sampling of each pig.

The DNA was extracted from follicles of the bristles by aliquoting 100 uL of PBS into each tube, centrifuging tubes for one minute at 13,300 g and placing tubes on a heating block set to 100 °C for 10 minutes. The presence or absence of the Mucin 4 allele was determined by a polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP) assay as detailed by Sterndale et al. (2019a). Briefly, Mucin 4 primers (5’-

GTGCCTTGGGTGAGAGGTTA/5'-CACTCTGCCGTTGTCTTTCC) (Sigma-Aldrich, Australia) were used to amplify a region of the MUC4 gene. Restriction enzyme digestion using *XbaI* (Promega, USA) and visualisation was then performed with resistant alleles remaining non-digested and viewed as a single band at 367 bp and susceptible alleles digested and viewed as two bands at 151 and 216 bp. Products with three bands detected underwent sanger sequencing of the muc4 PCR product with the presence of a C nucleotide at the *XbaI* digestion site classifying pigs as resistant and a G nucleotide as susceptible. The 36 susceptible pigs were selected for the trial. However due to low numbers of susceptible pigs, 37 partially susceptible (three bands with sanger sequencing showing C, G) and 7 resistant pigs (three bands with sanger sequencing showing C, C) were selected for the trial.

3.3.2 Animals, housing and experimental design

The five treatment groups were allocated to pens by a randomised block design with four replicate pens of each treatment. The treatments were (i) the control diet (CON), (ii) CON diet supplemented with 3,000 ppm ZnO (ZnO), (iii) CON diet supplemented with 2,000 ppm LFP (LFP), (iv) CON diet supplemented with 2,000 ppm SFP (SFP), and (v) CON diet supplemented with the combination of 2,000 ppm LFP and 2,000 ppm SFP (LAS). The base (CON) diet was (product details here) and pigs received feed and water ad libitum. The LFP and SFP feed additives used in the trial were Diamond V SynGenX™ and Diamond V Original XPC™, respectively.

Piglets from a commercial piggery were weaned at 21 days of age and 80 of these, selected on the basis of the pre-screening F4-ETEC susceptibility assay, were transported to the animal housing facility at Murdoch University. Piglets were allocated into pens according to weaning weight (6.04 ± 1.07 kg) and F4-ETEC susceptibility genetic testing with four piglets in each pen. Pens were constructed of metal with plastic flooring, and each contained a five-space

feeder, a nipple drinker, a manually filled water bowl containing electrolytes for the first seven days after F4-ETEC challenge, and plastic bottles for mental enrichment of the pigs. Pigs were acclimatised for five days on arrival and housed at 28.0 ± 1.0 °C. The holding temperature was reduced to 26.0 ± 1.0 °C the day prior to dosing and on both days of inoculation.

3.3.3 F4-ETEC inoculation

Following acclimatisation, pigs were inoculated with ETEC (serotype O157: F4: LT, STa, STb, EAST: β haemolytic) on two consecutive days, designated as Day 0 and 1, according to the gelatin capsule method as described by Sterndale et al. (2019b). Pigs received an average of 1.07×10^{10} and 3.07×10^{9} colony forming units on Day 0 and Day 1 of dosing, respectively.

3.3.4 Faecal sampling and processing

Rectal swabs were collected from all pigs on days 0, 1, 2, 3 and 7. All swabs were streaked onto 5% sheep blood agar plates (Edwards, Australia) and incubated overnight at 37 °C. Plates were examined for colonies showing morphology consistent with *E. coli* and haemolysis representative of the challenge ETEC strain. Swabs were frozen at -20 °C for microbiome analysis.

A single colony resembling the challenge ETEC strain was picked from each plate and inoculated into 500 uL of Luria Bertani broth in a 96 well format. These were grown overnight, and DNA extracted using 6% chelex (Bio-Rad, Australia). To confirm the ETEC extracted were most likely the challenge strain, a multiplex PCR using primers for the detection of fimbrial antigens K88 (F4), K99 (F5), 987 (F6), F41 and F18 and the enterotoxins STa, STb, and LTb and Shigatoxin Stx2e was performed (Casey and Bosworth, 2009). The PCR mix was prepared to a total volume of 15 µL consisting of 7.5 µL GoTaq Green Master Mix, 2 µL template DNA and 0.5 µM of each primer and water. The thermocycling conditions were as

described by Casey and Bosworth (2009). Products were on 2.5% gel at 80 V for 2-3 hours and imaged on a BioRad Gel Doc (Life Science, California, USA).

3.3.5 RASP ETEC and *E. coli* quantification

Fresh faecal samples were collected from pen floors on Days 1, 2, 3, 4, and 7 and were pooled by pen. The pooled pen faecal samples were processed using the Robotic Antimicrobial Susceptibility Platform (RASP) as outlined by Truswell et al. (2021) and Laird et al. (2021b). Briefly, one gram of faeces was added to 19 mL of PBS buffer and placed in a stomacher machine on high for 30 seconds. The contents were filtered upon pouring into sterile centrifuge tubes and then placed onto the RASP for dilutions and plating. Dilutions required for plating were estimated and then performed with two dilutions plated onto each agar plate. After overnight incubation at 37°C, plates were placed back on to RASP for imaging and counting of colonies. If the dilutions plated did not result in single colonies, samples were replated using calculated dilutions, and overnight incubation and next day robotic processing repeated.

Each sample was plated onto a 5% sheep blood agar plate and a Chromogenic ECC (MicroMedia, Edwards Group) agar plate for quantification of putative ETEC and total *E. coli*, respectively. Sheep blood agar was selected for visual identification of ETEC colonies due to ETEC typically producing alpha haemolysis when cultured on blood agar plates (Fairbrother et al., 2005). Meanwhile, Chromogenic ECC agar plates are selective for *E. coli* with this species presenting as a blue colony. Images of plates were digitally captured and Pickolo^{TM} software was calibrated to identify single colonies of ETEC and *E. coli* by image analysis based on colour, haemolysis, size and circularity. Pickolo^{TM} software (with manual assistance) was used to identify and count colonies, followed by species confirmation of representative colonies using Matrix assisted laser desorption ionisation time of flight (MALDI-TOF) mass

spectrometry (Bruker). Colony forming units per gram (CFU/g) of faeces were calculated from colony counts.

3.3.6 Faecal consistency scoring and weighing

Faeces were examined daily using a 5-point scale and scored as; (1) dry and granulated; (2) dry and firm shaped; (3) moist and soft with retained shape; (4) pasty; or (5) watery diarrhoea. Pigs were individually weighed weekly with weekly weighing of pen feed completed on the same days.

3.3.7 Microbiome diversity and abundance

DNA was extracted from all rectal swabs using the MagMax DNA Multi-Sample Ultra kit (ThermoFisher Scientific) following the faecal samples protocol on a Kingfisher 96 particle processor (Life Technologies). The V4 region of the 16S rRNA gene was amplified using the primers F515/R806 (Caporaso et al., 2011). Library preparation was performed using the Illumina 16s protocol as per manufacturer's instructions. Sequencing was performed on an Illumina Nextseq 500 platform using a 2 x 150 mid-output reagent kit. QIIME2 was used to process and analyse 16S rRNA gene sequencing data and to perform statistical calculations for alpha and beta diversity (Bolyen E, 2019). Reads with q score greater than 30 were imported into QIIME2 for analysis using the Deblur pathway. Sequences were grouped into operational taxonomic units (OTUs) based on 97% sequence similarity using the Greengenes reference database. The OTUs were filtered and those with less than 10,000 reads (additive across samples) were removed from the dataset.

3.3.8 Statistical analysis

Statistical analysis and graphing were conducted using STATA (v15.1 and v16.1), R Studio (v1.2.5033) and QIIME2 (Bolyen E, 2019). Liveweight (kg) of pigs from -5 to 27 days was

first analysed descriptively to assess the form of temporal trends and generalised additive models were used to fit smoothing splines to non-linear trends initially with pigs, pens and rooms as random effects in a full model. Simpler models were assessed for suitability based on the Akaike information criteria and the final model used to produce estimates of the mean effect of diet on liveweight through the experimental period with 95% confidence intervals relied on for interpreting the impact of sampling error on differences.

Faecal consistency days, a measure of FCS across time, was analysed using a multilevel mixed effects linear regression with pen as a random effect variable. Bacterial quantification was log transformed with a multilevel mixed effects linear regression used to analyse ETEC and total *E. coli* shedding across time, termed ETEC and ECC density, respectively.

The abundance of bacterial families in the faecal microbiome was checked for normality and analysed using the non-parametric Kruskal-Wallis H test. Post-hoc comparisons were conducted with the Dunn's test using Holm correction.

Analysis of the microbiome data was conducted using Qiime2 with treatment groups compared to the control and ZNO diet to determine if feed additives altered the faecal microbiota. The non-parametric Kruskall-Wallis one-way analysis of variance was used to compare alpha diversity between treatment groups at each timepoint sampled (Xia and Sun, 2017). Faith's phylogenetic diversity (Faith PD) was used to measure richness with Pielou's evenness used to measure evenness (Hagerty et al., 2020). Differences in beta diversity of the faecal microbiome between treatment groups were analysed across days using permutational multivariate analysis of variance (PERMANOVA). PERMANOVA of the diversity analysis was calculated with the 999 Monte Carlo permutation and Benjamini-Hochberg correction (FDR) (Xia and Sun, 2017)

3.4 Results

The *MUC4* genetic pre-screening resulted in the selection of 36 fully susceptible, 37 partially susceptible and 7 non-susceptible pigs for inclusion in the trial due to high numbers of ETEC-F4 resistance in pigs. Throughout the trial, 20 pigs were removed. This included 11 pigs removed before ETEC challenge due to the death of one pig and welfare concerns for 10 pigs, the death of one pig on day 4, and the removal of one pig on days 13 and 17 due to welfare concerns. These welfare concerns related to weight loss, poor health status and presence of diarrhoea. An additional six pigs were removed on day 2 due to discrepancies in the feed of pens 13 and 15.

The ETEC PCR was conducted on a single colony from each ETEC positive culture to determine presence of fimbrial antigens and enterotoxins. All colonies tested resembled the challenge strain through carriage of *lt, sta, stb and k88* (*east* was present in the challenge strain but not included in the PCR) with no additional genes detected.

3.4.1 No significant difference in faecal consistency scores of pigs supplemented with LFP and/or SFP

Prior to ETEC challenge, FCSs were low across all treatment groups with 82% of FCSs being 2 or less (Figure 11). This increased the day after challenge with 75%, 99% and 60% of pigs having a FCS of 3 or above on day 1, 2 and 3, respectively. Scores of 4 or higher were present in 72% (n=50) of pigs by day 2 with severe diarrhoea, a FCS of 5, present in over half of these pigs (n=29). The number of pigs with diarrhoea remained high for approximately four days, dropping to a FCS of 2 in 85% of pigs on day 5. Analysis of faecal score days, a measure of FCS weighted by time, using a linear mixed model demonstrated no significant difference

between treatment groups when analysed across the entirety of the study (Figure 11) and when analysed for the four-day period after challenge in which FCS increased.

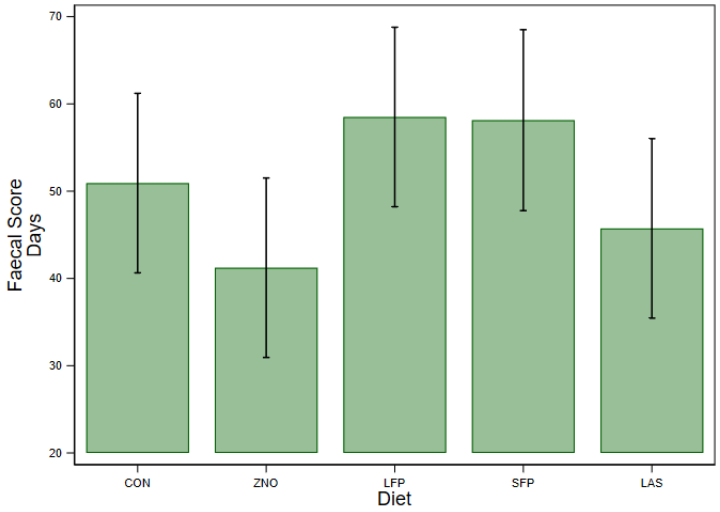

Figure 11. The mean faecal score days, a measurement relating to the faecal consistency score across the full duration of the study. Treatment abbreviations: CON control base diet, ZNO = base + 3000 ppm zinc oxide, LFP = base + 2000 ppm *Lactobacillus acidophilus* fermentation product (LFP), SFP = base + 2000 ppm *Sacchromyces cerevisiae* fermentation product (SFP), LAS = base + 2000 ppm LFP + 2000 ppm SFP.

3.4.2 No significant difference in faecal F4-ETEC concentration in pigs supplemented with LFP and/or SFP

Faecal F4-ETEC shedding was detected on days 1-4, with ETEC shedding cleared by day 7 in all groups (Figure 12). The average ETEC concentration across all pigs was 5.1, 5.3, 4.9 and 3.2 $\log_{10}$ CFU/g for days 1, 2, 3 and 4, respectively, showing a reduction in ETEC concentration over time. Analysis of ETEC density (ETEC shedding across time) demonstrated no significant difference between treatment groups with the shedding density of 15.6 (9.4, 21.8), 14.9 (8.7, 21.1), 20.2 (14.0, 26.4), 16.0 (9.8, 22.1) and 19.3 (13.1, 25.4) for the control, ZNO, LFP, SFP

and LAS groups, respectively (Figure 13). The total *E. coli* density also demonstrated no significant difference between treatment groups (Figure 13).

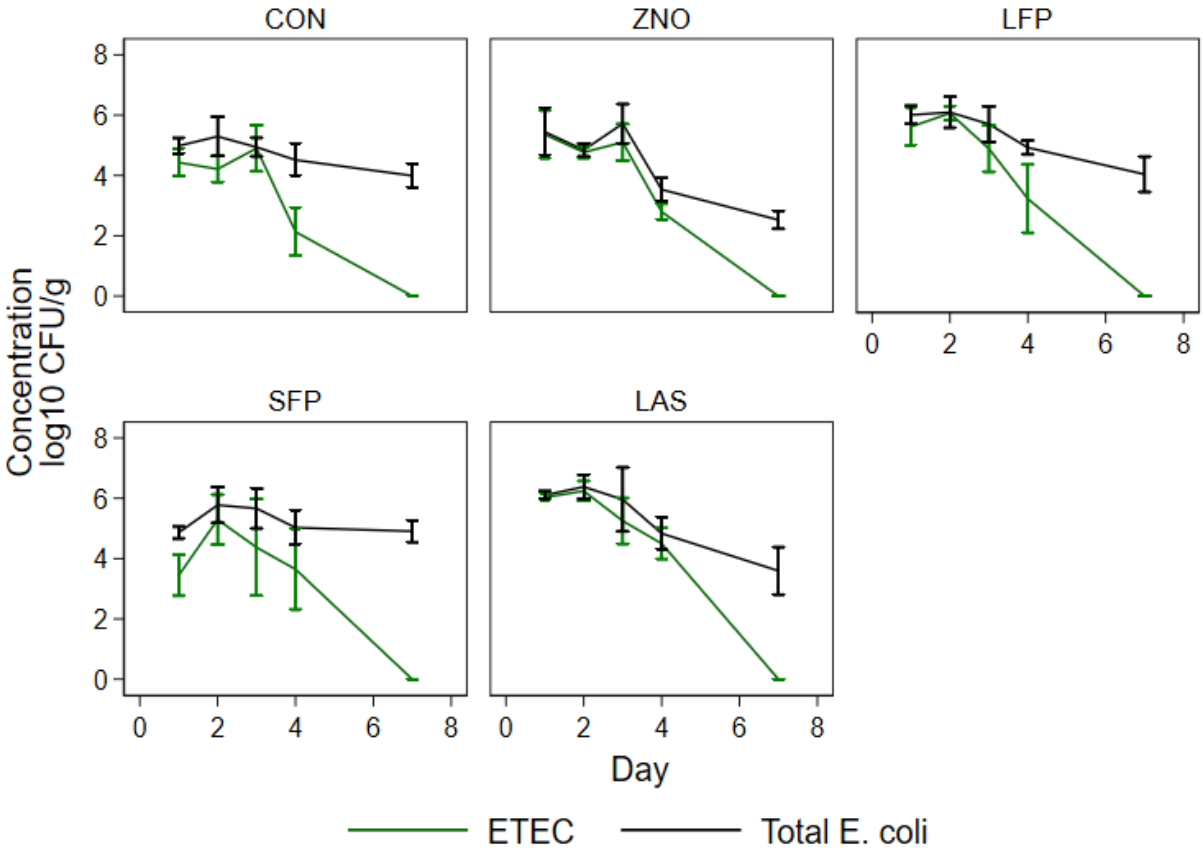

Figure 12. Quantification of faecal F4-ETEC and total putative *E. coli* in ETEC-challenged pigs (n=69) belonging to different treatment groups following challenge with ETEC. Error bars represent standard error of means. Pigs were inoculated with ETEC on Day 0 and 1. Treatment abbreviations: CON control base diet, ZNO = base + 3000 ppm zinc oxide, LFP = base + 2000 ppm *Lactobacillus acidophilus* fermentation product (LFP), SFP = base + 2000 ppm *Sacchromyces cerevisiae* fermentation product (SFP), LAS = base + 2000 ppm LFP + 2000 ppm SFP.

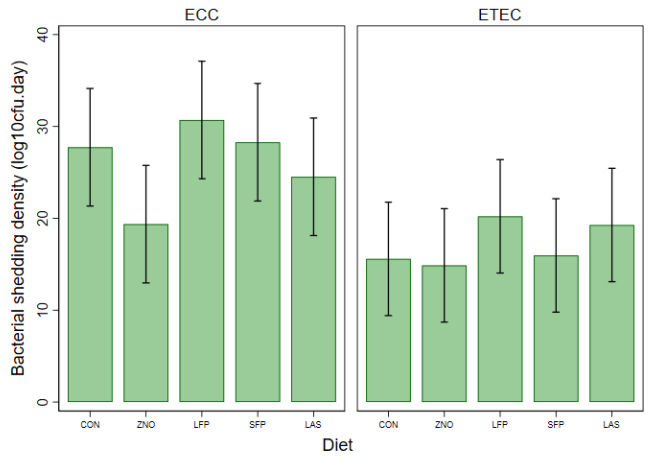

Figure 13. The mean F4-ETEC and total putative *E. coli* density, a measurement relating to the ETEC and putative total *E. coli* shedding across the seven days following challenge with ETEC. Pigs were challenged with ETEC on Day 0 and 1. Treatment abbreviations: CON control base diet, ZNO = base + 3000 ppm zinc oxide, LFP = base + 2000 ppm *Lactobacillus acidophilus* fermentation product (LFP), SFP = base + 2000 ppm *Sacchromyces cerevisiae* fermentation product (SFP), LAS = base + 2000 ppm LFP + 2000 ppm SFP.

Treatment groups demonstrated highest ETEC shedding within each treatment group on different days (Figure 12). This was observed on Day 1 for the ZNO group, Day 2 for the LFP, SFP and LAS groups, and Day 3 for the control group. The percent of *E. coli* comprised of ETEC was 92, 94, 87 and 70% across days 1, 2, 3 and 4 supporting the initial increase in ETEC before clearance. This percentage of ETEC was most similar between groups on day 3, between 77% and 99%, but showed large variation on day 4 in which ETEC compromised 47% of all *E. coli* in the control group compared to 66, 73, 79 and 93% in the LFP, SFP, ZNO and LAS groups, respectively.

3.4.3 Increased final liveweight of pigs supplemented with LFP and/or SFP

Starting bodyweights were similar between all groups with a mean weight of 6.08 ± 0.13 SEM (Figure 14). The mean liveweight (± 95% CI) of pigs at the end of the trial differed between treatment groups and was highest in the LAS group at 17.9 (17.4, 18.3) kg (

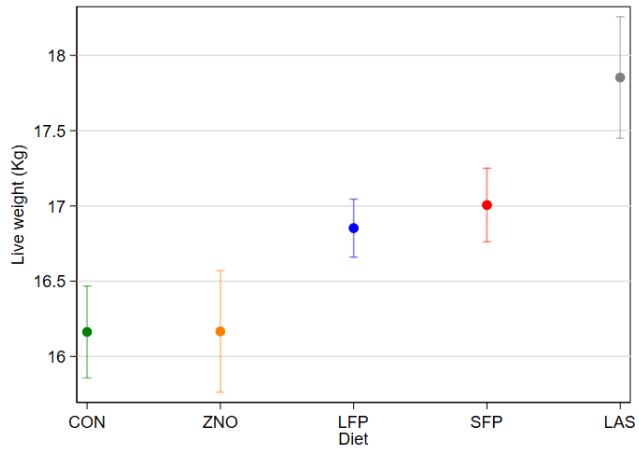

Figure *15*). In comparison, the ZNO and control groups had the lowest mean liveweight on the final day at 16.1 (15.8, 16.6) and 16.2 (15.9, 16.5) kg, respectively, approximately 1.6 kg lighter than pigs in the LAS group. On the final day of the trial, the LFP and SFP groups had a mean liveweight of 16.9 (16.7, 17.0) and 17.0 (16.8, 17.2) kg, respectively.

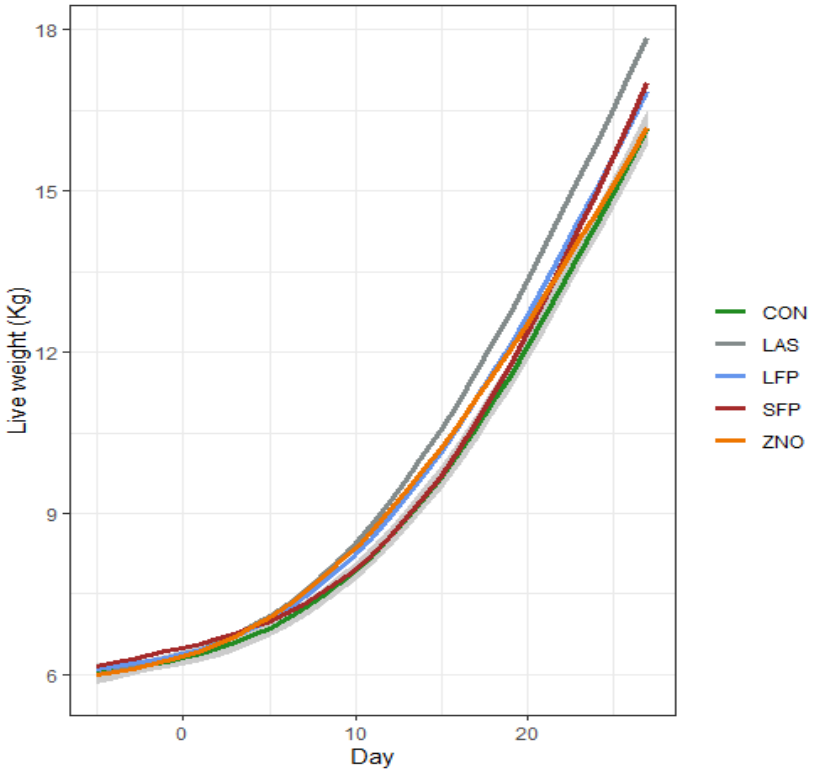

Figure 14. Mean effect of each of five diets on post-weaning pigs liveweight over the duration of the trial as estimated by generalised additive models fitting smoothing splines to non-linear changes in liveweight over time. Confidence intervals (shading) are provided for the control diet). Treatment abbreviations: CON control base diet, ZNO = base + 3,000 ppm zinc oxide, LFP = base + 2,000 ppm *Lactobacillus acidophilus* fermentation product (LFP), SFP = base + 2,000 ppm *Sacchromyces cerevisiae* fermentation product (SFP), LAS = base + 2,000 ppm LFP + 2,000 ppm SFP.

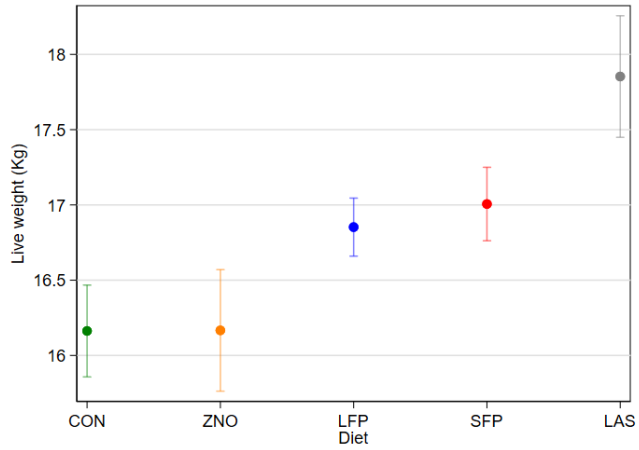

Figure 15. Mean effect of each of five diets on post-weaning pigs liveweight at the final timepoint (Day 27) of the trial as estimated by generalised additive models fitting smoothing splines to non-linear changes in liveweight over time. Confidence intervals (bars) are provided for each diet. Treatment abbreviations: CON control base diet, ZNO = base + 3,000 ppm zinc oxide, LFP = base + 2,000 ppm *Lactobacillus acidophilus* fermentation product (LFP), SFP = base + 2,000 ppm *Sacchromyces cerevisiae* fermentation product (SFP), LAS = base + 2,000 ppm LFP + 2,000 ppm SFP.

Average daily gain (ADG) increased over time for all treatment groups, with mean ADG across all pigs increasing from 60 grams in Week 1 to 208, 357 and 634 grams in Week 2, 3 and 4, respectively (Table 7). The ADG was highest in Week 2, 3 and 4 for pigs in the LAS group with the ADG 95.1 and 140.3 grams higher in the LAS group in Week 4 compared to the control and ZNO groups, respectively.

Average daily feed intake (ADFI) increased over time with mean ADFI of all groups starting between 86 and 94 grams in Week 1 and increasing to between 811 and 1,028 grams in the final week. The SFP group demonstrated the highest ADFI in week 4 (1,028 grams) with all other groups having an ADFI between 811 and 910 grams. The feed conversion ratio (FCR)

demonstrated the greatest variation in Week 1 (days -5 to 2) ranging from an average of 0.8 in the SFP group to 2.1 in the control group. The average FCR across treatment groups were less variant at other timepoints with ratios within 0.3, 0.2 and 0.4 for Week 2, 3 and 4, respectively.

3.4.4 Increased diversity and abundance of Lactobacillaceae in the faecal microbiome of pigs supplemented with LFP

Analysis of the faecal microbiota identified the dominant bacterial families across all groups to be Coriobactericaceae, Lactobacillaceae, Lachnospiraceae and Ruminococcaceae (Figure 16). The abundance of the two major bacterial families, Lactobacillaceae and Ruminococcaceae, differed between treatment groups on day 7. Lactobacillaceae abundance was statistically increased in the LFP ($p = 0.030$) and LAS ($p = 0.003$) groups compared to the control group. Further analysis of the Lactobacillaceae detected all OTU's identified to the genus level to belong to *Lactobacillus* and six different species composing this family. The dominant species across all treatment groups was *Lactobacillus reuteri* composing 50.5%, 64.3%, 72.9%, 76.9% and 81.2% for the ZNO, LFP, SFP, control and LAS groups, respectively. No species were identified in greater proportion between treatment groups. Meanwhile, the ZNO group had an increased abundance of Ruminococcaceae compared to the LFP ($p = 0.023$) and LAS ($p = 0.026$) groups. The two dominant species within this family were identified to be *Faecalibacterium prausnitzi* (*F. prausnitzi*) and *Ruminococcus bromii*, however no difference in percent abundance of these species was detected between these treatment groups. On day 1, the abundance of Clostridiaceae was also detected to be significantly increased ($p = 0.017$) in the ZNO group compared to the SFP group. This was attributed to the significantly increased proportion of the genus *SMB53* and reduction in the

genus *Sarcina* in the ZNO group compared to the SFP group. There was no statistical difference ($p > 0.05$) in the proportion of Enterobacteriaceae between treatment groups at any timepoint.

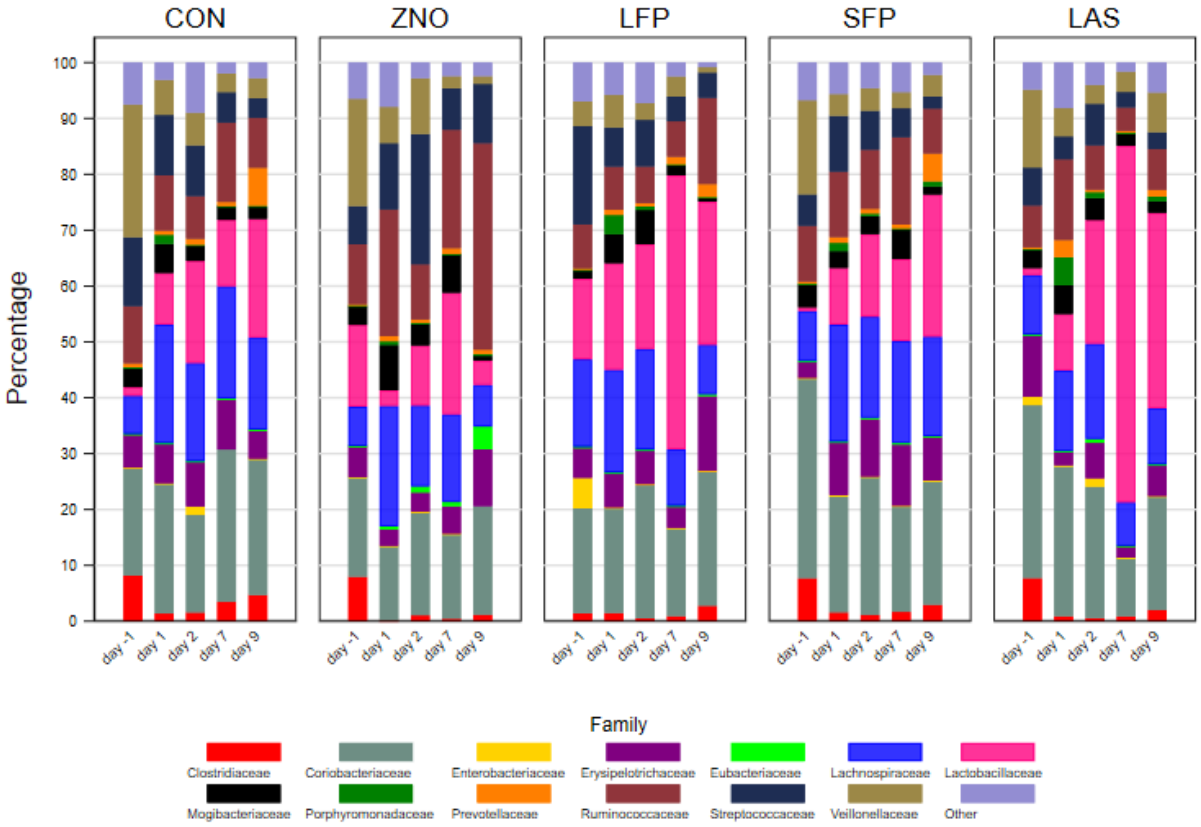

Figure 16. Proportion of bacterial families in faecal microbiota of ETEC-challenged weaners over multiple days and treatment groups. Treatment abbreviations: CON control base diet, ZNO = base + 3,000 ppm zinc oxide, LFP = base + 2,000 ppm *Lactobacillus acidophilus* fermentation product (LFP), SFP = base + 2,000 ppm *Sacchromyces cerevisiae* fermentation product (SFP), LAS = base + 2,000 ppm LFP + 2,000 ppm SFP.

Analysis of alpha diversity detected a greater richness, measured using Faith's PD index, in the LAS group compared to the control group on day 1 and compared to the ZNO group when all timepoints were analysed together (

Table 5). Meanwhile, the SFP group had greater richness than the ZNO group on day 2. No significant difference in evenness, measured using Pielou's evenness index, was detected between treatment groups at each timepoint analysed. The beta diversity was analysed using three indices; Jaccard distance, unweighted UniFrac and weighted UniFrac with analysis comparing treatment groups to the control and ZNO groups (Table 6). On day 1 and day 7, the ZNO group demonstrated difference in microbial composition compared to the control group. Differences in microbial composition were also detected between the ZNO group and the LFP and SFP groups on day 7. No differences in the microbial composition of treatments compared to the control group were detected on day -1, 2 or 9. Overall, the ZNO group differed to all other groups when all sample timepoints were analysed together.

Table 5. Pairwise comparison (Kruskal-Wallis test) of Faith's phylogenetic diversity index of treatment groups in ETEC-challenged weaners with significantly different alpha diversity when compared to the control and zinc oxide groups across timepoints.

Day	Group 1*	Group 2	Faith's PD	
			H	p
Overall	LAS (n=23)	ZNO (n=27)	4.138	0.042
1	LAS (n=6)	CON (n=9)	5.014	0.025
2	SFP (n=4)	ZNO (n=7)	4.321	0.038

* Group 1 is the treatment with greater alpha diversity detected. Treatment abbreviations: CON control base diet, ZNO = base + 3,000 ppm zinc oxide, LFP = base + 2,000 ppm *Lactobacillus acidophilus* fermentation product (LFP), SFP = base + 2,000 ppm *Sacchromyces cerevisiae* fermentation product (SFP), LAS = base + 2,000 ppm LFP + 2,000 ppm SFP.

Table 6. Pairwise comparisons (PERMANOVA test) of beta diversity indices of treatment groups in ETEC-challenged weaners with significantly different beta diversity when compared to the control and zinc oxide group across timepoints.

				Unweighted UniFrac	Jaccard distance	Weighted UniFrac
Day	Group 1	Group2	Sample size (n)	Q	q	q
Overall	CON	ZNO	64	0.010	0.007	0.083
	ZNO	SFP	46	0.010	0.005	0.070
	ZNO	LFP	45	0.218	0.048	0.180
	ZNO	LAS	50	0.010	0.005	0.180
1	CON	ZNO	16	0.067	0.020	0.477
7	CON	ZNO	12	0.010	0.020	0.126
	ZNO	LFP	9	0.273	0.047	0.217
	ZNO	SFP	9	0.195	0.020	0.131

q = q-value defined as the adjusted p-value following Benjamini & Hochberg correction.

Treatment abbreviations: CON = control base diet, ZNO = base + 3,000 ppm zinc oxide, LFP = base + 2,000 ppm *Lactobacillus acidophilus* fermentation product (LFP), SFP = base + 2,000 ppm *Sacchromyces cerevisiae* fermentation product (SFP), LAS = base + 2,000 ppm LFP + 2,000 ppm SFP

3.5 Discussion

The necessity to reduce the use of both antimicrobials and zinc oxide in the feed of newly-weaned pigs demands the development of novel strategies to negate the impact of bacterial disease in this class of animals. In this study, we evaluated two microbial derived products, separately and in combination, as postbiotics for amelioration of F4-ETEC infection.

The current study demonstrated no effect of LFP, SFP and the combination, on FCS, and the duration and quantity of F4-ETEC faecal shedding in F4-ETEC-challenged pigs, when compared to a standard diet or the established ZnO intervention. No previous studies have reported the effects of LFP or the combination of postbiotics in F4-ETEC-challenged weaner pigs. However, the current study supports Kiarie et al. (2011) who reported no effect of SFP on FCS and faecal ETEC concentration in F4-ETEC challenged weaner pigs. Meanwhile, a reduction of F4-ETEC was observed in ileal mucosa scrapings of pigs supplemented with SFP (Kiarie et al., 2011). Kiarie et al. (2011) also reported no effect of SFP, whether supplemented in-feed or in drinking water, on ADG in ETEC-challenged weaners. These data contrast with the current study, with pigs supplemented with LFP and SFP having an increased final liveweight compared to the control and ZnO groups. Furthermore, the combined effect of these two products was significantly superior to single administration with a final liveweight of 17.9 (17.4, 18.3). The discrepancies in the impacts of SFP between these studies may be due to the ETEC inoculation methods and pre-screening for selection of swine. Although no direct effect on ETEC infection was detected in the parameters measured, the increased growth performance suggests the feed additives may indirectly alleviate ETEC infection through variations to the faecal microbiota.

Identification and implementation of feed additives that manipulate the microbiome, improving defence against pathogens, may lead to a reduced reliance on antimicrobials in the future. Although the abundance of Enterobacteriaceae in the faecal microbiota demonstrated similarity between all treatment groups in this study, we identified advantageous changes to the faecal

microbiota of F4-ETEC-challenged weaners when receiving feed supplemented with LFP (LFP group), and LFP and SFP (LAS group). Firstly, bacterial diversity was increased in pigs receiving the combination of the LFP and SFP compared to the control and ZNO groups. Furthermore, the abundance of the bacterium family, Lactobacillaceae, was increased in pigs receiving feed supplemented LFP, whether alone or in combination with SFP, compared to the control and ZNO groups. An increased bacterial diversity and increased abundance of Lactobacillaceae have previously been demonstrated to be associated with increased health status and growth performance (Dou et al., 2017; Guevarra et al., 2019; Lu et al., 2018; Ober et al., 2017). The dominant species composing this bacterial family was *L. reuteri* with the probiotic effect of *L. reuteri* in the swine gut well documented. Studies have demonstrated its production of antimicrobial substances including reuterin and reutericyclin and its strong ability to adhere to and colonise the swine gut (Hou et al., 2015). This adhesion is pivotal for probiotic effects including pathogen exclusion and immune modulation. Furthermore, supplementation with *L. reuteri* has been to reported to enhance the intestinal mucosal barrier and immune stimulation in newborn piglets by increasing villous height, the crypt depth of the jejunum, the number of goblet and $CD3^+$ T cells, and the expression of antimicrobial peptides, IL-4 and IFN-γ (Wang et al., 2020b). Improved growth performance was also reported in these nursery pigs whilst other studies have reported increased growth performance in weaner pigs supplemented with *L. reuteri* (Hou et al., 2015; Wang et al., 2020b). While no reduction in ETEC shedding or FCS were reported in the current study, the direct effects of the postbiotics and the effect of the increased proportion of Lactobacillaceae on the small and large intestine were not measured, and may account for the increased weight in these pigs. An increased abundance of Ruminococcaceae was detected in pigs supplemented with ZnO compared to the LFP and LAS groups in the current study. This bacterial family has previously been detected at a higher abundance in healthy pigs compared to diarrhoeic pigs (Dou et al., 2017).

Ruminococcaceae have been reported at high levels within the caecum with *F. prausnitzi* associated with fibre fermentation and butyrate production (Benus et al., 2010). Fermentation of fibres in the large intestine lowers the pH value, with acidic conditions reducing the growth of pathogenic bacterial strains including ETEC (Heo et al., 2013). Despite the increased abundance of this bacterial family and bacterial species, no variation in ETEC faecal shedding density or faecal consistency was detected between treatments in the current study. Meanwhile, the functional role of the bacterial genera SMB53, detected at high levels in pigs supplemented with ZnO in the current study, remains unknown. However, it has previously been reported as a dominant genus within the small intestine of weaner pigs (Pollock et al., 2021). Overall, the alteration of the microbiome in pigs supplemented with the combination of both LFP and SFP, detected as an increased alpha diversity and increased abundance of Lactobacillaceae, may account for the increased liveweight in these pigs. Whilst our understanding of the microbiome in weaners requires further expansion, these postbiotics demonstrate potential to alleviate the reduced growth performance imposed by ETEC infections in weaners and requires further *in vivo* analysis.

Any realistic investigation into controlling ETEC-associated PWD needs to be performed *in vivo*. Traditionally, monitoring shedding and disease following an F4-ETEC infection has been achieved by assessing a faecal consistency score (FCS), or by laboratory-based culturing of F4-ETEC scored or described as pure, mixed or no ETEC growth. Both methods show low resolution and due to the subjective scoring nature, lack comparability between studies. Recently, Luise et al. (2019b) provided a highly detailed review of ETEC models, suggesting use of both clinical and ETEC specific biomarkers for the analysis of ETEC infection. The current study follows these suggestions, recording ETEC infection through enumeration of faecal ETEC shedding and the presence of diarrhoea through FCS. Bacterial quantification offers accurate and comparable data, removing subjectivity from traditional bacterial scoring

methods. However, this method can be highly laborious and being time sensitive, can be unachievable for large sample numbers during these already labour-intensive *in vivo* models. Implementation of high-through put robotic platforms, such as the RASP, can greatly reduce the labour costs associated with bacterial quantification. Accurate measurement of ETEC infection through methods including ETEC quantification is vital to analysing the effects of alternative ETEC control strategies in *in vivo* models.

In conclusion, this study demonstrates that the faecal microbiome is modified in ETEC-challenged weaner pigs supplemented with the combination of LFP and SFP, with these modifications previously associated with increased growth performance and health status in swine. Pigs receiving this combination of postbiotics also demonstrated an increased final liveweight, indicating that management of ETEC associated performance loss may not require the complete removal of ETEC from a production system.

3.6 Supplementary

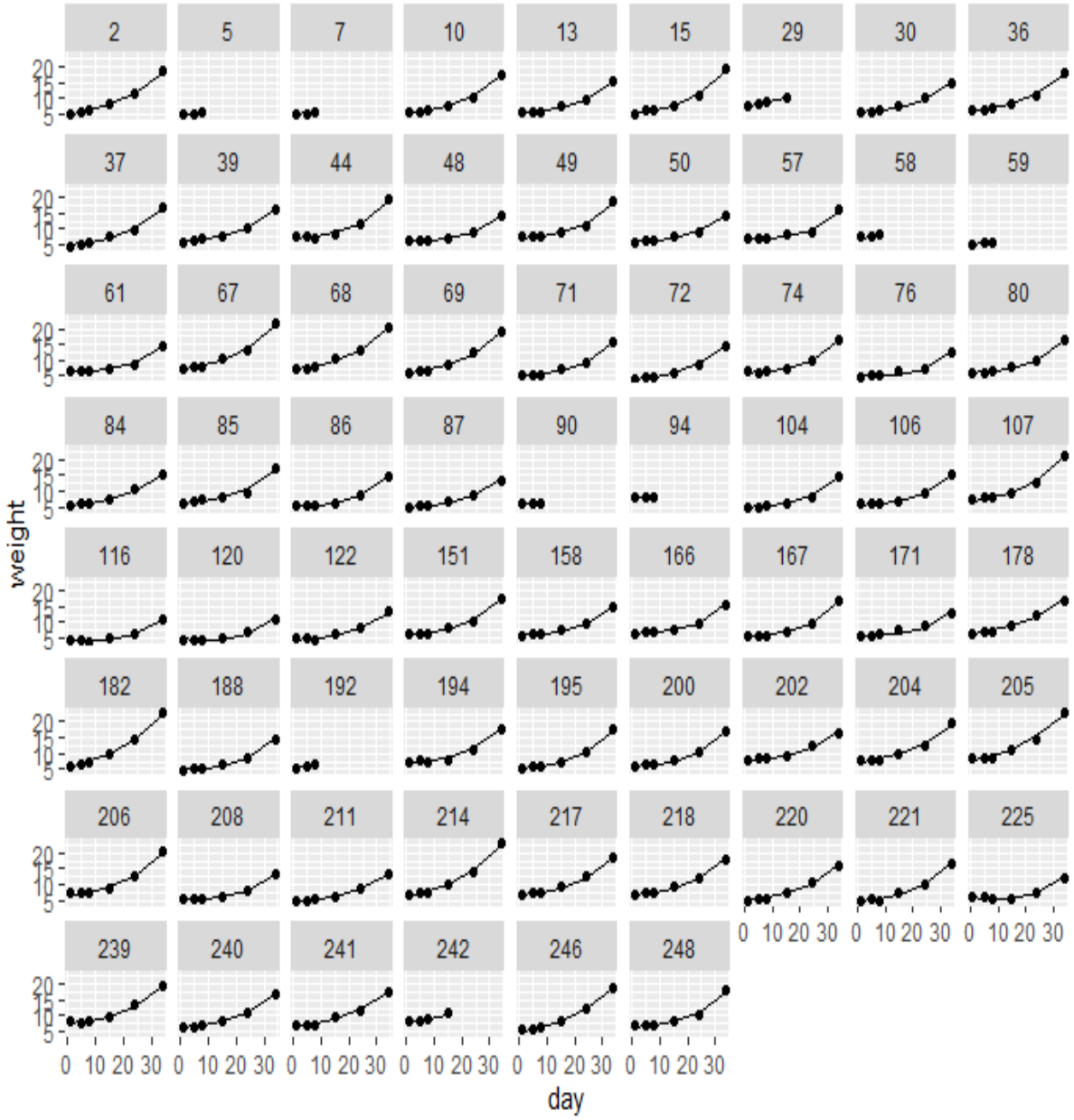

Figure 17 - Supplementary. Liveweight of individual pigs in ETEC-challenge trial. Pigs removed from trial prior to ETEC-challenge are not represented.

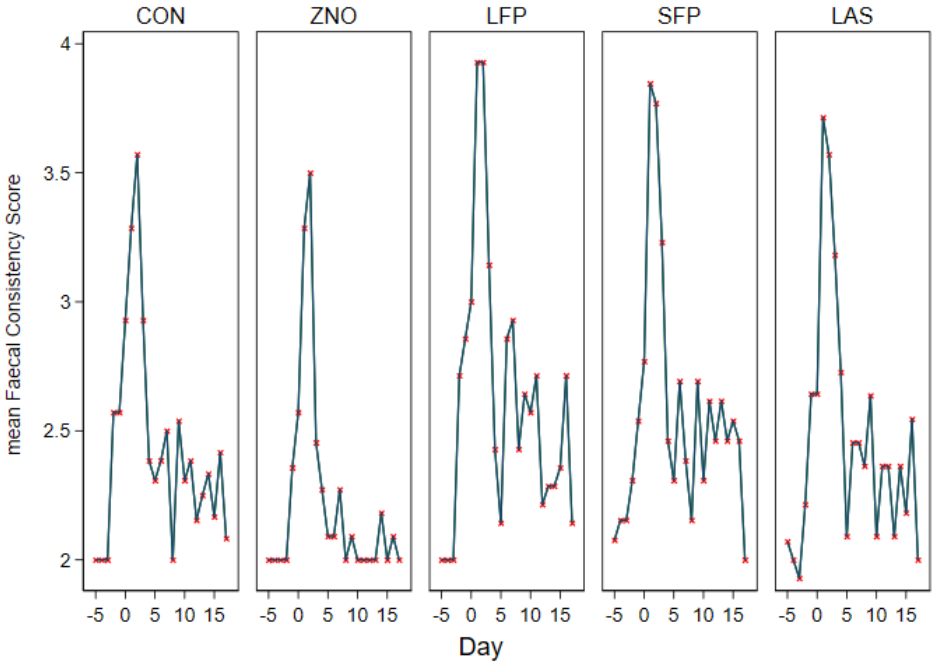

Figure 18 - Supplementary. Faecal consistency scores (FCS) of ETEC-challenged weaners belonging to different treatment groups over time. Treatment abbreviations: CON control base diet, ZNO = base + 3,000 ppm zinc oxide, LFP = base + 2,000 ppm *Lactobacillus acidophilus* fermentation product (LFP), SFP = base + 2,000 ppm *Sacchromyces cerevisiae* fermentation product (SFP), LAS = base + 2,000 ppm LFP + 2,000 ppm SFP.

Table 7 - Supplementary. Effects of postbiotics on average daily gain of F4-ETEC challenged weaner pigs.

	Week 1	Week 2	Week 3	Week 4
CON	63.3	169.0	347.9	601.5
ZNO	67.9	232.9	358.7	556.3
LFP	58.6	201.9	379.8	626.9
SFP	51.4	186.6	317.1	689.7

LAS	60.2	261.6	380.1	696.6
Overall	60.4	207.9	357.1	634.2

CON control base diet, ZNO = base + 3,000 ppm zinc oxide, LFP = base + 2,000 ppm *Lactobacillus acidophilus* fermentation product (LFP), SFP = base + 2,000 ppm *Sacchromyces cerevisiae* fermentation product (SFP), LAS = base + 2,000 ppm LFP + 2,000 ppm SFP.

Chapter 4 – Model to assess strategies in reducing extended-spectrum cephalosporin resistance in pigs: postbiotics effects on resistant *E. coli*

4.1 Abstract

Historic and current methods against antimicrobial resistance (AMR), a major health threat threatening today's society, focus on reducing the emergence of AMR by minimising antimicrobial usage. This is achieved through antimicrobial stewardship and a range of alternative control methods. While these control strategies require continued advancement, strategies that directly aim to reduce or eliminate existing antimicrobial resistant bacteria, specifically bacteria resistant to critically important antimicrobials (CIA), need to be investigated and established within the livestock sector. This study established an *in vivo* model for analysis of such strategies against extended-spectrum cephalosporin (ESC) resistant *E. coli* in weaner pigs. The model consisted of pigs intramuscularly administered a single dose of ceftiofur and orally challenged with ESC-resistant *E. coli* on the two following days. The model was used to evaluate the effects of postbiotics, in the form of *Lactobacillus acidophilus* fermentation products and *Saccharomyces cerevisiae* fermentation products, against ESC-resistant *E. coli*. Successful colonisation of ESC-resistant *E. coli* was detected in weaner pigs using this model with a reduction in ESC-resistant *E. coli* shedding over time. The postbiotics demonstrated no effect on this reduction rate whilst also demonstrating no effect on growth performance of pigs. Overall, this *in vivo* model enables future evaluation of ESC-resistant control strategies whilst increasing our knowledge on the carriage of ESC-resistant *E. coli* in pigs, both pivotal in reducing further dissemination of this One Health threat.

4.2 Introduction

Antimicrobial resistance (AMR) continues to be one of the greatest public health concerns in todays' society, and if no further actions are implemented, AMR is predicted to cause 10 million deaths per year by 2050 (O'Neill, 2016). Of particular concern is the more recent emergence and dissemination of resistance towards critically important antimicrobials (CIAs). Critically important antimicrobials are antimicrobials reserved as the last line of defence in life-threatening human infections with CIA resistance rendering antimicrobial therapy ineffective (WHO, 2019). Bacteria resistant to CIAs, including extended-spectrum cephalosporins (ESCs) and fluoroquinolones, have been detected globally in food-producing animals where the CIA resistance has the risk to spread further within animals and into humans via either direct or indirect transmission (Abraham et al., 2015; Ewers et al., 2012; Mukerji et al., 2017). The presence of this resistance is therefore of a One Health concern and urgently requires action.

ESC-resistant *E. coli* was first detected in food-producing animals in 1996 and despite tightened regulations of ESC usage in food-producing animals, has since been detected globally in swine (Abraham et al., 2015; Bradford et al., 1999; Dahmen et al., 2012). This resistance is predominantly attributed to variants of the $bla_{\text{CTX-M}}$ and bla_{CMY} genes (Ewers et al., 2012). Two factors heightening the One Health threat of this ESC-resistance is the ease of transmission through plasmid carriage, as demonstrated by global presence of the highly transferable IncI1-$bla_{\text{CTX-M-1}}$ plasmid, and its long-term persistence in the absence of direct selection pressures (Abraham et al., 2018; Börjesson et al., 2013; Dahmen et al., 2012). Although many prophylactic approaches have been implemented to reduce the emergence and dissemination of AMR, there are limited strategies directly targeting AMR, specifically ESC-resistance, upon its emergence on farms. Strategies to reduce current AMR prevalence under investigation include bacteriophage therapy, antimicrobial peptides and postbiotics (Skaradzińska et al., 2017)(Laird et. Al unpublished).

Postbiotics, the mixture of bioactive compounds resultant from fermentation of probiotic strains, were reported to reduce levels of antimicrobial resistance (Feye et al., 2016). Feye et al. (2016) observed that broilers challenged with a multi-drug resistant *Salmonella* strain and supplemented with *Saccharomyces cerevisiae* fermentation product (SFP) demonstrated a significant reduction in the percent of *Salmonella* resistant to chloramphenicol as compared to challenged broilers that were not supplemented. Postbiotics have also been reported to increase host health and protection against enteric pathogens through modulation of the gut microflora (Laird et al., 2021a). Contrasting impacts on weight gain have been reported in livestock supplemented with *Lactobacillus acidophilus* fermentation product (LFP) and SFP, with increased growth performance, increased diversity of faecal microflora and reduced pathogen levels in ileal mucosa being reported in swine (Kiarie et al., 2011)(Chapter 3). The effect of postbiotics on reducing levels of CIA-resistant bacteria requires further investigation, as this could potentially minimise the One Health threat of ESC-resistant *E. coli* as well as providing additional host health benefits in swine. Furthermore, models to assess the capacity of alternative strategies to reduce resistance levels in commensal bacteria need to be developed. Whereas many *in vivo* models for establishing disease have been established, the limited studies assessing alternative strategies for their direct impact on AMR are often confined to *in vitro* experiments (Skaradzińska et al., 2017).

We evaluated a novel model assessing the *in vivo* clearance of ESC-resistant *E. coli* in weaner pigs. This model consisted of challenge of pigs with an ESC-resistant *E. coli* (commensal strain) and application of the high-throughput Robotic Antimicrobial Susceptibility Platform (RASP) for quantification of feacal shedding of ESC-resistant *E. coli* in individual pigs at multiple timepoints. We hypothesised that in weaner pigs challenged with ESC-resistant *E. coli,* those receiving feed supplemented with LFP or SFP, alone or in combination, would demonstrate superior clearance of ESC-resistant *E. coli* to those not receiving the supplement.

4.3 Materials and methods

This experiment was approved by the Animal Ethics Committee of Murdoch University (R3181/19).

4.3.1 Animals, housing and experimental design

The treatment groups consisted of the control diet (CON), diet supplemented with 2000 ppm LFP (LFP), diet supplemented with 2000 ppm SFP (SFP), and diet supplemented with the combination of 2000 ppm of LFP and 2000 ppm SFP (LAS). Fermentation products were Diamond V SynGenX™ and Diamond V Original XPC™ for LFP and SFP, respectively.

Treatment groups were allocated to pens by a randomised block design with four replicate pens of each treatment. The 64 piglets were weaned at 21 days of age and moved from a commercial piggery in Western Australia to the animal housing facility at Murdoch University. Allocation of pigs to pens was based on weight with each pen housing 4 pigs. All pens were equipped with a 5-space feeder, a nipple drinker, plastic bottles for enrichment and were constructed of metal with plastic flooring. Pigs received feed and water ad libitum. Pigs were weighed on arrival with pigs and feed weighed on day 0, 7, 14, 21 and 28 for determination of weight gain and feed intake.

4.3.2 ESC-resistant *E. coli* inoculation

All pigs were treated with 25mg (50mg/mL) ceftiofur on day -1 to ensure colonisation with ESC-resistant *E. coli* upon inoculation. Pigs were inoculated with ESC-resistant *E. coli* strain SA13 (Abraham et al., 2018) on day 0 and 1 using gelatin capsules for delivery. This was prepared as described by Sterndale et al. (2019c) with the exception of the original strain grown on CHROMagar™ ESBL (MicroMedia, Edwards Group) and a single, blue colony picked. On days one and two of dosing, pigs received 2 capsules containing 1.92×10^8 colony forming units (CFU) per capsule.

4.3.3 Faecal sampling and processing

Rectal swabs were collected on days -1, 1, 2, 3, 5, 7, 14, 21 and 28. The average weight of the swab end was calculated by cutting off the swab end of ten clean swabs at the same point on and averaging the weight. All sample swabs ends were cut, weighed and then suspended in 15 mL centrifuge tubes containing 5 mL of PBS.

4.3.4 RASP quantification

These samples were then placed onto the RASP for quantification of ESC-resistant *E. coli* and total *E. coli* using CHROMagar™ ESBL and CHROMagar™ ECC (MicroMedia, Edwards Group) agar plates, respectively, as previously described (Laird et al., 2021b; Truswell et al., 2021) (Chapter 3). Briefly, samples were diluted to the expected concentrations with two dilutions plated onto each agar plate using dual spiral plating (Figure 19). Agar plates were incubated at 37 °C for 18 hours and placed back onto the RASP system for imaging of plates. Repeated plating at different dilutions was completed if too little or too many colonies were present, with quantification determined from repeated plates. Colonies were counted manually with distinction between species based on colour of colonies on chromogenic agar.

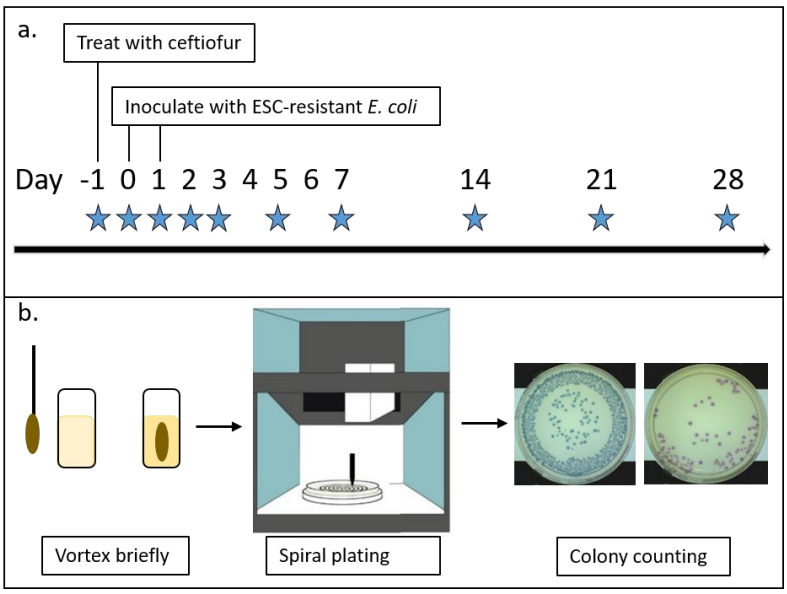

Figure 19. Overview of in vivo model for assessing effect of novel strategies on extended-spectrum cephalosporin resistant *E. coli*. a. Timeline of treatments and sampling of pigs. Blue stars represent days rectal swabs were collected. b. Laboratory processing of samples for quantification of total putative *E. coli* and extended-spectrum cephalosporin resistant *E. coli* from rectal swabs (calculated as CFU/g). Blue colonies are putative *E. coli* on CHROMagar™ ECC agar while pink colonies are putative ESC-resistant *E. coli* on CHROMagar™ ESBL agar.

4.3.5 Statistical analysis

Statistical analysis and graphing were conducted using STATA (v15.1) and R Studio (v1.2.5033). Liveweight (kg) of pigs from Day -7 to 28 was first analysed descriptively to assess the form of temporal trends and generalised additive models were used to fit smoothing splines to non-linear trends initially with pigs, pens and rooms as random effects in a full model. Simpler models were assessed for suitability based on the Akaike information criteria and the final model used to produce estimates of the mean effect of diet on liveweight through the

experimental period with 95% confidence intervals relied on for interpreting the impact of sampling error on differences.

Bacterial quantification was log transformed with a multilevel mixed effects linear regression used to analyse ESC-resistant *E. coli* and total *E. coli* shedding across time, termed ESC and ECC shedding density, respectively.

4.4 Results

4.4.1 ESC-resistant *E. coli* Quantification

No ESC-resistant *E. coli* was detected prior to challenge with the ESC-resistant *E. coli* strain. The concentration of ESC-resistant *E. coli* was highest on Day 1 (24 hours after first ESC-resistant *E. coli* inoculation) across all treatment groups (Figure 20). This was lowest in the LAS group at 3.4 $\log_{10}$ CFU/g in comparison to 4.0, 4.1 and 4.1 $\log_{10}$ CFU/g, in the SFP, LFP and control groups, respectively. A second peak in ESC-resistant *E. coli* was detected in all groups occurring on day 7 for the LAS and SFP groups and day 14 for the LFP and control groups. At the final time point, the concentration of ESC-resistant *E. coli* ranged from 0.1 $\log_{10}$ CFU/g in the SFP group to 0.2, 0.4 and 0.5 $\log_{10}$ CFU/g in the control, LAS and SFP groups, respectively.

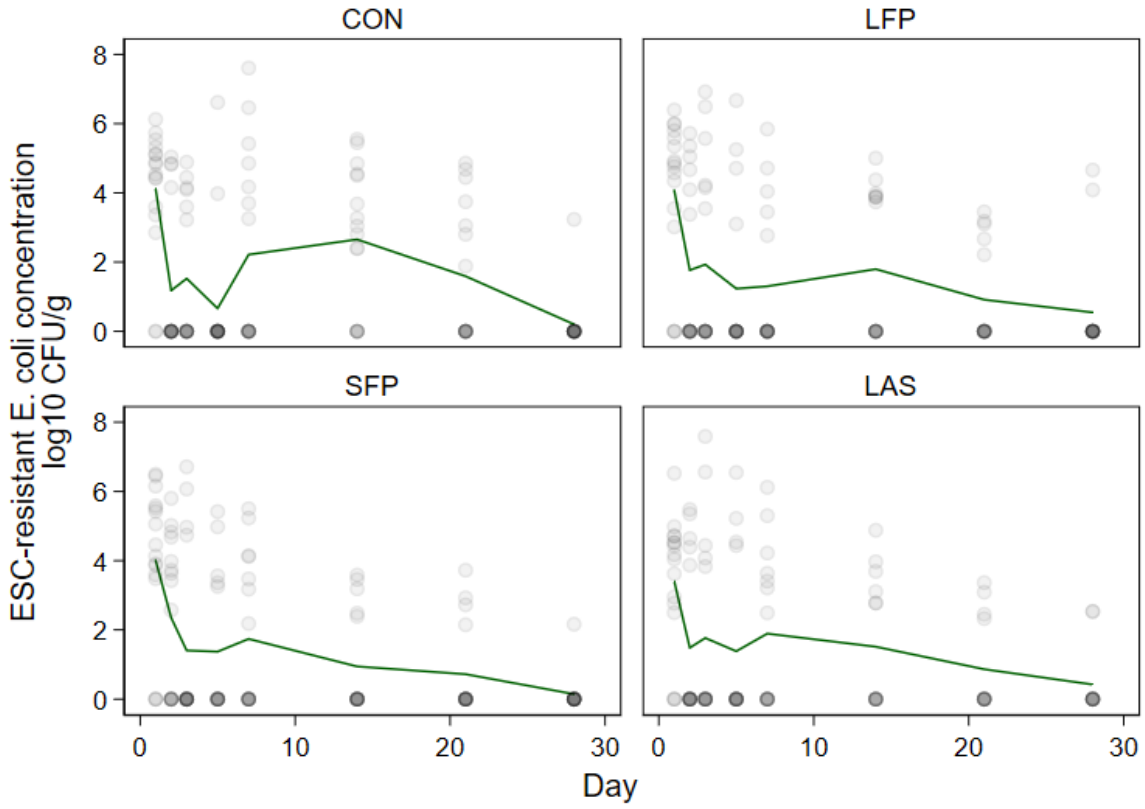

Figure 20. Mean concentration of extended-spectrum cephalosporin resistant *E. coli* in pig faeces (n=64) belonging to different treatment groups. Mean concentration is represented by the line with dots representing individual pigs. Line represents mean with individual pigs represented by dots. Treatment abbreviations: CON control base diet, LFP = base + 2000 ppm *Lactobacillus acidophilus* fermentation product (LFP), SFP = base + 2000 ppm *Sacchromyces cerevisiae* fermentation product (SFP), LAS = base + 2000 ppm LFP + 2000 ppm SFP.

The bacterial shedding density (a measure of bacterial shedding across the full duration of the trial) was used to statistically analyse the shedding of ESC-resistant *E. coli* and total *E. coli* (Figure 21). The mean ESC shedding density was highest in the control group at 47.3 (30.8, 63.7) compared to 28.7 (12.2, 45.1), 30.6 (14.2, 47.1) and 35.9 (19.5, 52.3) for the SFP, LAS and LFP groups, respectively. Despite the reduction of the ESC shedding density in the SFP

group, no treatments groups demonstrated significant differences compared to the control group. The total *E. coli* shedding was similar between treatment groups with the mean shedding density ranging from 141.9 (123.0, 160.7) in the LAS group to 166.5 (147.7, 185.3) and 173.8 (155.0, 192.6) in the control and LFP groups, respectively.

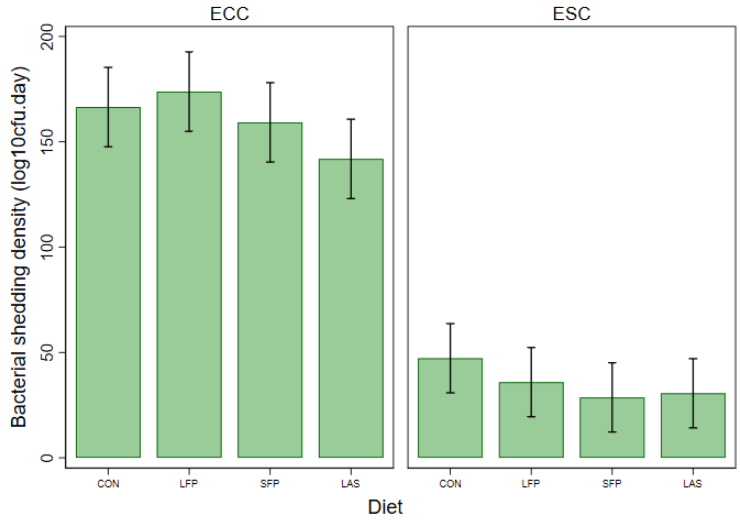

Figure 21. The total putative *E. coli* and extended-spectrum cephalosporin resistant *E. coli* shedding density, a measure of bacterial shedding across the full duration of the trial, in pigs (n=64) belonging to different treatment groups. Error bars are standard error of the means. Treatment abbreviations: CON control base diet, LFP = base + 2000 ppm *Lactobacillus acidophilus* fermentation product (LFP), SFP = base + 2000 ppm *Sacchromyces cerevisiae* fermentation product (SFP), LAS = base + 2000 ppm LFP + 2000 ppm SFP.

The number of pigs with ESC-resistant *E. coli* was highest in all treatment groups on Day 1 (24 hours after the first ESC-resistant *E. coli* inoculation) (Figure 22). This declined by 48 hours post-challenge with only 38% of pigs harbouring ESC-resistant *E. coli* on Day 2. The second peak detected in the quantification of ESC-resistant *E. coli* was also detected in the percent of pigs harbouring ESC-resistant *E. coli* with an increase from 13 to 69% of pigs in the

control group with ESC-resistant *E. coli* on Day 5 and 14, respectively. Over the entirety of the trial, ESC-resistant *E. coli* was undetected in two pigs. Meanwhile, only nine samples had no *E. coli* detected when grown on ECC agar.

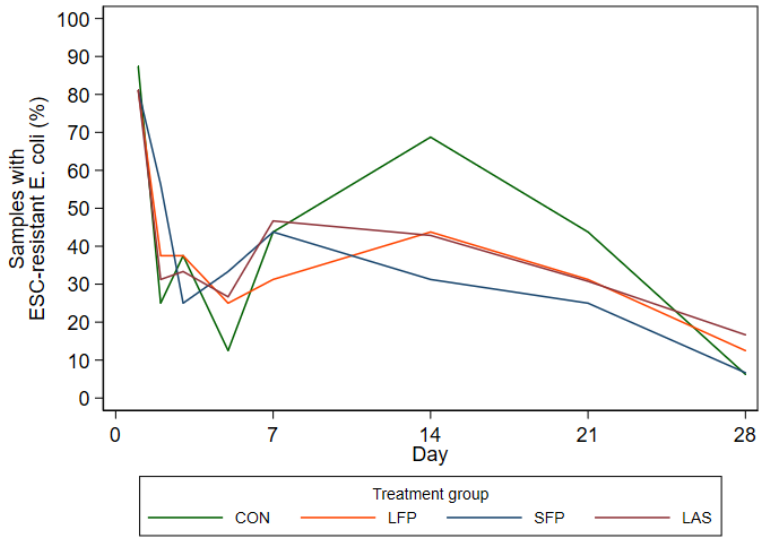

Figure 22. Percent of pigs harbouring ESC-resistant *E. coli* over duration of trial and belonging to different treatment groups. Treatment abbreviations: CON control base diet, LFP = base + 2000 ppm *Lactobacillus acidophilus* fermentation product (LFP), SFP = base + 2000 ppm *Sacchromyces cerevisiae* fermentation product (SFP), LAS = base + 2000 ppm LFP + 2000 ppm SFP.

4.4.2 Abundance of ESC-resistant *E. coli* relative to total *E. coli*

The abundance of ESC-resistant *E. coli* relative to the total *E. coli* population demonstrated similar trends to the ESC-resistant *E. coli* concentration (Figure 23). This was evident in the first peak of abundance of ESC-resistant *E. coli* followed by a second peak on days 7 or 14 depending on the treatment group. The lowest abundance of ESC-resistant *E. coli* occurred on the final day of the trial showing a natural clearance.

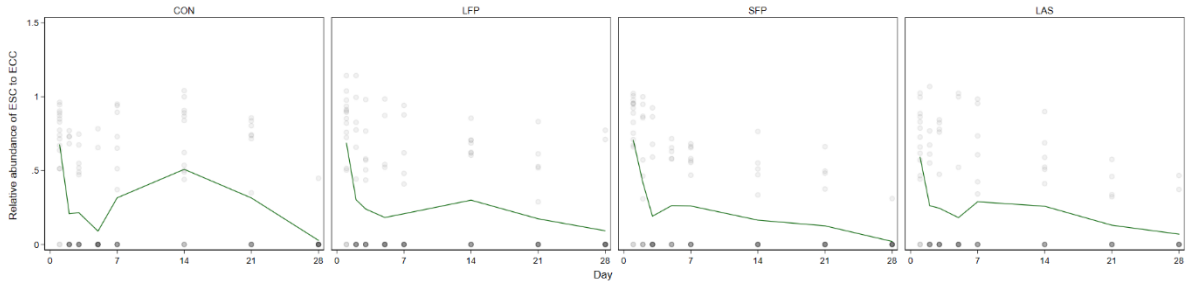

Figure 23. Abundance of ESC-resistant *E. coli* relative to total putative *E. coli* in faeces from ESC-resistant E. coli challenged weaners belonging to different treatment groups. Line represents median with individual pigs represented by dots. Treatment abbreviations: CON control base diet, LFP = base + 2000 ppm *Lactobacillus acidophilus* fermentation product (LFP), SFP = base + 2000 ppm *Sacchromyces cerevisiae* fermentation product (SFP), LAS = base + 2000 ppm LFP + 2000 ppm SFP.

4.4.3 Growth performance

The liveweight of pigs increased over the duration of the study, starting at an average of 6.62 kg across all pigs and reaching an average of 19.58 kg at day 28 (Figure 24). Low variation in liveweight was detected between treatment groups at all timepoints with the largest variation seen at the end of the trial. The SFP and control groups had an increased average liveweight at this timepoint at 20.0 (19.8, 20.2) and 19.8 (19.6, 20.0) kg, respectively. This was compared to 18.8 (18.5, 19.1) and 19.3 (18.1, 19.5) kg in the LAS and LFP groups, respectively. The rate of growth also increased over time with the mean average daily gain (ADG) across all pigs starting at 81.1 grams in week 0 and increasing to 220.4, 409.0, 517.5 and 619.9 grams in weeks 1, 2, 3 and 4, respectively.

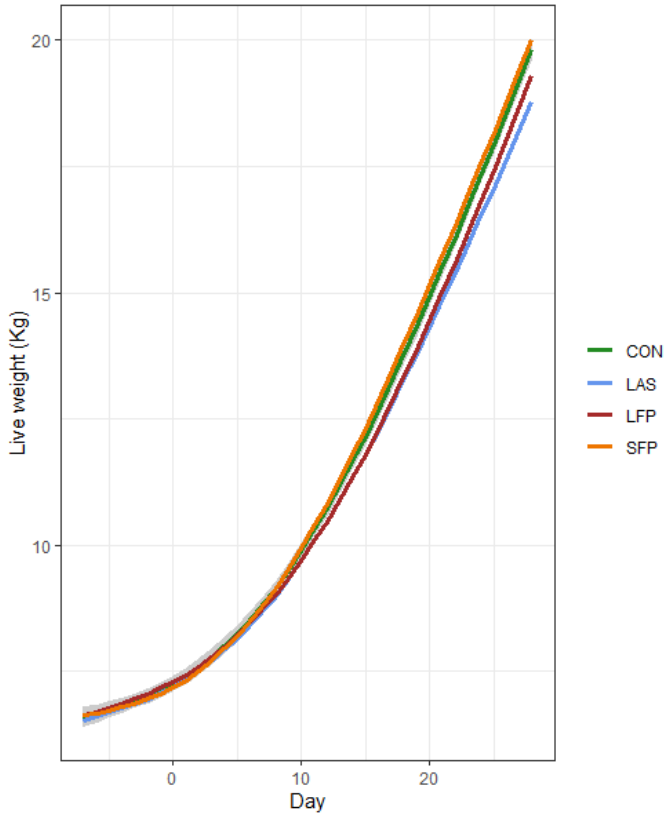

Figure 24. Mean liveweight of pigs (n=64) belonging to different treatment groups in ESC-resistant *E. coli* challenged weaners. Treatment abbreviations: CON = control base diet, LFP = base + 2000 ppm *Lactobacillus acidophilus* fermentation product (LFP), SFP = base + 2000 ppm *Sacchromyces cerevisiae* fermentation product (SFP), LAS = base + 2000 ppm LFP + 2000 ppm SFP.

4.4.4 Feed consumption

The ADFI increased over the duration of the trial with the mean ADFI for all pigs at 123, 279, 509, 731 and 871 grams for week 0, 1, 2, 3 and 4, respectively. The mean ADFI was similar between all treatment groups across weeks 0, 1, 2 and 4 with variation between treatment groups in week 3 (Figure 25). During this week, ADFI ranged from 662 grams in the LAS group to 713, 714 and 835 grams in the and SFP groups, respectively. This increased ADFI in

the SFP group didn't correlate to increased weight gain in pigs with the feed conversion ratio (FCR) at this time point being highest in this group at 1.49. In comparison, the FCR in the control group was the lowest at 1.34 whilst the FCR of the LAS and LFP group was 1.41 and 1.44, respectively. Similar levels of variation in mean FCRs between groups was detected across all weeks with mean FCRs within 0.07, 0.16, 0.10, 0.15 and 0.11 of each other at week 0, 1, 2, 3 and 4, respectively.

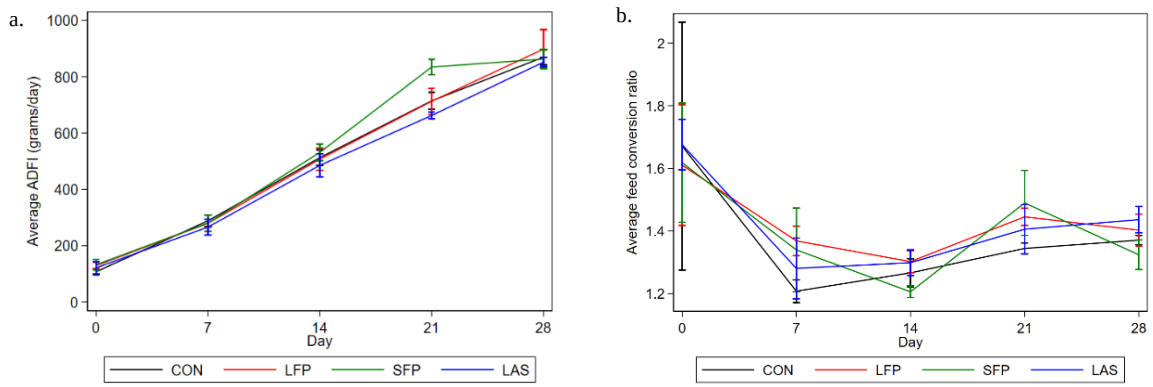

Figure 25. Feed intake analysis of ESC-resistant *E. coli* challenged weaners (n=64) belonging to different treatment groups. a. Average daily feed intake. b. Average feed conversion ratio. Error bars represent standard error of means. Treatment abbreviations: CON control base diet, LFP = base + 2000 ppm *Lactobacillus acidophilus* fermentation product (LFP), SFP = base + 2000 ppm *Sacchromyces cerevisiae* fermentation product (SFP), LAS = base + 2000 ppm LFP + 2000 ppm SFP.

4.5 Discussion

The introduction of human and bird isolates has recently been identified as another potential source of CIA resistance on swine farms (Mukerji et al., 2019; Sahibzada et al., 2017). Although many approaches to minimise the emergence of antimicrobial resistant bacteria have been established, sources including wild birds are currently and may remain uncontrolled.

Furthermore, there is a lack of strategies to decolonise CIA resistant bacteria or reduce carriage of CIA-resistant bacteria in food producing animals once detected. The development of these strategies is necessary in minimising the risk of this resistance spreading between animals, farms and to humans. This study tested an *in vivo* model to analyse the effects of strategies on reducing ESC-resistant *E. coli* in weaner pigs, whilst determining the effects of postbiotics in the form of LFP, SFP and the combination on ESC-resistant *E. coli* shedding. Analysis of postbiotics demonstrated no significant reduction in ESC-resistant *E. coli* and no improvement to growth performance in weaner pigs.

The *in vivo* analysis of strategies for decolonisation of ESC-resistant *E. coli* is dependent on a controlled colonisation of ESC-resistant *E. coli* within the gastrointestinal tract. An ideal model would demonstrate a pro-longed carriage of the resistant bacteria and detection of ESC-resistant *E. coli* across multiple days. The model used in the current study demonstrated success in inducing the shedding of ESC-resistant *E. coli*. This was demonstrated in an initial increase in the shedding of ESC-resistant *E. coli* 24 hours after challenge however, this concentration reduced by approximately 2 logs by 48 hours post-challenge. This small timeframe in high levels of ESC-resistant *E. coli* may hinder identifying strategies to control resistant *E. coli*, with an ideal model invoking high shedding for multiple days after challenge. Whilst this model provided insight into the dynamics of ESC-resistance, repeated treatment with ceftiofur or the use of an antimicrobial with longer duration of activity in future trials may prolong the shedding of high levels of ESC-resistant *E. coli*. Despite this short duration in high levels of ESC-resistant *E. coli* shedding, the persistence of low concentrations of ESC-resistant *E. coli* was also demonstrated in this study with 10% of pigs still harbouring ESC-resistant *E. coli* 28 days after challenge. Whilst this was at low concentrations (between 10^3 and 10^4 CFU/g) this persistence supports previous studies (Abraham et al., 2018) and highlights the importance in reducing, and ultimately eliminating ESC-resistant *E. coli*. Another point to consider in this

model is the use of swabs for bacterial quantification instead of faecal samples. Although collection of faecal samples is ideal and arguably more accurate than swabs, collection of freshly defecated faeces from 64 individual weaner pigs at multiple timepoints was considered unachievable due to time constraints. This was due to previous experience in the low occurrence of defecation from weaner pigs. In the current study, no *E. coli* was detected in only 1.6% of faecal swabs supporting the use of swabs for sampling. However, comparison between the concentration of *E. coli* recovered from the swabs and fresh faeces was not performed, with this required for full validation in the use of swabs for faecal collection. An alternative solution would be to collect samples from a proportion of pigs as representatives for that treatment group, however due to the relatively unstable gut microflora at this production stage, this technique would be unreliable in an experimental setting with low numbers of pigs.

ESC-resistant *E. coli* demonstrated a natural reduction over time, although postbiotics demonstrated no effect on the rate of reduction. In a previous study, broilers supplemented with SFP demonstrated a reduction in the virulence and resistance of the challenge *Salmonella* strain. This was attributed to the loss of the SGI1 integron (Feye et al., 2016). Integrons are mobile genetic elements that can move intra- and inter-molecularly, meaning integrons can move within a DNA molecule or between DNA molecules and can insert into chromosomal or plasmid DNA (Bennett, 2008). The resistance gene in the current study was carried on a IncI1-$bla_{\text{CTX-M-1}}$ plasmid. This plasmid is highly transferable, demonstrates long-term persistence in environments lacking direct selection pressures and has been detected globally (Abraham et al., 2018; Börjesson et al., 2013; Dahmen et al., 2012; Wang et al., 2014). These plasmids are also considered to be highly stable due to their conservation of coding regions and their genetic similarity when isolated from across continents. (Abraham et al., 2018). The stability of the plasmid, as well as the different bacterial strain that was used for challenge, may account for the contrasting results between these studies. Whilst the postbiotics didn't reduce ESC-resistant

E. coli in the current study, its previous success needs further exploration. Future studies to determine the effect of postbiotics in reducing antimicrobial resistance should focus on assessing the effects against different ESC-resistant *E. coli* strains, the effects on resistance against different antimicrobials and the use of different postbiotic strains.

Supplementation with postbiotics demonstrated no effect on growth performance. Studies investigating the effects of these postbiotics on growth performance of swine are heavily contradictory. While LFP, SFP and the combination demonstrated increased growth performance in ETEC-challenged weaner pigs (Chapter 3), other studies have demonstrated no significant effect to growth performance (Nordeste et al., 2017). Meanwhile, Bass and Frank (2017) reported an increased average daily gain in healthy weaner pigs in a study conducted by a feed company. This study contradicts these findings with no improvement in growth performance in pigs supplemented with postbiotics. The beneficial effects of postbiotics have been demonstrated to be through modulation of the gut microbiome, and therefore the efficacy of postbiotics may be dependant on the microbiome of pigs prior to supplementation. In the current study, pigs were treated with ceftiofur before challenge with ESC-resistant *E. coli,* and due to the broad-range nature of antimicrobials, this treatment may have disrupted to the gut microflora whilst also disrupting the effects of the postbiotics. Therefore the variation in the effects of postbiotics on growth performance may be attributed to the variation in the microbiome of pigs as impacted by environmental, host genetic factors and age (Bergamaschi et al., 2020). Heightening our understanding of both postbiotics, the microbiome and their interactive relationship may allow increased consistency between studies and determination of the potential of postbiotics in food-producing animals.

Overall, this study has demonstrated the applicability of an experimental model for analysing the effects on strategies against, but not restricted to, ESC-resistant *E. coli*. While this model can be used to evaluate these strategies through challenge with a single, commensal *E. coli*

strain, natural carriage of *E. coli* is highly diverse with strategies identified as having future potential, requiring further field-based trials (Laird et al., 2021b; Stoesser et al., 2015). Therefore, future field-based trials are required to determine the effects of this postbiotic, and other potential CIA control strategies, on the natural carriage of ESC-resistant *E. coli*. Furthermore, the prevalence and concentration of ESC-resistant *E. coli* also differs between farms (Laird et al., 2021b), potentially affecting postbiotic effects. While postbiotics, in the form of LFP and SFP, didn't affect the carriage of ESC-resistant *E. coli* in weaner pigs, this study describes an *in vivo* model for analysing strategies against ESC-resistant *E. coli*. The continued emergence and dissemination of ESC-resistant *E. coli* in livestock is a major One Health threat with the development of novel strategies that reduce resistance on farms urgently required to prevent its further dissemination.

Chapter 5 – Postbiotic supplementation of weaner pig diets and the effect on antimicrobial resistance carriage

5.1 Abstract

Reducing antimicrobial resistance (AMR) prevalence in food producing animals is essential in managing the associated One Health threat. *Saccharomyces cerevisiae* fermentation products (SFP), categorised as a postbiotic, has been demonstrated to reduce AMR in broilers. This study explores the effect of SFP and *Lactobacillus acidophilus* fermentation products (LFP), alone or in combination, on AMR and growth performance in healthy swine in a commercial facility.

High levels of tetracycline, ciprofloxacin, and extended-spectrum cephalosporin (ESC) resistant *E. coli* were detected at weaning before demonstrating a natural decline over the four-week period. The postbiotic supplementation had no effect on the rate of this reduction whilst also demonstrating no effect on growth performance of the weaner pigs.

5.2 Introduction

Antimicrobial resistant (AMR) bacteria continues to be a significant One Health threat faced by today's society (O'Neill, 2016). Due to selection pressures imposed by the use of antimicrobials, investigation into alternative control strategies is necessary. Postbiotics, the fermentation products of probiotic microorganism strains, have been reported to confer host health benefits and protection against pathogenic bacteria in food-producing animals (Laird et al., 2021a). Furthermore, *Lactobacillus acidophilus* fermentation products (LFP) and *Saccharomyces cerevisiae* fermentation products (SFP) have also demonstrated potential in reducing antimicrobial resistance levels in food-producing animals (Feye et al., 2016) (Chapter 3).

This study provides a detailed examination of the dynamics of resistant *E. coli* carriage in weaner pigs using the Robotics Antimicrobial Susceptibility Platform (RASP) (Truswell et al., 2021) (Chapter 3) to quantify extended-spectrum cephalosporin (ESC), ciprofloxacin and tetracycline-resistant *E. coli*. Additionally, the effects of the postbiotics on both carriage and growth performance were analysed based on the hypothesis that these feed additives would reduce carriage of antimicrobial resistant *E. coli* whilst increasing weight gain in pigs.

5.3 Materials and methods

5.3.1 Animals, housing and experimental design.

This experiment was approved by the Animal Ethics Committee of Murdoch University (R3251/20). Pigs were weaned at 21 days of age on a commercial piggery in Western Australia. The 1280 pigs were moved to pens with allocation based on gender and each pen housing 40 pigs. The four treatment groups consisted of the control diet (CON), diet supplemented with 1000 ppm LFP (LFP), diet supplemented with 1250 ppm SFP (SFP), and diet supplemented with the combination of 1000 ppm of LFP and 1250 ppm SFP (LAS). Feed additives were sourced from Feedworks with Diamond V SynGenX™ and Diamond V Original XPC™ used for the LFP and SFP, respectively. Treatment groups were allocated to pens using a randomised block design with eight replicate pens of each treatment. All pens were equipped with a feeder and nipple drinker and were constructed of metal with plastic grated flooring. The pigs received feed and water ad libitum.

Pens were weighed collectively on day 0, 14 and 25. The number of pigs were confirmed during weighing and average pig weight calculated. Feed consumption was measured on day 0, 14 and 25.

5.3.2 Faecal sampling and processing.

Fresh faecal samples were taken from ten pigs per pen on days 0, 7, 14, 21 and 25. Six faecal samples were randomly selected for AMR screening. One gram of faeces and 19mL of PBS buffer were placed in a stomacher machine on high for 30 seconds. The contents were filtered upon pouring into sterile centrifuge tubes with tubes then placed onto the RASP as described in Chapter 3. Dilutions required for plating were estimated, and then performed with two dilutions plated onto each plate (

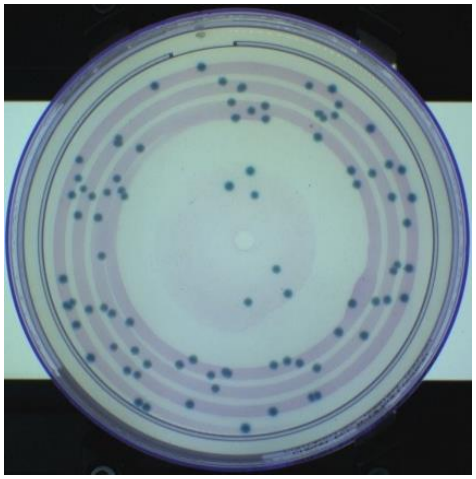

Figure 26). Each sample was plated onto four different agar plates produced by MicroMedia, Edwards Group: CHROMagar™ ECC; CHROMagar™ ECC with 16 µg/mL tetracycline; CHROMagar™ ECC with 4 µg/mL ciprofloxacin; CHROMagar™ ESBL. All media was incubated at 37°C for 18 hours. Plates were placed onto the robotic system for imaging and a manually assisted count of colonies was taken according to colony morphology and colour. If the dilutions plated did not result in single colonies, samples were replated using calculated dilutions.

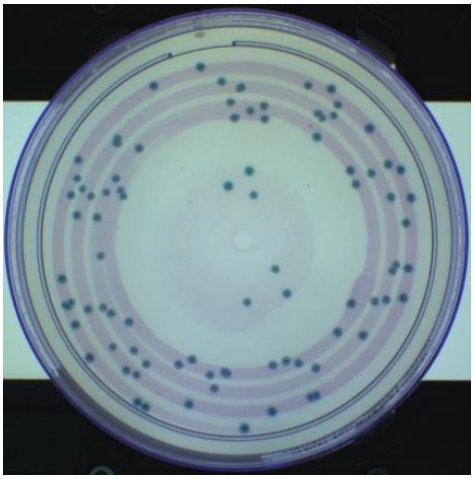

Figure 26. Spiral plating of sample at two different dilutions onto CHROMagar™ ECC with 4 µg/mL ciprofloxacin. Blue colonies represent putative *E. coli*. The outer circle contains the undiluted sample with the inner circle diluted 1 in 10.

5.3.3 Statistical analysis.

Graphing was conducted using STATA (v15.1).

5.4 Results

Carriage of *E. coli* was highest on day 0 across all treatment groups and *E. coli* types (Figure 27). The mean total *E. coli* concentration within treatment groups ranged from 7.3 to 7.6 $\log_{10}$ CFU/g on day 0 decreasing to between 5.0 and 5.3 $\log_{10}$ CFU/g on day 25. A reduction in all resistant *E. coli* was also demonstrated in all treatment and control groups over time, decreasing by 2.6, 2.9 and 4.3 $\log_{10}$ CFU/g over the 25 days for tetracycline, ESC, and ciprofloxacin-resistant *E. coli*, respectively.

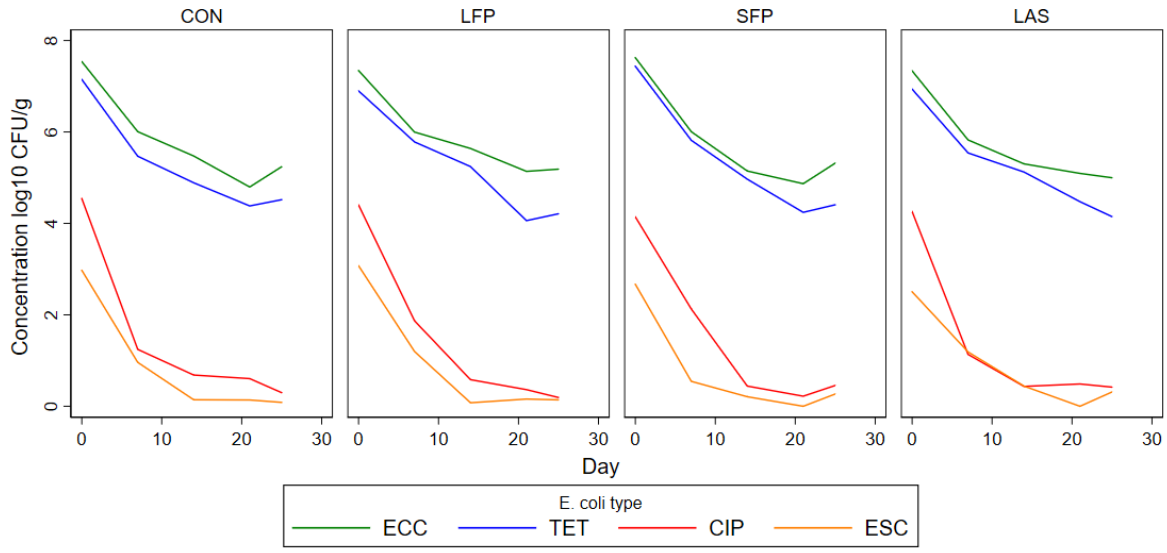

Figure 27. Mean concentration of different types of AMR *E. coli* in swine (n=1280) belonging to different treatment groups in a farm-based trial. E. coli abbreviations: ECC = total *E. coli*, TET = tetracycline resistant *E. coli*, ESC = extended-spectrum cephalosporin-resistant *E. coli*, CIP = ciprofloxacin-resistant *E. coli*. Treatment abbreviations: CON = control base diet, LFP = base + 1000 ppm *Lactobacillus acidophilus* fermentation product (LFP), SFP = base + 1250 ppm *Sacchromyces cerevisiae* fermentation product (SFP), LAS = base + 1000 ppm LFP + 1250 ppm SFP.

5.4.1 Tetracycline resistant *E. coli.*

Tetracycline resistance was the dominant resistance detected for all timepoints and across all treatment groups amongst the antimicrobials tested. The initial concentration showed the greatest variation between treatment groups ranging from 6.9 $\log_{10}$ CFU/g in the LFP group to 7.4 $\log_{10}$ CFU/g in the SFP group. The mean tetracycline-resistant *E. coli* concentration across all pigs at the final timepoint was 4.3 $\log_{10}$ CFU/g demonstrating a 2.8 log reduction over the duration of the trial.

5.4.2 Ciprofloxacin resistant *E. coli.*

Ciprofloxacin-resistant *E. coli* demonstrated the greatest reduction over time with a mean concentration of 4.3 $\log_{10}$ CFU/g on day 0, reducing to 1.6, 0.5, 0.4 and 0.3 $\log_{10}$ CFU/g on day 7, 14, 21 and 25, respectively. The greatest variation in the mean concentration of ciprofloxacin-resistant *E. coli* between treatment groups was detected on day 7. At this timepoint, the LAS group demonstrated one log difference in the mean ciprofloxacin-resistant *E. coli* concentration in comparison to the SFP group. This variation between treatment groups was also demonstrated in the percent of samples with ciprofloxacin-resistant *E. coli* detected (Figure 28). Whilst 29% of pigs belonging to the LAS group demonstrated no ciprofloxacin-resistant *E. coli* on day 7, 52% of pigs in the SFP group harboured ciprofloxacin-resistant *E. coli.* The percent of samples with ciprofloxacin-resistant *E. coli* decreased over time from 92.2% on day 0 to 9.4% on day 25.

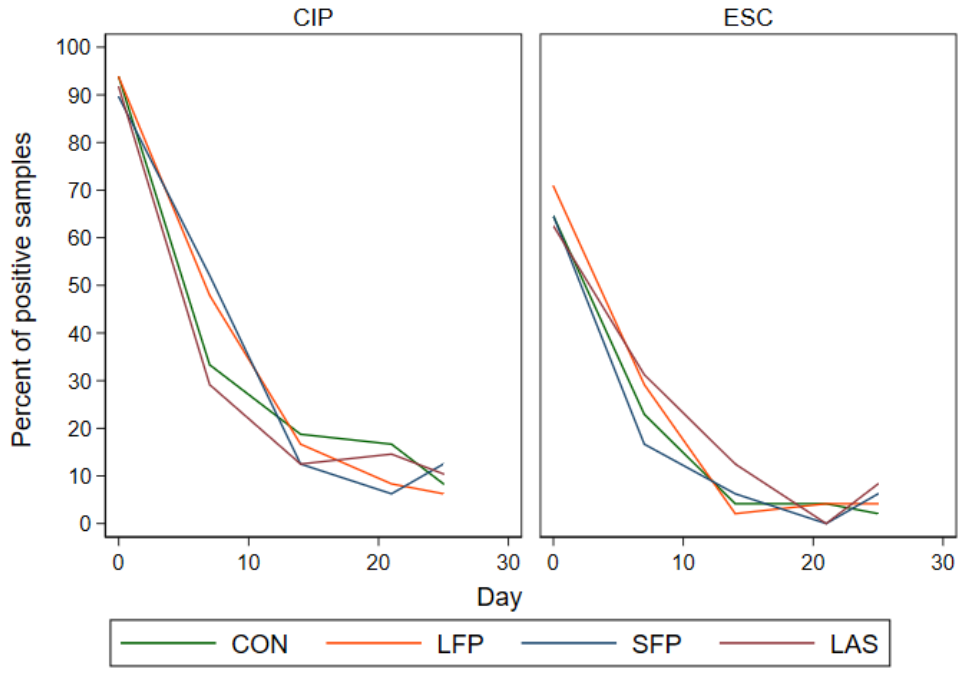

Figure 28. Percent of samples with resistant *E. coli* detected in swine (n=1280) belonging to different treatment groups in a farm-based trial. a. Ciprofloxacin-resistant *E. coli* b. Extended-

spectrum cephalosporin-resistant *E. coli*. Treatment abbreviations: CON = control base diet, LFP = base + 1000 ppm *Lactobacillus acidophilus* fermentation product (LFP), SFP = base + 1250 ppm *Sacchromyces cerevisiae* fermentation product (SFP), LAS = base + 1000 ppm LFP + 1250 ppm SFP.

5.4.3 Extended-spectrum cephalosporin resistant *E. coli.*

ESC-resistant *E. coli* was the least detected resistance across all timepoints, nevertheless demonstrating a natural reduction in concentration over time from a mean concentration of 2.8 $\log_{10}$ CFU/g across all pigs on day 0 to 0.07 $\log_{10}$ CFU/g and 0.2 $\log_{10}$ CFU/g on days 21 and 25, respectively. The percent of samples without ESC-resistant *E. coli* increased over time from 65.6% on day 0 to 2.1 and 5.2% on days 21 and 25, respectively (Figure 28). On day 7, the SFP group demonstrated the lowest mean ESC-resistant *E. coli* concentration at 0.55 $\log_{10}$ CFU/g with the control, LAS and LFP groups demonstrating a 1.75-, 2.18- and 2.20-fold higher concentration, respectively (Figure 27). The SFP group demonstrated the highest percent of samples with no ESC-resistant *E. coli* at this time point at 16.7% compared to 22.9, 29.2 and 31.2% for the control, LFP and LAS groups, respectively. No ESC-resistant *E. coli* was detected in the SFP and LAS groups on day 21.

5.4.4 Growth performance.

The weight of pigs increased in a non-linear relationship over time (Figure 29). The mean weight of pigs on day 0 was 6.22 ± 0.13 standard error of means (SE), 6.25 ± 0.10 (SE), 6.26 ± 0.12 (SE) and 6.29 ± 0.16 (SE) kg in the LFP, LAS, SFP and control groups, respectively. At the end of the trial (day 25), pigs supplemented with SFP demonstrated the highest liveweight at 14.61 ± 0.36 (SE) kg. In comparison, the LAS, control and LFP groups had a mean liveweight of 14.32 ± 0.43 (SE), 14.04 ± 0.58 (SE) and 13.87 ± 0.32 (SE) kg at the final

timepoint, respectively. There was no difference in liveweight detected between treatment groups.

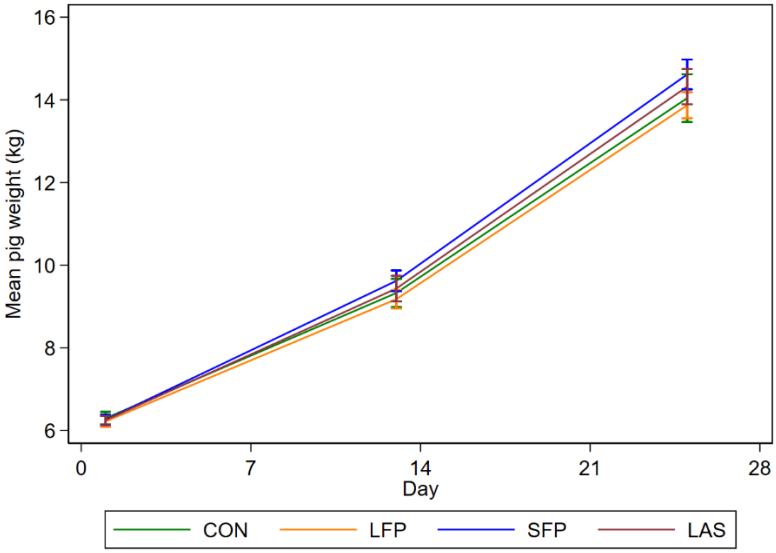

Figure 29. Mean liveweight of pigs (n=1280) over time and belonging to different treatment groups in farm-based trial. Error bars represent standard error of means. Treatment abbreviations: CON = control base diet, LFP = base + 1000 ppm *Lactobacillus acidophilus* fermentation product (LFP), SFP = base + 1250 ppm *Sacchromyces cerevisiae* fermentation product (SFP), LAS = base + 1000 ppm LFP + 1250 ppm SFP.

5.5 Discussion

The global dissemination and increasing prevalence of AMR in food producing animals, including resistance to CIA's, highlights the necessity of continued investigation and development of new strategies to control AMR. In this study, we evaluated the dynamics of antimicrobial resistant *E. coli* carriage and the effects of the postbiotics, LFP, SFP and the combination, on AMR levels in weaner pigs on a commercial facility. The major findings arising from this study are as follows: (i) resistant *E. coli* carriage naturally reduces after

weaning; and (ii) postbiotics had no effect on the faecal concentrations of tetracycline, ciprofloxacin, or ESC-resistant *E. coli* as well as no effect on growth performance of weaner pigs.

High levels of AMR were detected at weaning with 92.2% and 65.6% of pigs harbouring ciprofloxacin-resistant and ESC-resistant *E. coli*, respectively. Whilst resistance to these antimicrobials have previously been detected in Australian swine farms (Abraham et al., 2015), these studies reported significantly lower prevalence rates with ciprofloxacin-resistance in only 1.8-2% of *E. coli* isolates and no detection of ESC-resistant *E. coli* in finisher pigs (Kidsley et al., 2018; Laird et al., 2021b). Furthermore, investigation into the concentration of resistant *E. coli* in faeces from Western Australian finisher pigs (sampled across 2018 and 2019) detected no ESC-resistant *E. coli* whilst ciprofloxacin-resistant *E. coli* was detected at an average of 1 $\log_{10}$ CFU/g (Lee, unpublished). The higher prevalence in the current study may be due to the age of pigs with the prevalence of resistance demonstrated to decrease over the lifespan of pigs (Gaire et al., 2021; Lugsomya et al., 2018). The current study supported this reduction over time with ciprofloxacin-resistant *E. coli* reducing from 4.3 to 0.3 $\log_{10}$ CFU/g over a four-week period. These high levels at weaning are contrary to previous findings in which weaner pigs from a different piggery in Western Australia had no ESC-resistant *E. coli* detected 11 months earlier (Chapter 4). This may be a result of different antimicrobial usage patterns between these piggeries and highlights the importance of stewardship in the pre-weaning period. Reducing AMR prevalence during the nursery stage of production may reduce the risk of AMR disseminating within farms and potentially into pathogenic *E. coli*.

An inadvertent finding, made possible through the use of robotic processing which allowed high level granularity of AMR, was the natural decline in resistant *E. coli* over the weaning period. This reduction was evident in ciprofloxacin-resistant *E. coli* which reduced in concentration by 10^4 CFU/g and reduced from 92.2 to 9.4% of pigs harbouring *E. coli* resistant

to ciprofloxacin. It is possible that this period of decline could be utilised by producers as another AMR management tool, by minimising antimicrobial use and increasing the use of use of probiotics. Whilst resistant *E. coli* levels did demonstrate a significant reduction over the four weeks of the study, this rate of reduction was not affected by the administration of fermentation products. This supports previous findings in which the postbiotics LFP and SFP demonstrated no effect on the carriage of ESC-resistant *E. coli* carriage in swine treated with ceftiofur and challenged with ESC-resistant *E. coli* (Chapter 4). In comparison, this farm-based study examined the effects of postbiotics on the natural AMR carriage of *E. coli*, previously reported to be a highly heterogeneous population of bacteria (Laird et al., 2021b). These studies demonstrate that postbiotics, in the form of LFP and SFP, have no effect on resistant *E. coli* in weaner pigs. This contrasts to the reduced resistance of the *Salmonella* challenge strain reported in broilers supplemented with SFP (Feye et al., 2016). This suggests the postbiotics effects against resistance may be specific to bacterial species or specific to bacterial strains. Contradictory results have also been reported for the impact of LFP on growth performance with inclusion rates of LFP between 500-2000 ppm (Bass and Frank, 2017; Kiarie et al., 2011; Lan et al., 2016)(Chapter 3 and 4). The current study demonstrated no impact of LFP or SFP, as single modalities and combined treatment, on growth performance in healthy weaner pigs. Overall, the contrasting effects of postbiotics may be attributed to genetics of pigs, feed types and farm management practises. Further studies to determine the underlying mechanisms of postbiotics are required, potentially clarifying these contradictory results and identifying variables for their control.

Lastly, this study describes a model for measuring AMR carriage and the analysis of novel AMR control strategies through bacterial quantification. Whereas many studies focus on the presence or absence of resistant *E. coli*, quantification offers a more in-depth and accurate representation of AMR carriage. This enables higher resolution of the effects of novel control

strategies on existing AMR levels with development of strategies that reduce existing AMR urgently required. The continued growth in our understanding of AMR carriage requires parallel development of analytical methods for future studies, demonstrated in this study using the RASP for bacterial quantification.

In conclusion, whereas the postbiotics LFP and SFP demonstrated no effect on reducing the concentration of AMR *E. coli* or growth performance in healthy weaner pigs, this study provided detailed insight into dynamics of AMR *E. coli*. Importantly, the results of this study demonstrate that AMR carriage naturally reduces at weaning, providing a key period to manipulate AMR carriage in the herd.

Chapter 6 – In vitro demonstration of targeted phage therapy and competitive exclusion as a novel strategy for decolonisation of ESC-resistant *E. coli*

6.1 Abstract

Extended-spectrum cephalosporin resistant (ESC-R) *Escherichia coli (E. coli)* have disseminated in food-producing animals globally, attributed to horizontal transmission of *bla*$_{\text{CTX-M}}$ variants as seen in the InCI1-*bla*$_{\text{CTX-M-1}}$ plasmid. This ease of transmission, coupled with its demonstrated long-term persistence, presents a significant One Health antimicrobial resistance (AMR) risk. Bacteriophage (phage) therapy is a potential strategy in eliminating ESC-R *E. coli* in food-producing animals, however is hindered by the development of phage-resistant bacteria and phage biosafety concerns. Another alternative to antimicrobials is probiotics, with this study demonstrating that AMR-free commensal *E. coli*, termed competitive exclusion clones (CECs), can be used to competitively exclude ESC-R *E. coli*. This study isolated and characterised phages that lysed *E. coli* clones harbouring the InCI1-*bla*$_{\text{CTX-M-1}}$ plasmid, before investigation of the effect and synergy of phage therapy and competitive exclusion as a novel strategy for decolonising ESC-resistant *E. coli*. *In vitro* testing demonstrated superiority in the combined therapy, reducing and possibly eliminating ESC-R *E. coli* through phage-mediated lysis coupled with simultaneous prevention of regrowth of phage-resistant mutants due to competitive exclusion with the CEC. Further investigation into this combined therapy *in vivo* is warranted, with on-farm application possibly reducing ESC-R prevalence, whilst constricting newly emergent ESC-R *E. coli* outbreaks prior to their dissemination throughout food-producing animals or humans.

6.2 Importance

The emergence and global dissemination of resistance towards critically important antimicrobials, including extended-spectrum cephalosporins in the livestock sector, deepens the One Health threat of antimicrobial resistance. This resistance has the potential to disseminate to humans, directly or indirectly, nullifying these last lines of defence in life-threatening human infections. This study explores a novel strategy, the co-administration of bacteriophages (phages) and a competitive exclusion clone (antimicrobial susceptible commensal *E. coli*), to revert an antimicrobial resistant population to a susceptible population. Whilst phage therapy is vulnerable to the emergence of phage-resistant bacteria, no phage-resistant bacteria emerged when a competitive exclusion clone was used in combination with the phage. Novel strategies that reduce the prevalence and slow the dissemination of extended-spectrum cephalosporin resistant *E. coli* in food-producing animals, have the potential to extend the timeframe in which antimicrobials remain available for effective use in animal and human health.

6.3 Introduction

Antimicrobial resistance (AMR) has become one of the most pressing issues affecting human and animal health globally (Viens and Littmann, 2015). Without a concerted international effort to control AMR, it is predicted to cause 10 million human deaths per year by 2050, at a cumulative economic loss exceeding $100 trillion US (O'Neill, 2016). One of the most pervasive AMR threats are critically important antimicrobial-resistant (CIA-R) Gram-negative bacteria, especially sepsis-causing pathogens such as *E. coli* resistant to last-line-of-defence human therapies (extended-spectrum cephalosporins [ESCs], fluoroquinolones [FQs], carbapenems, and colistin). Livestock are a potential reservoir for the development and dissemination of CIA resistance which can be transmitted to humans through the food chain or via more diffuse ecological pathways (Abraham et al., 2015).

The emergence of ESC-R *E. coli* in food-producing animals has heightened concern regarding the use of CIAs in food animals and resulted in tightened regulations. Despite these efforts, diverse forms of ESC-resistance in *E. coli* have continued to spread globally (Abraham et al., 2015; Ewers et al., 2012). Concerningly, resistance towards ESCs is predominantly plasmid mediated and commonly attributed to variants of the antimicrobial resistance gene $bla_{\text{CTX-M}}$ (Ewers et al., 2012). Globally, genetically similar and highly transferable IncI1-$bla_{\text{CTX-M-1}}$ plasmids have been detected in genetically diverse *E. coli* from various animal species, including detection in potentially pathogenic *E. coli* lineages (Abraham et al., 2018; Börjesson et al., 2013; Dahmen et al., 2012). Furthermore, multiple studies have demonstrated the long-term persistence of ESC-resistance in environments despite these environments having no direct antimicrobial selection pressures (Abraham et al., 2018; Hansen et al., 2013; Mukerji et al., 2019). These two characteristics increase the One Health risk associated with the spread of ESC-resistance through livestock and to humans.

A significant decrease in CIA resistant bacteria in food-producing animals is required to thwart its dissemination and minimise the associated One Health threat. Whilst antimicrobial stewardship reduces selection pressures on bacteria, development of strategies to de-colonise CIA-resistance upon emergence are necessary. One such potential strategy is the use of lytic bacteriophages (phages). Phage therapy is increasing as a method for treatment of last line resistant infections in immunocompromised human patients (Aslam et al., 2019; Law et al., 2019) and has been investigated in swine for the clearance of pathogenic bacteria (Cha et al., 2012; Seo et al., 2018). Recent studies have also started to pave the way for phage treatment targeting CIA-R bacteria in healthy hosts through the isolation, characterisation and investigation of phages capable of lysing CIA-R bacteria (Bernasconi et al., 2020; Skaradzińska et al., 2017). The advancement of Next Generation Sequencing continues to advance our understanding of phage-bacteria interactions, and through phage genome

annotation can enhance selection of phages for therapeutic use through determining phage life cycle and the presence of any detrimental bacterial genes (Hyman, 2019). However, the main factors that continue to jeopardise the success of phage therapy, both against pathogens and CIA-R bacteria, are the *in vivo* efficacy of phage therapy, the high specificity of phages and the development of phage resistance among target bacteria. The efficacy of phages has been demonstrated to be dependent on the phage processing method, dose schedule, host-neutralisation, and administration method (Laird et al., 2021a; Malik et al., 2017; Melo et al., 2020). Recent studies have begun to optimise these variables evident in the increased retention time of liposome-encapsulated phages throughout the gastrointestinal tract (Malik et al., 2017). Meanwhile, the issue of narrow target range has largely been overcome through the design of phage cocktails; a preparation of multiple phages capable of targeting different bacterial strains (Laird et al., 2021a). Phage cocktails have also been demonstrated to lessen the emergence of phage-resistant bacteria, however the emergence of phage-resistant bacteria continues to occur and hinders the application of this novel CIA-R control method (Bernasconi et al., 2020; Laird et al., 2021a). Whilst phages also have the evolutionary potential to mutate and can potentially regain lytic activity against these mutated bacterial strains, this relies on random activity which is far from ideal for a therapeutic intervention, with an ideal control method preventing or minimising the emergence of phage-resistance.

Another approach worthy of investigation is the potential displacement of resistant bacteria by commensal bacterial strains capable of outcompeting resistant strains in the gastrointestinal tract. This concept, termed probiotics, has also previously been used against pathogenic bacterial strains, reducing the availability of nutrients to pathogens and host cell adhesion sites through competitive exclusion (Angelakis, 2017). The modification of this approach to target CIA-R *E. coli*, using fully susceptible commensal *E. coli* clones capable of outcompeting CIA-R clones, has potential to prevent colonisation by CIA-R bacteria or displace colonised

bacteria. Furthermore, these AMR-free clones, referred to in this context as competitive exclusion clones (CEC) could be selected to possess additional fitness advantages such the ability to produce bacteriocins, offering additional mechanisms for displacement of resistant *E. coli*. Bacteriocins are antimicrobial peptides, incorporating both colicins and microcins, and are produced in response to stressful conditions, targeting and killing *E. coli* strains that lack immunity proteins (Gordon et al., 2006). Whilst *E. coli* CECs may offer a CIA-R control method, a combination approach may have synergistic potential, through initial reduction of the target bacteria due to phage lysis, followed by prevention of re-colonisation due to the competitive exclusion provided by the CEC. Overall, upon emergence of CIA-R bacteria in a herd, specifically in countries with no or low prevalence of CIA-R bacteria, the possibility to revert the bacterial population to a CIA-susceptible population would reduce the risk of transfer to humans through the food chain, ultimately reducing the associated public health risk.

We here show the isolation and genomic characterisation of phages with specific lytic activity against ESC-R *E. coli*, all of which harbour the InCI1-$bla_{\text{CTX-M-1}}$ plasmid and belong to multiple *E. coli* lineages, followed by *in vitro* testing of phage, CEC and the combination, on ESC-R *E. coli* growth (Figure 30). We hypothesised that the combination of phage along with CEC isolates targeting ESC-R *E. coli* would demonstrate greater efficacy in controlling ESC-R *E. coli* growth than either intervention alone, offering a future ESC-R control method in livestock.

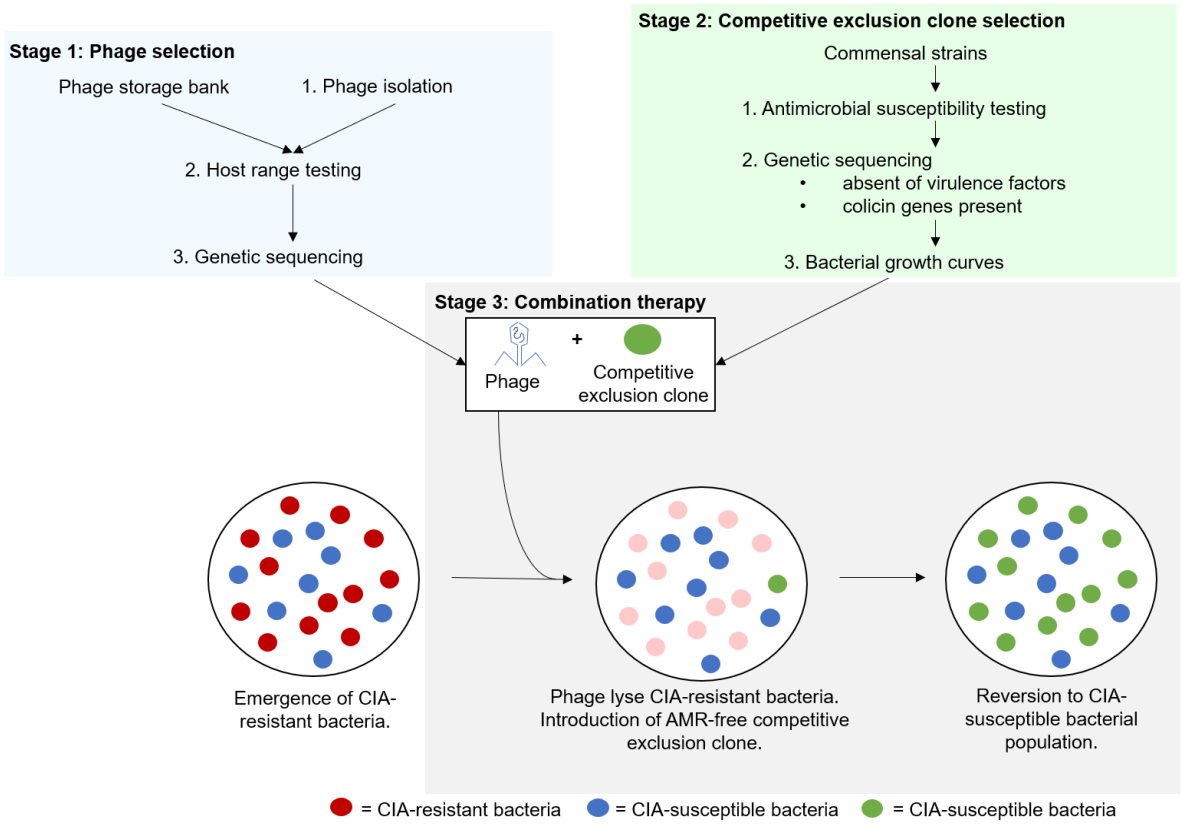

Figure 30. Process for selection of phage and competitive exclusion clone for use as combination therapy for controlling critically important antimicrobial resistant bacteria in livestock. Stage 1 and 2 can occur simultaneously followed by Stage 3 to analyse their combined efficacy.

6.4 Results

6.4.1 Phage isolation, specificity, and characterisation

Swine faecal samples were collected from two distinct locations and screened for phages capable of lysing the ESC-R *E. coli* strain *E.* coli_SA13. Overall, 20 phages targeting *E. coli*_SA13 were isolated from across both locations. The specificity of these phages against 14 ESC-R *E. coli* strains were analysed with the majority of phages (n=16) demonstrating a narrow host range, only capable of lysing the bacterial isolate used for phage isolation (Figure 31). In

comparison, phages 26 and 27 lysed 64.3% (n=9) of the ESC-R *E. coli* tested with phage 30 and 31 lysing 28.6% (n=4) and 14.3% (n=2) ESC-R *E. coli*, respectively. On average, phages isolated from the same environment as the ESC-R *E. coli* were capable of lysing 1.0 ESC-R *E. coli* whilst phages isolated from a separate location lysed 3.5 ± 1.3 SE different ESC-R *E. coli* isolates. These ESC-R *E. coli* belonged to different MLSTs however, all harboured the globally disseminated InCI1-$bla_{\text{CTX-M-1}}$ plasmid. Phages showed high specificity to ESC-R *E. coli* with 15 phages lysing only one ESC susceptible *E. coli* strain from the 44 ESC susceptible *E. coli* tested. These *E. coli* represent commensal and pathogenic strains from multiple hosts, including swine, cattle and companion animals, as well as laboratory strains. The lysed *E. coli* strain, *E. coli* J53, is a sodium azide resistant laboratory strain and laboratory mutant of *E. coli* K-12. No lysis of other bacterial genera or species was detected.

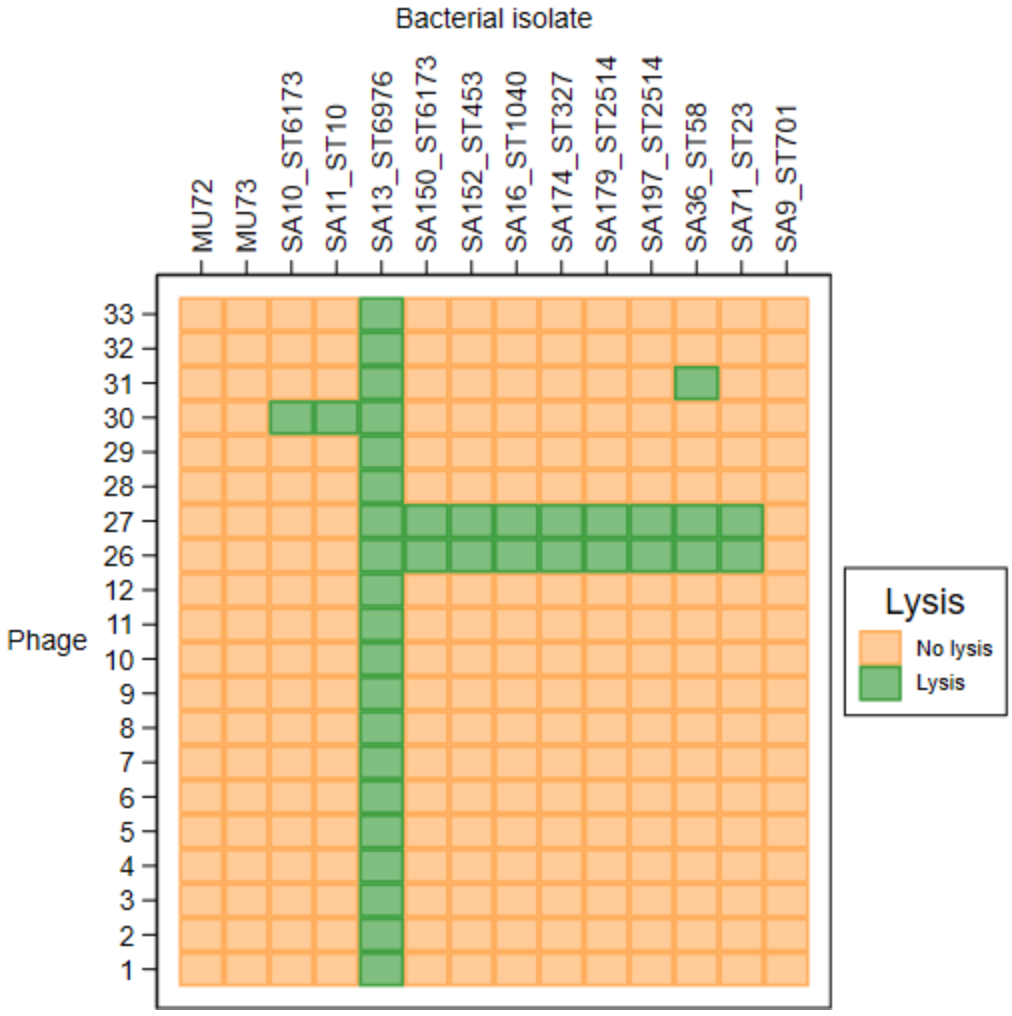

Figure 31. Overview of lytic activity of isolated phages (n=20) against extended-spectrum cephalosporin resistant *E. coli* isolates that harbour bla_{CTXM-1} (n=14). Multi-locus sequence type of *E. coli* are indicated after underscore in isolate name.

All phages were subjected to whole genome sequencing with 19 phage genomes extracted from 17 samples, with two different phages extracted from phage lysate 3 and 4. A single phage belonged to the viral family *Myoviridae* whilst nine belonged to the families *Siphoviridae* and *Drexlerviridae* each. The phages classed in the *Drexlerviridae* viral family were all isolated from location A whilst phages classified as *Siphoviridae* were isolated from both sources. None of the phage genomes contained the lysogenic-associated integrase gene. Phage 26, belonging

to the *Siphoviridae* family, was selected for all further testing based on its broad host range (Figure 31) and lack of lysogenic genes, bacterial virulence genes and antimicrobial resistance genes (Figure 32). The genome of Phage 26 was 42 262 bp in length with a G+C content of 50.4%. Seventy-three open reading frames (ORFs) were identified with annotation of ORFs identifying cell lysis related proteins, structural proteins and DNA metabolism associated proteins.

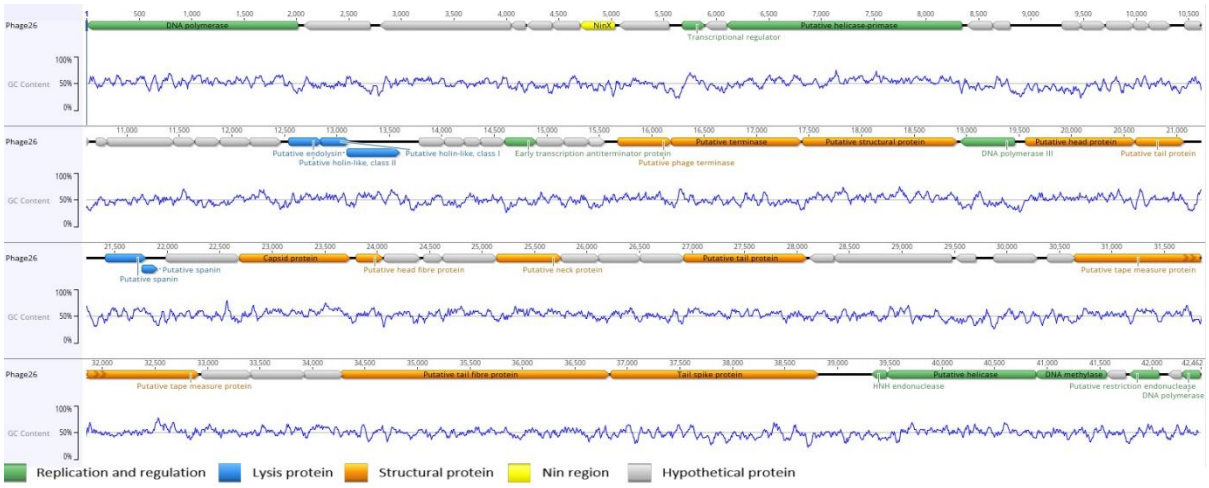

Figure 32. Annotated genome map of Phage 26. The blue line indicates the G+C content. The functional categories and annotation of ORFs are indicated by specific colours. The scale units are base pairs with genome length of 42 262 bp.

6.4.2 Phage assay

The growth of *E. coli*_SA13 was inhibited in the presence of Phage 26 as illustrated in Figure 33. Bacterial growth in cultures inoculated with phage showed an initial increase before declining by the end of the assay. In comparison, non-infected *E. coli*_SA13 concentration increased exponentially before plateauing. The inhibition level of *E. coli*_SA13 was related to phage concentration with inoculum of 30 PFU/mL of Phage 26 demonstrating the highest bacterial growth at T_{210} and the longest time to clear *E. coli*_SA13. In comparison, the ten-fold

increasing phage concentration series demonstrated lower bacterial growth rates and shorter durations to eliminate the ESC-R bacteria with minimal growth of *E. coli*_SA13 detected when inoculated with 3 x 10^6 PFU/mL of Phage 26.

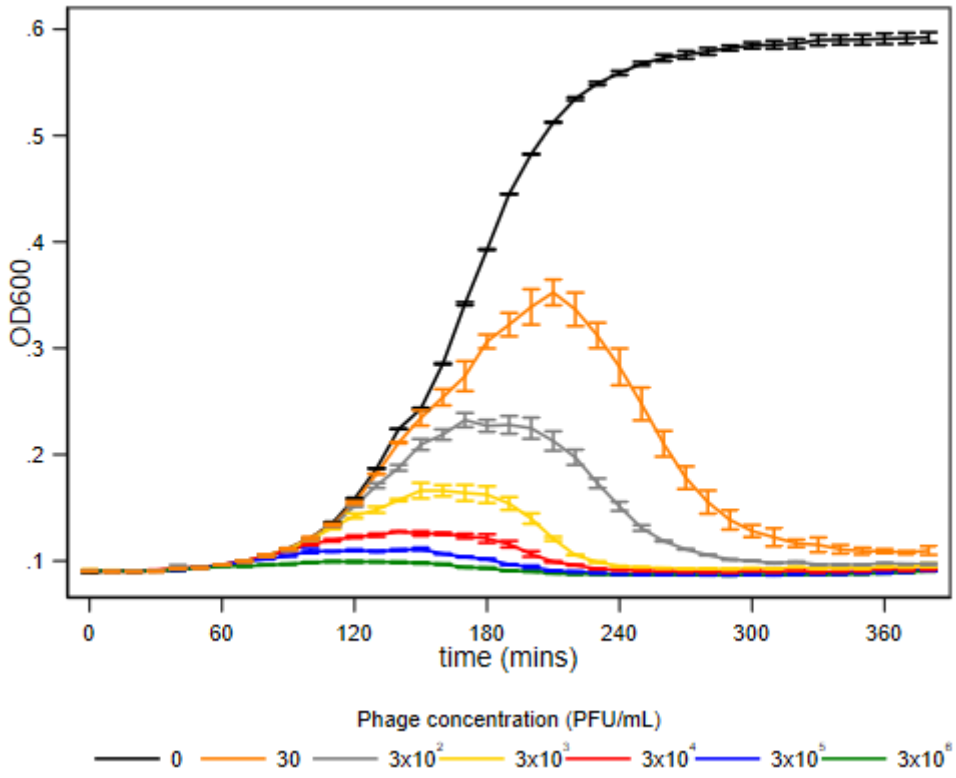

Figure 33. Bacterial growth curve of extended spectrum cephalosporin resistant *E. coli* strain SA13 when inoculated with ten-fold increasing concentrations of Phage 26. Error bars indicate standard error of the means. PFU/mL = Plaque forming units per mL. Experiment was performed in triplicate.

6.4.3 Competitive exclusion clone selection and assay

Selection of *E. coli* strains for use as CECs was based on their antimicrobial susceptibility, absence of known virulence genes, growth rate measures and presence of colicin genes. In a previous study, 800 commensal *E. coli* were isolated from Australian finisher pigs(Laird et al., 2021b) with 80 of these isolates selected for the current study based on antimicrobial

susceptibility data, availability of WGS data and the absence of the *E. coli* virulence genes *eltA, est, stx2e* and *eae*. Growth curves of these 80 isolates were analysed based on the rate of growth between absorbance readings of 0.2 and 0.5 and the concentration reached at plateau (Table 8). Isolate H10 was selected due to high performance in these two metrics whilst B1 was selected as a negative control due to low performance.

Table 8. Growth characteristics and presence of colicin genes of *E. coli* isolates selected for use as competitive exclusion clones.

Isolate	Growth rate (absorbance OD600/minute)	Concentration at plateau (absorbance OD600)	Colicin genes
B1	0.0036	0.59	
F1	0.0051	0.65	*cba, cma*
H10	0.0051	0.66	

Isolates were also screened for colicin and microcin genes with the colicin genes *cba* and *cma* and the microcin gene *mchB* identified in 20 of the potential CEC isolates with all three detected in a single isolate and *cba* and *cma* detected in eight isolates. No colicin genes were detected in Isolate H10 and B1 therefore a third isolate was detected to represent colicin producing strains. The growth curves of isolates with two or more colicin genes were compared with F1 selected for use in combination assays due to its high growth performance. These three selected CECs were tested *in vitro* for culture activity against *E. coli*_SA13. The culture supernatants of each isolate were tested for inhibitory activity against the growth of *E. coli*_SA13. The colicin supernatant from F1 demonstrated the strongest inhibition with ESC-R *E. coli* growth at the final timepoint reduced from an absorbance of 1.28 to 1.07. In comparison, Isolates B1

and H10 demonstrated mild inhibitory activity with both isolates having a final absorbance of 1.19 (Figure 34).

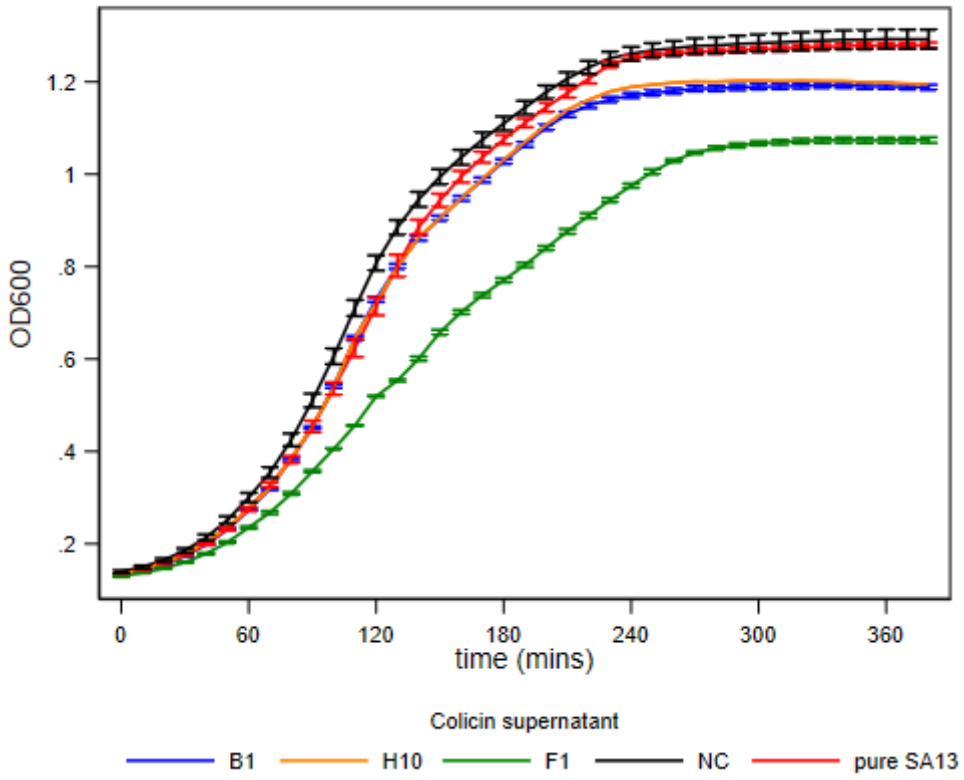

Figure 34. Inhibitory effect of culture supernatants, demonstrating colicin activity, from CECs on the growth of extended spectrum cephalosporin resistant *E. coli* strain SA13. The growth rate of pure ESC-R is illustrated in red. Error bars indicate standard error of the means. NC = Culture supernatant from non-colicin producing strain. Experiment was performed in triplicate.

The effect of selected CECs on the growth of ESC-R *E. coli* when grown in combination was investigated. Overall, all three CECs were demonstrated to reduce the growth of ESC-R *E. coli* (Figure 35). The growth of ESC-R *E. coli* was similar between cultures for the first six hours of the assay with divergence between concentrations first detected at T_{24} and again at T_{48}. The largest reduction in ESC-R *E. coli* concentration at T_{24} was detected when grown in combination with CEC H10 with a concentration of $\log_{10}$ 6.00 CFU/mL compared to the $\log_{10}$ 8.80 CFU/mL when grown as a pure culture. Furthermore, ESC-R *E. coli* concentration was

significantly reduced (p value < 0.001) when grown in combination with each CEC at the final time point (T_{48}), measured to be a reduction of 2.14, 1.74 and 1.39 $\log_{10}$ CFU/mL for CEC F1, B1 and H10, respectively. Competitive exclusion clones F1 and B1 were selected for use in the combination assay with F1 significantly (p value = 0.012) reducing ESC-R concentration compared to H10 at the final time point. The final concentration of the CECs was significantly increased by 0.84 $\log_{10}$ (p value = 0.002) and 0.73 $\log_{10}$ (p value = 0.032) CFU/mL for CEC H10 and CEC F1 when grown in the presence of the ESC-R *E. coli* strain compared to the pure culture, respectively. In comparison, on average the final concentration of CEC B1 was significantly reduced (p value = 0.033) by 1.07 $\log_{10}$ CFU/mL when grown in the mixed culture with ESC-R *E. coli*.

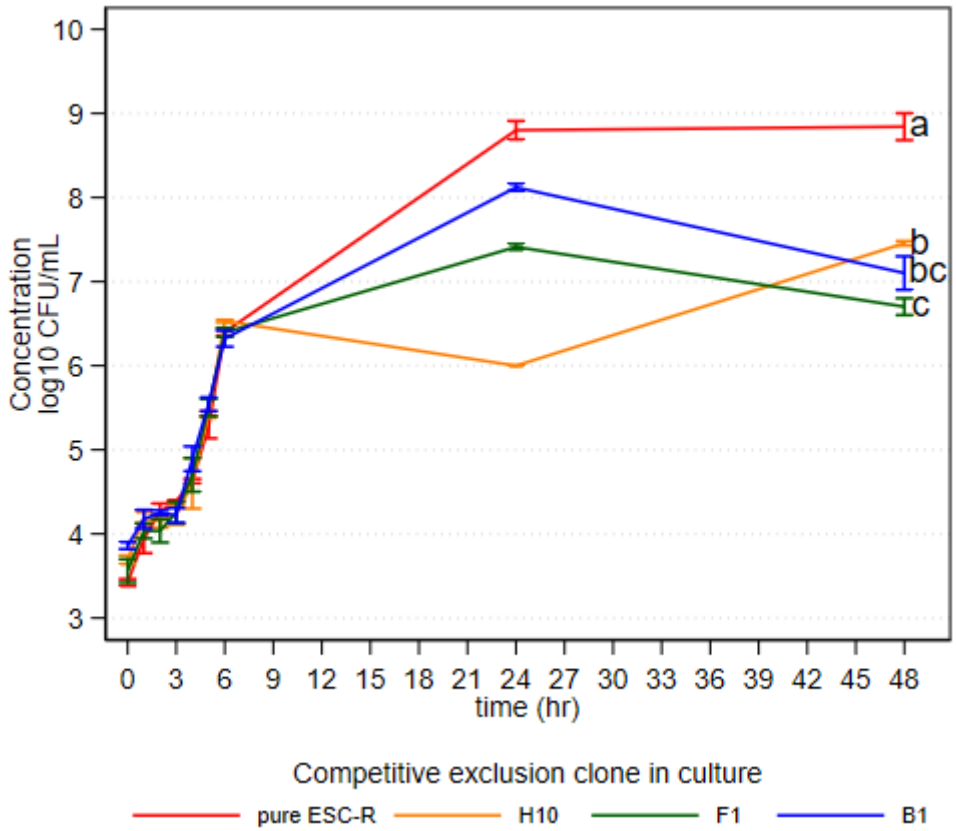

Figure 35. Effect of different *E. coli* competitive exclusion clones (CEC) on the growth of extended spectrum cephalosporin-resistant (ESC-R) *E. coli*. The growth rate of pure ESC-R is illustrated in red. Data points indicate average colony forming units per mL. Error bars indicate standard error of the mean CFU/mL.

6.4.4 Combination assay

The combined effects of phage therapy and CECs were tested against each treatment as a single modality and against a control of pure ESC-R *E. coli* (Figure 36). This was completed using the two different CECs, F1 and B1, due to these two CECs showing the lowest final ESC-R *E. coli* concentration in the above CEC assay. Treatment with CECs alone demonstrated similar results as the above CEC assays with exponential growth of the ESC-R *E. coli* strain for the

initial growth however a reduced concentration at T_{48} when compared to the pure ESC-R *E. coli* growth.

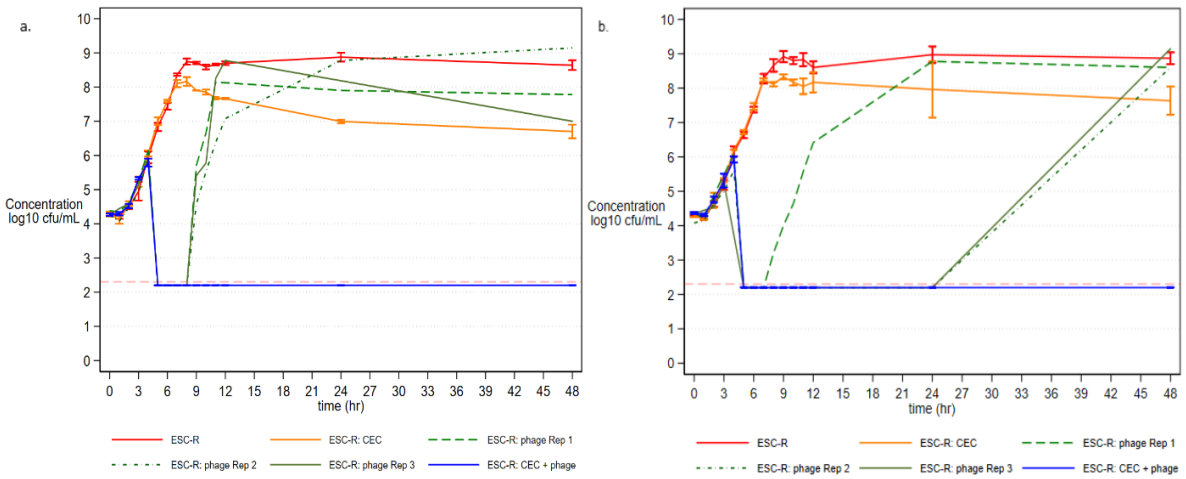

Figure 36. Effect of different methods on the growth of extended spectrum cephalosporin-resistant (ESC-R) *E. coli* in a series of in vitro experiments. Methods analysed were bacteriophages (green), *E. coli* competitive exclusion clones (CEC) (orange) and the combination of bacteriophages and CECs (blue). a. Isolate F1 used as CEC b. Isolate B1 used as CEC. Data points represent average $\log_{10}$ CFU/mL with error bar representing standard error of means with the exception of each biological replicate of phage treated ESC-R growth illustrated for observation of time-point in emergence of phage-resistant ESC-R *E. coli*. Inoculation of Phage 26 occurred at T_3 and T_6. Assay detection limit ($\log^{2.3}$) represented by red dashed line. Experiments were performed in triplicate.

*E. coli*_SA13 was no longer detectable across all replicates two hours after the first inoculation of phage (T_3). The concentration of *E. coli*_SA13 remained below the detection limit for three and four hours in one and three biological replicates, respectively, whilst ESC-R *E. coli* was detected again at the final time point (T_{48}) for two biological replicates. The average ESC-R *E. coli* concentrations returned to within 0.67 and 0.09 $\log_{10}$ CFU/mL of the negative control by the final time point. In comparison to treatment with CEC alone, phage therapy initially

demonstrated superior results through the clearance of *E. coli*_SA13. However, by the end of the assays, a greater reduction of ESC-R *E. coli* was detected when treated with CEC. Furthermore, treatment with both phages and CEC resulted in failure to detect ESC-R *E. coli* (<log 2.3 CFU/mL) by the final timepoint. An initial increase in *E. coli*_SA13 was observed before a sharp decline two hours after administration of Phage 26, similar to when treated with phages alone. However, *E. coli*_SA13 did not re-emerge with no detectable growth for the remainder of the assay. Minimal effects were detected on the concentration of CEC strains in treatments compared to when grown in pure culture, with final concentrations within 0.32 and 0.38 $\log_{10}$ CFU/mL of the concentration of the pure culture for all treatments for F1 and B1, respectively.

6.5 Discussion

In this study, we demonstrate *in vitro*, the efficacy of a promising novel strategy combining lytic phages and CECs for the control of CIA resistance in livestock. These therapies, having two different mechanisms of action demonstrated a synergistic effect when administered in combination. This resulted in the significant reduction (below detectable levels) and possible elimination of ESC-R *E. coli*, and was markedly more effective than when either were administered as single modalities.

Bacteria harbouring resistance towards CIAs have been detected globally in food-producing animals. This emergence has occurred despite the implementation of numerous actions by the industry (Ewers et al., 2012). Furthermore, studies have recently indicated human and bird isolates as sources of CIA resistance on farms, with this avenue of introduction difficult to control (Abraham et al., 2020; Ewers et al., 2012; Mukerji et al., 2019; Sahibzada et al., 2017). Upon emergence of CIA resistant bacteria, the carriage of additional antimicrobial resistance genes allows for co-selection by routinely administered antimicrobials, resulting in the long-

term persistence of CIA resistant bacteria in herds (Abraham et al., 2018). Together these demonstrate that strict regulation of CIA usage cannot be solely relied upon for the control of CIA-R bacteria in food-producing animals. Of particular One Health concern, are globally detected ESC-R *E. coli*, containing the dominant $bla_{\text{CTX-M-1}}$ gene detected within a highly transferable InCI1 plasmid. Development of novel strategies is urgently required to reduce current prevalence and further dissemination of resistance whilst remaining cost-effective for application at the farm level. In the current study, we isolated phages capable of lysing ESC-R *E. coli*. Initial experiments demonstrated greater effects on reducing ESC-R *E. coli* with increasing phage concentration with the highest phage concentration tested, 3×10^6 PFU/mL, demonstrating minimal bacterial growth (Figure 33). While these low phage concentrations demonstrated *in vitro* efficacy, the ideal concentration of phage and the probability of successful phage therapy *in vivo* are dependent on many factors within the complex environment of the gastrointestinal tract. These include phage-microbiome dynamics, gut motility and varying pH levels which may reduce the titre of active phage reaching the target bacterium (Malik et al., 2017; Nilsson, 2019). Therefore, whilst these low phage concentrations have the potential for successful phage therapy in optimum conditions, *in vivo* models commonly dose with increased phage titres ranging from 10^7 to 10^{10} PFU to increase the chances of successful phage therapy.

Bernasconi et al. (2020) also investigated the *in vitro* efficacy of phage therapy for the decolonisation of an ESBL-producing ST131 *E. coli* strain (*E. coli* 4901.28). Whilst repeated doses of the phage cocktail reduced *E. coli* 4901.28 to non-detectable concentrations ($\leq 10^1$ CFU/mL) in the first of two faecal pools tested, a phage-resistant strain emerged in the second experiment returning the ESBL-resistant *E. coli* concentration to $10^{3\text{-}4}$ CFU/mL (Bernasconi et al., 2020). In the current study we observed similar results with an initial reduction of the CIA-R bacteria two hours after inoculation with phage, before phage-resistant strains emerged,

returning ESC-R *E. coli* concentrations to similar levels to the untreated culture. In contrast, simultaneous administration of CECs prevented the re-emergence of CIA-R bacteria through competitive exclusion. These results establish this combined therapy as a promising strategy to modify and modulate the resistant *E. coli* population in herds towards AMR-susceptible population.

Currently, the requirement to access outbreaks of ESC-R *E. coli* for the isolation of phages has biosecurity implications and One Health risks due to the increased potential for dissemination between livestock enterprises. Recent studies have investigated overcoming this biosafety issue by isolating phages from locations separate from the occurrence of the target bacterium (LaVergne et al., 2018; Schooley et al., 2017; Skaradzińska et al., 2017). Skaradzińska et al. (2017) isolated phages from a Polish swine farm capable of lysing a range of *E. coli* resistant to extended spectrum beta-lactams (ESBL) and AmpC beta-lactam from German swine and turkey farms. The current study supports this with phages isolated from a distinct location having an increased host range against the ESC-R *E. coli*; an ideal characteristic for phage therapy. These broad range phages were capable of lysing a diverse range of *E. coli,* with the common target attribute being the presence of the InCI1-$bla_{\text{CTX-M-1}}$ plasmid. The development of phage storage banks, a strategy repeatedly relied upon in extensively drug-resistant bacterial infections in human medicine (LaVergne et al., 2018; Schooley et al., 2017), is another method to overcome the biosecurity concerns. Prior development of phage storage banks and pre-screening of potential CECs coupled with the evolution of robotic platforms for high-throughput screening (Truswell et al., 2021), also ensure timely development of the targeted therapy in response to any future ESC-R emergence. Eliminating this resistance prior to global dissemination, as seen in the InCI1-$bla_{\text{CTX-M-1}}$ plasmid, is an ideal and obtainable scenario.

Genomic sequencing of all phages and CECs prior to addition to storage banks will also reduce response times in developing the target therapy upon emergence of CIA-R bacteria in food-

producing animals. Secondly, WGS ensures therapeutic safety of both phages and CECs prior to administration. Analysis of phage genomes is considered an essential step in phage therapy, guaranteeing the absence of antimicrobial resistance genes and virulence genes whilst ensuring phages follow a strictly lytic life cycle (Hyman, 2019). Increasing publicly available phage sequencing data additionally allows continued research into phage-bacteria interactions and phage diversity (Grose and Casjens, 2014; Hyman, 2019). Software programs such as HostPhinder (Villarroel et al., 2016) and WIsH (Galiez et al., 2017) have begun to capitalise on this increased data collection, with the development of programmes predicting phage host ranges. Whilst current predictions are only 60-80% accurate (Galiez et al., 2017; Hyman, 2019; Villarroel et al., 2016), continued data collection and analysis will likely increase accuracy and aid in selection of phages from phage storage banks upon emergence of resistant bacterial strains on farm. Moreover, performing WGS on potential CECs is also required to ensure bacterial strains do not harbour specific antimicrobial resistance or virulence genes. Additionally, the identification of acquired or lost genes in *E. coli* strains that result in enhanced colonisation in guts of food-producing animals, may optimise selection of strains for use as a CEC (Abraham et al., 2012). This includes the presence of bacteriocins in *E. coli* and would require *in vivo* trials to determine their benefits.

The combination of these existing strategies against AMR, proposed in relation to enterotoxigenic *E. coli* harbouring CIA resistance in pigs (Laird et al., 2021a), offer promising and cost-effective approaches for application in food-animal production. With this industry subject to limited profit margins, economic feasibility of novel AMR strategies, both in cost and ease of administration, is imperative for their continued success. Both phage therapy and probiotics have demonstrated relatively low-economic production costs in intensive production systems with Torres-Acosta et al. (2019) calculating production costs of phage therapy at US$ 0.02 per chicken (Torres-Acosta et al., 2019; van Wagenberg et al., 2020). Meanwhile the

production cost of the combined therapy in this study is approximated at US$ 1.00 with application intended to be through freeze-drying and administration through drinking water or feed. This method for administration removes additional labour costs for repeated administration to individual animals whilst proving successful in previous studies of single modalities (Cha et al., 2012; Luise et al., 2019a). Furthermore, the economic burden of pathogens harbouring AMR may be alleviated through decolonisation of CIA-R bacteria prior to dissemination of CIA-R into pathogenic bacteria. Bacterial infections resistant to CIAs incur additional costs to industry due to extra laboratory tests, use of antimicrobials and the increased rate of mortalities (Laird et al., 2021a). Whilst novel CIA-R control methods will initially be an extra cost, the long-term benefit for the industry has potentially huge economic paybacks through downstream decrease of these AMR-associated costs.

Overall, this study highlights the necessity to continue exploring new avenues for the control of CIA-R bacteria with this combined therapy utilising the two different mechanisms of action of each single modality; the lytic capability of phages and the competitive exclusion of CECs. Further *in vitro* and *in vivo* studies are warranted to determine the efficacy and optimise this novel method in controlling other CIA-R bacteria. This includes evaluation of a phage cocktail in the combined therapy, the *in vivo* growth rate of the selected CEC, and the administration schedule of the combined therapy. Involvement of a phage cocktail would likely result in a broadened host range, and therefore widen the applicability of this novel method. Meanwhile, whilst the CECs used in the current study were originally isolated from swine, their competitive nature may not translate in *in vivo* models. The *E. coli* population within the swine gut has been demonstrated to be highly diverse (Laird et al., 2021b), therefore these CEC's will be competing against multiple *E. coli* lineages instead of a single ESC-R *E. coli*. The dosage and administration schedule would also need to be optimised to ensure the reduction observed in the *in vitro* model is achieved and maintained *in vivo*. Additional studies exploring phage

genetics are also required to further our understanding of phage-bacteria interactions. This study has demonstrated initial success of the *in vitro* efficacy of phages and CECs as a combined therapy for the control of CIA-R bacteria and prevented the occurrence of phage-resistant bacterial strains through competitive exclusion. Investigation of novel CIA-R control methods in food-producing animals is warranted in the battle against AMR, potentially prolonging the use of existing antimicrobials for life-threatening human infections. Combined with the lack of direct selection pressures arising from minimising or ceasing the use of CIAs, novel CIA-R control strategies may minimise the risk of the amplification and propagation of these forms of AMR within and beyond the livestock sector, yielding benefits for One Health.

6.6 Materials and Methods

6.6.1 Study overview

The study into the novel CIA-R control strategy was divided into three main components (Figure 30); (1) the isolation and characterisation of phages, (2) selection of a CEC and preliminary CEC testing, and (3) *in vitro* analysis of phages and CECs as single modalities and as a combined therapy. All three components required an ESC-R *E. coli* isolate with *E. coli*_SA13 selected. This isolate was selected from a previously characterised collection of ESC-R *E. coli* harbouring an InCI1-$bla_{\text{CTX-M-1}}$ plasmid (pCTXM1-MU2) (Abraham et al., 2018). Strain *E. coli*_SA13 belongs to ST6976, is phenotypically resistant to ampicillin, ceftiofur, ceftriaxone, tetracycline and trimethoprim/sulfamethoxazole, and harbours the antimicrobial genes; *aadA1*, *aadA5*, $bla_{\text{CTX-M-1}}$, *dfrA17*, *tetA*, *sul1* and *sul2*.

6.6.2 Phage isolation

Freshly defecated faecal samples were collected from swine from two distinct locations (over 3000km apart) and transported to Murdoch University on ice. Faecal samples were suspended in SM buffer (0.1 M NaCl, 1 mM $MgSO_4$, 0.2M Tris (pH 7.5), 0.01% gelatine) at a ratio of

1:10 and stirred using a magnetic stirrer for 24 hours at 4 °C. The suspensions were centrifuged at 17 000 g for 10 minutes followed by filtration through a 0.45 μm syringe driven membrane filter unit (Pall Corporation, USA). For overnight enrichment, an equal volume of phage preparation and 2 x Luria-Bertani (LB) (Thermo Fisher Scientific, Australia) broth were combined and inoculated with a single colony of the ESC-R *E. coli* isolate SA13. Samples were incubated overnight at 37 °C on an orbital shaker (80 rpm) before filtration through a 0.25 μm filter unit.

Phage lysates were then spot tested onto lawn plates of *E. coli*_SA13. Briefly, 4 mL of LB broth was inoculated with a single colony of *E. coli*_SA13 and incubated at 37 °C on an orbital shaker at 220 rpm for five hours. The culture was spread onto a LB agar plate, excess broth removed, and plates dried before 20 μL aliquots of phage lysates were dropped onto agar, allowed to dry and incubated overnight at 37 °C. Phage growth was indicated by the formation of phage plaques. A section of plaque was harvested using a sterile pasteur pipette and suspended into a solution containing 1 mL of SM buffer and 25 μL of chloroform (Sigma Aldrich, Australia). The phage suspension was held at room temperature for four hours before storage at 4 °C.

6.6.3 Phage stock preparation

Phage stock was prepared by incubating 100 μL of phage lysates with 100 μL of *E. coli*_SA13 culture for 20 minutes at 37 °C. This mixture was added to 3 mL of soft LB agar, mixed and then poured onto a LB agar plate. Plates were dried and incubated overnight at 37 °C. A solution containing 10 mL SM buffer and 200 μL chloroform (Sigma Aldrich, Australia) was poured on top of the soft agar. The plates were stored at 4 °C with constant agitation for four hours. The supernatant was then collected, centrifuged and filtered through a 0.25 μm

membrane filter. Phage stocks were immediately used for characterisation with the remainder stored at -80 °C.

6.6.4 Phage specificity

Host range of phages was tested against 14 ESC-R *E. coli* originating from location A and harbouring the InCI1-*bla*$_{\text{CTX-M-1}}$ plasmid (Abraham et al., 2018). Further specificity testing was completed against 44 *E. coli* isolates from in-house archival stores, including both commensal and pathogenic strains and originating from swine, cattle, seagulls, companion animals and laboratory strains. Additional testing was conducted testing phage lysis against multiple bacterial genera commonly comprising the gut microflora including; *Salmonella* spp. (n=1) (Abraham et al., 2016), *Streptococcus* spp. (n=2) (O'Dea et al., 2018), *Enterococcus* spp. (n=7) (Lee et al., 2021), *Staphylococcus aureus* (n=4) (Sahibzada et al., 2017) and in-house archival stores of *Bacillus* spp. (n=7). This was conducted using spot testing as described above with phage lysis measured as complete lysis or no lysis.

6.6.5 Phage characterisation

Phages were subjected to whole genome sequencing (WGS) for genetic characterisation. Phage stock was concentrated tenfold using 500 μL Vivaspin 10 kDa cutoff protein concentrator spin columns (GE Healthcare Life Sciences, Australia). DNA was extracted using a MagMAX Viral Isolation Kit (Ambion, Australia). Library preparation was performed using a Nextera XT DNA library preparation kit (Illumina, United States) with sequencing performed on an Illumina Miseq platform using a V3 2x300 flowcell. Sequencing data was *de novo* assembled using Geneious Prime 2021.1.1 (https://www.geneious.com) resulting in 12 contigs with a N50 of 42 589 and GC% content of 50.43. The single contig larger than 10 000 base pairs in length was characterised by contig sequence homology to known viral sequences in the BLASTn database. MultiPhATE2(Ecale Zhou et al., 2019) was used for gene calling and annotation

using the blastp, phmmer and jackhmmr databases. All phage sequences were screened for genes associated with lysogeny, antimicrobial resistance and bacterial virulence. The annotated genome of Phage 26 was deposited in the NCBI database under the Genbank accession number MZ832314.

6.6.6 Phage assay

Phage 26 was selected for use in the phage assay and combination therapy assays due to its strong lytic activity against *E. coli*_SA13 and broad host range against ESC-R *E. coli*. Phage stocks were prepared using the double agar method and stock was titrated by spotting ten-fold dilutions onto an *E. coli*_SA13 lawn LB agar plate in triplicate. Phage concentration was determined by analysing the growth of *E. coli*_SA13 when inoculated with varying concentrations of phage. This was conducted in a 96-well plate with wells containing 160 µL LB broth, 20 µL of an overnight culture of *E. coli*_SA13 diluted to a 0.5 McFarland standard and 20 µL of the phage inoculum. Ten-fold dilutions of phage were used starting at a concentration of 3 x 10^7 PFU/mL. Assays were completed in triplicate with positive (bacterial growth with no phage) and negative included. The plate was sealed and placed on a SPARK reader for 6 hours with continued incubation at 37°C, constant shaking at 270 rpm and absorbance (OD600nm) readings completed every 10 minutes.

6.6.7 Selection of competitive exclusion clones

Commensal *E. coli* from Australian finisher pigs (Laird et al., 2021b) were selected as potential CECs based upon the following selection criteria; (1) were either fully susceptible or harboured resistance to only ampicillin or tetracycline when tested against ampicillin, ceftriaxone, ciprofloxacin, gentamicin, tetracycline and trimethoprim-sulfamethoxazole, (2) had undergone WGS, (3) *E. coli* virulence genes, *eltA, est, stx2e* and *eae,* were not detected within the genome sequence.

Selection of isolates for inclusion in further assays was based on the bacterial growth rate with an additional isolate selected based on this isolate being the fastest growing strain that harboured two or more colicin or microcin genes. Colicin and microcin genes were determined by analysing WGS data using Abricate (Seemann T). The *E. coli* virulence finder database was used with cutoffs of $\geqslant$ 95% coverage and $\geqslant$ 99% identity. Growth curves were determined by growing bacterial isolates overnight at 37°C in individual wells of a 96 well deep well plate containing LB broth. Suspensions were diluted using a Tecan Evo liquid handling platform with an attached absorbance reader to an absorbance (OD620nm) equivalent to a 0.5 McFarland standard. A further one in ten dilution was completed using LB broth with transfer to a 200 µL 96-well u-bottom plate. Bacterial growth was measured using a Tecan SPARK reader programmed as described above. Colicin production and activity was tested *in vitro* as described by Abraham et al. (2011). The genomic sequences of the CECs are available in the NCBI database under BioProject number PRJNA720242. Isolates B1, F1 and H10 are isolates F1P10-008, F1P7-002 and F10P1-007, respectively (Laird et al., 2021b). Quality control was performed on all sequencing data using FastQC (v0.11.09) and Nullarbor with average base quality scores of 33.5, 33.2 and 33.1 and average depth of 33x, 40x and 51x for isolates B1, F1 and H10, respectively (Andrews, 2010).

6.6.8 Competitive exclusion clone assay

Overnight cultures of bacterial strains were standardised to an OD600nm of 0.1 using a SPARK (Tecan) reader followed by a 100 times dilution (approximately 1.6 x 10^6 CFU/mL). Assays were performed in 96-well deep-well plates with a starting assay volume of 2 mL. This consisted of 20 µL of the CEC and/or the ESC-R aliquoted into LB broth. Sampling was completed at the specified time intervals with 20 µL removed and used for 10-fold serial dilutions. These dilutions, and a sample directly from the assay plate, were used to quantify the bacterial strains, recorded as colony forming units (CFU)/mL. This was repeated in triplicate

by dropwise addition of 5 µL onto agar plates, followed by drying of the droplet and then overnight incubation of the agar plate. Two different agar plates were used: LB agar and LB agar + ceftriaxone (8 µg/mL). Cultures containing the CEC were spotted onto LB agar, cultures containing the ESC-R *E. coli* spotted onto LB agar containing ceftriaxone and cultures containing both isolates spotted onto both agar plates. All plates were incubated overnight at 37°C and colonies counted manually the next day. The CEC quantification in the mixed cultures was calculated using the ESC-R *E. coli* CFU/mL subtracted from the total bacterial CFU/mL.

6.6.9 Combination assay

Combination assays were completed using ESC-R *E. coli*, Phage 26 and either CEC F1 or B1. This was completed as described for CEC assays with the addition of phage inoculation at the three- and six-hour. Phage was inoculated into corresponding cultures, with 20 µL of 3 x 10^7 PFU/mL added. Biological replicates were completed for combination assays to detect potential mutation of the ESC-R *E. coli* strain.

6.6.10 Statistical analysis

All graphing and analysis was conducted using Stata 15.1(StataCorp, 2017). Bacterial concentrations (CFU/mL) were $\log_{10}$ transformed for graphing and statistical analysis. Comparisons of bacterial growth at final time points of the CEC assays were performed using linear regression model (ANOVA) using a continuous dependant variable ($\log_{10}$ CFU/mL) and the interacting effects of the 4-factor categorical variable (treatment) and continuous independent variable (time). ANOVA was followed by pairwise comparison of means with Tukey's adjustments. The alpha significance level was set to 0.05.

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Chapter 7 - Overall discussion and conclusion

Antimicrobial resistance (AMR) is one of the greatest One Health threats facing today's society worldwide. Establishment of national AMR surveillance programs in many countries was followed by implementation of interventions, including antimicrobial stewardship and the development of alternate control strategies, in human and animal health to combat antimicrobial resistance. However, despite these efforts, antimicrobial resistance is now a global health issue, moving from an emerging threat to a dominant public health concern spanning animal and medical industries. In order to maintain some control of this situation, and maintain the efficacy of the remaining classes of antimicrobials, quantum increases in AMR surveillance methods and capability, and development of novel control strategies are urgently required.

This thesis has endeavoured to understand the heterogeneity of commensal *E. coli* which may account for underrepresentation of AMR prevalence in national AMR surveillance programs. It also evaluated postbiotics, using *in vivo* experimental and field-based trials, as an alternative strategy for the control of enterotoxigenic *E. coli* (ETEC) and AMR-commensal *E. coli* in swine. A novel strategy for the control of CIA-resistance was also evaluated, consisting of combined therapy of phages and competitive exclusion clones (CECs) which were analysed *in vitro* for their effect on ESC-resistant *E. coli*. Lastly, the high through put robotics platform, RASP, was applied throughout this work highlighting its future potential in AMR surveillance and the analysis of novel control strategies in *in vivo* models.

Investigation into the diversity of commensal *E. coli* in swine demonstrated high levels of phenotypic and genotypic diversity. These findings enhance and inherently fit with our understanding of the complex gut microbiome, although little research has been performed to demonstrate such diversity, particularly as it related to AMR. Heterogeneity was demonstrated

within and between pigs and farms, evident in 89% of pigs harbouring isolates with multiple AMR indexes. In addition, 58 MLSTs were identified from the 151 isolates subjected to whole genome sequencing. This heterogeneity is most concerning in relation to CIA resistance in which only 23.8% of isolates from a single farm were detected as resistant to ciprofloxacin. This highlights the idea that CIA resistance is at high risk of remaining undetected if only a single isolate is sampled per farm, an approach that is currently used in national AMR surveillance programs. The sampling methodology of AMR surveillance programs requires improvement to account for these high levels of heterogeneity. Although the sampling methodology applied in this chapter demonstrates a higher accuracy in representation of AMR, there is a possibility that specific resistances may still have remained undetected. A different approach to ensure detection of all resistance types may be the quantification of resistance using selective agars. The selection of resistance towards antimicrobials of high public significance would ensure detection of these resistances. Furthermore, the combination of an increased sampling methodology, with samples collected from multiple pigs and across multiple farms, evaluating resistance at the host and farm level, as well as use of selective agar, would enable accurate determination of AMR prevalence whilst ensuring detection of resistance towards antimicrobials of concern. This supports the necessity to continuously review and advance current practises and methodologies in AMR surveillance. Overall, the high-through put robotics platform, RASP, allowed a detailed examination of the diversity within the *E. coli* population and an increased accuracy of AMR surveillance, demonstrating future potential in AMR research and surveillance.

The surveillance of AMR is essential in determining the success of AMR control strategies over time, including managerial practises and genetic screening. Neverthless, a greater focus on the development of novel strategies that minimise antimicrobial usage within the livestock sector is required. Although supraphysiological levels of ZnO demonstrated potential as an

alternative therapy against ETEC infection in weaner pigs, the co-selection of antimicrobial resistance and the environmental pollution resulting from these medicinal levels of ZnO, have resulted in its ban in the EU by 2022. Therefore, this thesis evaluated the alternate strategy of oral administration of postbiotics for its effects against ETEC infection in swine. Although no direct effects on ETEC infection were detected, measured using faecal consistency scores and quantification of faecal ETEC shedding from faeces pooled by pen, pigs supplemented with postbiotics demonstrated an increased final liveweight compared to the control and ZnO group, respectively. Furthermore, postbiotic supplementation led to modulation of the faecal microbiome, for instance, an increased abundance of Lactobacillaceae consisting mostly of *Lactobacillus reuteri* observed with diets containing LFP. This bacterial species has been reported to enhance the intestinal mucosal barrier and may account for the increase in growth performance within these pigs, perhaps providing a level of protection against ETEC which was below the threshold of our metrics. However, no explanation for the increased growth performance in pigs receiving SFP was found. This study was limited to assessment of these postbiotics on the ETEC faecal shedding, faecal consistency scores, weight and faecal microbiome of swine, whereas analysis of the postbiotics effects within the gastrointestinal tract requiring further study. Measuring the effects of the postbiotics within the gastrointestinal tract may shed light on the mechanisms leading to an increased weight gain. Overall, the results from this chapter suggest supplementation with postbiotics, in the form of LFP and SFP, may alleviate the reduced growth in ETEC-infected weaner pigs.

Research of strategies against AMR have largely focused on the reduction in emergence of AMR through minimising antimicrobial usage. Although the continued development of alternative methods is necessary, development of strategies that directly target the reduction of established AMR on farms requires increased attention. Accordingly, we evaluated the effect of postbiotics as single and combined control measures for resistant *E. coli.* Firstly, the effects

of postbiotics on ESC-resistant *E. coli* were evaluated using a novel *in vivo* model. This *in vivo* model was successful in the colonisation of ESC-resistant *E. coli* with this resistance persisting over the full duration of the trial despite its reduction in concentration. This supports previous studies in which ESC-resistant *E. coli* persisted on farms over an extended timeframe following withdrawal of ESC usage. A natural reduction in ESC-resistant *E. coli* was demonstrated in the current study with postbiotics having no impact on the rate of this clearance. Postbiotics also demonstrated no effect on the growth performance of the weaner pigs. This contrasts the findings of the previous chapter in which postbiotics increased growth performance in ETEC-challenged weaners. This may be attributed to the disease state of the weaner pigs in the previous chapter, with the postbiotics providing sufficient protective effect against ETEC to significantly effect weight gain. Another reason for this contrast may be due to the disruption of the gut microflora following treatment with ceftiofur, potentially impacting the effects of the postbiotics on growth performance and against ESC-resistant *E. coli*. This led to a study on the effect of these postbiotics on the natural carriage of AMR *E. coli* in a field-based trial in Chapter 5. Although this study demonstrated postbiotics had no effect on AMR *E. coli* or on growth performance, supporting previous findings, the study provided a detailed examination into the natural reduction of CIA-resistant *E. coli* with time after weaning. The period during and following this reduction would be an ideal window for AMR management, in which supplementation with probiotics may potentially prolong these low levels of resistance. Furthermore, this study suggests treatment with ceftiofur did not affect results from the experimental model and supports future development of the *in vivo* model.

One aspect to consider for future experimental trials is the method used for quantification of bacterial shedding in faeces. Due to the low occurrence of faecal excretion in weaning pigs and time constraints in sample collection and sample processing, collection of samples was difficult and limited in these trials with the first and second experimental trials using pooled pen faecal

samples and individual rectal swabs, respectively. These methodologies are inferior to those using individual faecal samples. In comparison, storage of samples at 4 °C following homogenisation in PBS allowed robotic processing the day after sample collection. This alleviated time constraints regarding sample collection and coupled with the high number of pigs in the field-based trial, permitted the inclusion of individual faeces collected from 192 representative pigs. Whilst this is the ideal method to alleviate time constraints and therefore increase sample number, a controlled experiment to determine the optimal sampling methodology is required, comparing the concentration of *E. coli* recovered from individual rectal swabs, individual faeces processed and plated on the day of collection, and individual faeces processed on the day of collection however plated after 24 hours of storage. This experiment would allow determination of the ideal sampling methodology, and if this sampling method is not achievable in following studies, the impact of alternative methodologies on bacterial quantifications would be known and could be accounted for.

Overall, the chapters involving analysis of postbiotics suggest that the postbiotics have no impact on commensal *E. coli* harbouring resistance to antimicrobials or on the growth performance of healthy weaner pigs. Nevertheless, supplementation with postbiotics led to an increased growth performance in ETEC-challenged weaners. This supports the idea that postbiotics impact gut health in diseased pigs without having any direct impact on ETEC infection and warrants further analysis.

Lastly, the combined therapy of phages and CECs was evaluated as a novel strategy for the control of CIA-resistant *E. coli*. These approaches demonstrated a superior effect on ESC-resistant *E. coli* when administered as a combination compared to their effects as single modalities. The significant reduction and possible elimination of ESC-resistant *E. coli* demonstrated *in vitro,* warrants further analysis and optimisation of this control strategy, both *in vitro* and *in vivo*. This would include investigation of phage cocktails instead of a single

phage, investigation into CECs that translate this competitive edge within the swine gut, and development of an administration schedule. Overall, this combined therapy provides a cost-effective method to reduce the prevalence of ESC-resistant *E. coli* on farms, reducing the risk of its further dissemination within and between farms, and into humans. Due to the emergence and global detection of CIA resistance despite the continued efforts of antimicrobial stewardship, strategies that focus on reducing and eliminating CIA resistant bacteria require increased attention and development.

In summary, this thesis advanced our understanding of the diversity of *E. coli* and its implications in AMR surveillance. Resulting from this chapter, it is recommended to further optimise and develop a new sampling methodology for AMR surveillance programs. The second recommendation for future research is to determine the effects of the postbiotics LFP and SFP within the gastrointestinal tract of ETEC-challenged weaner pigs, specifically the effects on the microflora, intestinal barrier, and immune stimulation. This would increase our understanding of postbiotics and their effect on the host health in this disease state, possibly leading to its future use on farms to alleviate growth reduction due to ETEC infection. Thirdly, the RASP has demonstrated use in experimental and field-based trials involving bacterial quantification however controlled experiments to determine the ideal sampling methodology for bacterial quantification by the RASP is required. Lastly, future development and analysis of the combined therapy *in vivo* is recommended with this strategy demonstrating exciting potential for reduction of ESC-resistant *E. coli* on farms.

In conclusion, this thesis detected the high level of phenotypic and genotypic diversity in commensal *E. coli* within and between pigs and farms. Secondly, the use of postbiotics, in the form of LFP and SFP, increased growth performance and the diversity of the faecal microflora in F4-ETEC challenged weaner pigs. Meanwhile, these postbiotics demonstrated no effect on growth performance in healthy weaner pigs. The effects of the postbiotics against resistant *E.*

coli was also analysed, with a field-based trial detecting a reduction in ESC-resistant *E. coli* in weaner pigs challenged with ESC-resistant *E. coli*. In comparison, the postbiotics had no effect on ciprofloxacin, tetracycline, or ESC-resistant *E. coli* in a farm-based trial. Lastly, an *in vitro* experiment demonstrated the combined therapy of phage and competitive exclusion significantly reduced and possibly eliminated ESC-resistant *E. coli*. Overall, this thesis furthered our understanding of AMR in commensal *E. coli* and our knowledge of novel and alternative ETEC and AMR control strategies.

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