Development and validation of assays to measure gut health in order to identify risk factors for E.coli disease in weaner pigs

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Executive Summary

Scouring caused by E.coli and other bacteria of the Enterobacteriaceae family are becoming more difficult to control in weaner pigs as many of these pathogens are resistant to commonly used antibiotics. Commensal bacteria in the intestine play an important role in preventing the colonisation of pathogens through competitive exclusion and excretion of bacteriocins capable of bacterial lysis. However, the use of broad spectrum antibiotics can alter the commensal and protective microflora, allowing growth of resistant pathogens and potentially exacerbating disease. The aim of this project was to develop qPCR assays to measure bacterial numbers in faeces and to investigate whether certain bacterial groups or ratios of bacterial groups provide an indicator of good intestinal health. The development of assays that could reliably measure intestinal health would assist producers in the monitoring of overall herd health and identify risk factors associated with scouring. Monitoring bacterial communities associated with healthy intestines could also be used to develop post-weaning management strategies which rationalize the use of antibiotics to control disease and evaluate alternative treatments such as probiotics, prebiotics and feeding strategies.

Faeces were submitted from both healthy and scouring sucker and weaner pigs from seven different farms (n = 121), along with information about antibiotic usage. Lactobacilli, total *E.coli,* F4 pathogenic *E.coli*, *Clostridium perfringens*, *Enterobacteriaceae* and total bacterial numbers were determined by quantitative PCRs (qPCR) and by culture. The water content of faeces from freeze-dried samples was used as an objective measure of scouring. Paired faecal and mucosal samples were collected from twelve pigs to investigate correlations between the bacterial populations (microflora) found in faeces and in the small intestine. The relative abundance and diversity of bacterial populations was also examined by next generation sequencing of bacteria found in faecal samples from both scouring and healthy weaner pigs.

The numbers of commensal and pathogenic bacteria detected in faeces correlated well between traditional culture methods and the new qPCR assays developed. The mean faecal water content was significantly higher in pigs identified as 'scouring' relative to 'healthy' pigs. However, increased water content in faeces did not correlate with increased pathogen numbers across all 121 samples, suggesting that water content is affected by other factors such as season, age of pigs, diet, environment, medication and pathogens. Expressing bacterial numbers as a proportion of the total bacterial population did not improve correlations. Neither did adjusting bacterial numbers on a dry weight basis. Although the relative abundance of bacterial groups differed between faeces and mucosal samples, the next generation sequencing from faeces has provided strong evidence of bacterial groups that are important in bacterial health, making bacterial profiling practical from live pigs. The sequencing studies also showed an increased abundance of commensals from *Lactobacillales* (*Weissella* spp., *Leuconostoc* spp., and *Streptococcus* spp) in scouring pigs. Increased pathogen numbers from the *Enterobacteriaceae* family and other genera (*Campylobacter spp., Clostridium spp.*) were observed in scouring pigs.

It was expected that measuring ratios of commensal to pathogenic bacteria would provide a good indicator of gut health as it overcame problems with differences in water content of faeces and the potential for overestimation of bacterial numbers by qPCR. Scouring pigs had reduced ratios of commensals to pathogens compared with healthy pigs in two herds. However, in the analysis of all herds, the numbers of

Lactobacilli increased relative to pathogenic and total *E.coli* as faecal water content increased.

Next generation microbial sequencing of the same faecal samples demonstrated an increased abundance of Lactobacillales and *E.coli* in scouring pigs. Previous studies had suggested that genera of the Lactobacillales (*Lactobacillus spp. Weissella spp., Leuconostoc* spp., and *Streptococcus* spp.) become more abundant in weaner pigs fed zinc oxide and in an antagonistic response to infection with *Enterobacteriaceae* such as *E.coli*. The next generation microbial sequencing also identified other commensals (*Lachnospiraceae, Ruminococcaceae, Veillonellaceae* and *Prevotellaceae*) that were less abundant in scouring pigs. Reduced abundance of these bacterial families could exacerbate disease in scouring pigs because the gut microflora would be less stable, have less energy for microbial fermentation and increased inflammation in response to *E.coli* infection. Reduced abundance of *Ruminococcaceae* and *Prevotella* spp. has previously been associated with increased excretion of *Salmonella typhimurium* in pigs.

The impact of antibiotics on the intestinal microflora was interesting, with decreased numbers of Lactobacilli and decreased total bacteria numbers in medicated pigs, along with increased numbers of F4 pathogenic *E.coli* and total *E.coli*. The development of antibiotic resistance by pathogens such as *E.coli* has been recognized for a number of decades; however, the effect of these same antibiotics on the commensal bacterial population has only been reported more recently. The reduced abundance of lactate producing bacterial families (*Streptococcaceae, Bifidobacteriaceae* and *Ruminococcaceae*) was also demonstrated with next generation microbial sequencing. *Bifidobacterium* spp. are known to decrease coliform shedding by acidifying the gut and excreting natural antibiotics against *E.coli* and *Clostridium* spp., so a loss in abundance could exacerbate disease. However, antibiotic use also increased the abundance of beneficial families such as *Lachnospiraceae*, which can confer resistance to scouring by producing bacteriocins capable of killing pathogens. A number of different types of antibiotics were used on these farms, so it is clear that further studies need to investigate the impact of specific antibiotics on both commensals and pathogens in the gut. In the past, antibiotics were only chosen on their activity against specific pathogens, but it now seems wise to also select antibiotics/antimicrobials on their inactivity against important protective commensal bacteria.

The next generation microbial sequencing studies have highlighted the importance of other groups of commensal bacteria, such as butyrate and lactate producing bacteria that may influence gut health and suppression of disease. Future studies in weaners are needed to confirm these results, along with the impact of antibiotics on commensal bacteria. Once the most important groups of commensal bacteria have been identified, it will be relatively easy to design qPCR assays to measure the "protective load" of bacteria in weaner pigs. Measures of protective bacteria can be used to evaluate the impact of antibiotics, vaccination, improved hygiene, other pathogens, prebiotics and probiotics on gut health. The development of qPCR assays to measure the intestinal health of pigs would assist producers in the monitoring of overall herd health and identification and control of risk factors associated with scouring. These outcomes would assist in the reduction of production losses associated with enteric disease.

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1. Introduction

Maintenance of intestinal health and prevention of outbreaks of enteric disease are dependent on the bacteria present in the pig's intestine, but also on interactions between these bacteria and the cells lining the intestine, along with the pig's immune system and nutrients provided in the diet (Yu et al. 2012). Intestinal health is optimal when these various factors complement each other in balance. Alterations in this balance have the potential to result in enteric diseases in the host.

Major changes occur in all of these systems in the first eight weeks of the pig's life, which coincides with significant disease challenges to the piglet both before and after weaning. Issues with intestinal health in pigs, such as severe diarrhoea or scouring, generally occur during the first two weeks of life or in the two weeks following weaning (Wada et al. 1996). Scouring leads to dehydration, toxaemia and sudden death in piglets, with the resulting mortality and reduction in growth rate responsible for significant financial losses within the pig industry.

Commensal bacteria, such as Lactobacilli, are common residents of the intestinal microflora and benefit the host by supporting normal development of the intestine, digestion, overall health and by preventing the colonisation of pathogenic bacteria (Brestoff & Artis 2013). Both commensal and pathogenic bacteria that colonise the piglet's intestine are initially transferred from the sow and the environment shortly after birth. Lactobacilli colonise the piglet's intestine early and help catabolise lactose in milk and prevent establishment of pathogenic bacteria. Commensal *E.coli* (non-pathogens) also colonise the piglet intestine within 24 hours of birth. Once the mix of bacteria in the intestine (microflora) becomes established, the intestine is relatively resistant to significant changes in bacterial populations, unless compromised by dietary changes (including weaning), pathogen challenge or antibiotics.

In contrast to virulence-specific diagnostics, this project aimed to use ratios of both beneficial (commensal) and pathogenic bacterial groups to measure 'intestinal health'. Changes in the ratios of these bacterial groups may indicate damage or disruption to the gut, scouring and potential production losses. The industry partner, Nutreco, have used conventional culture techniques to quantify ratios of beneficial and pathogenic bacteria in faecal samples. A shift in the proportion of commensal to pathogenic bacteria would indicate an imbalance in intestinal health. For example, if scouring is due to gram-negative bacteria such as pathogenic *E.coli*, the number of beneficial *Lactobacillus* species would decrease relative to the number of *E.coli*, producing a relatively low Lactobacilli to *E.coli* ratio. Disruptions to gut health may also be measured using the ratio of *Enterobacteriaceae* to *E.coli*. If the *Enterobacteriaceae* to *E.coli* ratio is low, it means that most of the *Enterobacteriaceae* are *E.coli*, suggesting an overgrowth of *E.coli* and imbalance in the microflora. If scouring is due to gram-positive bacteria such as *Clostridium* species, the ratio of Lactobacilli to Clostridia may provide a measure of intestinal damage or disruption. The proportions of these bacterial groups relative to total bacterial numbers may also provide a measure of species dominance within the gut microflora. If the proportion of *E.coli* relative to total bacteria was high, this would suggest an overgrowth of *E.coli* within the gut.

There are a number of advantages to focusing on broad groups of bacteria rather than each bacterial species. Kim et al. (2011) found that the proportions of different bacterial groups are relatively stable after colonisation, compared to the high variability in specific species within the gastrointestinal tract. Additionally, assays focusing on quantifying broad bacterial groups are simpler, less expensive and less time-intensive, compared to identifying and measuring bacterial numbers at the species level. This project will focus on four groups of bacteria that are important in pre- and post weaning health and disease of pigs: Lactobacilli, *Enterobacteriaceae*, *Escherichia coli* (including the F4 virulence factor) and *Clostridium perfringens*. As an extension to this project, numbers of total bacteria and pathogenic fimbrial type 4 (F4) *E.coli* were also quantified.

E.coli are important pathogens of weaned pigs and the overgrowth of enterotoxigenic (ETEC) strains of *E.coli* are a major cause of post weaning diarrhoea and oedema disease. Strains involved in post weaning diarrhoea mostly express F4 or F18 fimbriae, which are important virulence factors in bacterial colonisation. Selective breeding of pigs without the F18 fimbriae receptor can aid in the control of post weaning diarrhoea. However, control of pathogenic *E.coli* requires a combination of vaccination, hygiene, genetics and diagnostics to identify the serogroups and virulence genes causing disease. Not all *E.coli* found in the pig's gut are harmful; some also protect the intestine from pathogens or are antagonistic to pathogens. *E.coli* are a subset of the much larger *Enterobacteriaceae* bacterial family, which also includes the genera *Salmonella, Enterobacter, Shigella, Yersinia* and *Klebsiella*. A large subset of *Enterobacteriaceae* are commensal intestinal flora, however *Salmonella* species and some strains of *E.coli* are pathogenic and result in enteric diseases.

Another common pathogen of pre-weaned pigs is *Cl.perfringens*, which produces an enterotoxin responsible for pre-weaning diarrhoea. *Cl.perfringens* is present in both healthy and scouring pigs, but can cause disease when they replicate to large numbers. The organism is transferred by direct contact between infected piglets and the sow, but bacterial spores can also persist in soil and the environment. Scouring caused by *Cl.perfringens* is most common in 3-day-old piglets, but can affect piglets from 12 hours to 7 days old. Routine control of clostridial infections is by vaccination of the sows.

Nutreco have used these bacterial ratios to evaluate the efficacy of feed additives to improve productivity in weaned pigs on commercial farms in the Netherlands and other European pig-producing countries. This project will implement Nutreco's methods to objectively measure gut health in Australian pigs, however we will also develop new quantitative PCR (qPCR) assays to measure similar bacterial ratios. The qPCR assays are faster and less expensive to run (reduced labour costs) relative to the traditional culture assays. The qPCR assays will also overcome problems with survival of fastidious bacteria in faeces during transport from remote farms, as the qPCR can use frozen samples, unlike the culture techniques. However, due to Nutreco's experience in measuring gut health, we will use their techniques as the gold standard to evaluate new quantitative PCR assays.

This project aims to develop and validate the use of qPCR in the determination of ratios of commensal to pathogenic bacteria in pig faeces,

specifically including Lactobacilli, *Enterobacteriaceae*, total *E.coli*, F4 fimbriae *E.coli*, *Cl.perfringens* and total bacteria. The ratios to be investigated include Lactobacilli to *E.coli*, Lactobacilli to *Cl.perfringens*, *Enterobacteriaceae* to *E.coli* and the relative proportion of these bacterial groups to total bacterial numbers. The relationship between bacterial ratios and pig intestinal health will be investigated by correlating bacterial ratios to the faecal water content of scouring and healthy pigs. The project will also examine whether bacterial populations found in faeces are similar to those in the small intestine. The last part of the project will investigate the relative abundance and diversity of bacteria within the intestinal population of both scouring and healthy pigs to identify bacterial groups that differ in abundance during scouring. These bacteria may prove a useful indicator of gut health in weaner pigs. Developing a reliable indicator of intestinal health will enable further evaluation of dietary interventions and risks associated with scouring, all of which can potentially improve both economic viability and animal welfare within the Australian pig industry.

2. Methodology

2.1. Enumerate bacteria from reference bacterial cultures and faeces using standard bacterial culture techniques

Enumeration of bacteria was perfomed on pure cultures grown in liquid media. Bacterial type cultures of *E.coli*, *Salmonella typhimurium*, *Lactobacillus acidophilus, Clostridium perfringens, Clostridium difficile*, and *Shigella* species were grown from frozen stocks and streaked for single colonies on agar before sub-culturing in liquid media. The identity of each culture was confirmed by biochemical assays, latex agglutination or haemolysis patterns on blood agar plates. Following overnight growth, 10 ml of each broth culture was pelleted and washed in PBS before the final pellet was resuspended in one-tenth volume of PBS and stored at –20 °C for DNA extraction.

The numbers of *E.coli*, *S. typhimurium*, *L. acidophilus* and *Cl. perfringens* per mL of culture media were enumerated using the International Standard Operating procedures ISO 16649-2, ISO 21528-2, ISO 15214 and ISO 7937 respectively. Briefly, bacterial cultures were diluted in peptone water from 10^{-1} to 10^{-8}, and then 1mL of each dilution was pour plated in duplicate with the appropriate agar. The number of bacterial colonies was counted on duplicate plates at 3 different dilutions, and the mean number of bacteria per mL of each culture was determined.

2.2. Develop quantitative PCR assays for specific bacterial groups

2.2.1. DNA extraction procedures

A number of DNA extraction methods were evaluated to determine which method produced the best yield of DNA from each of our representative bacterial species. Alterations to the mechanical and enzymatic digestion of bacterial cell walls were included to the methods of the Qiagen DNeasy Blood and Tissue Kit to ensure optimal DNA yield from both Gram positive and Gram negative bacteria. Bacterial pellets were either pre-digested with 1mg/mL or 15mg/mL lysozyme, and then cell walls were either mechanically disrupted

with a bead beater and zirconia beads or left without bead beating. DNA concentrations and purity were compared between treatments using a spectrophotometer at OD_{260}, OD_{280} and OD_{230}.

In addition, the efficacy of two additional DNA extraction techniques was tested with pig faeces seeded with 4 x 10^7 *L. acidophilus*, 5 x 10^6 *Cl. perfringens*, 6 x 10^7 *E.coli* or 9 x10^8 *S. typhimurium*, in addition to the intestinal microflora present in this healthy pig. These samples were compared with unseeded faeces and faeces seeded with all four bacteria as above. The two commercial DNA extraction kits trialled were the MagMax 96 Viral RNA isolation kit and the Qiagen DNA stool kit. Both DNA extractions were performed according to the manufacturer's protocol except that the addition of 15mg/mL of lysozyme was added in this study. The presence of significant differences between DNA extraction kits and the inclusion of lysozyme were assessed using the Student's t-test for paired samples.

2.2.2. Design of primers and probes for quantitative PCR assays

Primers and TaqMan probes were selected from published literature or designed to target the 16S rRNA gene or the 16S-23S rRNA intergenic spacer region of Lactobacilli*, Cl. perfringens, E.coli*, *Enterobacteriaceae* and total bacteria. Primer and probe sequences were compared with the target DNA sequences from reference 16S rRNA genes and 16S-23S rRNA intergenic spacer regions submitted to the National Center for Biotechnology Information (NCBI) (Bethesda, USA). Sequences were aligned using Clustal Omega (European Bioinformatics Institute, Saffron Walden, UK) and Sequencher (Gene Codes Corporation, New York, USA). Proposed primer and probe sequences were compared to known sequences to test for specificity using NCBI BLASTN 2.2.28+ (Zhang et al. 2000). Sybr green was used to quantify amplicons from the F4 *E.coli* virulence gene qPCR using the primers developed by Franklin et al. (1996).

The *Cl.perfringens* qPCR used the primers and probes designed by Wise and Siragusa (2005). The *E.coli* and *Enterobacteriaceae* qPCRs both used the forward and reverse primer designed by Bartosch et al. (2004), which targeted the *Enterobacteriaceae* family (including *E.coli*). Two TaqMan hydrolysis probes were specifically designed to target *E.coli* and *Enterobacteriaceae* by Dr Yizhou Chen (Elizabeth Macarthur Agricultural Institute). The total bacteria qPCR used the primers and probes developed by Suzuki et al. (2000) with a modification to the forward primer.

Sequence variation between *Lactobacillus* species in the 16S-23S intergenic regions made it difficult to use published primer and probe sequences to amplify all *Lactobacillus* species expected to be found in pigs. Specific amplification was achieved after testing four different primer and probe sets (Haarman and Knol, 2006; Walter et al., 2001; Heilig et al., 2002; Delroisse et al., 2008).

2.2.3. PCR assay conditions

The initial cycling conditions for each PCR reaction were determined on the Eppendorf Mastercycler Gradient PCR machine where the annealing temperature could be varied between 51 °C to 71 °C. Once optimal conditions were established, the assays were transferred to an Applied Biosystems 7500 real time PCR machine.

The final reaction mixture for each real time PCR reaction was 1 x AgPath-ID RT-PCR buffer (Applied Biosystems, Foster City, USA), 1 U AgPath-ID RT-PCR enzyme mix containing Taq polymerase (Applied Biosystems, Foster City, USA), 5 pmol each primer, 1 pmol probe, 5 µl DNA and sterile water up to 25 µl total reaction volume. DNA from each reference bacterial species was serially diluted to 10^{-7} to produce standard curves. The optimal dilution of DNA (extracted from faeces) to fit within each bacterial standard curve was determined by testing undiluted DNA against 1:10 and 1:100 dilutions in each qPCR assay. Consequently undiluted faecal DNA was used for the quantification of *Cl. perfringens*, 1:10 dilutions of faecal DNA were used for the enumeration of Lactobacilli, *E.coli* and *Enterobacteriaceae*; and 1:100 dilutions were used for enumeration of total bacteria. All standards were assayed in triplicate and all faecal samples were assayed in triplicate (except for in the F4 *E.coli* PCR). A liquid handling robot was used to aliquot all DNA and pre-mixed PCR reagents to reduce operator errors associated with pipetting. Inter-run variation for the qPCR assays did not need to be calculated as each qPCR assay was run in a 384-well plate in a single qPCR reaction with all 121 samples. The cycling conditions for the *Cl. perfringens, E.coli, Enterobacteriaceae* and Lactobacilli qPCRs were an initial 95 °C for 10 mins followed by 40 cycles of 95 °C for 15 s and 63 °C for 45 s. The cycling conditions for the total bacteria qPCR were an initial 95 °C for 10 mins followed by 40 cycles of 95 °C for 15 s and 58 °C for 45 s. The F4 *E.coli* qPCR was performed on a Qiagen Rotor Gene thermal cycler, with 40 cycles of 95°C for 10s, 55°C for 30s and 72°C for 45s.

The standard curve and qPCR results were accepted if the linear regression had an $R^2 > 0.98$ and efficiency between 90 % and 110 %. Variation within each run was calculated as the coefficient of variation. The percentage of non-specific amplicons relative to specific amplicons was calculated according to the formula developed by Hargreaves et al. (2013). The identity of the PCR products obtained from the *Cl.perfringens, L.acidophilus, E.coli* and *S.typhimurium* reference bacteria were confirmed by sequencing (Australian Genome Research Facility) and by comparing the obtained sequence to reference sequences in the National Centre for Biotechnology Information (NCBI).

2.3. Comparison of bacterial ratios with bacterial culture and quantitative PCR

Once the bacterial culture conditions were established, 14 faecal samples were collected from sucker and weaner piglets for bacterial culture enumeration and qPCR quantification of *E.coli, Enterobacteriaceae, Clostridium perfringens* and Lactobacilli. No antibiotics were administered to the pigs from which the faecal samples were collected. For each faecal sample, a 10^{-1} faecal dilution was prepared by mixing 1.0 g faeces with 9 ml peptone water, and this was then serially diluted 1/10 in peptone water. Dilutions of 10^{-1} to 10^{-6} were plated in duplicate for the enumeration of *Cl.perfringens*, whereas dilutions of 10^{-3} to 10^{-8} were used to enumerate Lactobacilli, *E.coli* and *Enterobacteriaceae* according to international standards outline in section 2.1. Bacterial numbers were also quantified with the newly developed qPCR assays according to the methods outlined in section 2.2.

2.4. Determine bacterial ratios in herds/pigs with and without E.coli disease.

2.4.1. Collection of unknown porcine faecal samples

A total of 121 faecal samples were collected from seven Australian commercial piggeries from New South Wales, Victoria, South Australia and Western Australia. Veterinarians or farmers were asked to collect faecal samples from 5 healthy and 5 scouring piglets aged either 7 to 10 days old (suckers) or at 2 to 3 weeks post-weaning (weaners). Faecal samples were sent via overnight courier on ice. Information was provided concerning the age of the piglet, the presence of antibiotic usage (yes/no) and the health status of the pig (healthy/scouring).

In total 36 % of the 121 faecal samples were collected from suckers and 64 % from weaners. The health status of the pig was determined by the collector to be 'healthy' in 66 % of pigs and 'scouring' in 34 % of pigs. No antibiotic use was associated with 66 % of samples while antibiotics were routinely used in 34 % of pigs from which samples were collected.

2.4.2. Calculation of faecal water content

An objective scalar measure of scouring in piglets was determined by freeze-drying faecal samples to completion over a period of four to seven days. The difference between the initial 'wet' and the final 'dry' weight of the faeces was taken as a measure of the amount of water initially present within the sample.

2.4.3. Statistical analysis

Microsoft Excel, IBM SPSS Statistics and GenStat were used to plot graphs and to perform statistical analysis. The normality of each data set was visually assessed and the bacterial numbers were $\log_{10}$ transformed. Significant differences in faecal water content, the presence of scouring and antibiotic use were determined using the Student's t-test for independent samples. The presence of correlations between data sets was determined using the non-parametric measure of Spearman's rank correlation. The strength and significance of differences or correlations were based on probability (P) values of less than or equal to 0.05.

2.5. Next generation sequencing and analysis of microbial community diversity

The 16S ribosomal RNA genes from all the bacteria in our faecal samples were amplified with Golay barcode and Ion Torrent adapter primers V4/5 (515F and 806 R) as previously described (Jenkins et al., 2010). All PCR products were checked for size and specificity and concentrations were adjusted to 10 ng/µL for subsequent sequencing, which was performed on the Ion Torrent Personal Genome Machine (Life technologies, USA) using 400 base-pair chemistry as described in Whiteley et al. (2012). All data was analysed using the QIIME pipeline (Caporaso et al., 2010) with appropriate quality control of

sequence data, i.e. minimum quality score = 25, minimum/maximum length = 130/400bp, no ambiguous base calls, reverse primers were removed and no mismatches were allowed in the forward and reverse primer sequences. Chimera checking was done using usearch61 and only non-chimeric sequences were considered for assigning operational taxonomic units (OTUs, 97% sequence similarity with UCLUST). Species variation within habitats (Alpha rarefaction) was performed using the phylogenetic diversity and observed species metrics. Diversity between habitats (Beta diversity) was estimated by computing weighted and unweighted UniFrac distances between samples using QIIME. The relationship between environment (different farms), antibiotic use, presence of infection (scouring) and microbial diversity in the gastrointestinal tract of post weaned piglets was assessed using general linear model (GLM) and canonical correspondence analysis (CCA) approaches.

3. Outcomes

3.1. Enumerate bacteria from faeces using standard bacterial culture techniques

The mean numbers of each bacterial species cultured from fresh faeces of nursery and weaner pigs (scouring and non-scouring) from the same piggery are outlined in Figure 1. Numbers of *E.coli* ranged from 2.73×10^5 to 3.06×10^9 cfu / g faeces with the highest numbers of *E.coli* present in the nursery pigs and one of the scouring weaners (#6). The highest numbers of *Enterobacteriaceae* followed the same trend as *E.coli* with numbers ranging between 3.91×10^5 to 1.49×10^9 cfu / g faeces. *E.coli* can be both a pathogen and a normal part of the intestinal microflora (commensal), and the bacterial culture technique does not differentiate between pathogenic and commensal *E.coli*. Higher numbers of Lactobacilli were detected in all samples (5.95×10^8 to 5.60×10^9 cfu / g faeces) except for one nursery pig (#11). Numbers of *Cl. perfringens* ranged between 9.73×10^4 to 2.40×10^7 cfu / g faeces with higher numbers of *Cl.perfringens* in suckers than weaners.

Healthy suckers (7 to 10 days of age) had similar numbers of Lactobacilli and *E.coli* as reported in the literature (Muralidhara et al., 1977). Ratios of commensal to pathogenic bacteria agreed with Nutreco's work in the Netherlands. Healthy weaners had much higher numbers of Lactobacilli relative to *E.coli* (3500:1), but this ratio was reduced ten-fold in scouring weaners (Table 1). Healthy weaners also had higher numbers of Lactobacilli relative to *Cl. perfringens* with ratios greater than 100:1 indicative of average or good health, and ratios greater than 1000:1 indicative of very good health. Unfortunately, scouring suckers could not be found in close proximity to the laboratory, so Lactobacilli to *Cl. perfringens* ratios could not be determined by culture techniques in scouring sucker pigs.

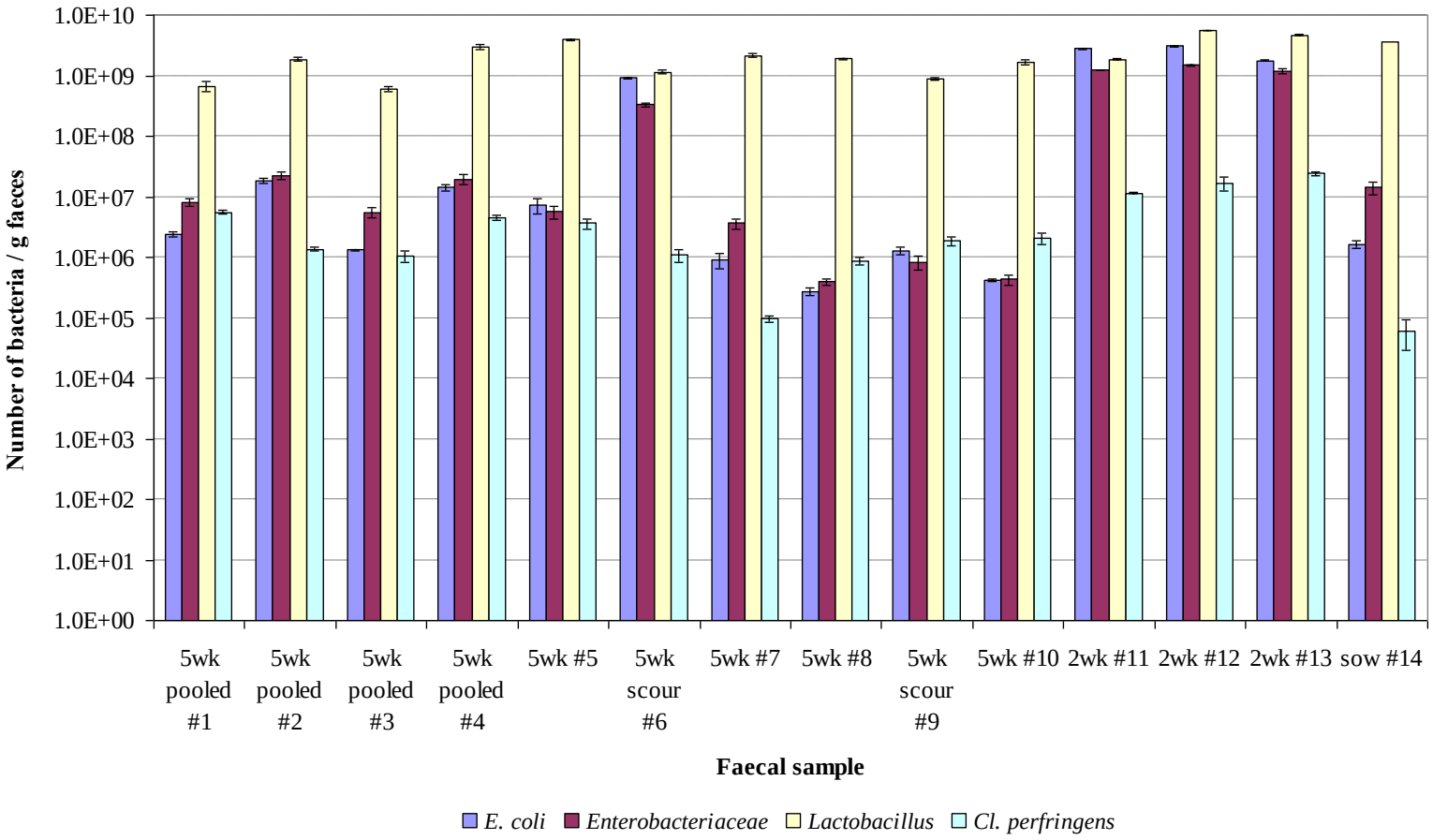

Figure 1 - Number of bacterial colony forming units (cfu) of *E.coli*, *Enterobacteriaceae*, Lactobacilli and *Cl. perfringens* in pig faecal samples as determined by microbial culture enumeration.

Table 1 - Mean ratios of Lactobacilli to *E.coli*, Lactobacilli to Cl.perfringens and E.coli to Enterobacteriaceae in faeces from healthy and scouring weaners and sucker pigs.

Bacterial ratios	Healthy weaners	Scouring weaners	Healthy suckers
Lactobacilli:*E.coli*	3500:1	347:1	1.7:1
Lactobacilli:Cl.perfringens	6500:1	770:1	229:1
E.coli: Enterobacteriaceae	0.6:1	2:1	1.9:1

The *E.coli* to *Enterobacteriaceae* ratios were aimed at determining what proportion of *Enterobacteriaceae* were *E.coli*. In the healthy weaner pigs, 60% of the cultured *Enterobacteriaceae* were *E.coli* (ratio *E.coli* to *Enterobacteriaceae* = 0.6:1). Ratios of greater than 1 indicated that *E.coli* formed a monoculture in the faeces and was probably the cause of scouring or poor health, as was observed in the scouring weaners. However, it is not possible for the ratio of *E.coli* to *Enterobacteriaceae* to be 2:1, because you cannot have more *E.coli* than *Enterobacteriaceae*. It is probable that there were some problems with the optimal culture of *Enterobacteriaceae* in this study. However it is still clear that *E.coli* was a significant problem in the scouring weaners.

3.2. Develop quantitative PCR assays for specific bacterial groups

3.2.1. DNA extraction from bacterial cultures

The addition of extra lysozyme (15 mg/ml compared to 1 mg/ml lysozyme) increased the total DNA yield in all cases except the DNA extraction of *E.coli*. Mechanical disruption through bead beating was trialled in this study to assist in the lysis of the bacterial cell walls and release of DNA. However, a reduction in total DNA yield was observed with bead beating (Figure 2). It is possible that loss of DNA occurred during the increased number of transfer procedures to remove the silica beads. Greater amounts of DNA were extracted from the *E.coli* and *Cl.perfringens* cultures compared to the *L.acidophilus* and *S.typhimurium* cultures. No statistical analysis was performed on these results as only one replicate was conducted.

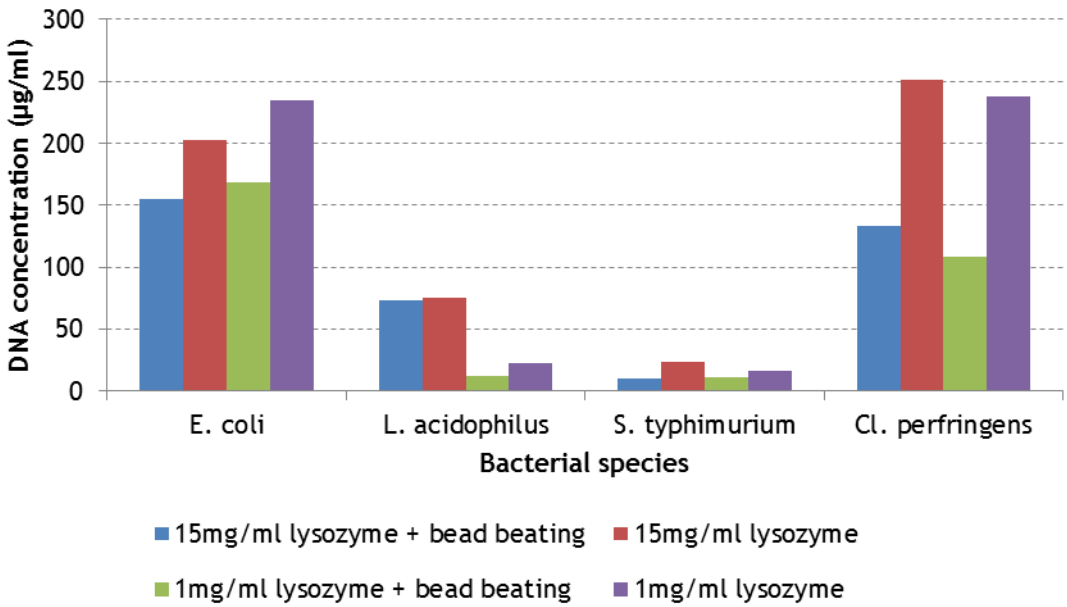

Figure 2 - Total DNA yield from reference bacterial cultures of *E.coli*, *L. acidophilus*, *S. typhimurium* and *Cl. perfringens* extracted with the DNeasy kit with 15 mg/ml or 1 mg/ml lysozyme, and with or without bead beating.

3.2.2. DNA extraction from seeded faeces

The MagMAX Viral Isolation kit resulted in greater total DNA yield from each faecal sample compared to the QIAamp DNA Stool Mini kit ($p < 0.0001$). However, faecal DNA extraction using the QIAamp DNA Stool Mini kit resulted in the quantification of significantly greater numbers of *Cl.perfringens* ($p < 0.001$), *E.coli* ($p = 0.003$) and *Enterobacteriaceae* ($p < 0.001$). The inclusion of additional lysozyme to seeded faecal samples led to the detection of significantly greater numbers of Lactobacilli ($p < 0.001$) and *Cl.perfringens* ($p = 0.007$). However, additional lysozyme had no significant effect on the quantification of *E.coli* ($p = 0.140$) or *Enterobacteriaceae* ($p = 0.125$) within each tested faecal sample.

The increased number of Lactobacilli and *Cl.perfingens* quantified with the inclusion of lysozyme is likely due to the effect of lysozyme on the cell wall structure of gram-positive bacteria. The inclusion of additional lysozyme aids the lysis and release of DNA from gram-positive bacteria which contain increased amounts of peptidoglycan (de Pedro 2009). Nechvatal et al. (2008) also found that the inclusion of lysozyme digestion resulted in no significant difference in the quantification of the gram-negative genus *Bacteriodes* within human faeces using qPCR.

3.2.3. Validation of qPCR assays

The *Cl. perfringens* real-time PCR specifically amplified *Cl. perfringens* DNA at an annealing temperature of 63 °C, and did not amplify DNA from *E.coli*, *Cl. difficile*, *L. acidophilus* or *S. typhimurium*, or in the no template control. The specificity of the *E.coli* real-time PCR was acceptable at an annealing temperature of 63 °C. However, the *E.coli* qPCR amplified small amounts of the *L. acidophilus* and *Cl. perfringens* reference bacteria, amounting to a very small error relative to the number of *E.coli* (Table 2). The *E.coli* qPCR also amplified the reference bacterial species of *Shigella* sp. with similar efficiency to the amplification of *E.coli*. This was expected due to the

minimal genetic difference between *E.coli* and *Shigella* species. The *Enterobacteriaceae* real-time PCR strongly amplified DNA extracted from *S. typhimurium, E.coli* and *Shigella* reference bacteria as expected; however small amounts of *L. acidophilus* and *Cl. perfringens* DNA were also amplified (Table 2). The specificity of the final modified Lactobacilli real-time PCR was acceptable at an annealing temperature of 63 °C. However, DNA from *S.typhimurium, Cl.perfringens* and *E.coli* was also amplified but at a much lower level (Table 2). The total bacteria qPCR amplified *Cl.perfringens, E.coli, L.acidophilus* and *S.typhimurium* as expected at an annealing temperature of 58 °C.

Table 2 - The dynamic range, coefficient of variation (CV) and the error due to non-specific amplification for each qPCR assay

qPCR target	Dynamic range (cfu / reaction)	Coefficient of variation (%)	Error due to non-specific amplification (%)
F4 E.coli	8.00×10^6 – 8.00×10^2	0.16 – 3.01 (intra-run) 1.38 – 6.72 (inter-run)	< 0.00002
Total E.coli	1.06×10^6 – 1.06×10^1	0.15 – 1.22	< 0.00009
Lactobacilli	8.46×10^6 – 8.46×10^1	0.23 – 1.01	< 0.003
Enterobacteriaceae	3.10×10^6 – 3.10×10^1	0.05 – 2.08	< 0.00009
Cl. perfringens	4.07×10^5 – 4.07×10^1	0.12 – 1.30	None
Total bacteria	1.06×10^7 – 1.06×10^2	0.05 – 2.05	< 0.0001

3.3. Comparison of bacterial ratios with bacterial culture and quantitative PCR

Strong and statistically significant correlations were observed between microbial culture techniques and qPCR for the quantification of *E.coli* (R^2 = 0.863, $p < 0.001$) and *Enterobacteriaceae* (R^2 = 0.912, $p < 0.001$). *Cl.perfringens* numbers were below the qPCR limit of detection in samples enumerated through culture, so no correlation between the two methods could be made. The correlation between the quantification of Lactobacilli using microbial culture and qPCR was close to significant (R^2 = 0.495, p = 0.072).

The number of Lactobacilli species within a sample was higher using qPCR in comparison to microbial culture. Similar differences in the quantification of bacterial numbers through microbial culture and qPCR have been reported by Huijsdens et al. (2002) and Nadkarni et al. (2002). Differences in the quantification of bacteria through culture compared to qPCR have been attributed to the presence of multiple copies of the 16S rRNA gene, the inability of microbial culture to enumerate dead or unculturable microorganisms and the selection of different bacterial species between the methods (Castillo et al. 2006). In particular, the 16S rRNA gene copy number of *Lactobacillus* species ranges from one to nine. Therefore, the observed difference in quantification of Lactobacilli using qPCR may in part be explained by differences in the average copy number within each faecal sample. Castillo et al. (2006) also found a significant correlation between bacterial numbers determined by culture compared to qPCR for Lactobacilli and total bacteria; however their correlation for *Enterobacteriaceae* numbers was not significant.

Comparing bacterial numbers enumerated using microbial culture and qPCR resulted in significant correlations for ratios of Lactobacilli to *E.coli* (R^2 = 0.684, p = 0.007) and ratios of Lactobacilli to *Enterobacteriaceae* (R^2 = 0.705, p = 0.005). The ratio of *Enterobacteriaceae* to *E.coli* obtained through microbial culture did not significantly correlate with the ratio calculated using qPCR (R^2 = 0.402, p = 0.154). Correlations were unable be made between *Cl.perfringens* numbers measured with the two methods due to the qPCR limit of detection.

3.4. Correlation between scouring and bacterial populations

3.4.1. Faecal water content and scouring

The faecal water content was measured to provide an objective scalar measure of scouring. Water content ranged from 46.27 % to 96.05 % from faecal samples obtained from 41 scouring piglets and 80 healthy piglets. The mean faecal water content from scouring pigs was significantly greater than that from healthy pigs within the sample population (p < 0.001) (Figure 3), with minimal variation within (0.05 %) and between runs (1.08 %).

The significant variation in water content between farms (Figure 4) may have been due to a number of factors including age of the pigs, the presence of disease, ambient temperature, season, piggery of origin, transport time for samples, pig diets and the antibiotics used. Water content of faeces may therefore not be the most accurate measure of intestinal disease and scouring in pigs. Comparing pathogen or commensal bacterial numbers with the total number of bacteria in faeces would overcome problems with accurate quantification in samples with significant variation in water content, and may be less affected by other on-farm factors that affect water content in faeces (Castillo et al, 2006).

A more accurate measure of scouring between piggeries may include the percentage water content, but should also include a more specific measure of clinical disease in the pigs such as dehydration, hairy coat, wasting or increased concentrations of pro-inflammatory cytokines.

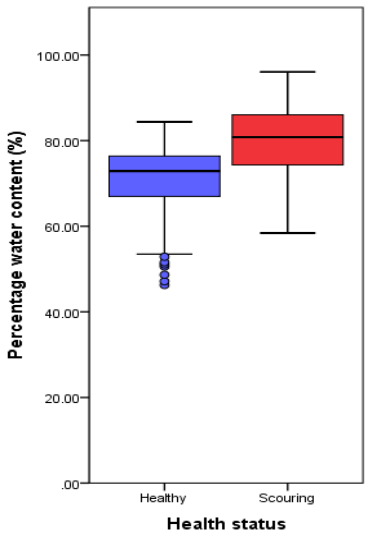

Figure 3 -Box plot of the percentage water content of healthy and scouring pigs

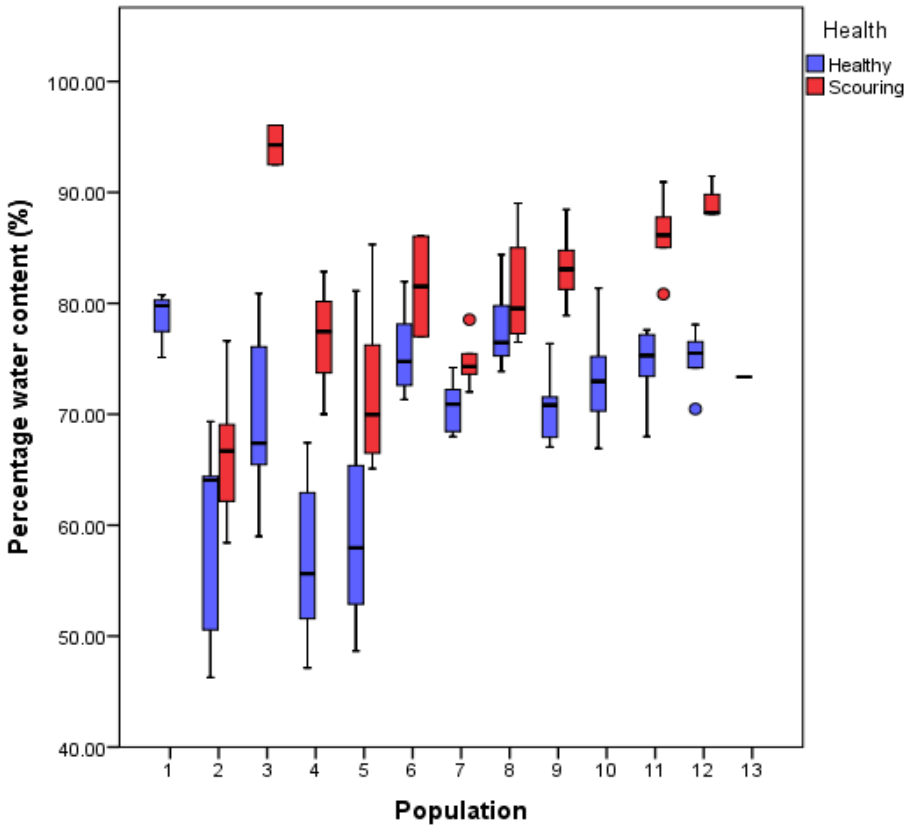

Figure 4 - Box plot showing the percentage water content of healthy and scouring pigs separated according to the population group of origin. Populations 1-5 are suckers, populations 6-12 are weaners and population 13 is an adult sow.

3.4.2. Correlation between scouring and numbers of each bacterial group

E.coli and *Cl.perfringens* are common causes of scouring in sucker and weaner pigs. Therefore, it was expected that increased faecal water content would be associated with increased numbers of *E.coli* and *Cl.perfringens* in the intestine. However, the opposite relationship was observed where water content was found to negatively correlate with numbers of *E.coli* (R^2 = -0.373, $p < 0.001$) and *Enterobacteriaceae* (R^2 = -0.380, $p < 0.001$), i.e. increased water content (scouring) correlated with decreased numbers of *E.coli* and *Enterobacteriaceae*. No significant correlation was found between scouring and numbers of *Cl.perfringens* or Lactobacilli.

It was hypothesised that quantifying the numbers of bacteria by faecal wet weight may have underestimated the numbers of bacteria in scouring faeces due to dilution with water. However, the negative correlations between pathogens and water content were still observed when bacterial numbers were expressed on a dry weight basis. A significant negative correlation between water content and numbers of *E.coli* (R^2 = -0.263, $p = 0.004$) and *Enterobacteriaceae* (R^2 = -0.269, $p = 0.003$) per gram of dry faeces were observed. Interestingly, when Lactobacilli numbers were expressed on a faecal dry weight basis, a significant correlation between water content and Lactobacilli numbers was observed (R^2 = 0.207, $p = 0.023$) i.e. as water content increased, the number of Lactobacilli increased.

Bacterial numbers and correlations between scouring and bacterial numbers were also examined on an individual farm basis, using Student's T tests and Spearman rank correlations. In all but one farm, higher Lactobacilli numbers were found in scouring nursery pigs relative to healthy pigs, along with significant positive correlations between Lactobacilli numbers and faecal water content (scouring). The trend was not as clear in weaner pigs, where no significant difference in Lactobacilli numbers were observed between scouring and healthy weaners on individual farms. Significant correlations between Lactobacilli numbers and water content were also variable in weaner pigs from different farms, with a positive correlation on three farms and a trend towards a negative correlation on three farms ($p < 0.10$). This variable response in weaner pigs may be explained by the presence of differing antibiotics, which will be discussed in more detail in section 3.6. No significant differences in numbers of total *E.coli*, F4 enterotoxigenic *E.coli* or *Enterobacteriaceae* were observed between scouring and healthy nursery pigs on any individual farm. However, there was a trend towards higher numbers of total *E.coli* and *Enterobacteriaceae* ($p \leq 0.1$) in scouring weaners on two of the farms.

The microbial sequence data of all bacteria found in faeces showed an increased prevalence of Lactobacilli, *E.coli* and *Enterobacteriaceae* in scouring pigs. The sequence analysis identified a total of 112 bacterial taxa (order/family/genera) belonging to 13 phyla in the faeces collected from post-weaned pigs. The faecal bacterial communities between different piggeries were relatively stable and dominated by the phyla Firmicutes, Bacteroidetes and Proteobacteria. These taxa are commonly found in gastrointestinal tracts of pig and are involved in a range of metabolic functions in the host including cellulolysis, proteolysis, fatty acid degradation, acidogenesis and acetogenesis. Some members of these phyla form syntrophic relationships (work together to break down a substrate) providing all the necessary microbial feeding groups for metabolism. Thus, these microbial interactions are crucial for digestion and absorption of nutrients in the host and for microbial stability in the gut.

Despite the overall community stability, there were differences in the relative abundance of many bacterial phyla between healthy and scouring pigs on each farm. Bacterial phyla that were more abundant in scouring pigs included phyla containing known pig pathogens such as *Enterobacteriaceae*, *Campylobacteraceae, Gammaproteobacteria* and *Lactobacillales* (Figure 4). Further analysis demonstrated that the *Enterobacteriaceae* were dominated by *Escherichia* and *Salmonella*, both of which are capable of causing severe scouring in pigs, as are *Campylobacter* spp. and *Brachyspira* spp.

The microbial sequence data supports the qPCR results that Lactobacilli numbers increased in scouring pigs. This was not expected, as lactate producing bacteria (Lactobacillales) have been implicated in good intestinal health. However, an overgrowth of Lactobacillales in response to scouring and repeated antibiotic therapy has been reported previously (Videnska et al., 2013) and was attributed to increased antagonistic activity of Lactobacilli against *Enterobacteriaceae* (Looft et al., 2014). This suggests that the presence of pathogenic *Enterobacteriaceae* and their interactions with *Lactobacillales* leads to changes in both community composition and function. The *Lactobacillale*s were largely dominated by *Weissella,* a known mucosal specialist that has pathogenic tendencies under certain circumstances (Looft et al., 2014). It is also possible that high zinc levels in the weaner diets caused the increased relative abundance of Lactobacillales,

including *Weissella* spp., *Leuconostoc* spp., and *Streptococcus* spp. (Vahjen et al., 2007). The Lactobacilli qPCR primers used did not differentiate between *Weissella* and Lactobacilli.

In contrast, *Lachnospiraceae, Ruminococcaceae, Veillonellaceae* and *Prevotellaceae* were more prevalent in healthy weaners on all farms. Further analysis revealed that these families were represented by lactate and butyrate producing genera *Pseudobutyrivibrio, Roseburia and Acidainococcus*. Butyrate producers play a key role in maintaining gut structure and function by providing energy for the microbial community; by influencing host gene expression and reducing inflammation associated with *E.coli* infection (Hamer et al., 2004). *Prevotella* spp. *(Prevotellaceae*) are frequently recovered from animal guts where they are involved in the breakdown of protein and carbohydrate, producing acetate and propionate (Metzler-Zebeli et al., 2010; Musso et al., 2011; Levine et al., 2013). A greater number of *Oxalobacteraceae* and *Methanobacteriaceae* were also recovered in the healthier weaners. *Methanobacteriaceae*, especially *Methanobrevibacter* are important for maintaining gut health since they consume the end products of bacterial fermentation (acetate, hydrogen and methane) and thereby confer community stability (Kong et al., 2014). Members of *Oxalobacteraceae* have been isolated from the large intestine where they play an important role in the metabolism of oxalate (Stewart et al., 2004).

Previously, lactate producing bacteria have been described as potentially beneficial bacteria because of their capacity to decrease the pH in the gut and to excrete natural antibiotics which inhibit the growth of potentially pathogenic or commensal bacteria, including *Escherichia coli, Streptococcus* and *Clostridium*. This suggests that butyrate and lactate production pathways may play an important role in maintaining microbial stability and piglet gut health, making competition of microbial pathogens more difficult within the intestinal environment. Experimental Salmonella challenge of pigs suggests that microbial populations also impact on subsequent disease severity. For example, increased numbers of *Ruminococcus* spp. and *Prevotella* spp. were reported in pigs shedding low numbers of *Salmonella* relative to pigs shedding higher *Salmonella* numbers (Bearson et al., 2013).

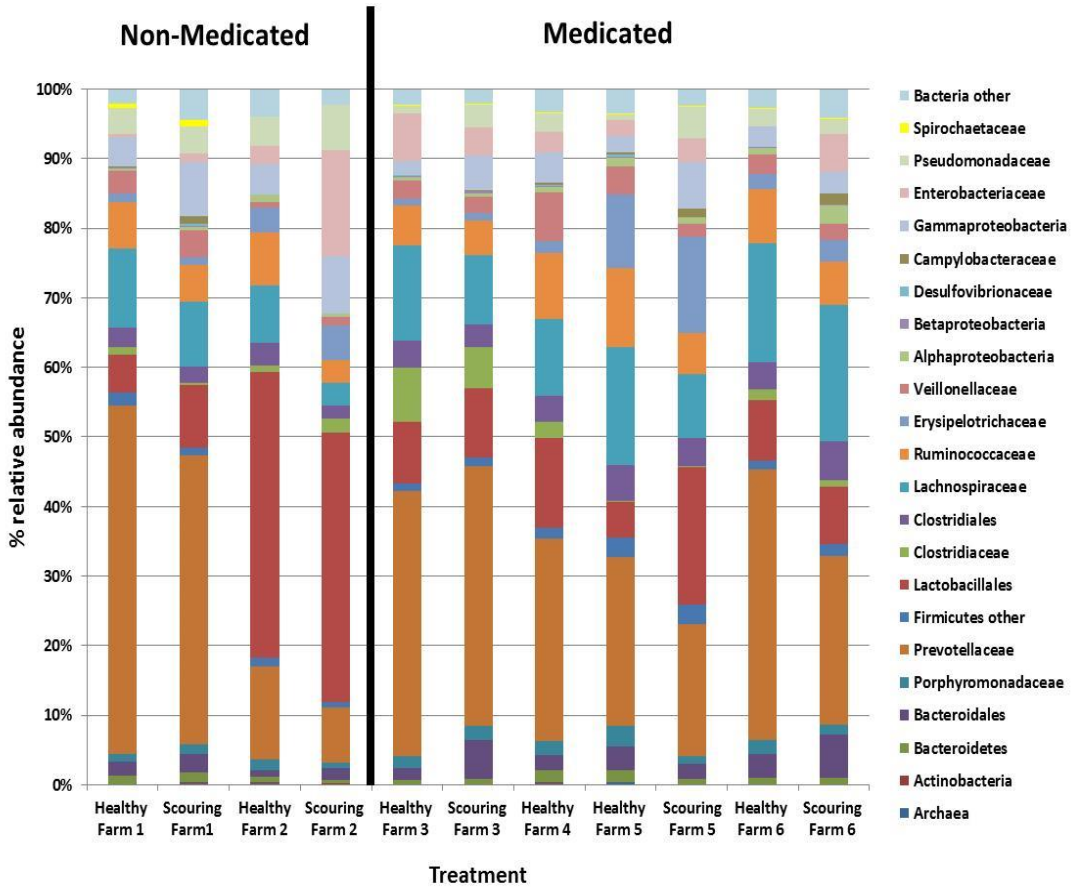

Figure 5 - Microbial community diversity for both Bacteria and Archaea in faecal samples from healthy and infected piglets at different farms across Australia. With the exception of Farm 1 and Farm 2, piglets were medicated at all other farms.

3.4.3. Correlation between scouring and pathogenic *E.coli* virulence types

The absence of a positive correlation between total *E.coli* and water content led to the hypothesis that scouring would correlate more strongly with solely pathogenic *E.coli*. The F4 fimbriae *E.coli* qPCR only detects pathogenic *E.coli*, as opposed to the total *E.coli* qPCR assay that measures both pathogenic and commensal strains. The virulence specific qPCR assay for F4 *E.coli* was performed at the University of Melbourne with validation and construction of standards performed by Dr Phillip Markham. The relative number F4 fimbriae genes ranged from 2.8 $x10^1$ to 8.65 $x10^6$ copies in the 121 faecal samples. An inverse correlation between water content and the numbers of the F4 fimbriae gene was observed (R^2 = – 0.230, p = 0.013) i.e. numbers of F4 *E.coli* decreased with increased water content. This was similar to the correlation between total *E.coli* numbers. It is possible that a number of other pathogenic *E.coli* strains such as the F18 fimbrial type might correlate more strongly with scouring, but this would need to be examined in a further study. It is also possible that lower numbers of pathogenic *E.coli* are detected in faeces because they are still attached to the mucosal surfaces in the small intestine. To examine this, bacterial numbers need to be compared between paired faecal and mucosal samples collected from the same pigs (Section 3.5)

3.4.4. Correlation between scouring and ratios of commensal to pathogenic bacteria

In the absence of a positive correlation between numbers of pathogens and scouring, we decided to investigate correlations between scouring and the balance of commensal to pathogenic bacteria in the intestine. It was hypothesised that ratios of commensal to pathogenic bacteria would be reduced in scouring pigs, ie. a decrease in commensal bacteria relative to an increase in pathogenic bacteria. However, the opposite relationship was found when correlating results from all 121 samples on 7 farms. Water content was positively correlated with the ratio of *Enterobacteriaceae* to total *E.coli* (ρ = 0.279, p = 0.002), Lactobacilli to total *E.coli* (ρ = 0.379, p < 0.001) and Lactobacilli to F4 pathogenic *E.coli* (ρ = 0.274, p= 0.003). That is, increased water content correlated with a decreased number of pathogens relative to commensals (adjusted for faecal dry weight). However, in two farms (farm 1 and 6) there was a trend towards reduced Lactobacilli to *E.coli* and *Enterobacteriaceae* ratios in scouring pigs. That is, increased pathogen numbers relative to commensal numbers led to reduced ratios in scouring weaner pigs, as was expected. Given higher sample numbers on each farm, these differences may have been significant. In addition, farm 6 showed significant correlations between scouring and ratios of Lactobacilli to *E.coli*, Lactobacilli to *Enterobacteriaceae* and *Enterobacteriaceae* to *E.coli* ($R^2 \geq$ - 0.69, p < 0.05) as hypothesised.

3.4.5. Correlation between scouring and bacterial numbers relative to total bacteria

Proportions of each bacterial group relative to total bacteria were calculated as a measure of their dominance within the intestinal microflora. Proportions of bacterial groups were determined by dividing $\log_{10}$ numbers (adjusted for dry weight) of each bacterial group by the total number of bacteria. It was expected that scouring would be associated with an increase in pathogenic bacterial numbers relative to total bacteria due to an increase in the prevalence of pathogens in the overall intestinal microflora. However, the opposite relationship was found in this study, where a significant inverse correlation was found between water content and the proportions of *E.coli* (ρ = –0.312, p < 0.001) and *Enterobacteriaceae* (ρ = –0.300, p < 0.001) relative to total bacterial numbers. That is, the relative proportion of pathogens in faeces decreased with increasing water content (scouring).

3.5. Microbial populations in faeces versus intestinal scrapings

The lack of a positive correlation between scouring and pathogen numbers may have been because faecal bacterial populations are not an accurate representation of the intestinal microflora. The adhesion of pathogens to the intestinal mucosa may mean that fewer pathogens are detected in faeces.

Paired faecal and intestinal scrapings from the same pigs showed that the total number of all bacterial species was greater in faeces than in the intestinal mucosa (p = 0.043). However, there were no significant differences in *E.coli*, *Enterobacteriaceae* or Lactobacilli numbers between paired faecal and mucosa samples (Table 7). The number of *Cl.perfringens* detected in the intestinal mucosa was significantly greater than the number detected in faeces (p = 0.019). The difference in numbers of *Cl.perfringens*

in faeces relative to mucosa suggests that faeces may not accurately represent the numbers of some bacteria in the intestine.

There were no significant correlations between the numbers of each bacterial group in faeces compared to the mucosa (Table 7), although correlations between *Enterobacteriaceae* numbers in faeces and mucosa was close to significant (p = 0.067). The absence of correlations between bacterial numbers in faeces and mucosa may partly explain why scouring did not correlate with numbers of pathogens or ratios of commensal to pathogenic bacteria as expected.

Table 7 - Comparisons between the numbers of commensal and pathogenic bacteria detected in faeces or intestinal mucosa and their significance.

	Mean Log_{10} bacteria mucosa	Mean Log_{10} bacteria faeces	T-test between sample types (p value)	Spearman's Rank Correlation value (R^2)	*P value*
Cl.perfringens	3.72	2.25	0.019*	0.167	*0.393*
E.coli	4.31	4.46	0.838	0.406	*0.191*
Enterobacteriaceae	5.01	5.14	0.861	0.545	*0.067*
Lactobacilli	2.21	3.73	0.053	0.490	*0.106*
Total bacteria	*9.30*	*10.03*	*0.043**	*0.336*	*0.286*

*Significance based on p < 0.05

3.6. Influence of antibiotic use

Overall, weaner pigs routinely medicated with antibiotics had significantly lower numbers of Lactobacilli (p = 0.010) and total bacteria (p = 0.015) compared to pigs not treated with antibiotics (Figures 5 and 6). More importantly, there were significantly higher numbers of pathogenic F4 *E.coli* (p < 0.001) and total *E.coli* (p < 0.001) in medicated pigs. Looft et al. (2012) also found increased *E.coli* numbers in medicated finishers compared to their non-medicated litter mates fed the same diet. Furthermore, Collier et al. (2003) showed a decrease in total bacteria numbers and more specifically a decrease in Lactobacilli, *Streptococcus* and *Bacillus* species with different antibiotic treatments. A decrease in numbers of Lactobacilli and total bacteria in medicated pigs may increase the susceptibility of the intestine to enteric disease due to loss of intestinal biodiversity and the protective commensal microflora. The loss of commensal bacteria combined with increased numbers of pathogenic *E.coli* may exacerbate scouring in compromised pigs.

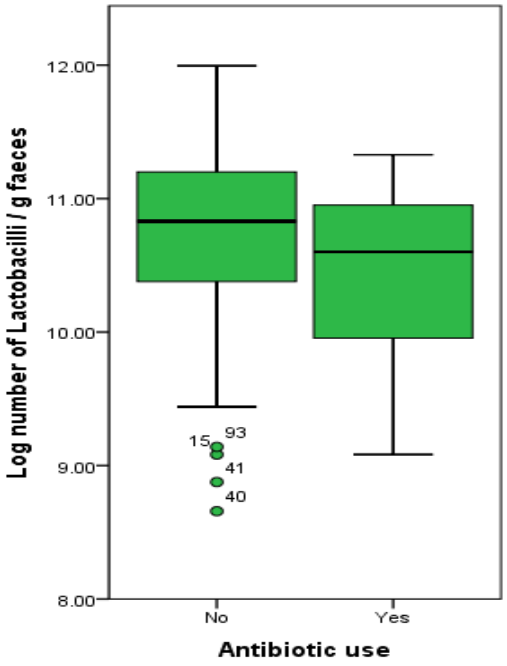

Figure 6 -Box plot showing the log number of Lactobacilli per gram of faeces in pigs with and without antibiotic use.

The influence of antimicrobials on relative abundance and diversity of bacterial populations was also examined using the microbial sequencing data. Bacterial diversity was studied using multivariate analysis modelling (Canonical correspondence analysis, CCA) to identify the impact of farm (diet, environment, genetics), scouring and antibiotics on bacterial communities. Samples that shared a similar microbial community composition clustered together. The direction and length of the arrow indicates the extent to which the community structure (Figure 7) and taxa distribution (Figure 8 and Table 8) can be explained by a given explanatory variable.

Faeces from medicated pigs had a higher abundance of butyrate-producing *Lachnospiraceae*, *Butyricimonas*, *Eubacteriaceae*, *Porphyromonadaceae* and certain *Clostridiaceae* (Figure 5 and 7), which play a major role in maintaining gut health in pigs (Metzler-Zebeli et al., 2010; Levine et al., 2013). Shifts in populations of Lachnospiraceae in piggery faecal samples following the administration of antibiotics has been shown to confer resistance to scouring (Kim et al., 2012; Looft et al., 2012; 2014). In fact, the abundance of *Lachnobacterium* spp. increased in both the lumen and mucosa with antibiotic use and members of this genus are known to produce bacteriocins (antimicrobial compounds) and butyrate (Looft et al., 2014) which inhibit the growth of pathogens. For this reason, *Lachnobacterium* could be considered a potential direct-fed probiotic for use on piggeries (McAllister et al., 2011).

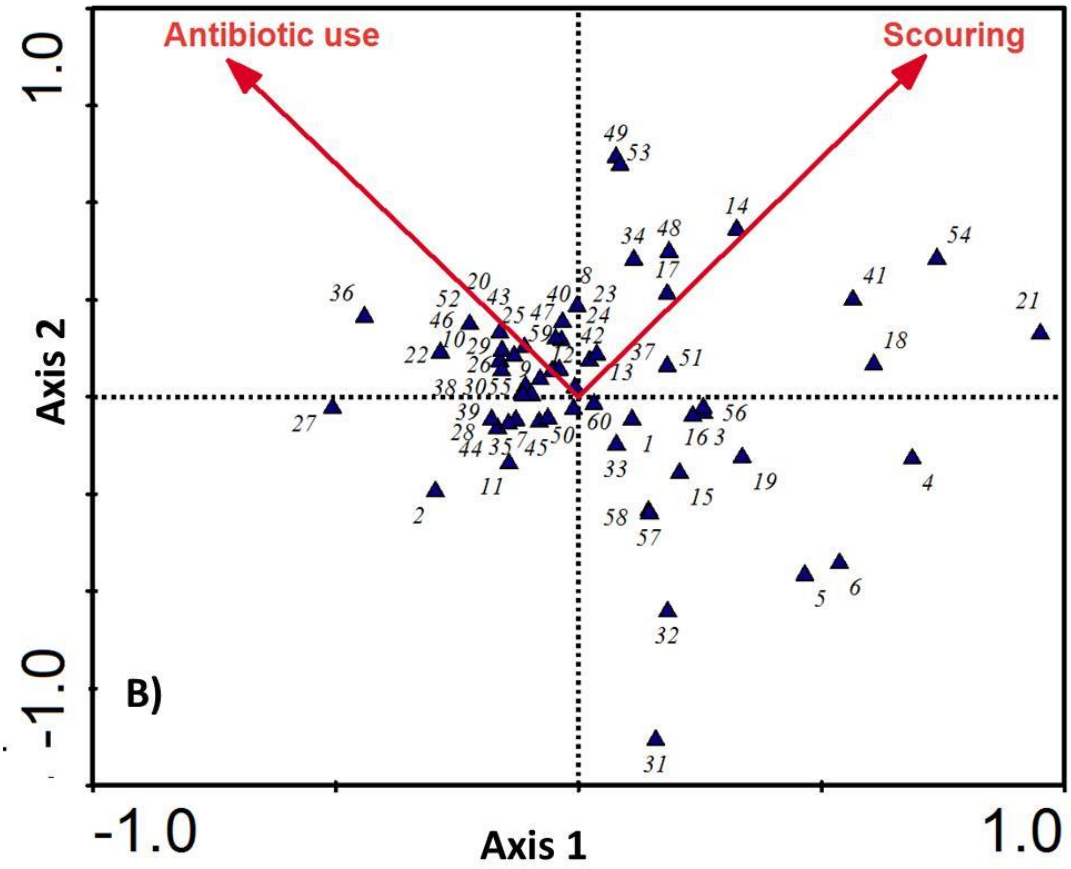

Figure 7 - CCA plot showing the relationship between individual bacterial taxa and explanatory variables in healthy and infected piglets at six farms. Plots on the graph represent the community composition for individual taxa (▲). Arrows represent the explanatory variables (antibiotic used and scouring).

Table 8 - Bacterial and archaeal taxa identified from sequencing piglet faecal samples

Code	Taxa	Code	Taxa	Code	Taxa
1	Archaea	21	Clostridium	41	Brevundimonas
2	Methanobacteriaceae	22	Proteiniclasticum	42	Betaproteobacteria
3	Actinobacteria	23	Sarcina	43	Burkholderiales
4	Microbacteriaceae	24	Clostridiales	44	Oxalobacteraceae
5	Micrococcaceae	25	Eubacteriaceae	45	Sutterella
6	Bifidobacteriaceae	26	Lachnospiraceae	46	Desulfovibrionaceae
7	Bacteroidetes	27	Pseudobutyrivibrio	47	Desulfovibrio
8	Bacteroidales	28	Roseburia	48	Campylobacter
9	Porphyromonadaceae	29	Peptostreptococcaceae	49	Arcobacter
10	Butyricimonas	30	Ruminococcaceae	50	Helicobacter
11	Prevotella	31	Faecalibacterium	51	Gammaproteobacteria
12	Firmicutes	32	Ruminococcus	52	Succinivibrionaceae
13	Staphylococcaceae	33	Sporobacter	53	Enterobacteriaceae
14	Lactobacillales	34	Erysipelotrichaceae	54	Escherichia
15	Enterococcaceae	35	Catenibacterium	55	Salmonella
16	Lactobacillaceae	36	Clostridium XVIII	56	Pseudomonadaceae
17	Weissella	37	Erysipelothrix	57	Spirochaetaceae
18	Leuconostocaceae	38	Selenomonadales	58	Treponema
19	Streptococcaceae	39	Veillonellaceae	59	Synergistaceae
20	Clostridiaceae	40	Alphaproteobacteria	60	Bacteria other

In contrast, the non-medicated pigs were mainly represented by lactate producing families *Streptococcaceae, Bifidobacteriaceae* and *Ruminococcaceae* (Figure 5 and 7 and Table 8). This finding is consistent with other studies, where increasing the populations of *Bifidobacterium* and other lactate-producing bacteria was linked with reduced coliform shedding and increased protection against *E.coli* (Kong et al., 2014; Looft et al., 2014). These lactate producing bacteria have also been described as potentially beneficial bacteria because of their capacity to decrease the pH in the gut and to excrete natural antibiotics which inhibit the growth of potentially pathogenic bacteria, including *Escherichia coli* and *Clostridium*. *Faecalibacterium*, a butyrate producer, was also more abundant in the healthy pigs not receiving antibiotics. This suggests that butyrate and lactate production pathways may play an important role in maintaining microbial stability and piglet gut health, making pathogen colonisation more difficult due to competitive inhibition or exclusion (Metzler-Zebeli et al., 2010; Levine et al., 2013). Interestingly, the non-medicated pigs also had a greater abundance of *Spirochaetaceae*, in particular *Treponema* spp., which may increase their risk of swine dysentery later in life.

These studies highlight the significant impact that antimicrobial medication has on the relative abundance and diversity of both commensal and pathogenic bacteria and on the metabolic function of the pig gut. Monitoring gut health using ratios of commensals to pathogens may be limited to situations where antimicrobials have not been used because many antimicrobials act against both commensal and pathogenic bacteria. Selecting narrow spectrum antibiotics that are active at the site of infection may help to reduce the unintended consequences of antimicrobial use. Ratios of commensal to pathogenic bacteria may prove useful in evaluating dietary additives such as the prebiotic inulin and organic acids to modulate bacterial colonisation and gut health.

3.7. Influence of diet, genetics and environment

The relative abundance of the bacterial phyla *Prevotellaceae, Clostridiaceae, Erysipelotrichaceae, Veillonellaceae, Campylobacteraceae* and *Spirochaetaceae* were significantly different between farms and regions (Figures 5 and 8 and Table 8). This suggests that the composition of gut microflora is strongly influenced by diet and environment and this is in agreement with previous findings (Pluske et al., 2007; Castillo et al., 2008; Lee et al., 2013).

A limitation of this study was that farm practices that impact on bacterial population in the gut such as diet, hygiene, vaccination, antibiotic medication, exposure to pathogens, age at weaning and mixing of pigs were not controlled in this study and insufficient data was collected to identify risk factors. Future work to address the issue of differing farm practices would involve sampling from medicated and non-medicated weaner pigs on the same farm and from different farms that use the same antibiotic to control for these extraneous variables.

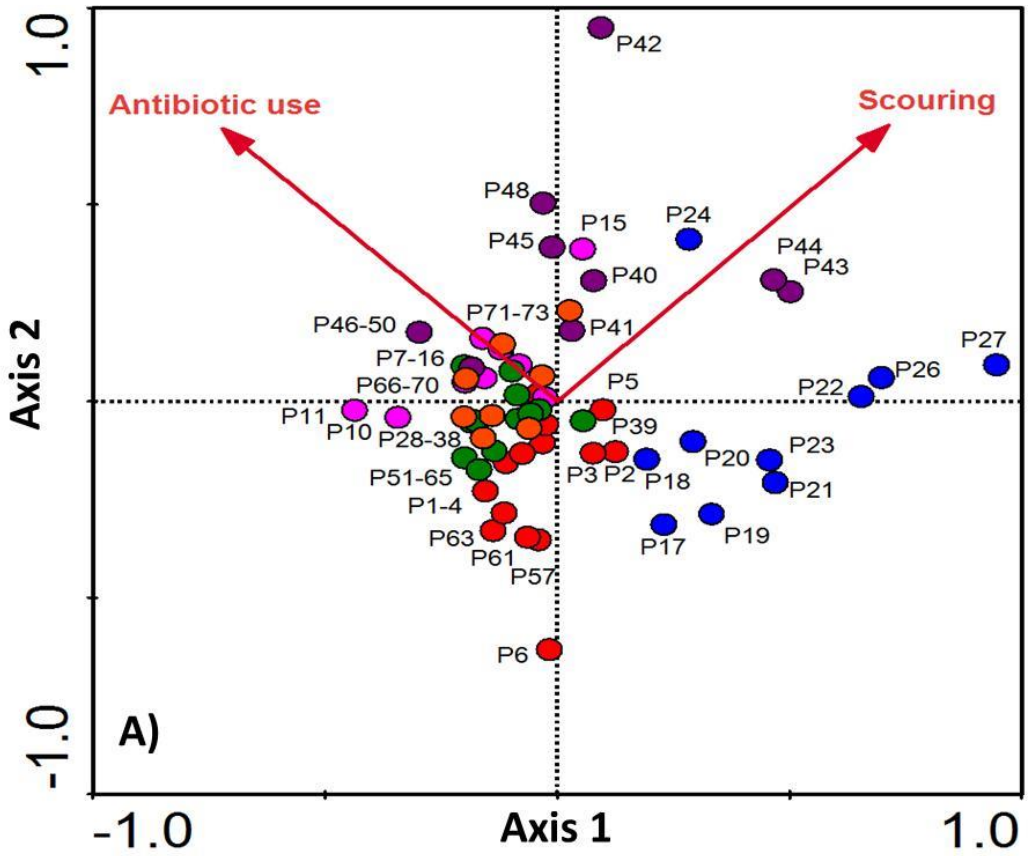

Figure 8 - CCA plot showing the relationship between microbial community composition and antibiotic use or scouring in healthy and infected piglets at six farms. Arrows represent the explanatory variables (antibiotic used and scouring) and data points of the same colour represent microbial communities from the same farm. Farm key is as follows:

Farm 1 Farm 2 Farm 3 Farm 4 Farm 5 Farm 6

4. Applications of Research

While the qPCR assays developed for *Lactobacilli, E.coli, Enterobacteriaceae* and F4 enterotoxigenic *E.coli* do not correlate as expected with water content in faeces; we have discovered additional parameters that need to be measured to get a better measure of gut health. We can therefore apply the knowledge gained from this study to help answer a much wider array of industry problems. It is clear that we need a more specific measure of scouring in faeces, which may include water content but also a measure of clinical disease in the pig. The next generation microbial sequencing has identified groups of other commensal bacteria, including butyrate and lactate producing bacteria that may play a more important role in protecting the gut from pathogens, maintaining microbial stability and encouraging microbial fermentation and short chain fatty acid production. The specific applications are outlined below.

1. Develop cost-effective diagnostic tests for bacterial species that are important in gut health, including qPCRs for the commensal families *Lachnospiraceae, Ruminococcaceae* and *Prevotellaceae*.

2. Evaluate the impact of prebiotics or other dietary manipulations/supplements (essential oils, organic acids, yeasts, beta glucan, zinc/copper and kaolin) to better control post weaning scours, and reduce the reliance on antibiotics for disease control. Pork CRC project 2C-114 Effect of a novel feed additive on post weaning diarrhoea and production in weanling pigs.
3. Identify management practices and risk factors associated with *E.coli* scours and their impact on the relative abundance of both commensal and pathogenic bacteria (temperature control, genetics, vaccination etc). Pork CRC 2A-106: A comprehensive risk factor analysis of *E.coli* disease in the piggery environment.
4. Evaluate the impact of antibiotics used in the field on the abundance of beneficial bacteria that protect against or inhibit pathogens in the gut.
5. Evaluate the potential for commensal bacterial groups such as *Lachnospiraceae, Ruminococcaceae* and *Prevotellaceae* to be used as probiotics in weaner pigs.
6. Identify commensal bacteria that are important in reducing pathogen shedding for other economically important enteric infections (*Lawsonia*, *Brachyspira* spp. *Salmonella* etc).
7. Evaluate the impact of prebiotics or other dietary manipulations/supplements to increase the abundance of energy and short chain fatty acid producing bacterial groups to better utilise feed ingredients.

5. Conclusion

This study demonstrated that enumeration of bacterial groups in faeces by quantitative PCR correlated well with traditional culture methods, and qPCR proved to be a more time efficient and robust procedure than traditional culture. The molecular techniques developed in this study can be used to quantify numbers of *E.coli*, Lactobacilli, F4 *E.coli*, *Enterobacteriaceae* and total bacteria in faeces or intestinal mucosa samples. This proof of concept study also demonstrates that similar qPCR tests can be developed for other bacterial groups associated with disease or gut health, without the need to compare the qPCR with traditional bacterial culture.

Calculating the percentage of water in faecal samples was an objective way to show significantly higher water content in 'scouring' faecal samples relative to 'healthy' pigs, as classified by the submitters. However, the poor correlation between pathogen numbers and water content indicates that there are other causes of watery faeces in pigs and that more specific clinical signs of infectious diarrhoea need to be included, such as dehydration with sunken eyes, inappetance and rough hair coat.

The water content of faeces and relative abundance of bacterial species differed between farms. Factors that impact on both bacterial numbers and faecal consistency include diet, genetics, immune status, antibiotic medication, environment, housing, season, pig age and the presence of enteric diseases. On some farms we observed the expected relationship between increased water content and decreased ratios of commensal to pathogenic bacteria. However, when samples from all seven farms were included, the opposite relationship was found; increased water content correlated to decreased numbers of total *E.coli* and pathogenic F4 *E.coli*

relative to Lactobacilli. An increased abundance of Lactobacilli and *E.coli* (Enterobacteriaceae) in scouring pigs was demonstrated in our next generation microbial sequencing studies. While it was somewhat surprising that Lactobacilli numbers would be higher in scouring pigs, previous studies had suggested that genera of the Lactobacillales (*Lactobacillus spp. Weissella spp., Leuconostoc* spp., and *Streptococcus* spp.) become more abundant in weaner pigs fed zinc oxide and in an antagonistic response to *Enterobacteriaceae* such as *E.coli*.

The next generation microbial sequencing studies enabled this study to identify other bacterial families that were less abundant in scouring pigs such as the butyrate producing *Lachnospiraceae, Ruminococcaceae, Veillonellaceae* and the protein and carbohydrate catabolising *Prevotellaceae*. A relative reduction in these bacterial families in scouring pigs could exacerbate disease because the gut microflora would be less stable, have less energy for microbial fermentation and increased inflammation in response to *E.coli* infection. A reduced abundance of *Ruminococcaceae* and *Prevotella* spp. was also associated with an increased magnitude of *Salmonella typhimurium* excretion in pigs. These studies indicate that the loss of bacterial families such as *Lachnospiraceae, Ruminococcaceae* and *Prevotellaceae* may be more important than other commensals like Lactobacilli in maintaining gut health/stability and recovery from scouring caused by bacterial pathogens, but this would need to be investigated in further studies.

This study also explored the possibility that scouring did not correlate with increased numbers of pathogenic bacteria in faeces because the pathogens remained attached to the intestinal mucosa in infected pigs. The abundance of commensal and pathogenic bacteria was compared in faecal and mucosal samples from the same pigs. However, paired faecal and mucosal samples showed no significant correlations between the numbers of *Cl.perfringens, E.coli*, *Enterobacteriaceae*, Lactobacilli or total bacteria. The differing bacterial numbers in faecal and mucosal samples suggests that faeces may not be an accurate representation of the intestinal microflora as has been demonstrated previously. However, next generation sequencing of faeces has provided strong evidence of bacterial groups that are important in bacterial health, making bacterial profiling practical from live pigs.

The significant impact of antibiotics on commensal microbial flora was an interesting outcome from this study. Pigs reared with antibiotics were found to have decreased numbers of Lactobacilli and decreased total bacteria numbers, along with increased numbers of F4 pathogenic *E.coli* and total *E.coli*. The reduced abundance of lactate producing bacterial families (*Streptococcaceae, Bifidobacteriaceae* and *Ruminococcaceae*) was also demonstrated with next generation microbial sequencing. The decrease in some commensal intestinal bacteria and increase in pathogenic bacteria may result in an exacerbation of scouring. Reduced abundance of *Bifidobacterium* spp. would reduce the effect of this protective bacterium, known to decrease coliform shedding by acidifying the gut and excreting natural antibiotics against *E.coli* and *Clostridium* spp. In this study antibiotic use also increased the abundance of beneficial families such as *Lachnospiraceae*, which can confer resistance to scouring by producing bacteriocins capable of killing pathogens. A number of different types of antibiotics were used on these farms, so it is clear that further studies need to investigate the impact of specific antibiotics used in weaner pigs on commensals and pathogens in the gut. In the past, antibiotics were only

chosen on their activity against specific pathogens, but it now seems wise to also select antibiotics on their inactivity against important protective commensal bacteria.

The next generation microbial sequencing studies have highlighted the importance of other groups of commensal bacteria, such as butyrate and lactate producing bacteria that may influence gut health and suppression of disease. Future studies in weaners are needed to confirm these results, along with the impact of antibiotics on commensal bacteria. Once the most important groups of commensal bacteria have been identified, it will be relatively easy to design qPCR assays to measure the "protective load" of bacteria in weaner pigs. Measures of protective bacteria can be used to evaluate the impact of antibiotics, vaccination, improved hygiene, prebiotics and probiotics on gut health. If reduced abundance of these protective bacteria coincided with increased excretion of a wider range of enteric pathogens in diseased animals (including swine dysentery, ileitis and salmonellosis), we could identify a useful measure of gut health in the growing pig. The development of assays to measure the intestinal health of pigs would assist producers in the monitoring of overall herd health and identification and control of risk factors associated with scouring. These outcomes would assist in the reduction of production losses associated with enteric disease.

6. Limitations/Risks

While Nutreco have used traditional culture techniques to quantify number and ratios of bacterial groups from faecal samples, there are inherent limitations in this method due to the need to keep bacteria alive, but not replicating over the time they are in transit from the farm to the lab. The new qPCR assays developed to replace culture techniques have significant advantages including the ability to freeze faeces and preserve bacterial populations during transit to the lab, the ability to quantify fastidious or non-culturable bacteria and the increased speed and reduced labour costs for testing (high throughput technology). However, one needs to be aware of two limitations of the qPCR technology. Firstly the qPCR can detect both live and dead bacteria, so it is likely that the qPCR may over-estimate bacterial numbers relative to culture. Apajalahti et al. (2003) estimated that somewhere between 17% and 34% of bacteria in human faeces are dead. In addition, many bacterial species have multiple copies of the 16S rRNA gene, which can also overestimate bacterial numbers. Using ratios of commensal to pathogenic bacteria, or expressing specific bacterial numbers as a proportion of total bacterial numbers will overcome both of these limitations as similar proportions of dead bacteria and 16S rRNA copy numbers would be expected in all bacterial groups and samples. New techniques also exist for qPCR detection of solely live bacteria, and preliminary trials at EMAI for *E.coli* show these live qPCR assays are more accurate for bacterial quantification than traditional culture techniques.

Ratios of commensal to pathogenic bacteria were difficult to determine with Gram positive bacteria such as *Cl. perfringens* because the sensitivity of this qPCR needs to be improved. This can be achieved by increasing the efficiency of the DNA extraction method, selection of an alternative gene with multiple copy numbers or by performing a nested PCR step to amplify the original PCR product.

A better measure of scouring due to infection is needed because increasing water content of faeces did not correlate with increased pathogen numbers. Considerable differences in water content were seen between scouring pigs on different farms. Faecal water content could be influenced by season, diet, pig age and other random factors. Future studies would therefore need a more specific measure of scouring caused by infection which could also include scores for descriptors of dehydration, wasting and rough hair coat or concentrations of pro-inflammatory cytokines.

The absence of the expected relationship between bacterial numbers and scouring may be due to the influence of factors other than the numbers of *Cl.perfringens* or *E.coli* in the faeces or may be due to the presence of other bacterial or viral cases of scouring in piglets. Whilst the most common causes of scouring in piglets are *Cl.perfringens* and *E.coli*, there are numerous alternative causes such as *Salmonella* species, *Lawsonia intracellularis, Brachyspira hydoysenteriae, Coccida* species and rotavirus (Schroeder et al. 2006).

Selecting only four bacterial groups to quantify from the multitude of bacterial species found in faeces was a significant limitation of this study. However, the next generation microbial sequencing of a subset of the same faecal samples highlighted a number of other groups of commensal bacteria that may be more important in maintaining gut health and speeding up recovery from infection.

Diet, medication, environment, genetics and immune status are likely to have a large impact on the relative abundance of both commensal and pathogenic bacteria. The results of this study need to be incorporated into other microbial population research studies being undertaken in Australia (Pork CRC 2C-114) and overseas to highlight the most important protective commensal bacteria in weaner and growing pigs across different diets, environments and genetics. Future studies in weaners may also be needed to confirm these results, along with the impact of antibiotics on commensal bacteria. Once the most important groups of commensal bacteria have been identified, it will be relatively easy to design qPCR assays to measure the "protective load" of bacteria in weaner pigs.

7. Recommendations

As a result of the outcomes in this study, the following recommendations have been made:

- Expand this preliminary research demonstrating that some antibiotics used to control scouring in weaner pigs actually increase pathogen numbers and reduce numbers of the important protective commensal bacteria. Extend this information to producers and veterinarians to help guide prudent use of antibiotics. Develop diagnostic qPCR assays to quantify the impacts of commonly used antibiotics on both pathogens and commensals in commercial pigs.
- Consolidate Australian and overseas data on the impacts of diet, genetics, immune status, pig age, scouring, antibiotics, environment and pathogens on gut microbial populations, especially with respect to the beneficial roles that microbes can play in the gut. Identify what knowledge is still needed and develop research projects for gaps in that knowledge.
- Fund a small research project to develop quantitative diagnostic assays for *Lachnospiraceae, Ruminococcaceae* and *Prevotellaceae* and evaluate the importance of these species in excretion of other enteric pathogens

(*Lawsonia, Brachyspira* spp., and *Salmonella*) using faecal samples already collected from enteric disease trials.

- Once microbial populations that are consistently important to pig gut health (across various management systems) are identified, develop qPCR assays to evaluate prebiotics, probiotics and dietary supplements (enzymes, beta glucans, essential oils, organic acids and yeasts) under field conditions.

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