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Analysis of temporal fecal microbiota dynamics in weaner pigs with and without exposure to enterotoxigenic *Escherichia coli* [1]

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ABSTRACT

The primary aim of this work was to study potential effects of sub-clinical enterotoxigenic *Escherichia coli* (ETEC) exposure on porcine fecal microbiota composition, with a secondary aim of profiling temporal shifts in bacterial communities over the weaning transition period. 16S rRNA gene metabarcoding and quantitative PCR (qPCR) were used to profile the fecal microbiota and quantify ETEC excretion in the feces, respectively. Temporal shifts in fecal microbiota structure and stability were observed across the immediate post-weaning period ($P < 0.05$), including significant shifts in the relative levels of specific bacterial phylotypes ($P < 0.05$). ETEC exposure did not change the fecal microbiota structure ($P > 0.05$), but significant variations in fecal community structure and stability were linked to variations in ETEC excretion level at particular time points ($P < 0.05$). In this study, marked temporal changes in microbiota structure and stability were evident over the short weaning transition period, with a relationship between ETEC excretion level and fecal microbiota composition being observed. This study has provided a detailed analysis of fecal microbiota dynamics in the pig, which should help to inform the development of novel management strategies for enteric disorders based on an improved understanding of microbial populations during the challenging post-weaning period.

Key words: Weaner pigs, 16S rRNA gene, metabarcoding, microbiome, ETEC.

INTRODUCTION

The importance of the gut microbiota in health and development is well documented in the pig (Leser et al., 2002; Isaacson and Kim, 2012), with marked changes in bacterial composition being observed throughout the production cycle (Kim et al., 2012; Holman and Chénier, 2014; Mach et al., 2015). The emergence of next-generation sequencing methodologies, such as 16S rRNA gene metabarcoding, now provides the opportunity to study complex microbial communities with high resolution. After weaning in pigs, there is an increased risk for the development of enteric disorders such as post-weaning colibacillosis, The symptoms which present as part of post-weaning colibacillosis range from fecal shedding of enterotoxigenic *Escherichia coli* (ETEC) (Fairbrother et al., 2005; Luppi et al., 2016) 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 variable reduction in performance may occur post-weaning (Hampson, 1994), which depending on its magnitude, can clearly be of economic importance. In this study, a previously developed sub-clinical ETEC exposure model (Athanasiadou et al., 2010) and 16S rRNA gene sequencing and quantitative polymerase chain reaction (qPCR) were utilized to assess the impact of ETEC exposure on fecal microbiome composition and ETEC shedding dynamics. In addition, temporal changes in microbiome composition were assessed over the weaning transition period. There are published studies which describe changes in the fecal microbiota during the weaning transition period specifically (Hu et al., 2016; Chen et al., 2017), but to our knowledge, this is the first study which focusses on the impact of ETEC exposure on the fecal microbiota using 16S rRNA gene metabarcoding.

MATERIALS AND METHODS

The animal experiment described was reviewed and approved by SRUC's Animal Welfare and Ethical Review Body (ED AE 23-2013) and carried out under Home Office regulations (PPL 60/4489).

Pigs and housing

Fifty-nine pigs (Large White × Landrace) were weaned at 26.7 ± 0.7 (mean ± SD) days of age and weighed 8.65 ± 1.77 kg, with 27 pigs being used in Round 1 (June 2013) and 32 pigs being used in Round 2 (August 2013). Pens were balanced as much as possible for sex, weaning weight and litter origin, with 8 litters being included across the trial. Pigs were housed in 4 m^2 square pens, groups of four maximum. The pens were bedded with sawdust as required, and a single feeder and nipple drinker were included. Water and feed were provided *ad libitum* for the trial duration. The environmental temperature was set at 25 °C for the first 4 days, and was decreased by 2 °C per week for the experiment duration. The shed lights were switched on between 0800 h and 1800 h and night lights were maintained between 1800 h and 0800 h. Pigs were fed a standard industry weaner diet for the first 14 days post weaning (digestible energy 16.9 MJ kg^{-1}; lysine 16.7 g kg^{-1}), before being moved onto a second phase weaner diet for the remainder of the trial (digestible energy 15.1 MJ kg^{-1}; lysine 15.0 g kg^{-1}), both of which did not contain antibiotics, organic acids or supra-nutritional levels of zinc oxide.

ETEC inoculum preparation

An ETEC O149:K91:F4 (ETEC F4) strain isolated from a weaner pig diagnosed with clinical post-weaning colibacillosis (SAC Veterinary Services, UK) was incubated in brain-heart infusion broth for 24 hours at 37 °C in an orbital shaker. Bacterial cells were harvested by centrifugation and the pellet was washed three times in 25 ml of sterile phosphate-buffered saline (PBS). The pellet was then re-suspended in 30 ml of PBS before preparation of an inoculum containing an estimated 10^8 colony forming units (cfu) ml^{-1}. The optical density of

the inoculum was measured using a spectrophotometer to estimate the concentration of ETEC cells. The inoculum was also serially diluted and enumerated on MacConkey agar for more accurate *post-hoc* confirmation of bacterial concentration.

ETEC exposure

Thirty-two pigs (16 in each of the two rounds) were administered 10^8 cfu of ETEC in PBS at five time points, i.e. days 4, 6, 8, 11 and 13 post-weaning as previously described (Athanasiadou et al., 2010), with weaning day defined as day 0. Briefly, 10 ml of the final inoculum was mixed with a further 10 ml of sterile PBS, before mixing with 20 g of feed. This mixture was then offered in small, discrete bins for each pig, with individual dosing being facilitated by temporarily splitting the pens in two for paired feeding. The remaining 27 sham-exposed (control) animals (11 in Round 1 and 16 in Round 2) were provided with feed in the same manner, mixed with 20 ml of sterile PBS only.

Fecal sampling and DNA extraction

Fecal samples were taken directly from the rectum on day 4 (before ETEC exposure), days 8 and 12 (during ETEC exposure), and days 15 and 19 (after ETEC exposure) using a spooned universal tube. The samples were immediately snap-frozen on dry ice prior to storage for a maximum of 2 weeks at -80 °C. DNA extraction was carried out using the MoBio PowerSoil® DNA Isolation kit (Cambio, United Kingdom), with modifications to the protocol. Briefly, 500 mg of feces were transferred into a 50 ml centrifuge tube, and 5 ml of MoBio PowerSoil® Bead Solution (Cambio, United Kingdom) were added to each tube. The feces was then homogenized using a vortex and 1 ml of supernatant was transferred into the provided bead tube, before being homogenized for 45 seconds at 5.0 m s^{-1} using a FastPrep FP120 Cell Disrupter (Qbiogene Inc, France). The homogenate was then processed according to the included kit protocol. The yield and quality of the DNA extracts were tested using a NanoDrop spectrophotometer (Thermo Scientific, UK) and by running the extracts on

a 2% agarose gel. The agarose gel visualisation confirmed the presence of intact, high molecular weight DNA.

ETEC quantification

Fecal excretion of ETEC F4 was determined using quantitative PCR (qPCR), which targeted the *faeG* major fimbrial subunit. Reactions were set up using Brilliant III Ultra-Fast SYBR® Green qPCR Master Mix (Agilent Technologies, United States) and primers F4-463F (5' – GGTTCTGAACTCTCGGCTGG – 3') and F4-597R (5' – AGAACCTGCGACGTCAACAA – 3'), which were designed as part of this study. All reactions were carried out in triplicate using a Stratagene MX3005P instrument (Agilent Technologies, United States), with 2 µl 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-exposed 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 target gene copy number in the fecal 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 ETEC F4 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, United Kingdom), and these values were used to estimate the number of gene copies per gram of wet feces.

144 ***Pig selection for DNA amplification and sequencing***

145 Thirty-two pigs were selected for 16S rRNA gene metabarcoding analysis (16 ETEC-
146 exposed and 16 control pigs) (Appendix 1), including one sample pre-exposure and four
147 samples post-exposure. Weaning weight, sex, litter origin and experimental round were
148 considered when recruiting particular pigs to the 16S rRNA gene metabarcoding study.

149 The V3 hypervariable region of the 16S rRNA gene was amplified using dual-indexed
150 primers which were previously used during a pig gut microbiome study - 341F (5' –
151 CCTACGGGAGGCAGCAG – 3') and 518R (5' – ATTACCGCGGCTGCTGG – 3') (Kim
152 et al., 2012), which incorporated TruSeq adapters (Appendix 2). Template DNA was
153 amplified in a one-step PCR using a high fidelity polymerase (Phusion®, New England
154 Biolabs, United Kingdom). A PCR mastermix was constructed to carry out 20 µl reactions,
155 including primers at a final concentration of 0.2 µM. The PCR conditions consisted of an
156 initial denaturation step at 98 °C (3 minutes), followed by 20 cycles of amplification at 98 °C
157 (30 seconds), 60 °C (30 seconds) then 72 °C (30 seconds), and a final extension step at 72 °C
158 (5 minutes). PCR products were purified using the AMPure XP PCR purification system
159 (Beckman Coulter, United States).

160 Reagent-only controls were amplified in parallel by adding 5 µl of DNA extract to the
161 PCR reaction mixture. The Human Microbiome Project mock community HM-782D (BEI
162 Resources, ATCC, Manassas, VA, United States) was also amplified by adding 1 µl of pre-
163 prepared DNA extract (containing 100,000 16S rRNA gene copies per organism per µl) to the
164 PCR reaction mixture. The presence of the correct sized product was confirmed by gel
165 electrophoresis and by use of a TapeStation instrument (Agilent Technologies, United
166 Kingdom). Before submission for sequencing (Edinburgh Genomics, United Kingdom),
167 double stranded DNA was quantified using a fluorometric assay (Qubit^{TM} dsDNA HS Assay
168 kit, Invitrogen, United Kingdom). Readings from this assay were used to create two pools

(80 samples per pool), using equimolar concentrations of each library. Sequencing was carried out using the Illumina MiSeq platform (Illumina, CA, United States), using V2 chemistry and producing 250 bp paired-end reads.

Sequence processing

Primer sequences were removed from raw sequence files 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. These sequences 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 135 bp in length or above 230 bp in length, contained over 8 homopolymers and if they contained ambiguous bases. Chimeras were identified and removed using UCHIME (Edgar et al., 2011). Sequences 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 which did not originate from bacteria were removed. The remaining sequences were binned into phylotypes based on their similarity to reference sequences and were subsampled for analysis.

Pig growth rate and fecal consistency scores

All pigs were weighed on days 0, 7, 14, 21 and 28 to assess growth rate over the trial duration. Consequently, the average daily weight gain (ADG) per pig was calculated over the trial duration. The general health and cleanliness of each pig was closely monitored and scored for the duration of the experiment. Throughout the experiment, pigs remained in good health, measured by active response to human presence and by the presence of pink skin, bright eyes and upright ears. Fecal consistency scores were recorded daily as described

194 previously (Wellock et al., 2006) on a pen basis using a subjective four-point scale (1,
195 normal; 2, normal diarrhoea; 3, watery diarrhoea and 4, dysentery).

196 ***Descriptive and statistical analysis of sequence data***

197 Descriptive and statistical analyses were carried out to describe temporal microbiota shifts
198 and to establish whether there was an effect of ETEC exposure on the fecal microbiota and/or
199 a link between ETEC excretion level and fecal microbial communities. Analyses were
200 carried out using the mothur software package (Schloss et al., 2009) unless stated otherwise.

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

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

217 To identify phylotypes that were expressed significantly differently between sample
218 groups, Metastats (Paulson et al., 2011) and analysis of composition of microbiomes

(ANCOM; Mandal et al., 2015) tools were used and the *P*-values were corrected for multiple observations.

To assess whether there were temporal effects of ETEC excretion level on microbiota composition, pigs were clustered into groups based on ETEC shedding level as measured by qPCR. Any links between ETEC excretion level and microbiota structure, stabilities and phylotype relative abundances were assessed using the AMOVA, HOMOVA, Metastats and ANCOM tools.

Statistical analysis of growth rate and fecal consistency score data

Statistical analyses were carried out using Genstat 16 (VSN International, UK) unless stated otherwise. The body weight data were assessed using repeated measures analysis of variance (RM-ANOVA) to establish any temporal effects of ETEC exposure. This analysis included ETEC exposure 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 ADG data was assessed using ANOVA to establish whether ETEC exposure had an effect on total weight gain between day 0 and day 28. To assess the consistency over time of the fecal scores, and whether there were any effects of ETEC exposure, an ordinal logistic regression (OLR) was performed using Minitab 17 (Minitab Inc, USA). The categorical indicator (i.e. fecal consistency score) was assigned as the response, and time point and ETEC exposure status were assigned as categorical predictors.

RESULTS

Sequencing quality control

After removing poor quality sequences and sequencing artefacts (17% of the original reads), a total of 16,816,541 reads were left for analysis. On average, 109,434 ± 43,035 (mean ± SD) reads were analyzed 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.

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, indicating that an estimated 99% of the bacteria present in the fecal samples were captured during sequencing.

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 1. *Acinetobacter baumannii*, *Bacillus cereus* and the streptococci were under-represented by sequencing, whereas *Clostridium beijerinckii* was over-represented.

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 were diverse with low read numbers within each phylotype.

Taxonomic classification of sequences

A total of 21 different phyla were identified across all fecal 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.

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 experimental rounds were combined for analysis.

Temporal changes in the fecal microbiota

The changes in relative abundances of dominant phyla and families are illustrated in Fig 1. A significant increase in relative abundance was observed at phylum level in the Bacteroidetes (RM-ANOVA: $P < 0.001$), with significant decreases in relative abundance in both the Proteobacteria and Spirochaetes (RM-ANOVA: $P < 0.05$). There were no significant temporal changes in relative abundances at family level (RM-ANOVA: $P > 0.05$). In addition, the richness (Chao 1) and diversity (ISI) of the fecal 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 (RM:ANOVA: $P < 0.01$; Table 2).

There was a change in fecal microbial community structure over time when considering both ETEC-exposed and control pigs (AMOVA: $P < 0.05$). Additionally, over the post-weaning period, the stability of the microbiota increased in both experimental groups (HOMOVA: $P < 0.05$). This is also visualized in a simplified NMDS plot, which combines all samples from day 4 (baseline) and day 19 (Fig 2).

A small number of phylotypes were differentially expressed between day 4 and day 8 in ETEC-exposed pigs (unclassified Clostridiales, *Prevotella* and Erysipelotrichaceae, Metastats: $P < 0.05$) and in control pigs (*Prevotella copri*, *Lactobacillus*, *Fecalibacterium 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

293 both ETEC-exposed and control pigs (Metastats: $P > 0.05$). Further changes in phylotype
294 relative abundances occurred between day 12 and 19, with a decrease in *Lactobacillus* being
295 evident in both ETEC-exposed and control pigs (Metastats: $P < 0.05$).
296 Changes in phylotype relative abundances between days 4 and 19 have been summarized
297 in Table 3. Phylotypes identified as *Prevotella copri*, *Prevotella stercorea* and *Prevotella*
298 showed significant increases in relative abundance in both experimental groups (Metastats: P
299 < 0.05). *Fecalibacterium prausnitzii* and Erysipelotrichaceae also showed a significant
300 increase in relative abundance in both experimental groups (Metastats: $P < 0.05$). The
301 relative abundance of S24-7 (family) decreased significantly in both groups of pigs
302 (Metastats: $P < 0.05$).
303 When considering all time points in the longitudinal analysis, there were no consistently
304 differentially expressed phylotypes over the course of the experiment (ANCOM: $P > 0.05$).
305 ***ETEC quantification***
306 All fecal samples from control pigs were confirmed as ETEC F4-negative by qPCR. The
307 dynamics of ETEC excretion were expressed over the trial period as log transformed
308 ($Log_{10}+1$) *faeG* gene copy number per gram of wet feces (Fig 3). On day 4 (pre-exposure),
309 all pigs tested negative when targeting the *faeG* gene. On day 8 post-weaning, the highest
310 levels of ETEC excretion were observed with 14 of 15 exposed pigs shedding detectable
311 levels of ETEC F4. Excretion levels dropped over the remaining sampling points with the
312 number of pigs having detectable levels of ETEC in their feces reducing over time.
313 ***ETEC exposure and fecal microbiota composition***
314 Although there was an indication that a decrease in richness occurred more rapidly in
315 ETEC-exposed pigs in comparison with the control pigs (RM-ANOVA: $P = 0.05$), the highly
316 significant decrease in richness and diversity over time was not affected overall by ETEC
317 exposure (RM-ANOVA: $P > 0.05$; Table 2).

318 The statistical significance of clustering in relation to ETEC excretion status was
319 assessed. Firstly, it was confirmed that there were no significant differences in community
320 structure when comparing ETEC-exposed and control pigs at baseline (AMOVA: $P = 0.65$).
321 Secondly, there were no significant differences in community structure at any of the sampling
322 points post-exposure when comparing pigs from both treatment groups (AMOVA: $P > 0.05$).
323 The stabilities of ETEC-exposed and control pig fecal communities were also compared
324 at each sampling point and no significant effects of ETEC exposure were observed
325 (HOMOVA: $P > 0.05$). Additionally, there were no differentially expressed OTUs over the
326 duration of the experiment when comparing ETEC- and sham-exposed pigs (ANCOM: $P >$
327 0.05).
328 ***ETEC shedding variation and microbiota composition***
329 Large variation was observed in ETEC F4 excretion level across all exposed pigs. In
330 order to explore potential links between microbiota composition and ETEC shedding levels,
331 all pigs exposed to ETEC were split into various groups based on the qPCR data and two
332 separate analyses were run.
333 Firstly, the cumulative area under the log curve (cumulative AULC) was calculated (Fig
334 4). Five "low shedders" and five "high shedders" were selected, based on their presence
335 within the lower and upper ranges of the AULC data. At baseline (day 4) and at all time
336 points post-exposure, there were no differences in community structure (AMOVA: $P > 0.05$),
337 stability (HOMOVA: $P > 0.05$) or phylotype relative abundances (Metastats: $P > 0.05$) when
338 considering ETEC excretion level. However, on day 8, the high shedders had a significantly
339 different community structure in comparison to the control pigs (AMOVA: $P = 0.013$),
340 whereas the low shedders had a more similar community structure to the control pigs
341 (AMOVA: $P > 0.05$), with no associated changes in phylotype relative abundances
342 (Metastats: $P > 0.05$). This community structure difference was not present for the remainder

of the experiment (AMOVA: $P > 0.05$). Additionally, there were no consistently differentially expressed phylotypes when comparing low and high shedders (ANCOM: $P > 0.05$).

Secondly, in order to investigate a clear contrast in shedding dynamics on day 12 (Fig 3), pigs were then retrospectively clustered into two groups - ETEC "clearers" (i.e. no shedding detected, n = 9) and ETEC "shedders" (i.e. shedding evident between 6.95×10^2 and 1.91×10^3 *faeG* copies g^{-1} wet feces, n = 6). No differences in community structure were evident in these groups prior to ETEC exposure on day 4 (AMOVA: $P > 0.05$), but the community structures were significantly different on days 12 (AMOVA: $P = 0.029$) and 19 (AMOVA: $P = 0.037$), as shown in Fig 5, but were not significantly different on days 8 and 15 (AMOVA: $P > 0.05$). Although there were observed changes in community structure, these were not linked with significant changes in relative abundance of particular bacterial phylotypes (ANCOM: $P > 0.05$, Metastats: $P > 0.05$). There were also significant differences in community stabilities on day 19, whereby the ETEC clearers had more variable bacterial communities in comparison with the ETEC shedders (HOMOVA: $P = 0.045$). No differences in community stability were observed on days 4, 8, 12 and 15 (HOMOVA: $P > 0.05$).

Growth rate and fecal consistency scores

The mean body weights for pigs included in the study and the subset selected for 16S rRNA gene metabarcoding are presented in Table 4. There were no significant effects of ETEC exposure on either body weight (RM-ANOVA: $P = 0.63$) or ADG (ANOVA: $P = 0.13$) when considering the total population of pigs (ETEC-exposed n = 32, control n = 27). When considering the pigs which were subject to 16S rRNA gene metabarcoding only (ETEC-exposed n = 16, control n = 16), there were again no significant effects of ETEC exposure on body weight (RM-ANOVA: $P = 0.80$) or ADG (ANOVA: $P = 0.56$).

Mean fecal consistency scores for both ETEC- exposed and control pens throughout the trial are presented in Fig 6. Overall, there was not a statistically significant effect of ETEC exposure on fecal score (OLR: $P = 0.822$), but statistically significant temporal effects on fecal consistency score were found (OLR: $P < 0.001$). On day 3 and 4 (pre-exposure), slightly elevated mean fecal consistency scores were observed in both ETEC-exposed and control pens. On day 5 (i.e. one day post-exposure), the mean fecal score in the ETEC-exposed pens increased to 1.5 ± 0.25 and to 1.29 ± 0.25 in the control pens. After day 5, the fecal scores gradually decreased and by day 20, all pens were assigned a fecal consistency score of 1. For the remaining 8 days of the trial, all pens were consistently assigned a fecal score of 1.

DISCUSSION

The primary aim of this work was to study potential effects of sub-clinical enterotoxigenic *Escherichia coli* (ETEC) exposure in pigs on fecal microbiota composition, with a secondary aim of studying temporal shifts in bacterial communities over the weaning transition period.

Sequencing controls

Contamination can arise from personnel and laboratory consumables, as well as DNA extraction kits and PCR reagents which can present during data analysis (Salter et al., 2014). In addition, it is good practice to include a known mock bacterial community to assess the methodology used for microbiome sequencing experiments. As a consequence, the inclusion of sequencing controls has been strongly encouraged (Pollock et al., 2018). In this study, low DNA yield was observed in the reagent-only control extracts and background DNA contamination was evident post-analysis. The sequences generated were diverse and low read numbers were observed within each phylotype. Since fecal samples have a high

biomass and are less sensitive to contamination biases, phylotypes that were identified in fecal samples and the reagent-only controls were not removed from the analysis.

When comparing the expected relative abundances with the measured relative abundances in the mock community, 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 (Schloss et al., 2011; Pinto and Raskin, 2012). Indeed, the calculated error rate as part of this sequencing run was low (i.e. 0.03%) which ensured that over-inflation of bacterial diversity due to sequencing error will have been minimized.

Temporal changes in the fecal microbiota

Over 80% of all DNA sequences generated in this study belonged to the Bacteroidetes and Firmicutes phyla, which is in line with existing literature (Kim et al., 2011; Costa et al., 2014; Mach et al., 2015; Hu et al., 2016; Chen et al., 2017). An increase in Bacteroidetes was evident over the immediate post-weaning period, which is also reflected in other work (Pajarillo et al., 2014; Hu et al., 2016; Chen et al., 2017). 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 found in the fecal samples (Kim et al., 2011; Lamendella et al., 2011; Kim et al., 2012; Liu et al., 2012; Looft et al., 2012; Holman and Chénier, 2014; Hu et al., 2016; Chen et al., 2017) and increases in relative abundance as the pig ages (Pajarillo et al., 2014; Mach et al., 2015; Hu et al., 2016; Chen et al., 2017). 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).

Significant changes in microbiota structure (i.e. bacterial membership and associated relative abundances) were observed over the immediate post-weaning period. Previous work

418 has established that suckling piglets have different gut microbial communities in comparison
419 to weaner piglets (Kim et al., 2011; Holman and Chénier, 2014; Pajarillo et al., 2014; Zhao et
420 al., 2015). These observed community shifts post-weaning are most likely associated with
421 the piglets' removal from a highly digestible milk source, the consequent removal of maternal
422 antibodies, and sudden introduction to a less digestible, solid, plant-based diet (Lallès et al.,
423 2007). The weaning process is also linked with social stressors, such as separation from the
424 sow and litter mixing, which contributes to greater susceptibility to enteric disorders (Lallès
425 et al., 2007). Additionally, the environment in which a piglet is housed has an impact on
426 mucosal immune function and microbiota composition (Mulder et al., 2009) and will likely
427 have a collective impact on the gut microbiota structure.

428 In this study, the fecal microbiota became more stable over the weaning transition period.
429 Differences in community stability have been linked with healthy and unhealthy states, and
430 temporal microbiota studies assist in linking variations in stability with variation in health
431 states (Schloss et al., 2012). Throughout the duration of this experiment, all pigs remained
432 clinically healthy which may assist in explaining why the fecal microbiota moved towards a
433 more stable composition in a relatively short time frame post-weaning.

434 ***ETEC excretion level and the fecal microbiota***

435 While there were no main effects of ETEC exposure on temporal fecal microbiota
436 structure, stability or relative phylotype levels, there were considerable variations in ETEC
437 excretion levels indicative of variable gastrointestinal tract colonization (Hampson et al.,
438 1985; Geenen et al., 2007). Specifically, on day 12, there were two clear groupings of pigs
439 defined as "ETEC clearers" and "ETEC shedders". The latter group showed a drastic
440 increased in ETEC shedding from the previous time point, which may be explained by better
441 adhesion and/or colonisation of ETEC F4 in the small intestine (Geenen et al., 2007). Pigs
442 which were more resistant to ETEC exposure may have shown lower excretion levels of this

443 bacterium, which would represent the time the organism took to pass through the pig without
444 significant interactions with the gastrointestinal tract (Hil et al., 2017). This level of variation
445 between individuals has been shown in previous studies on ETEC exposure (Hil et al., 2017)
446 and *Salmonella* exposure (Bearson et al., 2013) and may be attributable to the health status of
447 the animals and genetic variability underpinning innate and adaptive immune responses. It is
448 also proposed that competition with the commensal microbiota may play an important role in
449 ETEC colonization and excretion dynamics.

450 In this study, ETEC excretion level was associated with changes in fecal microbiota
451 structure and stability at specific sampling points. On day 8, when taking cumulative ETEC
452 shedding levels into account, the high shedders had a significantly different community
453 structure in comparison to the control animals. In addition, the low shedders had a more
454 similar community structure to the control animals rather than the high shedders at this time
455 point. Although structural differences were observed, there were no temporal differences in
456 phylotype relative abundances when comparing low and high shedders, suggesting that no
457 specific phylotypes were correlated with ETEC shedding variation. As previously described,
458 there were two clear groupings evident in terms of shedding levels on day 12 and when
459 animals were retrospectively clustered into these groups, their community structures were
460 significantly different on days 12 and 19. Although structural differences were observed on
461 days 12 and 19, these were not significantly linked with specific phylotypes, potentially
462 suggesting that several relative abundance shifts are occurring across many taxa.
463 Furthermore, higher fecal microbial diversity was observed in pigs that had no measurable
464 ETEC in the feces ("clearers") in comparison to pigs excreting ETEC ("shedders") on day 19.
465 Similarity to the low and high shedder pigs, there were no specific phylotypes which
466 correlated with this large variation in ETEC shedding level. Collectively, we consider that

the study does provide evidence of a relationship between fecal microbiota composition and ETEC excretion level.

In conclusion, a profound change in the fecal microbiota of pigs was observed over the short weaning transition period, with evidence of a relationship between ETEC excretion and fecal microbiota structure and stability being revealed.

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663 **FIGURES**

664 **Fig 1. Mean phylum- and family-level shifts in relative abundance**: The bacterial (a)
665 phyla and (b) families identified in highest mean abundances, showing changes in relative
666 abundances over a 19 day period post-weaning in both ETEC-exposed and sham-exposed
667 pigs.

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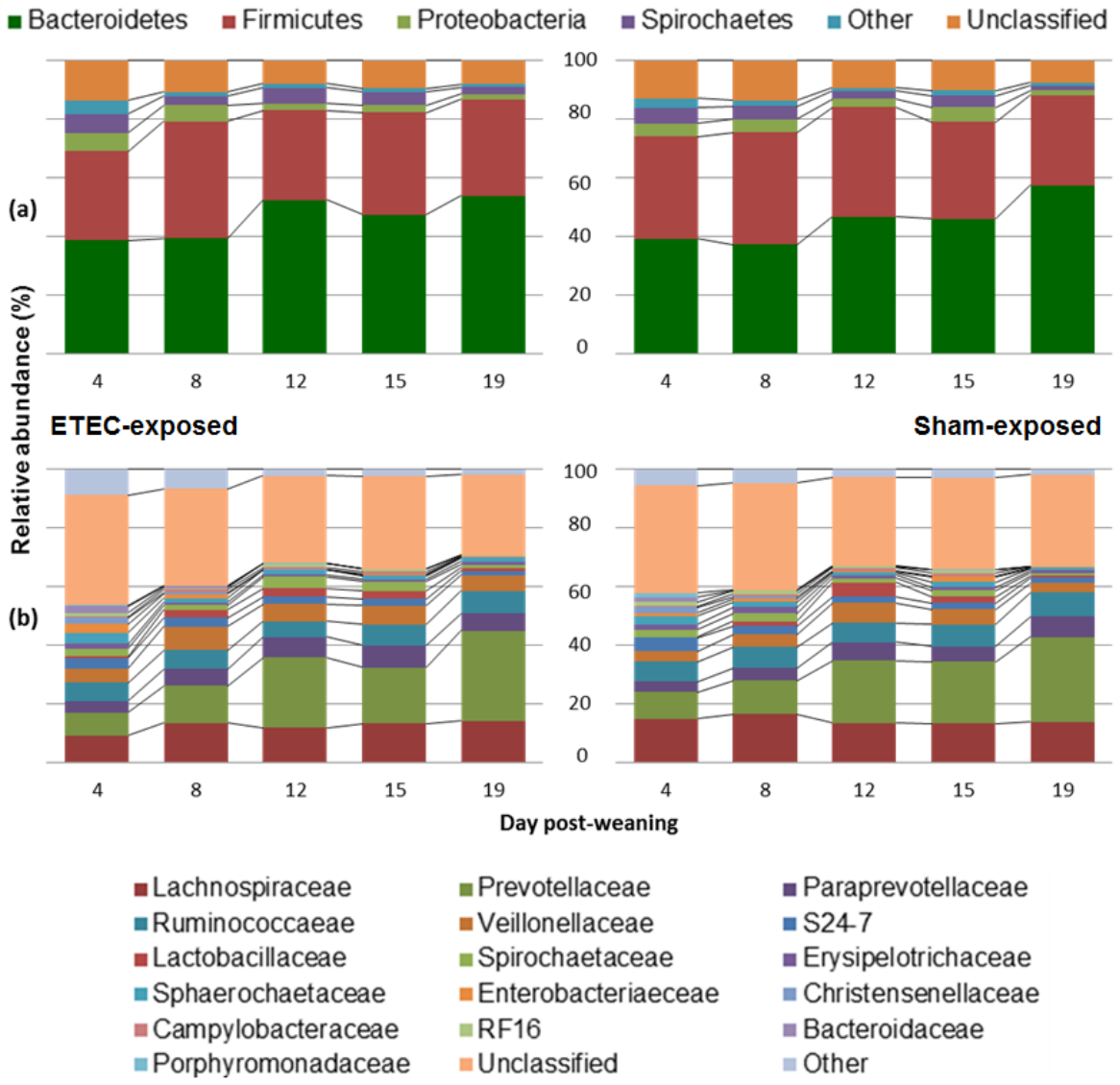
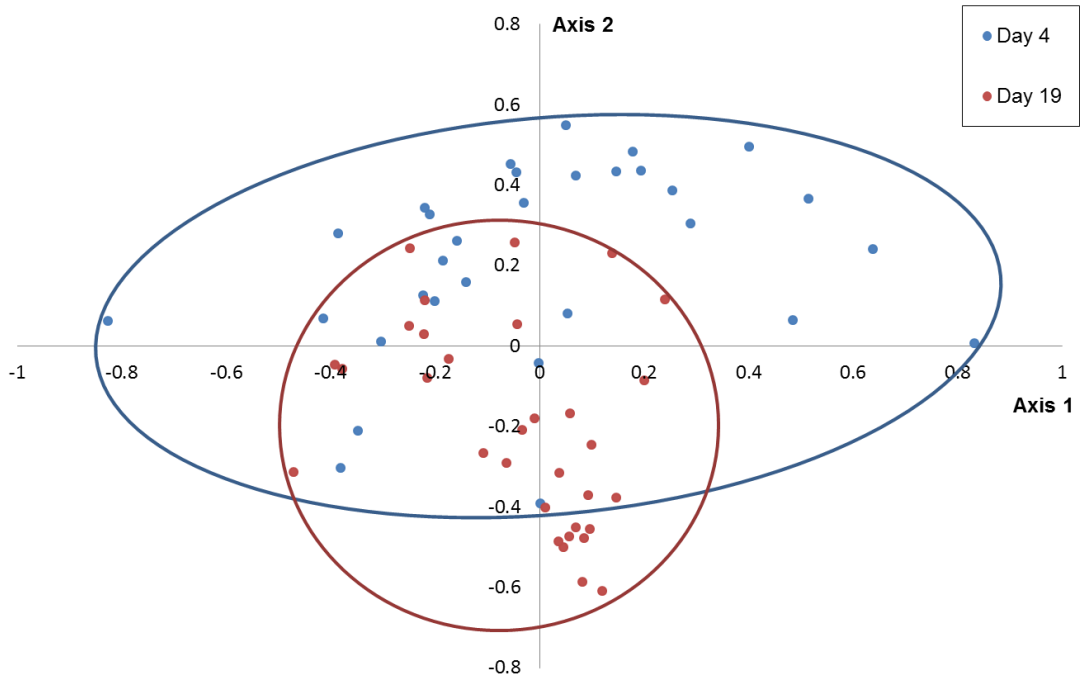


Fig 2. NMDS ordination comparing fecal communities on day 4 (pre-ETEC exposure) and day 19: Both ETEC- and sham-exposed pigs are included to visualize shifts in overall microbiota structure by comparing baseline samples (day 4) with the final samples (day 19). Shifts in community structure (AMOVA: $P < 0.05$) and a decrease in genetic diversity (HOMOVA: $P < 0.05$) were evident over time.



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688 **Fig 3. ETEC quantification in ETEC-exposed pigs by qPCR**: Fecal shedding of ETEC

689 over the trial duration for each ETEC exposed pig pre-exposure (day 4) and post-exposure (n

690 = 15, one pig missed out of analysis due to missing value).

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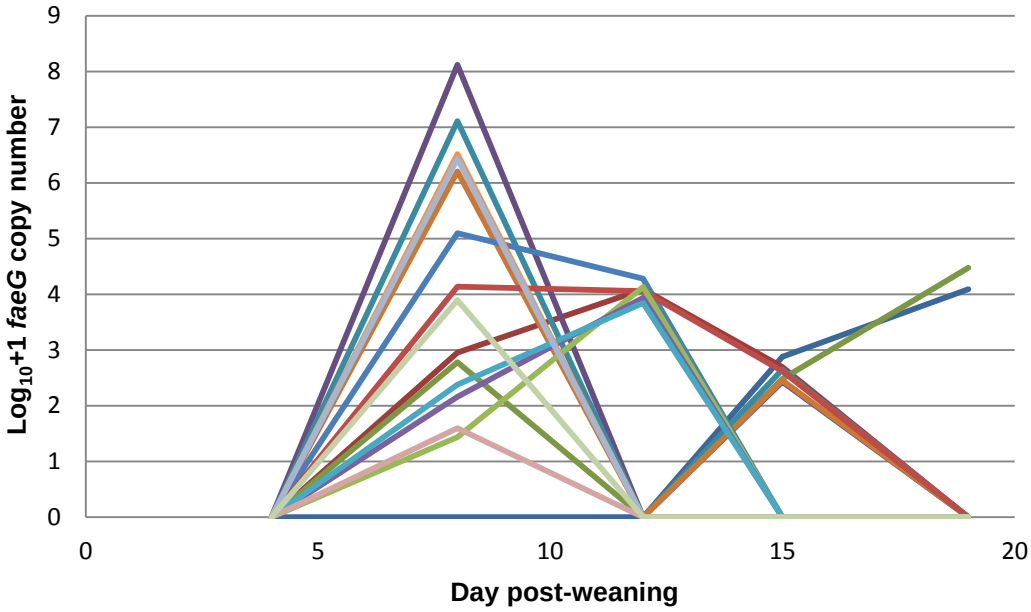

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704 **Fig 4. Variation in ETEC shedding levels:** Cumulative area under the log curve (AULC)
705 representing ETEC shedding level (i.e. low, intermediate and high shedding) by ETEC-
706 exposed pigs (± SD). The cumulative AULC was calculated using log normalized data for
707 each pig at each of the sampling points post-weaning.

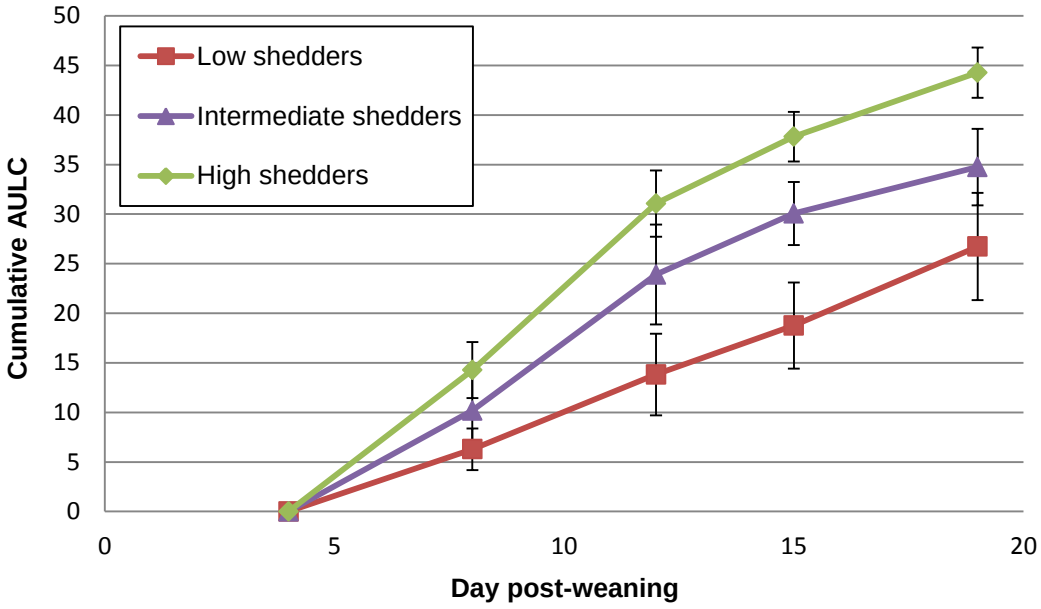
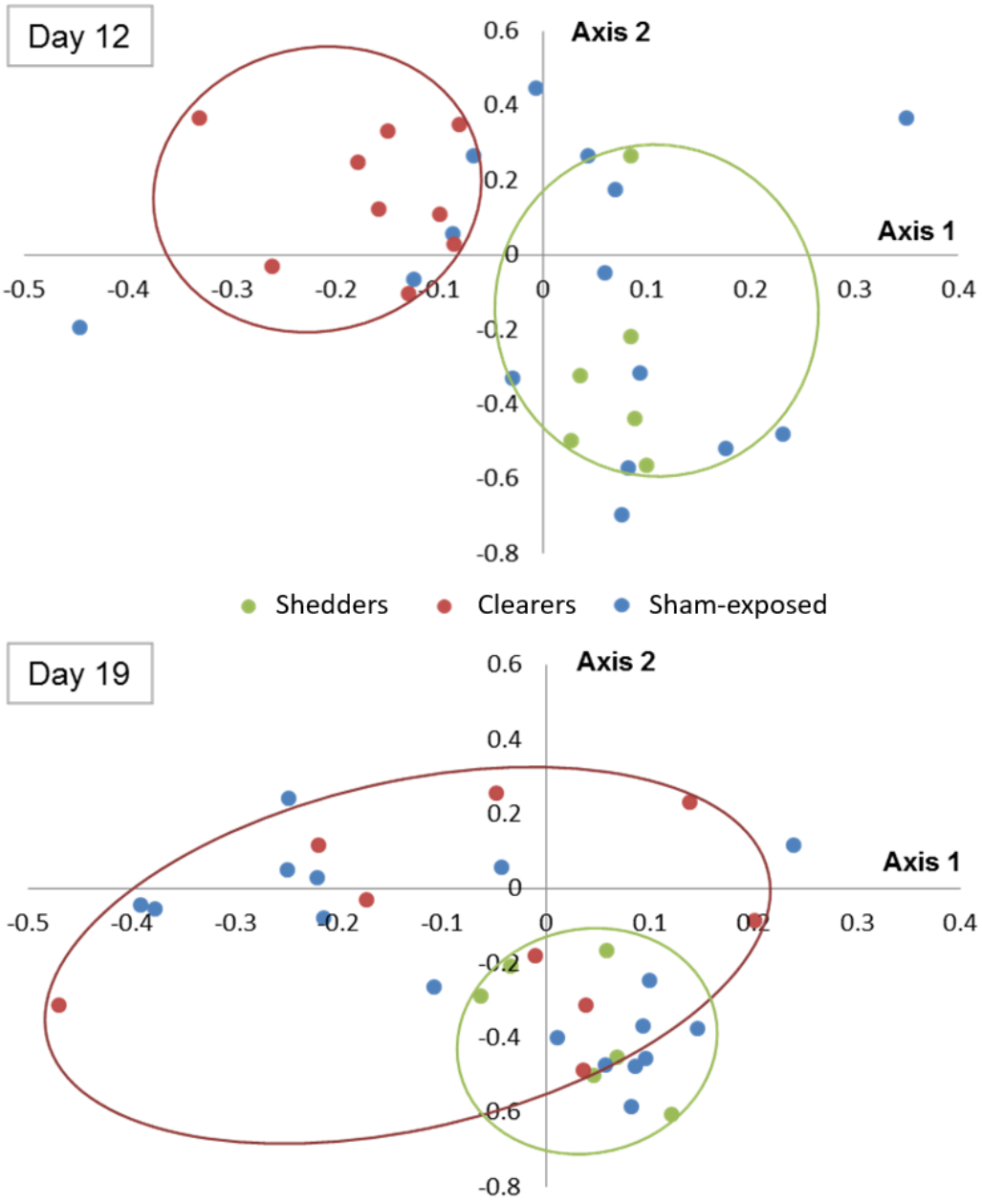


Fig 5. NMDS ordination of comparing fecal communities on days 12 and 19 by shedding status: Community structures were significantly different on days 12 (AMOVA: $P = 0.029$) and 19 (AMOVA: $P = 0.037$) based on shedding dynamics in day 12, where two distinct ETEC "clearer" and "shedder" groups were observed. On day 19, the pigs shedding no

724 detectable levels of ETEC had more variable bacterial communities in comparison with the
725 pigs shedding ETEC in the feces (HOMOVA: $P = 0.045$).



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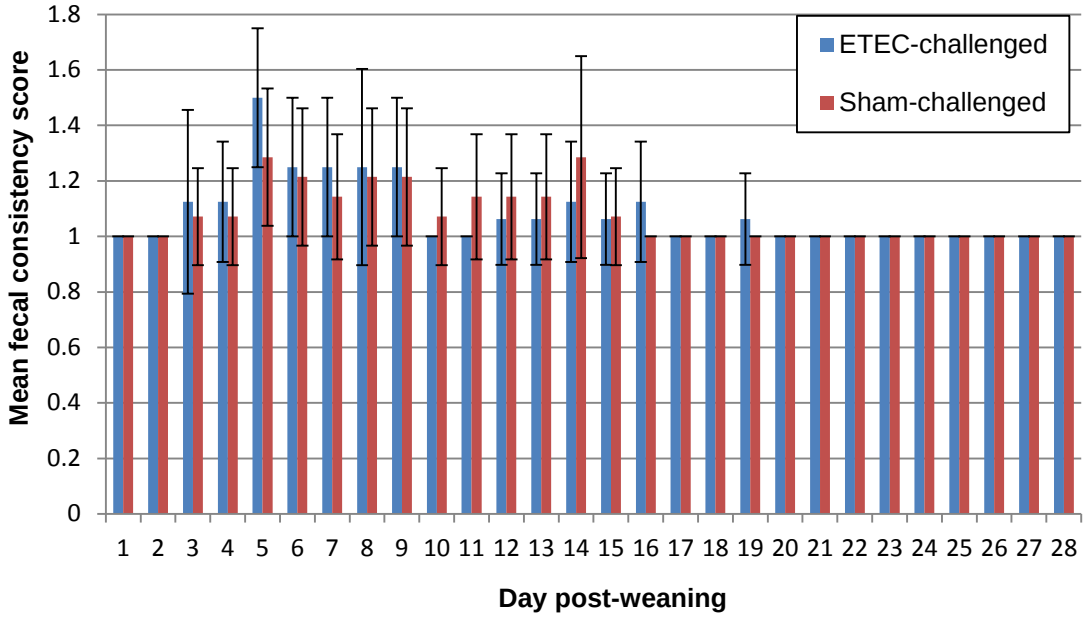
727 **Fig 6. Mean fecal consistency scores:** Bar graph showing the mean fecal consistency scores
728 (±SD) in both ETEC- and sham-exposed pens from day 1 to day 28 post-weaning, with no
729 significant effects of sub-clinical ETEC exposure being observed ($P > 0.05$).


ETEC-challenged
Sham-challenged
Mean fecal consistency score
Day post-weaning
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28

TABLES

Table 1. Measured and expected relative abundances of mock community strains. 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.

Mock community strain(s)	Level of identification	Relative abundance (%)	
		Measured	Expected
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 fecalis	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

Table 2. Mean richness (Chao 1) and diversity (Inverse Simpson's Index (ISI)) metrics. Alpha diversity of communities associated with ETEC-exposed (ETEC) and sham-exposed (Sham) pigs with temporal changes being assessed by repeated measures 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	
Exposure	0.242		0.918	
Time x Exposure	0.050		0.415	

768 **Table 3. Mean changes in relative abundances (± SEM) of dominant OTUs between day 4 and day 19.** Changes were tested using Metastats[1] in both

769 ETEC- and sham-exposed pigs. The OTU 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**
Fecalibacterium 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**

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771 [1] Statistically significant shifts in phylotype relative abundances are highlighted in bold (FDR corrected *P*-value: $P < 0.05$).

772 **Table 4.** Mean body weights for all ETEC- (ETEC) and sham- (Sham) exposed pigs included in this
773 study (all) and for pigs selected for 16S rRNA gene metabarcoding only (16S).

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	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

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APPENDICES

Appendix 1. Information that was considered upon selection of particular pigs for recruitment to the 16S rRNA gene metabarcoding study.

Pig ID	Sequencing ID	Litter ID	Sex	Treatment	Pen ID	Round	Weaning weight (kg)
2780	1	BF280	Female	Infect	Pen 1	Round 1	7.48
2756	2	BF219	Male	Infect	Pen 2	Round 1	10.24
2768	3	BF219	Female	Infect	Pen 3	Round 1	8.90
2782	4	BF208	Male	Infect	Pen 4	Round 1	8.36
843	5	36	Female	Infect	Pen 5	Round 2	6.40
795	6	1693	Female	Infect	Pen 6	Round 2	6.90
847	7	36	Male	Infect	Pen 7	Round 2	9.20
763	8	816	Male	Infect	Pen 8	Round 2	7.80
2749	9	BF212	Female	Infect	Pen 1	Round 1	9.14
2722	10	BF205	Male	Infect	Pen 2	Round 1	8.30
2779	11	BF208	Male	Infect	Pen 3	Round 1	6.66
2740	12	BF297	Male	Infect	Pen 4	Round 1	7.14
759	13	137	Female	Infect	Pen 5	Round 2	8.80
861	14	125	Female	Infect	Pen 6	Round 2	10.30
860	15	125	Female	Infect	Pen 7	Round 2	11.20
785	16	803	Male	Infect	Pen 8	Round 2	10.60
2777	17	BF208	Female	Sham	Pen 9	Round 1	4.90
2774	18	BF210	Male	Sham	Pen 10	Round 1	4.80
2750	19	BF212	Female	Sham	Pen 11	Round 1	11.00
2718	20	BF205	Male	Sham	Pen 9	Round 1	9.36
800	21	1693	Male	Sham	Pen 12	Round 2	8.20
827	22	454	Female	Sham	Pen 13	Round 2	6.40
789	23	803	Female	Sham	Pen 14	Round 2	7.50
768	24	816	Male	Sham	Pen 15	Round 2	8.00
2735	25	BF299	Male	Sham	Pen 9	Round 1	8.42
2766	26	BF219	Female	Sham	Pen 10	Round 1	10.52
2741	27	BF297	Male	Sham	Pen 11	Round 1	11.00
2752	28	BF212	Female	Sham	Pen 9	Round 1	10.22
820	29	126	Female	Sham	Pen 12	Round 2	9.90
837	30	459	Male	Sham	Pen 13	Round 2	10.10
838	31	459	Male	Sham	Pen 14	Round 2	9.70
863	32	125	Male	Sham	Pen 15	Round 2	11.70

798 **Appendix 2.** Primers including Illumina adapters and unique barcodes for sequencing.

Primer	Sequence (5'-3')
341-F1	AATGATACGGCGACCACCGAGATCTACACTATAGCCTACACTCTTTCCCTACACGACGCTCT TCCGATCTNNNNCCTACGGGAGGCAGCAG
341-F2	AATGATACGGCGACCACCGAGATCTACACATAGAGGCACACTCTTTCCCTACACGACGCTC TTCCGATCTNNNNCCTACGGGAGGCAGCAG
341-F3	AATGATACGGCGACCACCGAGATCTACACCCTATCCTACACTCTTTCCCTACACGACGCTCT TCCGATCTNNNNCCTACGGGAGGCAGCAG
341-F4	AATGATACGGCGACCACCGAGATCTACACGGCTCTGAACACTCTTTCCCTACACGACGCTCT TCCGATCTNNNNCCTACGGGAGGCAGCAG
341-F5	AATGATACGGCGACCACCGAGATCTACACAGGCGAAGACACTCTTTCCCTACACGACGCTC TTCCGATCTNNNNCCTACGGGAGGCAGCAG
341-F6	AATGATACGGCGACCACCGAGATCTACACTAATCTTAACACTCTTTCCCTACACGACGCTCT TCCGATCTNNNNCCTACGGGAGGCAGCAG
341-F7	AATGATACGGCGACCACCGAGATCTACACCAGGACGTACACTCTTTCCCTACACGACGCTC TTCCGATCTNNNNCCTACGGGAGGCAGCAG
341-F8	AATGATACGGCGACCACCGAGATCTACACGTACTGACACACTCTTTCCCTACACGACGCTCT TCCGATCTNNNNCCTACGGGAGGCAGCAG
518-R1	CAAGCAGAAGACGGCATACGAGATCGAGTAATGTGACTGGAGTTCAGACGTGTGCTCTTCC GATCTNNNNATTACCGCGGCTGCTGG
518-R2	CAAGCAGAAGACGGCATACGAGATTCTCCGGAGTGACTGGAGTTCAGACGTGTGCTCTTCC GATCTNNNNATTACCGCGGCTGCTGG
518-R3	CAAGCAGAAGACGGCATACGAGATAATGAGCGGTGACTGGAGTTCAGACGTGTGCTCTTCC GATCTNNNNATTACCGCGGCTGCTGG
518-R4	CAAGCAGAAGACGGCATACGAGATGGAATCTCGTGACTGGAGTTCAGACGTGTGCTCTTCC GATCTNNNNATTACCGCGGCTGCTGG
518-R5	CAAGCAGAAGACGGCATACGAGATTTCTGAATGTGACTGGAGTTCAGACGTGTGCTCTTCC GATCTNNNNATTACCGCGGCTGCTGG
518-R6	CAAGCAGAAGACGGCATACGAGATACGAATTCGTGACTGGAGTTCAGACGTGTGCTCTTCC GATCTNNNNATTACCGCGGCTGCTGG
518-R7	CAAGCAGAAGACGGCATACGAGATAGCTTCAGGTGACTGGAGTTCAGACGTGTGCTCTTCC GATCTNNNNATTACCGCGGCTGCTGG
518-R8	CAAGCAGAAGACGGCATACGAGATGCGCATTAGTGACTGGAGTTCAGACGTGTGCTCTTCC GATCTNNNNATTACCGCGGCTGCTGG
518-R9	CAAGCAGAAGACGGCATACGAGATCATAGCCGGTGACTGGAGTTCAGACGTGTGCTCTTCC GATCTNNNNATTACCGCGGCTGCTGG
518-R10	CAAGCAGAAGACGGCATACGAGATTTCGCGGAGTGACTGGAGTTCAGACGTGTGCTCTTCC GATCTNNNNATTACCGCGGCTGCTGG

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