Novel approaches for reducing antimicrobial resistant and pathogenic Gram-negative bacteria in the porcine gut

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By

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Executive Summary

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 enterotoxigenic *E. coli* (ETEC) infection in pigs and in reducing antimicrobial resistance (AMR). This in-depth project aimed to evaluate the use of novel ferment products as feed additives to (ultimately) reduce antimicrobial use (using ETEC as a target organism), while concurrently analysing the effects on the gut microbiome. This project also aimed to assess competitive probiotic solutions for reduction of critically important antimicrobial (CIA) resistant bacteria from herds where CIA resistant gut microbiota are established.

Specifically, the objectives of this project were to:

1. Evaluate the effect of specialised dried yeast ferments and specialised dried lactobacillus ferments in reducing the severity of ETEC infections in post-weaned pigs.

2. Evaluate the effect of specialised dried yeast ferments and specialised dried lactobacillus ferments in reducing antimicrobial resistant bacteria, focusing on CIAs, in the post-weaned pig gut.

3. Evaluate the impact of specialised dried yeast ferments and specialised dried lactobacillus ferments on overall performance and indices of gut health in post-weaned pigs.

The outcomes of this project showed that dietary supplementation with LFP and SFP increased growth performance of pigs challenged with ETEC and beneficially modulated the faecal microbiota, suggesting these postbiotics may have a role in the overall management of porcine ETEC. Overall, the studies demonstrated the applicability of an experimental model for analysing the effects on strategies against, but not restricted to, extended-spectrum cephalosporin (ESC)-resistant *E. coli*. This model can be used for other intervention studies for decolonization of resistant bacteria. Successful colonisation of ESC-resistant *E. coli* was detected in weaner pigs using an *in vivo* model established in this project, with a reduction in ESC-resistant *E. coli* shedding over time. However, the postbiotics demonstrated no significant effect on this reduction rate whilst also demonstrating no effect on growth performance of pigs. In a commercial nursery study, high levels of tetracycline, ciprofloxacin, and ESC-resistant *E. coli* were detected at weaning before demonstrating a natural decline over the 4-week post-weaning period. The postbiotic supplementation had no significant effect on the rate of this reduction whilst also demonstrating no effect on growth performance of the weaner pigs.

Given these findings, methods to remove CIA-resistant bacteria, once established in a herd, are needed. A possible approach is the use of a combination approach of decolonisation, which consists of the targeted displacement of CIA-resistant *E. coli* by competitive excluding clones, target specific bacteriophages (utilising a phage cocktail approach to address inherent resistance emergence), nutritional additives, and (or) removal of co-selection pressure.

Nevertheless, use of the postbiotic products in ETEC-inoculated post-weaned pigs showed promising benefits in terms of average daily growth and health scores in animals with experimentally-induced ETEC infection, despite continued shedding of bacteria. This may be due to the increase in the diversity of intestinal microbiota in pigs with postbiotic-supplemented diets.

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1. Introduction

Antimicrobial use (AMU) and antimicrobial resistance (AMR) in intensive pig production has come under heavy scrutiny due to its potential impact towards human and animal health. This is primarily due to the current reliance on antimicrobials for treating and controlling bacterial diseases in livestock also belonging to the same class as antimicrobials used in human medicine. This has become even more crucial since the emergence of resistance towards critically important antimicrobials (CIAs) in many livestock systems throughout the world, with Australia detecting its first such resistance in livestock in 2015 (Abraham et al., 2015). In Australia, the CIA ceftiofur (from the 3rd generation cephalosporin class) is used as an off-label antimicrobial to treat enterotoxigenic *Escherichia coli* (ETEC) that causes neonatal and post-weaning diarrhoea in pigs. The presence of multidrug resistant ETEC has thus become a cause of concern as it would increase the use of ceftiofur to treat resistant ETEC infections in pigs. In fact, a mid-2000s antimicrobial use survey reported that 25% of Australian pig farms used ceftiofur (Jordan et al., 2009). This is further exacerbated by the detection of pathogenic and commensal *E. coli* in healthy pigs with low frequency of resistance towards CIAs such as ceftiofur (2-5%), and high rates of resistance towards first-line antimicrobials such as ampicillin (50-60%) and tetracycline (60-75%) (Abraham et al., 2015; Kidsley et al., 2018; van Breda et al., 2018). Though the presence of CIA-resistance was at a low frequency, it highlights the need for prudent and careful use of CIAs to ensure that these low levels do not increase.

One of the major issues with AMR is the long-term persistence of resistant bacteria including those with resistance towards CIAs, in the absence of direct selective pressure (Hansen et al., 2013). Resistant bacteria can transfer resistance to other bacteria (across the genera and species boundary) using mobile genetic elements such as plasmids that possess genes conferring resistance to multiple antimicrobials. Therefore, once resistant bacteria including CIA-resistant bacteria emerges within a herd either through antimicrobial use or via reverse zoonosis, the resistant bacterial flora within the herd does not revert to being susceptible. This results in the herd remaining positive for resistant bacteria for protracted periods and reduces the efficacy of other antimicrobials due to co-resistance or multidrug resistance. The long-term persistence of resistant bacteria, especially to CIAs, has impacts on animal and public health as well as raising the risk of food safety. Therefore, it is imperative that the Australian pig industry research novel strategies for reversion of the gut microbiota from resistant to sensitive flora.

An important point towards the development of these novel strategies is the need for innovative action in reducing the reliance of antimicrobials in the current changing international climate of the post-antibiotic era. Novel alternatives include feed additives may have a negative impact on ETEC, and can shift resistant bacterial populations back to susceptible populations through various modes of action including immune modulation, gut secretion modifications, and (or) changes to the gut microenvironment. One alternative combination used extensively in the poultry industry consists of specialised dried yeast or lactobacillus ferments (commercially available under the product names XPC and SynGenX, respectively). A previous poultry study demonstrated that these ferments improve productivity, feed conversion and gut integrity, and reduce carriage levels of pathogenic *Salmonella* and other resistant

bacteria in the gastrointestinal tract while improving the overall immune function of birds (Feye et al., 2016). However, data pertaining to outcomes in pigs when fed with these ferments is currently lacking.

Competitive exclusion and establishment with susceptible bacteria are another key area in reducing overall resistance. Methods such as prebiotics and probiotics, and faecal transplanting beneficial bacterial populations from healthy pigs to unhealthy pigs, have previously been identified as potential alternatives assisting with the reversion of gut microbial population from resistant to susceptible. Faecal transplantation has been used in the medical sector to control *Clostridium difficile* infection after antimicrobial use (Brandt, 2012) although it is not feasible for implementation on a large-scale in commercial piggeries. For such large-scale application, we propose that commensal bacteria with limited resistance such as susceptible *E. coli* or *Enterococci* harvested from different sections of the gastrointestinal tract act as a surrogate for full faecal transplantation. The hypothesis examined is that increasing the numerical dominance of susceptible commensal bacteria (via oral supplementation or high carriage doses) will lead to competitive exclusion of resistant bacteria. Constant inoculation of susceptible bacteria with properties allowing attachment to the intestinal epithelium may have a competitive edge against resistant bacteria thus leading to a dilution effect of resistant bacteria within the gut.

This project thus aimed to analyse the effects of feed additives for treating ETEC infections, reduce shedding of CIA-resistant bacteria, and reversion of a resistant microbial population in the gut to a susceptible population within pigs. Ultimately, the results of this project will assist the Australian pig industry in developing novel approaches for treating ETEC and reducing carriage levels of CIA-resistant bacteria in pigs without resorting to antimicrobials including CIAs.

2. Methodology and Outcomes

2.1. Experiment 1: Effects of Lactobacillus *and* Saccharomyces *fermentation products on growth performance and faecal microbiome in ETEC-challenged weaners*

2.1.1. 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 AMR, use must be minimised and hence alternative control methods are needed. Medicinal levels of zinc oxide (ZnO) can alleviate ETEC infection, although this also has implications for resistance and environmental contamination. Postbiotics, the fermentation products of probiotic strains, have been reported to increase host health and defence against pathogens and in contrast to ZnO, have no impact on AMR or the environment. In a randomised controlled experiment, 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.

2.1.2. Methodology

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

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 (as per (Sterndale et al., 2019)).

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.

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) 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 manufactured (Specialty Feeds) and met the energy and nutrient requirements for pigs of this age. 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 based on 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 (SEM)) 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 enrichment. Pigs were acclimatised for 5 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.

F4-ETEC inoculation

Following acclimation, pigs were inoculated with ETEC (serotype O149: 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. (2019). 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 (Table 1).

Table 1. Experimental timeline.

	Pre-inoculation (ni)			Post-inoculation (pi)		
Day	NA	-5	-4 >-1	0	1	2-28
~ Age (days)	12	21	22-25	26	27	28-55
Trial Event	Genetic Pre-screening	Weaning and transport to facility	Acclimation	1st ETEC dose	2nd ETEC dose	Sample collection

Faecal sampling and processing

Rectal swabs were collected from all pigs on days 0, 1, 2, 3 and 7 post-inoculation (pi). All swabs were streaked onto 5% sheep blood agar plates (Edwards Group, 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 was 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).

RASP ETEC and *E. coli* quantification

Fresh faecal samples were collected from pen floors on Days 1, 2, 3, 4, and 7 pi 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. (2022). Briefly, one gram of faeces was added to 19 mL of PBS buffer and placed in a stomacher machine (high setting) 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 β-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.

Faecal consistency scores (FCS) and production data

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. Pen feed intake was determined on the same days as animals were weighed. The average daily feed intake (ADFI) was determined for individual pigs by averaging the pen feed intake based on the number of animals in a pen. Pigs exited the trial facility 34 days after weaning.

Microbiome diversity and abundance

The 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. The QIIME2 was used to process and analyse 16S rRNA gene sequencing data and to perform statistical calculations for alpha and beta diversity (Bolyen et al., 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.

Statistical analysis

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

A one-way ANOVA followed by Tukey post-hoc test was used to analyse performance data. Statistical significance was accepted at $p < 0.05$, and a trend was recognized at $p < 0.1$.

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 Kruskal-Wallis one-way analysis of variance test 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). The PERMANOVA of the diversity analysis was calculated with the 999 Monte Carlo permutation and Benjamini-Hochberg correction (FDR) (Xia and Sun, 2017).

2.1.3. Outcomes

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 experiment due to high numbers of ETEC-F4 resistance in pigs. Throughout the study, 20 pigs were removed. This included 11 pigs removed before ETEC challenge due to the death of one pig and welfare concerns for the other 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 clinical 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* genes *astA* was present in the challenge strain but not included in the PCR), with no additional genes detected.

No significant difference in FCS of pigs supplemented with LFP and/or SFP

Prior to ETEC challenge, FCS were low across all treatment groups with 82% of FCS being 2 or less (Figure 1). 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 4 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 1) and when analysed for the 4-day period after challenge in which FCS increased.

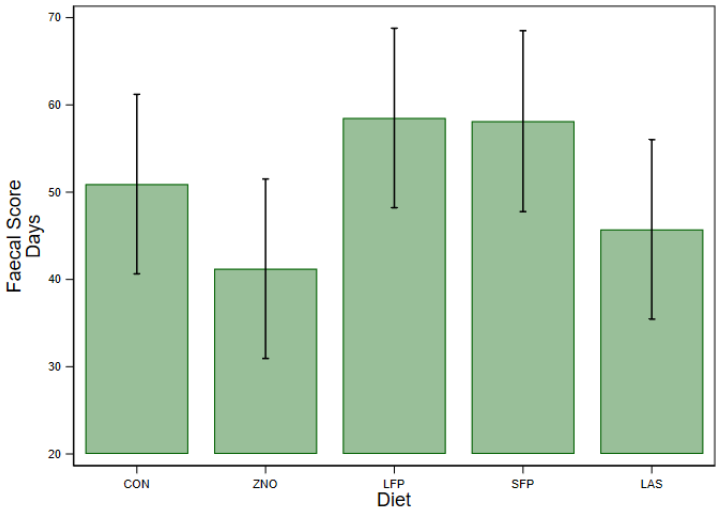

Figure 1: The mean faecal score days, a measurement relating to the faecal consistency score across the full duration of the study. Treatment abbreviations: CON control base diet, ZnO = base + 3000 ppm zinc oxide, LFP = base + 2000 ppm *Lactobacillus acidophilus* fermentation product (LFP), SFP = base + 2000 ppm *Sacchromyces cerevisiae* fermentation product (SFP), LAS = base + 2000 ppm LFP + 2000 ppm SFP.

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 pi, with ETEC shedding having cleared by day 7 in all groups (Figure 2). 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 3). The total *E. coli* density also demonstrated no significant difference between treatment groups (Figure 3).

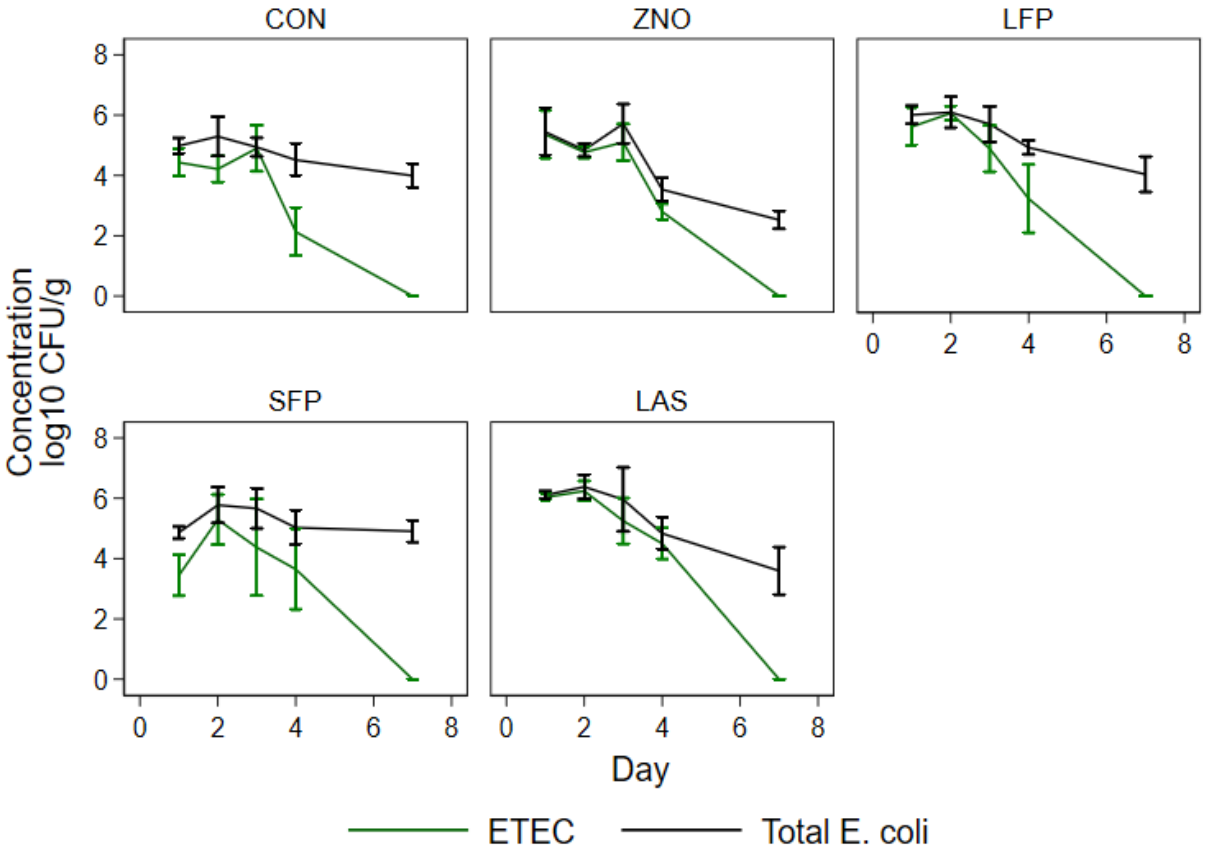

Figure 2: Quantification of faecal F4-ETEC and total putative *E. coli* in ETEC-challenged pigs (n=69) belonging to different treatment groups following challenge with ETEC. Error bars represent standard error of means. Pigs were inoculated with ETEC on Day 0 and 1. Treatment abbreviations: CON control 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.

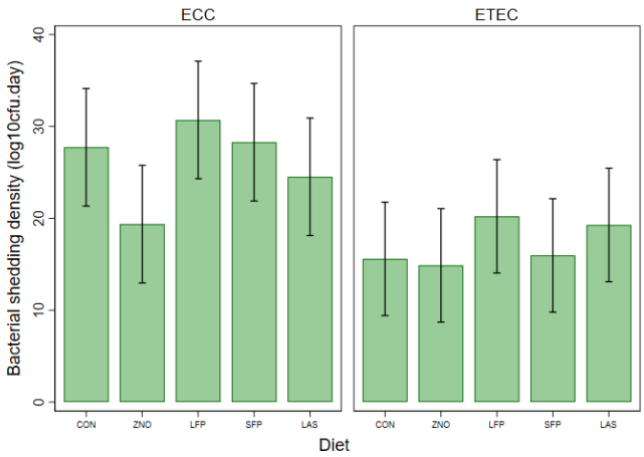

Figure 3: The mean F4-ETEC and total putative *E. coli* density, a measurement relating to the ETEC and putative total *E. coli* shedding across the seven days following challenge with ETEC. Pigs were challenged with ETEC on Day 0 and 1. Treatment abbreviations: CON control base diet, ZnO = base + 3000 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.

A difference in peak ETEC shedding was observed amongst treatments (Figure 2). Peak shedding 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 total *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.

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 4). 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 kg (17.4, 18.3 kg) (Figure 5). In comparison, the ZnO and control groups had the lowest mean liveweight on the final day at 16.1 kg (15.8, 16.6) and 16.2 kg (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 kg (16.7, 17.0) and 17.0 kg (16.8, 17.2) kg, respectively.

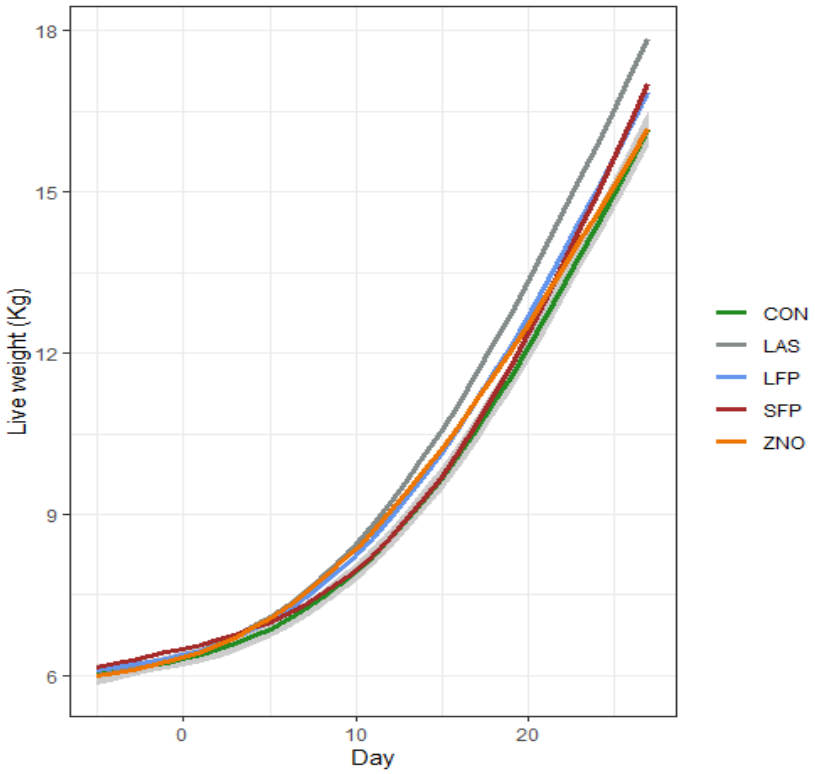

Figure 4: Mean effect of each of five diets on post-weaning pigs liveweight over the duration of the trial as estimated by generalised additive models fitting smoothing splines to non-linear changes in liveweight over time. Confidence intervals (shading) are provided for the control diet. Treatment abbreviations: 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.

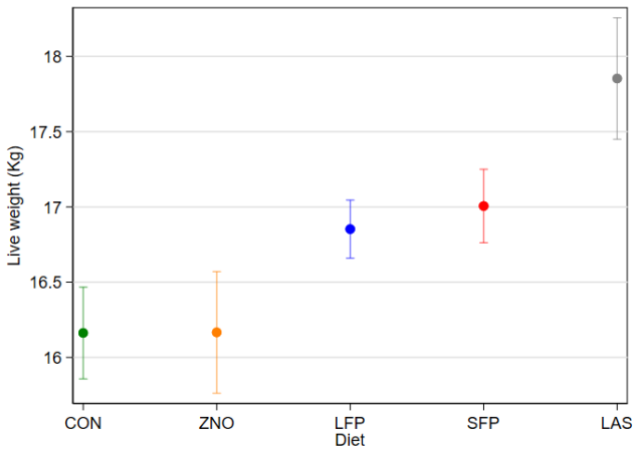

Figure 5: Mean effect of each of five diets on post-weaning pigs' liveweight at the final timepoint (Day 27) of the trial as estimated by generalised additive models fitting smoothing splines to non-linear changes in liveweight over time. Confidence intervals (bars) are provided for each diet. Treatment 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 2). The ADG was highest in Week 2, 3 and 4 for pigs in the LAS group, with the ADG being 95 and 140 grams greater in the LAS group in Week 4 compared to the control and ZnO groups, respectively. Based on one-way ANOVA, a statistically significant difference in ADG was noted between the CON and combined LAS treatment diet, but not between other diets at week 2. There was no significant difference in ADG at other timepoints however the LAS diet showed a trend to significance (adjusted p = 0.051) when compared to ZnO at week 4 (Table 2),

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, however there was no statistically significant difference between groups at any timepoint. 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, and no significant differences were detected.

Table 2. One way ANOVA analysis of average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCR).

	Treatment					
Item	CON	ZNO	LFP	SFP	LAS	*P* =
ADG, g						
wk 1	63±19.5	68±11.8	59±13.5	51±22.7	60±15.4	0.972
wk 2	169±15.7[a]	233±23.0 [ab]	202±19.9 [ab]	187±22.9 [ab]	262±31.8 [b]	0.049
wk 3	348±35.8	359±33.1	380±21.4	317±38.1	380±34.4	0.623
wk 4	602±40.8	556±37.9*	627±17.2	690±32.7	697±40.3*	0.029
ADFI						
wk 1	94±13.5	86±8.8	92±15.2	85±14.8	90±8.3	0.993
wk 2	255±56.2	297±28.8	271±28.0	250±52.4	290±35.6	0.929
wk 3	454±49.5	491±39.9	488±34.1	504±71.3	484±47.9	0.965
wk 4	872±69.8	811±19.0	893±27.8	1028±148.8	910±97.9	0.567
FCR						
wk 1	2.1±0.60	1.3±0.12	1.6±0.12	0.8±0.71	1.6±0.16	0.337
wk 2	1.40±0.11	1.3±0.03	1.3±0.08	1.3±0.14	1.1±0.04	0.394
wk 3	1.3±0.06	1.4±06	1.3±0.04	1.7±0.19	1.30±.10	0.090
wk 4	1.4±0.04	1.5±0.04	1.4±0.03	1.5±0.14	1.3±0.12	0.733

[a-b]Mean values within a row that have different superscripts are significantly different ($p < 0.05$).

* P-value = 0.051 following Tukey post-hoc test.

CON = control base diet, ZnO = base + 3,000 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.

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 6). 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 pi, 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.

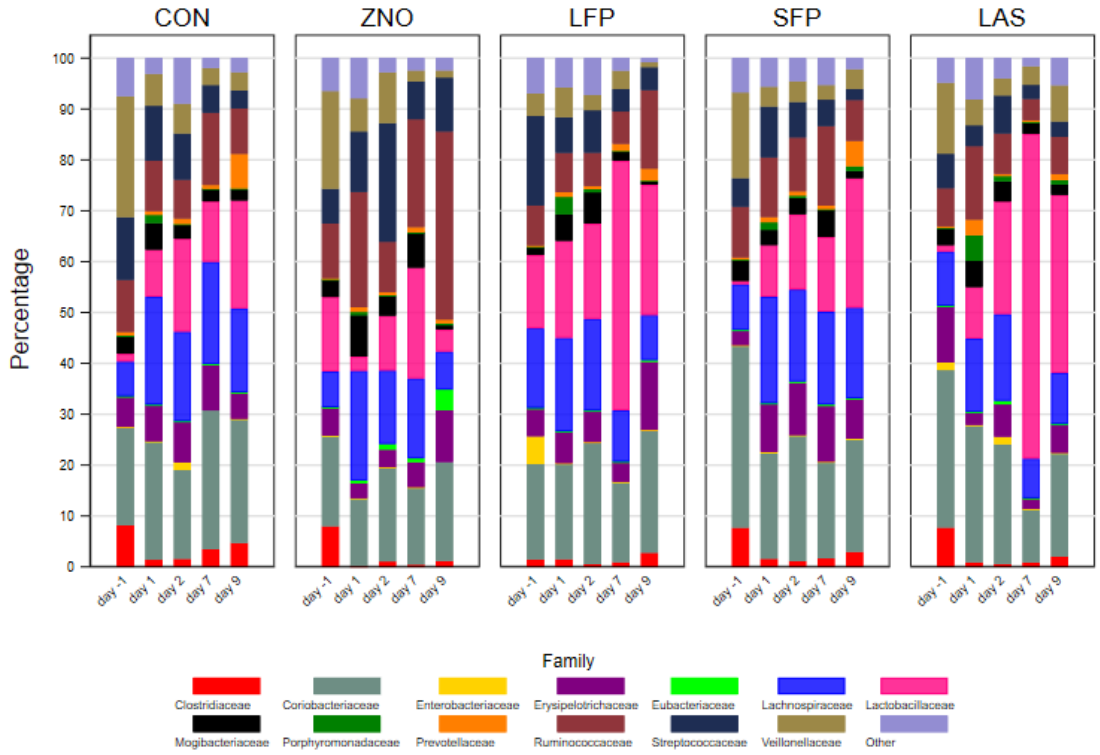

Figure 6: Proportion of bacterial families in faecal microbiota of ETEC-challenged weaners over multiple days and treatment groups. Treatment abbreviations: CON control base diet, ZnO = base + 3,000 ppm zinc oxide, LFP = base + 2,000 ppm *Lactobacillus acidophilus* fermentation product (LFP), SFP = base + 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 pi and compared to the ZnO group when all timepoints were analysed together (Table 3). 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 4). 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 3: 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 ZnO 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 4: 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 ZnO 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.

2.1.4. Discussion

The necessity to reduce the use of both antibiotics and ZnO 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 significant effects of LFP, SFP and their 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, although numerical trends for improvements were evident especially with the combination. However, the large variation between pigs in the number of faecal score days caused the lack of statistical significance. 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 demonstrated a trend towards significance when compared to ZnO, as opposed to single administration, with a final liveweight of 17.9 kg (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 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., 2020). 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., 2020). 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 unclear. 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 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 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. (2019) 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 followed 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 demonstrated 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.

2.2. Experiment 2: Development of an in vivo *extended-spectrum cephalosporin resistant* Escherichia coli *model in post-weaned pigs and its use in assessment of dietary interventions*

2.2.1. Background

Historic and current methods to tackle antimicrobial resistance (AMR), a major health threat threatening the livestock industries, focus on reducing the emergence of AMR by minimising AMU. This is achieved through antimicrobial stewardship (AMS) principles 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 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*. This study 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 more rapid and complete clearance of ESC-resistant *E. coli* to those not receiving the supplement.

2.2.2. Methodology

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

Animals, housing and experimental design

Dietary treatment groups were allocated to pens by a randomised block design with four replicate pens of each treatment. Pigs were recruited to the trial from a high health status Australian herd. Sows and gilts were vaccinated with ECOvac (MSD Animal Health) and PLEvac (MSD Animal Health). Weaners recruited for the trial were removed prior to receiving any vaccinations. Only male pigs were available as per the farm's breeding program. The 64 male piglets (Large White x Landrace) 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 entry weight with each pen housing 4 pigs resulting in 16 pigs representing each treatment group. 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 acclimatised for 7 days before inoculation with ESC-resistant *E. coli*, with this day designated as day 0. All pigs were weighed on arrival (day -7), with pigs and feed subsequently weighed on day 0 and then 7, 14, 21 and 28 days thereafter for determination of performance data. Pigs were therefore kept for a total period of 35 days.

Experimental diets, formulated to meet the energy and nutrient requirements of these pigs, comprised a control diet (CON), CON supplemented with 2,000 ppm LFP (LFP), CON supplemented with 2,000 ppm SFP (SFP), and CON supplemented with the combination of 2,000 ppm of LFP and 2,000 ppm SFP (LAS) (Table 5). The wheat- and barley-based diets comprised a mixture of vegetable and animal protein sources typi-cal for a diet fed to weaner pigs in Western Australia, and were formulated to contain 10.1 MJ/kg of net energy, 13.5 g/kg of standardised ileal digestible lysine, and 205 g/kg crude protein. Diets were fed in meal form. Fermentation products were Diamond V SynGenX™ and Diamond V Original XPC™ for LFP and SFP, respectively.

Table 5. A summary of the treatment used in the study.

Treatment	SynGenX, ppm	Diamond V Original XPC, ppm
CON	0	0
LFP	2,000	0
SFP	0	2,000
LAS	2,000	2,000

Treatment abbreviations: CON = control, LFP = *Lactobacillus acidophilus* fermentation product, SFP = *Saccharomyces cerevisiae* fermentation product, LAS = *Lactobacillus acidophilus* and *Saccharomyces cerevisiae* fermentation products.

ESC-resistant *E. coli* inoculation

All pigs were treated with 25 mg (50 mg/mL) ceftiofur via intramuscular injection on day -1 (i.e., 6 days after weaning) to promote colonisation with ESC-resistant *E. coli* upon inoculation. Pigs were inoculated with ESC-resistant *E. coli* strain SA13 (Abraham et al., 2018) on days 0 and 1 (i.e., days 7 and 8 after weaning) using gelatin capsules for delivery as described previously (Laird, 2021), except for the original strain grown on CHRO-Magar™ ESBL (MicroMedia, Edwards Group) and a single, blue colony being recultured. Strain SA13 is a commensal *E. coli* strain with a IncI1-*bla*$_{\text{CTX-M-1}}$ plasmid. On day one and two of inoculation, pigs received two capsules containing 1.92 x 108 colony forming units (CFU) per capsule

Faecal sampling and processing

Rectal swabs were collected from each pig on days -1, 1, 2, 3, 5, 7, 14, 21 and 28 pi. The average weight of the swab end was calculated by cutting off the swab end of 10 clean swabs at the same point and averaging the weight. All sample swabs ends were cut, weighed and then suspended in 15 mL centrifuge tubes containing 5 mL of PBS.

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., 2022; Truswell et al., 2021). Briefly, samples were diluted to the expected concentrations with two dilutions plated onto each agar plate using dual spiral plating. 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 the distinction between species based on colour of colonies on chromogenic agar (Figure 7).

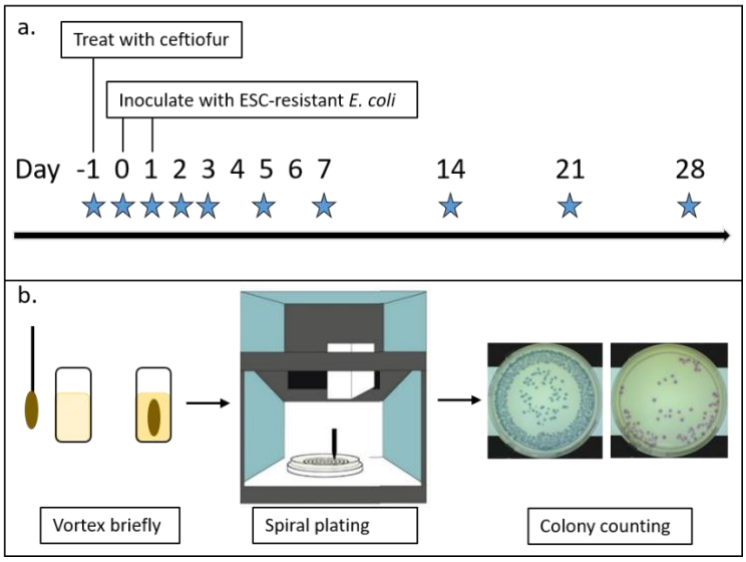

Figure 7. Overview of *in vivo* model for assessing effect of novel strategies on ESC-resistant *E. coli.* a. Timeline of treatments and sampling of pigs. The first inoculation with ESC-resistant *E. coli* was day 0 (i.e., 7 days after weaning). Blue stars represent days rectal swabs were collected. b. Laboratory processing of samples for quantification of total *E. coli* and ESC-resistant *E. coli* from rectal swabs (calculated as CFU/g). Blue colonies represent *E. coli* on CHROMagar™ ECC agar while pink colonies represent ESC-resistant *E. coli* on CHROMagar™ ESBL agar.

Statistical analysis

Statistical analysis and graphing were conducted using STATA (v15.1) and R Studio (v1.2.5033). Liveweight (kg) of pigs for 28 days starting on arrival at Murdoch Animal house 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. A one-way ANOVA followed by Tukey post-hoc test was used to analyse bacterial shedding and performance data. Statistical significance was accepted at $p < 0.05$, and a trend was recognized at $p < 0.1$.

2.2.3. Results

No ESC-resistant *E. coli* were detected prior to challenge. The concentration of ESC-resistant *E. coli* was highest on day 1 (i.e., 24 hours after the first ESC-resistant *E. coli* inoculation) across all treatment groups (Figure 8). 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, but there was no overall statistical difference between diets ($p > 0.05$). 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. The concentration of ESC-resistant *E. coli* showed a trend ($p < 0.1$) between the SFP and CON groups with the SFP group demonstrating a reduced ESC-resistant *E. coli* concentration. No difference in the concentration of ESC-resistant *E. coli* was detected at the final time point, with ESC-resistant *E. coli* concentration ranging 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.

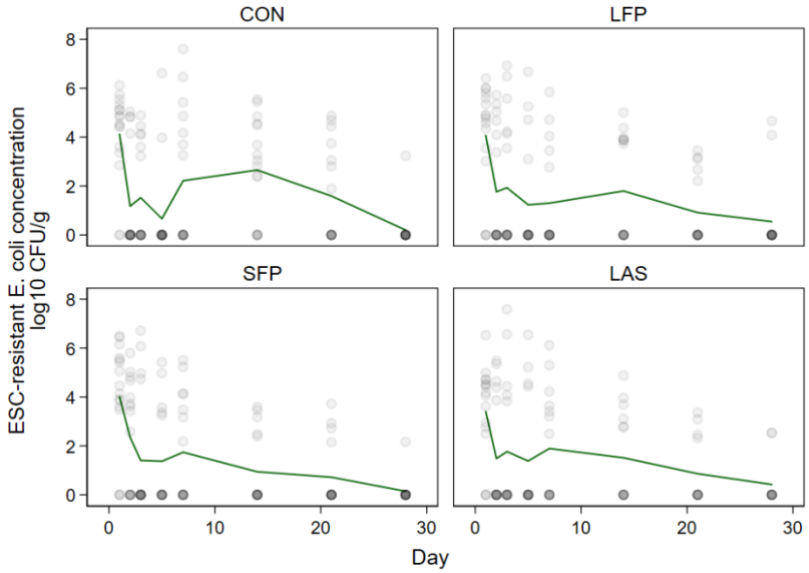

Figure 8. Mean concentration of ESC-resistant *E. coli* in pig rectal swabs (n=64) belonging to different dietary treatment groups. Mean concentration is represented by the line with dots representing individual pigs (overlapping of individual pig data results in darker dots in graph). Treatment abbreviations: CON: control diet, LFP = CON + 2,000 ppm *Lactobacillus acidophilus* fermentation product (LFP), SFP = CON + 2,000 ppm *Saccharomyces cerevisiae* fermentation product (SFP), LAS = CON + 2,000 ppm LFP + 2,000 ppm SFP.

The bacterial shedding density (a measure of bacterial shedding across the full duration of the study) was used to statistically analyse the shedding of ESC-resistant *E. coli* and total *E. coli* (Figure 9). The mean (with 95% CI) ESC shedding density was highest in the control group, 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, but these were statistically similar ($p > 0.05$) (Figure 9).

Total *E. coli* shedding was similar ($p > 0.05$) 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 9).

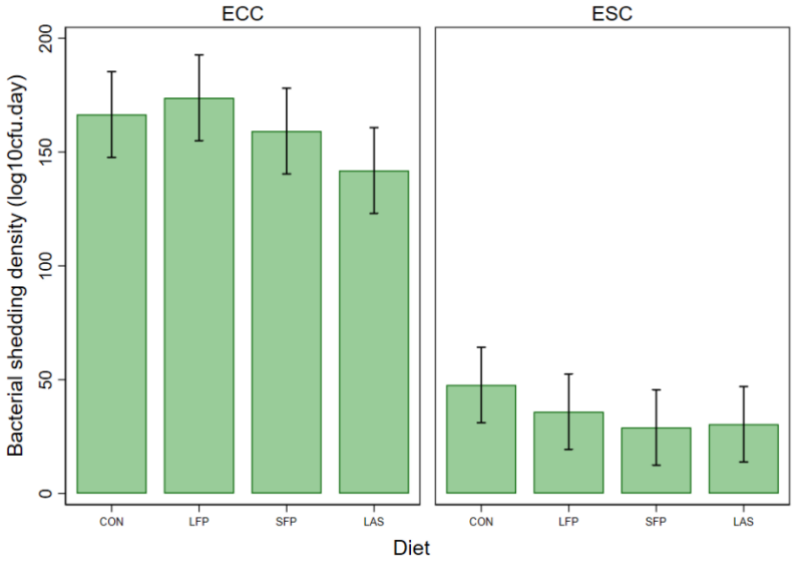

Figure 9. The total *E. coli* (ECC) and ESC-resistant *E. coli* (ESC) shedding density, a measure of bacterial shedding across the full duration of the trial, in pigs (n=64) belonging to different dietary treatment groups. Error bars are standard error of the means. Treatment abbreviations: CON = control diet, LFP = CON + 2,000 ppm *Lactobacillus acidophilus* fermentation product (LFP), SFP = CON + 2,000 ppm *Saccharomyces cerevisiae* fermentation product (SFP), LAS = CON + 2,000 ppm LFP + 2,000 ppm SFP.

The number of pigs with ESC-resistant *E. coli* was highest in all treatment groups on day 1 (i.e., 24 hours after the first ESC-resistant *E. coli* inoculation). This declined by 48 hours post-challenge with only 38% of pigs harbouring ESC-resistant *E. coli* on day 2 at detectable levels. The second peak detected in the quantification of ESC-resistant *E. coli* was also reflected in the percentage 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 (Figure 10). Over the entire study, ESC-resistant *E. coli* was undetected in two pigs. Meanwhile, only nine samples had no *E. coli* detected when grown on ECC agar.

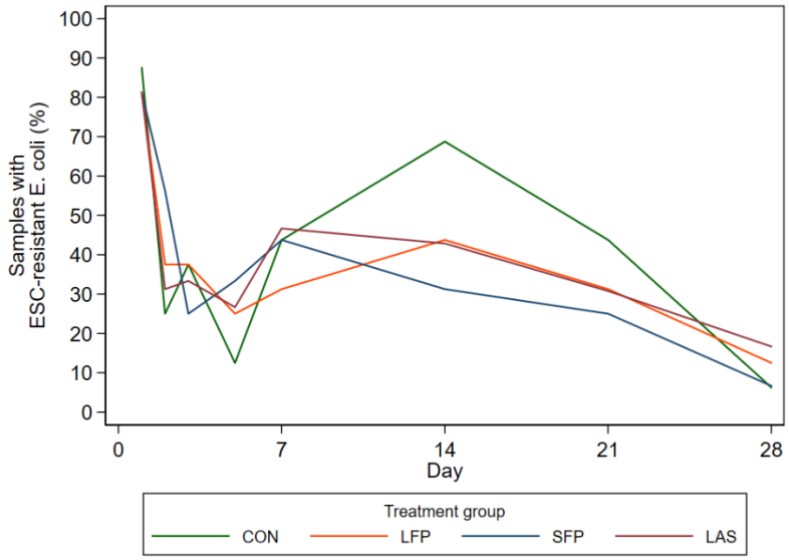

Figure 10. Percent of pigs harbouring ESC-resistant *E. coli* over duration of the study and belonging to different dietary treatment groups. Treatment abbreviations: CON = control diet, LFP = CON + 2,000 ppm *Lactobacillus acidophilus* fermentation product (LFP), SFP = CON + 2,000 ppm *Saccharomyces cerevisiae* fermentation product (SFP), LAS = CON + 2,000 ppm LFP + 2,000 ppm SFP.

2.2.4. 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 11). 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 demonstrating a natural clearance.

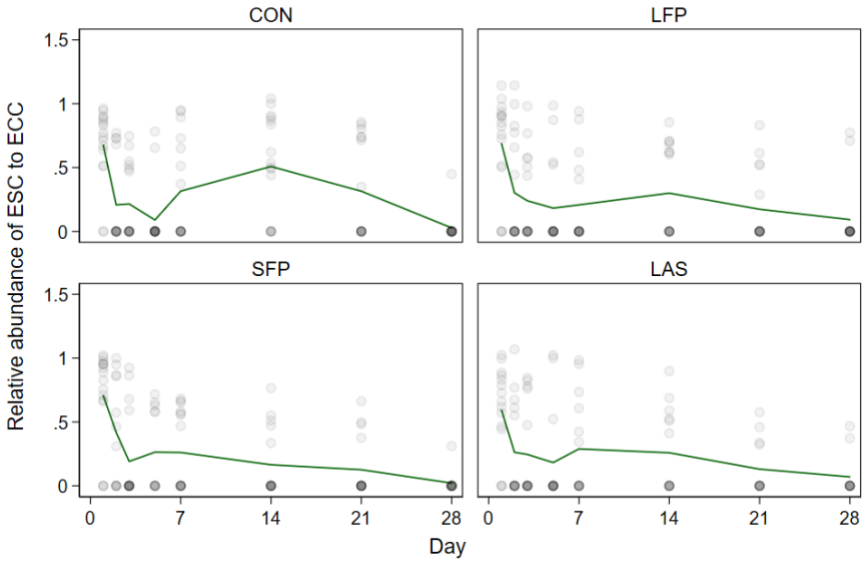

Figure 11. Abundance of ESC-resistant *E. coli* relative to total *E. coli* in rectal swabs from ESC-resistant *E. coli* challenged weaners belonging to different treatment groups. Line represents median with individual pigs represented by dots (overlapping of individual pig data results in darker dots in graph). Treatment abbreviations: CON = control diet, LFP = CON + 2,000 ppm *Lactobacillus acidophilus* fermentation product (LFP), SFP = CON + 2,000 ppm *Saccharomyces cerevisiae* fermentation product (SFP), LAS = CON + 2,000 ppm LFP + 2,000 ppm SFP.

2.2.5. Pig performance

Pig live weight increased over the duration of the study, starting at an average of 6.62 kg (day -7) across all pigs and reaching an average of 19.58 kg at 28 days after inoculation (Figure 12). 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 ($p < 0.05$) average (with 95% CI) liveweight at this timepoint of 20.0 kg [19.8, 20.2] and 19.8 kg [19.6, 20.0], respectively, compared to 18.8 kg [18.5, 19.1] and 19.3 kg [18.1, 19.5] kg in the LAS and LFP groups, respectively.

Accordingly, average daily gain (ADG) increased over time with the mean ADG across all pigs being 81 g in week 0 (i.e., entry to day -7) and increasing to 220, 409, 518 and 620 g in subsequent weeks 1, 2, 3 and 4, respectively. Pigs fed diet SFP grew faster (565 *vs* 473 g/day, $p < 0.05$) than pigs fed diet LAS between days 15 and 21 post-inoculation (Table 6).

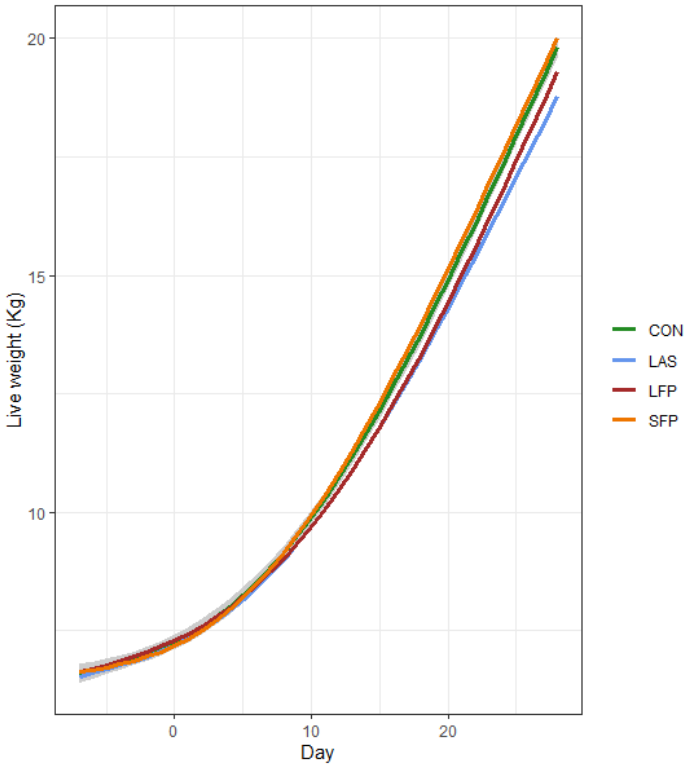

Figure 12. Mean liveweight of pigs (n=64) in different dietary treatment groups in ESC-resistant *E. coli* challenged weaner pigs. Treatment abbreviations: CON = control diet, LFP = CON + 2,000 ppm *Lactobacillus acidophilus* fermentation product (LFP), SFP = CON + 2,000 ppm *Saccharomyces cerevisiae* fermentation product (SFP), LAS = CON + 2,000 ppm LFP + 2,000 ppm SFP.

Average daily feed intake (ADFI) increased over the duration of the study with the mean ADFI for all pigs being 123, 279, 509, 731 and 871 grams for week 0 (i.e., entry to day -7), and subsequent weeks 1, 2, 3 and 4, respectively. Mean ADFI was similar ($p > 0.05$) between treatment groups in weeks 0, 1, 2 and 4, but in week 3, pigs fed diet SFP ate more feed than pigs fed diet LAS (835 vs 662 g/day, $p < 0.05$). There were no differences ($p > 0.05$) in FCR detected between any of the dietary treatment groups during any time period (Table 6).

Table 6. Effects of postbiotics on average daily gain (ADG), average daily feed intake (ADFI) and the feed conversion ratio (FCR) in ESC-resistant *E. coli*-challenged weaner pigs.

	Treatment				
Item	CON	LFP	SFP	LAS	*P* =
ADG, g					
d -7 to 0	77±14.3	85±14.2	87±16.9	75±17.7	0.928
d 1-7	240±16.6	205±19.3	215±24.9	222±24.4	0.705
d 8-14	406±27.3	393±28.0	440±19.0	396±26.1	0.530
d 15-21	531±19.4^{ab}	493±22.4^{ab}	565±22.5^{a}	473±25.6^{b}	0.028
d 22-28	635±22.9	639±30.6	608±36.0	593±25.4	0.673
ADFI					
d -7 to 0	107±10.9	129±11.8	132±18.4	121±22.0	0.717
d 1-7	288±20.4	280±14.6	280±28.6	266±27.9	0.926
d 8-14	513±27.7	507±39.8	531±29.3	485±41.3	0.831
d 15-21	714±29.9^{ab}	713±46.1^{ab}	835±27.3^{a}	662±12.0^{b}	0.013
d 22-28	870±27.6	898±69.1	863±33.0	852±16.4	0.875
FCR					
d -7 to 0	1.7±0.40	1.6±0.19	1.6±0.19	1.7±0.08	0.996
d 1-7	1.2±0.04	1.4±0.05	1.3±0.13	1.3±0.10	0.596
d 8-14	1.3±0.05	1.3±0.04	1.2±0.02	1.3±0.04	0.263
d 15-21	1.3±0.02	1.4±0.03	1.5±0.10	1.4±0.08	0.495
d 22-28	1.4±0.01	1.4±0.05	1.3±0.05	1.4±0.04	0.315

$^{a-b}$Mean values within a row that have different superscripts are significant different ($p < 0.05$). 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.

2.2.6. Discussion

Whilst many approaches in minimising the emergence of antimicrobial-resistant bacteria are being investigated and implemented in pork production, there is a lack of strategies to decolonise or reduce carriage of CIA-resistant bacteria once detected. The development of these strategies is necessary in minimising the risk of this resistance spreading between animals, farms and to humans. The current study aimed

to establish an *in vivo* model to analyse the effects of postbiotic dietary supplements on reducing ESC-resistant *E. coli* in weaner pigs, whilst determining the effects of postbiotics on ESC-resistant *E. coli* shedding and performance.

The model that was established in this study successfully induced the shedding and potential colonisation of ESC-resistant *E. coli* over an extended timeframe. There was an initial increase in the shedding of ESC-resistant *E. coli* 24 hours after challenge, with this concentration reducing by approximately two logs by 48 hours post-challenge. Whilst the second peak in ESC-resistant *E. coli* demonstrated its replication and colonisation within the pigs, the small timeframe of high shedding concentrations may hinder identifying strategies to control resistant *E. coli*, with an ideal model invoking high shedding for a longer period. Despite breeding stock receiving a vaccination against *E. coli*, the ECOvac vaccine targets *E. coli* fimbrial antigens K88, K99 and 987P, which were not present in the ESC-resistant *E. coli* challenge strain. Combined with the waning of maternal antibodies, it is unlikely this would have a confounding effect on the trial. Whilst this model provided insight into the dynamics of ESC-resistance, a repeat dosing schedule with ceftiofur or the use of an antimicrobial with a longer duration of activity in future studies may prolong the shedding of high levels of ESC-resistant *E. coli* via a reduction in competition from commensal gut flora. However, this would need to be carefully considered as a further confounding factor affecting gut stability.

Despite the 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 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. The collection of faecal samples is ideal and arguably provides more accurate data than swabs, but in the present study, faecal collection from 64 individual weaner pigs at multiple timepoints was considered unachievable. This was due to previous experience observing the sporadic time intervals between defecation in weaner pigs. In the current study, failure to detect *E. coli* only occurred in nine of the faecal swabs, supporting the use of swabs for sampling. 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 microbiome in the period following weaning (Angelakis, 2017), this technique would most likely be unreliable in an experimental setting with low numbers of pigs.

Despite the establishment of a successful experimental model, postbiotic supplementation with LFP and (or) SFP demonstrated no significant reduction ($p < 0.05$) in ESC-resistant *E. coli* and no general improvement in production in weaner pigs. This was most likely attributable to the high variation between pigs within a treatment, suggesting a larger sample size is required for future studies. Nevertheless, a positive trend ($p = 0.065$) of the SFP to reduce ESC-resistant *E. coli* carriage was demonstrated on day 14, compared to CON-fed pigs. 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). In contrast, the current study challenged weaner pigs with a commensal strain confirmed to harbour the IncI1-*bla*$_{CTX-M-1}$ plasmid. This plasmid is highly transferable, demonstrates long-term persistence in environments absent of direct selection pressures, and has been detected globally (Abraham et al., 2018; Borjesson et al., 2013; Dahmen et al., 2012; Wang et al., 2014). Coupled with its genetic similarity from isolates across continents and conservation of coding regions, the evidence suggests plasmids with an IncI1 backbone carrying the *bla*$_{CTXM-1}$ gene are highly stable (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.

Another aspect to consider in future studies, is the potential transfer of the plasmid to other commensal *E. coli* strains within the gastrointestinal tract. Genomic typing of ESC-resistant *E. coli* from multiple timepoints would determine if the plasmid had transferred to other strains, providing information on the transferability of the plasmid whilst verifying if all ESC-resistant *E. coli* quantified in the study was the original challenge strain. Whilst the postbiotics demonstrated no significant effect on ESC-resistant *E. coli* carriage in the current study, its previous success in boilers needs further exploration. Future exploration of the positive trend ($p = 0.065$) that the SFP postbiotics had on reducing ESC-resistant *E. coli* shedding is necessary using increased sample numbers and implementing mentioned strategies to increase the length of time in which high levels of ESC-resistant *E. coli* are shed. Furthermore, future studies using this model to need to assess the effect of postbiotics against different ESC-resistant *E. coli* strains and the effect of postbiotics on resistance against different antimicrobials. Lastly, while this model can be used to evaluate control strategies through challenge with a single, commensal *E. coli* strain, natural carriage of E. coli is highly diverse with these postbiotics, and other alternate control strategies, requiring field-based trials (Laird et al., 2022; Stoesser et al., 2015). The inclusion of pre- or postbiotics with these fermentation products may also increase the effects seen against the ESC-resistant *E. coli* with synbiotics, the combination of pre-, pro- or postbiotics, previously demonstrated to have a greater effect than the single additive (Wang et al., 2019). Lastly, the concentration of postbiotics in the diet requires optimisation. Whilst there are many studies demonstrating the effects of varying concentrations of a single probiotic (Lee et al., 2012), in contrast many of the studies investigating postbiotics investigate multiple postbiotics/combinations at a single concentration (Humam et al., 2019; Park et al., 2017). Determining the optimal concentration of the postbiotic, may demonstrated an in-creased capability of the postbiotic against antimicrobial-resistant bacteria.

Supplementation with postbiotics demonstrated no statistically positive overall effects on growth performance, although some positive impacts of feeding SFP compared to LAS were noted in week 3 following inoculation. Studies investigating the effects of these postbiotics on the growth performance of swine are contrasting. While LFP, SFP and the combination demonstrated increased growth performance in ETEC-challenged weaner pigs (Laird, 2021), other studies have demonstrated no significant effect on growth performance (Nordeste et al., 2017). Meanwhile, Bass and Frank

(Bass and Frank, 2017) reported an in-creased average daily gain in healthy weaner pigs. The beneficial effects of postbiotics have been demonstrated to be through modulation of the gut microbiome, and there-fore the efficacy of postbiotics may be dependent 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 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 in-creased consistency between studies and determination of the benefits of postbiotics in food-producing animals.

Conclusions

Overall, this study has demonstrated the applicability of an experimental model for analysing the effects of alternate control strategies against, but not restricted to, ESC-resistant *E. coli*. The *in vivo* model was used to assess the effects of postbiotics, in the form of LFP and SFP and their combination, against ESC-resistant *E. coli* levels in weaner pigs, with SFP demonstrating a positive statistical trend for a reduction in counts. 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.

2.3. Experiment 3: Postbiotic supplementation of weaner pig diets and the effect on the antimicrobial resistance carriage in a commercial nursery.

2.3.1. Background

Antimicrobial resistant (AMR) bacteria continue to be a significant threat faced by the Australian pork industry, and is interrelated to One Health (O'Neill, 2016). Due to selection pressures imposed using 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., 2021). Furthermore, *Lactobacillus acidophilus* fermentation products (LFP) and *Saccharomyces cerevisiae* fermentation products (SFP) have also demonstrated potential in reducing AMR levels in food-producing animals (Feye et al., 2016).

The purpose of this study was to provide a detailed examination of the dynamics of antibiotic-resistant *E. coli* carriage in weaner pigs on a commercial farm using the Robotics Antimicrobial Susceptibility Platform (RASP) (Truswell et al., 2021), 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 in a commercial farm.

2.3.2. Methodology

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 farm. The 1,280 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 1,000 ppm LFP (LFP), diet supplemented with 1,250 ppm SFP (SFP), and diet supplemented with the combination of 1,000 ppm of LFP and 1,250 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 after weaning. Feed consumption was measured on day 0, 14 and 25. Pigs exited the trial 26 days after weaning.

Faecal sampling and processing

Six freshly collected faecal samples per pen were randomly selected for AMR screening on days 0, 7, 14, 21 and 25 after weaning. One gram of faeces and 19 mL of PBS buffer were placed in a stomacher machine (high setting) for 30 seconds. The contents were filtered upon pouring into sterile centrifuge tubes with tubes then placed onto the RASP as previously described in Experiment 1. Dilutions required for plating were estimated, and then performed with two dilutions plated onto each plate (Figure 13). 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 repeated using calculated dilutions.

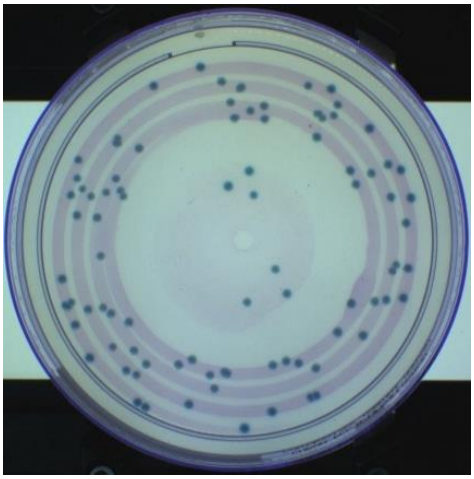

Figure 13: Spiral plating of sample at two different dilutions onto CHROMagar™ ECC with 4 µg/mL ciprofloxacin. Blue colonies represent putative *E. coli*. The outer circle contains the undiluted sample with the inner circle diluted 1 in 10.

Statistical analysis

Graphing and the mean and standard deviation of pig weight was calculated in STATA v15.1. A one-way ANOVA followed by Tukey post-hoc test was used to analyse performance data and bacterial shedding. Statistical significance was accepted at $p < 0.05$, and a trend was recognized at $p < 0.1$.

2.3.3. Results

Carriage of *E. coli* was highest on day 0, the day of weaning, across all treatment groups and *E. coli* types (Figure 14). 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 after weaning. 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.

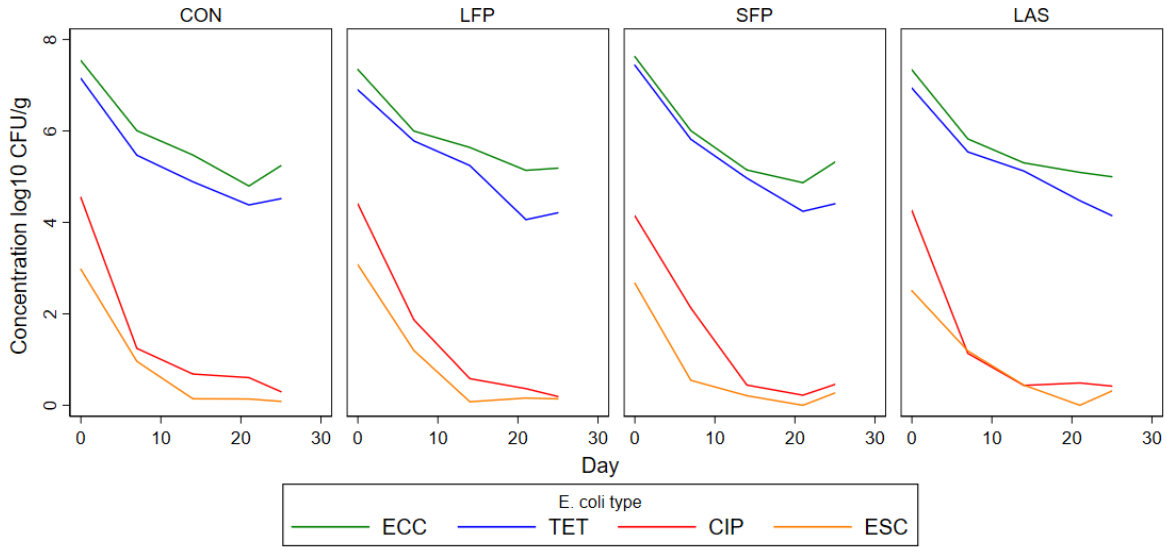

Figure 14: Mean concentration of different types of AMR *E. coli* detected in swine faeces (n=6 faecal samples/pen per time-point) belonging to different treatment groups in a farm-based trial. *E. coli* abbreviations: ECC = total *E. coli*, TET = tetracycline resistant *E. coli*, ESC = extended-spectrum 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.

Tetracylcine-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, with a significant difference between the LFP and SFP groups (Table 7). 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.

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 after weaning, 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 less in the mean ciprofloxacin-resistant *E. coli* concentration in comparison to the SFP group. This variation between treatment groups was also demonstrated in the percentage of samples with ciprofloxacin-resistant *E. coli* detected (Figure 15). 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 percentage of samples with ciprofloxacin-resistant *E. coli* decreased over time from 92.2% on day 0 to 9.4% on day 25. No statistically significant difference was noted between groups across the trial period, however there was a trend ($p = 0.065$, Table 7) between LAS and SFP on day 7.

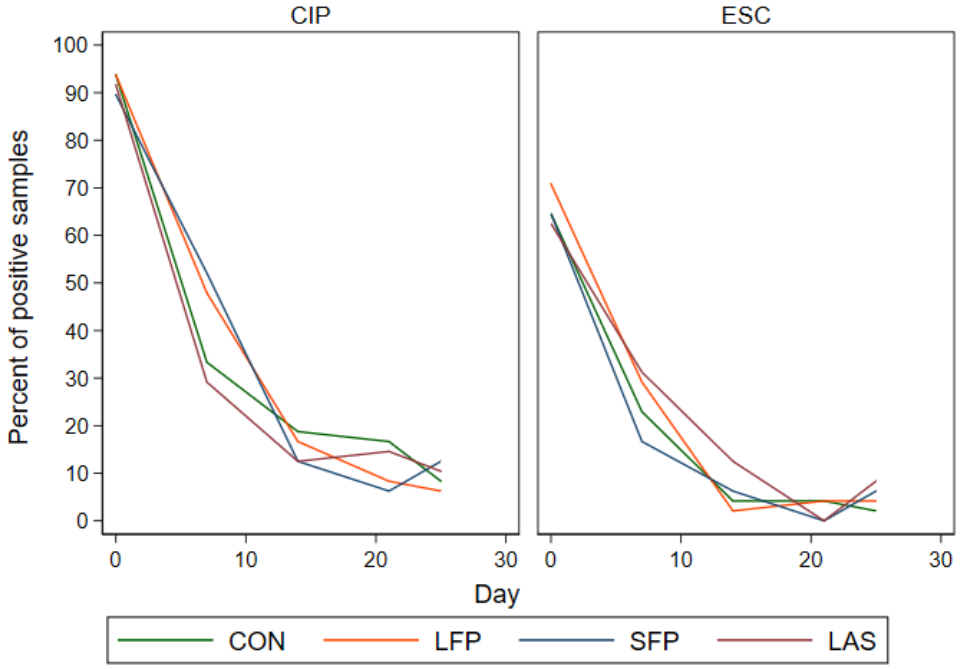

Figure 15: Percentage of samples with resistant *E. coli* detected in swine faeces (n=6 faecal samples/pen per time-point) belonging to different treatment groups in a farm-based trial. **CIP**. Ciprofloxacin-resistant *E. coli,* **ESC**. Extended-spectrum cephalosporin-resistant *E. coli*. Treatment 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.

Extended-spectrum cephalosporin resistant *E. coli*

The ESC-resistant *E. coli* showed 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 after weaning, respectively. The percentage of samples with ESC-resistant *E. coli* decreased over time from 65.6% on day 0 to 2.1 and 5.2% on days 21 and 25, respectively (Figure 15). On day 7, the SFP group demonstrated the lowest mean ($p > 0.05$) 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 14 and Table 7). No ESC-resistant *E. coli* was detected in the SFP and LAS groups on day 21. No statistically significant difference was noted between groups across the trial period (Table 7).

Table 7. One way ANOVA analysis of bacterial colony counts by resistance profile.

	Treatment				
Item	CON	LFP	SFP	LAS	*P* =
ECC					
d 0	7.54±0.10	7.35±0.12	7.63±0.13	7.34±0.15	0.286
d 7	6.00±0.11	6.00±0.13	6.00±0.12	5.83±0.12	0.646
d 14	5.47±0.13	5.64±0.12	5.15±0.13	5.30±0.15	0.061
d 21	4.80±0.10	5.14±0.12	4.87±0.08	5.09±0.16	0.104
d 25	5.24±0.12	5.19±0.11	5.32±0.14	5.00±0.10	0.264
ESC					
d 0	2.98±0.35	3.07±0.30	2.67±0.31	2.51±0.29	0.552
d 7	0.96±0.26	1.20±0.28	0.55±0.18	1.19±0.26	0.210
d 14	0.14±0.10	0.08±0.08	0.21±0.12	0.44±0.17	0.169
d 21	0.14±0.10	0.16±0.11	0	0	0.264
d 25	0.08±0.08	0.14±0.01	0.27±0.15	0.31±0.15	0.541
CIP					
d 0	4.55±0.21	4.40±0.19	4.14±0.23	4.26±0.22	0.569
d 7	1.24±0.26	1.87±0.29	2.13±0.31	1.13±0.26	0.036*
d 14	0.68±0.21	0.58±0.19	0.44±0.17	0.43±0.17	0.747
d 21	0.61±0.20	0.36±0.18	0.22±0.13	0.49±0.17	0.430
d 25	0.30±0.14	0.19±0.11	0.46±0.18	0.42±0.18	0.620
TET					
d 0	7.15±0.12^{ab}	6.90±0.14^{a}	7.44±0.14^{b}	6.94±0.17^{ab}	0.029
d 7	5.47±0.14	5.78±0.13	5.82±0.14	5.54±0.13	0.193
d 14	4.89±0.11	5.24±0.10	4.97±0.14	5.12±0.12	0.170
d 21	4.38±0.10	4.06±0.12	4.24±0.13	4.48±0.17	0.136
d 25	4.51±0.12	4.21±0.11	4.41±0.20	4.15±0.21	0.352

* P = 0.065 (SFP vs LAS) following Tukey post-hoc test.
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.

Growth performance

The weight of pigs increased in a non-linear relationship over time (Figure 16). The mean weight of pigs (weight of pen/number of pigs in pen) on day 0 was 6.22 ± 0.13 kg (standard error of means (SE)), 6.25 ± 0.10 kg, 6.26 ± 0.12 kg and 6.29 ± 0.16 kg in the LFP, LAS, SFP and control groups, respectively. At the end of the experiment (day 25), the liveweight of pigs was similar between groups, with the liveweight of SFP supplemented pigs 14.6 ± 0.36 kg. In comparison, the LAS, control and LFP groups had a mean liveweight of 14.3 ± 0.43, 14.0 ± 0.58 and 13.9 ± 0.32 kg at the final timepoint, respectively. There was no significant difference in liveweight detected between treatment groups.

Both ADG and ADFI were not significantly different between treatment groups (Table 8). Analysis of FCR at the final timepoint showed that CON was significantly better than LFP and LAS but not SFP. Unfortunately, analyses were confounded by the undocumented removal of pigs from pens during the 12-day intervals between measurements.

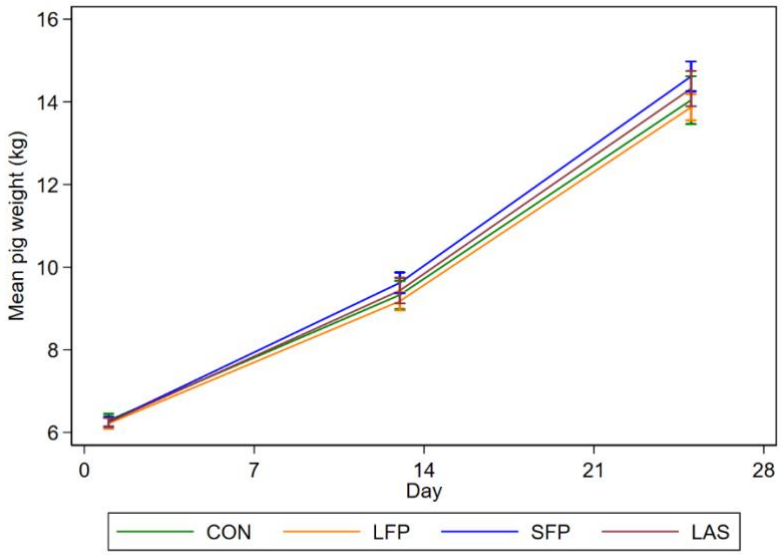

Figure 16: Mean liveweight of pigs (n=1,280) over time and belonging to different treatment groups in farm-based trial. Error bars represent standard error of means. Treatment abbreviations: CON = control base diet, LFP = base + 1000 ppm *Lactobacillus acidophilus* fermentation product (LFP), SFP = base + 1250 ppm *Sacchromyces cerevisiae* fermentation product (SFP), LAS = base + 1000 ppm LFP + 1250 ppm SFP.

Table 8. One way ANOVA analysis of average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCR).

	Treatment				
Item	CON	LFP	SFP	LAS	*P* =
ADG, g					
d 0-13	217±14.8	211±10.8	240±13.7	227±16.3	0.492
d 14-25	429±27.4	427±14.8	454±14.5	444±17.0	0.708
ADFI					
d 0-13	302±15.9	270±11.8	310±9.7	298±14.9	0.185
d 14-25	482±21.0	411±18.2	485±20.6	437±26.6	0.062
FCR					
d 0-13	1.4±0.84	1.3±0.05	1.3±0.05	1.3±0.08	0.546
d 14-25	1.2±0.07[a]	1.0±0.03[b]	1.1±0.03[ab]	1.0±0.03[b]	0.016

[a-b]Mean values within a row that have different superscripts are significantly different ($p < 0.05$).
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.

2.3.4. Discussion

The global dissemination and increasing prevalence of AMR in food producing animals, including resistance to CIAs, highlights the necessity of continued investigation and development of new strategies to control AMR. In this study, we evaluated the carriage dynamics of antimicrobial resistant-*E. coli* and the effects of the postbiotics, LFP, SFP and the combination, on AMR levels in weaner pigs in a commercial facility. The major findings arising from this study were as follows: (i) resistant *E. coli* carriage naturally reduces after weaning; and (ii) postbiotics had no significant effects 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. Resistance to these antimicrobials has previously been detected in Australian swine farms at very low prevalence in studies looking at weaner pigs. 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., 2022). 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 current study demonstrated a reduction over time with ciprofloxacin-resistant *E. coli* reducing from 4.3 to 0.3 $\log_{10}$ CFU/g over a four-week period. The current study suggests weaners acquire resistance, possibly before weaning, which reduces noticeably over time, however this hypothesis would require further investigation. The reduction in AMR regardless of feed type has been observed previously (Gaire et al., 2021; Lugsomya et al., 2018). However, the high levels of AMR 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. This may be a result of different AMU patterns between piggeries and highlights the importance of stewardship in the pre-weaning period and the carryover of AMU from sows to piglets through lactation and into weaning. Reducing AMR prevalence during the suckling stage of production may reduce the risk of AMR disseminating within farms and potentially into pathogenic *E. coli*.

An inadvertent finding, made possible using 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* resistance to ciprofloxacin. 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* (Laird et al., 2023). 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., 2022). This study demonstrated that postbiotics, in the form of LFP and SFP, had minimal 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 effects of postbiotics 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 (Kiarie et al., 2011; Lan et al., 2016; Bass and Frank, 2017). The current study demonstrated no impact of LFP or SFP, as single modalities and combined treatment, on growth performance in weaner pigs. Overall, the contrasting effects of postbiotics may be attributed to genetics of pigs, feed types and farm management practices. Further studies to determine the underlying mechanisms of postbiotics are required, potentially clarifying these contradictory results and identifying variables for their control.

Finally, 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 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, while the postbiotics LFP and SFP demonstrated no positive effects on reducing the concentration of AMR *E. coli* or on growth performance in weaner pigs, this study provided detailed insight into dynamics of AMR *E. coli*. Importantly, the results of this study demonstrate the high prevalence of AMR at weaning and a natural decline overtime, something which requires further investigation to understand the origins of AMR in the production chain.

3. Application of Research

Experiment 1 demonstrated that postbiotic dietary supplements LFP and SFP are associated with increased growth performance and health status in weanling pigs (measured by FCR, ADFG and faecal consistency) inoculated with F4-ETEC despite persistence of ETEC in treated pigs. While statistically significant differences were only noted at week 2, there was a trend to significance at the final weighing point for ADG for the postobiotics. In addition, there was a clearly beneficial effect on gut microbiota diversity when compared to ZnO supplementation. These observations demonstrate that supplementation with LFP and SFP, particularly in combination, may be beneficial during the stressful post-weaning period when ETEC associated disease and production loss is most prevalent.

In Experiment 2, a successful ESC-resistant *E. coli* infection model in pigs was developed. This model can be used to investigate and assess novel AMR control strategies in swine in the future. This experiment did not demonstrate effectiveness of LFP and SFP products in reducing ESC-resistant *E. coli* shedding.

In the large-scale commercial study (Experiment 3), a high prevalence of AMR *E. coli* was identified in pigs one day after weaning which declined over a 4-week period regardless of feed type. This prevalence of resistance was unexpected based on previous studies. This finding highlights the need to further investigate the role of breeder pigs and potential impact of historic antimicrobial use on farm in the persistence and dissemination of AMR in pigs.

4. Conclusions

Experiment 1: This study demonstrated 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 pigs. 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.

Experiment 2: Overall, this study has demonstrated the applicability of an experimental model for analysing the effects of alternate control strategies against, but not restricted to, ESC-resistant *E. coli*. The *in vivo* model was used to assess the

effects of postbiotics, in the form of LFP and SFP and their combination, against ESC-resistant *E. coli* levels in weaner pigs, with SFP demonstrating a positive statistical trend for a reduction in counts. 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.

Experiment 3: While the postbiotics LFP and SFP demonstrated no positive effects on reducing the concentration of AMR *E. coli* or on growth performance in weaner pigs, this study provided detailed insight into dynamics of AMR *E. coli*. Importantly, the results of this study demonstrate the high prevalence of AMR at weaning and a natural decline overtime, something which requires further investigation to understand the origins of AMR in the production chain and dynamic of resistant *E. coli* shedding over the various production stages.

5. Limitations/Risks

The F4-ETEC challenge model used in this project was successful in inducing disease. However, welfare concerns required the removal of numerous pigs from the study, which affected analysis of results. Despite this, there were demonstrated positive effects from the combination LAS diet, and no adverse effects noted from inclusion of the dietary supplements.

The natural clearance of ESC-resistant *E. coli* in the experimental challenge model (Experiment 2, Figures 9,11, 12) is one of the main limitations of the experimental challenge model as it reduces the time period to evaluate the effectiveness of intervention strategies. In addition, the *E. coli* strain used for the trial was commensal and non-pathogenic. Use of a more virulent strain in subsequent trials may demonstrate more obvious benefits in performance parameters.

Rapid natural decline in the colonisation rates of resistant and total *E. coli* in pigs in Experiment 3 (Figure 14 and 15) may influence the assessment of intervention strategies in reducing antimicrobial resistant *E. coli* in commercial farm settings. Another major limitation in this trial was the inadvertent removal of pigs from trial pens in the periods between weighing, confounding calculation of the performance data.

Finally, the low number of pigs used in Experiments 1 and 2 limited the statistical power during analysis, particular regarding the high variation of growth measurements and bacterial counts detected within treatment groups. This needs to be considered when interpreting the results, specifically results with a statistical trend ($p < 0.1$) and results with a visual trend detected and no statistical significance. Repetition of experiments with larger numbers of pigs can reduce this limiting factor.

6. Recommendations

The recommendations from each experiment within this study are stated below:

Experiment 1.

The use of in-feed postbiotics, *Lactobacillus acidophilus* fermentation products (LFP) and *Saccharomyces cerevisiae* fermentation products (SFP), to prevent F4-ETEC

infection in post-weaned pigs in place of ZnO was investigated, as the use of ZnO has negative environmental and antimicrobial resistance implications.

An increased final liveweight was observed in pigs supplemented with a combination of both postbiotics (LAS). This may be due to an increase in microbial diversity in comparison to no treatment or ZnO treatment.

No significant effects of postbiotics on the duration of ETEC infection in comparison to ZnO were observed, and this could be due to a large variation between pigs in the number of faecal score days. There was also no significant change in the shedding of the ETEC bacteria.

This experiment demonstrated that there is potential for postbiotics to replace ZnO in the management of ETEC in newly weaned pigs and that elimination of infection may not be required in management of pig health.

The recommendations based on Experiment 1:

1. Further investigation into the combination of LFP and SFP in the management of F4-ETEC infection in post-weaned pigs, particularly in relation to gut microflora, is warranted.

Experiment 2.
There is currently a lack of strategies to decolonise a herd when CIA is detected, raising concerns about the risk of spreading AMR between farms and to humans. This study aimed to establish a model of CIA infection in pigs and then investigate the potential of postbiotics to decolonise pigs with CIA infection.

A model of CIA using an ESC-resistant strain of *E. coli* was successfully established. This model provided quantification of infectious load using laboratory robotics. Abundance of ESC-resistant *E. coli* peaked at day 14 pi. This model can potentially be adapted for various antimicrobial resistance strains and utilized in further studies.

No significant effect on reduction of this particular ESC-resistant *E. coli* strain was observed between control and postbiotic treated animals.

The recommendations based on Experiment 2:

1. Use of the model established can be used to test other methods of decolonisation and novel AMR control strategies such as targeted displacement of CIA-R *E. coli* by competitive exclusion clones, target specific bacteriophages (utilising a phage cocktail approach to address inherent resistance emergence), nutritional additives and removal of co-selection pressure.

2. Test the effect of postbiotics on decolonisation of alternative CIA strains, including virulent strains associated with post-weaning disease.

Experiment 3.

The effects of postbiotics on carriage and growth performance of pigs was investigated using high throughput robotics platforms (RASP). The major findings arising from this study were (i) that resistant *E. coli* carriage naturally reduces after weaning. High levels of ciprofloxacin resistance were detected at weaning which reduced over the course of the study (from 92% to 9%); (ii) postbiotics had no significant effects on the faecal concentrations of tetracycline-, ciprofloxacin- or ESC-resistant *E. coli* as well as no effect on growth performance of weaner pigs.

The recommendations based on Experiment 3:

1. To use the RASP in management of AMR on a herd level and in the assessment of novel strategies of decolonisation due to the quantitative resolution of the data produced.
2. Further investigation into the high prevalence of antimicrobial resistance at weaning and the impact antimicrobial use in persistence and colonisation of antimicrobial resistant bacteria.

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Appendix I – Publications Arising

Laird, T.J., Abraham, S., Jordan, D., Pluske, J.R., Hampson, D.J., Trott, D.J., O'Dea, M., 2021. Porcine enterotoxigenic *Escherichia coli*: Antimicrobial resistance and development of microbial-based alternative control strategies. Vet Microbiol 258, 109117.

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