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Gut microbiota dynamics in the weaner pig in response to experimental *Escherichia coli* challenge and dietary manipulation

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Declaration

I declare that this thesis is my own composition, and the research contained within it is my own work, except where acknowledged. The work that has been described in this thesis has not been submitted for any other degree or professional qualification.

Jolinda Pollock

Acknowledgements and Dedication

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Dedication

For Granddad - We did not have time for that beer, so I wrote this book for you instead.

John Robertson Dawson

Publications

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Abstract

The weaning transition period in pigs is linked to increased vulnerability to enteric disorders, which is partly attributed to destabilisation of the gut microbiota. Post-weaning colibacillosis is an economically important disease of the small intestine, which is most commonly caused by enterotoxigenic *Escherichia coli* (ETEC) strains. This disease has been variably linked to a diarrhoeal phenotype and decreased growth rate under clinical or sub-clinical conditions, and has been associated with shifts in particular bacterial populations using culturing methods. The emergence of next-generation sequencing technologies such as 16S rRNA gene metabarcoding now allows higher resolution study of complex microbial communities, without being reliant on the ability to culture fastidious micro-organisms.

As part of this project, a 16S rRNA gene metabarcoding method was developed and validated to allow qualitative and quantitative measurement of gut microbiota shifts. A series of experimental ETEC challenge trials were carried out to monitor temporal faecal microbiota dynamics (**Chapter 2**), to further understand ETEC adhesion and shedding dynamics (**Chapter 3**) and to study potential changes in both ileal and faecal microbiota populations in response to dietary protein manipulation (**Chapter 4**). The effects of experimental treatments on pig health and performance were also measured as part of each experiment.

Temporal shifts in ileal and faecal microbiota structure and stability were observed over the post-weaning period, as well as shifts in relative abundances

of particular bacterial phylotypes ($P < 0.05$) (**Chapter's 2 and 4**). ETEC challenge had no effects on faecal microbiota composition, pig health and performance when comparing to samples obtained from sham-challenged pigs ($P > 0.05$). However, when taking ETEC shedding level into account, variations in both microbiota structure and stability were observed at specific time points ($P < 0.05$) (**Chapter 2**).

After a single-dose ETEC challenge, ETEC adhesion in the ileum and faecal shedding were evident up to 4 and 6 days post-challenge, respectively (**Chapter 3**). Changes in ileal microbiota structure and stability were observed in response to ETEC challenge ($P < 0.05$), with no changes exerted at faecal level ($P > 0.05$). Additionally, different dietary protein levels were linked to changes in ileal microbiota structure, stability and phylotype relative abundances ($P < 0.05$). Interestingly, significant differences in ileal microbiota structure were evident in samples obtained from ETEC-challenged pigs fed the low and high protein diets, with the pigs fed the high protein diet having significantly less stable ileal communities at population level ($P < 0.05$) (**Chapter 4**). The treatments had no effect on host performance ($P > 0.05$), but faecal consistency scores were higher in pigs fed the high protein diet ($P < 0.05$).

In conclusion, both ETEC challenge and manipulation of dietary protein level had profound effects on ileal microbiota composition and faecal microbial communities were variable according to ETEC shedding status. These findings have implications for the development of alternative management strategies for enteric diseases in weaner pigs.

Lay summary

The weaning period in pigs is a sudden process in the farming industry, whereby piglets are removed from the sow and from easily digestible milk and immediately introduced to a solid, less digestible diet. Consequently, the weaner pig is susceptible to a variety of infections which occur in the gut. Post-weaning colibacillosis is such an infection, which is usually caused by enterotoxigenic *Escherichia coli* (ETEC), which is a group of bacteria that produce a variety of toxins with the potential to damage the gut. This disease can lead to symptoms such as diarrhoea and weight loss, and in severe cases can result in death, though pigs can remain asymptomatic. It is thought that this vulnerability to infections is partly due to changes in bacterial community composition in the gut (i.e. the gut microbiota), as a result of the many stressors endured during the weaning process.

As part of this project, a method was developed to allow qualitative and quantitative measurement of changes in the gut microbiota. A series of experiments were carried out to monitor bacterial shifts in the faeces over time in response to ETEC exposure (**Chapter 2**), to further understand ETEC binding in the small intestine and release into the faeces (**Chapter 3**) and to study potential changes in microbial populations in the small intestine and in the faeces in response to ETEC exposure, changes in dietary protein levels and their combination (**Chapter 4**). The effects of these treatments on pig health, weight gain and feed intake were also measured throughout all experiments.

Significant changes in microbiota composition occurred after weaning in samples from the small intestine and in faecal samples (**Chapter's 2 and 4**). Although ETEC exposure had no effect on the faecal microbiota composition, variations were observed when taking the numbers of ETEC bacteria in the faeces into account (**Chapter 2**). When studying the microbiota in the small intestine, significant changes in these bacterial communities occurred as a consequence of changing dietary protein levels and in response to ETEC exposure. Interestingly, the microbiota in the small intestine changed significantly in pigs exposed to ETEC that were fed low and high protein diets, which suggests that the combination of ETEC exposure and a high protein diet can further destabilise the small intestinal microbiota (**Chapter 4**).

In conclusion, the bacterial communities in the gut change markedly after weaning and are sensitive to ETEC exposure and dietary protein level. This has implications for the development of alternative management strategies for enteric diseases in weaner pigs.

List of abbreviations

16S rRNA	16S ribosomal ribonucleic acid
ADFI	Average daily feed intake
ADWG	Average daily weight gain
AMOVA	Analysis of molecular variance
bp	Base pairs
cfu	Colony-forming units
DGGE	Denaturing gel gradient electrophoresis
DMM	Dirichlet multinomial mixture
DNA	Deoxyribonucleic acid
EAST1	Enteroaggregative heat-stable enterotoxin
ETEC	Enterotoxigenic *Escherichia coli*
F4	F4-type fimbriae (previously known as K88)
HOMOVA	Homogeneity of molecular variance
LT	Heat-labile enterotoxin
m/s	Metres per second
NMDS	Non-metric multidimensional scaling
OTU(s)	Operational taxonomic unit(s)
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PWC	Post-weaning colibacillosis
rcf	Relative centrifugal force
rpm	Revolutions per minute
SD	Standard deviation
SEM	Standard error of the mean
STa	Heat-stable enterotoxin A
STb	Heat-stable enterotoxin B
T-RFLP	Terminal restriction fragment length polymorphism
qPCR	Quantitative polymerase chain reaction

List of tables

List of figures

Table of contents

Chapter 1:
Introduction

1.1 General Introduction

The weaning transition period in the pig (*Sus scrofa domesticus*) is associated with a variety of stressors, which have been linked to increased vulnerability to enteric disorders. Post-weaning colibacillosis (PWC) is such an enteric disorder, which is most commonly caused by enterotoxigenic *Escherichia coli* (ETEC) strains. PWC is an economically important disease of the weaner pig, which can lead to a decreased growth rate, morbidity and mortality in severe cases (Fairbrother et al., 2005).

Traditionally, enteric disorders such as PWC were managed by using in-feed antimicrobials at prophylactic levels, until a ban was placed by the European Union in 2006 (European Union, 2005). Due to increasing concerns about dissemination of bacteria which are resistant to key antimicrobials, efforts are also now being made to reduce overall usage of antimicrobials in agricultural systems worldwide (Vondruskova et al., 2010).

Consequently, there is now a focus on developing alternative management strategies for enteric disorders such as PWC, including dietary manipulation and the use of probiotics. Such alternative strategies aim to influence microbial populations in the gut - the gut microbiota - which are a series of diverse, complex microbial communities. In recent years, DNA sequencing technologies have advanced which allows the study of complex microbial communities with greater resolution. Therefore, it is now timely to explore gut microbiota dynamics in the weaner pig to establish whether novel disease management

strategies can influence microbial populations and host performance in the face of ETEC challenge.

This chapter will introduce the key stressors associated with the weaning process which have an impact on gut health. Information on the development of the porcine gut microbiota from suckling through to the growing/finishing period will then be discussed. Post-weaning colibacillosis, the disease studied as part of this project, will be described with a focus on experimental models and the approaches being investigated as alternatives to antimicrobials. Finally, the technical aspects of how to study complex microbial communities will be presented, with an emphasis on 16S rRNA gene sequencing.

1.2 Weaning the pig

Weaning in wild pigs is a naturally slow process, but modern farming methods demand early and often sudden weaning. The weaning process is a stressful event for the pig, since they are suddenly forced to adapt in response to social, nutritional and immunological disturbances (Kim et al., 2012b). Although ongoing research and current farm-level management strategies aim to minimise the impact of such weaning stressors, interest in the study and "optimisation of gut health" of the weaner pig is a relatively new area (Pluske et al., 2003a). Key aspects of the weaning period which are linked to gut health will be discussed in this section.

1.2.1 Weaning age

The weaning age of pigs has been reduced from around 8 weeks in the 1950s and 1960s, to an average weaning age of 22-26 days in 2003 (Pluske et al., 2003a). Other authors have reported that weaning normally takes place between 16 and 30 days of age in most pig producing systems worldwide (Katouli and Wallgren, 2005; Varley and Wiseman, 2001). Early weaning has been associated with a more rapid onset and severity of diarrhoea in response to pathogen challenge, with growth rate being significantly reduced in early-weaned challenged pigs (i.e. at 16 and 18 days of age) (McLamb et al., 2013). Levast et al. (2010) showed that "ultra-early weaning" (i.e. at 7 days of age) was associated with a reduction in serum IgA concentration and induction of Th17 cells in the mesenteric lymph nodes, which could be related to changes in mucosal barrier function as a consequence of early removal from milk.

Importantly, previous research has shown that early weaning has an impact on gut structure and function. Moeser et al. (2007) have shown that earlier weaning (i.e. 15-21 days) can have a long-term impact on mucosal barrier function, since these pigs showed increased intestinal permeability, in comparison with pigs weaned later (i.e. 23-28 days). Further work by this group showed that the pigs weaned early showed impaired intestinal barrier function, which was explained by reductions in jejunal transepithelial electrical resistance in early-weaned pig gut tissue (Smith et al., 2010).

1.2.2 Housing

On separation from the sow, weaner pigs are handled, transported and mixed with piglets from other litters in a different physical environment. These stressors have been shown to increase both cortisol release and the expression of its receptor in the weaned pig intestine (Moeser et al., 2007). Post-weaning villus atrophy has been linked with the stress of moving the pigs from their sow and also moving them to different pens (Kelly and King, 2001). Villus atrophy can lead to a variety of problems, including malabsorption, dehydration, diarrhoea and high susceptibility to enteric infections (McCracken et al., 1999; Zijlstra et al., 1999). Crowding stress is another factor at this vulnerable time and in combination with pathogen challenge, has been shown to have a detrimental effect on feed intake and growth rate (Khafipour et al., 2014).

The importance of a stable environmental temperature has been highlighted by Laine et al. (2008). The authors found that the risk of post-weaning diarrhoea was reduced in the presence of a temperature-controlled environment and with a reduction in herd size. Earlier work also described that a stable environmental temperature has a positive effect on the health and performance of piglets in the first two weeks post-weaning (Le Dividich and Herpin, 1994) and that temperature fluctuations are associated with an increase in incidence of post-weaning diarrhoea (Le Dividich, 1981).

1.2.3 Feed intake

At weaning, the piglet is often abruptly removed from highly digestible milk and introduced to a less digestible, less palatable solid diet. Consequently, piglets may reduce their energy intake (Campbell et al., 2013) or completely refrain from eating (Kluess et al., 2010; Madec et al., 1998). It has been estimated that by the end of the first week after weaning, the metabolisable energy intake is only 60-70% of that consumed during the suckling period and that it takes a further two weeks to recover to the pre-weaning energy intake level (Campbell et al., 2013). The extent and duration of this reduction in energy intake is highly variable, as discussed in a review by Le Dividich and Sève (2000). A decreased feed intake has been linked to an increase in villus atrophy (van Beers-Schreurs et al., 1998), which may in part be explained by less energy being available for the maintenance of epithelial cell structure (Pluske et al., 1996). Villus atrophy and crypt hyperplasia have been shown to cause a decrease in digestive and absorptive capability within the small intestine (studies reviewed by Pluske et al., 1997) and exacerbate post-weaning diarrhoea.

Madec et al. (1998) carried out a cohort study to establish risk factors for the proliferation of post-weaning enteric disorders and found that low feed intake in the first week post-weaning was strongly correlated to the incidence of post-weaning diarrhoea. Despite the observed detrimental effects of low feed intake in weaner pigs, restrictive feeding after weaning has successfully been used to minimise the incidence and severity of diarrhoea (Ball and Aherne, 1982; Ball and Aherne, 1986; Rantzer et al., 1996). However, contrasting results have been

observed whereby episodes of post-weaning diarrhoea increased in pigs which were fed a restricted diet (Laine et al., 2008).

1.3 The gut microbiota

The "microbiota" has been defined as "the ecological community of commensal, symbiotic and pathogenic micro-organisms that literally share our body space" (Lederberg and McCray, 2001). The "gut microbiota" is a broad term for the series of complex microbial communities which are present throughout the gastrointestinal tract.

1.3.1 The importance of the gut microbiota

The gut microbiota has co-evolved with the host for millennia and has a variety of roles in the maintenance of host health. Several scientific approaches have been used to demonstrate the importance of commensal bacteria, specifically in the development of gut structure and the immune system. Among them, the study of germ-free animals has revealed both structural and functional defects in the gut which have been linked with immune system immaturity (Macpherson and Harris, 2004) and higher susceptibility to infection (review by Round and Mazmanian, 2009). Additionally, some authors have manipulated the gut microbiota through administration of antimicrobials. Exposure to antimicrobials has been shown to increase susceptibility to infection, with more profound microbial dysbiosis and intestinal damage post-infection (Sekirov et al., 2008) and alterations in immune cell homeostasis (Hill et al., 2010).

The epithelial layer in the intestinal mucosa encounters the largest microbial challenge of all body regions (McCracken and Lorenz, 2001). Arguably, one of the most important functions of the gut microbiota is to provide protection to the host against infection (Kim et al., 2011; Richards et al., 2005). The precise mechanisms by which protection is elicited are not fully understood, however production of antimicrobial compounds (including bacteriocins), direct stimulation of the immune system and competition for nutrients appear to be key mechanisms in this process (Corr et al., 2007; Ewing and Cole, 1994; Richards et al., 2005; Rolfe, 1996).

1.3.2 The development of the porcine gut microbiota

Bacterial colonisation of the porcine gut, like in other mammals, occurs at birth. Colonisation is influenced by a variety of factors, such as the surrounding environment, the mode of delivery, age of gestation and genetics (Bezirtzoglou, 1997; Ley et al., 2008). It is well established that there is spatial variability in the gut microbiota (Looft et al., 2014) and variation is present when comparing luminal and mucosal samples (Mann et al., 2014). Therefore, current literature on the porcine gut microbiota (at small intestinal and faecal level) during the suckling, weaning and growing/finishing periods will be discussed in this section.

1.3.2.1 Suckling period

The suckling pig's microbiota is established early on through a series of colonisation events, which appear in a similar manner in chicks, calves and humans (Mackie et al., 1999). Aerobic bacteria and facultative anaerobes

(including *Escherichia coli*, streptococci and lactobacilli) colonise immediately after birth after exposure to the sow's vaginal, faecal and skin microbiota and the external environment (Katouli and Wallgren, 2005; Mackie et al., 1999; Moughan et al., 1992). As a consequence, at this time, it has been shown that there is a high level of similarity between the sow and piglet gut microbiota (Katouli et al., 1997). The rapid proliferation of aerobic and facultative anaerobic bacteria leads to a decrease in oxygen availability in the intestine (Richards et al., 2005). This leads to the establishment of obligate anaerobes, which become part of the core bacterial community in the porcine gut (Inoue et al., 2005).

During the suckling period, a selective advantage is provided to particular groups of micro-organisms, such as *Bifidobacterium* and *Bacteroides* species, which can utilise milk. This has been well described in humans (Marcobal et al., 2011; Sela and Mills, 2011; Ward et al., 2007) but less so in pigs (Bian et al., 2016; Frese et al., 2015). Particular bacterial groups appear to dominate during the suckling period and decrease in numbers during later growth phases. Specifically, Holman and Chénier (2014) found that suckling pigs had a significantly higher proportion of Enterobacteriaceae in comparison with other growth phases. This finding was also presented in earlier work by Pieper et al. (2006) and is of particular interest, since this bacterial family contains pathogenic micro-organisms such as *Escherichia coli* and *Salmonella* species. Other dominant members of the suckling pig gut microbiota are lactobacilli which have been found in high numbers in all gut compartments prior to

weaning (Konstantinov et al., 2004, 2006; Pieper et al., 2006), as well as streptococci (Konstantinov et al., 2006). Indeed, recent work has shown that the suckling pig microbiota remains relatively stable over the first few weeks of life and has been termed a "milk-orientated microbiome" (Frese et al., 2015).

1.3.2.2 Weaning period

The transition into the weaning period has been associated with significant changes in microbiota composition, mostly attributed to the sudden withdrawal of milk and introduction to a solid diet (Frese et al., 2015). This solid diet has an increased chemical complexity, which provides a more suitable energy source to a greater range of bacterial species (Katouli et al., 1997; Konstantinov et al., 2006; Mackie et al., 1999). During the first week of the weaning transition period, the gut microbiota becomes destabilised with a significant decrease in bacterial diversity (Wallgren and Melin, 2001) and cultivable bacteria (Franklin et al., 2002). It is thought that this drastic change in the gut microbiota causes the piglet to become more vulnerable to gastrointestinal and respiratory diseases (Hopwood and Hampson, 2003). For example, post-weaning diarrhoea tends to occur in the weaner pig 3-10 days after weaning (Fairbrother et al., 2005; Hampson, 1994). At 2-3 weeks post-weaning, the microbiota begins to re-stabilise (Jensen, 1998) and an increase in microbial diversity has been observed (Inoue et al., 2005).

Bacteroides species are able to utilise monosaccharides and oligosaccharides which are present in the sow's milk. The Bacteroidaceae appear to be superseded by the Prevotellaceae during the weaning period, the former being

one of the most abundant families prior to weaning (Frese et al., 2015; Pajarillo et al., 2014). Prevotellaceae have been shown to increase 50-fold after weaning from an average of 0.3% in suckling piglets to 14.8% in weaner pigs (Frese et al., 2015). The abundance of Prevotellaceae in the weaner pig gut has been confirmed by many other studies (Hong et al., 2011; Kim et al., 2011, 2012a; Leser et al., 2002; Looft et al., 2012; Pajarillo et al., 2014). *Prevotella* species are widely associated with the breakdown of plant polysaccharides, which are rich in the diet post-weaning (Ivarsson et al., 2014).

The withdrawal of milk has also been associated with a maintained (Pajarillo et al., 2014) and an increased level (Frese et al., 2015) of *Lactobacillus* species in faeces. Members of the *Lactobacillus* species have the ability to consume plant-derived monosaccharides and disaccharides and simple milk sugars such as lactose, but not complex milk sugars (Schwab and Gänzle, 2011; Ward et al., 2006). However, a decrease in absolute and relative levels of lactobacilli has been shown in small intestinal samples post-weaning (Konstantinov et al., 2006; Pieper et al., 2006; Su et al., 2008), as well as a decrease in richness and diversity of *Lactobacillus* species immediately post-weaning (Janczyk et al., 2007).

Although there are overlapping members of both the ileal and faecal microbiota, such as Enterobacteriaceae (Dowd et al., 2008; Frese et al., 2015; Hong et al., 2011; Jensen, 1998; Leser et al., 2002; Pieper et al., 2006), Clostridiaceae (Dowd et al., 2008; Frese et al., 2015; Kim et al., 2015; Leser et al., 2002; Mulder et al., 2009) and Bacteroidaceae (Dowd et al., 2008; Frese et al., 2015; Leser et al.,

2002; Mulder et al., 2009; Swords et al., 1993), there are large variations in the relative and absolute abundances of these taxa across gut sections. This highlights the importance of studying multiple gut compartments in microbiota studies, particularly if the aim of the study is to explore the functional roles of the microbiota.

1.3.2.3 Growing and finishing period

Moving into the growing period, a core microbiota or "climax community" is established due to a series of further bacterial succession events (Isaacson and Kim, 2012; Simpson et al., 2000). This core bacterial community appears to maintain a relatively stable association with the host. This is presented by Kim et al. (2015), whereby a close overlap between grower and finisher pigs was shown, with 80% of operational taxonomic units being shared. However, microbial shifts will still occur as a result of further microbial exposure, disease episodes, stress and dietary alterations (Conway, 1996; Kim et al., 2011).

Prevotellaceae remain dominant in the faecal microbiota of growers/finishers, which has been described in several studies (Heinritz et al., 2016b; Kim et al., 2011, 2012a; Lamendella et al., 2011; Leser et al., 2002; Looft et al., 2012). Lamendella et al. (2011) carried out one of the first 16S rRNA gene sequencing studies, aiming to explore the faecal microbiota in 8 pigs at 6 months of age. *Prevotella* species were found to be the most abundant species, comprising 22% of the total number of sequences. Kim et al. (2011) also found that *Prevotella* was the most abundant member of the microbiota at 10 weeks of age, with 30% of classifiable bacteria being a member of this genus. However, as the pigs aged,

a consistent decrease in *Prevotella* was observed and at 22 weeks of age, only 3.5-4% of classifiable bacteria belonged to the *Prevotella* genus.

Another core feature of the grower/finisher pig faecal microbiota are members of the Lactobacillales order (Lamendella et al., 2011), which includes the *Lactobacillus* and *Streptococcus* genera. *Lactobacillus* has been identified as a key member of the adult pig ileal (Leser et al., 2002; Mølbak et al., 2008) and faecal (Leser et al., 2002) microbiota. Kim et al (2011) found that as pigs aged, a consistent decrease in *Lactobacillus* was observed in faecal samples. *Streptococcus* has been found to represent 5% of the total number of bacterial sequences in faecal samples (Lamendella et al., 2011).

Clostridiales again appear to dominate in the grower/finisher phase in both small intestinal and rectal samples (Heinritz et al., 2016b; Kim et al., 2015; Lamendella et al., 2011; Leser et al., 2002; Pajarillo et al., 2015; Wang et al., 2007). Specifically, Heinritz et al. (2016) found that Clostridiaceae, Lachnospiracaeae, Ruminococcaceae and Veillonellacaeae were abundant in faeces (the former three reflecting previous cloning work by Wang et al., 2007), with a significant increase in Veillonellaceae being observed over this growth phase, echoing a previous study (Kim et al., 2015). Lamendella et al. (2011) found that *Clostridium* represented at least 5% of the bacterial sequences in grower/finisher pig faeces and *Clostridium* has also been found to be a dominant member of the small intestinal microbiota (Leser et al., 2002; Metzler-Zebeli et al., 2010a; Mølbak et al., 2008).

Other members of the Clostridiales order appear to be dynamic during this growth phase, with consistent increases in levels of *Oscillibacter*, *Sarcina* and *Sporacetigenium* and *Anaerobacter* species being observed (Kim et al., 2011), with *Anaerobacter* becoming the most abundant member of the community as *Prevotella* decreased. Looft et al., (2012) described the faecal microbiotas of three pigs which were sampled at 18 and 20 weeks of age. The findings showed some overlap with the Kim et al. (2011) study, whereby the dominant genera were found to be *Anaerovibrio*, *Succinivibrio*, *Oscillibacter*, *Parabacteroides*, *Hallella*, and *Coprococcus* species. *Oscillibacter*, for example, is a bacterium capable of producing valerate (Iino et al., 2007) which has been established as an important short-chain fatty acid which is linked with increased feed efficiency in cattle (Guan et al., 2008). This may suggest that this bacterium may also have an important digestive role in the pig (Pajarillo et al., 2015). However, many of the bacteria mentioned which have been consistently linked with the grower/finisher pig microbiota have not been studied further to establish the potential role of these members in host health and digestion.

1.4 Post-weaning colibacillosis

PWC is a multifactorial disease (Hampson, 1994; Madec et al., 2000) primarily caused by ETEC strains (Francis, 1999). In this section, relevant ETEC pathotypes will be discussed before describing PWC as observed in the field and as part of experimental challenge models.

1.4.1 Enterotoxigenic *Escherichia coli*

1.4.1.1 ETEC in farm animals

Enterotoxigenic *Escherichia coli* (*E. coli*), or ETEC, is an important causative agent of diarrhoeal diseases in both humans and farm animals worldwide. The key virulence factors which define ETEC strains are adhesins (which mediate bacterial attachment and initiate colonisation) and enterotoxins (which disrupt fluid homeostasis in the small intestine) (Duan et al., 2012). Enteric diseases caused by ETEC strains on-farm mostly occur as colibacillosis in both pigs and calves and a general characteristic of ETEC infections is host specificity due to the expression of specific receptors in a limited number of animal species (Fairbrother et al., 2005; Nagy and Fekete, 1999). However, a recent study highlighted that ETEC strains containing porcine-specific virulence factors were found in water and sediment samples after a manure spill, showing potential for transmission via the environment to animals and even humans (Haack et al., 2015). In calves, the vast majority of ETEC strains which cause diarrhoea are linked with the F5 adhesin and STaP enterotoxins, and ETEC strains causing diarrhoea in post-weaned pigs are linked with the F4 adhesin and STa, STb and LT toxins (Nagy and Fekete, 1999).

1.4.1.2 ETEC virulence factors linked with PWC

Adherence to porcine enterocytes is mediated by colonisation factors, such as flagella (Zhou et al., 2013), fibrilliae and fimbriae (**Figure 1.1**). In porcine ETEC strains, F4 fimbriae (previously known as K88 fimbriae) are an important colonisation factor associated with infection in weaner pigs (Nagy and Fekete,

1999) and are expressed by the majority of colibacillosis-associated isolates (de la Fé Rodríguez et al., 2011; Moon et al., 1999; Wang et al., 2006). F4 are flexible fimbriae, which have three naturally occurring antigenic variants – F4ab, F4ac and F4ad – with differing primary sequences of the major adhesive subunit (*faeG*) that forms the major structural component of the F4 fimbriae. The F4ac-type is by far the most common variant associated with PWC outbreaks worldwide (Fairbrother et al., 2005), with 96% of isolates (Choi and Chae, 1999) and 98% of isolates (Alexa et al., 2001) being found to carry the F4ac fimbrial genes. One of the critical factors in the host that has been established for ETEC pathogenesis is the presence of the F4-receptor. However, Madec et al. (2000) found that one-third of pigs that did not express the F4-receptor did develop diarrhoea, showing that this is not the only key virulence adhesin.

ETEC strains are associated with the production of heat stable (ST) and heat labile (LT) toxins (**Figure 1.1**). Studies describing ETEC isolates from young animals, as well as those of young and adult humans, have shown that there is a considerable amount of variation in terms of the enterotoxins produced. For example, STa is typically the sole enterotoxin expressed by ETEC-infected lambs and calves, and LT is the only toxin expressed by ETEC strains causing diarrhoeal disease in chickens (Zhang et al., 2006). STb is predominantly associated with porcine ETEC strains only, however it has occasionally been detected in ETEC strains of human origin (Fairbrother et al., 2005).

In porcine ETEC strains, a series of enterotoxin combinations are produced, whereby the most common strains express both LT and STb (with or without STa) (Moon and Bunn, 1993). A study in the United States examined the pathotypes of porcine ETEC strains causing PWC from 1999 through 2001, and found that half of all strains isolated possessed genes for expression of F4 fimbriae, LT and STb (Francis, 2002). Interestingly, Berberov et al. (2004) found that gnotobiotic pigs exposed to ETEC LT knockouts showed a reduced severity of diarrhoea, as well as a reduction in colonisation. It has been proposed that LT stimulates the adhesion of ETEC to porcine intestinal cells *in vitro* (Johnson et al., 2009) and that LT enhances ETEC colonisation in gnotobiotic piglets (Zhang et al., 2006). Zhang et al. (2006) also showed that the effect of LT on the host was significantly greater than that of STb, with previous work by Casey et al. (1998) showing that STb did not significantly contribute to diarrhoeal symptoms in ETEC-infected piglets.

Enteroaggregative heat-stable enterotoxin (EAST1) is a low molecular weight enterotoxin, which belongs to the STa family. EAST1 was discovered as a product of diarrhoea-causing *E. coli* isolated from humans, but has since been shown to be widespread among porcine ETEC strains (Choi et al., 2001; Ngeleka et al., 2003; Noamani et al., 2003; Osek, 2003; Yamamoto and Nakazawa, 1997)

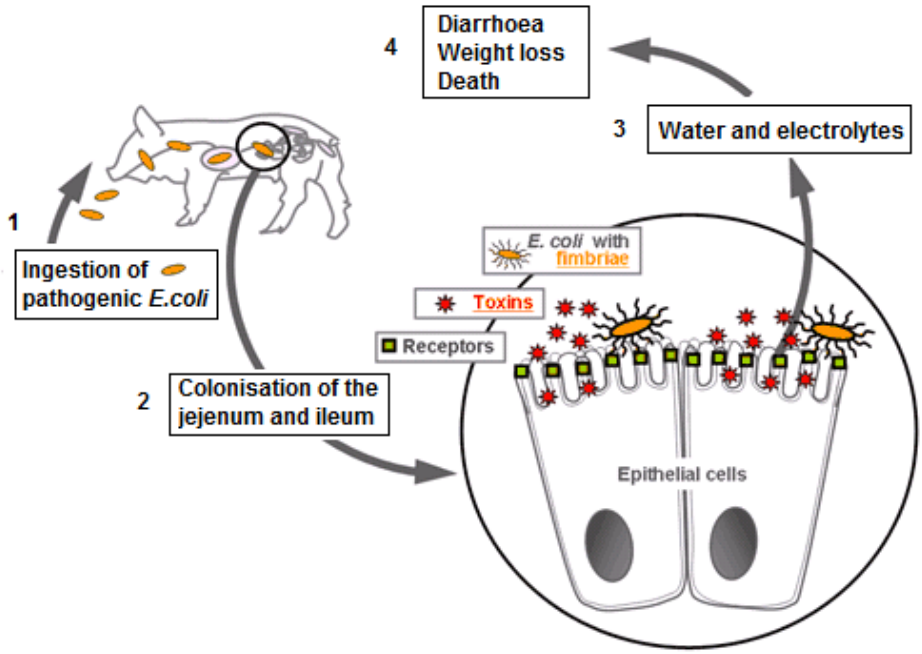

Figure 1.1: Diagram explaining the pathogenesis of enterotoxigenic *Escherichia coli*. Pathogenic bacteria are ingested from the environment and travel to the gastrointestinal tract (1), whereby fimbrial adhesins mediate the adherence of the bacteria to specific receptors in the jejenum and/or ileum (2). Bacteria then produce enterotoxins, which leads to water and electrolyte loss into the intestinal lumen (3). This can lead to dehydration and in extreme cases can lead to death (4). Image modified and reproduced with permission from The *Escherichia coli* Laboratory (EcL), The University of Montréal.

Specifically, a high prevalence of *astA* (i.e. the gene that confers the expression of EAST1) has been found in diarrhoeic pigs (Frydendahl, 2002; Kim et al., 2010; Noamani et al., 2003; Osek, 2003; Vu-Khac et al., 2007) and also in *E. coli* isolated from non-diarrhoeic pigs (Ngeleka et al., 2003; Vu-Khac et al., 2007). To date, the role of EAST1 in ETEC pathogenesis (specifically in PWC) is not well understood (Francis, 2002; Kim et al., 2010; Vu-Khac et al., 2007).

1.4.2 Natural cases of PWC

1.4.2.1 Diagnosis and presentation of PWC

PWC was first described by Richards and Fraser (1961), whereby a proliferation of beta-haemolytic *E. coli* was observed in the proximal small intestine of diseased pigs. In later studies, large numbers of ETEC bacteria (up to 10^9 colony-forming units (cfu) per gram of tissue) have been detected from the mid-jejenum through to the ileum (Fairbrother et al., 2005) and the ileum only (Francis, 1983) of infected pigs. Growth of ETEC bacteria has been observed on the apical surface of villus epithelial cells in the ileum, and less consistently, in the jejenum (Sarmiento et al., 1988), with mild to moderate intestinal hyperaemia being observed (Moxley and Duhamel, 1999). Generally, ETEC-infected pigs do not exhibit observable lesions (Nagy and Fekete, 1999) aside from those which are associated primarily with dehydration (Francis, 2002). Since PWC is a disease of the small intestine, definitive diagnosis must come from histopathological examination of the organ itself and usually involves culturing specimens from the ileum or preparing Gram-stained impression smears of the mucosal surface, with or without DNA-based screening for key virulence factors (Francis, 2002).

The symptoms which present as part of PWC range from faecal shedding of ETEC with no diarrhoea to peracute fatal diarrhoea (Hodgson and Barton, 2009). The disease can be present at a sub-clinical level, whereby diarrhoea is absent but a reduction in performance occurs post-weaning (Hampson, 1994). In cases of clinical disease, piglets commonly develop yellow/grey diarrhoea

which begins 3-10 days post-weaning and normally lasts for 1-5 days (Fairbrother et al., 2005; Hampson, 1994). Pigs affected by the disease may also suffer from loss of appetite, show a rough coat, develop a swollen abdomen, appear depressed and begin to shiver (Hampson, 1994; Hodgson and Barton, 2009).

1.4.2.2 Impact on the farming industry

It has been observed that cases of PWC are increasing worldwide (Fairbrother et al., 2005). Although the factors involved with this increase are not currently understood, increases of disease incidence may be partly attributable to the in-feed antimicrobial ban enforced by the European Union (Casewell et al., 2003), which is discussed later in this chapter. The disease can cause substantial morbidity and mortality in infected animals, thereby creating potential for significant economic losses (van Beers-Schreurs et al., 1992; Elsinghorst, 2002; Fairbrother et al., 2005). Amezcua et al. (2002) found that mortality (on average) increased from 2% to 7%, as a consequence of post-weaning diarrhoea. However, in severe cases, mortality rates were as high as 20-30% over a period of 1-2 months. In the same study, it was reported that daily growth rates were lower on farms that had problems with PWC in comparison with farms that were clear of the disease. In surviving pigs, some remain chronically affected and consequently fail to thrive (Madec et al., 2000).

1.4.2.3 Treatment and prevention strategies

The reduction in the use of prophylactic antimicrobials worldwide means that there has been an associated increase in therapeutic use of aminoglycosides,

trimethoprim-sulphamethoxazole, macrolides and lincosamides for the treatment of PWC (Fairbrother et al., 2005).

Less is known about prevention strategies for PWC in comparison to neonatal colibacillosis and ETEC F4 strains are generally not easily controlled by prophylaxis due to the emergence of antimicrobial resistance (Fairbrother et al., 2005). Therefore, alternative management strategies are being explored such as vaccination, changes in animal management, selective breeding of ETEC F4-resistant pigs and dietary manipulation and the administration of probiotics (Fairbrother et al., 2005; Laine et al., 2008), with the latter two being discussed later in this review.

1.4.3 Experimental modelling of PWC

Due to the economic importance of PWC, many attempts have been made to model the disease in a controlled, experimental setting. Only a small number of ETEC serotypes have been used in such experiments, which is mostly due to the difficulty in reproducing the disease in an experimental environment to imitate disease as observed in the field (Fairbrother et al., 2005). Attempts to reproduce PWC experimentally have involved the use of a variety of different methods, including different inoculation doses, methods of inoculation, varying ages of inoculation and presence/absence of pre-treatment, such as antimicrobial administration or a pre-infection. The authors of such studies have described a wide range of disease states of varying severity and duration

(Cox et al., 1991; Krsnik et al., 1999; Molist et al., 2012; Montagne et al., 2004; Opapeju et al., 2009; Rossi, 2012; Wellock et al., 2008a).

Both Cox et al. (1991) and Madec et al. (2000) carried out extensive studies to develop a reproducible, clinical PWC model. Cox et al. (1991) carried out several experiments with different combinations of pre-treatments prior to exposure to ETEC. In the first two experiments (i.e. ETEC inoculation only and pre-treatment with chloramphenicol followed by ETEC inoculation), consistent episodes of diarrhoea were not observed. Later work by the same group also failed to produce diarrhoeal disease after pre-treatment with florfenicol and administration of 10^{10} cfu of ETEC F4 (Verdonck et al., 2005). However, Cox et al. (1991) carried out a final experiment with a variety of pre-treatments/infections (i.e. pre-treatment with chloramphenicol, inoculation with transmissible gastroenteritis virus, followed by ETEC inoculation) which resulted in reproducible induction of diarrhoea (93%) and dehydration resulting in an 80% mortality rate. Indeed, other authors also utilised pre-infections (Nakamine et al., 1998) and have shown an increased severity of disease which led to a high mortality rate after a short period of diarrhoea.

Madec et al. (2000) carried out 6 sequential experiments which contained 23 treatment groups, with 17 of these groups being challenged orally or intragastrically with ETEC F4. These challenge events were carried out as a single infection or as multiple dosing events, with the inoculation doses ranging between 10^{8} and 10^{12} cfu. Across the whole study, 14.5% of challenged pigs developed a severe illness and died, with gastrointestinal morphology being

indicative of PWC. Transient diarrhoea was induced a few hours after challenge in 50% of the weaner pigs exposed, with the average duration of diarrhoea being 1.7 days. However, when the authors increased the infection dose to 10^{12} cfu, 10 of the 16 piglets died, showing that the severity of infection was primarily linked with the inoculation dose.

In addition to work on the induction of clinical disease, work has been published on sub-clinical infection models of PWC, which are defined here as experimental models which did not induce severe or prolonged diarrhoea but did have a negative effect on performance parameters. Opapeju et al. (2009) carried out an ETEC challenge study to explore the impact of dietary protein level on pig performance and health. The pigs were administered 5 x 10^{10} cfu by oral gavage, and diarrhoea was not observed in any of the challenged pigs. However, there was an impact on pig performance in challenged pigs which were fed a high protein diet, with a 3% reduction in average daily weight gain being observed from day 3 to day 7 of the trial.

Work in our group has been carried out which also implements sub-clinical models of PWC (Athanasiadou et al., 2010; Houdijk et al., 2007), particularly in the context of dietary management of the disease. Houdijk et al. (2007) administered 10^{8} cfu of ETEC on day 5 post-weaning to establish whether this had an impact on pig performance, health and levels of acute phase proteins, specifically haptoglobin (Hp) and C-reactive protein (CRP). There was no impact on diarrhoeal score, but an 11% reduction in growth rate was evident post-challenge with a mean 3-fold increase in Hp and a mean 10-fold increase in

CRP. Wellock et al. (2008a) carried out a study to examine the effect of weaning age and protein source on gut health in the presence of ETEC challenge (10^9 cfu on day 3 post-weaning). After challenge, prolonged ETEC shedding in the faeces was observed and feed intake and growth rate were impaired (Wellock et al., 2008b) with a transient effect on faecal consistency and cleanliness scores.

1.5 Management strategies for PWC

As previously stated, the rise in PWC cases (Fairbrother et al., 2005) has been partly attributed to the ban of food animal growth-promoting antimicrobials in the EU (Casewell et al., 2003), since such compounds also exhibited prophylactic activity. Consequently, alternatives to antimicrobials are now being explored to improve weaner pig gut health, with key methods being dietary manipulation and administration of probiotics. The use of antimicrobials and antimicrobial resistance patterns will be discussed in this section, with previous work on dietary and probiotic strategies being reviewed.

1.5.1 Antimicrobials and resistance

Antimicrobials have been used in swine production for over 50 years at sub-therapeutic levels to improve growth rate (Hodgson et al., 2009), and the mechanisms by which this occurs are still not well understood. However, with the observed increase in antimicrobial resistance in bacterial isolates from livestock, the use of particular growth promoting antimicrobials was placed under restriction by the EU starting in 1997 (Casewell et al., 2003) and a complete ban on all prophylactics in feed was put in place on 1st January 2006

(European Union, 2005). This ban was implemented due to a combination of consumer and political opinion, with scientific concern that resistance conferred to bacteria in livestock may be transmitted to humans, either directly (resistant bacteria) or indirectly (resistance genes) with a negative impact on human health (Casewell et al., 2003).

Since the ban on use of growth promoters, it has become clear that these also had a prophylactic effect and an increase in the use of therapeutic antimicrobials has been partly linked to this ban. In the UK, a report was published in 2014 which showed that the number of tonnes of antimicrobials sold specifically for treatment in pigs has increased from 47 tonnes/active substance in 2010 to 64 tonnes/active substance in 2014 (Veterinary Medicines Directorate, 2014). Additionally, this report showed that overall sales of macrolides, fluoroquinolones and 3rd and 4th generation cephalosporins have increased from 2008 to 2014 for use in food producing animals, which are important antimicrobials in human medicine (Collignon et al., 2009).

In the specific case of ETEC F4 isolates collected in Canada, the presence of antimicrobial resistance genes has risen between 1978 and to 2000, with the number of organisms being resistant to at least 3 antimicrobials increasing over time (Maynard et al., 2003). Interestingly, the isolates were most commonly resistant to tetracyclines and sulphonamides which are commonly used as growth promoters in Canada and the USA (Hodgson et al., 2009).

Soluble antimicrobials administered in water (such as apramycin and neomycin) and injectable antimicrobials (such as apramycin and trimethoprim-sulphamethoxazole) are frequently used on farms with problems with PWC (Amezcua et al., 2002). Indeed, *E. coli* isolates linked to PWC in pigs have demonstrated multi-drug resistance to antimicrobials including apramycin, co-trimoxazole, spectinomycin and neomycin (Amezcua et al., 2002; Fairbrother et al., 2000; Lanz et al., 2003; Maynard et al., 2003). According to Fairbrother et al. (2000), one of the most commonly used antibiotics for PWC treatment is apramycin administered in water, and a study by Amezcua et al. (2002) showed that 23.5% of *E. coli* isolates were resistant to apramycin with no resistance to amikacin being evident, which is a drug that is not normally used in swine production. Additionally, Amezcua et al. (2008) also found that co-trimoxazole was the most commonly used injectable antimicrobial on farms with high levels of PWC, with farms being affected by PWC using more injectable antimicrobials than farms not affected by PWC, which poses a significant cost to the farmer.

1.5.2 Dietary manipulation

The known, complex interactions between dietary composition, the gut microbiota and host health has led to many studies which have been focussed on manipulation of standard diets to maximise the health and performance of weaner pigs (Hodgson et al., 2009). A change in feeding behaviour has been found to be one of the most important influences on the gut microbiota (Rist et al., 2013) and specifically, alterations in dietary protein and non-starch polysaccharide levels have been shown to affect the proliferation of disease.

1.5.2.1 Dietary protein

The source and the level of dietary protein provided in the diet have been found to have an effect on the enteric health of piglets (Kim et al., 2012b), which is associated with changes in fermentation levels and microbial ecology (Rist et al., 2013). The young piglet is unable to fully digest dietary proteins, due to the presence of underdeveloped proteolytic enzyme systems just after weaning (Pluske et al., 2003b). Consequently, bacterial fermentation occurs in the small intestine, which leads to an increase in intestinal pH, proliferation of pathogens and subsequent production of irritant by-products such as ammonia, phenols and biogenic amines (Halas, 2007; Hodgson and Barton, 2009).

As a result, studies have been carried out to examine the effect of lowering protein levels in weaner pig diets on enteric health. Bikker et al. (2006) showed that ammonia concentrations in the small intestine decreased in pigs fed a low crude protein diet, in comparison to pigs fed a high crude protein diet. It was concluded that protein fermentation was reduced in response to a decreased dietary protein supply. Heo et al. (2010) showed that blood plasma urea nitrogen levels and ammonia nitrogen content in the gastrointestinal tract were reduced in response to a lower protein diet. In the same study, it was presented that nitrogen digestibility at the terminal ileum was not affected by dietary protein. However, in animals infected with ETEC, nitrogen digestibility at the terminal ileum was impaired, which resulted in an increase of ileal nitrogen flow and fermentation of by-products in both the small and large intestine. It

has been hypothesised that reducing dietary protein levels lowers nitrogen flow in the gut, and thereby protein fermentation is lowered (Nyachoti et al., 2006).

The effect of dietary protein level on ETEC pathogenesis has been studied during controlled enteric challenge. Opapeju et al. (2009) carried out an ETEC challenge model study on weaner pigs fed a corn-soybean meal-fish meal diet supplemented with either 225 or 176g/kg of crude protein. Three days post-challenge, ETEC was not isolated from pigs fed the lower crude protein diet, whereas ETEC was isolated from the ileal digesta in 80% of the pigs fed the higher crude protein diet. Wellock et al. (2008a) also found that by reducing dietary protein from 230 to 130g/kg in both soy bean meal-based or dried skimmed milk powder-based diets, lower levels of ETEC were isolated from weaner pig faeces.

Studies have also been carried out to assess whether protein quality has an impact on severity of PWC disease. Owusu-Asiedu et al. (2003) orally challenged pigs with ETEC that were fed either a plant protein (pea protein) or animal protein (spray-dried porcine plasma) diet. The piglets on the pea protein diet showed a higher incidence of diarrhoea and an increase in mortality, in comparison with piglets fed the higher quality plasma diet. A later study by Bosi et al. (2004) showed that a spray-dried porcine plasma diet improved growth performance of ETEC-challenged pigs, inhibited ETEC excretion in faeces and reduced ETEC-induced inflammatory status.

Undigested fermentable protein substrates which are present in the gut have the ability to affect microbial populations (Bhandari et al., 2010; Wellock et al.,

2006). Some authors have reported that faecal samples contained a higher lactobacilli to coliform ratio when dietary protein was reduced (Jeaurond et al., 2008; Wellock et al., 2006). Wellock et al. (2009) also found that less coliforms were isolated from pigs on a high quality diet, with increased lactobacilli numbers. However, these culture-based techniques provide limited information on the composition of the complex gut microbiota. Opapeju et al. (2009) utilised a culture-independent technique (i.e. terminal-restriction fragment length polymorphism, T-RFLP) to investigate the composition of the faecal microbiota in pigs fed a diet containing different levels of crude protein in the presence of ETEC challenge. Pigs fed a higher protein diet contained a higher level of Clostridiaceae and *Clostridium* species but a lower level of the order Clostridiales, specifically the Lachnospiraceae family and *Roseburia* species. Additionally, faecal samples obtained from pigs fed a higher protein diet contained more bacterial cells and had a higher microbial diversity on day 7 post-challenge.

1.5.2.2 Dietary non-starch polysaccharides

Many associations have been made between dietary non-starch polysaccharides (NSP) and pathogen proliferation in the weaner pig gut. NSP are fermented primarily in the terminal ileum and in the large intestine of piglets, and have been found to have an influence on gut health and enteric disease susceptibility (Pluske, 2001). NSPs can be split into two groups: soluble NSPs which are soluble in water and insoluble NSPs which are not. There has been a great deal

of interest in the inclusion of NSPs in weaner pig diets due to potential prebiotic properties.

There is conflicting evidence in the literature as to whether soluble NSP inclusion exerts a positive or negative effect on host health (Mosenthin et al., 2001; Wellock et al., 2008c). McDonald et al. (1999) studied the effects of soluble NSPs on healthy and ETEC challenged weaner pigs. The challenged pigs with soluble NSP-supplemented diets showed a significantly lower weight gain and an increase in ETEC proliferation in the small intestine. Further work by McDonald et al. (2001) showed a positive correlation between dietary levels of soluble NSPs and proliferation of ETEC in the small intestine in weaner pigs. However, Wellock et al. (2008c) found that inclusion of soluble NSPs significantly lowered diarrhoeal incidence and caecal digesta pH and increased the lactobacilli: coliform ratio in the faeces. Insoluble NSPs have been studied extensively in the context of enteric disease (Kim et al., 2012b), but the precise mechanisms of action are not fully understood. Mateos et al. (2007) and Kim et al. (2008) supplemented rice-based diets with 20g of oat hulls/kg, consequently improving performance and decreasing ETEC load respectively. A later study by Molist et al. (2010) described an ETEC F4 challenge study whereby the inclusion of coarse or finely ground wheat bran significantly decreased total ETEC numbers in the ileal digesta and mucosa.

1.5.3. Probiotics

Probiotics have been defined as living micro-organisms which are non-pathogenic to the host, which can provide beneficial effects with respect to disease prevention and general host health (Vanderpool et al., 2008). On administration to the host, the probiotic must survive transit to the gastrointestinal tract, as well as being able to withstand exposure to low pH conditions and bile salts. Probiotics can be composed of one specific bacterial strain, or can be a mixture of bacterial strains or species. The most commonly used probiotic bacteria belong to the lactic acid bacteria group (i.e. *Lactobacillus, Bifidobacterium* and *Enterococcus* species) and the bacilli (de Lange et al., 2010; Stein and Kil, 2006). In the pig industry, lactobacilli are of particular interest, since these are key residents in the pig's gastrointestinal tract (Richards et al., 2005) and therefore may have a selective advantage in binding to the porcine gut. Several probiotic strains including *Lactobacillus sobrius* DSM 11698 (Roselli et al., 2007), *Enterococcus faecium* 18C23 (Jin et al., 2000) and *Bifidobacterium lactis* Bb12 and *Lactobacillus rhamnosus* GG (Collado et al., 2007) reduce ETEC F4 adhesion to intestinal mucus *in vitro*. *Lactobacillus sobrius* 001T appears to inhibit ETEC F4 and promotes growth in pigs (Konstantinov et al., 2008).

Vahjen et al. (2010) investigated the timing of probiotic administration, since current practices involve several applications of probiotics which is not commercially feasible. By administering a single dose of 3 x 10^{10} cfu of *Lactobacillus plantarum* at weaning and following this with ETEC F4 challenge,

the clinical symptoms of PWC were reduced in comparison to pigs which were not given the probiotic and to pigs which were administered a lower dose of the probiotic (3×10^9 cfu).

Daudelin et al. (2011) evaluated the effect of administering *Pediococcus acidilactici* and *Saccharomyces cerevisiae boulardii* on the intestinal colonisation of ETEC F4. The authors found that the adhesion of ETEC F4 to the ileum was reduced in pigs which received either of these probiotics, in comparison to the control groups. A porcine competitive exclusion culture (the caecal contents of a healthy 6 week old pig) reduced shedding of ETEC in the faeces of neonatal pigs (Genovese et al., 2000). In commercial farm conditions, administration of this probiotic reduced ETEC-related mortality and morbidity (Harvey et al., 2013).

Although several studies highlight positive effects on host performance and health in response to probiotic supplementation, some studies do not show any such effects (Heo et al., 2012; Walsh et al., 2007). Walsh et al. (2007) administered a bolus dose of both *Lactobacillus acidophilus* and *Enterococcus faecium* at weaning, and found there were minimal impacts of probiotic administration of pig performance in comparison to the controls. The inconsistency in the results in probiotic studies has been partly attributed to differences in dosage, strain of probiotic, environmental conditions and dietary composition (Bontempo et al., 2004) and more work is needed to fully elucidate modes of action.

1.6 Studying complex microbial communities

1.6.1 Culturing and early molecular methodologies

For many years, complex microbial communities such as the gut microbiota have been studied using traditional laboratory methods such as culturing, microscopy and phenotypic identification. The major limitations of these techniques are that they are time-consuming and only allow study of a small sample of the microbial world, as it is estimated that only a very small proportion of bacteria are cultivable due to unknown or complex growth requirements under laboratory conditions (Stewart, 2012).

Culture-independent methods involve DNA-based approaches which either include the analyses of whole genomes or marker genes, such as the 16S rRNA and 18S rRNA genes (ribosomal RNA) in prokaryotes and eukaryotes, respectively. Using molecular methodology, the 16S rRNA gene (around 1500 bp in length) is the most common target for phylogenetic study of bacterial communities. The rationale for this is that this gene is present in all prokaryotes (i.e. bacteria and archaea), and is highly conserved because of the essential function of this gene in expressing the 16S ribosomal subunit and therefore has a very low mutation rate in critical gene sections. Sections of this gene which are not essential for forming the structure of the 16S ribosomal subunit have a higher mutation rate, thereby forming regions with high genetic diversity. These regions are termed conserved and hypervariable regions

respectively (**Figure 1.2**), and the latter can be targeted to enable discrimination between bacterial groups (Chakravorty et al., 2007).

When using next-generation sequencing platforms, unlike Sanger sequencing, the whole 16S rRNA gene cannot be sequenced due to fragment size limitations. Consequently, research has been carried out to reveal that a small fragment of the 16S rRNA gene can be used sufficiently as a proxy for the study of microbial communities (Liu et al., 2007). Due to the structure of the 16S rRNA gene, universal primers can be developed to bind to the flanking conserved regions in order to sequence the hypervariable region(s) of interest.

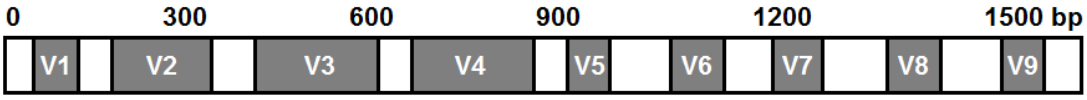

Figure 1.2: Schematic diagram of the 16S rRNA gene, highlighting conserved regions (white) and hypervariable regions (grey) and their approximate positioning.

Denaturing Gradient Gel Electrophoresis (DGGE) is such a culture-independent method, whereby 16S rRNA gene fragments are targeted in order to compare microbial communities (Janczyk et al., 2007; Konstantinov et al., 2004; Simpson et al., 2000). 16S rRNA gene amplicons are generated using PCR and fragments are then separated in a polyacrylamide gel, in which a gradient of denaturing

compounds is incorporated. This technique exploits the fact that 16S rRNA gene sequences will vary between bacterial species, and therefore the chemical stability will vary accordingly. As well as being a qualitative technique, like many other gel electrophoresis techniques, this is semi-quantitative when referring to band intensities. DNA fragments can also be cut from the gel and sequenced to allow identification. However, a single band does not necessarily represent a single genus or species, which is a limitation of this method.

Terminal-Restriction Fragment Length Polymorphism (T-RFLP) analysis is another technique that can be applied to study complex microbial communities (Jensen et al., 2011; Metzler-Zebeli et al., 2010b). The 16S rRNA gene is amplified from DNA extracts using fluorescently labelled primers and the PCR products are purified and subjected to restriction enzyme digestion. As a result, this step generates labelled terminal restriction fragments which are separated using capillary electrophoresis and detected using a fluorescence detector. The data output are a series of peaks (which represent the fragments) of various sizes and heights which represent the microbial profile of a particular sample. T-RFLP analysis has a similar key drawback to DGGE analysis, whereby a single terminal fragment peak does not necessarily represent a single bacterial genus or species. Additionally, it is not possible to retrieve sequences from T-RFLP analysis and in order to identify sequences, it is necessary to construct clone libraries in parallel for this purpose.

1.6.2 16S rRNA gene metabarcoding

In recent years, the emergence of next-generation sequencing platforms such as Illumina MiSeq have revolutionised the study of complex microbial communities. Most commonly in such studies, marker genes (i.e. 16S rRNA and 18S rRNA genes) are amplified and sequenced, providing both qualitative and quantitative (i.e. relative abundance) measurements. Prior to sequencing, there are a series of critical steps which need to be undertaken which are subject to potential biases. Therefore, it is paramount to use consistent methodology throughout the workflow to minimise potential biases and maximise the comparative power between studies using the methodology. Key steps are discussed below.

1.6.2.1 Sampling and storage

Studies have been carried out to assess whether sampling and storage conditions can have an impact on microbiota sequencing results. There is conflicting evidence on whether different storage conditions alone can have an impact on sequencing results (Fouhy et al., 2015; Lauber et al., 2011; McKain et al., 2013; Rubin et al., 2013).

Many studies have been carried out which compare the microbial compositions of fresh and frozen samples using 16S rRNA gene sequencing. Freezing of faecal samples weakens the Gram-positive bacteria cell well and lead to a larger yield of Gram-positive DNA being extracted in comparison to fresh faecal samples (Bahl et al., 2012). McKain et al. (2013) found that freezing samples without

glycerol as a cryoprotectant caused a significant loss in Bacteroidetes in ruminal digesta. Earlier work by Bahl et al. (2012) also found that freezing samples caused an increase in Firmicutes: Bacteroidetes ratio in comparison with fresh samples. Conversely, Fouhy et al. (2015) found that there were little significant differences in the biological findings in samples which were processed immediately without freezing, snap frozen and stored at -80°C or stored immediately at -80°C. The only differentially expressed groups were the *Faecalibacterium* and *Leuconostoc* genera when comparing the fresh samples and the snap frozen samples.

The impact of storage duration has also been explored in various studies. Lauber et al. (2011) stored soil, faeces and skin samples at various temperatures and found that storage duration had no significant impact on microbiota structure or diversity. Indeed, the relative abundances of the majority of taxa were not affected even after 14 days of storage. However, Shaw et al. (2016) found that storage of samples at room temperature for only 2 days caused significant changes in the microbial communities. In samples which were stored at -80°C for 2 years, relatively few changes in the microbial communities were observed with increased abundances of lactobacilli and bacilli, and a reduction in the total number of operational taxonomic units (OTUs).

1.6.2.2 DNA extraction

The next step is DNA extraction, for which there are a series of kit-based extractions available. Some authors have carried out studies to compare the

performance of various DNA extraction kits. Nelson et al. (2010) tested two commercial faecal extraction kits and also carried out a phenol/chloroform extraction using human faecal samples, and then used qPCR to quantify *Escherichia coli* and *Enterococcus* species. Although levels of *Escherichia coli* were not significantly impacted by extraction method, *Enterococcus* species were significantly lower in samples prepared with one of the commercial kits. Kennedy et al. (2014) found significant differences in relative abundances in several bacterial families when comparing two commercially available kits. Like in the majority of comparative DNA extraction studies, Gerasimidis et al. (2016) found that different methods produced a varying yield and quality of DNA which led to different results in downstream analyses.

One key step in the DNA extraction that has been linked with variation is a bead beating, or mechanical lysis, step. Walker et al. (2015) found that *Bifidobacterium* was not detected when using an extraction kit without a mechanical lysis step (i.e. QIAamp DNA Stool Minikit), which was reflected in the findings of a previous study (Maukonen et al., 2012). Additionally, Maukonen et al. (2012) also found that with methods which implement a bead beating step, higher bacterial cell numbers and diversity become apparent. Guo and Zhang (2013) also later found that kits which did not implement a bead beating step yielded much less DNA and less operational taxonomic units (OTUs) were assigned from these samples and Gram-positive bacteria were under-estimated as a result of using these kits. DNA yields and microbial

communities were again different when comparing 9 different DNA extraction methods on rumen samples (Henderson et al., 2013).

Salter et al. (2014) described the introduction of DNA contamination from personnel and laboratory consumables, as well as contamination within DNA extraction kits and PCR reagents. This can be problematic when the original sample has a low bacterial biomass (e.g. the lung microbiota), but samples containing a high bacterial biomass are less sensitive to the impact of background contamination (e.g. the gut microbiota). In order to address background contamination, some authors have included reagent-only controls as part of microbiota sequencing experiments (Glendinning et al., 2016; Wolfe et al., 2012).

As a result of these findings, many authors have advised that samples which have been subject to different DNA extraction methods should not be studied collectively due to the variations in microbial composition being observed (Gerasimidis et al., 2016; Henderson et al., 2013; Wesolowska-Andersen et al., 2014).

1.6.2.3 Selection of hypervariable region

Since the entire 16S rRNA gene cannot be sequenced using next-generation sequencing platforms, a short region of the gene must be selected for amplification and sequencing. At present, there is no consensus on which hypervariable region is the most suitable for study of microbial communities and a variety of single or multiple hypervariable regions have been used across

studies (Glendinning et al., 2016; Kim et al., 2012a). Others have carried out systematic studies using multiple 16S rRNA hypervariable regions to explore how the biological findings vary according to primer selection. For example, Chakravorty et al. (2007) explored which regions would best diagnose pathogenic bacteria and found, for example, that the V2 and V3 hypervariable regions were the most suitable for identification down to genus level with a great deal of variability in results being shown between hypervariable regions. Importantly, the selection of hypervariable region(s) to target and the design of the universal PCR primers also have an effect on phylogenetic resolution in microbiota studies (Cruaud et al., 2014; Ghyselinck et al., 2013; Tremblay et al., 2015; Yang et al., 2016).

1.6.2.4 PCR amplification

Biases associated with PCR include inhibition by contaminating compounds such as humic acids. This can be minimised by DNA dilution, but this has an impact on PCR efficiency. Additionally, generation of PCR artefacts can influence the final sequencing results, which can over-inflate the diversity of the microbial community (Schloss et al., 2011).

Very recently, the inclusion of mock bacterial communities as controls has become more common and has highlighted the effects of biases throughout the library preparation process (Fouhy et al., 2016) and allows the calculation of sequencing error rate (Kozich et al., 2013). Since a mock community is compiled with known organisms, all possible chimeras can be predicted and removed before establishing the closest reference sequence for each of the

remaining reads. The ratio of mismatched bases to total bases can then be calculated to reveal the sequencing error rate. A systematic study was carried out by Gohl et al. (2016) to establish sources of error using mock bacterial communities, and the authors concluded that others should use high-fidelity polymerases and lower the number of PCR cycles to minimise errors.

1.6.2.5 Sequencing

Over the last 5 years, rapid improvements in sequencing technologies have been made which have led to the release of benchtop sequencers from a variety of companies (D'Amore et al., 2016). Until very recently, the 454 series of platforms were the most commonly used for 16S rRNA gene metabarcoding studies but Roche have announced that the 454 platform will be withdrawn in 2016. The other sequencing platforms which are available have differing attributes when it comes to read length, accuracy, speed and throughput (D'Amore et al., 2016). The choice of sequencing platform has an impact on the biological findings (Frey et al., 2014; Quail et al., 2012; Tremblay et al., 2015).

Due to improving technologies, read length is being extended which may eventually lead to the ability to sequence multiple hypervariable regions with complete overlap. When this PhD project began (October 2012), generation of 2x150bp reads was possible but the capabilities of the Illumina MiSeq machine have since been extended to 2x250bp reads, with 2x300bp reads being possible with the Illumina V3 chemistry. As previously discussed, there is still no consensus on which hypervariable region(s) are best targeted. However, due to amplicon size limitations some authors recommend using a single hypervariable

region rather than multiple regions (Hamady and Knight, 2009) to ensure that a complete overlap of paired-end reads is possible to lower error rates and so to not over-inflate diversity of microbial communities (Kozich et al., 2013).

1.6.2.6 Sequence analysis

Analysis of large and complex 16S rRNA gene sequencing data sets requires the use of bioinformatics tools to gain a taxonomic overview of the data. There are many tools available to analyse 16S rRNA gene sequencing data, and the software packages QIIME (Caporaso et al., 2011), MG-RAST (Meyer et al., 2008) and mothur (Schloss et al., 2009) contain a comprehensive set of tools for complete analysis of 16S rRNA gene data, which are accessible for biologists. Plummer and Twin (2015) analysed a single data set using the aforementioned software packages and found that there were few differences in the results when considering taxonomic classification and diversity. However, there were differences in the ease of use of each of these packages and the time required for analysis.

Typically, 16S rRNA gene sequence analysis workflows begin by removing primers, assessing the quality of the data and removing noise (Barriuso et al., 2011). For paired-end reads, these are aligned to form one contiguous DNA sequence and those which do not overlap or have a corresponding sequence are discarded. Quality filtering consists of removing sequences which are of unexpected length, have long homopolymers, contain ambiguous bases or do not align to the correct 16S rRNA gene region. Sequences are then screened for chimeras, using algorithms such as UCHIME (Edgar et al., 2011).

After reducing sequencing and PCR errors, sequences are then aligned to a reference alignment prior to taxonomic assignment. There are several reference alignments available, but Schloss (2009) showed that the SILVA database provided more accurate alignments than the other available alignments (i.e. RDP and Greengenes). The database selected for taxonomic assignment is also likely to have an impact on final results, and classification depth can be improved by trimming the database information to the specific hypervariable region(s) of interest (Werner et al., 2012). Werner et al. (2012) also found that by comparing the SILVA, RDP and Greengenes databases, the dominant phyla were similarly assigned but the rarer members of the community were assigned taxonomy more successfully using Greengenes. The databases are also biased towards clinically relevant bacteria in humans (Poretsky et al., 2014), which can mean that it is more difficult to assign taxonomy to sequences derived from agricultural or environmental samples.

Sequences which are identified as anything other than bacteria are then removed from further analysis, before using either a database-dependent or database-independent approach to cluster sequences into phylotypes or OTUs, respectively. In the former method, sequences are compared to databases and are binned according to similarity to the database. This database-dependent approach has the advantage of being less sensitive to sequencing errors and taxonomic names are provided directly. However, the disadvantages mostly stem from being reliant on databases, which are incomplete and may contain inaccurate taxonomic information.

When using a database-independent approach, sequences are compared with each other and are binned into OTUs based on similarity to other sequences in the dataset. The advantages of this approach are that the potential inaccuracies and incompleteness of the databases are not as critical, taxonomic names can be added if desired, and resolution is greatly improved. For example, there may be several OTUs which are assigned as "*Lactobacillus*" using a database-independent approach and may provide "sub-genus" taxonomic information (Patrick D. Schloss, personal communication), but a database-dependent approach would only yield one "*Lactobacillus*" phylotype. However, the primary disadvantages of the database-independent approach are that it is more computationally challenging and is more sensitive to sequencing error rates.

In addition to taxonomic analysis of sequences, descriptive and statistical methods are now being proposed to study the entire composition (or structure) of microbial communities (Schloss, 2008). In such analyses, distance matrices are constructed using 16S rRNA gene sequences which can take both community membership (i.e. qualitative information: presence and absence of OTUs/phylotypes) and relative abundance (i.e. quantitative information) into account. Descriptive methods such as non-metric multidimensional scaling (NMDS) and statistical methods such as analysis of molecular variance (AMOVA) (Escoffier et al, 1992) can then be used to visualise any clustering and assess the statistical significance of clustering, respectively. Changes in the relative abundances of particular phylotypes or OTUs can also be assessed statistically using methods such as Metastats (Paulson et al, 2011). Such

methods are advantageous in hypothesis-driven research, as ecologically meaningful information can be derived from studying the dynamics of microbial community structure, in combination with the study of changes in relative abundances over time or in response to particular treatments.

1.7 Thesis outline and main objectives

As discussed throughout **Chapter 1**, weaning exerts a variety of stressors on the pig, which have an impact on gut health and host performance. The gut microbiota has been shown to have a pivotal role in gut health and is the target of many disease management strategies. In the development of such management strategies, it is important to consider that there are temporal (Frese et al., 2015; Holman and Chénier, 2014) and spatial (Looft et al., 2014) variations in gut microbiota composition, highlighting the importance of targeted study of individual growth phases and gut compartments. ETEC challenge has been shown to have an impact on pig performance (Houdijk et al., 2007; Wellock et al., 2008b; Opapeju et al., 2009) and recent evidence has emerged that there are links between the gut microbiota and pig performance (Mach et al., 2015; Ramayo-Caldas et al., 2016).

In **Chapter 2**, a previously developed multiple-dosing ETEC challenge model (Athanasiadou et al., 2010) was implemented to study the effects of ETEC exposure on weaner pig performance, health and faecal microbiota composition. 16S rRNA gene metabarcoding was utilised to carry out a temporal analysis of faecal microbiota dynamics in response to ETEC challenge, with ETEC shedding being measured using quantitative PCR. Comparisons were also made between

pigs with differing growth rates and ETEC faecal shedding levels to establish whether there were variations in microbial composition associated with these measures.

In **Chapter 3**, a pilot study is presented whereby a single-dose ETEC challenge model was implemented with the central aim of studying ETEC adhesion and shedding in weaner pigs. ETEC isolates used in previous studies were characterised using PCR, targeting the genes responsible for the expression of key ETEC F4 virulence factors (i.e. F4, STa, STb, LT and EAST1). Additionally, both ileal digesta, ileal mucosa and faecal samples were obtained to quantify ETEC adhesion and shedding, with the former not previously being addressed in production-based studies in our group (Athanasiadou et al., 2010; Houdijk et al., 2007; Wellock et al., 2008a).

In **Chapter 4**, a 2 x 2 factorial experiment was carried out to study the interactive effects of dietary protein level and ETEC challenge on pig health, performance and both ileal and faecal microbiota composition. ETEC quantification was carried out for both ileal digesta and faecal samples to establish ETEC load at the infection site and ETEC shedding level, respectively. 16S rRNA gene metabarcoding was utilised to profile both ileal and faecal samples to establish whether there were treatment effects on microbiota composition. Additionally, any links between pig performance and ETEC cell counts (at ileal and faecal level) and microbiota composition were assessed.

Finally, in **Chapter 5**, a general discussion of the research findings from this project will be presented, with suggestions for potential future work.

Chapter 2:

The effects of ETEC challenge on temporal faecal microbiota dynamics, pig health and performance

2.1 Introduction

Due to the economic importance of post-weaning colibacillosis (PWC), several studies have been carried out to study the effects of experimental enterotoxigenic *Escherichia coli* (ETEC) challenge on weaner pig health and performance (discussed in **Chapter 1**). Many authors have reported that experimental ETEC challenge caused a reduction in average daily weight gain and feed intake (Ren et al., 2014; Rossi, 2012), which also occurred in the absence of diarrhoea (Opapeju et al., 2009; Wellock et al, 2008b).

The microbiology of the porcine gut has been a focus of study for decades due to evidence supporting the multifaceted role of commensal bacteria in host health and development (Isaacson and Kim, 2012; Leser et al., 2002). Indeed, studies have been carried out which have included analysis of the gut microbiota in the presence of ETEC challenge using culturing methods to measure lactobacilli to coliform ratio (Wellock et al., 2008a) and using early molecular methodologies (**Chapter 1**) such as T-RFLP (Jensen et al., 2011). The development of next-generation sequencing technologies, such as 16S rRNA gene metabarcoding, now allows higher resolution identification and quantification of bacteria as part of complex microbial communities.

Using 16S rRNA gene sequencing, temporal effects (Kim et al., 2011; Zhao et al., 2015) and the impact of enteric infections (Bearson et al., 2013; Costa et al., 2014; Dowd et al., 2008) on gut microbial communities have been described. However, the majority of published studies that explore temporal microbiota

shifts in pigs were carried out over a large time frame whereby small numbers of samples were taken at multiple stages of development (Holman and Chénier, 2014; Kim et al., 2012a; Mach et al., 2015).

Also of significant economic interest is the performance (e.g. growth rate and feed efficiency) of farm animals and recent studies using next-generation sequencing have highlighted that particular bacterial groups are associated with obesity in pigs (Pedersen et al., 2013), feed efficiency in cattle (Jewell et al., 2015) and various performance parameters in poultry (Rubio et al., 2015). However, there are limited studies on pig growth performance and only recent work has started to reveal links between microbiota composition and growth traits in pigs (Ramayo-Caldas et al., 2016; Mach et al., 2015).

In this experiment, a previously developed ETEC challenge model (Athanasiadou et al., 2010) was used to study the temporal effects of challenge on weaner pig health, performance and faecal microbiota dynamics using 16S rRNA gene metabarcoding. To our knowledge, this is the first ETEC challenge study which utilises this method to study gut microbiota dynamics, particularly in the context of growth performance. The specific aims of this experiment were to characterise the weaner pig faecal microbiota over the immediate post weaning period, ascertain whether ETEC challenge had an impact on faecal microbiota dynamics, pig performance and health, and establish whether there is a link between faecal microbiota composition and pig performance.

2.2 Methods

2.2.1 Animals and housing

A subset of pigs from a larger trial were selected for this experiment which took place across two rounds starting in June 2013 and August 2013. Pigs were selected for this study on the basis that these animals were fed an industry-standard weaner pig diet (**Appendix A**). Fifty-nine pigs (Large White x Landrace) were weaned at 26.7 ± 0.7 (mean ± SD) days of age and weighed 8.65 ± 1.77kg, with 27 pigs being selected from round 1 and 32 pigs being selected from round 2. Pens were balanced as much as possible for sex, weaning weight and litter origin, with 8 litters being included across the trial.

The selected pigs were housed in groups of four in 4 m^2 square pens. The pens were bedded with sawdust as required, and a single feeder and nipple drinker were included. The pens were cleared of faecal material and wet sawdust daily. Water and feed were provided *ad libitum* for the trial duration. The environmental temperature was set at 25^{o}C for the first 4 days, and was decreased by 2^{o}C per week for the experiment duration. The shed lights were switched on between 8:00 and 18:00 and night lights were maintained between 18:00 and 8:00.

2.2.2 ETEC strains and inoculum

Two ETEC strains containing the gene encoding for the expression of F4 fimbriae, *faeG*, were obtained from pigs diagnosed with post-weaning

colibacillosis (SRUC Veterinary Services Laboratory, UK). Nalidixic acid-resistant variants were produced from each of these strains by serial passage of the isolates in brain-heart infusion broth containing increasing concentrations of nalidixic acid from 0 to 15µg/ml at 37°C for 24 hours. Organisms which were tolerant of 15µg/ml of nalidixic acid were then stored in glycerol.

To produce the inoculum, each strain was recovered by streaking directly onto MacConkey agar plates and incubated at 37°C for 18-24 hours. A nalidixic acid-infused enrichment broth was then prepared by addition of 50µl of 15mg/ml nalidixic acid to 50ml of brain-heart infusion broth. 5ml of nalidixic acid-infused broth was aliquoted into 5 universal tubes. A single, well-isolated bacterial colony was then immersed into each of the universal tubes, prior to incubation in an orbital shaker at 37°C for 18 hours. The contents of each universal were combined prior to centrifugation at 3000rpm for 10 minutes, before being suspended in 25ml of sterile phosphate-buffered saline (PBS) for two further centrifugation washes at 3000rpm for 5 minutes. The pellets were then resuspended in 30ml of PBS and combined before using a spectrophotometer (Ultrospec 2100 Pro, Fisher Scientific, UK) to measure optical density, thereby estimating the number of colony forming units (cfu) in the stock suspension. The stock suspension was then diluted according to the spectrophotometer reading, to aim for a final inoculum concentration of 1.00 x 10^8 cfu. The stock solution was also serially diluted five times and 100µl of each dilution was pipetted and spread onto MacConkey agar plates and incubated at 37°C for 18 hours for standard enumeration.

2.2.3 ETEC challenge

Thirty-two pigs were repeatedly challenged with ETEC O149:F4 on days 4, 6, 8, 11 and 13 as highlighted in previous work (Athanasiadou et al., 2010). Feed was withdrawn 30 minutes prior to challenge to stimulate the appetite. Briefly, 10ml of the final inoculum were then mixed with a further 10ml of sterile PBS, before mixing with 20g of feed. This feed was then offered in small, individual bins to each of the 4 pigs in each pen (**Figure 2.1**). Twenty-seven pigs were provided with feed in the same manner, mixed with 20ml of sterile PBS as a sham challenge. Throughout the experiment, biosecurity measures were strict to minimise potential cross-contamination of the challenge pathogen. Changes of nitrile gloves and overshoes were enforced when moving between pens, and foot baths containing disinfectant were used regularly. Regular disinfection of equipment and shed corridors was also carried out.

2.2.4 Pig performance and health

The mass of feed offered and refused (on a pen basis) was recorded daily from day 1 until day 14 of the trial, and then on a weekly basis until day 28. This allowed assessment of the average daily feed intake (ADFI) per pen throughout the experiment. All pigs were weighed on days 0, 7, 14, 21 and 28 to assess growth performance over the trial duration. Consequently, the average daily weight gain (ADWG) per pig was calculated over the trial duration.

Figure 2.1: Photograph of an ETEC challenge event showing individual feeding, which was facilitated by splitting the pen into two using a wooden board for paired feeding.

Faecal consistency, cleanliness and overall health scores were taken every morning using a subjective four-point scale as shown in **Table 2.1**. Briefly, for faecal consistency scoring, an increase in score represents an increase in faecal fluidity. Increases in both cleanliness and overall health scores represent an increase in faecal contamination and a deterioration of health, respectively.

Table 2.1: Description of faecal consistency, cleanliness and health scores which were assigned daily on a pen basis.

Measure	Score	Description
Health	1	Pigs lying, sitting, standing or walking and actively respond to human presence; skin pink, bright eyes, upright ears.
	2	Pigs lying/sleeping but responding normally to human presence following gentle stimulation; skin pink, bright eyes, upright ears.
	3	Pigs lying and slightly shivering, not responding normally following gentle stimulation, skin pink-greyish, eyes somewhat sunken, ears slightly dropping.
	4	Pigs showing pain, e.g. kicking at abdomen, lying twisted, hunched back, skin grey, sunken eyes, ears completely dropping.
Faeces	1	Firm stool shape (1.5 for soft faeces but compact, clay-type).
	2	No formed stool, little spreading ('normal diarrhoea').
	3	No formed stool, watery, readily spreading ('watery diarrhoea').
	4	No formed stool, very watery, flecks of blood, rapidly spreading ('dysentery').
Cleanliness	1	All pigs are clean.
	2	1 or 2 pigs are a little dirty
	3	3 or 4 pigs are a little dirty
	4	All pigs are very dirty

2.2.5 16S rRNA gene sequencing

2.2.5.1 Faecal sampling and DNA extraction

Faecal samples were taken directly from the rectum on day 4 (before challenge), days 8 and 12 (during challenge), and days 15 and 19 (after challenge) post-weaning using spooned universal tubes. The samples were immediately snap-frozen on dry ice prior to storage for a maximum of 2 weeks at -80^{o}C.

DNA extraction was carried out using the MoBio PowerSoil® DNA Isolation kit (Cambio, UK), with modifications to the protocol. Briefly, 500mg of faeces were transferred into a 50ml centrifuge tube, and 5ml of MoBio PowerSoil® Bead Solution (Cambio, UK) were added to each tube. The faecal suspensions were then homogenised using a vortex mixer and 1ml of supernatant was transferred into the provided bead tube, before being homogenised for 45 seconds at 5.0 m/s using a FastPrep FP120 Cell Disrupter (Qbiogene Inc, France). The homogenate was then processed according to the manufacturer's instructions. The yield and quality of the DNA extracts were tested using a NanoDrop 1000 spectrophotometer (Thermo Scientific, UK) and gel electrophoresis. For the latter, DNA extracts were visualised using an ultraviolet imager (Gel Doc XR+ System, Bio-Rad, UK) which confirmed the presence of intact, high molecular weight DNA.

2.2.5.2 Library preparation and sequencing

Thirty-two pigs were selected for 16S rRNA gene metabarcoding (16 ETEC-challenged and 16 sham-challenged pigs), including one sample pre-challenge and four samples post-challenge. Pigs were selected on the basis of

performance (i.e. 16 pigs with a "low" growth rate and 16 pigs with a "high" growth rate, based on ADWG/kg weaning weight) and the selection was balanced on the basis of weaning weight, sex, pen origin, litter origin, challenge status and experimental round.

The V3 hypervariable region of the 16S rRNA gene was amplified using dual-indexed primers 341F (5' – CCTACGGGAGGCAGCAG – 3') and 518R (5' – ATTACCGCGGCTGCTGG – 3') (Kim et al., 2012a), which incorporated TruSeq adapters (see **Appendices B and C**). Equimolar concentrations of template DNA were amplified in a one-step PCR using a high fidelity polymerase (Phusion®, New England Biolabs, UK). A PCR mastermix was constructed to carry out 20µl reactions, including primers at a final concentration of 0.2µM. The PCR conditions consisted of an initial denaturation step at 98^{o}C (3 minutes), followed by 20 cycles of amplification at 98^{o}C (30 seconds), 60^{o}C (30 seconds) then 72^{o}C (30 seconds), and a final extension step at 72^{o}C (5 minutes). PCR products were then purified using the AMPure XP PCR purification system (Beckman Coulter, USA).

Reagent-only controls were amplified in parallel by adding equimolar concentrations of DNA extract to the PCR reaction mixture. The Human Microbiome Project mock community HM-782D (BEI Resources, ATCC, Manassas, VA, USA) was also amplified by adding 1µl of pre-prepared DNA extract (containing 100,000 16S rRNA gene copies per organism per µl) to the PCR reaction mixture. PCR products were purified in the same manner as described above.

To confirm the presence of a 300bp product, the purified DNA samples were subject to gel electrophoresis and products were visualised using an ultraviolet imager (Gel Doc XR+ System, Bio-Rad, UK). In addition, the product sizes were assessed using a TapeStation instrument (Agilent Technologies, UK) and double stranded DNA was quantified using a fluorometric kit (Quant-iT PicoGreen, Invitrogen, UK). Readings from this assay were used to create two 3.5nM pools (80 samples per pool), using equimolar concentrations of each library and were submitted to the sequencing centre (Edinburgh Genomics, UK). Sequencing was carried out using the Illumina MiSeq platform (Illumina, CA, USA), using V2 chemistry and producing 250bp paired-end reads.

2.2.5.3 Sequence processing

The raw data was processed using CASAVA 1.8 (Illumina, USA) and files were delivered in FASTQ format, which contained sequences which were de-multiplexed according to the unique barcode combinations and primer sequences were removed using cutadapt (Martin, 2011). The following processing steps were carried out using the open source software, mothur (Schloss et al., 2009), based on a protocol written by the developers (Kozich et al., 2013). Briefly, contiguous sequences were constructed from the paired-end reads. Reads were then aligned to reference sequences from the SILVA small-subunit rRNA sequence database (Pruesse et al., 2007), and those which did not map to the correct position in this file were removed. Sequences were also removed if they were below 135bp in length or above 230bp in length, contained over 8 homopolymers and if they contained ambiguous bases.

Sequences were screened for chimeras using UCHIME (Edgar et al., 2011) and were classified using the Greengenes database (DeSantis et al., 2006) which was trimmed to the V3 hypervariable region of the 16S rRNA gene to improve classification depth (Werner et al., 2012). Sequences were binned into phylotypes using a database-dependent approach and were subsampled for analysis.

2.2.6 ETEC quantification from faeces

2.2.6.1 Faecal sampling and bacteriology

A freshly-voided faecal sample was collected from each pen on days 4, 8, 12, 15, 19 and 21 post-weaning for pen-based detection of nalidixic acid – resistant *E. coli* (i.e. the challenge isolate). Approximately 0.5g of faeces was weighed out and transferred to a bijou tube. The exact mass of faeces weighed out was recorded to factor this into the final calculations. 4.5ml of sterile PBS was added to each bijou, before homogenisation using a vortex for 10 seconds. Serial dilutions were then carried out to a dilution of 1×10^{-5}. 100µl of each dilution was then pipetted onto a nalidixic acid-enriched (15mg/L) MacConkey agar plate, and a spreader was used to distribute the sample evenly across the agar plate. The plates were then incubated at 37^{o}C for 18-24 hours. After incubation, the number of colonies on each countable plate were recorded to calculate the number of colony forming units per gram of wet faeces (cfu/g wet faeces).

2.2.6.2 DNA extraction and PCR confirmation

A subsample of bacterial colonies were subject to DNA extraction using the Wizard® Genomic DNA Purification kit (Promega, UK) following the

manufacturers instructions, prior to PCR to confirm the presence of the *faeG* gene using the following PCR primers – *faeG*-F 5'-ATCGGTGGTAGTATCACTGC-3' and *faeG*-R 5'- AACCTGCGACGTCAACAAGA-3') (Ojeniyi et al., 1994). A PCR mastermix was constructed using JumpStart™ REDtaq® ReadyMix™ Reaction Mix (Sigma Aldrich, UK) in a final volume of 20µl, including primers at a final concentration of 0.2µM. The PCR run conditions consisted of an initial denaturation step at 94°C (2 minutes), followed by 30 cycles of amplification at 94°C (30 seconds), 55°C (30 seconds) then 72°C (30 seconds), and a final extension step at 72°C (5 minutes). PCR product presence and size was confirmed by gel electrophoresis.

2.2.6.3 Quantitative PCR

Faecal shedding of ETEC F4 was determined using quantitative PCR (qPCR), which targeted the *faeG* major fimbrial subunit. The samples utilised for 16S rRNA gene sequencing only were taken forward for this screening. Reactions were set up using Brilliant III Ultra-Fast SYBR® Green qPCR Master Mix (Agilent Technologies, USA) and primers F4-463F (5' – GGTTCTGAACTCTCGGCTGG – 3') and F4-597R (5' – AGAACCTGCGACGTCAACAA – 3'), which were designed and optimised as part of this study.

All reactions were carried out in triplicate using a Stratagene MX3005P (Agilent Technologies, USA), with 2µl of DNA extract being added to each reaction. The qPCR run conditions consisted of an initial denaturation step at 95°C (5 minutes), followed by 40 cycles of amplification at 95°C (30 seconds) then 65°C (15 seconds). A melt curve was generated using the following cycling

conditions - 95°C (60 seconds), 55°C (30 seconds) and 72°C (30 seconds). A subsample of purified PCR products from ETEC-challenged animals were sequenced to test the specificity of the designed primers (Eurofins, Germany), and the sequences were then matched using the NCBI BLAST reference database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

To enable calculation of *faeG* gene copy number in the faecal samples, absolute quantification using a standard curve was carried out. The standard curve was constructed using purified PCR products from tenfold serial dilutions of the challenge strain. In order to convert the quantity given by the qPCR output to the number of *faeG* gene copies, it was calculated that one nanogram of DNA contained 6.86×10^9 copies of the target gene. The original concentration of the standards was determined using a spectrophotometer (NanoDrop 1000, Thermo Scientific, UK), and these values were used to estimate the number of gene copies/gram wet faeces.

2.2.7 Statistical analysis of performance data

Statistical analyses were carried out using Genstat 16 (VSN International, UK) unless stated otherwise. The body weight and ADFI data were assessed using repeated measures analysis of variance (RM-ANOVA) to establish any temporal effects of ETEC challenge on these parameters. These analyses included challenge as a main factor and experimental round as a block. Day 0 values for body weight were used as co-variates for assessment of changes in body weight. The ADWG data was assessed using analysis of variance (ANOVA) to establish

whether ETEC challenge had an effect on total weight gain between day 0 and day 28.

To assess the consistency over time of the health scores, and whether there were any effects of ETEC challenge, an ordinal logistic regression (OLR) was performed using Minitab 17 (Minitab Inc, USA). The categorical indicators (i.e. health scores) were assigned as the response, and time point and challenge status were assigned as categorical predictors.

2.2.8 Descriptive and statistical analysis of sequence data

Descriptive and statistical analyses were carried out to describe temporal microbiota shifts and to establish whether there was an effect of ETEC challenge/shedding level or growth rate on faecal microbiota composition. Analyses were carried out using the mothur software package (Schloss et al., 2009) unless stated otherwise.

The Inverse Simpson's Index (ISI) was calculated for each sample to measure diversity, and the Chao 1 index was calculated to assess richness. To test whether there were significant differences in diversity and richness over time and between ETEC- and sham-challenged pigs, RM-ANOVA was carried out using Genstat 16 (VSN International, United Kingdom). The values for day 4 were initially included as co-variates, but these had no significant effect and were therefore not included as co-variates in the final analysis. Temporal changes in relative abundances at both phylum and family levels were also assessed using RM-ANOVA with logit-transformed data.

A distance matrix was compiled using Yue and Clayton theta similarity coefficients (Yue and Clayton, 2005), which take into account both community membership and relative abundance. Non-Metric Multidimensional Scaling (NMDS) plots were constructed in two dimensions with co-ordinates generated using the NMDS function to visualise community similarities over time and between treatment groups. The statistical significance of any clustering was assessed by analysis of molecular variance (AMOVA) (Excoffier et al., 1992). The statistical significance of variation between populations was assessed using homogeneity of molecular variance (HOMOVA) (Stewart and Excoffier, 1996).

To identify phylotypes that were expressed significantly differently between sample groups, Metastats (Paulson et al., 2011) was used and the P-values were corrected using false discovery rate (FDR) to correct for multiple observations. The subsampled dataset was simplified to only include phylotypes which were equal to or more than 0.1% abundant at each time point examined. Dirichlet multinomial mixture (DMM) models were run to group samples into enterotypes based on the relative abundances of bacterial genera in each sample (Holmes et al., 2012).

To assess whether there were temporal effects of growth rate and ETEC shedding level on microbiota composition, pigs were firstly clustered into "low" (52.51 ± 5.38g/day/kg weaning weight, n = 10) and "high" (74.40 ± 4.55g/day/kg weaning weight, n = 10) growth rate groups and "low" and "high" shedder groups (using qPCR data), respectively. Relative abundance values at phylum and family levels were logit-transformed and analysed using RM-

ANOVA with an average relative abundance cut-off set at 1%. Transformed relative abundances for the five sampling points were entered as the data at successive time points, with either growth rate or ETEC shedding status being entered as the treatment and the experimental round was included as a block. In addition, any effects of ETEC challenge or growth rate on microbiota structure, phylotype relative abundances and enterotype clustering were assessed using AMOVA, Metastats and DMM modelling, respectively.

2.3 Results

2.3.1 Body weight and ADWG

The mean body weights for all ETEC- and sham-challenged pigs included in the study and the pigs selected for 16S rRNA gene metabarcoding only are presented in **Table 2.2**. There were no significant effects of ETEC challenge on either body weight (RM-ANOVA: P = 0.63) or ADWG (ANOVA: P = 0.13) when considering the total population of pigs (ETEC-challenged n = 32, sham-challenged n = 27). When considering the pigs which were subject to 16S rRNA gene metabarcoding only (ETEC-challenged n = 16, sham-challenged n = 16), there were again no significant effects of ETEC challenge on body weight (RM-ANOVA: P = 0.80) or ADWG (ANOVA: P = 0.56).

Table 2.2: Mean body weights for all ETEC- and sham-challenged pigs included in this study (all) and for pigs selected for 16S rRNA gene metabarcoding only (16S). No significant effects of ETEC challenge were observed (P = 0.63).

	Mean body weight (kg) ± SD				
Group	**Day 0**	**Day 7**	**Day 14**	**Day 21**	**Day 28**
ETEC (all)	8.59 ± 1.52	10.01 ± 1.63	13.23 ± 1.90	17.94 ± 2.39	23.43 ± 3.00
ETEC (16S)	8.71 ± 1.39	10.29 ± 1.62	13.33 ± 1.93	18.01 ± 2.57	23.43 ± 3.26
SHAM (all)	8.82 ± 1.77	10.12 ± 2.15	13.24 ± 2.67	18.33 ± 3.47	24.34 ± 4.14
SHAM (16S)	8.80 ± 1.99	10.14 ± 2.43	13.32 ± 3.21	18.15 ± 4.23	24.20 ± 5.12

2.3.2 Average daily feed intake

The effect of ETEC challenge on ADFI from day 0 to day 27 is shown in **Figure 2.2**. There was no effect of ETEC challenge on feed intake throughout the experiment (RM-ANOVA: P = 0.25), although the ADFI in the sham-challenged pigs was markedly higher on day 20 (926 ± 159g) in comparison to the ETEC-challenged pigs (634 ± 261g). Interestingly, on day 8, both ETEC- and sham-challenged pigs showed a marked drop in ADFI of 49.5 ± 44.5g and 70.7 ± 49.1g per pig (mean ± SD), respectively.

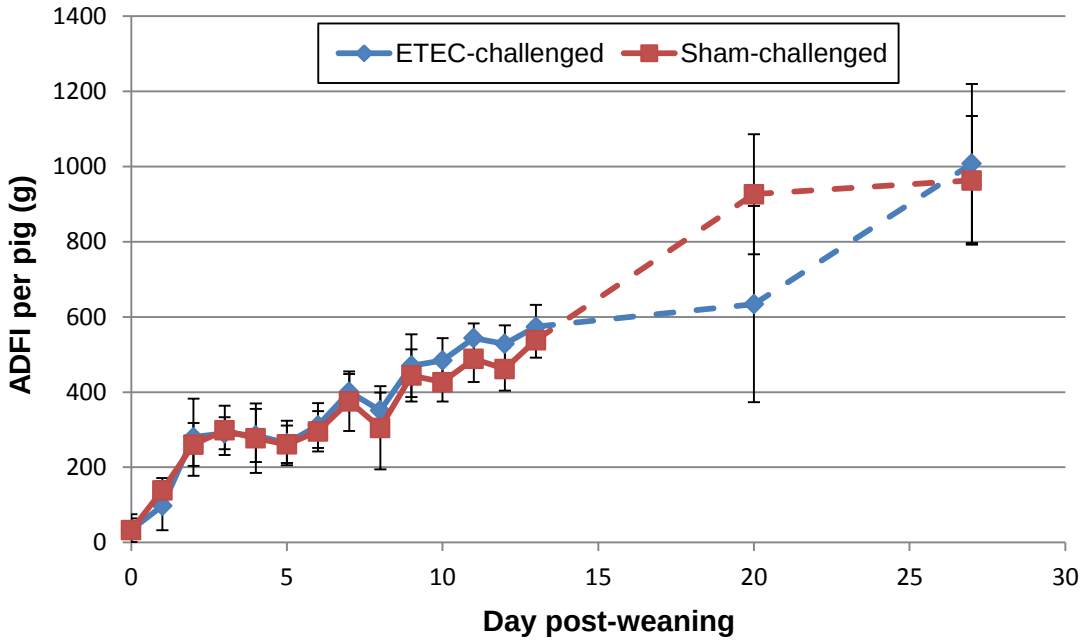

Figure 2.2: Average daily feed intake (±SD) estimated per pig using pen-level measurements. Weekly measurements were made after day 14, highlighted by the broken lines. No treatment effects were observed at any time point (P > 0.05).

2.3.3 Faecal consistency, cleanliness and health scores

All pigs were assigned a cleanliness and health score of 1 throughout the experiment, and so these will not be discussed any further in this section. The temporal mean faecal consistency scores in both ETEC- and sham-challenged pigs are shown in **Figure 2.3**. On days 1 and 2, all pens were assigned a faecal score of 1. On days 3 and 4 (pre-challenge), slightly elevated faecal scores were observed in both ETEC- and sham-challenged pens. On day 5 (i.e. one day post-challenge), the mean faecal score in the ETEC-challenged pens increased to 1.5 ± 0.25 and to 1.29 ± 0.25 in the sham-challenged pens. After day 5, the faecal scores gradually decreased and by day 20, all pens were assigned a faecal score

of 1. For the remaining 8 days of the trial, all pens were consistently assigned a faecal score of 1. Overall, there was not a statistically significant effect of ETEC challenge on faecal score (OLR: $P = 0.822$), but significant temporal effects on faecal score were found (OLR: $P < 0.001$).

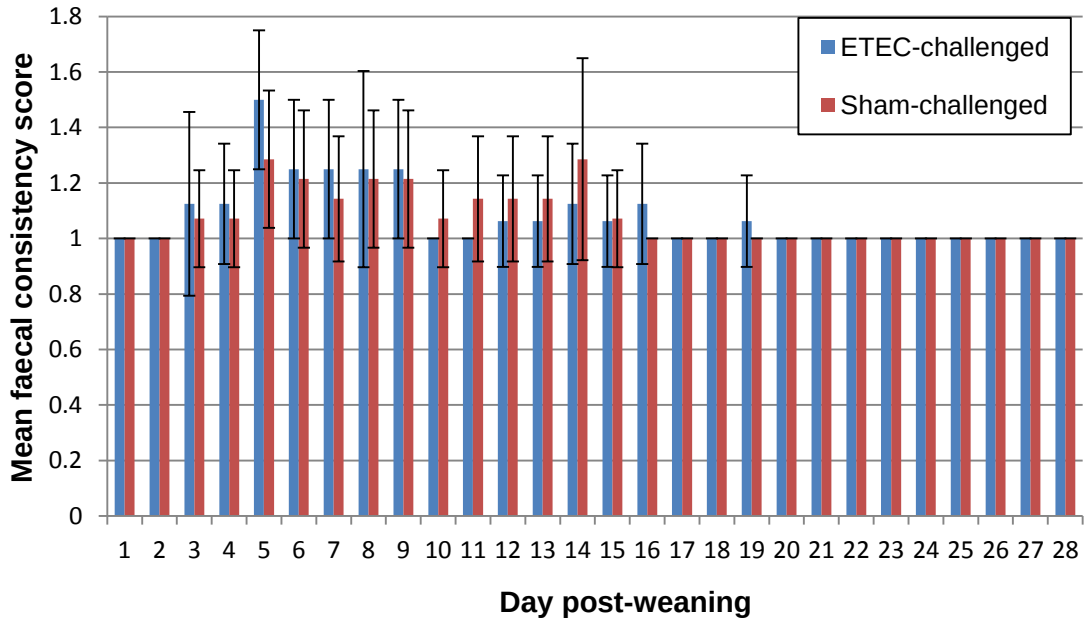

Figure 2.3: Bar graph showing the mean faecal consistency scores (±SD) in both ETEC- and sham-challenged pens from day 1 to day 28 post-weaning, with no significant effects of ETEC challenge being observed ($P > 0.05$).

2.3.4 ETEC quantification from faeces

2.3.4.1 Bacteriology

No nalidixic acid-resistant organisms were isolated at baseline (day 4) in pigs from round 1. However, background growth of nalidixic acid-resistant micro-organisms were ubiquitous in baseline samples during round 2. All sham-challenged pigs tested negative for nalidixic acid-resistant organisms

throughout the trial during round 1 of the experiment, but the sham-challenged pigs in round 2 showed growth of nalidixic acid-resistant micro-organisms on the agar plates at several dilutions. Due to the presence of background nalidixic acid-resistant organisms in baseline and sham-challenged pig faecal samples throughout the second round of the trial, the data could not be considered for analysis.

2.3.4.2 Quantitative PCR

All faecal samples from sham-challenged pigs were confirmed as ETEC F4-negative by qPCR. The dynamics of ETEC shedding were expressed over the trial period as log transformed (Log_{10}+1) *faeG* gene copy number per gram of wet faeces (**Figure 2.4**). On day 4 (pre-challenge), all pigs tested negative when targeting the *faeG* gene. On day 8 post-weaning, the highest levels of shedding were observed with 14 of 15 tested ETEC-challenged pigs shedding detectable levels of ETEC F4. Shedding levels dropped over the remaining sampling points with the number of pigs having detectable levels of ETEC in their faeces reducing over time.

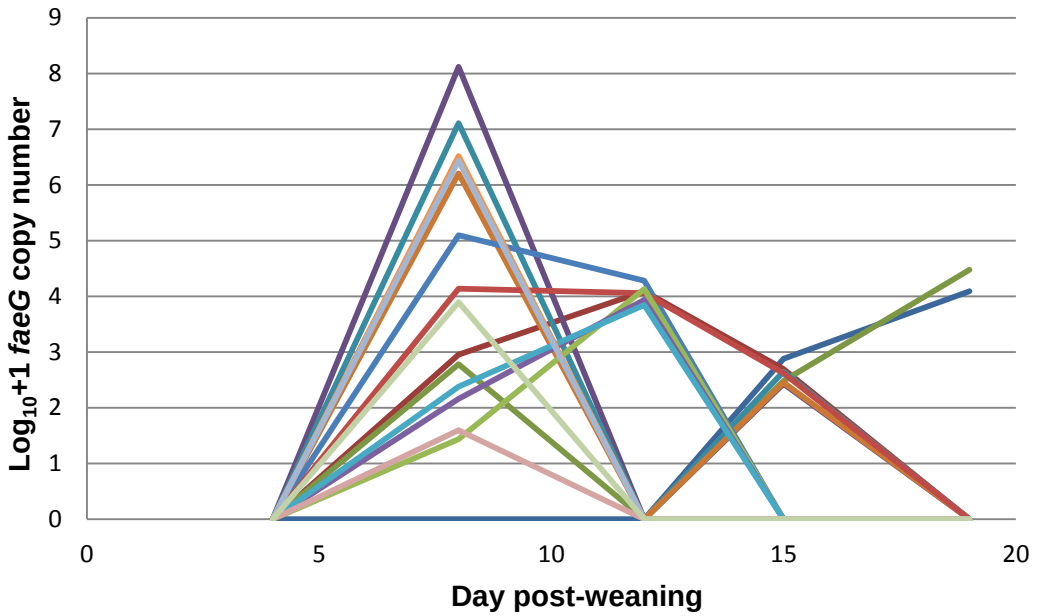

Figure 2.4: Faecal shedding of ETEC between day 4 and day 19 for each challenged pig pre-challenge (day 4) and post-challenge (n = 15, one pig missed out of analysis due to missing value).

2.3.5 Quality control of sequences

After carrying out quality control steps to minimise the number of poor quality sequences and sequencing artefacts, 17% of the original reads were removed, which left 16,816,541 reads in total for analysis. On average, 109,434 ± 43,035 (mean ± SD) reads were analysed per sample and 590 phylotypes were identified, with 90% of reads being classified at phylum level, 68% at family level, 51% at genus level and 24% at species level.

Co-sequencing a mock community allowed assessment of sequencing error rate and how well the developed method captured a known bacterial community. Using the mock community data, the sequencing error rate was calculated as 0.03%. All bacteria in the mock community were identified to genus level, and

45% of the strains were identified at species level. The proportions of expected and measured relative abundances are highlighted in **Table 2.3**. *Acinetobacter baumannii*, *Bacillus cereus* and the streptococci were under-represented by sequencing, whereas *Clostridium beijerinckii* was over-represented. The single hypervariable region, however, did manage to capture the diversity of the mock bacterial community.

Table 2.3: List of bacterial strains included in the mock bacterial community (including two *Staphylococcus* and three *Streptococcus* species), the obtained level of taxonomic classification post-sequencing, and both measured and expected relative abundances (relabund).

Mock community strain(s)	Level of identification	Measured relabund (%)	Expected relabund (%)
Acinetobacter baumannii	Genus	0.01	5.00
Actinomyces odontolyticus	Genus	4.99	5.00
Bacillus cereus	Species	0.01	5.00
Bacteroides vulgatus	Genus	6.52	5.00
Clostridium beijerinckii	Genus	13.83	5.00
Deinococcus radiodurans	Genus	4.20	5.00
Enterococcus faecalis	Genus	5.19	5.00
Escherichia coli	Species	6.97	5.00
Helicobacter pylori	Species	8.89	5.00
Lactobacillus gasseri	Genus	6.24	5.00
Listeria monocytogenes	Species	8.00	5.00
Neisseria meningitidis	Genus	6.51	5.00
Propionibacterium acnes	Species	8.05	5.00
Pseudomonas aeruginosa	Genus	2.48	5.00
Rhodobacter sphaeroides	Species	2.78	5.00
Staphylococcus spp.	Species	13.41	10.00
Streptococcus spp.	Genus	5.68	15.00

Co-sequencing reagent-only controls assists in identifying background contamination that may have been introduced during sample processing. Low DNA yield was evident from the reagent-only control extracts according to

spectrophotometer measurements (NanoDrop 1000, Thermo Scientific, UK), however background DNA contamination was detected post-sequencing. These sequences appeared to be diverse with low read numbers within each phylotype. Therefore, it is unlikely to affect the study results since faecal samples have a high biomass and therefore are less sensitive to contamination biases.

To ensure that sequencing depth was adequate for this study, Good's coverage was calculated. All samples had an estimated Good's coverage over 0.99. This indicated that an estimated 99% of the bacteria present in the faecal samples were captured during sequencing.

2.3.6 Taxonomic classification of sequences

A total of 21 different phyla were identified across all faecal samples. The majority of sequences were members of the Bacteroidetes (46%) or Firmicutes (34%), comprising 80% of all sequences. Spirochaetes and Proteobacteria were the third and fourth most dominant phyla, each comprising 4% of the total number of sequences. At family level, 50% of all sequences belonged to (in decreasing order of abundance) the Prevotellaceae, Lachnospiraceae, Ruminococcaceae, Paraprevotellaceae and Veillonellaceae.

A phylotype-based analysis was carried out, whereby sequences were binned according to taxonomic information. A total of 590 bacterial phylotypes were identified, with 14 of these phylotypes each representing over 1% of the total number of sequences. The most abundant phylotype was assigned as *Prevotella*,

comprising 25% of the total number of sequences. This phylotype was present almost consistently throughout all samples at a cut-off of 0.1% relative abundance (153 out of 154 samples).

There were no effects of experimental round, weaning day weight or sex on microbiota structure or phylotype relative abundances at any time point ($P < 0.05$). Therefore, data from both rounds were combined for analysis.

2.3.7 Temporal changes in the faecal microbiota

The changes in relative abundances of dominant phyla and families are illustrated in **Figure 2.5**, and the data was logit-transformed for statistical analysis. A significant increase in relative abundance was observed at phylum level in the Bacteroidetes (RM-ANOVA: $P < 0.001$), and significant decreases in relative abundances were observed in both the Proteobacteria and Spirochaetes (RM-ANOVA: $P < 0.05$). There was no impact of ETEC challenge on relative abundances at family level (RM-ANOVA: $P > 0.05$).

The richness (Chao 1) and diversity (ISI) of the faecal microbiota decreased significantly from Day 4 (Chao 1 = 74.31 ± 10.11, ISI = 7.09 ± 2.23) to Day 19 (Chao 1 = 65.29 ± 8.58, ISI = 5.57 ± 1.72) of the trial, inclusive of all pigs (**Table 2.4**).

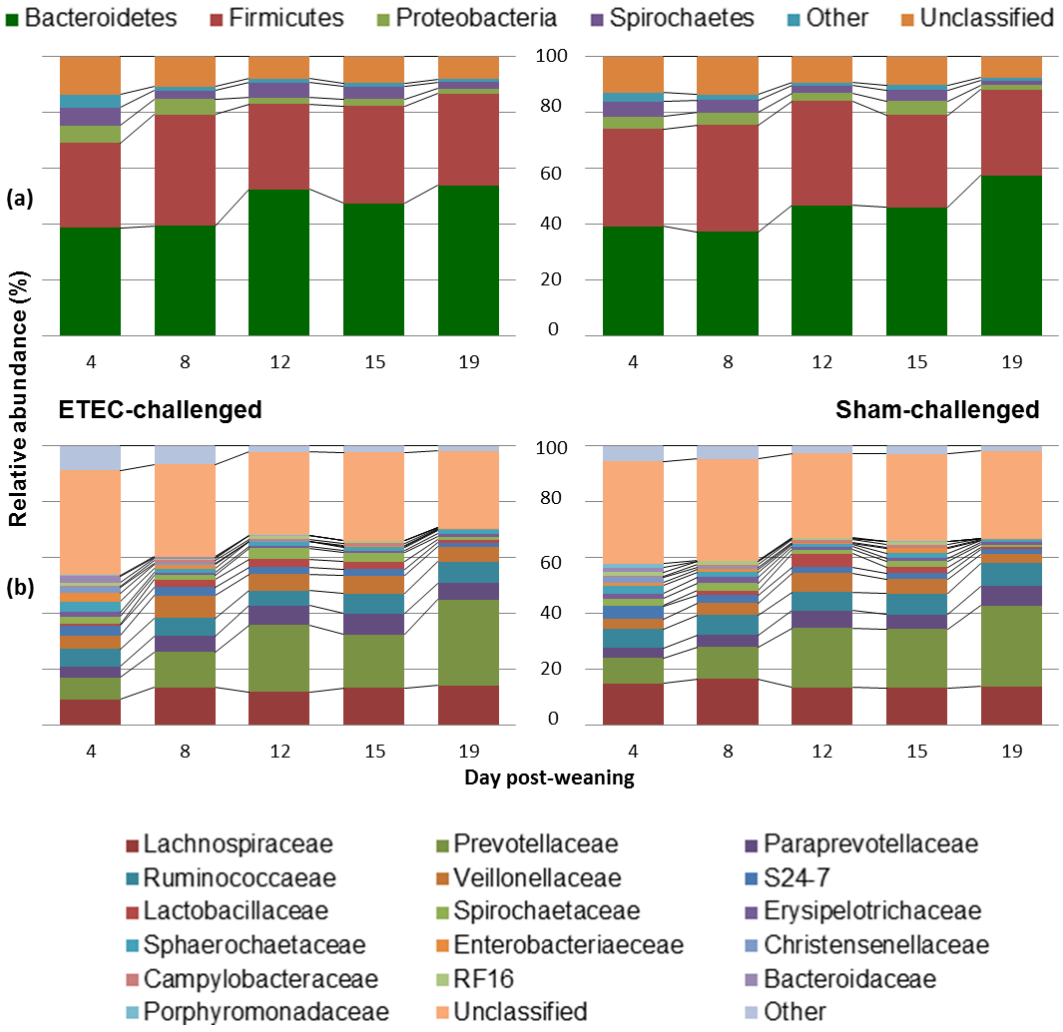

Figure 2.5: The dominant bacterial (a) phyla and (b) families identified, showing changes in relative abundances over a 19 day period post-weaning in both ETEC-challenged (n = 16) and sham-challenged (n = 16) pigs.

Table 2.4: Average richness (Chao 1) and diversity (ISI) metrics of ETEC-challenged (ETEC) and sham-challenged (SHAM) pigs, with changes over time being assessed by RM-ANOVA.

	Richness (CHAO 1) (± SD)		Diversity (ISI) (± SD)	
Day post-weaning	**ETEC**	**SHAM**	**ETEC**	**SHAM**
4	72.88 ± 11.39	75.64 ± 8.92	6.61 ± 2.24	7.54 ± 2.20
8	65.49 ± 7.95	76.22 ± 14.33	8.84 ± 1.63	8.43 ± 2.18
12	68.67 ± 11.90	68.63 ± 10.51	7.14 ± 2.31	6.87 ± 1.74
15	70.25 ± 11.01	70.44 ± 8.96	7.84 ± 1.97	7.15 ± 2.30
19	66.42 ± 9.63	64.16 ± 7.54	5.44 ± 1.58	5.45 ± 1.90
P-values				
Time	0.008		< 0.001	
Challenge	0.242		0.918	
Time x Challenge	0.050		0.415	

There was a change in faecal microbial community structure over time when testing both ETEC- and sham-challenged pigs (AMOVA: $P < 0.05$). Additionally, over the post-weaning period, the stability of the microbiota increased in both ETEC- and sham-challenged pigs (HOMOVA: $P < 0.05$). This is also visualised in a simplified NMDS plot, which combines all samples from Day 4 (baseline) and Day 19 (**Figure 2.6**).

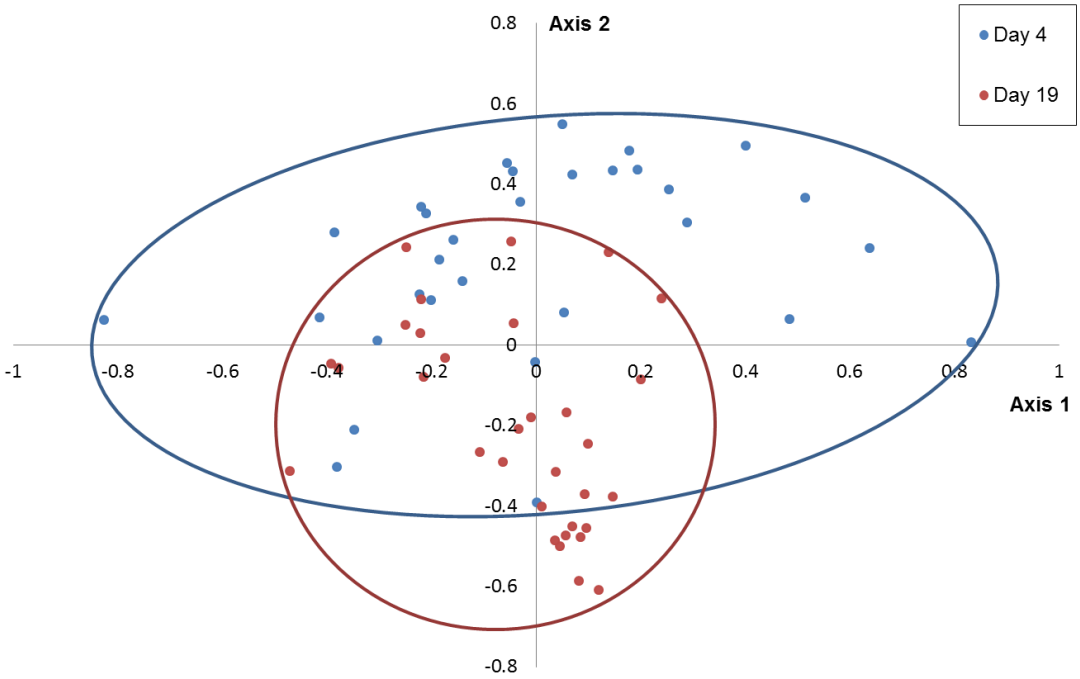

Figure 2.6: NMDS ordination of Yue and Clayton dissimilarity indices from faecal microbial communities pre-challenge (day 4) and 15 days post-challenge (day 19). Both ETEC- and sham-challenged pigs are included to visualise shifts in overall microbiota structure. Shifts in community structure (AMOVA: $P < 0.05$) and a decrease in genetic diversity (HOMOVA: $P < 0.05$) were evident over time. Stress value = 0.18.

To further assess changes in the microbiota composition over time, samples were clustered into community types using a DMM model. The DMM model with the highest likelihood partitioned all of the faecal samples into two enterotypes. The majority of faecal samples from day 4 (30/31, 1 missing value) belonged to enterotype 2. By day 19, the majority of faecal samples had switched to enterotype 1 (24/30, 2 missing values). The pigs which were still clustered into enterotype 2 by the end of the trial had clustered into enterotype

2 for all previous samples. Enterotype 1 was most substantially enriched for Prevotellaceae, with other enriched bacterial groups being Erysipelotrichaceae, Clostridiales and a member of the Clostridiales, *Faecalibacterium prausnitzii*. Enterotype 2 was most substantially enriched for Lachnospiraceae, with *Campylobacter* and *Lactobacillus* also being enriched in this group.

Changes in phylotype relative abundances were also examined over time using Metastats. A small number of phylotypes were differentially expressed between day 4 and day 8 in ETEC-challenged pigs (unclassified Clostridiales, *Prevotella* and Erysipelotrichaceae, Metastats: $P < 0.05$) and in sham-challenged pigs (*Prevotella copri*, *Lactobacillus*, *Faecalibacterium prausnitzii* and Erysipelotrichaceae, Metastats: $P < 0.05$). No significant changes in phylotype relative abundances occurred between day 8 and 12 and between day 12 and 15 in both ETEC- and sham-challenged pigs (Metastats: $P > 0.05$).

Further changes in phylotype relative abundances occurred between day 12 and 19, with a decrease in *Lactobacillus* being evident in both ETEC- and sham-challenged pigs (Metastats: $P < 0.05$). Changes in phylotype relative abundances over the entire post-weaning period (between day 4 and 19) have been summarised in **Table 2.5**. Phylotypes identified as *Prevotella copri, Prevotella stercorea* and *Prevotella* showed significant increases in relative

Table 2.5: Mean changes in relative abundance (± SEM) in dominant phylotypes between day 4 and day 19 in both ETEC-challenged (ETEC) and sham-challenged (SHAM) pigs. The significance of these changes were assessed using Metastats and significant findings are highlighted with an asterisk (FDR corrected P-value: P > 0.05). The phylotype relative abundance cut-off was assigned at 0.1% for any given time point.

	ETEC			SHAM		
Phylotype	**Day 4**	**Day 19**	**P-value**	**Day 4**	**Day 19**	**P-value**
unclassified Bacteria	14.5 ± 2.8	8.0 ± 1.0	0.077	12.9 ± 1.9	7.6 ± 0.7	0.001*
unclassified Lachnospiraceae	7.8 ± 1.0	7.5 ± 1.4	0.787	13.6 ± 2.2	6.7 ± 0.6	0.001*
Prevotella copri	4.7 ± 1.5	21.6 ± 2.8	0.006*	6.2 ± 2.0	20.0 ± 3.2	0.001*
unclassified Clostridiales	4.1 ± 0.6	2.5 ± 0.4	0.059	3.5 ± 0.4	2.6 ± 0.4	0.015*
Prevotella	1.7 ± 0.3	6.1 ± 1.7	0.006*	1.8 ± 0.5	5.7 ± 0.9	<0.001*
unclassified Bacteriodetes	2.8 ± 0.7	3.6 ± 0.4	0.403	2.2 ± 0.4	3.5 ± 0.5	0.006*
[Prevotella]	2.3 ± 1.1	3.9 ± 0.7	0.270	2.3 ± 0.7	4.9 ± 0.6	0.002*
S24-7 (Bacteroidetes)	3.5 ± 0.8	1.4 ± 0.3	0.010*	4.7 ± 1.3	1.8 ± 0.4	0.002*
Prevotella stercorea	1.2 ± 0.4	3.3 ± 0.5	0.009*	0.9 ± 0.2	2.9 ± 0.3	<0.001*
unclassified Ruminococcaeae	2.5 ± 0.5	2.1 ± 0.2	0.485	3.1 ± 0.5	2.2 ± 0.3	0.015*
Anaerovibrio	2.8 ± 1.5	2.5 ± 0.5	0.818	1.8 ± 0.5	1.3 ± 0.2	0.040*
Lactobacillus	0.4 ± 0.3	0.3 ± 0.1	0.838	0.0 ± 0.0	0.2 ± 0.0	0.002*
Treponema	2.6 ± 1.0	1.0 ± 0.5	0.229	2.5 ± 0.6	1.0 ± 0.3	0.006*
Phascolarctobacterium	1.3 ± 0.2	1.2 ± 0.2	0.647	1.4 ± 0.2	0.8 ± 0.2	0.006*
Sphaerochaeta	3.5 ± 0.9	1.3 ± 0.5	0.067	2.9 ± 0.6	0.8 ± 0.2	0.001*
Faecalibacterium prausnitzii	0.4 ± 0.3	2.2 ± 0.6	0.009*	0.3 ± 0.1	2.9 ± 0.7	<0.001*
Erysipelotrichaceae	0.0 ± 0.0	3.5 ± 0.7	0.006*	0.1 ± 0.1	3.6 ± 0.6	<0.001*

abundance in both ETEC- and sham-challenged pigs (Metastats: $P < 0.05$). *Faecalibacterium prausnitzii* and a phylotype classified as part of the Erysipelotrichaceae family also showed a significant increase in relative abundance in both ETEC- and sham-challenged pigs (Metastats: $P < 0.05$). The phylotype identified as S24-7 (family) decreased significantly in both groups (Metastats: $P < 0.05$).

2.3.8 ETEC challenge and the faecal microbiota

2.3.8.1 Main effects of challenge

Although there was an indication that a decrease in richness occurred more rapidly in ETEC-challenged pigs in comparison with sham-challenged pigs (RM-ANOVA: $P = 0.05$), the highly significant decrease in richness and diversity over time was not affected overall by ETEC challenge (RM-ANOVA: $P > 0.05$; **Table 2.4**).

AMOVA was used to test the statistical significance of clustering when comparing ETEC- and sham-challenged pig faecal microbial communities. Firstly, it was confirmed that there were no significant differences in community structure between pigs in the ETEC- and sham-challenged treatment groups at baseline (AMOVA: $P = 0.65$). Secondly, there were no significant differences in community structure at any of the sampling points post-challenge when comparing ETEC- and sham-challenged pigs (AMOVA: $P > 0.05$).

The stability of ETEC- and sham-challenged faecal communities were also compared at each sampling point and no significant effects of ETEC challenge were observed (HOMOVA: $P > 0.05$). Additionally, there were no differentially expressed phylotypes at baseline (i.e. day 4) or any time points post-challenge when comparing ETEC- and sham-challenged pigs (Metastats: $P > 0.05$), with no main effects of ETEC challenge on enterotype partitioning using DMM modelling.

2.3.8.2 ETEC shedding level and faecal microbiota structure

Large variation was observed in ETEC shedding levels across all challenged pigs. In order to identify low and high shedders, the cumulative area under the log curve (cumulative AULC) was calculated (**Figure 2.7**). Five high shedders and five low shedders were selected, based on their presence within the lower and upper ranges of the AULC data. At baseline (day 4) and at all time points post-challenge, there were no differences in community structure (AMOVA: $P > 0.05$) or phylotype relative abundances (Metastats: $P > 0.05$) when considering ETEC shedding level. However, on day 8, the high shedders had a significantly different community structure in comparison to the sham-challenged pigs (AMOVA: $P = 0.013$), whereas the low shedders had a more similar community structure in comparison with the sham-challenged pigs (AMOVA: $P > 0.05$), with no associated changes in phylotype relative abundances (Metastats: $P > 0.05$). This community structure difference was not present for the remainder of the experiment (AMOVA: $P > 0.05$).

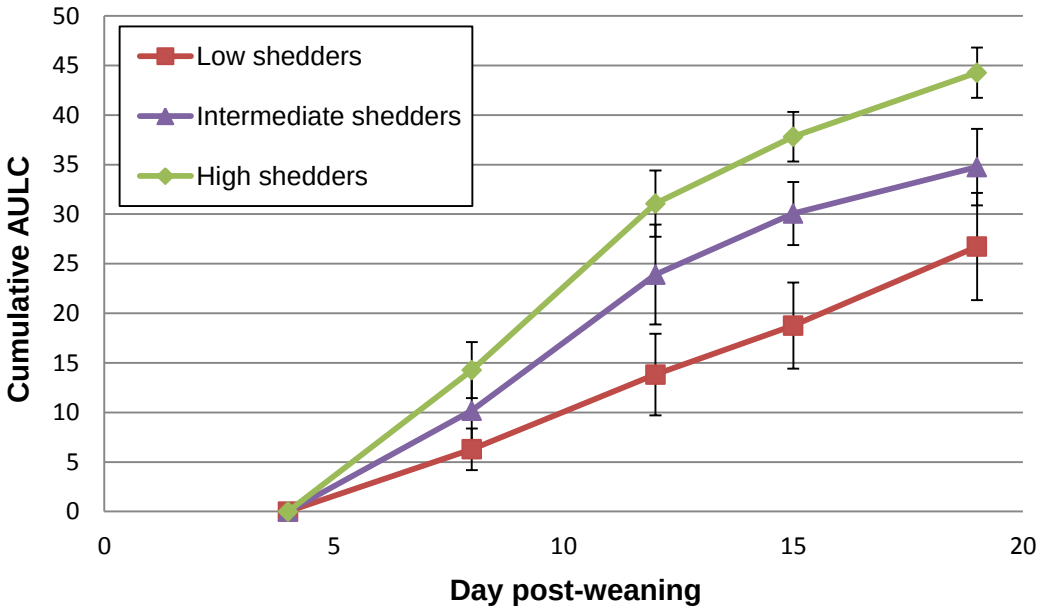

Figure 2.7: Cumulative area under the log curve (AULC) representing ETEC shedding level (i.e. low, intermediate and high shedding) by challenged pigs (± SD). The cumulative AULC was calculated using log normalised data for each pig at each of the sampling points post-weaning.

In order to investigate the possible relationships between ETEC shedding dynamics and faecal population structure, pigs were retrospectively clustered into two groups based on shedding dynamics on day 12 (**Figure 2.4**), where two clear groupings were evident in ETEC-challenged animals – ETEC "clearers" (i.e. no shedding detected, n = 9) and ETEC "shedders" (i.e. shedding evident between 6.95 x 10^2 and 1.91 x 10^3 *faeG* copies/g wet faeces, n = 6). No differences in community structure were evident in these groups prior to challenge on day 4, but the community structures were significantly different on days 12 (AMOVA: P = 0.029) and 19 (AMOVA: P = 0.037), as shown in **Figure 2.8**, but were not significantly different on days 8 and 15. There were also

significant differences in community stabilities on day 19, whereby the clearers had more variable bacterial communities in comparison with the shedders (HOMOVA: P = 0.045). No differences in community stability were observed on days 4, 8, 12 and 15 (HOMOVA: P > 0.05).

2.3.8.3 Pig performance and faecal microbiota composition

When comparing the logit-transformed relative abundance data from pigs with a low and high growth rate, at phylum level, there were no significant differences in the relative abundances of Bacteroidetes, Firmicutes or Spirochaetes when taking both growth rate and challenge status into account (RM-ANOVA: P > 0.05). However, the logit-transformed relative abundance of Proteobacteria was differentially expressed in pigs with a low and high growth rate over the trial duration (RM-ANOVA: P = 0.001), whereby pigs with a lower growth rate showed a markedly higher mean relative abundance of Proteobacteria on day 4 (low = 6.03 ± 2.41%, high = 4.57 ± 4.95%), day 8 (low = 6.74 ± 6.87%, high = 3.22 ± 2.55%) and day 15 (low = 5.17 ± 6.25%, high = 2.15 ± 1.15%).

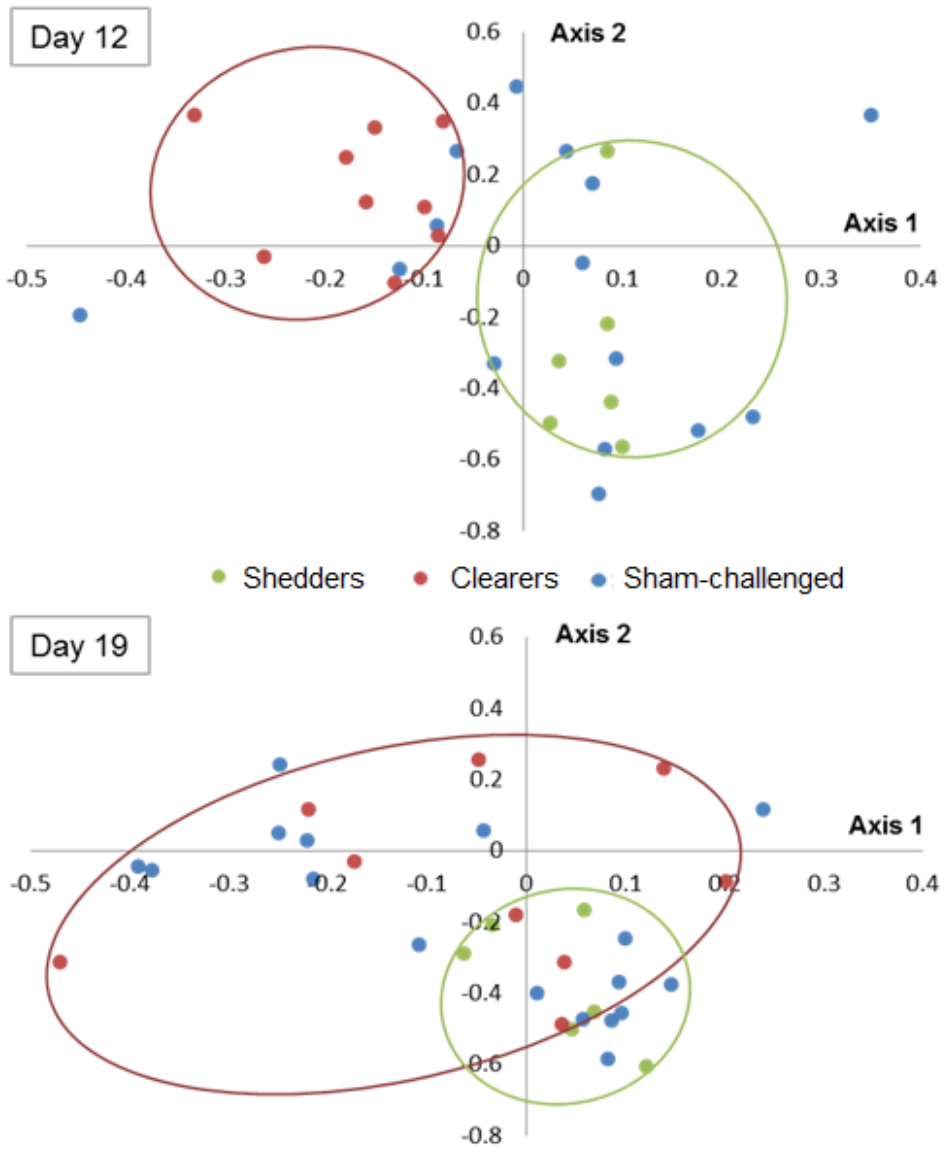

Figure 2.8: NMDS ordinations of Yue and Clayton dissimilarity indices from faecal microbial communities obtained on days 12 and 19 in relation to shedding status. Community structures were significantly different on days 12 (AMOVA: P = 0.029) and 19 (AMOVA: P = 0.037) based on shedding dynamics in day 12, where two distinct "clearer" and "shedder" groups were observed. On day 19, the clearers had more variable bacterial communities in comparison with the shedders (HOMOVA: P = 0.045).

The majority of bacterial families tested (see **Figure 2.5**) were not differentially expressed when comparing pigs with a low and high growth rate. However, the relative abundance of Enterobacteriaceae was differentially expressed over the duration of the trial when comparing pigs with a low and high growth rate (RM-ANOVA: $P < 0.001$), with the former showing markedly higher mean relative abundances on day 8 (low = 2.27 ± 3.44%, high = 0.26 ± 0.53%) and day 15 (low = 1.97 ± 6.27%, high = 0.06 ± 0.05%). There were no effects of growth rate status on microbiota structure (AMOVA: $P > 0.05$), phylotype relative abundances (Metastats: $P > 0.05$) or enterotype partitioning (DMM) at any time point.

2.4 Discussion

The aims of this experiment were to characterise the weaner pig faecal microbiota over the immediate post-weaning period, to ascertain whether experimental ETEC challenge had an impact on faecal microbiota composition, pig performance and health and to establish whether there was a link between faecal microbiota composition and pig growth rate. To our knowledge, this is the first study which implements 16S rRNA gene sequencing to study porcine gut microbiota dynamics in response to ETEC challenge, particularly when considering ETEC shedding levels and growth rate.

2.4.1 Temporal changes in the faecal microbiota

In the current study, over 80% of all DNA sequences generated belonged to the Bacteroidetes and Firmicutes phyla, which is in line with existing literature (Costa et al., 2014; Kim et al., 2011; Mach et al., 2015). An increase in Bacteroidetes was evident over the immediate post-weaning period, which is also reflected in other recent work (Pajarillo et al., 2014). This increase in Bacteroidetes was clearly driven by increases in Prevotellaceae, which contains the *Prevotella* genus. Similarly, as in other previous work, *Prevotella* was the most dominant genus in pig faeces (Holman and Chénier, 2014; Kim et al., 2011, 2012a; Lamendella et al., 2011; Liu et al., 2012; Looft et al., 2012). Additionally, other studies have highlighted that the relative abundance of *Prevotella* tends to increase post-weaning (Pajarillo et al., 2014; Mach et al., 2015), which was also a finding of this study whereby the climax community enterotype was most noticeably enriched for Prevotellaceae. The increase in *Prevotella* after weaning is likely due to the ability of this bacterium to produce enzymes that can break down polysaccharides in the cereal cell wall (Flint et al., 2008), which is discussed in more detail in **Chapter 1**.

Significant changes in microbiota structure were observed over time when taking both bacterial membership and relative abundances into account. Previous work has shown that suckling piglets have different gut microbial communities in comparison to weaner piglets (Pajarillo et al., 2014; Holman and Chénier, 2014; Kim et al., 2011; Zhao et al., 2015). These observed community shifts post-weaning are most likely associated with the piglets' removal from a

highly digestible milk source, the consequent removal of maternal antibodies, and sudden introduction to a less digestible, solid diet (Lallès et al., 2007). The weaning process is also linked with social stressors, such as separation from the sow and litter mixing, which results in higher susceptibility to enteric disorders (Lallès et al., 2007). Additionally, as discussed in **Chapter 1**, the environment in which a piglet is housed has an impact on mucosal immune function and microbiota composition (Mulder et al., 2009) and will likely have a collective impact on the gut microbiota structure.

It was also found that the faecal microbiota became more stable over time when comparing samples from day 4 and day 19 post-weaning. The stability of a microbial community is thought to be an important consideration for aiding understanding of the effects of dietary change and other perturbations (such as disease states) on host health (Schloss et al., 2012). Additionally, differences in community stability have been linked with healthy and unhealthy states, and temporal microbiota studies assist in linking variations in stability with variation in health states (Schloss et al., 2012). Throughout the duration of this experiment, pigs remained healthy which may assist in explaining why the faecal microbiota moved towards a more stable composition in a relatively short time frame post-weaning.

2.4.2 Effects of ETEC challenge on pig performance and health

In this study, there were no significant effects of ETEC challenge on the health scores of pigs. This was expected, since the implemented model previously resulted in a sub-clinical state, whereby a minimal increase in diarrhoeal scores

and a transient reduction in ADFI and ADWG were observed (Athanasiadou et al., 2010). In the current study, however, there were no effects of ETEC challenge on ADFI and ADWG. These findings may suggest that the piglets utilised for this study were perhaps more resilient and therefore did not suffer any ill effects after exposure to ETEC. Indeed, it has been shown on many occasions that it is difficult to reproduce the effects of experimental PWC challenge due to the multifactorial nature of the disease (Madec et al., 2000).

2.4.3 Relationship between ETEC shedding level and microbiota structure

In this study, there were no treatment effects of experimental ETEC challenge on faecal microbiota structure, stability or phylotype relative abundances at any time point. However, there was a great deal of inter-individual variation when measuring ETEC shedding levels which has been reflected in previous studies (Geenen et al., 2007; Hampson et al., 1985). One factor that has been linked to variation in colonisation and consequently shedding is the presence of the ETEC F4 receptor, with significant increases in shedding being associated with the expression of this receptor (Geenen et al., 2007). The variations in ETEC shedding level could also be partly attributed to the health status of the pig, variations in innate and adaptive immune responses, genetic variation and competition with the commensal microbiota.

During this study, variations in shedding level were associated with changes in faecal microbiota structure and stability. When taking cumulative ETEC shedding levels into account, on day 8, the high shedders had a significantly

different community structure in comparison to the sham-challenged pigs. However, the low shedders had a more similar community structure to the sham-challenged pigs than to the high shedder pigs. Additionally, pigs were retrospectively clustered into groups based on shedding dynamics on day 12, where two clear groupings were evident. Interestingly, the community structures were significantly different on day 12 and day 19, and more genetic diversity was observed in the "clearers" in comparison to the "shedders" on day 19. Collectively, the data suggests that to address the long-term impact of ETEC challenge on faecal microbiota composition, ETEC shedding level should be taken into account.

2.4.4 The faecal microbiota as a representative

The majority of studies on the gut microbiota, in both humans and animals, have been focussed on analysis of faecal samples for a variety of reasons. Firstly, earlier studies on the gut microbiota may have been focussed on faeces due to initial thoughts that the faecal microbiota was representative of the populations upstream in the gastrointestinal tract (Moore et al., 1978). Secondly, on a more technical note, faeces can be collected fairly non-invasively and contain large numbers of microbial cells which are consequently easier to process and culture (Gerritsen et al., 2011). Thirdly, when considering experimental designs, longitudinal studies can be carried out using the same individuals which adds power to experiments. However, it is now well established that microbial populations vary significantly in different gut compartments (Leser et al., 2002; Looft et al., 2014).

For this study, there were both technical and biological reasons for focussing on the faecal microbiota. To address the former, this experiment was part of a larger study during which post-mortem was not possible due to the assessment of longer term performance parameters. Biologically, there was an interest in establishing whether there was a relationship between faecal ETEC shedding and faecal microbiota composition. Specfically, there was interest in addressing whether there were "marker" phylotypes in the faeces which were sensitive to ETEC challenge. Although the microbiota composition changed significantly over time, there was no treatment effect of ETEC challenge on the faecal microbiota. Collectively, however, it must be considered that the site of colonisation for ETEC F4 is the small intestine (Daudelin et al., 2011; Fairbrother et al., 2005), so any further effects exerted on the microbiota may have been localised and not detectable at faecal level.

2.4.5 Growth rate and faecal microbiota composition

As part of this study, pigs were selected for 16S rRNA gene sequencing on the basis of differences in growth rate. No significant differences in bacterial community structure or stability were observed when comparing pigs with a "low" and "high" growth rate, with no associated differences in phylotype relative abundances. However, at phylum level, the relative abundance of Proteobacteria was differentially expressed in the two groups. Overall, the levels of Proteobacteria decreased significantly over time (around 5-fold) but pigs with a low growth rate had a markedly higher level of Proteobacteria on days 4, 8 and 15. At family level, the relative abundances of Enterobacteriaceae

were differentially expressed in pigs with a low and high growth rate over the trial duration. Although relative abundances in this family were particularly dynamic over the trial, the pigs with a lower growth rate showed a mean relative abundance at least 10-fold higher than the pigs with a higher growth rate on days 8, 15 and 19.

Traditionally, Proteobacteria (and specifically, the Enterobacteriaceae) are of interest since this phylum contains a number of pathogenic bacteria such as *Salmonella* species and *Escherichia coli*. In many studies, an increased level of Enterobacteriaeceae is used as an indicator of poorer gut health (Buzoianu et al., 2012; Heinritz et al., 2016a; Vigors et al., 2016). Specifically, Hermann-Bank et al. (2015) found that *Enterococcus* species were around 24-times more abundant in pigs with diarrhoea. Conversely, opportunistic pathogens such as *Enterococcus cloacae*, *Klebsiella pneumoniae* and *Citrobacter freundii* have also been found to dominate the porcine gastrointestinal tract and did not appear to affect host health despite these high numbers (Guenther et al., 2010; Schierack et al., 2007). However, the Enterobacteriaceae family also contains many commensal organisms and are distributed throughout the gastrointestinal tract of healthy piglets in the current study and in previous studies (Gordon and FitzGibbon, 1999; Schierack et al., 2007). Currently, there is little knowledge on the distribution of non-pathogenic or facultatively pathogenic Enterobacteriaceae in the pig gut, since many studies are focussed on pathogenic bacteria. Therefore, although differential relative abundances of Proteobacteria and Enterobacteriaceae were observed in pigs with a low and

high growth rate as part of this study, this must be carefully interpreted at such high taxonomic levels.

As part of this study, like in other recent work (Ramayo-Caldas et al., 2016; Mach et al., 2015), phylotype-level analyses were carried out to establish whether there were links with growth performance. In this study, no relationships were found between phylotype relative abundances and growth rate, and enterotype partitioning and growth rate. Conversely, Ramayo-Caldas et al. (2016) found that pigs that clustered into a *Prevotella* enterotype performed better than pigs clustered into a *Ruminococcus* enterotype and earlier work by Mach et al. (2015) showed that *Prevotella* abundance was positively correlated with body weight.

2.4.6 Nalidixic acid-resistant bacteria

Standard enumeration techniques using agar plates have the advantage of being economical, practical and relatively fast, since viable bacterial counts can be obtained without pre-enrichment. However, there are also many disadvantages to agar plating methods. There is a great deal of variation in the composition and concentration of the commensal microbiota and this can cause problems when trying to isolate a pathogen of interest. As well as this, generally the pathogen of interest in an experimental challenge study has a known antimicrobial resistance pattern to enable the use of antimicrobial-infused agars for selection purposes.

In previous experiments run by our group, the use of nalidixic acid-enriched agar plates has been successful in eliminating background growth and enumerating the ETEC F4 challenge strain. However, in round 2 of this experiment, background growth of another nalidixic acid-resistant organism occurred. As a result, an alternative enumeration method had to be considered which led to the development of a qPCR assay for the detection of ETEC F4 to eliminate this contamination. The contaminant was identified as nalidixic acid-resistant *Enterococcus* species by Sanger 16S rRNA gene sequencing, which is an abundant member of the porcine gastrointestinal tract.

2.4.7 Reagent-only controls and contamination

Sampling and DNA extraction are crucial steps in microbial community analysis experiments. A previous study has described contamination which arose from personnel and laboratory consumables, as well as contamination within DNA extraction kits and PCR reagents (Salter et al., 2014). Some of these factors, at least in part, were addressed in this study by including reagent-only controls. In this study, low DNA yield was observed in the buffer-only control extracts and background DNA contamination was evident on sequencing. The sequences generated were diverse and low read numbers were observed within each phylotype. Since faecal samples have a high biomass and are less sensitive to contamination biases, phylotypes that were identified in faecal samples and the reagent-only controls were not removed from the analysis. Many bacteria that are associated with the gastrointestinal tract were amplified from the reagent-only controls (e.g. *Faecalibacterium* spp. and *Anaerovibrio* spp.), and therefore it

is difficult to justify removal of these from the analysis due to the potential of introducing more bias.

2.4.8 Mock bacterial community and sequencing errors

In this study, a mock bacterial community was sequenced in parallel with the faecal samples to assess how well the developed 16S rRNA gene metabarcoding method captured a known bacterial community and to allow calculation of sequencing error rate. When comparing the expected relative abundances with the measured relative abundances, it was found that some bacterial species were over-represented or under-represented by this method. This misrepresentation may be caused by a variety of factors, including primer biases and the bioinformatics pipeline used (Pinto and Raskin, 2012; Schloss et al., 2011).

As part of this study, the V3 hypervariable region of the 16S rRNA gene was targeted for sequencing. There are limitations in using a small 16S rRNA gene fragment, since it can be more difficult to accurately identify bacteria at genus or species level. However, the key advantage of targeting a small fragment is the minimisation of sequencing error rate due to partial (or in this case, complete) overlap of contiguous sequences. Indeed, the calculated error rate as part of this sequencing run was low (i.e. 0.03%) which ensures that over-inflation of bacterial diversity due to sequencing error will have been minimised.

Finally, it must also be considered that different bacterial taxa contain variable copy numbers of the 16S rRNA gene (Kembel et al., 2012), which further complicates the analysis of this sequencing data. However, the same methodology was applied across all samples and it can therefore be assumed that these biases will occur evenly across the entire data set and so comparisons between sample groups are valid.

2.5 Conclusion

In conclusion, changes in community structure and stability were observed over a 19 day period post-weaning. Although there were no observed effects of ETEC challenge on pig performance and health, when taking ETEC shedding level into account, variations in microbiota structure and stability were observed at specific time points. Differential expression of both Proteobacteria and Enterobacteriaceae was evident in pigs with a low and high growth rate. Using a 16S rRNA gene metabarcoding method developed as part of this study, a better understanding of faecal microbiota dynamics post-weaning has been obtained which will lead to further ETEC challenge studies focussing on both the ileal and faecal microbiota.

Chapter 3:

Intestinal adhesion
and faecal shedding of ETEC
in experimentally challenged
weaner pigs

3.1 Introduction

Due to the economic importance of post-weaning colibacillosis (PWC), numerous studies have been carried out to attempt to empirically model the disease in an experimental setting, which have been discussed in detail in **Chapter 1.** The symptoms which present due to ETEC infection in pigs range from faecal shedding of the organism to peracute fatal diarrhoea (Hodgson and Barton, 2009), and sub-clinical cases of PWC (i.e. absence of diarrhoea) can have a negative impact on host performance (Hampson, 1994).

An ETEC challenge model was previously developed in our group (Athanasiadou et al., 2010) which did not result in a significant change in faecal consistency score, but did lead to a decrease in average daily feed intake and average daily weight gain. This multiple-dose model was used in the experiment described in **Chapter 2**. However, a multiple-dose model has the disadvantage of limiting the study of pathogen shedding dynamics, since detection of the challenge pathogen in the faeces may be due to the organism simply passaging through the gastrointestinal tract.

A single-dose infection model has also been developed by our group, whereby an impact on average daily feed intake and average daily weight gain was also observed (Wellock et al., 2008b; Houdijk et al., 2007). However, when both the multiple-dose and single-dose challenge models were developed, the aims of these studies were to address nutrition and production effects of ETEC challenge. Consequently, it was not assessed whether ETEC had adhered to the

small intestine. In addition to the expression of F4 fimbriae, which are key adhesion factors, the majority of ETEC F4 strains are associated with enterotoxins, such as LT, STa and STb, which are key virulence determinants (Nataro and Kaper, 1998). Therefore, it is important that the ETEC isolates used as part of a challenge model are well characterised to establish the presence or absence of the genes that encode for these virulence factors.

In this pilot study, a single-dose challenge model was used in order to gain a better understanding of ETEC adhesion in the small intestine and associated faecal shedding. The primary aims of this study were to characterise the ETEC F4 strains which have been used in previous challenge models and to assess adhesion and shedding dynamics during a single-dose challenge. A secondary aim was to assess the impact of the single-dose challenge on health parameters and aspects of growth performance.

3.2 Methods

3.2.1 Animals and housing

Twenty-one pigs (Large White x Landrace) were weaned at 27 days of age and weighed 8.66 ± 1.10kg, with 7 pigs being selected from 3 litters. Pigs were housed in 4m^2 pens in a single room and were bedded with shavings. The pens were cleared of faecal material and wet sawdust daily. Pens were equipped with a single feeder and nipple drinker for *ad libitum* water access. Environmental temperature was set as required for young pigs; at 25^oC for the first 4 days, with a reduction of 2^oC per week until the end of the experiment.

Lights were on from 08.00 until 17.00, and night lights were maintained at other times. The pigs were fed a commercial weaner pig diet *ad libitium*, which did not contain antibiotics.

3.2.2 Experimental design

On day 1 (i.e. one day post-weaning), 3 pigs (one pig from each litter) were subject to post-mortem to provide baseline gut content samples for analysis. The remaining 18 pigs were housed in four pens and allocated to treatments as shown in **Table 3.1**, which were balanced for sex, litter origin and weaning weight. After ETEC challenge on day 2, further post-mortems were carried out on days 4, 6 and 8 (pen 1-3 only) and on day 11, all remaining pigs were subject to post-mortem, including the sham-challenged pigs.

Table 3.1: Pig allocation to treatments after baseline post-mortems. Pigs in pens 1-3 were ETEC-challenged (ETEC) and pigs in pen 4 were sham-challenged (SHAM). Post mortems were carried out on days 4, 6, 8 and 11 as highlighted in the table, with selections being balanced for litter.

Pen ID	Litter 1	Litter 2	Litter 3
Pen 1	ETEC, day 4	ETEC, day 6	ETEC, day 8
	ETEC, day 11		ETEC, day 11
Pen 2	ETEC, day 6	ETEC, day 8	ETEC, day 4
	ETEC, day 11	ETEC, day 11	
Pen 3	ETEC, day 8	ETEC, day 4	ETEC, day 6
		ETEC, day 11	ETEC, day 11
Pen 4	SHAM, day 11	SHAM, day 11	SHAM, day 11

3.2.3 ETEC strains

Frozen bead stocks of two ETEC F4 strains (ETEC 239 and ETEC 591) were obtained, which were isolated from pigs diagnosed with post-weaning colibacillosis (SRUC Veterinary Services, UK) and nalidixic acid-resistant variants were produced as described in **Chapter 2**. To further characterise the virulence factors within these strains, well-characterised ETEC F4 strains (GIS26 - O149: F4ac+, LT+ STa+ STb+ EAST+, GIS26 Δ F4 - O149: F4ac-, LT+ STa+ STb+ EAST+) were obtained to use as controls (University of Ghent, Belgium). Additionally, an *E. coli* K12 strain was used as a negative control for this screening.

3.2.4 ETEC strain characterisation

All bacterial strains were recovered from glycerol or bead stocks by direct plating onto 5% sheep blood agar and the plates were incubated at 37^{o}C for 18-24 hours. A single, well-isolated colony was selected from each of the plates and these were suspended in 1ml of nuclease-free water (Qiagen, UK) and vortexed for 5 seconds. Bacterial genomic DNA was extracted from the bacterial suspension using a MoBio PowerSoil® DNA Isolation Kit (Cambio, UK) with some modifications. Specifically, 500µl of bacterial suspension was aliquoted into the bead tubes and bead solution prior to following the enclosed instructions. A negative control sample (i.e. 500µl of nuclease-free water) was also prepared in the same manner.

Conventional PCR was then carried out (G-Storm Thermal Cycler, Labtech, UK) using previously published primers (**Table 3.2**) to screen the SRUC strains for the presence of key virulence factors. PCR mastermixes were set up using JumpStart™ REDtaq® ReadyMix™ Reaction Mix (Sigma Aldrich, UK) in a final volume of 20µl, with inclusion of primers at a final concentration of 0.2µM. The PCR conditions consisted of an initial denaturation step at 94°C (2 minutes), followed by 30 cycles of amplification at 94°C (30 seconds), 55°C (30 seconds) then 72°C (30 seconds), and a final extension step at 72°C (5 minutes). PCR product size was confirmed by gel electrophoresis, using a 2% agarose gel with products being visualised using an ultraviolet transilluminator (Gel Doc XR+ System, Bio-Rad, UK).

Table 3.2: Complete primer list for this experiment, including the original reference from which they were obtained.

Primer target	Forward Primer (5 ' – 3')	Reverse Primer (5' – 3')	Reference
F4	ATCGGTGGTAGTATC ACTGC	AACCTGCGACGTCAACA AGA	Ojeniyi et al, (1994)
STa	TCCCCTCTTTTAGTC AGTCAACTG	GCACAGGCAGGATTACA ACAAAGT	Ngeleka et al. (2003)
STb	GCAATAAGGTTGAGG TGAT	GCCTGCAGTGAGAAATG GAC	Lortie et al. (1991)
LT	TTACGGCGTTACTAT CCTCTCTA	GGTCTCGGTCAGATATG TGATTC	Furrer et al. (1990)
EAST1	CCATCAACACAGTAT ATCCGA	GGTCGCGAGTGACGGCT TTGT	(Yamamoto and Nakazawa (1997)
16S rRNA	AGAGTTTGATCCTGG CTCAG	GGTTACCTTGTTACGAC TT	Weisburg et al. (1991)

3.2.5 ETEC inoculum and challenge

Bead stocks of the ETEC challenge isolates were revived by picking an individual bead from the appropriate storage tube, and streaking directly onto 5% sheep blood agar, prior to incubation at 37^{o}C for 18 hours. A well-isolated bacterial colony was immersed in 5ml of brain-heart infusion broth containing nalidixic acid (15µg/ml) for 24 hours (with shaking) to produce a stationary phase culture. Bacterial cells were then harvested via centrifugation (Heraeus Labofuge 400, Thermo Scientific, UK) and the pellet was washed three times in 25ml of PBS. The pellet was resuspended and an inoculum containing an estimated 10^8 cfu/ml was prepared. The optical density of this inoculum was then measured using a spectrophotometer (Ultrospec 2100 Pro, Fisher Scientific, UK) to estimate bacterial cell concentration. For a more accurate *post-hoc* confirmation of ETEC cell concentration, the inoculum was serially diluted and enumerated on MacConkey agar plates. On day 2, pigs were orally administered 8 x 10^8 ETEC bacterial cells in 3ml of PBS using a syringe. Control animals were given 3ml of PBS in the same manner. This dose was trickled slowly at the back of the mouth, to ensure that a swallowing reflex took place.

3.2.6 Faecal sampling

Faecal samples were collected by carefully inserting the small spoon in a spooned universal tube into the rectum of the pig and around 0.5g of faecal material was collected. The faecal samples were placed immediately onto wet ice prior to bacteriological analysis. Faecal samples were collected from all pigs on day 1, prior to ETEC challenge on day 2. Further faecal samples were then

taken daily between day 3 and day 11 from both ETEC- and sham-challenged pigs.

3.2.7 Post-mortem sampling

At post-mortem, pigs were first sedated using a mixture of medetomidine (0.01 ml/kg at 1mg/ml), midazolam (0.1ml/kg at 5mg/ml), ketamine (0.1ml/kg at 100mg/ml) and azaperone (0.025ml/kg at 40mg/ml). After sedation, the pigs were euthanised by intracardiac injection of pentobarbital (0.7ml/kg at 200mg/ml). The abdomen was opened up from pubis to sternum to reveal the gastrointestinal tract, which was then separated into 3 sections (jejenum, ileum and large intestine) using string as ligatures to minimise the movement of gut contents. The terminal ileum was measured out cranially from the ileal-caecal junction (around 25cm in length). From this point, a further 10cm was measured out cranially and tied off, and was classified as the jejenum. A 10cm section of the proximal large intestine was tied off, measured caudally from the caecal-colonic junction.

After the required parts of the gastrointestinal tract were tied off, they were cut from the rest of the tract before emptying the contents into individual universal tubes before being stored on wet ice for bacteriological analysis. The sections of tissue were then carefully cut open longitudinally using forceps and scissors, and washed three times with ice-cold phosphate buffered saline (PBS). Five sections from the ileum, and one section from the jejenum and large intestine, were fixed in 10% formalin for histopathological analysis. Five sections from

the ileum, and one section from the jejenum and large intestine, were also stored in PBS and stored on wet ice prior to bacteriological analysis.

3.2.8 Bacteriology and ETEC identification

To enumerate ETEC F4 from the ileal digesta and faeces, samples were weighed and homogenised in PBS. For the detection of ETEC F4 on the tissue mucosa, 500mg of tissue was homogenised in ice-cold PBS for 10 seconds. Five serial dilutions were carried out per sample and 100µl of each dilution was plated out onto MacConkey agar infused with of 15µg/ml nalidixic acid. The plates were incubated at 37^{o}C for 18-24 hours, prior to observation of colony morphology and counting. A small sub-sample of suspected *E. coli* colonies and any atypical colonies were picked off of the plates and were subject to direct DNA extraction using the Wizard® Genomic DNA Purification kit (Promega, UK) (following the protocol as written for Gram-negative bacteria), 16S rRNA gene amplification (**Table 3.2**) and Sanger sequencing (Eurofins, Germany) for identification. Additionally, a single suspected ETEC colony was selected from each sample, and were subject to PCR to confirm the presence of the *faeG* gene (**Table 3.2**) as described previously.

3.2.9 ETEC detection by conventional PCR

The faecal samples prepared for bacteriology were also taken forward for total DNA extraction using the MoBio PowerSoil® DNA Isolation Kit (Cambio, UK), with modifications to the provided manufacturer instructions. Briefly, the homogenised faecal samples were centrifuged at low force (1500 rcf) for 5

minutes to spin down large particulate matter (i.e. undigested food particles and shavings), and 1ml of the supernatant was added to the provided bead tubes. The manufacturer's instructions were then followed as written.

3.2.10 Histopathology

Tissue sections from the jejenum, ileum and large intestine which were fixed in 10% formalin were embedded in paraffin and sections were cut before staining with haematoxylin and eosin. The stained sections were examined by light microscopy and were scored by a veterinary pathologist (SRUC Veterinary Services, UK) blinded to challenge status, but aware of the associated time points. Scores were assigned for congestion, oedema, inflammation, epithelial degeneration, bacterial attachment, villus or crypt damage, Peyer's patch depletion and lymphadenitis. Scores were assigned as follows: 1 - no difference from expected level for age group, 2 - very mild and/or localised changes, 3 – moderate changes, 4 – marked changes and 5 – severe changes.

3.2.11 Pig performance and health

Average daily feed intake (ADFI) was calculated per pen by measuring the feed offered and refused daily. All pigs were weighed on days 0, 2, 4, 6, 8 and 11. The weights were then used for the calculation of average weight gain (ADWG) per pig. Faecal consistency, cleanliness and overall health scores were taken each morning using a subjective four-point scale as described in **Chapter 2** (**Table 2.1**).

3.2.12 Statistical analyses

This study was not designed to be analysed statistically, as temporal post-mortem samples were taken from ETEC-challenged pigs only. Instead, data have been described using descriptive statistics. Growth data could have been analysed statistically with the pig as the experimental unit for ADWG, but the pigs in the single sham-challenged control group lagged behind for several days in terms of starting to consume sufficient food. Such delays are not uncommon but in this case resulted in ADWG data not being representative of normal post-weaning performance. Furthermore, for pen-based observations on feed intake, there were three ETEC-challenged pens but only one sham-challenged pen, which prevented the effective use of statistical tests due to lack of replication.

3.3 Results

3.3.1 Characterisation of ETEC strains

Using conventional PCR, the virulence factor profile of the previously characterised GIS26 and GIS26 Δ F4 strains were confirmed (**Table 3.3; see Appendix D for gel images**), and the F4 knockout strain tested negative for the *faeG* gene. The *E. coli* K12 strain tested negative for all virulence determinants. The SRUC strain, ETEC 239, was shown to have the full complement of virulence determinants as seen in GIS26, whereas ETEC 591 tested negative for STa. Consequently, SRUC ETEC 239 was taken forward as the challenge strain for this and further experiments.

Table 3.3: PCR screening results for SRUC strains, and University of Ghent strains with the inclusion of a negative control (*E. coli* K12).

Strain ID	F4	STa	STb	LT	EAST1
ETEC 239	+	+	+	+	+
ETEC 591	+	-	+	+	+
GIS26	+	+	+	+	+
GIS26 Δ F4	-	+	+	+	+
***E. coli* K12**	-	-	-	-	-

3.3.2 Bacteriology

Unexpectedly, the baseline samples (including faecal, mucosal and ileal content samples) yielded nalidixic acid-resistant colonies at high levels. These colonies were morphologically dissimilar to ETEC, but since the levels of growth were high, it is likely that this had a competitive effect and affected ETEC F4 growth and confounded this measurement. Consequently, the quantification of ETEC was not possible using agar plating methods and colony counts will not be presented. A selection of both suspect ETEC colonies and atypical colonies were identified by Sanger sequencing (Eurofins, Germany). All suspected ETEC colonies were identified to species level as *Escherichia coli*, and all atypical colonies were identified at genus level as *Enterococcus* species.

3.3.3 ETEC detection in faeces

Presence or absence of ETEC F4 was established using conventional PCR and gel electrophoresis, using both DNA prepared from individual colonies and total

DNA as template. The comparison of both tests is presented in **Figure 3.1**. On day 1 (pre-challenge), all pigs tested negative by both direct colony and total DNA PCR. Throughout the experiment, all sham-challenged pigs tested negative at faecal level for the presence of the *faeG* gene. On day 3 (1 day post-challenge), 9 out of 15 pigs challenged with ETEC tested positive using both PCR screening methods. On day 7, all pigs tested positive using the direct colony PCR, with 6 out of the 9 pigs testing positive using total DNA PCR. On day 8, using both methods, 1 pig out of 9 tested positive and on the remaining sampling days (i.e. day 9 - 11), all pigs tested negative for ETEC F4 using both methods.

3.3.4 ETEC detection in post-mortem samples

Presence or absence of ETEC F4 was established in both ileal digesta and ileal mucosa, again using both DNA prepared from individual colonies and total DNA as template. The results are described in **Figure 3.2**. All baseline faecal, ileal digesta and mucosa samples tested negative for the presence of the *faeG* gene using both screening methods.

Two days post-challenge (i.e. day 4), one pig out of a total of three pigs tested positive for the *faeG* gene in all post-mortem samples, including all five ileal mucosa samples. On day 6, all three pigs tested positive for the *faeG* gene at ileal level (in both digesta samples and all five ileal mucosa samples), and two of

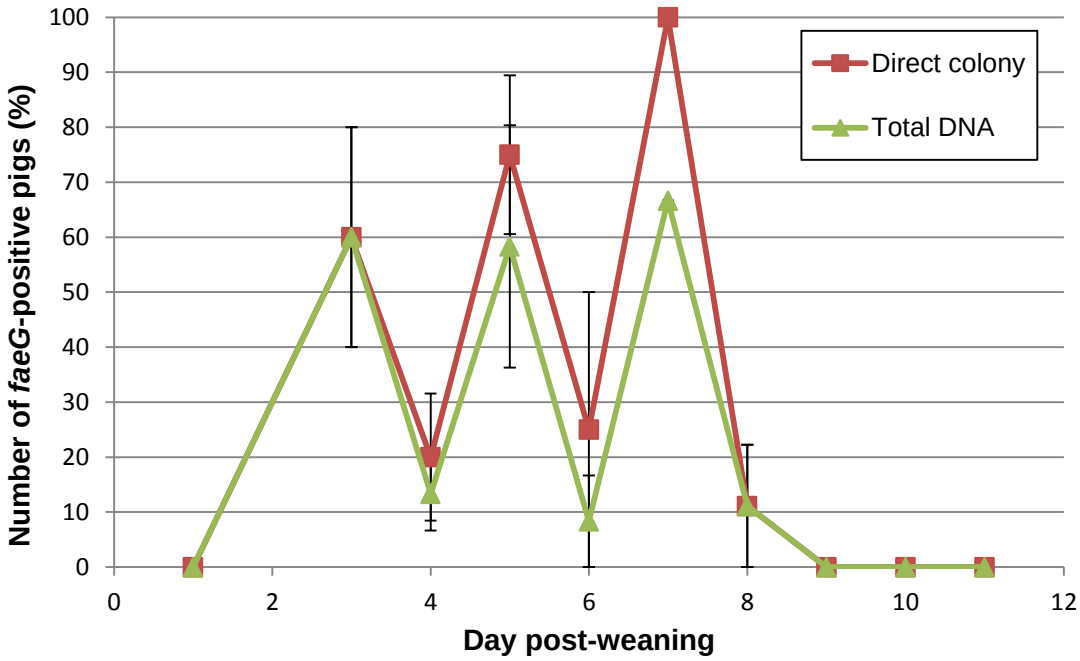

Figure 3.1: Graph describing the proportion of remaining pigs (±SE) that tested positive per pen when running the *faeG* PCR over the trial duration, using both direct colony and total DNA PCR. Sham-challenged pigs tested negative throughout the experiment using both methodologies.

the three pigs were shedding ETEC F4 in the faeces. By day 8 (i.e. 6 days post-challenge), no ETEC F4 was detected in the ileal mucosa samples, although two of three pigs tested positive when testing the ileal digesta. Additionally, one of three pigs was still shedding ETEC F4 6 days post-challenge. On the final sampling day (i.e. 9 days post-challenge), all remaining ETEC-challenged pigs tested negative at both ileal and faecal level when screening for the *faeG* gene.

3.3.5 Histopathology

At baseline and on day 4, for all histopathological observations, all gut tissues were assigned a score of 0. On day 6, two pigs showed signs of epithelial degradation and light to heavy bacterial colonisation in both ileal and jejenal

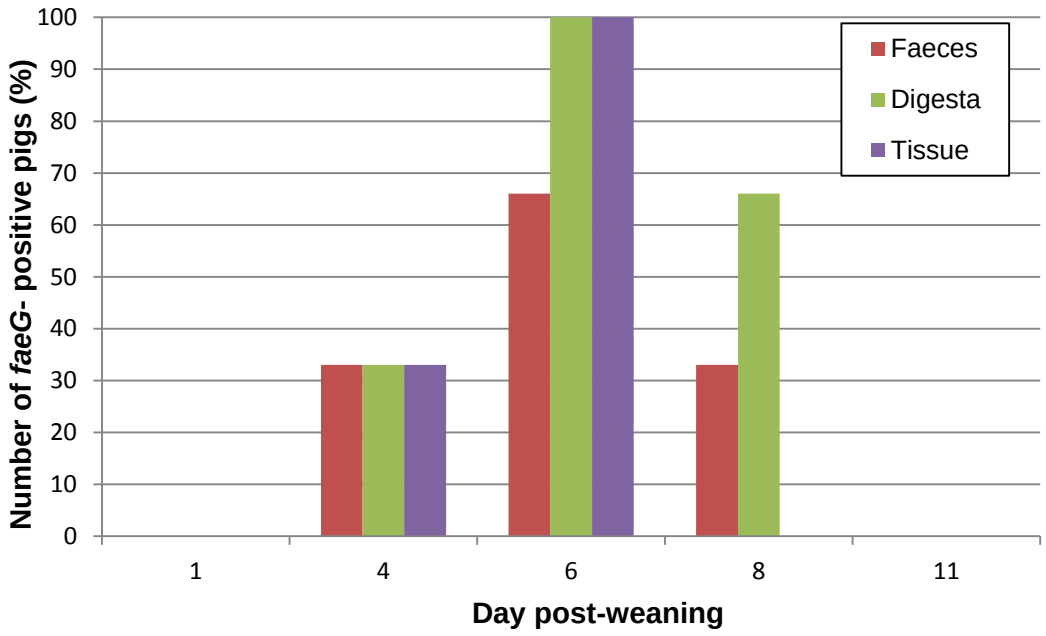

Figure 3.2: Graph describing the proportion of pigs at post-mortem that tested positive when running the *faeG* PCR in faecal samples, ileal digesta and ileal mucosa samples.

sections (**Table 3.4** and **Appendix E**). On day 8, signs of mild inflammation were observed in the ileal segments of a single pig, with sporadic and low level observations of epithelial degradation, bacterial colonisation and villus or crypt damage (**Table 3.4**). Nine days post-challenge, there was no evidence of bacterial attachment or tissue damage in both ETEC- and sham-challenged pigs. However, mild inflammation was present in some ileal sections in both ETEC-challenged and sham-challenged pigs on the final sampling day (score 1-2).

Table 3.4: Histopathology scores assigned to small intestinal tissues on days 6 and 8. Oedema, Peyer's patch depletion and lymphadentis were not evident (score = 0) and are not presented. Increases in congestion (Con), inflammation (Inf), epithelial degradation (Epi), bacterial colonisation (Bac) and villus or crypt damage (V/CD) were observed in tissue sections.

Pig ID	PM day	Tissue	Con	Inf	Epi	Bac	V/CD
A	6	Ileum	0	0	0	0	0
A	6	Ileum	0	0	0	0	0
A	6	Ileum	0	0	0	1	0
A	6	Ileum	0	0	0	1	0
A	6	Ileum	0	0	1	1	0
A	6	Jejunum	0	0	0	0	0
B	6	Ileum	0	0	0	0	0
B	6	Ileum	0	0	0	0	0
B	6	Ileum	0	0	0	0	0
B	6	Ileum	0	0	0	0	0
B	6	Ileum	0	0	0	0	0
B	6	Jejunum	0	0	0	0	0
C	6	Ileum	0	1	1	2	0
C	6	Ileum	0	1	1	3	0
C	6	Ileum	0	1	1	3	0
C	6	Ileum	0	2	2	3	0
C	6	Ileum	0	2	2	3	0
C	6	Jejunum	2	2	2	3	0
D	8	Ileum	0	0	0	0	0
D	8	Ileum	0	0	0	0	0
D	8	Ileum	0	0	0	0	0
D	8	Ileum	0	0	0	0	0
D	8	Ileum	0	0	0	0	0
D	8	Jejunum	0	0	0	0	0
E	8	Ileum	0	0	0	0	0
E	8	Ileum	0	0	0	0	0
E	8	Ileum	0	0	0	0	0
E	8	Ileum	0	0	0	0	0
E	8	Ileum	0	0	0	0	0
E	8	Jejunum	0	0	0	0	0
F	8	Ileum	0	1	1	0	1
F	8	Ileum	0	1	0	0	0
F	8	Ileum	0	1	1	1	1
F	8	Ileum	0	1	0	0	0
F	8	Ileum	0	1	0	1	0
F	8	Jejunum	0	0	0	0	0

3.3.6 Average daily feed intake

ADFI was estimated per pig based on the pen-level measurements, and the results are presented in **Figure 3.3**. The sham-challenged pigs (n = 3) refrained from eating during the first 3 days post-weaning, but feed intake increased rapidly after day 5 after the introduction of a small quantity of feed mixed with warm water to encourage eating. The ETEC-challenged pigs feed intake showed a general steady increase over the trial duration. However, two of the three ETEC-challenged pens showed a decreased feed intake on day 7 post-weaning which quickly recovered the next day. By the end of the trial (day 11), ETEC-challenged pigs showed an ADFI of 521 ± 58g, with the sham-challenged pigs recovering well from the low intake phase and consuming 444 g/day on average.

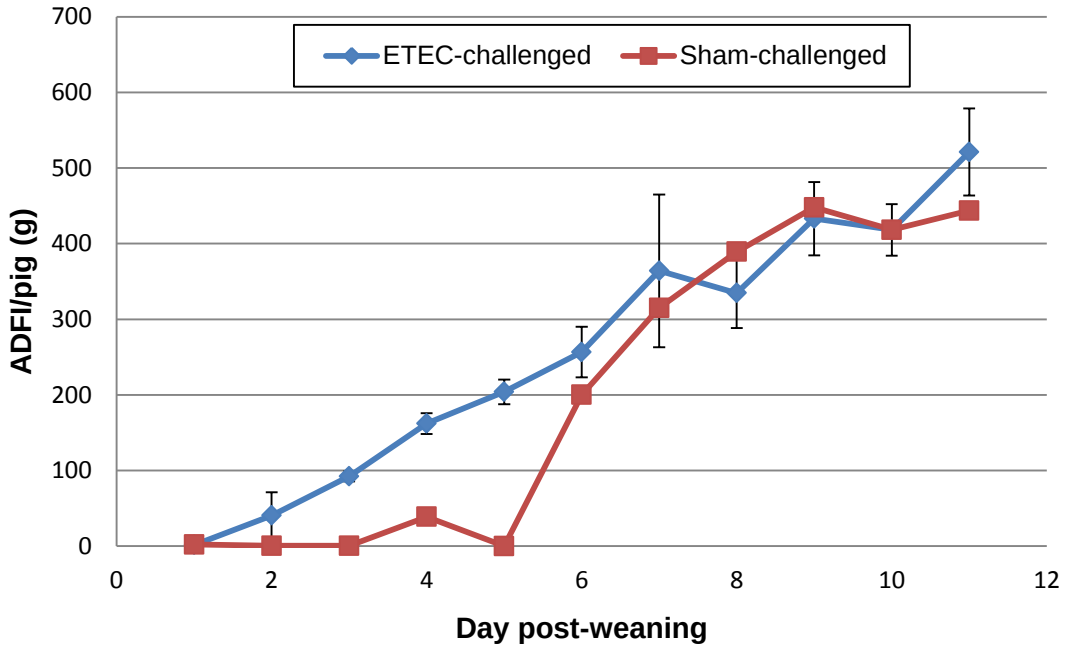

Figure 3.3: Average daily feed intake per pig (±SD) estimated from pen-based measurements in ETEC- and sham-challenged pigs.

3.3.7 Weight gain

The weight gain per pig over specific time intervals in the ETEC- and sham-challenged treatment groups are shown in **Figure 3.4**. Over the first 4 days post-weaning, 16 of the 18 pigs lost weight with the most noticeable weight losses occurring in the sham-challenged pigs (873 ± 121g). Between day 4 and 8, the sham-challenged pigs showed the most marked weight gain (1111 ± 721g) in comparison to the ETEC-challenged pigs (2087 ± 594g), but a large variation in ADWG was observed between individuals during this phase. Between day 8 and 11, the weight gain reduced in both ETEC- and sham-challenged pigs with the most marked decrease occurring in the sham-challenged pigs. The sham-challenged pigs gained the least weight over the trial (1400 ± 673g) in comparison to the ETEC-challenged pigs (2038 ± 375g) (day 0 to day 11).

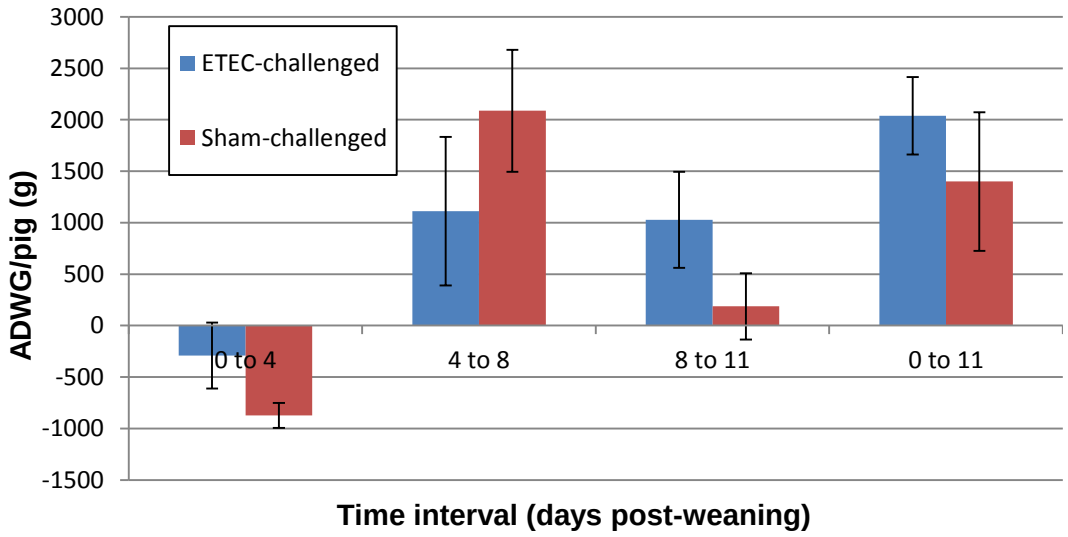

Figure 3.4: Weight gain per pig (mean ± SD) over specific time intervals in the ETEC- and sham-challenged treatment groups.

3.3.8 Faecal consistency, cleanliness and health scores

Health scores (i.e. faecal consistency, cleanliness and overall health) were taken throughout the duration of the experiment. Cleanliness and overall health scores were consistently excellent (score of 1) throughout the experiment in both ETEC- and sham-challenged pigs and so are not discussed further in this section.

Faecal consistency scores are presented in **Figure 3.5**. On days 1 and 2 (i.e. pre-challenge), two of three ETEC-challenged pens had loosely formed faeces. Mean faecal consistency scores peaked on days 5 and 6 of the trial (i.e. 3 and 4 days post-challenge) in the ETEC-challenged pens. On these days, all ETEC-challenged pens showed signs of normal diarrhoea, with one pen showing both normal and watery diarrhoea (score 2.5). On day 7, two of three pens showed normal diarrhoea, and the faecal scores remained between 1 and 1.5 for the rest of the trial. Throughout the experiment, the sham-challenged pigs had well-formed faeces and were consistently allocated a score of 1.

3.4 Discussion

The primary aims of this pilot study were to characterise ETEC F4 strains which have been used in previous challenge models and to assess adhesion and shedding dynamics in response to a single-dose ETEC challenge. A secondary aim of this study was to assess the impact of the single-dose challenge on pig performance and health parameters. This work was essential to establish

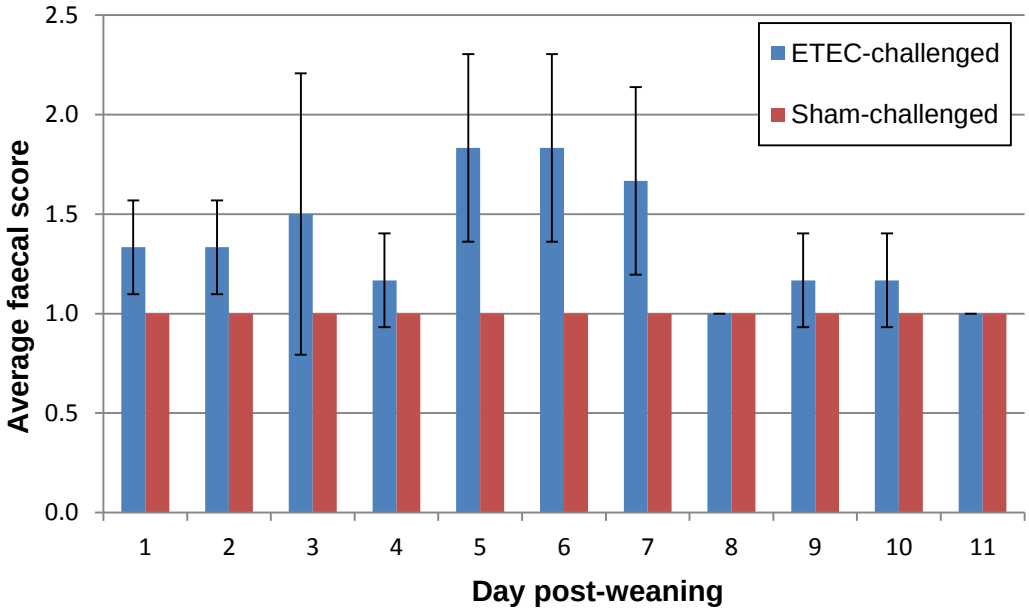

Figure 3.5: Bar graph describing the mean faecal consistency scores (±SD) measured on a pen basis in both ETEC- and sham-challenged groups.

whether the available ETEC strains had the appropriate virulence genes and whether the selected ETEC strain adhered to the infection site (i.e. the small intestine) or simply passaged through the gastrointestinal tract.

3.4.1 ETEC strain characterisation

Of the two ETEC F4 strains available for these studies, ETEC 239 was selected on the basis that it contained the full complement of virulence genes of interest – F4, STa, STb, LT and EAST1. ETEC 591 tested negative for the virulence gene *estA*, which encodes for production of STa. Since the mechanisms for each enterotoxin are different at intestinal level (Nataro and Kaper, 1998), it is thought that the presence of STa should enhance the pathogenesis of ETEC F4 (Noamani et al., 2003), thereby supporting the use of strain ETEC 239.

In porcine ETEC strains, a series of enterotoxin combinations have been observed. Noamani et al. (2003) used PCR screening to assess the distribution of virulence genes in "recent" and "old" strains and found that 92% of the more recent isolates tested positive for *estA*, but the older strains did not possess this gene. Therefore, the strain utilised in our study more closely resembled the more recently isolated strains in the Noamani et al. (2003) study.

3.4.2 Nalidixic acid-resistant bacteria

As part of this study, faecal samples were prepared for direct agar plating in order to enumerate nalidixic acid-resistant Gram-negative bacilli, with the assumption that the ETEC F4 challenge isolate would be the only nalidixic acid-resistant organism in the population. This selective method has the advantage of being both practical and cost effective, without the need for an enrichment step. However, the main disadvantage is the inherent variability of the background microbial bacterial population in the samples. If antimicrobial-resistant organisms are present in the gut microbiota, high numbers of atypical bacterial colonies can present on the plates. This was the case in this study, whereby large numbers of nalidixic acid-resistant *Enterococcus* species were part of the gastrointestinal population and therefore prevented accurate quantification of ETEC F4 using agar plating methodology.

In order to gain a better understanding of ETEC F4 dynamics and to eliminate the issue of competitive exclusion by *Enterococcus* species on the agar plates, a PCR assay targeting the *faeG* gene was used. The faecal homogenates and individual colonies from the agar plates were subject to DNA extraction for PCR

amplification. When examining the results, it was clear that the PCR using colony extracts was more sensitive than using total DNA. This is unsurprising, as the isolated colonies were present on the agar plates at the lowest dilution level, suggesting that ETEC F4 were likely present in small numbers in the faecal samples. Since incubation on an agar plate is an enrichment technique, direct PCR using bacterial colonies can be lot more sensitive than using total DNA as template, which has not been enriched and contains a dense, mixed population of bacterial DNA.

3.4.3 Histology and bacteriology

In this study, mild inflammation and mild epithelial degeneration of the ileal tissue was observed in 6/15 tissue sections 4 days post-challenge (day 6) with inter-individual variation being present. In this pilot study, only a small number of pigs (i.e. n = 3) were subject to post-mortem at each time point. Additionally, this experimental design did not allow for temporal tissue sampling of sham-challenged pigs, but this was not a core aim of this study. Future studies focusing on the histopathological effects of ETEC challenge should incorporate more pigs to account for large differences in mucosal responses and include sham-challenged pigs as controls at each post-mortem point. However, large changes in gut structure were not expected as previous evidence shows that pigs which have been exposed to ETEC bacteria may show no observable lesions (Francis, 2002), with PWC being linked with mild to moderate intestinal hyperaemia (Moxley and Duhamel, 1999).

Critically, in the current study, large numbers of ETEC bacteria were cultured successfully from mucosal samples and were confirmed as ETEC F4 by Sanger sequencing. Interestingly, one pig showed a heavy colonisation of Gram-negative bacteria on the ileal tissue submitted for histopathological analysis. Although this not been confirmed serologically from the tissue section, it is likely that these colonising bacteria are ETEC F4, based on the bacteriological findings presented. Indeed, histological study of the ETEC-infected small intestine generally reveals large numbers of Gram-negative bacilli on the villus surface which can be cultured easily from these tissues (Francis, 2002) which occurred as part of this work.

3.4.4 Effects of ETEC challenge on pig performance and health

In this study, ETEC challenge had a variable impact on pigs, as highlighted by the faecal consistency scores and the PCR results for the detection of ETEC at both ileal and faecal levels. Due to the complex, multifactorial nature of this disease, it has proven difficult to reproduce in an experimental setting and has caused a variety of disease states, as discussed in **Chapter 1**.

The faecal scores taken as part of this study were likely affected by a variety of factors. Most importantly, the sham-challenged pigs refrained from eating at the start of the trial and feeding did not commence until day 5 when a small amount of feed mixed with warm water was introduced. The sham-challenged pigs showed consistently low faecal consistency scores, which is likely linked with a lower feed intake as well as the absence of ETEC exposure. The ETEC-challenged pigs ate well throughout the experiment, and the sudden

introduction to a solid diet and a high feed intake can exacerbate diarrhoea, as discussed in **Chapter 1**. Indeed, in this study, a raised faecal score was already evident prior to ETEC challenge, so it is not possible in this case to establish whether ETEC exposure alone had an impact on faecal consistency score.

3.5 Conclusion

In conclusion, the results of this single-dose challenge model study have confirmed ileal adhesion (up to 4 days post-challenge) and faecal shedding (up to 6 days post-challenge) of ETEC F4, which suggests that the organism is colonising the small intestine. Using this model, further ETEC-challenge experiments will be carried out to improve our understanding of ETEC pathogenesis and the effects on weaner pig health and performance.

Chapter 4:

Interactive effects of ETEC challenge and dietary protein level on ileal and faecal microbiota dynamics, pig health and performance

4.1 Introduction

As a consequence of the EU-wide ban of prophylactic antimicrobials, alternative management strategies for enteric disorders are now being considered which were discussed in detail in **Chapter 1**. Specifically, dietary strategies are under investigation with the aim to manipulate the microbiota of the porcine gut to improve gut health (Hodgson and Barton, 2009; Rist et al., 2013). An important component of the weaner pig diet is protein, and the quality and quantity of protein provided around weaning has been found to have a profound effect on enteric health (Kim et al., 2012b). Protein is essential for growth, but if provided in excessive quantities, bacterial fermentation of protein and the consequent production of irritant by-products such as ammonia can occur (Bikker et al., 2006; Halas et al., 2007).

Studies have been undertaken to ascertain the impact of dietary protein level on performance parameters in ETEC-challenged pigs, which have been reviewed in **Chapter 1**. Specifically, studies have shown that by lowering dietary protein content, levels of ETEC were reduced in the ileal digesta (Opapeju et al., 2009) and in the faeces (Wellock et al., 2008a). Interestingly, differences in dietary protein levels have also been linked to changes in lactobacilli: coliform ratio using culturing methods (Jeaurond et al., 2008; Wellock et al., 2006) and faecal microbiota composition using early molecular methods (Opapeju et al., 2009).

In this study, the interactive effects of dietary protein level and ETEC challenge on pig performance, health and gut microbiota dynamics were assessed.

Although studies have been carried out to study the effects of dietary protein level and ETEC challenge on various gut health and performance parameters in the presence of ETEC challenge, to our knowledge, this is the first study to implement 16S rRNA gene metabarcoding, which allows the study of total microbial community dynamics with better resolution than culturing methods or earlier molecular methodologies. The main aims of this study were to assess temporal ileal and faecal (gut) microbiota shifts over the post-weaning period, to evaluate the main and interactive effects of ETEC challenge and dietary protein level on pig health, performance and the gut microbiota and to establish any links between growth performance and ETEC counts and gut microbiota dynamics.

4.2 Methods

4.2.1 Animals and housing

Pigs were weaned at 25.0 ± 0.8 days (mean ± SD) of age and weighed 9.10 ± 1.27kg at weaning (day 0). A total of 144 pigs (Large White x Landrace) were used for this trial, which were housed in groups of four in 4 m^2 square pens (in two rooms) and were bedded with sawdust daily. The pens were cleared of faecal material and wet sawdust daily. A single feeder and nipple drinker were included, with both water and feed being provided *ad libitum* for the trial duration.

The environmental temperature was set at 25^{o}C for the first 4 days, and was decreased by 2^{o}C for the remainder of the experiment. The shed lights were switched on between 8:00 and 18:00 and night lights were maintained between 18:00 and 8:00.

4.2.2 Experimental design and diet formulations

This experiment was carried out over four experimental rounds which took place between 9th July and 14th October 2015. A total of 16 litters were included in the trial (4 litters from each round), with pens being balanced for sex and litter origin as much as possible.

The experiment consisted of a 2 x 2 factorial combination of dietary crude protein (CP) level (low protein - LP vs. high protein - HP) and experimental ETEC challenge (ETEC-challenged - ETEC vs. sham-challenged control - SHAM). After baseline samples were obtained (i.e. immediate post-mortem on day -1, n = 16), remaining pigs were assigned to one of four treatments – ETEC LP (n = 32), ETEC HP (n = 32), SHAM LP (n = 32) and SHAM HP (n = 32).

Each treatment was present in each of the two rooms for each of the four rounds with 4 pigs being housed per pen initially. A serial slaughter design was implemented whereby pigs were removed for post-mortem on days 5, 9 and 13 as described in **Table 4.1**. At each post-mortem point, pigs were selected from each pen to balance for litter origin, weaning weight and sex.

Table 4.1: Description of serial slaughter design, highlighting post-mortem day (day post-weaning), the number of pigs per treatment and the appropriate crude protein (CP)/challenge combination for each treatment (low protein (LP) and high protein (HP), ETEC-challenged (+) and sham-challenged (-)).

Treatment ID	PM day(s)	Number of pigs	CP level	Challenge
Baseline	-1	16	N/A	N/A
5-ETEC LP	5	8	LP	+
5-ETEC HP	5	8	HP	+
5-SHAM LP	5	8	LP	-
5-SHAM HP	5	8	HP	-
9-ETEC LP	9	8	LP	+
9-ETEC HP	9	8	HP	+
9-SHAM LP	9	8	LP	-
9-SHAM HP	9	8	HP	-
13-ETEC LP	13	16	LP	+
13-ETEC HP	13	16	HP	+
13-SHAM LP	13	16	LP	-
13-SHAM HP	13	16	HP	-

Prior to weaning, all piglets were provided access to a standard creep feed (digestible energy (DE) = 16.3 MJ/kg, CP = 230g CP/kg) from around 7 days pre-weaning. The ingredients included in the LP and HP experimental diets are listed in **Table 4.2**, and were modelled to reflect the diets presented in Wellock et al. (2009). Both diets were formulated to balance for digestible energy and essential amino acid composition (i.e. relative to lysine) as much as possible.

Feed was sampled during daily feed weighing and was pooled per treatment for proximate feed analysis for key ingredient and amino acid compositions which are described in **Table 4.3.**

Table 4.2: Table listing ingredients included in the two experimental diets.

Ingredients (g/kg)	Low Protein	High Protein
Micronized wheat	314.9	237.2
Cooked dehulled oats	125.0	125.0
Micronized maize	125.0	125.0
Fishmeal	32.5	50.0
Full fat soya	20.0	20.0
Dried-skim milk powder	91.0	140.0
Soya protein concentrates	96.7	149.0
Fat-filled whey	32.5	50.0
Vegetable oil	34.4	10.1
Lactose	101.0	74.2
Limestone	0.8	1.5
Dicalcium phosphate	14.7	7.8
Salt	1.9	0.3
l-Lysine-HCl	1.6	1.1
dl-Methionine	1.4	1.8
l-Threonine	1.2	1.5
l-Tryptophan	0.2	0.3
Premix	5.2	5.2

Table 4.3: Table showing measured chemical composition and calculated digestible energy for experimental diets.

Component (g/kg)	Low Protein	High Protein
Crude protein	180.9	228.8
Lysine	10.3	13.2
Methionine	5.7	6.0
Threonine	8.3	9.9
Tryptophan	1.7	2.6
Ash	47.7	46.1
Acid hydrolysed ether extract	51.9	53.8
Neutral detergent fibre	69.1	59.8
Digestible energy (MJ/kg)[1]	16.0	16.0

[1] Calculated from in-house matrix at feed mill

4.2.3 ETEC inoculum and challenge

ETEC 239 (SRUC Veterinary Services Laboratory, UK) was used for the trial and the inoculum was prepared as described in **Chapter 3**, prior to estimating the bacterial cell concentration using a spectrophotometer (Ultrospec 2100 Pro, Fisher Scientific, UK). The culture was then diluted in PBS to aim for a final stock concentration of 1.00×10^8 cfu/ml. The final inoculum was serially diluted and plated out at 5 dilutions for standard enumeration and confirmation of the appropriate bacterial concentration. On day 2 of the trial (i.e. two days post-weaning), pigs were orally administered 3ml of the stock solution using a

syringe. Sham-challenged pigs were administered 3ml of PBS in the same manner. This dose was trickled slowly at the back of the mouth, to ensure that a swallowing reflex took place. The achieved mean dose across the four experimental rounds was 3.81 ± 0.85 x 10^8 cfu (mean ± SD).

Throughout the experiment, biosecurity measures were strict to minimise potential spread of the challenge pathogen. Changes of nitrile gloves and overshoes were enforced when moving between pens, and foot baths containing disinfectant were used regularly. Regular disinfection of equipment and shed corridors was also carried out.

4.2.4 Pig performance and health

The mass of feed offered and refused (at pen level) was recorded daily for the trial duration. From these measurements, the average daily feed intake (ADFI) per pen per pig during the experiment was calculated. Pigs were weighed on days -1 (pre-challenge baseline pigs only), 0, 2, 5, 9 and 13 post-weaning. All pigs which were subject to post-mortem on day 13 were used to assess average weight gain (ADWG) over the trial duration.

Faecal consistency, cleanliness and overall health scores were recorded daily, using a subjective four-point scale described in **Chapter 2** (see **Table 2.1**). Briefly, for faecal consistency scoring, an increase in score represents an increase in faecal fluidity. Increases in both cleanliness and overall health scores represent an increase in faecal contamination and a deterioration of health, respectively.

4.2.5 Post-mortem sampling and DNA extraction

Prior to euthanasia, pigs were firstly sedated using a mixture of medetomidine (0.01 ml/kg at 1mg/ml), midazolam (0.1ml/kg at 5mg/ml), ketamine (0.1ml/kg at 100mg/ml) and azaperone (0.025ml/kg at 40mg/ml). The pigs were then euthanised by intracardiac injection of pentobarbital (0.7ml/kg at 200mg/ml). The abdomen was opened from pubis to sternum to reveal the gastrointestinal tract. The caecum was isolated and tied off at the ileal-caecal junction, before measuring out 10cm cranially and tying off again with string. The ileal segment was then cut from the rest of the tract and the digesta was emptied into a universal tube before being snap-frozen on dry ice.

A faecal sample was also taken directly from the rectum at post-mortem and was snap-frozen on dry ice. These samples were then stored at -80^{o}C for a maximum of 2 weeks before DNA extraction using the MoBio PowerSoil® DNA Isolation kit (Cambio, UK) with modifications as described in **Chapter 2**. The ileal digesta samples were prepared with the removal of one modification, with the centrifugation step after homogenisation being excluded to ensure optimal DNA yield.

4.2.6 Faecal sampling and ETEC enumeration

Faecal samples were taken directly from the rectum of all pigs present daily between days 2 and 7, and on days 9, 11 and 13. These samples were immediately snap-frozen on dry ice prior to storage for a maximum of 4 weeks at -80^{o}C. DNA extractions were carried out using the MoBio PowerSoil® DNA

Isolation kit (Cambio, UK), with modifications to the protocol as described in **Chapter 2**.

ETEC-challenged pigs that underwent post-mortem on day 13 were subject to faecal screening for ETEC F4 in order to monitor individual shedding levels over the trial duration (n = 32). Two sham-challenged pigs from each round were selected as controls, with the selection being balanced for treatment across the four experimental rounds (n = 8). Faecal shedding of ETEC F4 was measured using qPCR, using primers designed as part of this project to target the *faeG* gene, as described in **Chapter 2**. Additionally, all ileal digesta samples from each post-mortem point were included for qPCR analysis. All reactions were carried out in triplicate using a Stratagene MX3005P (Agilent Technologies, USA), with 2µl of DNA extract being added to each reaction. The qPCR run conditions consisted of an initial denaturation step at 95°C (5 minutes), followed by 40 cycles of amplification at 95°C (30 seconds) then 65°C (15 seconds). A melt curve was generated using the following cycling conditions - 95°C (60 seconds), 55°C (30 seconds) and 72°C (30 seconds). To calculate *faeG* gene copy number, absolute quantification using a standard curve was carried out and qPCR output was converted into the number of *faeG* gene copies/gram wet digesta or faeces as previously described in **Chapter 2**.

4.2.7 16S rRNA gene sequencing

Ileal digesta and faecal samples obtained at post-mortem were subject to 16S rRNA gene metabarcoding. The PCR and purification steps involved in library preparation are described in detail in **Chapter 2**. Briefly, the V3 hypervariable

region of the 16S rRNA gene was amplified using dual-indexed primers (see **Appendices B and C**) which incorporated TruSeq adapters. Equimolar concentrations of template DNA were amplified in a one-step PCR using a high fidelity polymerase (Phusion®, New England Biolabs, UK) using the mastermix composition and PCR conditions described in **Chapter 2**. PCR products were then purified using the AMPure XP PCR purification system (Beckman Coulter, USA).

DNA concentrations of the purified TruSeq libraries were then measured using a Qubit® 3.0 Fluorometer (Thermo Fisher Scientific, United Kingdom) using the Qubit® double-stranded DNA High Sensitivity Assay Kit (Thermo Fisher Scientific, United Kingdom). Using the readings obtained by the Qubit® instrument, 4 library pools were constructed using equimolar concentrations of DNA from each sample.

Two reagent-only controls and two mock community samples (BEI Resources, ATCC, Manassas, VA, USA - detailed in **Chapter 2**) were included (one per sequencing run) to assess background contamination, sequencing error rate and inter-run variability. On submission to the sequencing centre (Edinburgh Genomics, United Kingdom), library pools were quantified using the Quant-iT™ PicoGreen® double-stranded DNA Assay Kit (Thermo Fisher Scientific, United Kingdom) to ensure sufficient yield for sequencing. Sequencing was carried out using the Illumina MiSeq platform (Illumina, United States), using V2 chemistry and producing 250bp paired-end reads. Sequences were processed using

CASAVA 1.8 (Illumina, United States) and the mothur software package (Schloss et al., 2009) as described in **Chapter 2**.

4.2.8 Statistical analysis of performance and ETEC shedding data

Statistical analyses were carried out using Genstat 16 (VSN International, UK) unless stated otherwise. The body weight and ADFI data were assessed using repeated measures analysis of variance (RM-ANOVA) to establish any main or interactive temporal effects of ETEC challenge and dietary protein level on these parameters. These analyses included challenge status and dietary protein level as main factors and experimental round as a block. Day 0 values were used as co-variates for assessment of changes in body weight. The ADWG data was assessed using analysis of variance (ANOVA) to establish whether there were any main or interactive effects of ETEC challenge and dietary protein level on total weight gain between day 0 and day 13.

To assess the uniformity over time of the faecal consistency scores, and whether there were any main effects of ETEC challenge or dietary protein level, an ordinal logistic regression (OLR) was performed using Minitab 17 (Minitab Inc, USA). The categorical indicators (i.e. faecal consistency scores) were assigned as the response, and time point, challenge status and dietary protein level were assigned as categorical predictors. To assess whether there were statistically significant effects of dietary protein level on both ileal ETEC load and faecal ETEC shedding, ANOVA and RM-ANOVA were carried out, respectively, with dietary protein level being included as a main factor and experimental round as a block.

4.2.9 Descriptive and statistical analysis of sequence data

Descriptive and statistical analyses were carried out to describe the main and interactive effects of ETEC challenge and dietary protein level on ileal and faecal microbiota composition. The following steps were carried out using the mothur software package, unless stated otherwise.

The Inverse Simpson's Index (ISI) was calculated for each sample to measure diversity, and the Chao 1 index was calculated to assess richness. To test whether there were significant differences in diversity and richness over time and between treatments, RM-ANOVA was carried out using Genstat 16 (VSN International, United Kingdom). The values for day 4 were initially included as co-variates, but these had no significant effect and were therefore not included as co-variates in the final analysis.

Distance matrices were compiled using Yue and Clayton theta similarity coefficients (Yue and Clayton, 2005), which take into account both community membership and relative abundance. Two distance matrices were created – one for all faecal samples and one for all ileal digesta samples. Non-Metric Multidimensional Scaling (NMDS) plots were constructed in two dimensions with co-ordinates generated using the NMDS function to visualise community similarities over time. The statistical significance of any clustering by time point or treatment was assessed by analysis of molecular variance (AMOVA) (Excoffier et al., 1992). The statistical significance of variation between populations was assessed using homogeneity of molecular variance (HOMOVA) (Stewart and Excoffier, 1996).

To identify bacterial phyla, families or phylotypes that were expressed significantly differently over time or between treatment groups, Metastats (Paulson et al., 2011) was used and the P-values were corrected using false discovery rate (FDR). The subsampled dataset was simplified to only include phyla, families or phylotypes which were equal to or more than 0.1% abundant at each time point examined. Dirichlet multinomial mixture (DMM) models were run to group both ileal and faecal samples into enterotypes based on the relative abundances of bacterial genera in each sample (Holmes et al., 2012).

In order to explore potential links between growth rate and microbiota composition, pigs subject to post-mortem on day 13 only were clustered into "low" (22.20 ± 5.79g/day/kg weaning weight, n = 20) and "high" (46.17 ± 10.65g/day/kg weaning weight, n = 20) growth rate groups. To establish whether there were any links between growth rate and community structure and phylotype expression, AMOVA and Metastats tests were carried out respectively, with the inclusion of a DMM model analysis to establish whether there were effects of growth rate on enterotype clustering.

To explore links between ETEC counts at ileal and faecal level on gut microbiota composition, pigs subject to post-mortem on day 13 only were clustered into "low ETEC count" (n = 10) and "high ETEC count" (n = 10) groups on the basis of the qPCR data at faecal and ileal level on day 13. These selections were

balanced for experimental round, sex, challenge status and dietary treatment. To establish whether there were any links between ETEC count level and microbiota structure and phylotype relative abundances, AMOVA and Metastats tests were carried out, respectively.

4.3 Results

4.3.1 Mean body weight and ADWG

There were no significant main effects of ETEC challenge or dietary protein level, or any interactive effects, on mean body weight throughout the duration of the trial when considering pigs which were subject to post-mortem on day 13 (RM-ANOVA: $P > 0.05$). Additionally, there were no significant main effects of ETEC challenge or dietary protein level, or any interactive effects, on ADWG (ANOVA: $P > 0.05$). When considering both mean body weight (**Table 4.4**) and ADWG data from all pigs assigned to all post-mortem points, there were again no significant effects of ETEC challenge and dietary protein level on these parameters ($P > 0.05$).

Table 4.4: Mean body weights (± SD) for all ETEC- and sham-challenged pigs fed the low (LP) and high (HP) protein included in this study. There were no main or interactive effects of ETEC challenge and dietary protein level on mean body weight (P > 0.05).

	Mean body weight (kg) ± SD				
Group	**Day 0**	**Day 2**	**Day 5**	**Day 9**	**Day 13**
ETEC LP	9.11 ± 1.11	9.10 ± 1.06	9.91 ± 1.15	11.50 ± 1.30	13.43 ± 1.70
ETEC HP	9.15 ± 1.11	9.18 ± 1.19	10.03 ± 1.37	11.41 ± 1.32	12.84 ± 1.48
SHAM LP	9.03 ± 1.47	8.87 ± 1.38	9.80 ± 1.43	11.30 ± 1.45	12.85 ± 1.72
SHAM HP	9.11 ± 1.35	9.10 ± 1.35	9.85 ± 1.44	11.52 ± 1.77	13.15 ± 1.90

4.3.2 Average daily feed intake

The ADFI per pig was estimated using the pen-level feed measurements and the data are visualised in **Figure 4.1**. The feed intakes across the four experimental treatments were comparable from day 0 to 9. On day 10, the ADFI in the ETEC LP group was markedly higher in comparison to the remaining three treatment groups and remained higher for the remainder of the trial. However, there were no significant main or interactive effects of ETEC challenge or dietary treatment on ADFI throughout the experiment (RM-ANOVA: P > 0.05).

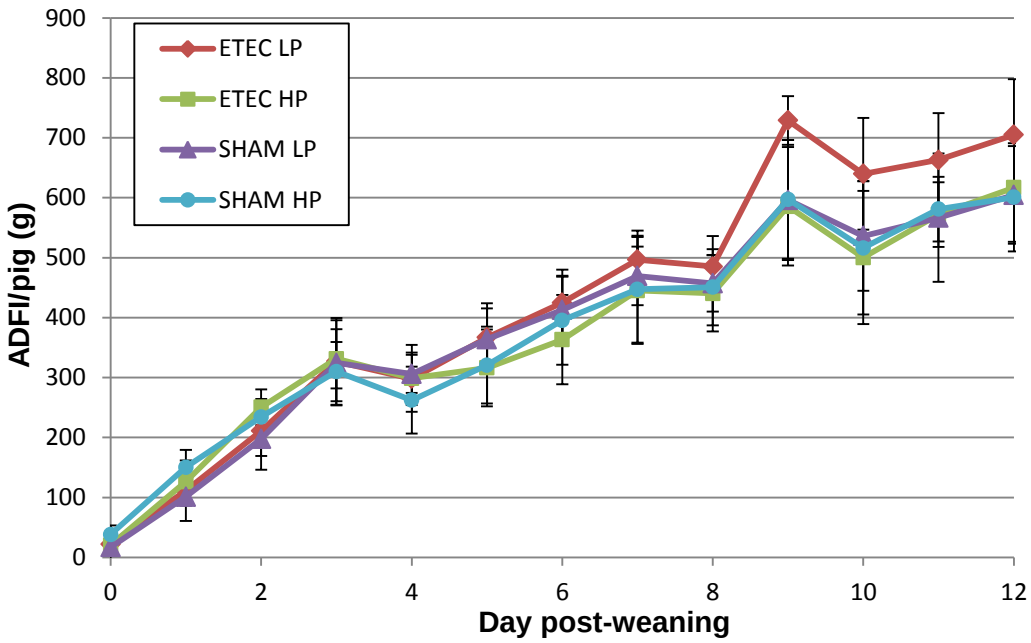

Figure 4.1: Average daily feed intake (ADFI) per pig over the trial duration (mean ± SD). There were no main or interactive effects of ETEC challenge or dietary protein level on ADFI (P > 0.05).

4.3.3 Faecal consistency, cleanliness and health scores

Throughout each of the experimental rounds, both cleanliness and health scores were consistent (i.e. score = 1) across all treatments and so are not discussed any further in this section. The mean faecal consistency scores over the trial duration across the four treatments are shown in **Figure 4.2**. Prior to challenge (i.e. days 1 and 2), the mean faecal consistency scores across all treatment

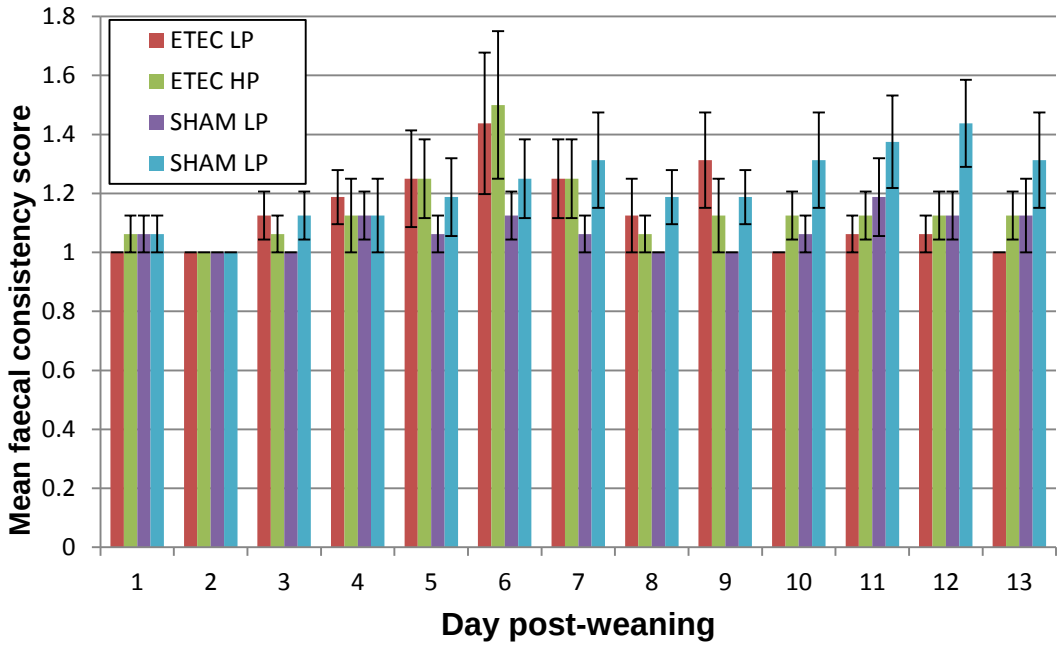

Figure 4.2: Mean faecal consistency scores (±SEM) across treatments. There were no main effects of challenge on faecal consistency score (P > 0.05), but dietary protein level had a significant effect, with the pigs fed the HP diet having a higher faecal score (P = 0.03).

groups ranged between 1 and 1.06, highlighting that the faeces was generally well formed. There was no effect of ETEC challenge on faecal consistency score across the entire trial (OLR: P = 0.67). However, diet was found to significantly affect mean faecal consistency score (mean ± SD) over the course of the trial (OLR: P = 0.03) with the scores for pigs fed the high protein diet (overall mean = 1.19 ± 0.09) being significantly higher than those in the low protein treatment (overall mean = 1.11 ± 0.07).

4.3.4 ETEC quantification

4.3.4.1 Ileal digesta

All ileal digesta samples obtained at post-mortem were screened using qPCR targeting the *faeG* gene. At baseline (i.e. day -1), digesta samples (n = 15, 1 missing observation due to absence of digesta at sampling) tested negative for the presence of the *faeG* gene, with the exception of 1 sample which tested positive (pig ID 1721, 6.22 $\log_{10}$ +1 *faeG* copies/g wet digesta). The PCR product obtained from this sample was sequenced by Sanger sequencing (Eurofins, Germany), and the qPCR-positive result was deemed a false-positive reaction. Additionally, this PCR product was run out on an agarose gel and no band was evident at the correct size (i.e. 135bp). Therefore, at baseline, all sham-challenged pigs on both the LP and HP diets tested negative for the presence of ETEC F4 at ileal level. Additionally, throughout the experiment, all pigs within the sham-challenged LP and HP treatments tested negative for ETEC F4.

On days 5 and 9, ETEC-challenged pigs fed the HP diet showed a higher mean ETEC load at ileal level in comparison to those fed the LP diet (**Figure 4.3**). On day 13, mean levels of ETEC in ileal samples from pigs fed the LP diet increased from day 9 and a small reduction was observed in mean ETEC load in the pigs fed the HP diet (5.61 ± 0.95 to 5.18 ± 0.54 $\log_{10}$ +1 *faeG* copies/g wet digesta). However, there were no significant differences in *faeG* copy number when comparing pigs fed the LP and HP diets at any of the post-mortem points (ANOVA: $P > 0.05$).

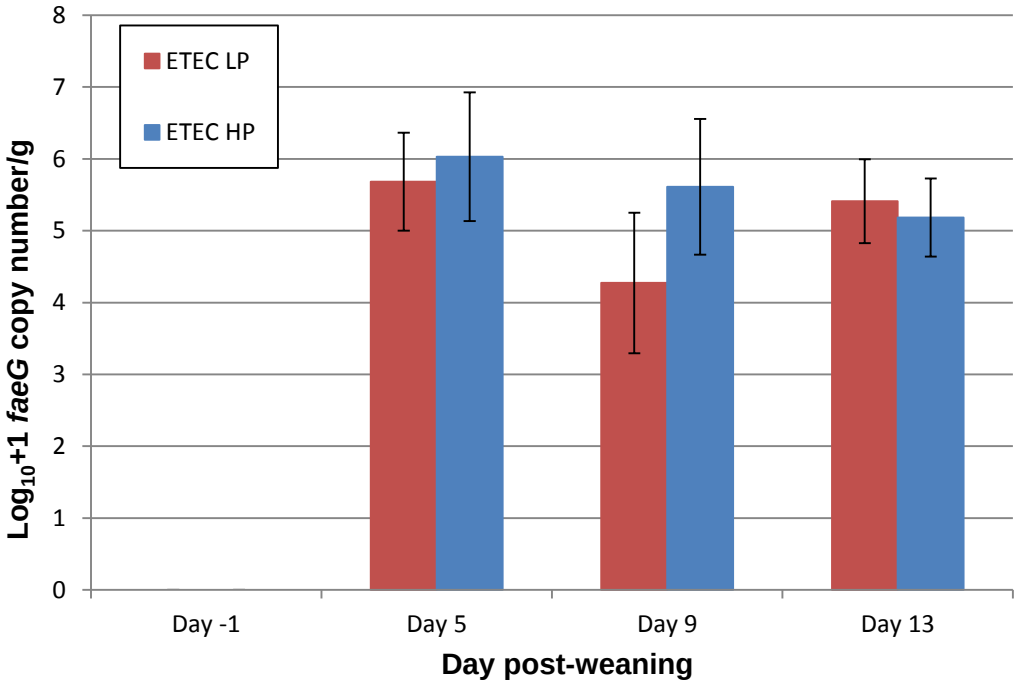

Figure 4.3: Mean log_{10}+1 *faeG* copy number (±SEM) per gram of wet digesta at each post-mortem point.

4.3.4.2 Faeces

Pigs which were subject to post-mortem on day 13 only were screened throughout the trial to establish temporal ETEC shedding dynamics in the faeces. As for ileal samples, faecal samples obtained prior to challenge tested negative for ETEC F4. Additionally, all faecal samples obtained from pigs in the SHAM LP and SHAM HP treatment groups tested negative for ETEC F4 throughout.

The mean log_{10}+1 *faeG* copy number/g wet faeces values obtained from ETEC LP and ETEC HP pigs subject to post-mortem on day 13 are presented in **Figure 4.4**. On day 3 (i.e 1 day post-challenge), ETEC HP pigs were shedding higher numbers of ETEC in comparison to ETEC LP pigs. On day 7 (i.e. 5 days post-challenge), the greatest difference between the ETEC LP and ETEC HP treatment

groups was observed, with the ETEC HP pigs shedding almost 10-fold more ETEC F4 than the ETEC LP pigs. From day 7 to day 13, both treatment groups showed a steady decrease in mean ETEC counts. Overall, there was no effect of dietary protein level on ETEC shedding in the faeces over the trial duration (RM-ANOVA: P = 0.24).

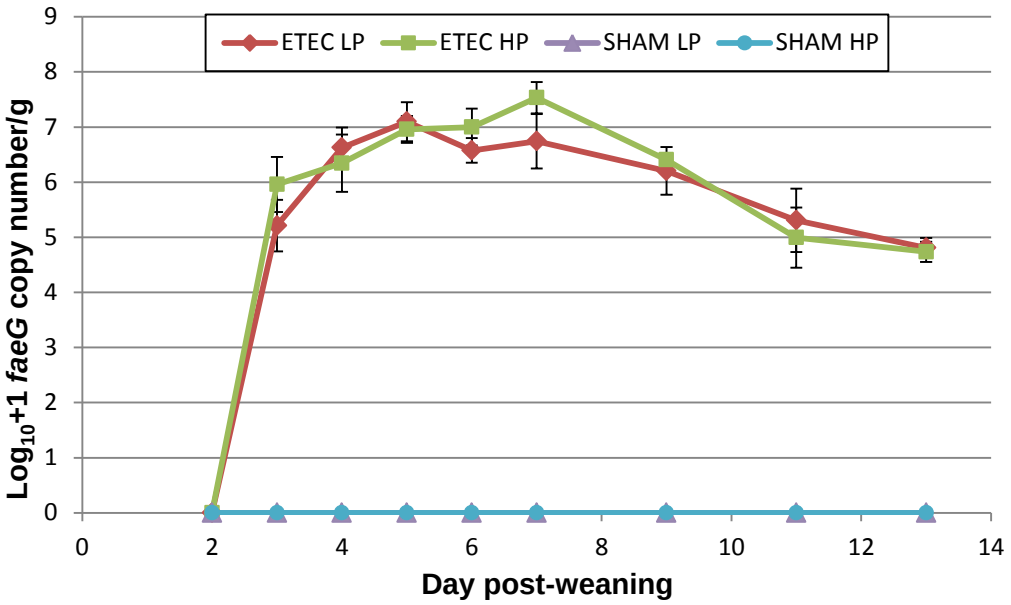

Figure 4.4: Average log_{10}+1 *faeG* copy number/gram wet faeces (±SEM) in faecal samples prior to challenge (day 2) and after challenge (day 3-7, 9, 11 and 13).

4.3.5 Quality control of sequences

After contiguous DNA sequences were constructed from the forward and reverse reads, various quality control steps were carried out to minimise the number of poor quality sequences and sequencing artefacts. When considering both ileal and faecal samples, this resulted in a 15% loss of sequences which left 27,245,438 reads in total for analysis. On average, 102,674 ± 55,780 (mean ± SD) reads were generated per sample and 681 phylotypes were identified.

Seven ileal digesta samples failed sequencing, which were balanced equally across treatments. Therefore, these samples were not re-sequenced as part of this analysis.

Two mock community samples were included in this experiment, with one sample being included on each Illumina MiSeq run to allow assessment of inter-run variability, in addition to calculating sequencing error rate and assessing the performance of the developed methodology. Using the mock community data, the sequencing error rate in both Illumina MiSeq runs was calculated as 0.01%. Additionally, of the 20 bacteria which were present in the mock community, all were taxonomically identified at genus level and 45% of these were identified at species level. This indicates that the primers selected for this experiment were able to amplify a wide range of bacteria.

The proportions of expected versus measured relative abundances within the mock community are presented in **Table 4.5**. When comparing the measured relative abundances gained from both mock community runs, the quantitative data were similar which suggests that there was not a pronounced difference between Illumina MiSeq runs. *Clostridium beijerinckii* was over-represented by this method with a mean (± SD) relative abundance of 10.24 ± 0.25%, and *Pseudomonas aeruginosa* and *Rhodobacter sphaeroides* were under-represented with mean relative abundances of 2.31 ± 0.07% and 2.26 ± 0.12%, respectively.

Table 4.5: Measured and expected relative abundances of mock community strains over two MiSeq lanes. Two staphylococci (*S. aureus and S. epidermidis*) and three streptococci (*S. mutans, S. agalactiae and S. pneumoniae*) were present in the mock community. Some members of the community were over-represented (i.e. *Clostridium beijerinckii) or* under-represented *(*i.e. *Pseudomonas aeruginosa* and *Rhodobacter sphaeroides)* in this workflow.

Mock community strain(s)	Level of identification	Measured relative abundance 1 (%)	Measured relative abundance 2 (%)	Expected relative abundance (%)
Acinetobacter baumannii	Genus	2.83	3.71	5.00
Actinomyces odontolyticus	Genus	4.19	3.94	5.00
Bacillus cereus	Species	6.39	6.62	5.00
Bacteroides vulgatus	Genus	3.38	4.49	5.00
Clostridium beijerinckii	Genus	9.98	10.49	5.00
Deinococcus radiodurans	Genus	4.93	3.99	5.00
Enterococcus faecalis	Genus	3.34	3.78	5.00
Escherichia coli	Species	4.90	5.23	5.00
Helicobacter pylori	Species	7.56	6.89	5.00
Lactobacillus gasseri	Genus	3.97	4.40	5.00
Listeria monocytogenes	Species	5.54	5.70	5.00
Neisseria meningitidis	Genus	4.64	4.71	5.00
Propionibacterium acnes	Species	7.92	6.73	5.00
Pseudomonas aeruginosa	Genus	2.37	2.24	5.00
Rhodobacter sphaeroides	Species	2.37	2.14	5.00
Staphylococcus spp.	Species	9.98	9.64	10.00
Streptococcus spp.	Genus	15.26	14.58	15.00

Although biases were observed, it was assumed that any biases would present evenly across ileal and faecal samples, which means that any statistical tests between samples should still be valid.

In the reagent-only controls, low DNA yields were evident according to the spectrophotometer measurements (NanoDrop 1000, Thermo Scientific, UK), but background DNA contamination was detected post-sequencing. These sequences appeared to be diverse with low read numbers within each phylotype. Therefore, it is unlikely to affect the study results since ileal and faecal samples have a high biomass and therefore are less sensitive to contamination biases, unlike samples with very low bacterial levels.

To ensure that sequencing depth was adequate for this experiment, Good's coverage was calculated. All samples had a Good's coverage over 0.99, highlighting that an estimated 99% of the bacteria contained in both ileal digesta and faecal samples were captured during sequencing.

4.3.6 Taxonomic classification of sequences

When considering the total data set, 94.6% of sequences were classified at phylum level, 92.1% at class level, 91.9% at order level, 82.0% at family level, 56.3% at genus level and 29.4% at species level. These levels varied markedly when considering the classification of sequences from the ileum and rectum separately, with a higher proportion of sequences from the ileum being assigned taxonomy at phylum level in comparison to faecal samples (e.g. 0.4% of ileal

digesta sequences were not classified at phylum level, in comparison to 9.7% of sequences from faecal samples).

The dominant bacterial phyla and families isolated from both ileal digesta and faecal samples are illustrated in **Figure 4.5** with the associated mean relative abundances. In both ileal digesta and faecal samples, 29 phyla were identified with 7 phyla forming a core community in both compartments (98.69% in ileal digesta and 89.40% in faeces). In the ileal digesta samples, the Firmicutes and Proteobacteria comprised 89.96% of all sequences. In the faecal samples, the dominant phyla were Bacteroidetes and Firmicutes, which comprised 82.62% of all sequences. In both ileal and faecal samples, 138 bacterial families were identified. Of these 138 bacterial families, 16 core bacterial families comprised 95.33% of the ileal digesta and 67.11% of the faecal bacteria. Although core bacterial phyla and families were identified, quantitative differences were clearly apparent within these.

4.3.7 Temporal shifts in the gut microbiota

4.3.7.1 Phylum- and family-level shifts

Temporal shifts in both the ileal and faecal microbiota at both phylum and family level (with all treatment groups combined) are visualised in **Figure 4.6**. In the following section, statistically significant changes in relative abundance from baseline to day 13 (i.e. the final post-mortem point) will be discussed.

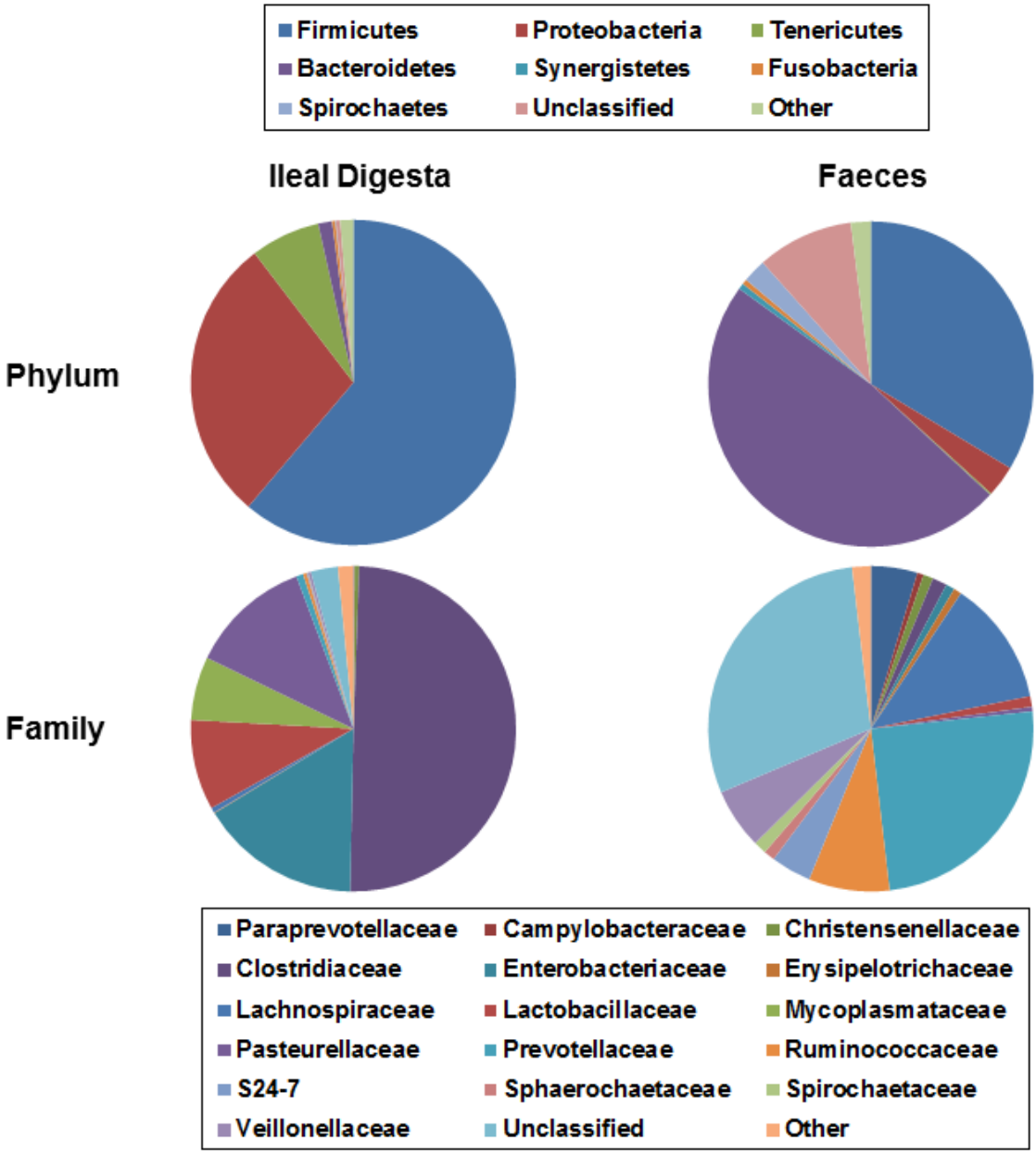

Figure 4.5: Mean relative abundances of core bacterial phyla and families in both ileal digesta and faecal samples. Although there are qualitative similarities, the quantitative differences are clearly evident. All phyla and families which represented less than an average of 0.5% were clustered into the "other" category. All sequences which were not classifiable at phylum and/or family level are binned into the "unclassified" category.

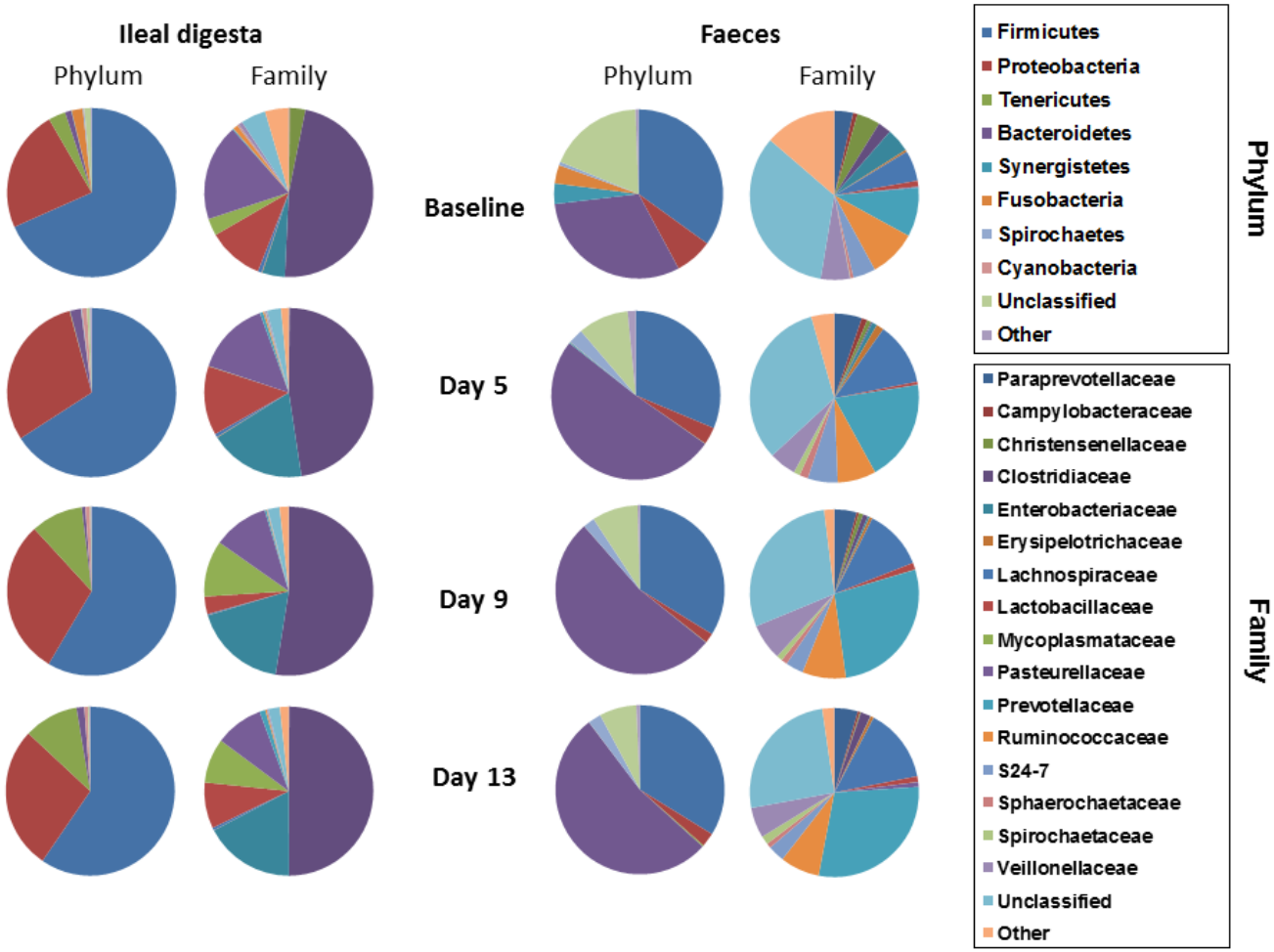

Figure 4.6: Temporal changes in relative abundance of core phyla and families in ileal digesta and faecal samples at each post-mortem point. All phyla and families which represented less than 0.5% of a community at all time points were classified as "other".

In the ileal digesta, the only statistically significant shift at phylum level was a mean (± SD) decrease in Fusobacteria from baseline (2.10 ± 2.52%) to day 13 (0.01 ± 0.03%) (Metastats: P = 0.02). At family level, a significant increase in Mycoplasmataceae occurred over the trial duration showing an increase by two orders of magnitude (Metastats: P = 0.04).

In the faeces, a significant increase in the most abundant phylum Bacteroidetes was observed from 30.91 ± 18.88% at baseline to 52.96 ± 14.96% on day 13 (Metastats: P = 0.02). Both Synergistes and Fusobacteria phyla decreased by two orders of magnitude from baseline to day 13 (Metastats: P = 0.02) and a significant increase in Spirochaetes was also observed (Metastats: P = 0.03). At family level, the relative abundance of Enterobacteriaeceae and Christensenellaceae decreased significantly over the trial duration (Metastats: P < 0.05). Finally, significant increases in both Lachnospiraceae and Prevotellaceae were observed (Metastats: P < 0.05).

4.3.7.2 Phylotype-level shifts

Any changes in phylotype-level relative abundances between post-mortem points were assessed using Metastats and DMM models. In the ileal digesta samples, there were no significant changes in phylotype relative abundances on day 5 in comparison to the baseline samples. Additionally, there were no further step-wise differences in relative abundances observed between day 5 and 9 and day 9 and 13 (Metastats: P > 0.05).

Comparisons between baseline samples and day 13 samples were made in order to capture overall changes in relative abundances over the experiment, with significant changes in particular phylotype relative abundances being evident (**Table 4.6**).

Table 4.6: Statistically significant changes in particular phylotypes in ileal samples between baseline and day 13 post-weaning. Mean changes in relative abundances (± SEM) of core phylotypes (relative abundance cut-off set at 0.1%) with FDR-corrected P values (P < 0.05).

OTU phylotype	Baseline	Day 13	P-value
Mycoplasma	0.0 ± 0.0	11.3 ± 2.5	0.05
Phascolarctobacterium	2.5 ± 0.9	0.0 ± 0.0	0.05
Anaeroplasma	0.5 ± 0.0	0.0 ± 0.0	0.05
Veillonella	0.4 ± 0.1	0.0 ± 0.0	0.05
Parvimonas	0.5 ± 0.2	0.0 ± 0.0	0.05

To further assess changes in the ileal microbiota composition over time, samples were clustered into community types using a DMM model. The DMM model with the highest likelihood partitioned all of the ileal samples into three enterotypes. At baseline, 35.7% of ileal samples were partitioned into enterotype 1 and 64.3% into enterotype 2. On day 5, enterotypes 1 and 2 were equally dominant with 44.8% and 41.4% of samples being partitioned into these enterotypes, respectively. On day 5, the remaining samples were partitioned into enterotype 3, which is an enterotype enriched for *Escherichia*

coli (13.8%). On day 9, enterotype 2 again becomes dominant with 66.7% of samples being assigned this enterotype, with 22.2% and 11.1% of the remaining samples being assigned to enterotypes 1 and 3, respectively. Finally, on day 13, enterotype 2 increases in dominance with 83.7% of samples belonging to this enterotype. Enterotype 1 was most enriched for Pasteurellaceae in comparison to the other two enterotypes, and enterotype 2 was most enriched for Clostridiaceae, with *Clostridium* and *Lactobacillus* also enriching this enterotype.

In the faecal samples, significant changes in relative abundances were observed between baseline and day 5 (Metastats: $P < 0.05$). Increases in unclassified Bacteroidales (7.3 ± 1.5% to 15.4 ± 1.2%), *Prevotella* (0.8 ± 0.4% to 7.1 ± 1.2%), *Treponema* (0.1 ± 0.0% to 1.2 ± 0.3%) and *Faecalibacterium prausnitzii* (0.1 ± 0.0 % to 1.4 ± 0.2%) were observed. No further step-wise differences in relative abundances were observed between days 5 and 9 and between days 9 and 13 (Metastats: $P > 0.05$). When comparing baseline samples with day 13 samples, several phylotypes were differentially expressed as shown in **Table 4.7**.

To further assess changes in the faecal microbiota composition over time, samples were clustered into community types using a DMM model. The DMM model with the highest likelihood partitioned all of the faecal samples into three enterotypes. The majority of baseline samples belonged to enterotype 3 (15/16 samples), which was most enriched for *Escherichia coli*. On day 5, the majority of samples (87.1%) belonged to enterotype 1 with the remaining 12.9% of samples belonging to enterotype 2. On day 9, enterotypes 1 and 2 contained similar numbers of samples with 46.9% and 53.1% of samples, respectively.

Table 4.7: Statistically significant changes in particular phylotypes in faecal samples between baseline and day 13 post-weaning. Mean changes in relative abundances (± SEM) of core phylotypes (relative abundance cut-off set at 0.1%) with FDR-corrected P values (P < 0.05). When assigned species-level taxonomy, the percentage similarity was ≥ 97%.

OTU phylotype	Baseline	Day 13	P-value
Prevotella	0.8 ± 0.4	4.9 ± 0.4	0.01
Prevotella copri	1.3 ± 0.9	17.7 ± 1.6	0.01
Treponema	0.1 ± 0.0	1.6 ± 0.3	0.01
CF231 [Paraprevotellaceae]	0.3 ± 0.1	0.9 ± 0.1	0.02
Faecalibacterium prausnitzii	0.1 ± 0.0	2.5 ± 0.6	0.01
Desulfovibrio	0.6 ± 0.1	0.2 ± 0.0	0.01
Lachnospira	0.0 ± 0.0	0.9 ± 0.1	0.01
Bacteroides	2.2 ± 0.8	0.0 ± 0.0	0.01
Ruminococcus	0.0 ± 0.0	0.2 ± 0.0	0.01
Succinivibrio	0.0 ± 0.0	0.4 ± 0.2	0.01
Butyricimonas	0.5 ± 0.1	0.0 ± 0.0	0.01
Escherichia coli	4.6 ± 2.5	0.0 ± 0.0	0.01
Fusobacterium	3.5 ± 0.9	0.0 ± 0.0	0.01
Pyramidobacter piscolens	3.5 ± 1.8	0.0 ± 0.0	0.01
Campylobacter subantarcticus	0.6 ± 0.3	0.0 ± 0.0	0.01
Parabacteroides distasonis	0.3 ± 0.1	0.0 ± 0.0	0.01

By day 13, enterotype 2 becomes dominant which contained 60.9% of samples in comparison to enterotype 1 (37.5%). On day 13, one sample out of a total of 64 was assigned to enterotype 3, which was the dominant enterotype at baseline. Enterotype 1 was the most enriched for Lachnospiraceae, with enterotype 2 being dominated by *Prevotella copri* and *Prevotella stercorea*.

When assessing temporal changes in community structure, communities in the ileal digesta samples changed significantly over time (AMOVA: $P = 0.046$) and this was also the case in faecal samples (AMOVA: $P < 0.001$). However, there were no changes in community stability at ileal or faecal level (HOMOVA: $P > 0.05$). The structural changes are visualised in simplified NMDS plots, which combine all samples from day -1 (baseline) and day 13 (**Figure 4.7**).

4.3.8 Main effects of ETEC challenge on the gut microbiota

Both descriptive and statistical analyses were carried out to establish main effects of ETEC challenge on the ileal and faecal microbiotas.

There were no significant main effects of ETEC challenge on faecal microbiota richness or diversity at any time point (ANOVA: $P > 0.05$). When considering faecal samples, there were no significant differences in community structure (AMOVA: $P > 0.05$) or stability (HOMOVA: $P > 0.05$) on days 5, 9 or 13 when comparing ETEC- and sham-challenged pigs (AMOVA: $P > 0.05$). There were also no significant differences in the expression of relative abundances at any time point when comparing ETEC- and sham-challenged pigs (Metastats: $P > 0.05$).

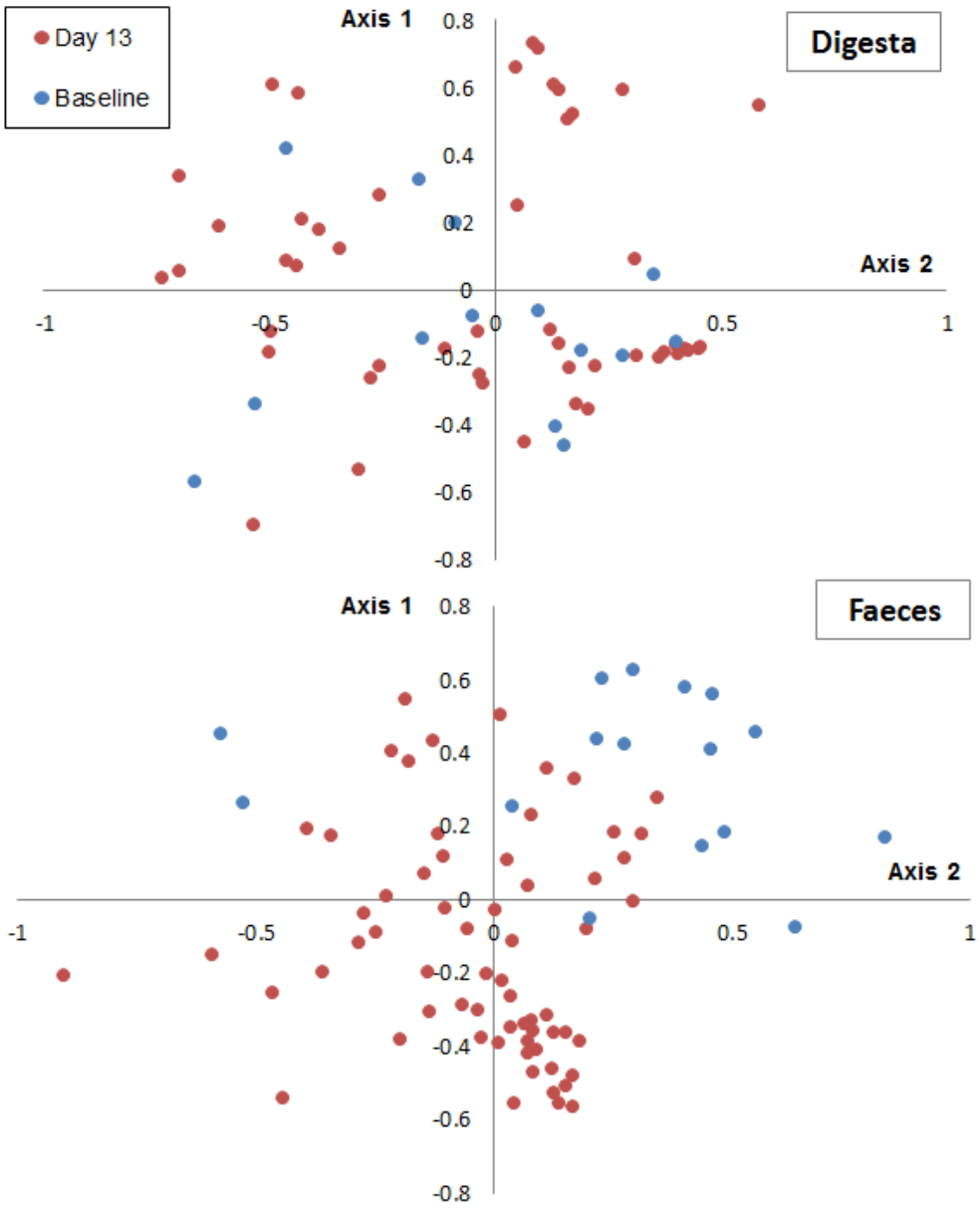

Figure 4.7: NMDS ordinations of Yue and Clayton dissimilarity indices from both ileal digesta and faecal microbial communities at baseline prior to challenge (day -1) and 11 days post-challenge (day 13). Both ETEC- and sham-challenged pigs on both LP and HP diets are included to visualise shifts in overall microbiota structure. Stress values: digesta= 0.26, faeces = 0.21.

In the ileal digesta samples, there were no significant main effects of challenge on either community richness or diversity on days 5 or 13 (ANOVA: $P > 0.05$). However, on day 9, significant main effects of ETEC challenge on both community richness (ANOVA: $P = 0.03$) and diversity (ANOVA: $P = 0.003$) were evident. Both the richness and diversity of microbial communities were higher in the ETEC-challenged pigs (Chao 1 = 59.7 ± 27.0, ISI = 3.95 ± 2.48) compared with the sham-challenged pigs (Chao 1 = 39.9 ± 20.1, ISI = 2.0 ± 0.8) on day 9.

On days 5 and 9 (i.e. 3 days and 7 days post-challenge, respectively), there was no impact of ETEC challenge on ileal microbiota structure (AMOVA: $P > 0.05$). However, on day 13 (i.e. 11 days post-challenge), ETEC- and sham-challenged pigs had a significantly different ileal microbiota structure (AMOVA: $P = 0.024$). Additionally, when comparing ileal communities from ETEC- and sham-challenged pigs, the ETEC-challenged ileal microbiota was significantly less stable on day 5 (HOMOVA: $P = 0.03$) and day 13 (HOMOVA: $P = 0.04$). There were no associated phylotype-level changes in relative abundances at any of the sampling points when comparing ileal communities in ETEC- and sham-challenged pigs (Metastats: $P > 0.05$).

4.3.9 Main effects of dietary protein level on the gut microbiota

There were no significant main effects of dietary protein level on faecal microbiota richness or diversity at any time point (ANOVA: $P > 0.05$). There were also no effects of dietary treatment on the faecal microbiota structure (AMOVA: $P > 0.05$) or stability (HOMOVA: $P > 0.05$) of pigs on days 5, 9 and 13 of the trial. Likewise, there were also no differentially expressed phylotype

relative abundances when comparing faecal samples obtained from pigs on the low and high protein diet (Metastats: $P > 0.05$).

In ileal digesta samples, on day 5, there were no significant differences in community richness or diversity when comparing pigs fed the low and high protein diets (ANOVA: $P > 0.05$). On day 9, significant differences in both richness (ANOVA: $P = 0.03$) and diversity (ANOVA: $P = 0.003$) were found when comparing pigs on the two different diets. The ileal communities obtained from pigs fed the HP diet had a higher mean richness and diversity (Chao 1 = 59.2 ± 28.0, ISI = 3.9 ± 2.6) in comparison to the samples obtained from pigs fed the LP diet (Chao 1 = 40.6 ± 19.3, ISI = 2.1 ± 0.8). On day 13, there were no significant differences in diversity, but significant differences in richness were evident (ANOVA: $P = 0.02$) with the pigs fed the HP diet still showing a higher mean Chao 1 index (HP = 50.1 ± 16.5, LP = 36.5 ± 22.3).

There were no effects of dietary treatment on ileal microbiota structure or stability on day 9 (AMOVA: $P < 0.05$, HOMOVA: $P < 0.05$). However, there were significant differences in both community structure (AMOVA: $P < 0.05$) and stability (HOMOVA: $P < 0.05$) on days 5 and 13 when comparing pigs on the low and high protein diets. These changes in community structure were also associated with changes in relative abundances of particular bacterial phylotypes on day 13 only. Pigs fed the high protein diet had a higher level of the order Burkholderiales, *Lactobacillus* and *Lactobacillus salivarius* in the ileal digesta (Metastats: $P < 0.05$). The pigs fed the low protein diet also showed

higher levels of *Campylobacter fetus* in the ileal digesta, in comparison with the pigs fed the high protein diet (Metastats: P = 0.027).

4.3.10 Interactive effects of challenge and dietary protein level on the gut microbiota

When considering faecal samples, there were no interactive effects of dietary protein level and ETEC challenge on community richness or diversity at any time point (ANOVA: P > 0.05). There were also no interactive effects of ETEC challenge and dietary protein level on the faecal microbiota structure (AMOVA: P > 0.05) or stability (HOMOVA: P > 0.05) at any time point.

When considering ileal digesta samples, there were no interactive effects of treatment on microbial community richness or diversity on days 5 and 13 (ANOVA: P > 0.05). However, on day 9, there were significant interactive effects of challenge and diet on community diversity (ANOVA: P = 0.04), whereby ileal samples obtained from pigs in the ETEC HP treatment showed the highest mean ISI (5.85 ± 2.81) and samples from pigs in the SHAM LP treatment had the lowest mean ISI (1.66 ± 0.54).

When comparing ileal microbial communities from samples obtained from pigs in all four treatment groups, significant differences in community structure and stability were observed on day 13 only (AMOVA: P = 0.017, HOMOVA: P = 0.04). There were no differences in community structure or stability when comparing the sham-challenged pigs fed the LP and HP diets at any time point (AMOVA: P > 0.05, HOMOVA: P > 0.05). However, there were differences in both community

structure (AMOVA: $P < 0.05$) and stability when comparing ETEC-challenged pigs fed the LP and HP diets on both days 5 and 13, with the ETEC HP pigs having a less stable ileal community at both time points at population level (HOMOVA: $P < 0.05$).

4.3.11 Growth rate and microbiota composition

To explore links between growth rate and microbiota composition, pigs subjected to post-mortem on day 13 were classified as those with a "low" and "high" growth rate on the basis of ADWG/kg weaning weight. Firstly, there were no significant differences in relative abundances at phylum and family level in both ileal and faecal samples when comparing pigs with a low and high growth rate (Metastats: $P > 0.05$). Additionally, no link was established between growth rate and ileal or faecal microbiota structure or stability (AMOVA: $P > 0.05$, HOMOVA: $P > 0.05$) or phylotype-level relative abundances (Metastats: $P > 0.05$) on day 13. When considering the DMM data, there did not appear to be enterotype clustering according to growth rate, with the majority of ileal communities clustering into partition 2 in both slow growers (89%) and fast growers (79%), and the majority of faecal communities clustering into partition 2 in both slow growers (65%) and fast growers (78%).

4.3.12 ETEC counts and microbiota composition

To explore links between ETEC counts in ileal digesta samples and microbiota composition, pigs subject to post-mortem on day 13 were grouped into two groups ("low ETEC count" and "high ETEC count") when taking ileal ETEC levels

on day 13 into account. When comparing these two groups, there were no significant differences in community structure or stability at ileal or faecal level (AMOVA: $P > 0.05$, HOMOVA: $P > 0.05$). There were also no differences in relative abundances at phylum, family or phylotype-level (Metastats: $P > 0.05$).

To explore links between ETEC shedding level and faecal microbiota composition, pigs subject to post-mortem on day 13 were split into two groups ("low shedders" and "high shedders") when taking ETEC counts on day 13 only into account. There were no differences in community structure or stability on day 13 when comparing pigs with the lowest and highest ETEC counts on day 13 (AMOVA: $P > 0.05$, HOMOVA: $P > 0.05$). Additionally, there were no differences in relative abundances at phylum, family or phylotype-level (Metastats: $P > 0.05$).

4.4 Discussion

The aims of this experiment were to characterise the weaner pig ileal and faecal (gut) microbiota over the immediate post-weaning period, to ascertain whether experimental ETEC challenge and/or dietary protein level had an impact on gut microbiota composition, pig performance and health and to establish whether there was a link between gut microbiota composition and pig growth rate or ETEC counts. Since the majority of gut microbiota studies focus only on faecal microbial communities, a key goal of this study was to identify microbial shifts at the site of ETEC infection (i.e. the ileum) and also at the primary site of where effects of dietary protein may be more pronounced.

4.4.1 Comparing ileal and faecal microbial communities

When classifying sequences from both ileal digesta and faecal samples, it was evident that a higher proportion of sequences obtained from ileal samples were assigned taxonomy in comparison to the faecal samples (e.g. only 0.4% of ileal digesta samples were not classified at phylum level, in comparison to 9.7% of sequences from faecal samples). This may be partly attributable to known biases in the sequence database for clinically relevant bacteria in humans (Poretsky et al., 2014) as mentioned in **Chapter 1**.

When comparing ileal and faecal communities, qualitative similarities were observed at phylum- and family-level. In both ileal and faecal samples, 29 bacterial phyla were identified with 7 of these phyla forming a core microbial community in both compartments. This was also the case at family-level, whereby 138 bacterial families were identified with 16 of these forming a core microbial community in both gut compartments. However, within these core groups, quantitative differences were clearly evident which has been the case in other studies where different gut compartments have been compared (Stanley et al., 2015). As discussed in **Chapter 2**, faecal samples are often studied as part of gut microbiota experiments which is mostly due to ease of sample collection. However, it is now well established that there are distinct microbial communities that reside in each gut section (Looft et al., 2014) and as part of this study, no changes at faecal level were observed in response to ETEC challenge or dietary protein level but profound changes at ileal level were

detected. This suggests that the choice of sampling site should be carefully considered as part of future experiments.

4.4.2 Temporal changes in the ileal microbiota

When considering changes in the ileal microbiota between baseline and day 13, considerable shifts in relative abundances at phylum- and family-level were evident and significant changes in community structure were found. The most considerable change in relative abundance was observed in the most dominant phylum, Firmicutes, which decreased from 68.3 ± 24.2% to 59.6 ± 30.4%. At family-level, the most sizeable change in mean relative abundance occurred in the Enterobacteriaceae from 4.3 ± 6.8 % at baseline to 17.4 ± 27.1 % on day 13. These changes, however, were not statistically significant due to large inter-animal variation which was also the case across other several other bacterial families isolated from the ileum. Indeed, it has been found that the ileal microbiota is subject to considerably more inter-individual variation in comparison to the faecal microbiota in humans (Booijink et al., 2010) and in pigs (Rettedal et al., 2009). Therefore, it is clear that inter-animal variation needs to be taken into account when studying the ileal microbiota in future studies.

At phylotype-level, significant changes in ileal microbiota composition were observed over the trial duration. The baseline samples were mostly enriched with Clostridiaceae, *Clostridium* and *Lactobacillus* species which have been shown to dominate in suckling pigs (Bian et al., 2016; Frese et al., 2015). By day 13 post-weaning, the majority of ileal digesta samples were enriched for

Pasteurellaceae, with an increased relative abundance of *Mycoplasma* which are both common members of the upper respiratory tract (Kielstein et al., 2001). However, both of these groups have also been isolated from the porcine GI tract (Leser et al., 2002) with the roles of these organisms being unclear. Finally, the anaerobic bacteria *Phascolarctobacterium*, *Anaeroplasma*, *Veillonella* and *Parvimonas* phase out completely by day 13, which represent significant reductions from baseline. Although the role of these bacteria as members of the gastrointestinal tract are not well understood, 16S rRNA gene metabarcoding studies are leading to further work to attempt to isolate, cultivate and characterise gastrointestinal microbes of interest (Watanabe et al., 2012).

4.4.3 Temporal changes in the faecal microbiota

When considering changes in the faecal microbiota between baseline and day 13, significant changes in community structure and phylotype relative abundances were evident. A significant increase in relative abundance was evident in the Bacteroidetes from 30.9 ± 18.9% to 53.0 ± 15.0% which has also been reported in previous work (Pajarillo et al., 2014). Additionally, both the Synergistes and Fusobacteria phyla showed a 100-fold decrease in relative abundance between baseline and day 13, with the roles of these bacteria in the porcine gut remaining unclear. However, an increased level of Fusobacteria has been linked with an increased incidence in neonatal diarrhoea in pigs (Hermann-Bank et al., 2015). At family level, the mean relative abundances of both the Enterobacteriaeceae and Christensenellaceae decreased significantly, with significant increases in both Lachnospiraceae and Prevotellaceae being

observed over the trial duration which links with a small number of bacterial species at phylotype-level.

At baseline, the majority of faecal samples were enriched with *Escherichia coli* which has been shown in previous studies (Holman and Chénier, 2014) and a significant decrease in *E. coli* was observed between baseline and day 13. By day 13, the majority of faecal samples were enriched with *Prevotella copri* and *Prevotella stercorea*. Increases in *Prevotella* post-weaning has been observed in other studies (Mach et al., 2015; Pajarillo et al., 2014) and is most likely driven by the change in diet (Flint et al., 2008) which is discussed in **Chapter 1**. Other phylotype-level changes between baseline and day 13 included an increase in relative abundance of *Treponema* (in agreement with Dicksved et al., 2015) and *Faecalibacterium prausnitzii* which has been linked to decreased inflammation in the gut (Martín et al., 2015) and is currently being targeted as a potential probiotic for livestock (Foditsch et al., 2014).

4.4.4 Impact of treatments on pig performance and health

Previous work utilising ETEC challenge models have shown a sub-clinical disease state, whereby diarrhoea did not present and minimal and transient effects on ADWG and ADFI were observed (Athanasiadou et al., 2010; Opapeju et al., 2009; Wellock et al., 2008b). In the current study, however, there were no transient or prolonged effects on any of these parameters in response to ETEC challenge. These findings may suggest that the piglets utilised for this study were more resilient and therefore did not suffer any ill effects after exposure to ETEC. Indeed, it has been shown on many occasions that it is difficult to

reproduce the effects of experimental PWC challenge due to the complex and multifactorial nature of the disease (Madec et al., 2000).

Although there was not a significant main effect of ETEC challenge on pig health or performance, there was a significant main effect of dietary protein level on faecal consistency score in pigs fed the HP diet showing a higher overall mean faecal consistency score (1.19 ± 0.09) in comparison to pigs fed the LP diet (1.11 ± 0.07). Although statistically significant, this does not indicate a major biological change in faecal consistency score. Although there was sufficient variation in dietary protein level to induce a consistent effect on faecal score, a larger variation may have been required to have a more profound impact on health and performance (Wellock et al., 2008b). As discussed in **Chapter 1**, higher protein diets have been linked to bacterial fermentation of excess protein in the gut which can lead to production of irritant by-products such as ammonia, phenols and biogenic amines (Halas, 2007; Hodgson and Barton, 2009) which have the potential to exacerbate diarrhoea.

4.4.5 Impact of treatments on the gut microbiota

In the faecal samples, there were no main or interactive effects of ETEC challenge and dietary protein level on community richness, diversity, structure, stability or phylotype relative abundances at any time point. Therefore, the faecal microbiota was not sensitive to ETEC exposure or an increased dietary protein level, or their combination. Since the site of both ETEC colonisation and protein fermentation is the small intestine, it is not surprising that communities in the distal gut are not significantly impacted by these factors. Hermes et al.,

(2009) studied the faecal microbiota composition of weaner pigs fed a low and high protein diet and no changes were observed. However, previous work has shown faecal-level alterations in microbial composition in response to diets containing varying levels of fat and fibre (Heinritz et al., 2016a) and varying levels of dietary protein (Cho et al., 2015) in grower pigs.

In contrast to the faecal-level observations, significant effects of treatment were observed at ileal level. In ileal digesta samples, there was a significant main effect of challenge on both community richness and diversity on day 9, with both indices being higher in the ETEC-challenged pigs. On day 9, however, there were no significant differences in community structure, stability or phylotype relative abundances when comparing ETEC- and sham-challenged pigs. However, on day 13 (i.e. 11 days post-challenge), ETEC- and sham-challenged pigs had a significantly different ileal microbiota structure and stability, suggesting that experimental ETEC challenge had an impact on community membership and relative abundances. These data showed that ETEC-challenged pigs had less stable ileal communities, meaning that there is significantly more genetic variation at population level in response to ETEC exposure. The stability of microbial communities is thought to be an important consideration for aiding understanding of the effects of dietary change and has been linked with healthy and unhealthy states (Schloss et al., 2012).

Dietary protein level also had a significant impact on richness and diversity indices on day 9. The pigs fed the HP diet had a higher mean richness and diversity value in comparison to pigs fed the LP diet, which was also found in

previous work at faecal level (Opapeju et al., 2009). This difference in richness was maintained on day 13, with the pigs fed the HP diet showing a higher mean Chao 1 index. An increased richness and diversity in the HP group may be partly explained by increased substrate availability for protein fermenting bacteria (Macfarlane and Macfarlane, 1995).

Interestingly, on days 5 and 13, dietary protein level had a highly significant effect on microbial community structure and stability, with pigs fed the HP diet having significantly less stable communities. On day 13 only, this change in structure was associated with differences in phylotype relative abundances. Pigs which were fed the HP diet showed higher levels of the order Burkholderiales and *Lactobacillus* in the ileal digesta. Wellock et al. (2009) also found that pigs fed a high protein diet had greater numbers of lactobacilli in faecal samples in comparison to those fed a low protein diet, with similar dietary formulations as described in this study. Specifically, members of the *Lactobacillus* genus have proteolytic properties (Kim et al., 2007) and may have increased in response to excess protein availability. Pigs which were fed the LP diet showed higher levels of *Campylobacter fetus* in the ileal digesta, whereby the LP gut environment may have provided more favourable growth conditions or resulted in lower level of competitive exclusion for this organism.

When exploring the interactive effects of ETEC challenge and dietary protein level on the ileal microbiota, again on day 9, there were effects on both richness and diversity. Additionally, there were interactive effects of challenge and dietary protein level on microbiota structure and stability when comparing the

four treatment groups on day 13. Interestingly, there was no impact of dietary protein level on both ileal microbiota structure and stability in the sham-challenged pigs, but significant differences in the ileal communities of ETEC-challenged pigs on the LP and HP diets were apparent on days 5 and 13. The ETEC-challenged pigs fed the HP diet had significantly less stable ileal communities and significantly different microbiota structures. Clearly, the combination of ETEC exposure and a high protein diet has caused a shift in microbiota structure and phylotype relative abundances, and the data indicate that the HP diet induced a more pronounced microbial dysbiosis at population level in ETEC-challenged pigs only.

4.4.6 Growth rate and gut microbiota composition

In order to explore whether there were links between growth rate and gut microbiota composition at ileal and faecal level, pigs were clustered into "low performer" and "high performer" groups on the basis of ADWG/kg weaning weight. As a result of this comparison, no links were established between growth rate and gut microbiota structure or phylotype relative abundances. Additionally, an enterotype analysis was carried out to establish whether pigs which performed at varying levels were enriched for particular bacterial phylotypes and no clustering was observed when comparing pigs with a low and high growth rate. Previous work has shown that pigs at 60 days of age cluster into two enterotypes which were significantly associated with body weight and ADWG (Ramayo-Caldas et al., 2016). Previous work by the same group also studied 31 pigs across 5 age ranges between day 14 and day 70 of

age and found that after weaning, almost half of the animals moved into a *Prevotella*-enriched cluster. Pigs which were clustered into the *Prevotella* group presented better growth rates post-weaning which is thought to be due to the essential role of *Prevotella* in the digestion of complex dietary polysaccharides as previously described. However, the authors also found that these enterotype groups were not fixed over time (only 11 pigs remained in the same cluster in 80% of time points), highlighting the complexity of microbiota dynamics post-weaning and the complexity and repeatability issues of linking this with growth rate.

4.4.7 ETEC quantification and the gut microbiota

In order to explore links between ETEC counts and gut microbiota composition, pigs were grouped into "low ETEC count" and "high ETEC count" groups and "low shedder" and "high shedder" groups on the basis of qPCR screening of ileal and faecal samples, respectively. When making comparisons, no differences in microbiota structure, phylotype relative abundances or enterotype clustering were evident in pigs with low and high ETEC *faeG* copy number in each compartment. In previous work, shedding level has been linked with microbiota composition. Bearson et al. (2013) found that "low" and "high" *Salmonella* shedders had significantly different faecal microbiota structures. However in the current study, and in the Bearson et al. (2013) study, it is clear that there is a high level of inter-individual variation in shedding dynamics. Additionally, after the acute stage of infection in the Bearson et al. (2013) study, the microbiota of both low and high shedders were not significantly different.

Therefore, as part of a sub-clinical infection model, any changes may also have been minimal and therefore undetectable at treatment level.

4.4.8 Reagent-only controls and mock bacterial community

As discussed in **Chapter's 1 and 2**, sampling and DNA extraction are critical steps in 16S rRNA gene metabarcoding experiments. Reagent-only controls were included as part of this experiment to establish background contamination introduced by personnel and laboratory consumables, as identified in previous work (Salter et al., 2014). During this experiment, low DNA yield was observed in both reagent-only controls and background DNA contamination was evident post-sequencing. However, the sequences generated were diverse and low read numbers presented in each bacterial phylotype. Therefore, as justified in detail in the discussion of **Chapter 2**, no phylotypes were removed as part of this experiment due to the potential introduction of more bias during sequence analysis.

In this study, a mock bacterial community was sequenced in parallel with the ileal and faecal samples to assess how well the developed 16S rRNA gene metabarcoding method captured a known bacterial community, to allow calculation of sequencing error rate and to establish inter-run variation. When comparing the expected relative abundances with the measured relative abundances, it was found that some bacterial species were over-represented or under-represented by this method. This misrepresentation may be caused by a variety of factors, including primer biases and the bioinformatics pipeline used (Pinto and Raskin, 2012; Schloss et al., 2011). There were only slight variations

in relative abundances when comparing both mock community samples, which suggests that the inter-run variability was low which ensures that biases introduced by running on a different Illumina MiSeq were low as part of this experiment.

4.5 Conclusion

In conclusion, both the ileal and faecal microbiota change significantly over the post-weaning period. There were no main or interactive effects of ETEC challenge or dietary protein level on faecal microbial communities, and there were no links established between host growth rate and ETEC load on faecal or ileal microbiota composition. Although ETEC exposure or manipulation of dietary protein level did not have a significant impact on host performance, significant main and interactive effects were exerted on ileal microbiota structure, stability and phylotype relative abundances, which is the site of both ETEC colonisation and protein fermentation in the porcine gut.

Chapter 5:
General discussion

5.1 Introduction

The weaning period exerts a variety of stressors on the pig, which can have an impact on host health and performance, as described in **Chapter 1**. The gut microbiota undergoes profound changes post-weaning, which is mostly due to the sudden change to a less digestible, solid diet. The study of germ-free animals has revealed the pivotal role of the gut microbiota in immune system development (Macpherson and Harris, 2004) and host protection against infection (Kim et al., 2011; Richards et al., 2005; Round and Mazmanian, 2009).

Due to the clear role of the gut microbiota in host health, it has become a potential target in the development of future management strategies for enteric diseases, such as dietary manipulation and administration of probiotics. Specifically, lowering dietary protein levels has been linked with a lowered level of protein fermentation (Halas, 2007; Hodgson and Barton, 2009), changes in microbial ecology (Rist et al., 2013) and a reduction in ETEC load in the ileum in the presence of experimental ETEC challenge (Opapeju et al., 2009). Although links have been made between dietary protein level and microbiota composition in the past, these observations were based on findings from culturing studies and the use of early molecular methodologies. The recent emergence of 16S rRNA gene metabarcoding allows higher resolution study of changes in complex microbial communities, which has been utilised as part of this project to gain a clearer insight into the impact of ETEC challenge and

dietary manipulation on weaner pig health, performance and gut microbiota dynamics.

In this chapter, the challenges underlying the development of both experimental 16S rRNA gene metabarcoding methods and experimental ETEC challenge models will be discussed, including data from **Chapter's 2-4**. Additionally, temporal changes in the weaner pig microbiota in the presence and absence of ETEC challenge at both ileal and faecal level will be discussed in light of findings from **Chapter's 2 and 4**. The main and interactive effects of ETEC challenge and dietary protein manipulation on both the ileal and faecal microbiota will be discussed using data from **Chapter 4**. Finally, considering both data obtained from this project and the published literature, potential directions for future research will be proposed.

5.2 16S rRNA gene metabarcoding

5.2.1 Minimising biases

As part of this project, a 16S rRNA gene metabarcoding method was developed and validated. As addressed in **Chapter 1**, there are several steps in 16S rRNA gene library preparation which have the potential to introduce biases. Considering the literature presented in **Chapter 1**, steps were taken throughout the studies presented in this thesis in an attempt to minimise biases at some of these stages.

Samples were snap-frozen on dry ice immediately on collection, and were stored for a maximum of 2 weeks at -80^{o}C prior to DNA extraction to minimise variation introduced due to differing storage conditions and duration. As discussed in **Chapter 1**, significant differences in microbial composition are detected when using different commercially available DNA extraction kits. Therefore, as part of the work presented in this thesis, only one brand of DNA extraction kit was utilised.

A limitation of the method used as part of this study was the use of both wet ileal and faecal samples in downstream processing, rather than drying the samples to standardise the dry mass taken forward for DNA extraction. When this study was designed, there was no published work which highlighted any impact of drying samples on 16S rRNA gene metabarcoding results. However, a recent study has described that freeze-drying had a limited impact on 16S rRNA gene sequencing results (Fouhy et al., 2015). In the current study, relative abundance data were calculated using the data obtained from sequencing and so should not be subject to a large amount of bias. However, the same samples were used for absolute quantification of ETEC load which would have been subject to biases as a consequence of variations in dry matter. Therefore, in future studies to minimise biases further, drying of ileal and faecal samples should be considered to standardise for dry weight.

5.2.2 Classification of sequences

When classifying sequences from both ileal digesta and faecal samples, it was evident that a higher proportion of sequences obtained from ileal samples were

classified at phylum level in comparison to the faecal samples. In **Chapter's 2 and 4**, 10.0% and 9.7% of sequences were not classified at phylum level respectively, in comparison to only 0.4% of sequences from ileal samples in **Chapter 4**. As mentioned in previous chapters, this may be partly due to the known biases in the sequence databases for clinically relevant bacteria in humans (Poretsky et al., 2014). However, when considering other studies, the levels of unclassifiable bacteria at phylum level are much lower. For example, in one longitudinal study, only 1.4% of sequences were unclassified at phylum level (Slifierz et al., 2015). Although it was initially hypothesised that the small V3 hypervariable region was not providing enough taxonomic resolution, Slifierz et al. (2015) targeted the V4 hypervariable region which is of comparative size. However, in this example study, Firmicutes dominated the porcine faecal microbiota (i.e. 70% relative abundance) showing that this study is describing a markedly different bacterial community, so it is not directly comparable to the current study.

A database-dependent approach was used in this study to bin sequences into phylotypes. Initially, a database-independent approach was trialled, but the resultant distance matrix was too large to use in downstream analysis. This is most likely due to the large number of samples used as part of the studies presented in **Chapter 2** and **Chapter 4,** whereby marked inter-individual variation was evident. In future studies, a database-independent approach could be considered using smaller datasets to gain better classification depth without being reliant on information from sequence databases.

5.2.3 The importance of sequencing controls

To address background contamination introduced by personnel and laboratory reagents, reagent-only controls were included as part of each sequencing run. It was brought to light recently by Salter et al. (2014) that background contamination can have a significant impact on the findings of 16S rRNA gene sequencing studies, particularly those which are focussed on samples with low biomass. Since this study included samples with high biomass (i.e. ileal digesta and faeces), it was hypothesised prior to sequencing that background contamination was not likely to have an effect on the sequencing results. Background contamination was evident post-sequencing (which was likely to have originated from the DNA extraction kit and PCR reagents), and the reagent-only controls contained a wide range of bacterial species at relatively low levels. Many bacteria that are associated with the porcine gastrointestinal tract (e.g. *Faecalibacterium* and *Anaerovibrio* species) were identified in the reagent-only controls. In light of these points, no bacterial phylotypes were removed from the analyses to minimise the introduction of more bias.

In order to calculate sequencing error rate and to establish the performance of the developed 16S rRNA gene metabarcoding method, a mock bacterial community was also included during each sequencing run. The sequencing error rates were calculated as 0.03% in the first run (**Chapter 2**) and as 0.01% in the second and third runs (**Chapter 4**). In other published work which included mock bacterial communities, the sequencing error rate has been reported as 0.39% (Glendinning et al., 2016, V2-V3 regions), 0.29% (Kozich et

al., 2013, V3-V4 regions), 0.58% (Kozich et al., 2013, V4-V5 regions) and 0.01% (Kozich et al., 2013, V4 region). As part of this project, the V3 hypervariable region was targeted to ensure a complete overlap in forward and reverse reads to reduce error rate. By targeting multiple hypervariable regions, better resolution can be obtained when assigning taxonomy (Yang et al., 2016). However, an inflated number of OTUs/phylotypes can present due to the increased error rate, with an increased difficulty in identifying chimeras.

As well as allowing calculation of error rate, sequencing a mock bacterial community can provide information on how well a 16S rRNA gene sequencing methodology has captured a bacterial community. Although a mock bacterial community is artificially produced and is not representative of a true bacterial community, nonetheless it can provide information on whether biases were introduced during library preparation. **Figure 5.1** shows the measured relative abundances of the three mock communities run as part of this project (Mock 1 – **Chapter 2**, Mock 2 and 3 – **Chapter 4**), which are compared with the expected relative abundances provided by the manufacturer of the mock community.

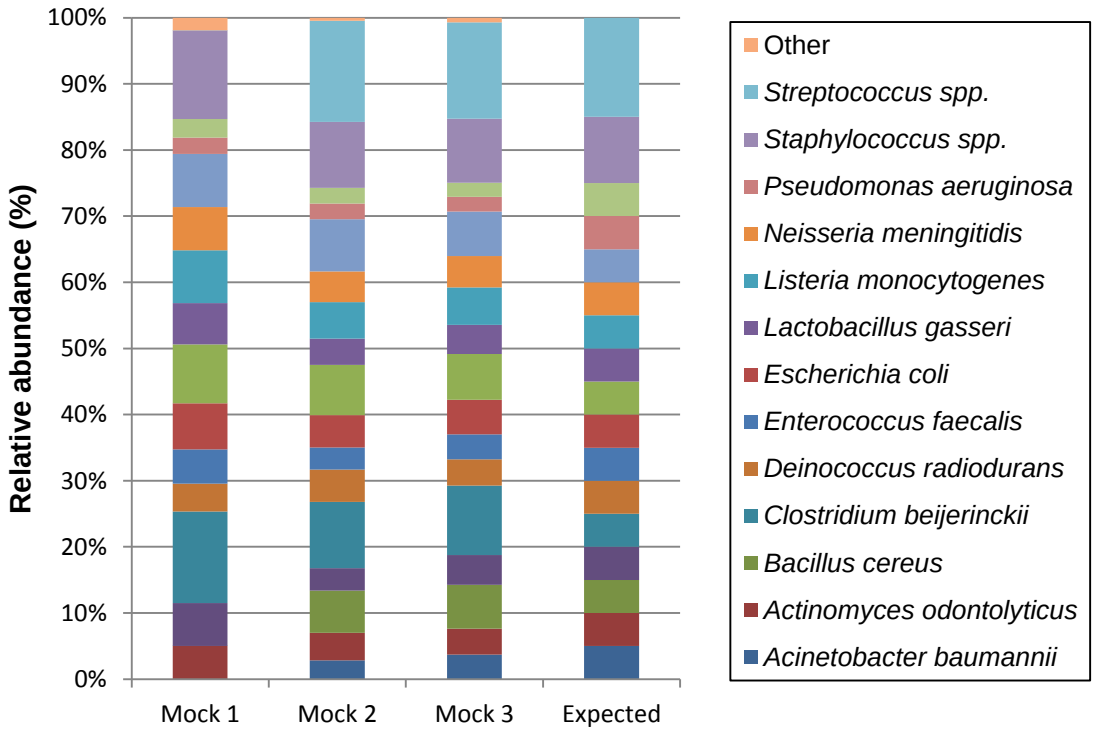

Figure 5.1: Measured and expected relative abundances in three mock bacterial community reactions.

When comparing the expected relative abundances with the measured relative abundances, in both **Chapter's 2 and 4**, it was found that some bacterial strains were over-represented or under-represented using the developed method. This will have been driven by a variety of factors, but will likely be most influenced by primer biases, since no 16S rRNA gene primer set is truly universal. However, the method used throughout this project identified all of the bacterial strains at genus level and 45% of these strains were identified at species level. Additionally, the inter-run variability (Mock 2 and Mock 3) in relative abundances was low which confirmed that biases introduced by running on a different Illumina MiSeq run were minimal.

Therefore, targeting a single hypervariable region in this study showed a good level of discriminatory power and captured a wide range of bacteria. In light of these results, inclusion of sequencing controls are essential to provide information on 16S rRNA gene sequencing experiments and should be included as part of every sequencing run.

5.3 Experimental PWC models

5.3.1 Single and multiple-dose models

Throughout this project, both multiple-dose (**Chapter 2**) and single-dose (**Chapter's 3 and 4**) ETEC challenge models were used. The multiple-dose model used was previously developed in our group (Athanasiadou et al., 2010) to address nutrition, production and inflammatory effects of ETEC challenge. Consequently, it was not assessed whether ETEC had adhered to the small intestine which was a critical factor as part of this project when linking ETEC challenge to gut microbiota dynamics. In **Chapter 3**, a single-dose model was trialled in a pilot study in order to assess ETEC adhesion and shedding dynamics by eliminating the possibility that the organism may have simply been passaging through the gastrointestinal tract and masking true shedding. In this pilot study, ileal adhesion was confirmed up to 4 days post-challenge and faecal shedding occurred up to 6 days post-challenge, which suggested that the organism was colonising the small intestine after a single-dose of ETEC inoculum. Therefore, this model was taken forward into the experiment presented in **Chapter 4**. In **Chapter 4**, ETEC was detected in both ileal and

faecal samples 11 days post-challenge, clearly showing the persistence of this organism as part of a single-dose challenge model.

5.3.2 ETEC challenge and host performance and health

For this work, sub-clinical PWC models were utilised whereby diarrhoeal episodes were not expected but an impact on performance parameters such as feed intake and weight gain were anticipated as shown in previous work in our lab and elsewhere (Athanasiadou et al., 2010; Houdijk et al., 2007; Opapeju et al., 2009; Wellock et al., 2008a). In **Chapter 3**, in the single-dose pilot study, a transient increase in faecal consistency score was evident in ETEC-challenged pigs in comparison to the sham-challenged pigs. However, in **Chapter's 2 and 4**, there were no significant effects of ETEC challenge on faecal consistency scores, feed intake or growth rate.

It is well known that it is difficult to reproduce the effects of PWC experimentally (Fairbrother et al., 2005). PWC is a multifactorial disease and clearly exposure to ETEC alone is not sufficient to reproduce the disease. As described in **Chapter 1**, there are many predisposing factors to PWC including weaning age, housing conditions and feed intake immediately post-weaning. The pigs sourced for this study were obtained from a high welfare and hygiene unit, and were housed to an equally good standard when included in the described experiments. Therefore, the pigs included in this study were perhaps not exposed to the variety of stressors which are present in a commercial setting. When interpreting the results, it must be considered that the management practices on a commercial farm are not comparable to those on an

experimental farm, with consequent implications for informing future disease management strategies. Consequently, it is paramount that work continues on generating a reproducible challenge model to continue work on the effects of ETEC on growth performance and health in weaner pigs.

5.3.3 ETEC challenge and the gut microbiota

Although ETEC challenge did not exert any significant effects on host performance and health, exposure to the pathogen did exert effects on the gut microbiota. In **Chapter 2**, the impact of ETEC challenge on the faecal microbiota only was studied. Although there were no significant treatment effects of ETEC challenge on faecal microbiota composition, when taking ETEC shedding levels into account, variations in both microbiota stability and structure were observed at specific time points. Interestingly, the microbiota structure of pigs shedding low levels of ETEC over the course of the trial had a more similar community structure to the sham-challenged pigs than the pigs shedding high levels of ETEC. In **Chapter 4**, the faecal microbiota was again studied, but only one faecal sample per animal was analysed for this purpose, and so it was not possible to link temporal shedding with faecal microbiota dynamics within the same animals. Due to the large inter-animal variation in ETEC shedding levels in this study and in other studies (Geenen et al., 2007; Hampson et al., 1985), it appears essential that ETEC shedding level should be taken into account to assess the long-term impact of ETEC exposure on the gut microbiota.

In **Chapter 4**, the main effects of ETEC challenge on the ileal microbiota were also studied. Although there were no significant main effects of challenge on the

faecal microbiota, clear changes in microbiota structure and stability were evident at ileal level on day 13 of the trial, which equated to 11 days post-challenge. Interestingly, these data showed that ETEC-challenged pigs had a less stable ileal microbiota at population level. As discussed in previous chapters, the stability of microbial communities has been linked with healthy and unhealthy states (Schloss et al., 2012), and it has been shown that pigs with diarrhoea have less stable communities in comparison to those that do not in a commercial setting (Hermann-Bank et al., 2015). Therefore, although in the absence of clinical signs of PWC, ETEC challenge did have an effect on the ileal microbiota composition, which is the site of ETEC colonisation and infection. This could suggest that at the site of colonisation, ETEC bacteria are interacting with the commensal microbiota resulting in the observed changes in microbiota structure and stability.

5.4 Temporal shifts in gut microbiota composition

5.4.1 Comparing gut compartments

It was originally proposed in the literature that the bacterial populations in the faeces are representative of those further upstream in the gastrointestinal tract (Moore et al, 1978). However, more recent work has suggested that distinct microbial communities exist in different compartments of the gastrointestinal tract (Looft et al., 2014). A primary aim of this study was to use 16S rRNA gene metabarcoding to gain a deeper understanding of ileal and faecal microbiota

shifts in the immediate post-weaning period, with data being presented in **Chapter's 2 and 4**.

When comparing the ileal and faecal microbiota, qualitative similarities but quantitative differences were observed. At phylum level, 24% of the total identified phyla and 11% of the total identified families were shared between ileal and faecal samples. However, within these core groups, sizeable quantitative differences were observed which has been reported in other work (Stanley et al., 2015).

In this study, significant differences were observed in ileal microbiota structure and phylotype relative abundances in response to ETEC challenge and dietary manipulation (discussed in the following sections), but the faecal microbiota did not change in response to either of these treatments. As part of this project, it was of interest to establish whether there were "faecal phylotypes" which were sensitive to both ETEC challenge and dietary protein manipulation, which was not the case at treatment level. Therefore, findings from this study and previous work collectively highlight that the choice of sampling site is crucial as part of future experiments and is dependent on the biological question which is being addressed.

5.4.2 Shifts in faecal microbiota composition

In **Chapter 2**, temporal faecal microbiota dynamics were studied in the same individuals at five time points over a 19 day period post-weaning. Therefore, powerful descriptive and statistical analyses could be carried out to track faecal

microbiota dynamics over the immediate post-weaning period. In **Chapter 4**, ileal and faecal microbiota compositions were compared as part of a serial slaughter design until day 13 post-weaning with the consequent loss of repeated measurements. Data from both of these chapters will be discussed here to compare and contrast key findings from these two separate experiments.

At phylum level, a significant increase in the relative abundance of the Bacteroidetes was observed over time in both experiments and in previous work (Pajarillo et al., 2014), with significant decreases in both Proteobacteria and Spirochaetes being observed in **Chapter 2** and significant decreases in Synergistes and Fusobacteria phyla in **Chapter 4**. No significant changes in family-level relative abundances were observed in **Chapter 2**, but the mean relative abundances of both the Enterobacteriaeceae and Christensenellaceae decreased significantly in **Chapter 4**, with significant increases in both Lachnospiraceae and Prevotellaceae also being observed. It was established in **Chapter 2** that both the richness and diversity of the faecal microbiota decreased significantly over the trial duration, with the likely driver of these changes being the increasing dominance of the Bacteroidetes (specifically Prevotellaceae) over time.

There were also significant changes in faecal microbial community structure in both studies over time, with faecal community stability significantly increasing over time in **Chapter 2** only. At baseline in **Chapter 4** (i.e. one day pre-weaning), the majority of faecal samples were enriched for *Escherichia coli*. At

baseline in **Chapter 2** (i.e. four days post-weaning), the samples were primarily enriched for Lachnospiraceae. This difference in enterotype clustering between studies is most certainly due to the 5 day gap between baseline sampling, meaning that the faecal microbiota in the pigs sampled later had already undergone changes in response to the solid diet. This highlights how quickly the weaner pig microbiota changes in response to this diet change. However, in both trials, the changes in community structure are most likely explained again by the increased dominance of the Prevotellaceae, specifically *Prevotella copri* and *Prevotella stercorea*, which increased significantly over time in both studies.

5.4.3 Shifts in ileal microbiota composition

The ileal microbiota was studied as part of **Chapter 4** only. Although clearly repeated measurements from the same animal were not possible in our experimental design, core changes in the ileal microbiota over time were established using samples from each post-mortem point.

A statistically significant decrease in the Fusobacteria phylum from baseline to day 13 was observed, with a significant increase in Mycoplasmataceae also occurring over the same time period. As mentioned in **Chapter 4**, due to inter-animal variation, many sizeable shifts in relative abundance were not statistically significant at population level. Previous work has highlighted that the ileal microbiota is subject to more inter-individual variation in comparison to the faecal microbiota in humans (Booijink et al., 2010) and in pigs (Rettedal et al., 2009). Inter-animal variation has been observed in other porcine gut microbiota studies and although a relatively conserved core microbiota is

apparent, the variable microbiota appears to carry out many unique functions (Lamendella et al., 2011). In light of data from this study, careful consideration of sample sizes must occur for future work to ensure sufficient statistical power to establish core shifts in microbiota composition. Additionally, it is important to investigate the drivers of inter-individual variation in future work in a controlled, experimental setting to establish potential effects on host performance and health.

In the current study, the baseline samples were mostly enriched with organisms which dominate the suckling pig gastrointestinal tract (Clostridiaceae, *Clostridium* and *Lactobacillus*)(Bian et al., 2016; Frese et al., 2015). By day 13 post-weaning, the samples were mostly enriched for Pasteurellaceae with an increased relative abundance of *Mycoplasma*, which are primarily both members of the upper respiratory tract (Kielston et al., 2001) but have been isolated in the porcine gut (Leser et al., 2002). Finally, the anaerobic bacteria *Phascolarctobacterium*, *Anaeroplasma*, *Veillonella* and *Parvimonas* are members of the ileal microbiota at baseline but are no longer members of the ileal microbiota by day 13. Although the roles of the listed bacteria are not well understood, 16S rRNA gene metabarcoding studies are leading to targeted work in attempts to isolate, cultivate and characterise gastrointestinal microbes of interest (Watanabe et al., 2011).

5.4.4 Gut microbiota and performance

As part of this project, it was of interest to establish whether there was a link between gut microbiota composition (at ileal and faecal level) and host

performance. In **Chapter's 2 and 4**, there were no enterotypes identified which were linked to variation in growth rate, with no associated changes in microbiota structure or phylotype relative abundances being evident at ileal or faecal level when comparing pigs with a low and high growth rate.

In **Chapter 2**, it was found that differential expression of both Proteobacteria and Enterobacteriaceae were evident in faecal samples when comparing pigs with a slow and fast growth rate. Interestingly, slower growing pigs had a markedly higher relative abundance of Proteobacteria on days 4, 8 and 15 and a higher relative abundance of Enterobacteriaceae on days 8, 15 and 19. In previous work, an increased abundance of Proteobacteria (and specifically, members of the Enterobacteriaceae family) are used as indicators of poorer gut health (Buzoianu et al., 2012; Heinritz et al., 2016; Hermann-Bank et al., 2015; Vigors et al., 2016) since these bacterial groups contain several well-characterised pathogenic species. However, several opportunistic pathogens such as *Enterococcus cloacae*, *Klebsiella pneumoniae* and *Citrobacter freundii* have also been found to be dominant members of the porcine gut and did not appear to have a negative impact on host health (Guenther et al., 2010; Schierack et al., 2007). Additionally, the Enterobactericaeae family contains many commensal organisms which are present in the gastrointestinal tract of healthy weaner pigs in the current study and in pigs in previous studies (Gordon and FitzGibbon, 1999; Schierack et al., 2007). Therefore, although differential levels of Proteobacteria and Enterobacteriaceae were found in slow

and fast growing pigs in this study, this finding must be carefully interpreted at such a high taxonomic level.

5.5 Manipulation of dietary protein level

5.5.1 Formulation of diets

In the context of animal production, a key strategy to improve host health and performance is to aim to manipulate the microbiota through a variety of different methods, such as manipulation of dietary components and administration of probiotics (Richards et al, 2005).

In **Chapter 4**, two diets were formulated to have different levels of dietary protein in order to assess effects on gut microbiota composition, host health and performance. However, results from studies such as these are often confounded by the use of varying levels of other ingredients. In this study, the increased protein levels were largely balanced with a reduction in micronized wheat and oil to ensure that the diets were iso-energetic, with an associated reduction in neutral detergent fibre levels being observed in this diet. In future work, it should be considered that any effects that are exerted may not only be due to the reduction in protein level, and it would be appropriate to include additional iso-nitrogenous diets with manipulated neutral detergent fibre levels to substantiate the findings presented in **Chapter 4**.

5.5.2 Dietary protein level and pig performance and health

In **Chapter 4**, it was found there was not a significant main effect of dietary protein level on pig performance. Some studies have reported that the growth performance of weaner pigs decreased with a reduced crude protein level in the diet (Nyachoti et al., 2006; Wellock et al., 2009). However, other work has also shown that growth performance increased with a reduced dietary protein level in the diet (Wu et al., 2015). In the latter study, the higher protein diets exacerbated diarrhoea in pigs included in this treatment, so lowering dietary protein level improved performance by lowering diarrhoeal incidence. However, when comparing the current study with previous work, it is important to note that the weaning ages across these studies varied. As highlighted in **Chapter 1**, earlier weaning has been associated with increased incidences of diarrhoea and a decreased growth rate (McLamb et al., 2013) and a decreased mucosal barrier function (Levast et al., 2010; Moeser et al., 2007; Smith et al., 2010) and so the pigs weaned at a mean age of 25 days as part of the current study may have tolerated the high protein diet to a higher degree in comparison to early-weaned pigs.

Although no significant impact on host performance was observed when manipulating dietary protein level, pigs fed the high protein diet showed a significantly higher faecal consistency score over the course of the experiment. As discussed in previous chapters, a high level of crude protein has been linked to increased levels of bacterial fermentation in the gut which can lead to the production of irritant by-products such as ammonia, phenols and biogenic

amines (Bikker et al., 2006; Halas, 2007; Hodgson and Barton, 2009) which can potentially exacerbate diarrhoea, which may have led to the looser faecal consistency scores observed as part of this study.

Although severe diarrhoeal episodes were not observed as part of this trial, which were not expected due to the sub-clinical nature of the models used, the significant decrease in faecal consistency score in response to decreased dietary protein level reflects findings from previous work (Opapeju et al., 2009; Wellock et al., 2008a) and is of important commercial interest, since it has been proposed that an increase in dietary protein level is likely to have a higher impact on post-weaning diarrhoea proliferation (and pig growth performance) in a commercial setting (Göransson et al., 1995). Therefore, the manipulation of dietary protein level has been put forward as an important nutritional strategy in the management of post-weaning diarrhoea (Wu et al., 2015) and should be considered in light of the data obtained in the current study. The observation that growth performance was not impacted at the lower protein level used may further indicate that pigs weaned under perhaps more robust conditions in an experimental setting can better cope with lower protein diets. Although the weaner phase is relatively short in terms of total feed input for producing pigs, a reduction in dietary protein level has the potential to lower the cost of feed, nitrogen excretion and thus environmental load, provided that there that are no negative consequences on life-time host performance (Wellock et al., 2009).

5.5.3 Dietary protein level and the gut microbiota

In **Chapter 4**, it was found that altering dietary protein level did not have an impact on the faecal microbiota composition, in the presence or absence of ETEC challenge. Therefore, from this work, it has been concluded that the faecal microbiota was not sensitive to both ETEC exposure and dietary protein manipulation. The site of colonisation for ETEC is the small intestine and protein fermentation primarily occurs in same gut compartment, so this may be the biological reason for the lack of impact on more distal microbial communities. However, there are conflicting findings in the literature on the impact of dietary protein level on the faecal microbiota. Hermes et al. (2009) found that there was no impact of dietary protein level manipulation on the faecal microbiota, but Wellock et al. (2009) found smaller levels of faecal lactobacilli in pigs fed LP diets, and Cho et al. (2015) found that differing protein levels resulted in differentially expressed phylotype relative abundances in the faecal microbiota. However, it must be considered that the latter study included grower pigs and additionally, the levels of dietary protein inclusion vary between the current and previous studies.

During this project, profound effects of dietary protein level were observed on alpha diversity indices (Chao 1 and ISI indices). Pigs fed the HP diet showed a higher mean richness and diversity 7 days post-weaning in comparison to those pigs fed the LP diet, which was also the case in previous work at faecal level (Opapeju et al., 2009). This increased richness in samples from pigs fed the HP diet was maintained 11 days post-weaning. The increase in both of these alpha

diversity indices may be partly explained by increased substrate availability for protein fermenting bacteria (Macfarlane and Macfarlane, 1995) which could be explored in future work by carrying out functional metagenomic studies.

Additionally, the manipulation of dietary protein level had highly significant effects on microbial community structure and stability, with pigs being fed the HP diet having less stable communities at population level. On day 13, changes in structure were associated with differing levels of Burkholderiales and *Lactobacillus* (higher relative abundance in HP diet) and *Campylobacter fetus* (higher relative abundance in pigs fed LP diet). The potential roles of these bacteria in the context of dietary manipulation can only be speculated using 16S rRNA gene metabarcoding data and again could be further explored using functional metagenomics studies in future work.

Interestingly, there was no impact of dietary protein level on both the ileal microbiota structure and stability in the sham-challenged pigs, but the ileal community stability in the ETEC-challenged pigs fed the HP diet was significantly lower in comparison to ETEC-challenged pigs fed the LP diet both 3 days and 11 days post-challenge. Additionally, significant differences in microbiota structure were evident at the same time points. Interpreting the data, it appears that the combination of ETEC exposure and a high protein diet has caused profound microbiota shifts, with pigs on the HP diet having a more pronounced microbial dysbiosis at population level. Interestingly, in previous work, dietary protein level had more prominent effects in the presence of an experimental challenge, with larger levels of ETEC being isolated from pigs fed

high protein diets (Opapeju et al., 2009; Wellock et al., 2008a) with an associated reduction in growth performance (Opapeju et al., 2009). When considering data from the current study and published work, clear interactive effects exist between dietary protein level and exposure to ETEC, and highlights the importance of the study of total microbial communities as well as focussing on quantifying particular bacterial groups.

5.6 Concluding remarks

Collectively, the experiments presented in this thesis have provided further insight into the temporal gut microbiota dynamics in the weaner pig, showing the profound changes that occur in gut microbiota composition in a short time frame post-weaning. Although sub-clinical experimental ETEC challenge alone did not have a significant effect on host health and performance, dietary protein level appears to be a critical driver of ileal microbiota dynamics and pigs exposed to ETEC in the presence of a high protein diet have a different microbiota structure and less stable microbial communities in comparison to those fed a low protein diet.

The work presented in this thesis has provided a step forward using 16S rRNA gene metabarcoding with the aim to increase current knowledge on the weaner pig microbiota composition and dynamics in response to both ETEC exposure and the manipulation of dietary protein levels. As sequencing technologies continue to develop and improve, future studies should progress to functional metagenomic studies to establish the roles of crucial organisms in the

gastrointestinal tract and to advance understanding on the complex interactions between the gut microbiota, host health and performance. This research should provide insight into which bacterial groups should be targeted as sentinels for dietary and probiotic interventions to promote host health and performance and to protect weaner pigs from ETEC infection.

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Appendices

Appendix A: Diet ingredients with calculated and analysed nutrient levels.

Component	Unit	
Raw Material		
Skim milk powder	%	5.000
betaGro™	%	0.000
IgG-Plus	%	0.000
Proglobulin 80 P	%	0.000
Dairy crest whey	%	7.500
Micro ground wheat	%	30.288
Porridge oats	%	16.000
Micro ground maize	%	10.000
Potato protein	%	1.250
Full fat soya extruded	%	17.500
Fishmeal	%	7.500
L Lysine HCl	%	0.510
DL Methionine	%	0.252
L Threonine	%	0.190
Tryptophan	%	0.050
Soya oil	%	2.500
Limestone	%	0.300
Monocalcium phosphate	%	0.450
Salt	%	0.200
Min-Vit premix	%	0.500
Vitamin E	%	0.010
Nutrients (calculated)		
DE	MJ/kg	16.85
T Lysine	%	1.67
Methionine	%	0.64
Threonine	%	1.04
Tryptophan	%	0.30
Crude protein	%	22.10
Oil EE	%	8.08
Calcium	%	0.98
Phosphorus	%	0.75
Salt	%	0.64
Lactose	%	7.86
Vit A	I.U./kg	12.50
Vit D3	I.U./kg	2.00
Vit E	I.U./kg	250.00
Copper	mg/kg	165.65
Nutrients (analysed)		
Dry matter	%	89.6
Crude protein	%	19.9
Ash	%	5.5
Crude fibre	%	1.3
NDF	%	4.5
Oil B (AH EE)	%	7.5
Oil A (EE)	%	6.5
DE (wet chemistry)	MJ/kg	16.0

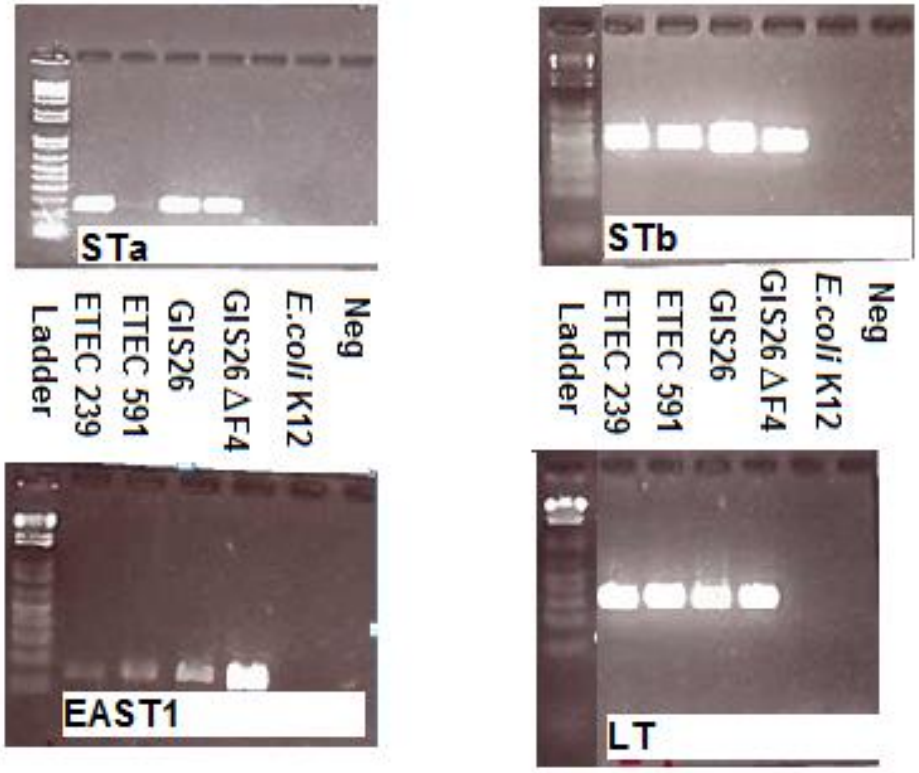
Appendix B: Forward primers including Illumina adapters and unique barcodes for sequencing.

Primer	Sequence (5'-3')
341-F1	AATGATACGGCGACCACCGAGATCTACACTATAGCCTACACTCTTTCCCTA CACGACGCTCTTCCGATCTNNNNCCTACGGGAGGCAGCAG
341-F2	AATGATACGGCGACCACCGAGATCTACACATAGAGGCACACTCTTTCCCTA CACGACGCTCTTCCGATCTNNNNCCTACGGGAGGCAGCAG
341-F3	AATGATACGGCGACCACCGAGATCTACACCCTATCCTACACTCTTTCCCTA CACGACGCTCTTCCGATCTNNNNCCTACGGGAGGCAGCAG
341-F4	AATGATACGGCGACCACCGAGATCTACACGGCTCTGAACACTCTTTCCCTA CACGACGCTCTTCCGATCTNNNNCCTACGGGAGGCAGCAG
341-F5	AATGATACGGCGACCACCGAGATCTACACAGGCGAAGACACTCTTTCCCT ACACGACGCTCTTCCGATCTNNNNCCTACGGGAGGCAGCAG
341-F6	AATGATACGGCGACCACCGAGATCTACACTAATCTTAACACTCTTTCCCTA CACGACGCTCTTCCGATCTNNNNCCTACGGGAGGCAGCAG
341-F7	AATGATACGGCGACCACCGAGATCTACACCAGGACGTACACTCTTTCCCTA CACGACGCTCTTCCGATCTNNNNCCTACGGGAGGCAGCAG
341-F8	AATGATACGGCGACCACCGAGATCTACACGTACTGACACACTCTTTCCCTA CACGACGCTCTTCCGATCTNNNNCCTACGGGAGGCAGCAG

Appendix C: Reverse primers including Illumina adapters and unique barcodes for sequencing.

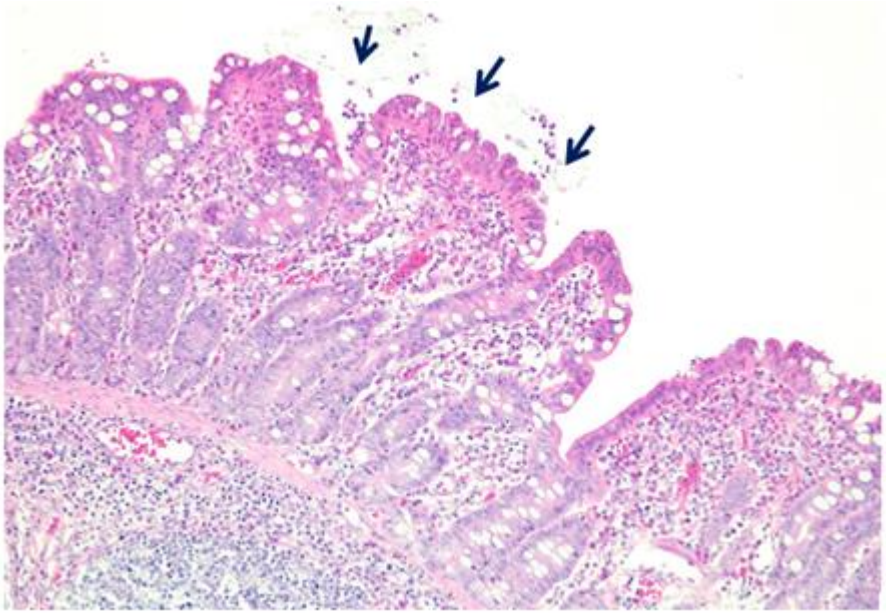
Primer	Sequence (5'-3')
518-R1	CAAGCAGAAGACGGCATACGAGATCGAGTAATGTGACTGGAGTTCAGACG TGTGCTCTTCCGATCTNNNNATTACCGCGGCTGCTGG
518-R2	CAAGCAGAAGACGGCATACGAGATTCTCCGGAGTGACTGGAGTTCAGACG TGTGCTCTTCCGATCTNNNNATTACCGCGGCTGCTGG
518-R3	CAAGCAGAAGACGGCATACGAGATAATGAGCGGTGACTGGAGTTCAGACG TGTGCTCTTCCGATCTNNNNATTACCGCGGCTGCTGG
518-R4	CAAGCAGAAGACGGCATACGAGATGGAATCTCGTGACTGGAGTTCAGACG TGTGCTCTTCCGATCTNNNNATTACCGCGGCTGCTGG
518-R5	CAAGCAGAAGACGGCATACGAGATTTCTGAATGTGACTGGAGTTCAGACG TGTGCTCTTCCGATCTNNNNATTACCGCGGCTGCTGG
518-R6	CAAGCAGAAGACGGCATACGAGATACGAATTCGTGACTGGAGTTCAGACG TGTGCTCTTCCGATCTNNNNATTACCGCGGCTGCTGG
518-R7	CAAGCAGAAGACGGCATACGAGATAGCTTCAGGTGACTGGAGTTCAGACG TGTGCTCTTCCGATCTNNNNATTACCGCGGCTGCTGG
518-R8	CAAGCAGAAGACGGCATACGAGATGCGCATTAGTGACTGGAGTTCAGACG TGTGCTCTTCCGATCTNNNNATTACCGCGGCTGCTGG
518-R9	CAAGCAGAAGACGGCATACGAGATCATAGCCGGTGACTGGAGTTCAGACG TGTGCTCTTCCGATCTNNNNATTACCGCGGCTGCTGG
518-R10	CAAGCAGAAGACGGCATACGAGATTTCGCGGAGTGACTGGAGTTCAGACG TGTGCTCTTCCGATCTNNNNATTACCGCGGCTGCTGG

Appendix D: Gel images generated after running the key virulence factor PCRs (Chapter 3) targeting STa, STb, EAST1 and LT. An *E.coli* K12 strain was included as a negative control, and a water-only negative control was also included.



Appendix E: Low (a) and high (b) power images of an H&E-stained ileal section (Pig C) which was obtained from a post-mortem on Day 6 (i.e. 4 days post-challenge), showing (a) acute damage to the surface epithelium of the villi and some inflammatory cells exuding into the lumen and (b) bacterial attachment to the outer membrane of the luminal epithelial cells.

(a)



(b)