Microbial profiling of liquid feed for pigs and the impact of strategies to optimise feed microbial quality on the feed and pig gut microbiome

A thesis presented in fulfilment of the requirements for the degree of

Doctor of Philosophy

By

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Declaration

No element of the work described in this thesis has been previously submitted for a degree at this or any other institution. The work in this thesis has been performed by the author, with supervision and guidance/support as outlined in the Acknowledgements section.

Signature: 

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List of abbreviations

Abbreviation	Explanation
ADFI	Average daily feed intake
ADG	Average daily gain
AHDB	Agriculture and Horticulture Development Board
AHL	N-acyl-homoserine lactone
AI-2	Auto-inducer-2
ALF	Acidified liquid feed
ANCOMBC	Analysis of Compositions of Microbiomes with Bias Correction
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
ARG	Antibiotic resistance gene
ASV	Amplicon sequence variant
ATP	Adenosine triphosphate
BB	Bead-beating
BD	BigDutchman
BP	Base pair
BW	Body weight
BWG	Body weight gain
CAZyme	Carbohydrate active enzyme
CE	Carbohydrate esterase
CEC	Competitive exclusion culture
CFU	Colony forming units
CoA	Coenzyme A
CRISPR	Clustered regularly interspaced short palindromic repeats
DA	Differential abundance
DADA2	Divisive Amplicon Denoising Algorithm 2
DE	Digestible energy
DM	Dry matter
DMI	Dry matter intake
EFSA	European Food Safety Authority
EHEC	Enterohemorrhagic *E. coli*
ENA	European Nucleotide Archive
ENZ	Xylanase and β-glucanase enzyme complex
EPS	Extracellular polymeric substances
ESBL	Extended-spectrum β-lactamase
ETEC	Enterotoxigenic *E. coli*
EU	European Union
FAD	Flavin adenine dinucleotide
FCE	Feed conversion efficiency
FCR	Feed conversion ratio
FE	Feed efficiency
FID	Flame ionisation detector
FLC	Fermented liquid cereal
FLF	Fermented liquid feed
FMT	Faecal microbiota transplantation
G:F	Gain to feed ratio
GC	Gas chromatography
GE	Gross energy
GH	Glycoside hydrolases

GI	Gastrointestinal
GIT	Gastrointestinal tract
HAM	Hamamelitannin
HDAC	Histone deacetylase
HGT	Horizontal gene transfer
HMDS	Hexamethyldisilazane
HPLC-FLD	High-performance liquid chromatography fluorescence detector
HSD	Honestly significant difference
ISO	International Organisation for Standardisation
ITS	Internal transcribed spacer
LAB	Lactic acid bacteria
LDA	Linear discriminant analysis
LFC	Log fold change
LOD	Limit of detection
LPS	Lipopolysaccharide
LW	Live weight/Liquid whey
MBC	Minimum bactericidal concentration
MCT1	Monocarboxylate transporter isoform 1
MDR	Multi-drug resistant
MGE	Mobile genetic element
MIC	Minimum inhibitory concentration
MPN	Most probable number
MRD	Maximum recovery diluent
MRSA	Methicillin-resistant Staphylococcus aureus
NAD	Nicotinamide adenine dinucleotide
ND	Not detected
NM	Not measured
NMDS	Non-metric multidimensional scaling
NR	Not reported
NRC	National Research Council
NSD	No significant difference
NSP	Non-starch polysaccharide
OTU	Operational taxonomic unit
PAS	Pot-ale syrup
PBS	Phosphate buffered saline
PC	Post-cleaning
PCR	Polymerase chain reaction
PCR-DGGE	Polymerase chain reaction-denaturing gradient gel electrophoresis
PERMANOVA	Permutational analysis of variance
PFLF	Partially fermented liquid feed
PL	Polysaccharide lyases
PVC	Polyvinyl chloride
QIIME	Quantitative Insights into Microbial Ecology
qPCR	Quantitative polymerase chain reaction
QQ	Quorum quenching
QS	Quorum sensing
RA	Relative abundance
RFI	Residual feed intake
RLU	Relative light units
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SCFA	Short chain fatty acid

SD	Standard deviation
SE	Standard error
SEM	Scanning electron microscopy/Standard error of the mean
SLC	Soaked liquid cereal
SMCT1	Sodium-coupled monocarboxylate transporter 1
STEC	Shiga toxin-producing *E. coli*
TCA	Trichloroacetic acid
TLC	Thin-layer chromatography
TPP	Thiamine diphosphate
TRFLP	Terminal restriction fragment length polymorphism
VFA	Volatile fatty acid
WWDG	Wet wheat distillers' grains

Microbial profiling of liquid feed for pigs and the impact of strategies to optimise feed microbial quality on the feed and pig gut microbiome

James Cullen

Abstract

The objectives of this thesis were to (1) develop an optimal methodology for simultaneous profiling of liquid feed and pig gut bacteriome and mycobiome; (2) profile the bacteriome and mycobiome of liquid feed on commercial pig farms; (3) determine the impact of different feed form and delivery methods on the feed and pig gut bacteriome; (4) determine whether intensive sanitisation of liquid feeding systems improves system hygiene and the microbiological and nutritional quality of liquid feed and if it impacts the bacteriome and mycobiome of feed and feeding system surfaces. All experiments were conducted with grow-finisher feed/pigs. The optimal method for simultaneously profiling the bacteriome and mycobiome of liquid feed and pig faeces included a 20-minute bead-beating step to minimise lysis bias. Application of this methodology on liquid feed samples from commercial pig farms revealed increased relative abundances of lactic acid bacteria (LAB) (*Lactobacillus*, *Weissella* and *Leuconostoc*) and yeasts (*Kazachstania* and *Dipodascus*) between liquid feed in the mixing tank and troughs, consistent with spontaneous fermentation. This was supported by moderate concentrations of biogenic amines, the products of amino acid degradation, which likely contributes to the poorer feed efficiency of liquid-fed pigs. The LAB, *Leuconostoc*, which was associated with spontaneous fermentation in liquid feed, was enriched in the ileal and faecal bacteriome of liquid-fed pigs and correlated with higher feed intake and poorer feed efficiency. This may, in part, explain the poorer feed efficiency of liquid-fed pigs observed in the study. Intensive sanitisation of the liquid feeding system removed pipeline biofilms, improved system hygiene and reduced concentrations of acetic acid, ethanol and biogenic amines in the liquid feed. Bacteriome and mycobiome profiling revealed differences in liquid feed post-cleaning, including predominance of fermentative *Weissella* and *Kazachstania* in the residual trough-sampled feed, however, fermentation-associated metabolites remained below pre-cleaning concentrations at 10 weeks post-cleaning.

Methodological considerations

This thesis expands on the work of O'Meara (2020a), who investigated strategies to improve the microbial quality of liquid feed and to optimise growth of liquid-fed grow-finisher pigs. In fact, samples banked from O' Meara et al.'s studies were used in Chapters 3 (O'Meara et al., 2020b) and 4 (O'Meara et al., 2020c). O'Meara et al.'s work demonstrated the occurrence of unintentional spontaneous (uncontrolled) fermentation in 'fresh' (not deliberately fermented) liquid feed on commercial pig farms. However, these studies were solely culture-based and the impact of liquid feed on the pig gut microbiome was not investigated. For these reasons, and due to the overall lack of DNA sequence-based studies of the liquid feed microbiome, high-throughput amplicon sequencing was selected in this thesis as the appropriate method to profile the bacteriome and mycobiome of liquid feed and the pig gut. Although shotgun metagenomics would provide more insight into taxonomy and the functionality of the microbiome, amplicon sequencing is more cost-effective for large-scale compositional profiling, especially considering the large number of samples analysed here. Additionally, although other microbial groups such as archaea and protists are known to be important components of the pig gut microbiome, this thesis focuses only on the bacterial and fungal communities in liquid feed and the pig gut. Samples previously banked from commercial pig farms were selected in order to profile liquid feed quality in Chapter 3 because a research farm-based study alone would not be representative of different commercial farms. Chapters 4-6, however, required a controlled environment and were therefore performed on a research farm/samples taken from the research farm to minimise variability in experimental conditions when assessing the impact of different experimental diets and of a liquid feeding system sanitisation programme.

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Chapter 1: Literature Review (Part 1): Microbiological services provided by the pig gut microbiome

Cullen, J.T., Lawlor, P. G., & Gardiner, G. E. (2022). Microbiological services delivered by the pig gut microbiome. In M. Bailey & C. Stokes (Eds.), *Understanding Gut Microbiomes as Targets for Improving Pig Gut Health*. Burleigh Dodds Science Publishing Ltd., Cambridge, UK.

1.1 Introduction

The pig gastrointestinal tract (GIT) is a complex and diverse microbial ecosystem inhabited by bacteria, viruses and archaea, as well as eukaryotes including fungi and protists, i.e. the gut microbiota, which, together with their genomes are collectively referred to as the gut microbiome (Ilhan, 2018; Ramayo-Caldas et al., 2020). An increasing body of research has highlighted the fundamental role of the gut microbiome in pig health and growth (Guevarra et al., 2019; Nowland et al., 2019). This chapter will focus primarily on the role of the resident bacterial communities in the pig gut and will explore their relationships, interactions, and contributions to the host. An estimated 100 trillion bacterial cells in the mammalian GIT contribute to host health, with the pig colon alone estimated to contain between 10 billion and 100 billion bacteria per gram of content (Gaskins et al., 2002; Guevarra et al., 2019; Isaacson & Kim, 2012). These microorganisms deliver microbiological services such as prevention of pathogen colonisation and production of VFAs and vitamins from food components that are typically indigestible to the host (Holman et al., 2017). This chapter also examines bacterial quorum sensing (QS) as well as the pig gut antibiotic resistome, and its implications as a reservoir of antibiotic resistance genes (ARGs).

1.2 Pig gut microbiome: abundance and diversity

The co-evolution of gut microbes with pigs has allowed for a synergistic relationship to develop between the host and 500-1000 distinct bacterial species that have adapted to perform a range of beneficial functions related to modulation of pig health (Patil et al., 2019). The pig gut microbiome is highly dynamic and is determined, and subsequently influenced by, several factors including age, diet and antibiotic administration, for example (Niu et al., 2015). This section will serve as an introduction to the pig gut microbiome and will discuss the microbial shifts that occur in the pig GIT from birth to slaughter and along different regions of the tract, as well as recent developments in identifying the core microbiome of pigs.

1.3 Development of intestinal microbiota over the lifetime of a pig

It has long been held that during gestation, the piglet gut is sterile, and that immediately following birth, microbial colonisation begins (Guevarra et al., 2019). However, studies in mice and humans suggest that some *in utero* bacterial colonisation occurs but whether this happens in pigs is currently open to debate (Ardissone et al., 2014; Jiménez et al.,

2008; Nowland et al., 2019). The nature of initial colonisation is influenced by environmental factors including the sow as well as the timing of exposure to different inocula, with repeated compared to single exposures reportedly resulting in different microbiomes (Fouhse et al., 2016).

One of the most crucial periods for pigs is weaning, as around this time the gut microbiota is most susceptible to change (Nowland et al., 2019). This period is characterised by a range of stressors for piglets including separation from the sow and littermates as well as the transition from milk to a solid cereal-based diet (Guevarra et al., 2019). These weaning stressors contribute to disruption of the gut microbiota, termed 'dysbiosis', allowing for the proliferation of pathogenic microorganisms, thereby increasing the incidence of diseases such as diarrhoea and enteritis (Yang et al., 2019).

Sun et al. (2019) found that *Enterobacteriaceae* dominated the faecal microbiota of diarrhoetic piglets during suckling, while *Bacteroidales* family *S24-7 group* was identified as a biomarker of diarrhoetic piglets at the early weaning stage. Furthermore, *Escherichia-Shigella* was identified as the core component of the diarrhoetic piglet microbiota, while *Prevotellacea*e *UCG-003* was the dominant genus in non-diarrhoetic piglets. Yang et al. (2019) also suggested that an alteration in relative abundance of *Escherichia* and *Prevotella* may be associated with pre-weaning diarrhoea.

De Rodas et al. (2018) observed age-related changes in the gut microbiota of pigs from birth to market, including increasing abundances of *Clostridia* and decreasing abundances of *Gammaproteobacteria*. However, at 24 hours post-weaning (21 days of age), there was a significant reduction in *Lactobacillaceae*, followed by a subsequent dramatic increase at day 33. This coincided with the introduction of solid feed and had the greatest impact on gut microbiota composition compared to age, changes in solid feed type, and pig movement (De Rodas et al., 2018). Motta et al. (2019) found that the weaning period resulted in a shift from a high relative abundance of *Bacteroidaceae* and *Enterobacteriaceae*, to a *Prevotellaceae*- and *Ruminococcaceae*-dominated microbiota post-weaning. Functional metagenomic analysis indicated that high concentrations of long-chain fatty acids in the sow's milk may serve as an energy source for *Enterobacteriaceae* in suckling piglets (Motta et al., 2019).

Zhao et al. (2015) found that the ratio of *Firmicutes* to *Bacteroidetes* in the faeces of older pigs (2, 3 and 6 months old) was 10-fold higher than that of piglets at one month old (Figure 1.2). As the pigs matured, they developed a more stable microbiota, in agreement

with previous findings (Nowland et al., 2019; Schmidt et al., 2011). Han et al. (2018) reported that diversity and richness of the gut microbiota decreased with age, especially in finishing pigs. They also found compositional differences, with *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* dominating for the first 42 days post-weaning, followed by *Bacteroidetes*, *Firmicutes* and *Spirochaetes* during the growing stage, and *Bacteroidetes*, *Firmicutes*, and interestingly the archaeal phylum *Euryarchaeota* during the finishing stage. Principal coordinate analysis (PCoA) also shows distinct clustering of pig gut microbiota across development stages (Han et al., 2018; Figure 1.1).

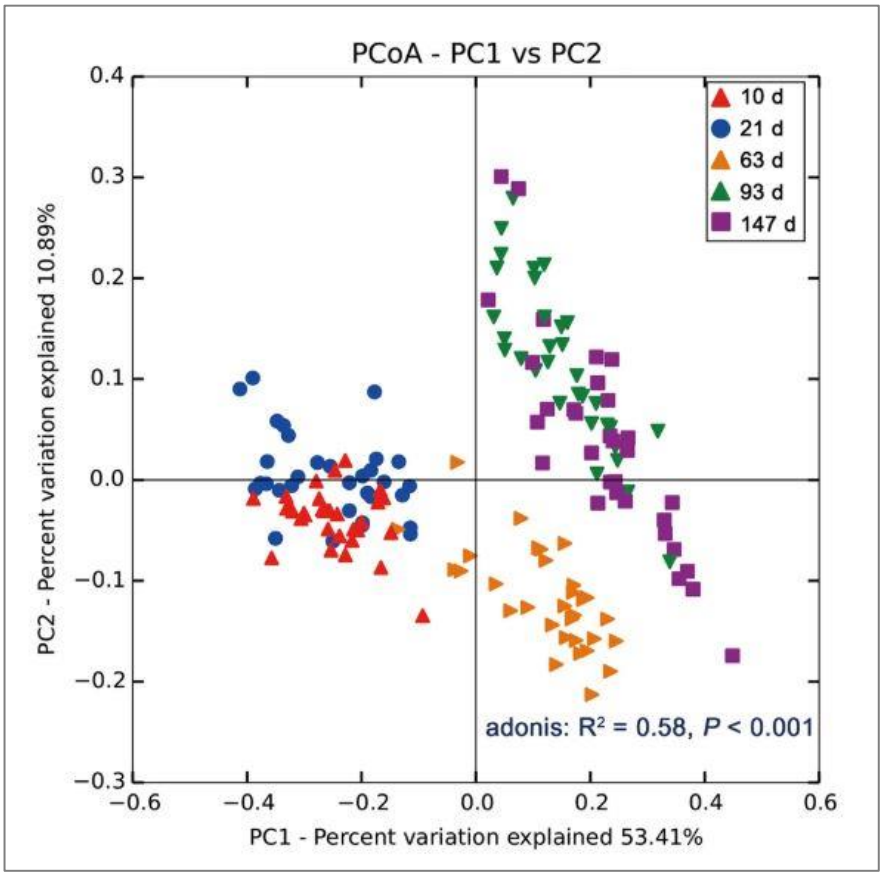

Figure 1.1: Principal coordinate analysis (PCoA) plot of weighted UniFrac distances displaying the diversity of faecal microbiota of commercial pigs (*n* = 32) at various growth stages (pigs weaned at 26 days of age). The effect of growth stage on microbial community was analysed using Adonis statistical tests with 999 permutations. Adapted from Han et al. (2018) distributed under terms of the Creative Commons Attribution 4.0 International License.

Overall, these data demonstrate distinct age-related microbiota composition, with microbiota maturation occurring over time and weaning leading to the most dramatic microbial shifts.

1.4 Core gut microbiome of pigs and variance between intestinal sites

The conditions of the GIT vary from proximal to distal regions and between the mucosa and lumen, resulting in differing bacterial populations (Figure 1.2 and Figure 1.3) (Kelly et al., 2017). Zhao et al. (2015), when investigating whether faecal samples were representative of the intestinal microbiome, found that the dominant phylum in faeces was *Firmicutes*, while *Proteobacteria* predominated in the small intestine (Figure 1.2). However, as expected, the microbial composition of the large intestine was more like that of faeces, in agreement with McCormack et al. (2017).

Zhao et al. (2015) reported that *Proteobacteria* and *Firmicutes* constituted > 70 % and ~20 % of the microbiota in the jejunum and ileum, respectively (Figure 1.2). Conversely, others have reported that *Firmicutes* predominate in the small intestine, with variable proportions of *Bacteroidetes* and *Proteobacteria* (Crespo-Piazuelo et al., 2018; De Rodas et al., 2018; Quan et al., 2018). In the caecum and colon, Zhao et al. (2015) concluded that *Firmicutes* are the dominant phylum, representing > 75 % of the bacterial population followed by *Proteobacteria*; however, Quan et al. (2018) found that the relative abundance of *Bacteroidetes* was as high as 46 % in the caecum.

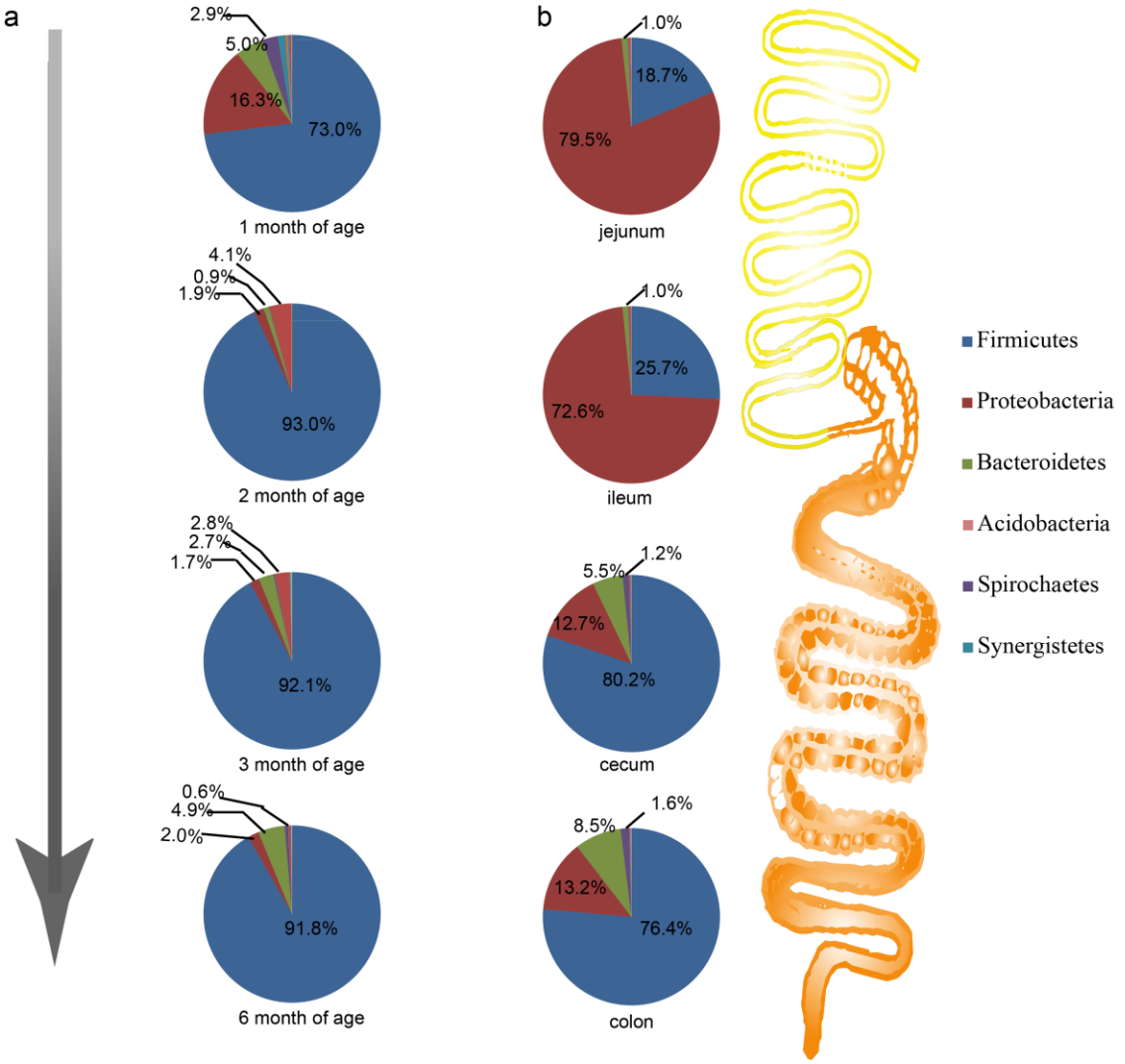

Figure 1.2: Microbial profile within distinct sections of the pig intestinal tract and faeces at phylum level. (a) Faecal microbiota of pigs at 1, 2, 3, and 6 months of age. (b) Microbial profile in the small and large intestines at 6 months of age (slaughter). Adapted from Zhao et al. (2015) distributed under terms of the Creative Commons Attribution 4.0 International License.

An interesting concept that has emerged over the last number of years is whether a 'core' pig gut microbiome exists, independent of age, breed, origin and diet. A meta-analysis carried out by Holman et al. (2017) analysed 20 published datasets of 16S ribosomal ribonucleic acid (rRNA) gene sequences of pig gut and faecal samples, in order to determine if certain bacterial taxa prevailed, irrespective of age, gut location etc. *Firmicutes* and *Bacteroidetes* were the dominant phyla, representing almost 85 % of all 16S rRNA gene sequences detected across all gut locations, with *Proteobacteria* the only other phylum present at all locations. They also found a number of genera that were present in > 90 % of samples including (in order of decreasing relative abundance) *Prevotella, Lactobacillus, Clostridium, RC9 gut group* and *Blautia* (Figure 1.3). Wang et al. (2019) also found *Prevotella* to be the most dominant and the most diverse genus

within the faecal microbiota, particularly after the introduction of solid feed **to** the diet at weaning. Interestingly, *Prevotella* has gained considerable attention recently as a key genus within the pig gut microbiota, having been linked with increased piglet growth rates (Mach et al., 2015).

Lactobacillus is also dominant within the core pig gut microbiome, accounting for up to 15 % of 16S rRNA gene sequences in faeces, independent of age (Niu et al., 2015), and is reportedly the dominant genus found in the stomach (Mann et al., 2014). Holman et al. (2017) also found certain genera to be differentially abundant in specific areas of the pig gut, as summarised in Figure 1.3. Mucosa-associated bacterial populations are represented here, but many studies focus on the lumen contents and/or faeces, as reflected in the sample numbers indicated. This may be an oversight considering that mucosa-associated bacteria are more likely to be autochthonous than taxa found in the digesta which may merely be passing through. Mann et al. (2014) studied the mucosa-associated microbiota of the pig GIT and found a similar composition to that reported by Holman et al. (2017).

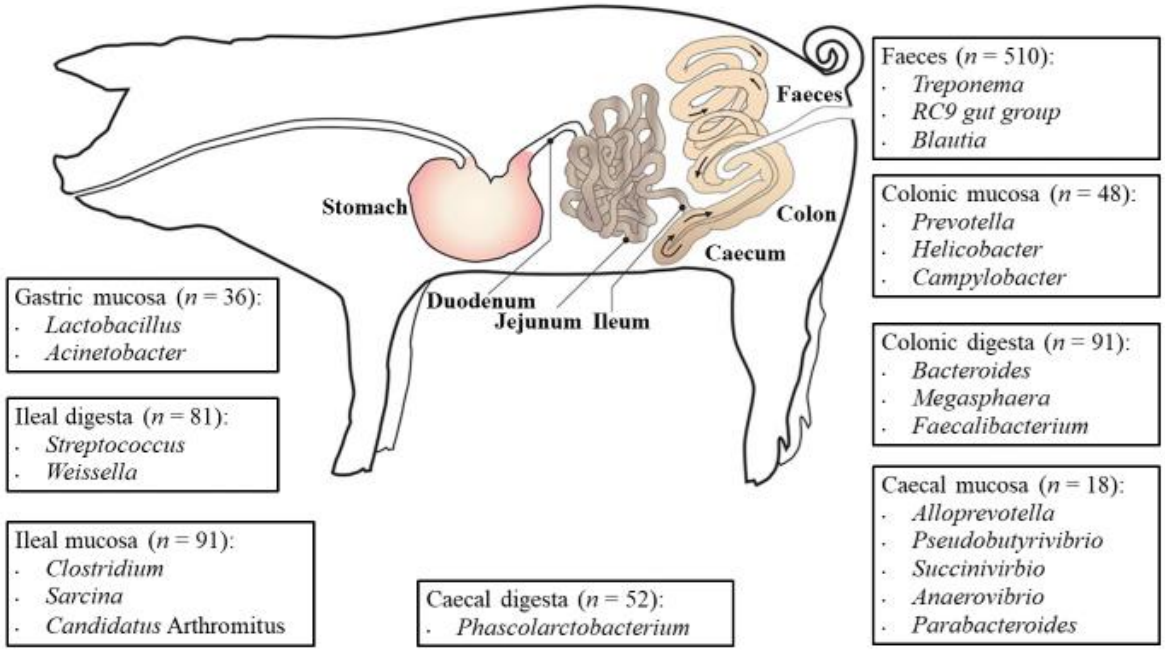

Figure 1.3: Diagram indicating major sections of the pig gastrointestinal tract and direction of movement of digesta in the colon. Boxes detail the differentially abundant genera in each distinct gastrointestinal section as determined by linear discriminant analysis (LDA) with effect size (LEfSe) measurements. Genera with an LDA score (log10) > 4.0 are displayed. Duodenum and jejunum mucosa and digesta samples were excluded from this analysis as sample numbers were insufficient. Adapted from Holman et al. (2017) distributed under the terms of the Creative Commons Attribution 4.0 International License.

A considerable amount of research is still required to elucidate whether a core pig gut microbiome exists. Perhaps, identifying the core functionality of the microbiota, through functional metagenomic and metabolomic studies, may provide a clearer picture, as opposed to identifying the predominant taxa alone. An additional challenge in identifying the core gut microbiome of pigs is that many studies have focused primarily on faecal samples, as outlined above (Guevarra et al., 2018; Han et al., 2018; Kim et al., 2015; Kubasova et al., 2018; Motta et al., 2019) (Figure 1.3). Some of the reasons for this include the relatively high rearing cost and long growth cycle of pigs from birth to slaughter, when compared to poultry, for example, and the ease of obtaining repeated faecal samples from the same pig (De Rodas et al., 2018).

There is also considerable study-to-study variation in the deoxyribonucleic acid (DNA) extraction methods used, the 16S rRNA gene hypervariable region sequenced, and the sequencing platforms employed, all of which are likely to impact the reported microbial composition. In fact, Holman et al. (2017) reported that study-level effects were the strongest predictors of microbiome structure, followed by intestinal location and age, respectively. It should be noted though, that age, among other metadata categories, was also associated with study-level effects as several studies sampled at only one time point.

1.5 Colonisation resistance

As outlined above, early microbial colonisation of the piglet GIT plays a crucial role in establishing the resident microbiome, which subsequently influences host phenotype, nutrient utilisation and immunity (Mulder et al., 2011; Mach et al., 2015; Umu et al. 2017). One of the microbiological services provided by the pig gut microbiome is colonisation resistance. This concept refers to the ability of the commensal microbiota to act as a barrier, thereby offering intestinal protection as a result of direct competition between commensals and potential pathogens, for intestinal niches and the limited nutrients available (Iacob et al., 2019; Lawley & Walker, 2013; Spees et al., 2013). A number of mechanisms of colonisation resistance exist (Fons et al., 2000; Pickard et al., 2017). These include ‘bacterial antagonism’, via the production of bacteriocins and other antimicrobial compounds (Fons et al., 2000; Hu et al., 2018) which will be discussed in Section 1.5. Other mechanisms include competition for nutrients and/or receptor sites along the GIT, generally referred to as ‘competitive exclusion’, as well as microbiota-mediated upregulation of mucin secretion by goblet cells which prevents pathogen binding (Iacob et al., 2019; Liao & Nyachoti, 2017; Sicard et al., 2017; Spees et al., 2013).

These mechanisms of colonisation resistance, among other beneficial pig gut microbiota-mediated microbiological services are summarised in Figure 1.4.

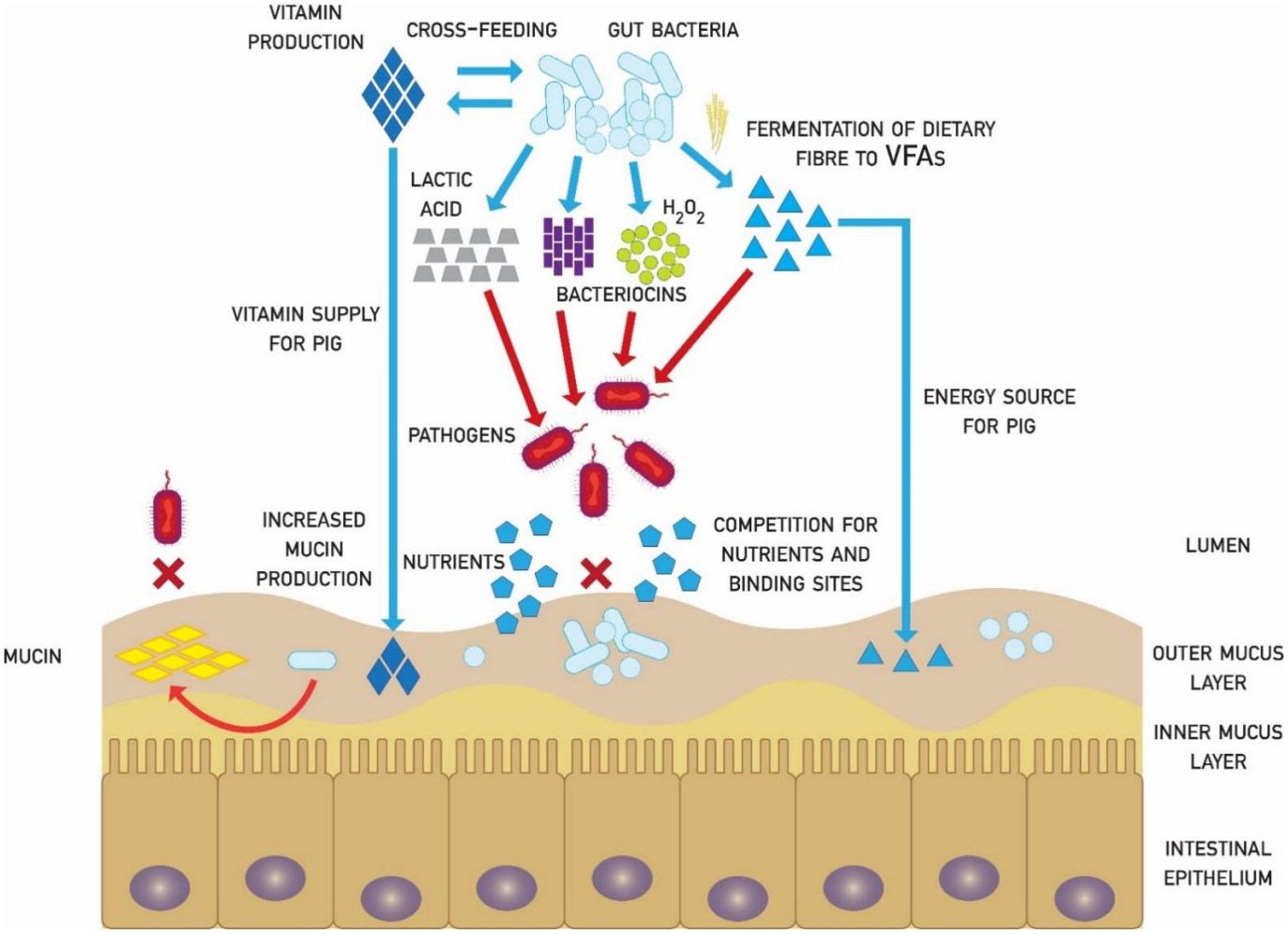

Figure 1.4: Schematic diagram of mechanisms of colonisation resistance and beneficial microbiological services provided by the pig gut microbiome. Straight red arrows denote inhibitory/bactericidal activity; curved red arrows denote stimulatory activity; red crosses denote inhibition of pathogen binding (Credit: Jonathan Brazil).

It should be noted that much of the research on the mechanisms of competitive exclusion to date has been carried out in murine models and refers to the human gut microbiome. However, it is reasonable to assume that similar mechanisms of competitive exclusion occur within the pig gut microbiome, considering the physiological similarities of the GIT, and that pigs are often used as a model for humans (Zhang et al., 2013).

It is widely reported that the resident gut microbiota competitively excludes pathogens by competing for nutritive sources. As outlined by Pereira & Berry (2017), in a stable, mature gut microbiome, all available nutritional niches would be expected to be occupied. Subsequently, new potential colonisers, whether commensal or pathogenic, would have to either outcompete a resident species, colonise a new nutritional niche arising from a change in host diet or take the place of an eliminated resident species, such as in the case of dysbiosis induced through antibiotic treatment.

The metabolic pathways to which commensals have adapted are also a key factor in maintaining colonisation resistance. For instance, some strains of *Escherichia coli* have developed to utilise specific carbon sources that some commensal *E. coli* cannot metabolise. For example, in the presence of two commensal *E. coli* strains, Maltby et al. (2013) demonstrated that enterohemorrhagic *E. coli* **(**EHEC**)** failed to colonise the gut in a mouse model. They hypothesised that this occurred because the commensal strains occupy slightly different nutritional niches to each other, but both use five sugars determined to be necessary for EHEC colonisation, indicating that the commensal *E. coli* had competitively excluded EHEC via direct competition for specific sugars. In addition, one of the commensal *E. coli* strains (Nissle 1917) used in the study by Maltby et al. (2013) has been shown to out-compete *Salmonella* Typhimurium in mouse models due to superior iron uptake ability (Deriu et al., 2013). Maldonado-Gómez et al. (2016) demonstrated that a strain of *Bifidobacterium longum* was capable of colonising and persisting in the human gut, but only in the absence of metabolically similar competitors. If present, these competitors occupied its niche and competitively excluded *B. longum*.

One of the other key mechanisms of competitive exclusion is competition for adhesion sites along the intestinal mucosa (Fons et al., 2000; Monteagudo-Mera et al., 2019) (Figure 1.4). However, much of the research on the mechanisms of pathogen exclusion through competition for binding sites in pigs comes from probiotic studies (Liao & Nyachoti, 2017; Plaza-Diaz et al., 2019; van Tassell & Miller, 2011; Yang et al., 2015). The mucus layer of the mammalian GIT is known to protect against pathogen invasion

by preventing colonisation and aiding in the removal of bacteria by peristalsis (Singh et al., 2018). Although the mechanisms of bacterial adhesion to the gut mucosa are not well understood, it has been proposed to be mediated by a number of surface adhesion proteins such as the mucus-binding protein MUB, fibronectin-binding protein, S-layer protein, and collagen binding protein (Monteagudo-Mera et al., 2019; Singh et al., 2018).

Enterotoxigenic *E. coli* **(**ETEC**)** and several other intestinal pathogens are known to initiate colonisation through surface adhesins, which interact with various receptors on the surface of intestinal epithelial cells in order to mediate bacterial binding (Singh et al., 2018). Resident bacterial communities and pathogens compete for these cell surface receptors for colonisation of the GIT. Competitive exclusion via inhibition of adhesion was first hypothesised by Chan et al. (1985), where human *Lactobacillus* isolates were found to inhibit the adhesion of uropathogenic bacteria to uroepithelial cells *in vitro*. They suggested that lipoteichoic acid was involved in the attachment of *Lactobacillus* to the cells but that steric hindrance most likely played a role in preventing uropathogen attachment (Chan et al., 1985; Reid et al., 1985).

Competitive exclusion cultures (CECs) have been developed for use in pigs to inhibit enteropathogen colonisation. Genovese et al. (2003) administered a caecum-derived mixed bacterial CEC to piglets twice within 24 hours of birth, prior to challenge with *Salmonella* Choleraesuis 48 hours after birth. These piglets shed *Salmonella* at a lower rate and had reduced *Salmonella* counts in the GIT compared to a control group, with effects persisting for up to 10 days post-weaning.

In addition to directly competing for attachment sites, there is also *in vitro* evidence to suggest that members of the commensal microbiota can promote mucin production, thereby enhancing the barrier function of the mucous layer and preventing pathogen binding (Sicard et al., 2017) (Figure 1.4). For example, a well-studied commensal bacterium, *Bacteroides thetaiotaomicron* increased goblet cell differentiation and gene expression related to mucous production in a mouse model (Wrzosek et al., 2013). Although it is difficult to determine the exact mechanisms by which competitive exclusion occurs and there is a lack of data for pigs, it is most likely through a complex combination of competitive interactions between the resident microbiota and pathogens for nutrients and binding sites along the GIT, some of which have been outlined above. The gut microbiome also confers colonisation resistance to the host via a range of other mechanisms, one of which is the production of antimicrobial substances.

1.6 Production of antimicrobial substances

Members of the gut microbiome secrete a wide range of antimicrobial substances capable of altering the composition of the resident microbiota, amongst other functions (Figure 1.4). These bacterial metabolites may be generated either as intermediates or end products (Engevik & Versalovic, 2017) and include bacteriocins, hydrogen peroxide, lactic acid and VFAs. In fact, because of the abundance and diversity of antimicrobials produced by members of the gut microbiome, it is considered a bountiful source of novel antimicrobials for potential therapeutic applications (Garcia-Gutierrez et al., 2019).

1.6.1 Bacteriocins

Bacteriocins are classified as small, heat-stable peptides that are synthesised ribosomally and secreted by bacteria, with narrow- or broad-spectrum bactericidal activity against competing bacteria, to which the producer has "immunity" (Lawley & Walker, 2013; Umu et al., 2017). Although they differ widely in terms of chemical structure and mode of action, many bacteriocins target bacterial cell membrane phosphate groups and disrupt the structural integrity of the membrane by decreasing the potential and/or the pH gradient across the membrane, forming pores which leads to cellular leakage (Engevik & Versalovic, 2017).

Many microorganisms including both Gram-positive and Gram-negative bacteria, as well as certain archaea, produce bacteriocins (Umu et al., 2017). Lactic acid bacteria (LAB) and members of the genus *Bacillus* are known to produce a large number of bacteriocins which have been better characterised than those produced by many other bacterial groups in light of their use as probiotics (Abriouel et al., 2011; Hu et al., 2018; Liao & Nyachoti, 2017; Plaza-Diaz et al., 2019). Therefore, many of the taxa found within the pig gut microbiota are capable of producing bacteriocins, and in fact, a number of bacteriocins produced by porcine gut-derived bacteria have been described in the literature (Barrett et al., 2007; Du Toit et al., 2000; Han et al., 2014; Lin et al., 2020a; O'Connor et al., 2015; O'Shea et al., 2009, 2011, 2013; Robredo & Torres, 2000) (Table 1.1). The range of activity of these bacteriocins can be seen from Table 1.1, with a number of significant pig pathogens (or human pathogens carried by pigs), such as *E. coli, Salmonella,* and methicillin-resistant *Staphylococcus aureus* (MRSA), amongst the targets. This highlights the potential microbiological service offered by bacteriocin-producing members of the pig gut microbiome.

It is important to note though that *in vitro* production of bacteriocins by gut-derived bacteria does not necessarily imply production in the gut or that they are mediators of anti-infective activity. However, a few studies to date have demonstrated production of bacteriocins *in vivo*. For example, Corr et al. (2007) showed in a mouse model of *Listeria* infection that the bacteriocin-producing strain *Lactobacillus salivarius* UCC118 protected the mice, while a non-bacteriocin-producing mutant did not, demonstrating that the anti-infective activity was mediated primarily by the bacteriocin. Following on from this, Riboulet-Bisson et al. (2012) showed, via administration of the wild-type alongside a mutant lacking bacteriocin production, that *Lb. salivarius* UCC118 had a 'significant but subtle' impact on the pig gut microbiota, including inhibition of potentially pathogenic Gram-negative taxa, mediated, at least partially, by bacteriocin production.

Table 1.1: Range of bacteriocin-producing bacteria isolated from the pig gut or faeces and their spectra of inhibition.

Strain (bacteriocin produced)	Source of strain	Bacteria inhibited	References
Lactobacillus animalis 30a-2[1]	Pig ileal mucosa	Methicillin-resistant *Staphylococcus aureus* (16 isolates) *Bacillus cereus* *Listeria monocytogenes* *Acinetobacter baumannii* *Escherichia coli* K12 *Pseudomonas aeruginosa* including MDR[2] *Salmonella* Choleraesuis *Salmonella* Enteritidis *Salmonella* Typhimurium S*higella flexneri* *Shigella sonnei* *Yersinia enterocolitica* MDR *Acinetobacter baumannii* Extended-spectrum β-lactamase *Escherichia coli*	(Lin et al., 2020)
Lactobacillus salivarius DPC6005 (Salivaricin P and Bactofencin A)	Pig caecum	*Enterococcus faecalis* *Enterococcus faecium* *Lactobacillus casei* *Lactobacillus helveticus* *Lactobacillus delbrueckii* subsp. *bulgaricus* *Leuconostoc* sp. *Listeria innocua* *Pediococcus pentosaceus*	(Barrett et al., 2007; O'Connor et al., 2015; O'Shea et al., 2009, 2011, 2013)
Streptococcus hyointestinalis DPC6484 (Nisin H)	Pig caecum	*Bacillus cereus* *Enterococcus faecalis* *Listeria innocua*	

		Listeria monocytogenes *Staphylococcus aureus*	
Enterococcus faecium BFE 1072 (Enterocin L50A and L50B)	Pig faeces	*Lactobacillus helveticus* *Lactobacillus acidophilus* *Pediococcus pentosaceus* *Leuconostoc cremoris* *Enterococcus faecalis* *Listeria monocytogenes* *Clostridium sporogenes* *Clostridium tyrobutyricum* *Propionibacterium acidopropionici*	(Du Toit et al., 2000)
Enterococcus faecalis AP 45[1]	Pig faeces	*Clostridium perfringens* *Enterococcus faecalis* *Lactobacillus brevis* *Lactobacillus delbruekii* *Lactobacillus plantarum* *Listeria monocytogenes*	(Han et al., 2014)
Enterococcus faecalis AP 216[1]	Pig faeces	*Clostridium perfringens* *Listeria monocytogenes*	
Lactobacillus salivarius X13[1]	Pig faeces	Methicillin-resistant *Staphylococcus aureus* *Staphylococcus epidermidis* (Methicillin-resistant and susceptible) *Micrococcus luteus* *Lactobacillus salivarius* *Lactobacillus fermentum* *Lactobacillus paracasei* *Pediococcus acidilactici* *Pediococcus pentosaceus*	(Robredo & Torres, 2000)

[1] Bacteriocin responsible for antibacterial activity has not been defined/identified
[2] MDR - Multidrug-resistant

A study by Hu et al. (2018) highlighted the importance of gut microbiota-derived bacteriocins in maintaining gut health in pigs. They identified two bacteriocin-producing *Lactobacillus* strains, *Lb. gasseri* LA39 and *Lb. frumenti,* as mediators of the diarrhoea resistance conferred by faecal microbiota transplantation (FMT) from diarrhoea-resistant to susceptible piglets. Moreover, they demonstrated that the diarrhoea resistance was facilitated by the bacteriocin gassericin A, which was found to be essential for modulating diarrhoea-associated fluid absorption and secretion across the intestine through binding to Keratin 19 on the plasma membrane of the host's intestinal epithelial cells (Hu et al., 2018). The results also indicated that this plasma protein may mediate signal transduction from gassericin A to the cell, with the bacteriocin acting as a signalling molecule. There is also other evidence to show that bacteriocins may act as signalling molecules, either from one bacterium to another via QS or to host cells (Dobson et al., 2012).

It is also interesting to note that many of the pig gut microbiota-derived bacteriocin-producing strains identified to date also inhibit closely related genera/species (Table 1.1). This is a common finding for bacteriocin-producers, most likely due to the fact that bacteriocins are thought to confer a competitive advantage on producing strains by enabling them to colonise a particular niche. This potentially occurs in the pig GIT, with Walsh et al. (2008) concluding that one of the strains within a 5-strain *Lactobacillus*/*Pediococcus* probiotic mixture predominated in the ileum, possibly due to the production of salivaricin P, a bacteriocin active against *Listeria* but also other *Lactobacillus* species (Barrett et al., 2007).

Therefore, when considering the microbiological services provided by the gut microbiota of pigs, it is not only the anti-pathogen activity of bacteriocins produced by members of the gut microbiome that is important, but also their role in aiding colonisation and their physiological activity in the gut. Overall, the findings outlined here highlight the significant contribution that bacteriocin secretion from the commensal microbiome plays in conferring colonisation resistance and promoting the health of pigs.

1.6.2 Hydrogen peroxide

Hydrogen peroxide (H_2O_2) which is produced by many microbes, is a reactive oxygen species (ROS) capable of creating breaks in the phosphate backbone of DNA, which leads to the release of nucleotides, thereby inhibiting DNA replication (Engevik & Versalovic, 2017; Finnegan et al., 2010; Gough & Cotter, 2011). Additionally, the dissociation of H_2O_2 produces other ROS such as hydroxyl radicals which can attack the methyl group

of thymine, resulting in DNA damage (Engevik & Versalovic, 2017; Li et al., 2020). There is a lack of information on H_2O_2 production by members of the pig gut microbiota and research into its role within the pig gut microbiome.

However, many bacterial taxa found within the pig gut microbiota, for example, members of the LAB (which are all catalase-negative), can produce H_2O_2, leading to inhibition of pathogenic bacteria that lack catalase, the enzyme responsible for breakdown of H_2O_2 (Vieco-Saiz et al., 2019) (Figure 1.4). For example, Lin et al. (2020b) isolated a strain of *Lb. animalis* from pig ileal mucosa which had antimicrobial activity against a range of pathogens including *S. aureus*. Upon addition of catalase, *Lb. animalis* lost its *S. aureus* inhibitory activity, indicating that it was mediated, at least in part, by H_2O_2. It should be noted that *S. aureus* usually produces catalase; however, protease was added to degrade any antimicrobial peptides and therefore most likely inactivated *S. aureus*-secreted catalase (Lin et al., 2020). However, whether this gut-derived *Lb. animalis* has any H_2O_2-mediated anti-pathogen activity *in vivo* remains to be investigated.

Anaerobic bacteria generally lack catalase and are therefore usually more sensitive to H_2O_2. In addition, Gram-negative bacteria are more sensitive compared to Gram-positives (Engevik & Versalovic, 2017). Bacterially produced H_2O_2 is known to act synergistically with lactic acid, the antimicrobial properties of which will be discussed in Section 1.6.3. Lactic acid disrupts the outer membrane of Gram-negative bacteria, rendering the cells sensitive to H_2O_2 and other antimicrobial substances (Engevik & Versalovic, 2017; Garcia-Gutierrez et al., 2019).

In addition to the inter-bacterial interactions mediated by ROS, the host gut epithelium plays a key role in influencing the microbiome via production of antimicrobials and ROS which may act as signalling molecules in the communication between gut microbiota and the intestinal mucosa (Berstad et al., 2016). The enzyme dual oxidase 2 (Duox2) produces H_2O_2 in the GIT, and its expression is induced by the microbiome via different signalling pathways (Sommer & Bäckhed, 2015).

However, it should be noted that some inflammatory diseases of the GIT are associated with high levels of H_2O_2 (Basu Thakur et al., 2019; Garcia-Gutierrez et al., 2019). In addition, H_2O_2 production is not limited to beneficial commensals; pathogenic bacteria such as *Streptococcus pneumoniae* are also thought to produce H_2O_2 to inhibit competing organisms (Engevik & Versalovic, 2017). In fact, Erttmann & Gekara (2019) have shown

that H_2O_2 released by *S. pneumoniae* inhibits inflammasome-dependent innate immunity, and thus may contribute to pathogen colonisation.

1.6.3 Lactic acid

Lactic acid is an organic acid and is a major metabolic end-product of carbohydrate fermentation by LAB, the group of Gram-positive aerotolerant anaerobic bacteria named as such due to their fermentative metabolism (Tannock, 2004; Yang et al., 2015). Lactic acid bacteria are classified into three different groups: obligately homofermentative which produce lactic acid as their sole metabolite (e.g. *Lb. acidophilus*, *Lb. delbrueckii*, *Lb. salivarius*), facultatively heterofermentative (e.g. *Lb. plantarum*, *Enterococcus*, *Lactococcus*, *Pediococcus*, *Streptococcus*) and obligately heterofermentative (e.g. *Leuconostoc*, *Weissella*), which generate less lactic acid but produce other end-products, including acetic acid, formic acid, ethanol and carbon dioxide (Du Toit et al., 2001; Endo & Dicks, 2014).

Lactobacillus alone, many species of which are homofermentative, has been reported to account for up to 15 % of the pig faecal bacterial community (Niu et al., 2015). Hence, a relatively large quantity of lactic acid can be assumed to be produced by the pig gut microbiota. For instance, in pigs fed a dry diet, lactic acid concentrations in the stomach are ~70 mmol kg^{-1} while pigs fed fermented liquid feed can have concentrations as high as 120 mmol kg^{-1}, with a decreasing trend observed along the GIT, in both cases (Højberg et al., 2003). Lactic acid production in the stomach of suckling and newly weaned pigs is particularly relevant. At this time, the pig has a poorly developed ability to produce gastric acid and relies on the fermentation of lactose to lactate to maintain a low pH in the stomach, which is the first line of defence against ingested pathogens (Lawlor et al., 2020).

Lactic acid is known to inhibit the growth of, and also to directly kill, pathogens (Figure 1.4). Wang et al. (2015) determined, *in vitro*, that exposure to 0.5 % lactic acid for 1 hour was sufficient to completely inactivate the Gram-negative pathogens *Salmonella* Enteritidis and *E. coli*, while *L. monocytogenes* (Gram-positive) required 2 hours of exposure. However, lactic acid does not generally affect host epithelial cells due to the secretion of bicarbonate by the mucus layer, creating a pH gradient with a pH close to neutral (Allen & Flemström, 2005; Vieco-Saiz et al., 2019).

Apart from acidification of the gut, the antimicrobial effects of lactic acid produced by the gut microbiota are achieved through several mechanisms. Alakomi et al. (2000) demonstrated that lactic acid effectively permeabilises the outer membrane of Gram-negative bacterial cells, thereby inducing lipopolysaccharide (LPS) release and rendering the cell susceptible to antimicrobial substances including lactic acid itself. Lactic acid can also penetrate the cytoplasmic membrane of bacteria in its undissociated form. Once inside the cell, the higher cytosolic pH causes the acid to dissociate into lactate, releasing protons, which reduces intracellular pH, disrupting enzymatic activity, protein function and DNA structure (Stanojević-Nikolić et al., 2016; Suiryanrayna & Ramana, 2015).

In addition, in order to counteract the low pH, the cell must use adenosine triphosphate (ATP) to pump protons out of the cell, which depletes cellular energy and upon prolonged exposure to lactic acid, this can result in cell death (Suiryanrayna & Ramana, 2015). Another antimicrobial mechanism of lactic acid involves inhibition of substrate transport as a result of the aforementioned changes in membrane permeability. In addition, the changes in pH within the cell can suppress the oxidation of the co-enzyme nicotinamide adenine dinucleotide (NADH) which is critical for fermentation and the electron transport chain during cellular respiration and thus can lead to death of the bacterium (Stanojević-Nikolić et al., 2016).

As mentioned in Section 1.6.2, lactic acid also acts synergistically with other antimicrobial substances including H_2O_2 and bacteriocins to inhibit the growth of pathogens (Atassi & Servin, 2010; Engevik & Versalovic, 2017). This is likely the result of the outer membrane-permeabilising activity of lactic acid which renders the cell susceptible to the antimicrobial action of H_2O_2, which is exacerbated by the pH-associated damage mediated by lactic acid. In addition to the antimicrobial properties of lactic acid, the associated reduction in gastric pH due to the high abundance of *Lactobacillus* in the pig stomach, particularly in the *Pars oesophagea*, may also increase the activity of pepsin, thereby enhancing protein utilisation (De Witte et al., 2019; McGillivery & Cranwell, 1992; Suiryanrayna & Ramana, 2015). This is particularly important in suckling and newly weaned pigs, as they have insufficient gastric acid production, as outlined above. An additional beneficial effect of lactic acid is that lactate can be converted by members of the gut microbiota, into butyrate, the beneficial properties of which will be discussed in Sections 1.6.4 and 1.8 (Esquivel-Elizondo et al., 2017).

1.6.4 Volatile fatty acids

Short-chain fatty acids (SCFAs), particularly acetate (C2), propionate (C3) and butyrate (C4), are the major VFAs produced by the gut microbiota, and therefore will be the focus of this section (Figure 1.4). They are produced primarily in the large intestine of hindgut fermenters including pigs, in which they have been estimated to contribute between 10-25 % of basal energy requirements (Agyekum, 2016; Bergman, 1990; Nakatani et al., 2018), which will be discussed in Section 1.8.1. Short-chain fatty acids are carboxylic acids, generally classified as having less than 6 carbon atoms, produced in the gut lumen by bacterial fermentation of primarily undigested dietary carbohydrates. Short-chain fatty acids concentrations are generally highest in the proximal colon, where most fermentable substrates are available, with a decline towards the distal colon (Liu et al., 2018; Venegas et al., 2019; Yoon et al., 2018).

Butyrate is mostly produced by *Firmicutes* in the colon, while acetate and propionate are produced mainly by members of the phylum *Bacteroidetes* (Iacob et al., 2019; Venegas et al., 2019). *Clostridium*, *Blautia*, and *Ruminococcus* (*Firmicutes*) typically produce butyrate from acetate through the butyryl coenzyme A (CoA): acetate CoA transferase pathway. *Prevotella* (*Bacteroidetes*) among other genera, produce acetate, and therefore act as an energy source for butyrate producers via a process known as cross-feeding (Holman et al., 2017). Additionally, as previously mentioned, butyrate can be formed from lactate; specifically from the conversion of lactate to pyruvate through either the butyrate kinase or butyryl-CoA: acetate-CoA transferase pathways (Esquivel-Elizondo et al., 2017). Cross-feeding also occurs here, as lactate is a major end-product of many LAB found within the pig gut, as outlined above. While SCFAs have a range of functions in the host (Sun & O'Riordan, 2014; Venegas et al., 2019) (see Section 1.8), this section will focus on their antimicrobial properties.

Short-chain fatty acids directly acidify the GIT, aiding in colonisation resistance (Iacob et al., 2019). Like lactic acid, the non-ionised form of SCFAs can exhibit antibacterial activity once inside the bacterial cytoplasm. Upon entry, dissociation of the acid leads to an accumulation of protons, resulting in pH reduction and subsequent disruption of the transmembrane proton motive force. Additionally, the dissociation of acids results in a build-up of SCFA anions which interferes with osmotic balance. The combination of these factors ultimately leads to disruption of critical cellular processes including ATP generation, resulting in death of the bacterial cell (Sun & O'Riordan, 2014).

Jacobson et al. (2018) showed that the anti-*Salmonella* activity of *Bacteroides* was mediated by propionate which directly inhibited growth *in vitro* via disruption of intracellular pH. Other pig pathogens that are susceptible to the antibacterial effects of VFAs include *E. coli*, *Salmonella* spp., *Clostridium perfringens* and *Campylobacter coli* (Beier et al., 2018; Gómez-García et al., 2019). Gómez-García et al. (2019) determined the minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of propionic acid and sodium butyrate against some of these pathogens (Table 1.2; the lower the values the more active the compound). Propionic acid had a more pronounced inhibitory and bactericidal effect on all tested pathogens compared to sodium butyrate; however, both acids were inhibitory as well as bactericidal. Gómez-García et al. (2019) reported MIC_{50} values of 1,200 and 125,000 ppm for propionic acid and sodium butyrate, respectively, against *Salmonella* (Table 1.2), which compares well to the MIC of 3,750 ppm reported for butyric, propionic, and valeric acids against poultry-derived *Salmonella* (Lamas et al., 2019).

Table 1.2: Antimicrobial activity (MIC_{50}[1], MBC_{50}[2] and MBC_{50}/MIC_{50} ratio[3]) of propionic acid and sodium butyrate against pig pathogens. Adapted from Gómez-García et al. (2019) distributed under the terms of the Creative Commons Attribution 4.0 International License.

		Propionic acid (ppm)	Sodium butyrate (ppm)
E. coli	MIC_{50}	1,200.0	50,000.0
	MBC_{50}	9,600.0	125,000.0
	MBC_{50}/MIC_{50}	8.0	2.5
Salmonella spp.	MIC_{50}	1,200.0	125,000.0
	MBC_{50}	2,400.0	125,000.0
	MBC_{50}/MIC_{50}	2.0	1.0
C. perfringens	MIC_{50}	2,400.0	31,250.0
	MBC_{50}	2,400.0	62,500.0
	MBC_{50}/MIC_{50}	1.0	2.0

[1] MIC_{50} = lowest concentration that inhibited the growth of 50 % of the strains of each bacterial species tested; [2] MBC_{50} = MBC was the lowest concentration which kills 99.9 % or more of the bacteria in the original inoculum (less than 5 colonies). Median of the MBC (MBC_{50}) was estimated for each bacterial species. [3] MBC_{50}/MIC_{50} ratio = $^{3}MBC_{50}/MIC_{50}$.

Interestingly, SCFAs are also known to help maintain the integrity of intestinal epithelial tight junctions. By decreasing intestinal permeability in this way, they aid in the prevention of bacterial translocation across the gut barrier, thereby preventing infection (Kelly et al., 2015). Overall, the findings outlined here indicate that the production of certain VFAs by the pig gut microbiota may have a pronounced impact on colonisation resistance via antimicrobial activity against pathogens.

1.7 Production of enzymes

The pig gut microbiome contributes to host metabolism by providing a plethora of enzymes that the host does not produce. Many of these enzymes are essential for the digestion of complex polysaccharides (Mohammed & Guda, 2015). This section will focus primarily on the enzymatic capacity of the pig gut microbiota for dietary fibre digestion. In commercial pig production, dietary carbohydrates account for 60-70 % of total energy intake (Bach Knudsen et al., 2012). Specific microbial taxa have developed specialised enzyme-catalysed metabolic pathways for nutrient digestion and energy harvest from these host-indigestible polysaccharides, thereby providing an indispensable service to the host (Wang et al., 2019). The majority of these dietary fibres, such as resistant starch, arabinoxylan and β-glucan are fermented in the proximal colon, leading to the production of SCFAs which are used as an energy source by the pig, in addition to having a range of benefits for host health (Tiwari et al., 2019) (see Sections 1.6.4 and 1.8; Figure 1.4).

Evidence of how the gut microbiome provides a service to the host via production of enzymes comes from studies comparing the microbiota of suckling versus weaned pigs. It has been widely reported that the transition from sow's milk to solid feed promotes an increase in the relative abundance of plant polysaccharide-degrading *Prevotellaceae* and *Ruminococcaceae*, with a concomitant decrease in the abundance of milk glycan-degrading *Bacteroidaceae* and *Enterobacteriaceae* (Chen et al., 2017; Frese et al., 2015; Motta et al., 2019; Wang et al., 2019). This diet-associated adaptation of gut microbial enzymatic activity is also evidenced by a study which utilised 16S rRNA gene sequencing and whole-metagenome shotgun sequencing to examine compositional and functional differences within the faecal microbiome of nursing versus weaned piglets (Guevarra et al., 2018).

Through functional annotation of sequence reads, they found that genes mapped to metabolism of carbohydrates such as xylose and mannose, as well as genes for L-rhamnose utilisation were more prevalent within the gut microbiome of weaned piglets, associated with increased relative abundances of *Lactobacillus* and *Prevotella*. This was expected as these sugars are the end-products of non-starch polysaccharide (NSP) hydrolysis and are present in solid feed ingredients in post-weaning diets such as soybean meal and cereals. Conversely, the microbiome of the nursing piglets was enriched in genes associated with lactose and galactose utilisation (lactose and galactose being two

of the main sugars present in sows' milk), along with increased relative abundance of *Bacteroides* (Guevarra et al., 2018).

The degradation of simple and complex carbohydrates is generally catalysed by three broad enzyme classes: glycoside hydrolases (GHs), carbohydrate esterases (CEs) and polysaccharide lyases (PLs), collectively known as carbohydrate active enzymes (CAZymes). These CAZymes are further categorised into families and sub-families in the CAZy database (Bhattacharya et al., 2015). Wang et al. (2019) used *de novo* metagenomic binning to reconstruct 360 high-quality genomes as a metagenomic reference for the pig gut microbiome. This metagenomic reference was used against the CAZy database to predict carbohydrate metabolism within the faecal microbiome of pigs, fed six experimental diets from weaning to 21 days post-weaning. This study provided many insights into the enzymatic capacity of the pig gut microbiome in relation to carbohydrate metabolism. It showed that the microbial communities responsible for degrading starch, fructans and lactose in the post-weaning piglet are substantially different from those within the human microbiome. *Firmicutes* and *Bacteroidetes* were found to use different starch-degrading systems. *Firmicutes* used an extracellular 1,4-alpha-glucan branching enzyme (*GlgB*) and pullulanases (*Amy12*), with the majority carrying only the *GlgB* gene. *Bacteroidetes,* on the other hand, harboured multiple genes for extracellular and periplasmic starch degradation (Wang et al., 2019). *Firmicutes* and *Bacteroidetes* also harboured distinct enzymes for fructan hydrolysis, with the former using intracellular β-fructofuranosidase and extracellular fructansucrases and the latter, fructan by β-2,6-endo-fructanases. Most of the bacterial genomes encoding lactose degradation within the pig gut microbiome (the majority of which are *Firmicutes* including *Lactobacillus*, *Subdoligranulum* and *Ruminococcus)* hydrolyse lactose by intracellular GH2 β-galactosidase or GH42 β-galactosidase (Wang et al., 2019).

These findings highlight the diversity of the enzymatic repertoire of the pig gut microbiome and its key role in nutrient utilisation in pigs. Other metagenomic studies of the pig gut microbiome have revealed interesting FE-associated findings, linked with the enzymatic and metabolic capacity of the pig gut microbiome. For example, unsurprisingly, Quan et al. (2020) reported that the pig caecum and colon had higher polysaccharide-metabolising capacity compared to the ileum. Additionally, taxa that were more abundant in the caecum of highly feed efficient pigs had a greater abundance of genes associated with polysaccharide and protein metabolism pathways, in agreement with the findings of Tan et al. (2017).

McCormack et al. (2017) found that some of the predicted pathways at higher relative abundance in the ilea of low RFI (high FE) pigs were related to biosynthesis of amino acids. In a more recent study they found that most of the enriched predicted pathways in the feed efficient pigs were associated with core metabolism, including carbohydrate and nucleotide metabolism (McCormack et al., 2019).

In summary, the pig gut microbiome provides the host with an indispensable contribution to the metabolism of dietary constituents, in particular fibre, providing an abundance of critical enzymes that are not expressed by the host. Members of the gut microbial community have developed specialised enzyme-catalysed metabolic pathways that are critical for the promotion and maintenance of host health and productivity.

1.8 Benefits of volatile fatty acids (apart from antimicrobial activity)

As detailed in Section 1.7, dietary fibre in the pig GIT is resistant to degradation by endogenous host enzymes but can be partially or completely fermented by the hindgut microbiota to produce VFAs that play an important role in colonisation resistance (Figure 1.4). They are also a key energy source for the host and are involved in regulation of host metabolism, immune modulation and cell proliferation (Mohammed & Guda, 2015; Wang et al., 2019; Zhao et al., 2020). These services will be discussed here.

1.8.1 Contribution to host metabolism: energy source for colonocytes

The majority of SCFAs are produced in the large intestine of pigs, and are absorbed and used as an energy source for the pig, with an estimated 95 % of those produced by the luminal microbiota absorbed by the mucosa and the remaining 5 % excreted in the faeces (den Besten et al., 2013b; Nakatani et al., 2018). Absorption of SCFAs across the apical membrane of colonocytes occurs via two main mechanisms: passive diffusion of the undissociated acid and SCFA transporter-mediated active transport of the dissociated form. Short-chain fatty acid transporters include hydrogen-coupled monocarboxylate transporter isoform 1 (MCT1), and sodium-coupled monocarboxylate transporter 1 (SMCT1) (Engevik & Versalovic, 2017; Liu et al., 2018).

Despite being the least abundant of the three aforementioned main SCFAs, butyrate is the primary energy source for colonocytes, with as much as 90 % of butyrate metabolised by these cells (Bedford & Gong, 2018; Rowland et al., 2018; Venegas et al., 2019). Colonocytes have a higher affinity for butyrate compared to acetate and propionate. A large proportion of butyrate is metabolised through the oxidation pathway resulting in the

production of acetyl co-enzyme A (CoA) following several intermediate steps. Measurements in isolated colonocytes have shown that they obtain up to 70 % of their energy supply from SCFA oxidation (Astbury & Corfe, 2012; den Besten et al., 2013b).

Donohoe et al. (2011) demonstrated *in vitro* that the colonocytes of germ-free mice exhibited an energy-deficient state characterised by decreased expression of metabolic enzymes involved in the tricarboxylic acid (TCA) cycle, resulting in decreased oxidative phosphorylation and ATP levels. Upon introduction of the butyrate-producing strain *Butyrivibrio fibrisolvens*, mitochondrial respiration was restored, preventing autophagy, indicating that microbially-derived butyrate acted as a direct energy source for colonocytes (Donohoe et al., 2011).

den Besten et al. (2013) found that mice infused with labelled SCFAs utilised 62 % of propionate as a substrate for gluconeogenesis, with glucose synthesis from propionate accounting for almost 70 % of total glucose production, with acetate and butyrate acting as substrates for palmitate and cholesterol in the liver (den Besten et al., 2013a; LeBlanc et al., 2017). Although these data were not generated in pigs, they indicate that VFAs produced by the pig gut microbiota, particularly acetate, propionate, and butyrate, play an intrinsic role in host metabolism, particularly as an energy source for colonocytes.

1.8.2 Other beneficial effects on gut health

Volatile fatty acids also exhibit a wide range of additional intestinal health-enhancing properties in the pig gut. Literature regarding the role of acetate and propionate in pigs is less abundant compared to butyrate, for which there is a broad range of research focusing on its impacts in the GIT. These impacts include gut health-promoting properties such as anti-inflammatory and antioxidant roles and improved intestinal morphology and immunomodulatory capacity, many of which are related to regulatory effects on host gene expression (Bedford & Gong, 2018; Tugnoli et al., 2020; Xiong et al., 2016).

Butyrate and to a lesser extent, propionate, are known to function as epigenetic substances, acting as histone deacetylase (HDAC) inhibitors and hence may modulate disease and immune homeostasis, altering the expression of many genes with diverse functions, including cell proliferation, apoptosis, and differentiation (Li et al., 2018; Marks et al., 2000; Vinolo et al., 2011). HDACs remove the acetyl groups from histones which results in condensed and transcriptionally inactive chromatin. However, HDAC inhibitors suppress this activity and can result in hyper-acetylation of histones which is

thought to increase accessibility of the transcriptional machinery to promote gene transcription, and therefore may have a profound impact on gene expression (Bedford & Gong, 2018; Koh et al., 2016).

Due to the offensive odour of butyrate and its potential absorption in the upper GIT, alternative forms, such as sodium butyrate and butyrate glycerides are often fed to pigs (Bedford & Gong, 2018). Feng et al. (2018) found that a sodium butyrate-supplemented diet alleviated diarrhoea symptoms and decreased intestinal permeability in early-weaned piglets without impacting growth. From experiments with the Caco-2 epithelial cell line, the mechanism was suggested to be due to upregulation of tight junction proteins, including claudin-3 and occludin (Feng et al., 2018).

Many other studies in pigs have reported similar improvements in gut barrier function and intestinal health as a result of butyrate supplementation (Wang et al., 2018; Zhong et al., 2019). Diao et al. (2019) showed that intra-gastric administration of a mixture of acetate, propionate and butyrate increased SCFA concentrations in both sera and digesta, and increased expression of occludin and claudin-1 genes in the duodenum and ileum, indicating improved barrier function. Moreover, intestinal morphology was also improved, with increased villus height observed in the jejunum and ileum, and increased villus height to crypt depth ratio found in the duodenum and jejunum, and this was associated with an increase in nutrient digestibility.

In summary, bacterially-derived VFAs, particularly butyrate, acetate and propionate contribute significantly to host metabolism, with butyrate serving as the primary energy source for colonocytes in the pig gut, as well as performing numerous health-promoting functions from regulation of gene expression and gut tissue development to immune modulation and disease prevention. The production of VFAs by the pig gut microbiome exemplifies the mutualistic relationship that exists between the resident gut microbiota and the host; commensals thrive on substrates provided by the host, while the host benefits from a range of microbially-derived regulatory, metabolic and immunomodulatory services.

1.9 Production of vitamins

Vitamins are essential organic micronutrients that are critical for cellular function, primarily required as co-enzymes for nutrient metabolism, most of which the host itself cannot synthesise. Pig diets are, therefore, always supplemented with vitamin premixes, although many vitamins are synthesised endogenously by the pig gut microbiome, and

therefore, may not need to be supplemented in the diet (Engevik & Versalovic, 2017; Gaudré & Quiniou, 2009; NRC, 2012) (Figure 1.4). Bacterially synthesised vitamins of note include fat-soluble vitamin K and water-soluble B-group vitamins including biotin (B_7, B_8 or H), cobalamin (B_{12}), folate (B_{11}, B_9 or M), niacin (B_3), panthothenate (B_5), pyridoxine (B_6), riboflavin (B_2), and thiamine (B_1) (Engevik & Versalovic, 2017; Rowland et al., 2018). This section will review the services that the pig gut microbiome provides to the host via endogenous production of vitamins.

1.9.1 Production of vitamin K

Vitamin K is a general term used for a group of fat-soluble compounds that are essential for the conversion of inactive blood clotting factors into biologically active compounds. It may also play a role in calcium metabolism, which requires vitamin K-dependent proteins (Akbari & Rasouli-Ghahroudi, 2018; National Research Council, 2012). In plants, vitamin K exists as phylloquinone (vitamin K_1), while bacteria synthesise a family of compounds known as menaquinones (vitamin K_2) which act as electron carriers during cellular respiration (Dairi, 2009; Hiratsuka et al., 2008; NRC, 2012). Synthetic forms of menadione (vitamin K_3) are often used as vitamin K supplements in pig feed (European Food Safety Authority, 2014). It has been reported that vitamins synthesised by the gut microbial community are mostly absorbed in the colon, with dietary vitamins being absorbed primarily in the small intestine (LeBlanc et al., 2013).

Rowland et al. (2018) reviewed several studies examining vitamin K deficiency in animal models including a study by Gustafsson et al. (1962) in which inoculation of germ-free vitamin K-deficient rats with either *E. coli* or a presumptive *Micrococcus* strain, both isolated from healthy rats, was found to reverse the deficiency within 48 hours, indicating that the microbiota played a key role in vitamin K production. Interestingly, Frick et al. (1967) found that humans receiving low vitamin K diets did not develop vitamin deficiency; however, treatment with a broad-spectrum antibiotic decreased plasma prothrombin levels, indicating that the gut microbial community plays an important role in supplementing low dietary vitamin K intake.

However, despite the role that gut bacteria play in synthesising menaquinone, there is evidence from germ-free rat studies to suggest that menaquinone synthesis is not fully dependent on the gut microbiota (Ravcheev & Thiele, 2016). Furthermore, a recent metagenomic analysis of vitamin synthesis pathways of the human gut microbiome revealed that the number of taxa encoding menaquinone biosynthetic pathways was fewer

compared to those encoding B-group vitamins (Das et al., 2019). The authors suggested that the host may have only a limited dependence on microbially-derived menaquinone.

However, to our knowledge there has been little research characterising vitamin K production within the pig gut microbiome but considering the similarities between the pig and human intestinal microbiome, some of the findings from humans can perhaps be extrapolated to pigs. Menaquinone-producing microorganisms that have been described in the human gut have been identified primarily by thin-layer chromatography (TLC). Ramotar et al. (1984) found that many species of *Bacteroides* produced menaquinone, as well as *E. coli, K. pneumoniae, Propionibacterium, Eubacterium* and *Veillonella*. Cooke et al. (2006) analysed lipid extracts of bacteria isolated from the human neonatal GIT and found that *Enterobacter agglomerans, Serratia marcescens* and *Enterococcus faecium* produced various forms of menaquinone. Certain LAB such as *Lactococcus lactis* and *Leuconostoc lactis* have also been found to be high-producers of menaquinone (Morishita et al., 1999). The wide range of menaquinone producing species isolated from the human GIT, which are also found in pigs, implies that the pig gut microbiota could be an abundant source of vitamin K. However, further research is needed to determine the extent to which the pig gut microbiota contributes to host vitamin K utilisation.

1.9.2 B-group vitamins

B-group vitamins act as important co-factors for a range of biological processes, including metabolism of lipids and carbohydrates and synthesis of nucleic acids. Most B-group vitamins are either not synthesised by the host or are synthesised in insufficient amounts, and therefore, must be obtained from the diet (Magnúsdóttir et al., 2015; Yoshii et al., 2019). Moreover, the intestinal microbiome is now also recognised as an important source of B vitamins. However, not all bacteria produce B vitamins, and many also require dietary or bacterially-derived B-group vitamins and therefore, competition may occur between the host and the intestinal microbiota for these essential nutrients (Yoshii et al., 2019).

The majority of B-group vitamins are directly involved in energy metabolism; the biologically active forms of the vitamins act as co-factors for key enzymes catalysing various reactions in the Krebs cycle, as outlined in Figure 1.5. Thiamine (B_1), in its active form (TPP), aids in the cleavage of pyruvate, the main product of glycolysis. Riboflavin (B_2) is phosphorylated into flavin adenine dinucleotide (FAD) which acts as a proton acceptor and catalyses the decarboxylation of pyruvate to acetyl-CoA and the conversion

of α-ketoglutarate to succinyl-CoA. Nicotinamide adenine dinucleotide (NAD) is the active form of Niacin (B_3) and acts as an electron acceptor for several important enzymatic steps of the cycle, while pantothenic acid (B_5) is required for synthesis of CoA required for multiple steps. Lastly, cobalamin (B_{12}) and biotin (B_7) both function as enzyme co-factors for the catabolism of fatty acids and some amino acids in the Krebs cycle (LeBlanc et al., 2017).

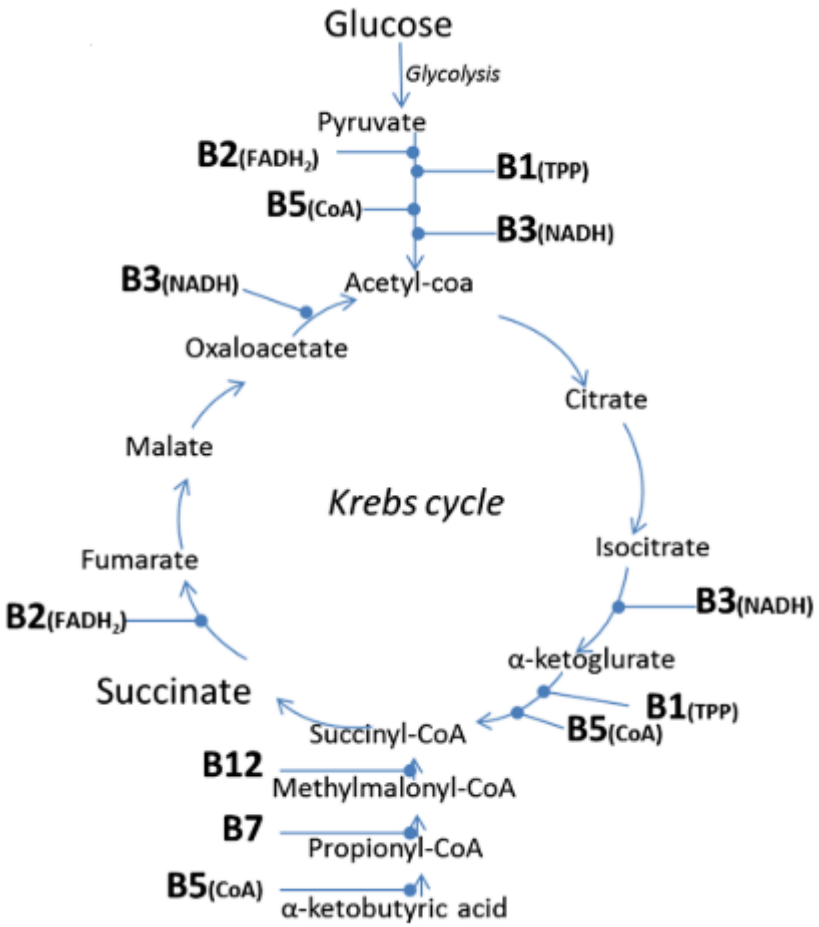

Figure 1.5: Diagram representing some of the key roles of bacterially-synthesised B-group vitamins (B1 - thiamine, B2 - riboflavin, B3 - niacin, B5 - panthothenic acid, B7 - biotin, and B12 - cobalamin) in energy metabolism. Abbreviations in brackets refer to active forms of the co-factors necessary for each enzymatic step: FADH2 (flavin adenine dinucleotide); CoA (acetyl coenzyme A); TPP (thiamine pyrophosphate); NADH (nicotinamide adenine dinucleotide). Adapted from LeBlanc et al. (2017) distributed under terms of the Creative Commons Attribution 4.0 International License.

With regard to the capacity of the pig gut microbiome to produce B vitamins, Crespo-Piazuelo et al. (2018) found that pathways related to metabolism of co-factors and vitamins, including folate, vitamin B_6 and vitamin B_2 were most abundant in the proximal colon. McCormack et al. (2017) found that the relative abundance of pathways associated with thiamine (vitamin B_1) metabolism was higher in the caecal digesta of high RFI (low

FE) pigs than in low RFI (high FE) pigs, albeit relative abundances of most of the predicted pathways were low (0.001 - 0.99 %). Conversely, Quan et al. (2020) found pathways associated with the metabolism of co-factors and vitamins to be more abundant in pigs with high FE.

Although, to our knowledge, there is no information on the microbes within the pig gut that are responsible for the synthesis of B-group vitamins, some evidence exists for humans. Magnúsdóttir et al. (2015) mined the genomes of 256 common human gut microbiome inhabitants for B-group vitamin biosynthesis pathways. Overall, between 40-65 % of the genomes analysed were predicted to harbour all necessary pathways for production of the eight analysed vitamins. The proportion of each bacterial phylum predicted to synthesise each vitamin is shown in Table 1.3. Vitamins predicted to be the most abundant, in terms of the presence of the necessary genes, were vitamin B_3, with 166 predicted producers and vitamin B_5, with 162 predicted producers. For vitamins B_3 and B_7, the vast majority of *Bacteroidetes*, *Fusobacteria* and *Proteobacteria* possessed the genes encoding the necessary synthesis pathways, with *Firmicutes* and *Actinobacteria* generally having a lower tendency for B-group vitamin biosynthesis. Regarding vitamin B_{12}, all *Fusobacteria* were predicted to be producers, with proportions of producers in the other four phyla ranging from ~10–50 %. However, it should be noted that fewer *Fusobacteria* genomes were analysed compared to the other phyla. Excluding vitamin B_{12}, in excess of 90 % of *Bacteroidetes* genomes were predicted to produce the other seven analysed B-group vitamins (Magnúsdóttir et al., 2015). Due to similarities between the human and pig gut microbiota, similar findings for pigs would be expected.

An interesting outcome of the study was the identification of organisms that had vitamin biosynthesis pathways that were complementary to other microbes, indicating that some bacteria synthesise B-group vitamins that are directly utilised by neighbouring commensals in a symbiotic relationship i.e. cross-feeding (Figure 1.4). Interestingly, for four of the analysed B-group vitamins, the gut microbiome was estimated to have the capacity to contribute more than a quarter of the recommended dietary requirements, without taking into consideration microbial utilisation. However, these estimations were based on intracellular vitamin concentrations of organisms cultured *in vitro* and hence do not necessarily reflect what is happening in the GIT where substrates may be less abundant (LeBlanc et al., 2017). Nonetheless, these results indicate that the gut microbiome is an important source of these micronutrients in humans (Magnúsdóttir et al., 2015; Rowland et al., 2018) but also in the pig gut (Crespo-Piazuelo et al., 2018;

McCormack et al., 2017; Quan et al., 2020). However, further research is required to investigate the extent of B vitamin production by bacteria within the pig gut microbiota.

Table 1.3: Proportion of bacterial phyla (%) predicted to synthesise eight B group vitamins via PubSEED subsystem gene function annotation of 256 human gut microbiome organism genomes. Adapted from data published in Magnúsdóttir et al. (2015) distributed under terms of the Creative Commons Attribution 4.0 International License.

Vitamin	Annotated subsystems analysed	Proportion (%) of bacterial phyla predicted to synthesise B-group vitamins				
		Bacteroidetes (*n* = 51 genomes)	*Fusobacteria* (*n* = 14 genomes)	*Proteobacteria* (*n* = 38 genomes)	*Firmicutes* (*n* = 130 genomes)	*Actinobacteria* (*n* = 23 genomes)
Biotin (B7 or H)	"Biotin biosynthesis"	96	100	84	5	ND
Cobalamin (B12)	"Co-enzyme B12 biosynthesis"	51	100	26	43	9
Folate (B11, B9 or M)	"Folate Biosynthesis"	92	79	71	14	26
Niacin (B3)	"NAD and NADH cofactor biosynthesis global"	98	86	76	38	65
Panthothenate (B5)	"Co-enzyme A Biosynthesis"	100	ND[1]	95	32	13
Pyrodoxine (B6)	"Pyridoxin (Vitamin B6) Biosynthesis"	94	21	92	25	87
Riboflavin (B2)	"Riboflavin, FMN, and FAD metabolism Extended"	100	100	95	50	9
Thiamin (B1)	"Thiamin biosynthesis"	98	100	74	28	65

[1] ND: Not detected

Overall, despite the lack of studies in pigs, human studies suggest that the pig gut microbiota is likely a valuable source of vitamins, particularly vitamin K and B-group vitamins, for both the host and the gut microbial community itself, and that dysbiosis may significantly impact vitamin requirements of the host. In addition to nutritional functions, many vitamins have also been implicated in the development and function of host immunity with a link between vitamin intermediates derived from commensal bacteria and immune cells that directly recognise these intermediates (Caballero & Pamer, 2015; LeBlanc et al., 2017; Yoshii et al., 2019). However, further research is needed into the importance of the pig gut microbiome as a source of vitamins.

1.10 Quorum sensing and manipulation

Gut microbial community structure is regulated by QS, a system of communication between bacterial cells, which relies on the production, secretion and sensing of chemical signals called auto-inducers (Jimenez & Sperandio, 2019; Xavier, 2018). It allows bacteria to sense the population density and synchronise different behaviours and expression of genes (Krzyżek, 2019), with these QS-mediated effects more efficient at high cell densities, such as those found within the GIT (Xavier, 2018). Quorum sensing is known to be involved in a range of bacterial activities including virulence factor production, toxin production and secretion, sporulation, biofilm formation, and enzyme secretion (Jimenez & Sperandio, 2019; Krzyżek, 2019). Therefore, bacterial behaviours within the gut microbiome regulated by QS can be either beneficial or detrimental.

Commensals utilise QS to ensure gut homeostasis, as signalling molecules are involved in many of their vital processes including metabolism-related gene expression, cell division and DNA repair; hence, the production of auto-inducers can be seen as a microbiological service (Iacob et al., 2019; Xavier, 2018). Quorum sensing research in the pig gut is less studied than in humans; however, Yang et al. (2018) recently isolated and characterised, for the first time, an N-acyl-homoserine lactone (AHL)-producing bacterium, *Aeromonas hydrophila* strain YZ2, from pig intestinal scrapings (AHLs are auto-inducers that mediate QS in Gram-negative bacteria).

In vitro research also suggests that pig pathogens, such as *S.* Typhimurium, ETEC and Shiga toxin-producing *E. coli* (STEC), use QS to mediate pathogenicity (Smith et al., 2011; van Parys et al., 2011; Yang et al., 2014; Zhu et al., 2011). This section will explore both the beneficial and detrimental roles of QS within the pig gut microbiome and how they may be manipulated. We will focus primarily on auto-inducer-2 (AI-2), as it is one

of the most widely studied QS signalling molecules, primarily because it is synthesised and recognised by a wide range of bacteria and is involved in inter-species signalling.

1.10.1 Control of pathogenesis and biofilm formation

The diverse microbial communities within the mammalian gut consist of both planktonic and free-living bacteria as well as exopolysaccharide-coated biofilms which allow bacteria to thrive in microhabitats and nutritional niches. An example of a gut microbial biofilm can be seen in Figure 1.6. Biofilms provide protection from antimicrobial substances and enzymes, and facilitate QS and horizontal gene transfer (HGT) (Buret et al., 2019; Macfarlane & Dillon, 2007). Hence, they can be beneficial to gut commensals. However, biofilm formation involving pathogens is often associated with chronic infections, owing to their propensity to acquire and to confer antibiotic resistance within the population (Jensen et al., 2017).

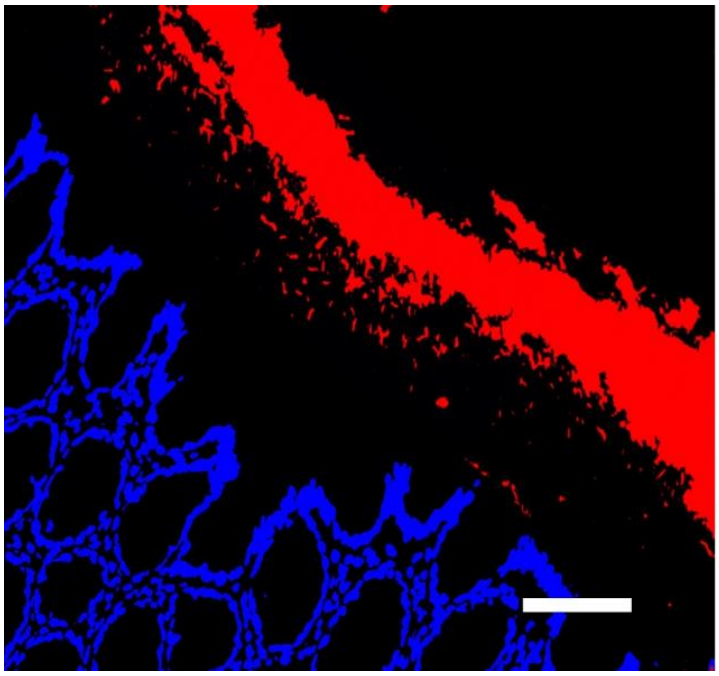

Figure 1.6: Example of a biofilm formed by the commensal colonic microbiota (red) of a healthy rat, separated from the epithelial surface (blue) by the intestinal mucus barrier (not stained). Scale bar = 50 μm. Adapted from Buret et al. (2019) distributed under terms of the Creative Commons Attribution 4.0 International License.

The role of QS in pathogenesis including expression of virulence factors, production and secretion of toxins, as well as biofilm formation, has led to the concept of anti-QS therapy, also referred to as quorum quenching (QQ), as a means of controlling pathogen proliferation. However, in a recent review, Krzyżek (2019) highlighted the need for caution with such therapies, as the same targeted signalling molecules are involved in many vital processes of commensal microbes as outlined above, and therefore disruption

of signalling may result in a disturbance of microbiota homeostasis (Krzyżek, 2019). Nonetheless, several studies have investigated the potential of QQ therapy for the disruption of pathogenesis, with some research also performed on the endogenous QQ potential of the resident gut microbiota, albeit very few QQ studies have been performed in pigs.

An *in vivo* feeding trial carried out by Kim et al. (2018) investigated the QQ effects of supplementing weaned pigs with a probiotic pig gut-derived *Lb. acidophilus* strain, shown *in vitro* to reduce AI-2 production and biofilm formation by EHEC O157:H7, albeit this is not a pig pathogen. Using traditional culturing, the authors found reduced coliform counts in the faeces, although it is difficult to attribute this to QQ activity of the administered strain as the pigs were not challenged with EHEC. Increased lactobacilli were also observed in the faeces and polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) analysis showed a difference in the 16S rRNA gene products after administering the *Lb. acidophilus* strain, most of which were identified as uncultured clones, *Lactobacillus* or *Bifidobacterium*. The authors concluded that bacteria with QQ properties can beneficially modulate the composition of the pig gut microbiota.

In conclusion, although data for pigs is scarce, QS potentially plays a dichotomous role in the pig GIT. Firstly, it can serve as a mechanism for maintaining gut health by mediating gene expression related to metabolism, cell division, DNA repair and biofilm formation in commensals, although this has not been specifically shown for pigs. Conversely, QS is also a key mechanism in facilitating pathogenesis through the control of sporulation, biofilm formation and the production of virulence factors, with the latter shown for pig pathogens. Hence, there is potential to manipulate QS within the pig gut microbiome with the use of anti-QS or QQ treatments.

1.11 Antibiotic resistance genes

Antimicrobial resistance is a natural phenomenon that the gut microbiota has developed in order to survive in the presence of antimicrobial substance-producing competitors (Zeineldin et al., 2019a). Resistance to antibiotics, a broad group of naturally, as well as chemically, synthesised antimicrobial agents, is a concern due to their widespread use for the treatment and prevention of infections in both humans and animals (Sultan et al., 2018). Antibiotics have long been used in pig production for therapeutic and sub-therapeutic purposes. However, there is now widespread agreement that these practices contribute to the selection of antibiotic-resistant bacteria by transfer of the associated

ARGs between populations leading to both public health and environmental concerns (Liu et al., 2019; Zeineldin et al., 2019a).

The ARG profile, or 'antibiotic resistome' of the pig gut microbiome has been well characterised through high-throughput metagenomic sequencing (Hu et al., 2017). It has been shown to harbour a highly genetically diverse microbial community that facilitates HGT of ARGs between and within resident commensal organisms and pathogens (Sengupta et al., 2013; Zeineldin et al., 2019a). Focusing on ARGs, this section will discuss some of the undesirable microbiological services offered by the pig gut microbiome, namely its ability to act as a reservoir of ARGs and the transfer of these between commensal microbes and pathogens. We will also outline ways in which ARGs may offer a beneficial service to the host and possible ways in which ARG-harbouring bacteria may be excluded from the gut microbiome.

1.11.1 The gut microbiome as a reservoir of antibiotic resistance genes

Antibiotic administration has significant impacts on the pig gut microbiota and subsequently, the associated antibiotic resistome (Zeineldin et al., 2019). When an antibiotic is administered, susceptible microbial populations are eliminated, with only those harbouring resistance remaining. This selective pressure gives resistant organisms an evolutionary advantage, and ultimately allows them to evolve, divide and confer their antibiotic resistance (Zeineldin et al., 2019a). However, it should be noted that antibiotic use is not the sole driver of antibiotic resistance, as several studies have shown that the antibiotic resistome is established prior to and/or in the absence of antibiotic exposure (Joyce et al., 2019; Knöppel et al., 2017; Wright, 2007; Zeineldin et al., 2019). Joyce et al. (2019) identified 56 core (present in all samples) and 201 accessory ARGs, within healthy pigs without selective antibiotic pressure, suggesting highly diverse antibiotic resistomes. Sets of ARGs suggested by Bengtsson-Palme (2018) also correlated well with those identified by Joyce at al. (2019).

A metagenomic study by Ghanbari et al. (2019) found that 41 ARGs were significantly enriched within the faecal microbiome of weaned pigs administered therapeutic levels of in-feed oxytetracycline for 7 days (followed by 14 days on a standard starter diet) compared to the control group, fed a standard starter diet for 21 days. Increases in the relative abundances of the genera *Escherichia* and *Prevotella* were identified 7 days post-antibiotic treatment, which may be attributed to their propensity to carry ARGs such as *tetQ*, which may, in turn, be transferred to other susceptible bacteria within the GIT. Looft

et al. (2012) also reported an increase in *E. coli* abundance in weaned pigs 14 days after administering a diet supplemented with chlortetracycline, sulfamethazine and penicillin.

Another interesting finding of the study by Ghanbari et al. (2019) was that, in addition to enrichment of tetracycline resistance genes, some ARGs unrelated to oxytetracyline were also enriched. This is in agreement with the findings of Looft et al. (2012) who proposed that this may be due to co-occurrence of ARGs on mobile genetic elements (MGEs) such as plasmids and integrons. The majority of the ARGs found to be enriched by Ghanbari et al. (2019) were located on MGEs carrying at least two other resistance genes. This co-occurrence of ARGs on MGEs may facilitate HGT of ARG clusters to other commensals but also human pathogens such as *E. coli* (see Section 1.10.1), thereby explaining the importance of the pig gut microbiome as a reservoir of ARGs.

One drawback of metagenomic studies of ARGs is that the abundance of certain genes does not necessarily reflect their expression. Wang et al. (2020) performed a metatranscriptomic study of 330 ARGs identified within the gut microbiome of pigs, humans and chickens relating to 21 classes of antibiotics. This revealed that 56.6 % of the ARGs were expressed in pigs suggesting that a substantial proportion of ARGs are transcriptionally inactive. Additionally, the authors found that the β-lactam, tetracycline and aminoglycoside ARG transcripts were primarily a result of ARG acquisition.

Antibiotic resistance genes may also have other roles in the pig gut microbiome, influencing FE, for example. In a metagenomic analysis of different intestinal regions of pigs with contrasting FE, Quan et al. (2020) found that *macB* was the most abundant ARG, attributed primarily to *Prevotella* and *Treponema* in the low and high FE pigs, respectively. The authors found that the *macB* gene may affect energy metabolism of the microbiota and could be involved in regulating community composition, thereby affecting host FE (Quan et al., 2020). *Prevotella*, which was highly enriched in the caecum of pigs with poor FE, and to which *macB* abundance was linked, is associated with NSP degradation (Flint & Bayer, 2008; Wu et al., 2011). Nonetheless, it has also been suggested to be antagonistic towards some microbiota members such as *Bacteroides*, which also ferment dietary fibre but have additionally been associated with protein degradation (Chen et al., 2017; Ley, 2016).

The authors, therefore, suggested that excessive *Prevotella* abundances may impede the development of an efficient nutrient-utilising microbiota, thereby decreasing FE (Quan et al., 2020). However, likewise, the abundance of *macB* attributed to *Treponema* in highly

feed efficient pigs may implicate members of this genus as having a positive effect on FE, suggesting that some bacteria that harbour ARGs may provide a beneficial microbiological service to the host. *Treponema* has been previously associated with improved FE in pigs and has been positively correlated with digestibility and negatively correlated with fatness (He et al., 2016; McCormack et al., 2017; Yang et al., 2016; Niu et al., 2015; Yang et al., 2017). However, more extensive research is required to elucidate the potentially beneficial roles of *macB* and ARGs in general within the pig gut microbiome.

1.11.2 Transfer of ARGs between commensals and pathogens

Many studies have investigated the movement of ARGs between commensal and pathogenic bacteria in pigs and the pig farm environment, focusing on *E. coli*, as the pig gut harbours many commensal *E. coli* (Mazurek et al., 2018; Pérez Gaudio et al., 2018). A study by Reid et al. (2017) highlighted the role of commensal *E. coli* in the pig gut as contributors to the mobilisation of ARGs and the conferring of antibiotic resistance. A total of 103 *E. coli* isolates from the faeces of healthy pigs were all found to carry class 1 integrons, genetic elements capable of integrating and expressing ARGs, with 97 % of the strains found to be MDR. Moreover, most isolates carried virulence genes associated with human infection.

Pérez Gaudio et al. (2018) performed a conjugation assay to investigate HGT via class 1 integrons from a pig-derived antibiotic resistant commensal *E. coli* to pathogenic STEC O157:H7. Following 4 hours of co-culture, STEC had acquired the class 1 integron, and presumably ARGs; however, antibiotic resistance was not investigated following the transfer. Nonetheless, the study demonstrates that commensal *E. coli* may serve as an important source of ARG transfer to pathogens in a short period of time.

Blake et al. (2003) performed a similar study where MDR commensal *E. coli* and a *Salmonella* isolate from the pig ileum were assessed for their ability to confer antibiotic resistance to antibiotic-susceptible pathogenic *E. coli* strains and a *Salmonella* Poona isolate under simulated ileal conditions. A bovine-derived pathogenic *E. coli* O157 strain dominated and persisted in the system as well as an antibiotic resistant sub-population of this strain, which had obtained ARGs from a ‘donor’, co-inoculated resistant commensal *E. coli*. This, and the studies outlined above, demonstrate the ability of commensal bacteria to confer antibiotic resistance to pathogenic bacteria within the pig GIT.

1.11.3 Targeting the pig gut microbiome to reduce antibiotic resistance

Research on the exclusion or re-sensitisation of ARG-harbouring bacteria is mounting but is still in its infancy. Earlier, we discussed colonisation resistance via competitive exclusion as a means of inhibiting pathogen colonisation. Kim et al. (2005) performed the first study to examine the ability of a pig-derived mucosal CEC, previously shown to exclude *Salmonella* in pigs (Fedorka-Cray et al., 1999) to reduce antibiotic resistance in commensal *E. coli* in piglets. However, they found that resistance of *E. coli* to tetracycline and streptomycin was higher in the CEC-treated group, although streptomycin resistance returned to baseline at weaning. The authors indicated that the tetracycline resistance was most likely influenced by a combination of resistant *E. coli* from the sows, the environment and the CEC, all of which were found to harbour tetracycline resistance. Although mechanisms of transfer such as MGEs were not investigated, these results highlight a safety concern regarding the administration of CECs and their potential to confer resistance to the commensal gut microbiota. Consequently, guidance from the European Food Safety Authority (EFSA) requires comprehensive characterisation of microbial feed additives to avoid adding to the gut antibiotic resistome and to decrease the risk of transfer of antibiotic resistance (EFSA, 2018).

A more recent study in rabbits by Achard et al. (2019) yielded more promising results. They evaluated the effect of oral delivery of a faecal suspension, or faecal pellets added to nests (both derived from three different antibiotic-naive does) on the antibiotic resistome of kits from antibiotic-exposed dams. The three different faecal inocula differed widely in their impact on the microbiome and associated antibiotic resistome, with one inoculum reducing the proportion of resistant *Enterobacteriaceae* from 93 to 9 % and reducing the relative abundance of eight ARGs. Conversely, the least effective inoculum had no impact on ARGs or the microbiota composition. Interestingly, the authors found that exposure to faecal pellets was more effective than oral inoculation. This suggested that coprophagy, the behaviour of consuming faeces, is important in the transmission of microbes and associated ARGs to offspring. Coprophagy has been widely reported in pigs and recently, piglets that were deprived of maternal faeces for seven days after birth, showed poorer immune function and growth performance (Aviles-Rosa et al., 2019). Further studies are required to replicate these findings in pigs and to elucidate the mechanism and components of the inocula responsible for the competitive exclusion of ARG-harbouring microbes.

Pigs are known to be reservoirs of several species of staphylococci including *Staphylococcus suis* and *S. aureus*; the former is an important pig pathogen and an emerging zoonotic pathogen, while antibiotic resistant strains of the latter, namely MRSA are considered a serious public health threat. A potential route of increasing antibiotic susceptibly of MDR-bacteria is targeting bacterial QS. For example, hamamelitannin (HAM) is a QQ molecule that affects the susceptibility of *S. aureus* biofilms to antibiotics by suppressing cell wall synthesis and extracellular DNA release; two mechanisms facilitating vancomycin resistance in *S. aureus*. There is also *in vitro* evidence to suggest that HAM increases the susceptibility of *S. aureus* to other classes of antibiotics (Brackman et al., 2016).

Several other technologies have also shown promise in tackling antibiotic resistance. One is the revolutionary genome editing tool: the clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9 (CRISPR/Cas9) system. This is a natural prokaryotic defence mechanism that acts as a nuclease and can be guided to cleave any target DNA (Goren et al., 2017). Kim et al. (2015) applied the CRISPR/Cas9 system to kill extended-spectrum β-lactamase (ESBL)-producing *E. coli* which are generally MDR and harbour plasmid-encoded ARGs which are transferred via HGT. However**,** the frequency of mutations on ESBL gene sequences meant that finding a target for one mutant would be therapeutically impractical. For this reason, the authors used a highly conserved sequence in ESBL mutants as a CRISPR/Cas9 target and successfully cleaved the ESBL plasmid of a clinical isolate, restoring susceptibility to both ampicillin and ceftazidime; the latter was not specifically targeted but was disarmed because it was encoded on the same plasmid. This technology has potential as an effective method for combatting plasmid-carrying MDR bacteria (Kim et al., 2015).

There are, nonetheless, significant challenges with applying such technologies to complex microbial ecosystems such as the pig gut where individual species or strains may contain lineages with highly diverse antibiotic resistomes, carrying a variety of different plasmids and MGEs. Another challenge of using genome editing tools such as CRISPR/Cas9 is the risk of undesirable knock-on effects within the microbial community. For example, like the microbiota perturbations that occur following antibiotic administration, removal of a particular strain from the ecosystem may promote the proliferation of other potentially pathogenic species. The consequences of antibiotic resistance manipulation with CRISPR/Cas9 have not been well studied to date, and must be considered for any potential therapeutic applications (Pursey et al., 2018).

1.12 Conclusions

The resident pig gut microbial community, dominated by the phyla *Firmicutes*, *Bacteroidetes* and *Proteobacteria*, is provided with a hospitable habitat that provides protection and a continuous supply of nutrients. The gut microbiota, in turn, provides a plethora of beneficial services to the host, including conferring of colonisation resistance through competitive exclusion and the production of antimicrobial substances, production of enzymes, metabolism of dietary fibre and the production of VFAs and vitamins (Figure 1.4). Quorum sensing can also be considered a beneficial service offered by the gut microbiome, as it can act as a mechanism for maintaining gut health by mediating the expression of genes controlling essential functions in commensals. However, the pig gut microbiome can also deliver negative microbiological services; for example, it can act as a reservoir of ARGs which can be transferred to pathogens and disseminated to other animals, humans, food and the environment.

Recently, the concept of a 'core' pig gut microbiome, independent of age, origin, breed and diet, has emerged. This provides insights into the most prevalent genera colonising different sections of the GIT, which may act as potential markers of gut health. As pig gut microbiome data becomes more abundant and as advances in functional metagenomics continue to provide valuable insights into the role of gut microbes, there is huge potential to identify microbial targets and mechanisms that can be exploited to improve gut health. The focus should be on enhancing the beneficial services offered by the pig gut microbiome, while reducing/eliminating services with negative impacts.

Specific approaches could include the administration of probiotic microorganisms as a means of implanting microbes that can offer beneficial services within the gut microbiome or alternatively, prebiotics or other feed additives which can increase the numbers of microbes already providing benefits. In terms of reducing/eliminating negative gut microbiome-related services, there is potential to manipulate QS within the pig gut microbiome with the use of anti-QS treatments, as QS facilitates pathogenesis in gut microbes, as well as benefitting commensals. Using microbiota-derived CECs for the exclusion of ARGs or technologies such as CRISPR/Cas to restore antibiotic susceptibility in MDR bacteria are other options. Some of these approaches are already being exploited by commercial pig producers, while the more novel strategies are only at the research stage and safety and efficacy must be demonstrated before they can be adopted commercially.

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Chapter 1: Literature Review (Part 2): Microbial quality of liquid feed for pigs and its impact on the pig gut microbiome

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1.14 Introduction to liquid feeding

In this review, liquid (or wet) feed refers to a mixture of dry feed components (cereals, protein sources and pre-mixes containing vitamins, minerals, synthetic amino acids and other feed additives) combined with either water and/or liquid food industry co-products (e.g., dairy and distillery co-products), in a mixing tank to a pre-defined water:feed ratio, prior to feed-out. The homogenised liquid feed is pumped from the mixing tank to troughs located in pig pens via a network of pipes (Brooks et al., 2001; Sol et al., 2019). Such feeding systems are computer-controlled and frequently referred to as automated liquid feeding systems.

Liquid feeding is generally carried out using either long or short troughs. The former allows all pigs in the pen to eat simultaneously and because of this, the feed allowance to pigs can be restricted. However, with the latter, only a portion of the pigs in a pen (normally 30–40 %) can eat at any one time and so pigs must be provided with *ad libitum* access to feed which is controlled by readings from sensor(s) or probe(s) within the trough to maintain a reservoir of feed within the trough (de Lange & Zhu, 2012). Figure 1.7 illustrates a typical modern liquid feeding system. The distinction between liquid feeding and wet/dry feeding should be noted; with wet/dry feeding, dry feed and water remain separate before entering the trough, where the pig can then mix them at its desired water:feed ratio (Hurst et al., 2008).

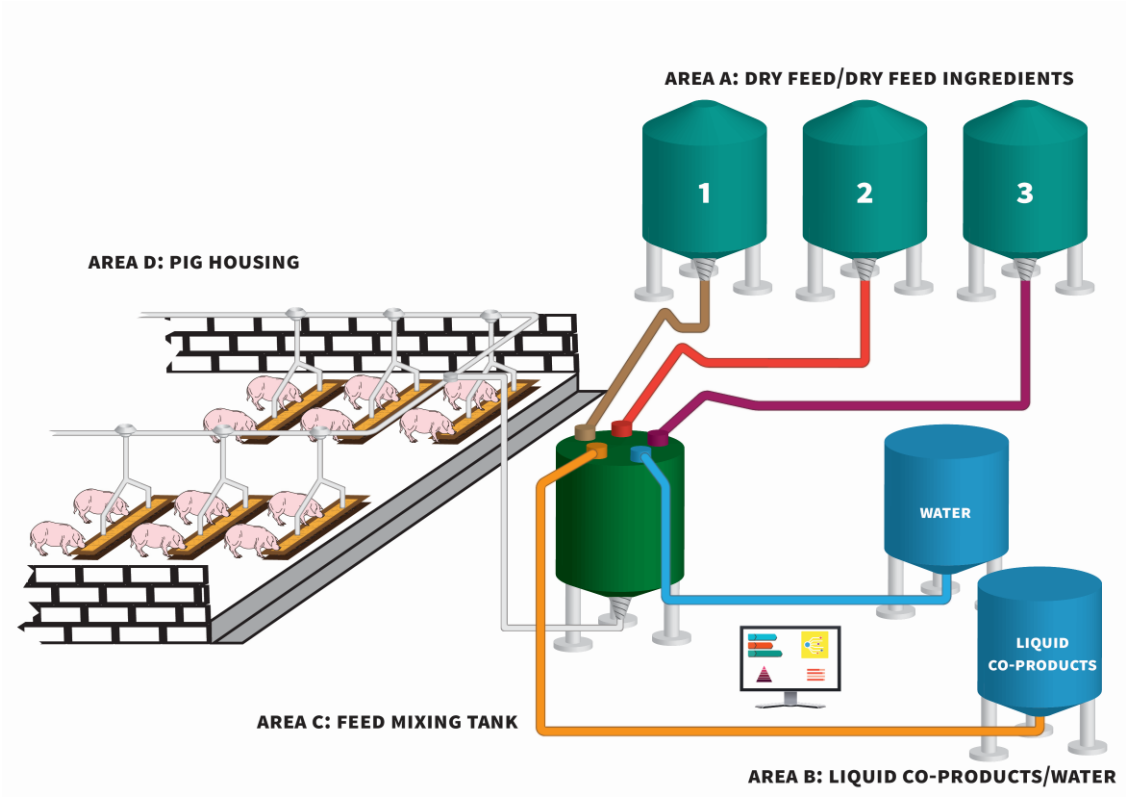

Figure 1.7: Diagram of an automated liquid feeding system demonstrating how dry feed/dry feed ingredients from feed bins (Area A) and water and/or liquid co-products (Area B) are delivered to a central mixing tank (Area C) and agitated, followed by the delivery of liquid feed to pens via a series of pipes for consumption by pigs (Area D). On farms where fermentation is practiced, an additional fermentation tank may be included before the mixing tank, where the whole diet or the cereal fraction of the diet are fermented for a period of time prior to pumping to the mixing tank for delivery to the pens (Credit: Jonathan Brazil).

Liquid feeding is common in many parts of the world, most notably in western Europe (de Lange & Zhu, 2012). However, there is a lack of up-to-date accurate information regarding the number of liquid-fed pigs in Europe. Approximate figures from Best (2009) indicate that > 60 % of Danish and Swedish finishers, as well as the majority of sows, are liquid-fed. Best (2009) also reported that approximately one third of grow-finisher pigs in the Netherlands and France are liquid-fed, but in the main pig regions this figure is between 50–60 %; however, Martineau et al. (2008) reported that ~70 % of finishers in France are liquid-fed. About 40 % of grow-finishers in Germany receive liquid diets but the figure is much lower for sows (Best, 2009). Data collected from a survey of 56 Irish farrow-to-finish pig farms found that 37.5 % of these fed a liquid diet from weaning to slaughter (Rodrigues da Costa, 2018). However, it should be noted that in Ireland it is on the farms with large herd sizes that liquid feeding is most prevalent. Therefore, in order to obtain a truer picture of the prevalence of liquid feeding, rather than basing it on the number of herds/farms using the practice, it should be calculated based on the number of

pigs that are liquid-fed. In Ireland, this figure is estimated to be ~70 % (Lawlor & O'Meara, 2018). Liquid feeding is less popular in North America, compared to Europe. However, an exception to this is Ontario, Canada, where in 2012, 20 % of grow-finishers were fed liquid diets, but with the difference being that these are primarily corn-based, compared to the mainly wheat- and barley-based diets fed in Europe (de Lange & Zhu, 2012). Liquid feeding has been adopted more readily in Europe due to the widespread availability of inexpensive nutrient-rich co-products from the food, beverage and biofuel industries, which aid in reducing feed costs by up to 17 % compared to dry feed (Canibe & Jensen, 2012; de Lange & Zhu, 2012; Scholten et al., 1999). These will be discussed in more detail in Section 1.17.2.

1.15 Types of liquid feed

The two main types of liquid feed (LF) are fresh LF and fermented LF (FLF). Liquid feed is characterised as fresh when the whole diet is mixed with water/liquid co-products, usually at a ratio of 1:1.5 to 1:4, immediately prior to feeding. It has been well documented, however, that some degree of unintentional 'spontaneous' fermentation occurs in fresh LF once mixing begins. This may have a negative impact on the microbial quality of the feed as a result of malfermentation. This occurs due to the selection for, and proliferation of, undesirable microbes and subsequent microbial degradation of crystalline amino acids in the diet, leading to the production of undesirable metabolites such as biogenic amines (Brooks et al., 2001; Canibe & Jensen, 2003; Missotten et al., 2010; O'Meara et al., 2020a). On the other hand, FLF, is deliberately fermented, either with/without the use of a microbial inoculant. Water/liquid co-products are mixed with the whole diet (or the cereal fraction alone), at a similar ratio to fresh LF, and the mixture is allowed to ferment for a period of time prior to feeding, with the addition of the remaining dietary components just prior to feed-out in the case of fermentation of the cereal fraction (Brooks, 2008; Missotten et al., 2010).

A common method of producing FLF is the mixing of fresh feed and liquid with a proportion of a previously successful fermentation; a process known as 'backslopping'. The production of organic acids, such as lactic acid, produced by lactic acid bacteria (LAB) fermentation is considered one of the key benefits of FLF, as it reduces the pH of the feed and the pig gastrointestinal tract (GIT), resulting in a reduction in the levels of *Enterobacteriaceae* (Plumed-Ferrer & Von Wright, 2009; Van Winsen et al., 2001). An alternative to microbial fermentation of LF is the direct addition of organic acids to fresh LF to produce acidified LF (ALF). The potential benefits and disadvantages of the

aforementioned LF types will be discussed in detail throughout this review, along with their microbial quality, and their impact on the pig gut microbiome and pig growth.

1.16 Desirable characteristics of liquid feed

The widely accepted desirable characteristics of FLF include low pH (generally <4.5), high numbers of LAB, low numbers of *Enterobacteriaceae*, high concentrations of lactic acid and low concentrations of acetic acid (Canibe et al., 2007b; Dujardin et al., 2014; Plumed-Ferrer et al., 2004). Fungal communities are also an important component of LF, with positive or negative impacts on feed quality, depending on the species dominating (Missotten et al., 2015a); however, excessive yeast fermentation is generally undesirable as high levels can impact feed palatability due to the production of acetic acid and ethanol in addition to a loss of energy from the feed (Brooks, 2008; Brooks et al., 2001). Olstorpe et al. (2010) suggested adding a desirable yeast strain to LF starter cultures that could dominate the feed without reducing feed microbial and nutritional quality. Certain fungal species have potential benefits in LF including acting as a protein source (Olstorpe et al., 2008; Urubschurov et al., 2018) and inhibiting moulds and *Enterobacteriaceae* (Druvefors & Schnürer, 2005; Olstorpe et al., 2012). The potential benefits of fungi need to be further explored as there is evidence to suggest that yeast species such as *Kazachstania slooffiae* are beneficial to pig gut health, providing amino acids for microbial as well as pig growth and exhibiting a potential symbiotic relationship with *Lactobacillus* (Arfken et al., 2019, 2020; Summers et al., 2021).

Van Winsen et al., (2001) described a successful batch of FLF as having: pH < 4.5; lactic acid > 150 mmol/L; acetic acid < 40 mmol/L; butyric acid < 5 mmol/L; ethanol < 0.8 mmol/L; total lactobacilli > 9 $\log_{10}$ CFU/mL; *Lactobacillus plantarum* > 9 $\log_{10}$ CFU/mL; *Enterobacteriaceae* < 1.8 $\log_{10}$ CFU/mL; and no detectable *Salmonella*/25 mL. Standard microbiological and physicochemical properties of 'residue-free' and 'residue-containing' LF can be seen in Table 1.4. The former refers to a situation where the pipelines are rinsed with water prior to delivery of the new batch and is akin to fresh LF. The latter refers to a situation where residual feed from a previous batch remains in the pipelines and is recirculated with the new batch of feed, thus acting as an inoculum for fermentation (in a similar fashion to backslopping). Therefore, it is essentially FLF, as evidenced by its properties (Table 1.4). However, in practice, farms operating both of these systems will likely consider that they are both feeding fresh LF as neither intentionally sets out to produce FLF.

Table 1.4: Standard pH values, microbiological parameters and organic acid levels in residue-free and residue-containing liquid feed (Fisker & Jørgensen, 2010; Vils et al., 2018).

Liquid Feed Characteristic/Component	Residue-Free Liquid Feed (Fresh LF) [1]	Residue-Containing Liquid Feed (FLF) [2]
pH	5.0–6.0	4.5–5.0
Lactic acid bacteria (CFU/g)	10^6–10^8	10^8–10^9
Yeast (CFU/g)	10^4–10^6	10^6–10^7
Enterobacteria (CFU/g)	10^4–10^5	$<10^3$–10^4
Fungi (CFU/g)	10^3–10^4	$<10^3$
Clostridium perfringens (CFU/g)	$<10^3$–10^4	$<10^2$
Lactic acid (mmol/kg)	0–10	40–150
Acetic acid (mmol/kg)	0–10	10–50
Formic acid (mmol/kg)	0–10	0–40
Ethanol (g/kg)	0–0.5	0.1–4

[1] Residue-free liquid feed refers to fresh LF that is delivered to troughs immediately after the mixing of feed and water, following rinsing of the pipes. [2] Residue-containing liquid feed refers to fresh LF that is delivered to troughs immediately after the mixing of feed and water; however, feed from the previous batch remaining in the pipes acts as a fermentation inoculum (essentially producing FLF but differing from feed that is deliberately fermented).

1.17 Potential benefits of liquid feed for pigs

Liquid feeding has several potential advantages compared to conventional dry feeding of pigs. These include improved gut health, utilisation of inexpensive industry co-products, flexibility and ease of delivery, and the ability to optimise microbial and nutritional quality via the addition of feed additives such as microbial inoculants for controlled fermentation and enzyme preparations to improve nutrient digestibility (de Lange & Zhu, 2012; Jakobsen et al., 2015; Torres-Pitarch et al., 2020a, 2020b). These advantages can improve the growth and feed efficiency (FE) of pigs, while different liquid feeding strategies may also act as suitable alternatives to the traditional inclusion of sub-therapeutic levels of in-feed antibiotics and pharmacological levels of zinc oxide (Brooks et al., 2001; Canibe & Jensen, 2003; Missotten et al., 2015a).

1.17.1 Improved gut health and pathogen inhibition

As outlined earlier, the mixing of feed and water during LF preparation allows for the proliferation of naturally occurring fermentative LAB and yeast present in feed ingredients. The phase of fermentation at which FLF is fed is important, as during the initial phase, conditions are conducive to a rapid surge in coliforms and other potential pathogens (Canibe & Jensen, 2012). Optimal conditions are achieved once the fermentation reaches ‘steady state’ at the end of the second phase and into the third phase

of fermentation, when there are high levels of LAB and lactic acid, moderate levels of yeast, low pH, and low numbers of enterobacteria (Brooks, 2008; Canibe & Jensen, 2003; Jakobsen et al., 2015). At this stage, the production of lactic acid, acetic acid and ethanol by the dominant microbiota, has reduced the pH, preventing pathogens such as *Escherichia coli* and *Salmonella* from proliferating (Missotten et al., 2015a). During the third phase, however, yeasts can continue to proliferate in the feed (Brooks, 2008). There is also evidence that when fed to pigs, low pH liquid feed can increase lactic acid concentrations in the stomach, reduce gastric pH, and reduce enterobacteria along the GIT (Canibe & Jensen, 2003; Mikkelsen & Jensen, 1998). The effect of liquid feeding on the pig gut microbiota will be discussed in Section 1.22. The health benefits for liquid-fed pigs have been reviewed by Brooks (2008; 2003) and include reduced *Salmonella* prevalence, reduced diarrhoea incidence and a reduction in antibiotic-resistant *E. coli.*

1.17.2 Use of industry co-products

The inclusion of inexpensive food and beverage industry co-products in animal diets has traditionally been used as a means of decreasing feed cost and as an alternative to disposal, which has an associated economic, as well as environmental impact (Canibe et al., 2010; Scholten et al., 1999). The use of industry co-products, however, requires careful management of diet formulation as co-products such as whey can be high in salt, and may also increase water requirements of pigs (de Lange & Zhu, 2012). Another challenge of co-product inclusion is the variability in microbial and nutritional composition between different products and indeed batches of the same co-product (Sol et al., 2016). Nonetheless, depending on availability, continuity and consistency in supply and proximity of co-products to a given farm, it can be a viable means of reducing feed costs. Another potential benefit of liquid co-product inclusion is that many sugar-rich food and beverage industry co-products have undergone fermentation by LAB and/or yeast and, therefore, have a pH of ~3.5–4.5 resulting from the formation of organic acids. The resultant lactic acid, and to a lesser extent acetic acid, are known to exhibit antimicrobial activity against pathogenic and spoilage microorganisms (Brooks, 2008; Scholten et al., 1999). In a survey of liquid feeding practices in the finisher section of commercial Irish pig units, O'Meara et al., (2020a) found that 3 of the 8 units surveyed included either pot-ale syrup and/or liquid whey in their diets. The study found that on these pig units, *Enterobacteriaceae* counts in LF delivered freshly to troughs tended to be lower compared to units that did not use co-products. Additionally, *E. coli* counts were reduced in residual LF remaining in troughs just prior to the next feed-out on the units

that used liquid co-products. Dietary co-product inclusion also reduced mould counts in the mixing tanks, as well as in fresh and residual LF in troughs, while also reducing the pH of the feed in the mixing tanks and of the fresh feed in the troughs (O'Meara et al., 2020a).

1.18 Other benefits of liquid feed

1.18.1 Reduced feed costs

Recently, Lawlor (2021) examined the effect of different feeding systems on margin over feed per pig during the growing finishing stage. This analysis was based on finisher feed price and pig meat price in Ireland in August 2021 (Teagasc feed and pig-meat price monitor, 2021) and data from O'Meara et al. (2020d). It was found that in order to reach a target slaughter weight of 105 kg, where finisher space is not limited, dry and wet/dry feeding resulted in a higher margin over feed than liquid feeding because of superior FE in the former. However, where farms are limited by space and, therefore, maximising growth rate is essential to reach a target slaughter weight, as is the case on many finisher units, then liquid feeding is as cost-effective as dry and wet/dry feeding, due to the increased growth rate observed with liquid feeding. These results are in agreement with a similar analysis performed in 2018 (Lawlor & O'Meara, 2018). The real benefit of liquid feeding over dry and wet/dry feeding, however, is that it allows the inclusion of low to medium dry matter (DM) co-products in the diet which can greatly reduce feed cost.

1.18.2 Practical benefits

In addition to the improved DM intake and growth rates observed with liquid feeding (Brooks, 2003; Missotten et al., 2015a; Moran, 2001), there are a number of practical benefits over dry feeding which include the ability to optimise microbial and nutritional quality via addition of feed additives such as starter cultures for controlled fermentation, enzyme preparations to improve nutrient digestibility, and direct acidification of feed using organic acids (Plumed-Ferrer & Von Wright, 2009; Rudbäck, 2013). Liquid feeding systems also allow for increased accuracy of feeding, as more appropriate feeding curves can be achieved (Brooks, 2003) while different diets can also be fed to different pens allowing for phase feeding (Missotten et al., 2010; Moran, 2001). If home compounding, ingredients can be mixed to form a diet prior to feeding, and thus the liquid feeding system acts as both a feed mixing and distribution system (Moran, 2001). Liquid feeding also reduces dust during handling and feeding compared to dry feeding, resulting in less feed

loss and a healthier environment for the stockperson and the pigs (Brooks, 2003; Missotten et al., 2010).

1.18.3 Environmental benefits

As mentioned in Section 1.17.2, one of the environmental benefits of liquid feeding is the ability to include food and beverage industry co-products in pig diets. Firstly, it avoids unnecessary disposal of these products but also due to their nutritive value, it improves sustainability by reducing reliance on ingredients such as soybean meal (Brooks, 2001). The high growth rates achievable with liquid feeding (as outlined in Section 1.18.1) are also an environmental benefit, as the number of days to slaughter is reduced, thereby reducing the environmental footprint of pig production. Phase feeding, mentioned earlier, also allows for the protein requirements of pigs to be met more accurately based on growth stage, i.e. reducing nitrogen output in effluent (Missotten et al., 2010; Brooks, 2001).

1.19 Disadvantages of liquid feed for pigs

1.19.1 Formation of biogenic amines/loss of nutritional value

Concerns over biogenic amines in LF include decreased nutritional value of feed due to microbial decarboxylation of free amino acids as well as toxicity and reduced feed palatability with a subsequent reduction in feed intake (Brooks et al., 2001; Canibe et al., 2007a; Niven et al., 2006). However, the only relevant EU legislation is concerned with histamine content of food, and no clear guidance exists on acceptable levels of biogenic amines in LF (EFSA., 2011). A recent cytotoxicity study by del Rio et al. (2019) found that the highest levels of putrescine and cadaverine that elicited no adverse effects on an intestinal cell line were 440.75 and 255.45 mg/kg body weight/day, respectively. A French survey of 33 finishing units reported 310 and 1182 ppm (mg/kg) as the maximum detected levels of putrescine and cadaverine in LF for pigs, respectively, indicating that amine levels in LF, particularly cadaverine, may be a concern. They also suggested that biogenic amine levels in LF are highly variable and linked to individual farms, with the use of industry co-products, for example, being a risk factor for high levels (Le Treut, 2012).

The microbial decarboxylation of free lysine, which is usually added to pig diets as lysine-hydrochloride (lysine HCl) to fulfil nutritional requirements, results in the formation of cadaverine, with some cadaverine formation also occurring due to ornithine

decarboxylation (Özogul & Özogul, 2019). Putrescine is also formed from the decarboxylation of ornithine and/or arginine (Barbieri et al., 2019). The amino acid decarboxylase enzymes required for biogenic amine formation are widely distributed among different bacterial groups including undesirable and spoilage-associated microbes such as members of the *Enterobacteriaceae* family. However, they are also produced by many desirable microorganisms such as LAB which are naturally present in feed, or may be intentionally added as an inoculum; however, this characteristic is strain-specific (Barbieri et al., 2019; EFSA., 2011; Yazgan et al., 2021). This highlights the need for careful consideration when choosing a microbial inoculum for FLF.

1.19.2 Bile salt hydrolase activity

Bile salt hydrolases are enzymes produced by gut bacteria that result in the deconjugation of conjugated bile acids. Hence, they have a major influence on host lipid metabolism, energy harvest, and body weight (Negga, 2015). In fact, evidence has linked reduced bile salt hydrolase activity associated with reductions of intestinal *Lactobacillus* with the growth-promoting effect of antibiotics in livestock (Geng & Lin, 2016). As such, the proliferation of *Lactobacillus* and other bacteria in LF as a result of spontaneous fermentation may result in negative effects on the health of liquid-fed pigs due to seeding of the gut with bile salt hydrolase-producing bacteria and hence increased bile salt hydrolase activity in the gut. For example, He et al. (2017) found that feeding *Bacillus subtilis*-fermented liquid feed to piglets during the pre- and early post-weaning period promoted the growth of LAB and other bile salt hydrolase-active bacteria in the gut, leading to higher concentrations of unconjugated bile acids and greater diarrhoea incidence. Although research is lacking on the impact of increased bile salt hydrolase activity on growth and feed efficiency in pigs, its association with poorer lipid absorption and energy harvest may contribute to the poorer feed efficiency of liquid-fed pigs.

1.20 Environmental implications

Some environmental drawbacks to liquid feeding include the fact that water usage and subsequently manure volume is typically higher compared to dry feeding, especially so with older liquid feeding systems where high water to feed ratios are often required to push liquid feed through the systems. Undesirable fermentation in feed can also have negative environmental effects; for example, heterofermentative LAB and yeast fermentation results in CO_2 production, adding to greenhouse gas emissions. This also reduces the energy content of the diet, potentially contributing to poorer feed efficiency.

1.21 Microbial quality of feed

1.21.1 Dry feed: meal versus pellets

Liquid feed is generally prepared using meal, while pellets are often used when dry feeding in order to optimise FE. However, pelleting can reduce particle size, and it is important that the proportion of particles < 400 µm is minimised to prevent ulceration (Patience et al., 2015; Vukmirović et al., 2017). There are also distinct microbiological differences between feed forms, and their impact on pig gut microbial communities. For example, O'Meara et al. (2020d) found that LAB, *Enterobacteriaceae*, yeast, and mould counts were lower in a pelleted compared to a meal diet while Burns et al. (2015) also found that *Enterobacteriaceae* counts were lower in pelleted feed than in meal (Table 1.5). This is likely due to the high temperatures and pressure used during the pelleting process. Mikkelsen et al. (2004) also reported lower counts of total anaerobic bacteria, coliforms and yeast in pelleted compared to non-pelleted diets; however, there were no significant differences in LAB counts (Table 1.5).

Table 1.5: Microbiological data (log_{10} CFU/g) from experimental meal and pelleted diets (finely or coarsely ground) (Canibe et al., 2005; Mikkelsen et al., 2004; O'Meara et al., 2020d).

Reference	(O'Meara et al., 2020d)		(Mikkelsen et al., 2004)				(Canibe et al., 2005)	
Feed Form/Diet	Meal	Pellet	F-NP [a]	F-P	C-NP	C-P	F-NP	C-NP
Lactic acid bacteria	3.30	2.29	3.29	3.31	3.76	3.42	<3.81 [c]	<3.00
Enterobacteriaceae	5.24	3.26	NM	NM	NM	NM	<3.00	5.28
Yeast	3.92	3.12	4.33	4.43	3.30	3.14	<3.49	3.76
Mould	3.75	3.00	NM	NM	NM	NM	NM	NM
Total anaerobic bacteria	NM [b]	NM	5.64	5.66	4.64	4.07	NM	NM
Total aerobic bacteria	NM	NM	NM	NM	NM	NM	4.74	6.08
Coliforms	NM	NM	5.10	4.88	3.45	3.14	NM	NM

[a] F-NP = Fine non-pelleted diet; F-P = Fine pelleted diet; C-NP = Coarse non-pelleted diet; C-P = Coarse pelleted diet. [b] NM = Not measured. [c] The less than symbol (<) denotes that some observations from which the mean was calculated had values less than the detection level of 3 log_{10} CFU/g or had no colonies detected, in which case the detection limit was used as the value to calculate the mean.

Canibe et al. (2005) reported higher levels of enterobacteria, yeast, and total aerobic bacteria in a coarsely ground meal diet compared to a finely ground pelleted diet (Table 1.5). Mikkelsen et al. (2004) also investigated fine and coarse grinding of the feed and found that feeding coarsely ground meal to pigs reduced the pH and increased the lactic acid concentration in the gastric content, and consequently decreased the survival of *Salmonella* Typhimurium in the stomach (Mikkelsen et al., 2004). A systematic review

of the effect of feed characteristics and management practices on *Salmonella* prevalence in finisher pigs also associated non-pelleted diets with reduced *Salmonella* prevalence, albeit with a low degree of confidence (O'Connor et al., 2008). Increased viscosity of the gastric content, and the associated slower gastric passage rate of coarsely ground meal is thought to facilitate the proliferation of LAB in the stomach and small intestine, along with the subsequent increase in lactic acid concentration and decrease in pH. This lower pH then exerts a barrier effect against pathogens i.e., conferring colonisation resistance (Canibe et al., 2005; Mikkelsen et al., 2004; Vukmirović et al., 2017).

It is also important to consider the impact of feed form on the microbial communities present in the pig GIT. Interestingly, using quantitative polymerase chain reaction (qPCR), Lebel et al. (2017) found that only *Bifidobacterium* (a genus used as a probiotic) was enriched in the faeces of pigs fed a meal diet and/or a diet with a large particle size (Lebel et al., 2017). Mikkelsen et al. (2004) observed a decline in coliform counts in the distal small intestine, caecum and colon of pigs fed a coarsely ground as opposed to finely ground feed, with the lowest counts observed in pigs fed coarsely ground meal (Table 1.6). Canibe et al. (2005) also found benefits in feeding a coarse meal diet, with higher counts of total anaerobic bacteria and LAB in the stomach and small intestine and lower enterobacteria counts in the caecum and colon compared to feeding a finely ground pelleted diet (Canibe et al., 2005) (Table 1.6).

Table 1.6: Microbiological counts (log_{10} CFU/g) in the gastrointestinal contents of pigs fed either meal or pelleted diets (finely or coarsely ground) (Canibe et al., 2005; Mikkelsen et al., 2004).

	Reference	(Mikkelsen et al., 2004)				(Canibe et al., 2005)	
GI Section	Microbial Group	Microbial Counts (Log_{10} CFU/g)					
		Diet [a]					
		F-NP	F-P	C-NP	C-P	F-NP	C-NP
Stomach	Lactic acid bacteria	6.81	6.98	7.88	7.39	<6.6 [c]	8.3
	Enterobacteria	NM [b]	NM	NM	NM	<3.7	<3.7
	Coliforms	4.98	4.55	4.35	4.73	NM	NM
	Yeast	4.57	3.95	4.60	4.02	<4.1	<4.6
	Total anaerobic bacteria	7.06	7.16	8.53	7.59	<6.6	8.5
Distal small intestine	Lactic acid bacteria	7.89	8.42	8.50	8.31	8.4	8.9
	Enterobacteria	NM	NM	NM	NM	<6.3	<5.8
	Coliforms	6.65	6.46	6.01	6.05	NM	NM
	Yeast	5.54	4.89	5.20	5.12	<5.1	<5.3
	Total anaerobic bacteria	8.39	8.64	8.67	8.24	8.5	8.9
Caecum	Lactic acid bacteria	8.43	9.10	9.05	8.64	9.1	9.2
	Enterobacteria	NM	NM	NM	NM	7.1	6.4
	Coliforms	6.84	7.25	5.92	6.33	NM	NM
	Yeast	5.61	5.80	5.82	5.29	5.2	<5.2
	Total anaerobic bacteria	9.69	9.95	9.63	9.69	9.5	9.5
Mid-colon	Lactic acid bacteria	8.88	8.85	9.42	8.94	9.5	9.4
	Enterobacteria	NM	NM	NM	NM	6.8	<6.3
	Coliforms	7.53	6.44	5.87	6.45	NM	NM
	Yeast	5.39	4.65	5.58	5.48	5.2	<5.0
	Total anaerobic bacteria	9.66	10.22	9.91	9.91	9.9	<9.7

[a] F-NP = Fine non-pelleted diet; F-P = Fine pelleted diet; C-NP = Coarse non-pelleted diet; C-P = Coarse pelleted diet. [b] NM = Not measured. [c] The less than symbol (<) denotes that some observations from which the mean was calculated had values less than the detection level of 3 log_{10} CFU/g or had no colonies detected, in which case the detection limit was used as the value to calculate the mean.

These studies indicate that coarsely ground meal is beneficial for gut health, facilitating the proliferation of LAB, production of lactic acid, and a subsequent decrease in pH, and inhibiting the growth of potentially pathogenic bacteria and their transit through the GIT. These results, however, are in conflict with pig growth and nutritional data. Smaller feed particle size is known to improve pig growth due to increased digestibility, and pelleted diets (which usually have smaller particle sizes) are generally preferred due to the improved feed conversion ratio (FCR) observed (Jong et al., 2016; O'Meara et al., 2020d; Vukmirović et al., 2017). Considering that meal has generally higher microbial counts compared to pellets, and that meal is the usual starting material for producing LF, it is reasonable to assume that its higher microbial load contributes to the spontaneous fermentation observed in LF. Differences in LF produced with meal or pellets may also impact on the gut microbiota of pigs fed these diets. The microbiota of liquid-fed pigs will be discussed in Section 2.10.

1.21.2 Fresh and fermented liquid feed

As outlined earlier, the two main types of LF are fresh LF and FLF. The microbiology of FLF has been studied more extensively than that of fresh LF. However, due to the occurrence of unintentional spontaneous fermentation, it is likely that many farmers intending to feed fresh LF are in practice feeding a diet in which some degree of fermentation has occurred along the feed circuit. This is particularly the case with short trough *ad libitum* feeding, where feed remaining in troughs continues to ferment and acts as an inoculum for freshly delivered feed (O'Meara et al., 2020a; Plumed-Ferrer & Von Wright, 2009; Russell et al., 1996). The degree of fermentation occurring in fresh LF is exemplified in a study by O'Meara et al. (2020a) which sampled finisher feed on commercial pig units feeding 'fresh' LF. They found that LAB, yeast and *E. coli* counts increased from the mixing tank to the residual feed remaining in the troughs, resulting in reduced pH, indicating the occurrence of spontaneous fermentation (Figure 1.8). The negative impact of this fermentation on the nutritional quality of the feed was evidenced by reduced levels of lysine, methionine, threonine and gross energy in the residual feed (O'Meara et al., 2020a). Canibe & Jensen (2003) reported similar findings in fresh LF, with spontaneous fermentation evidenced by a loss of low molecular weight sugars and increases in LAB, yeasts and total anaerobes compared to dry feed, although they did not sample at different locations. High levels of *Enterobacteriaceae* and a pH of ~6 in the fresh LF, compared to dry feed and FLF, also indicated that the fresh LF was in the first phase of fermentation (Canibe & Jensen, 2003). This spontaneous fermentation was likely

accelerated by inoculation from residual feed remaining from the previous batch of feed. Additionally, the time lag between feeding and actual consumption of the feed likely contributed to the poor microbial quality, similar to the decline in the quality of residual feed in troughs reported by O'Meara et al. (2020a).

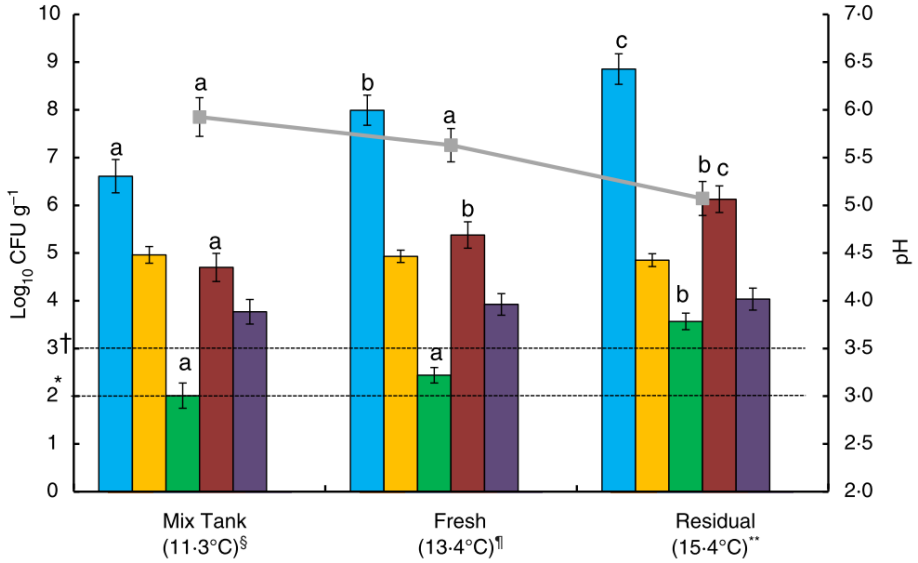

Figure 1.8: Mean (± SEM) counts of (blue) lactic acid bacteria; (orange) *Enterobacteriaceae*; (green) *Escherichia coli;* (brown) yeast and (purple) mould; and (grey line) feed pH ‡ in liquid feed samples from the mixing tank, fresh feed from troughs and residual feed from troughs on eight Irish commercial finisher pig units. * Detection limit for lactic acid bacteria, *Enterobacteriaceae* and *E. coli* (2 $\log_{10}$ CFU/g). † Detection limit for yeast and mould (3 $\log_{10}$ CFU/g). ‡ Sample pH is displayed on the secondary *y*-axis. § Mix Tank sample temperature = mean of data from eight samples. ¶ Fresh sample temperature = mean of data from 24 samples. ** Residual sample temperature = mean of data from 21 samples. [a,b,c] Within each bar colour and the line representing pH, bars and data points, respectively, that do not share a common letter are significantly different ($p < 0.05$). Adapted from O'Meara et al. (2020a).

As a result of the potential for malfermentation and the subsequent issues with microbial and nutritional quality, different strategies have been employed to control and optimise the quality of LF for pigs. These range from simply backslopping (mixing fresh feed and liquid with a proportion of a previously successful fermentation as described in Section 1.15) to inoculation with a suitable LAB starter culture and can include whole diet fermentation, fermentation, or soaking of only the cereal component of the diet and/or enzyme supplementation. Physicochemical and microbiological data from studies examining the impact of these strategies on the microbial quality of LF are summarised in Table 1.7. Strategies are often combined. For example, Olstorpe et al. (2010) investigated the effect of adding a four-strain LAB silage starter culture to cereal and wet wheat distillers' grain in addition to 80 % backslopping daily for 5 days, inoculating either

at the start of the fermentation only, or at the start as well as daily at each backslopping. The latter appeared to be preferable as higher numbers of *Lactobacillus plantarum* (a component of the inoculant) were found; however, even with this approach, levels of acetic acid were high and lactic acid levels did not reach the desired concentration to completely exclude *Enterobacteriaceae* (Beal et al., 2002; Olstorpe et al., 2010). One reason for this was that the dominant organisms in the feed were *L. plantarum* (from the starter culture) and *Lactobacillus panis* (likely from the wet wheat distillers' grain). Both of these species are heterofermentative and therefore likely responsible for the lower lactic acid and increased acetic acid production. Canibe et al. (2010) surveyed 'naturally produced' FLF (where microbial inoculants were not used) on 40 Danish piglet farms. Despite LAB counts in the FLF being > 8 $\log_{10}$ CFU/g, average lactic acid concentrations were ~90 mmol/kg, which is less than the desired concentration for successful fermentation (> 150 mmol/L). This may be explained by the prevalence of four *Lactobacillus* phylotypes in the FLF, which were predominantly heterofermentative. Nonetheless, van Winsen et al. (2001) achieved a lactic acid concentration of 261 ± 20 mmol/L with a *L. plantarum* starter (Table 1.7).

Table 1.7: Microbial and physicochemical properties of fresh, fermented, cereal fraction only fermented, soaked, acidified, and enzyme-supplemented liquid feed.

Type/Description of Liquid Feed [Microbial Inoculant if Applicable]	Microbial Counts (log_{10} CFU/g)					Organic Acids, Biogenic Amines and Other Microbial Metabolites	References
	pH	LAB	Enterobacteria / Coliforms [h]	Yeast	Moulds		
FLC [a]	5.00 ± 0.18	8.90 ± 0.33	<3.10 ± 0.30	7.80 ± 0.21	NM	Acetate: 13.00 ± 1.40 mmol/kg Lactate: 40.00 ± 5.70 mmol/kg Ethanol: 26.00 ± 4.10 mmol/kg Tyramine: 95.00 ± 13.00 mg/kg DM Putrescine: 75.00 ± 8.50 mg/kg DM Cadaverine: 153.00 ± 18.70 mg/kg DM Histamine: <11.00 ± 0.50 mg/kg DM	(Canibe et al., 2007a)
FLF [b]	4.45 ± 0.11	9.30 ± 0.26	<3.50 ± 0.71	7.20 ± 0.24	NM	Acetate: 24.00 ± 2.40 mmol/kg Lactate: 160.00 ± 16.00 mmol/kg Ethanol: 17.00 ± 5.00 mmol/kg Tyramine: 40.00 ± 8.40 mg/kg DM Putrescine: 199.00 ± 123.30 mg/kg DM Cadaverine: 890.00 ± 151.30 mg/kg DM Histamine: 57.00 ± 2.20 mg/kg DM	
Fresh LF [c] (ENZ −) [d]	4.50	8.50	5.50	6.60	<3.00	Acetate: 34.60 ppm Butyrate: 0.32 ppm Propionate: 0.42 ppm Cadaverine: 18.50 ppm Tyramine: <5.00 ppm	(Torres-Pitarch et al., 2020a)

						Putrescine: 27.80 ppm Spermine: <5.00 ppm Spermidine: 34.90 ppm Histamine: <5.00 ppm
Fresh LF (ENZ +)	4.70	8.80	5.60	7.50	<3.00	Acetate: 39.30 ppm Butyrate: 0.40 ppm Propionate: 0.55 ppm Cadaverine: 19.30 ppm Tyramine: <5.00 ppm Putrescine: 62.10 ppm Spermine: 7.30 ppm Spermidine: 31.20 ppm Histamine: 36.00 ppm
FLC (ENZ −) [*Lactobacillus plantarum* DSMZ16627 and *Pediococcus acidilactici* NCIMB3005]	4.50	9.10	5.10	7.20	<3.00	Acetate: 34.60 ppm Butyrate: 0.28 ppm Propionate: 0.56 ppm Cadaverine: 186.60 ppm Tyramine: <5.00 ppm Putrescine: 156.00 ppm Spermine: <5.00 ppm Spermidine: 31.60 ppm Histamine: 89.90 ppm
FLC (ENZ +)	4.40	8.70	4.60	7.60	<3.00	Acetate: 65.40 ppm

[*Lactobacillus plantarum* DSMZ16627 and *Pediococcus acidilactici* NCIMB3005]						Butyrate: 0.32 ppm Propionate: 0.65 ppm Cadaverine: 6.50 ppm Tyramine: <5.00 ppm Putrescine: 20.70 ppm Spermine: <5.00 ppm Spermidine: 36.00 ppm Histamine: 86.10 ppm	
Fresh LF (ENZ −)	5.70	8.80	6.90	5.60	<3.00	Acetate: 22.50 ppm Butyrate: 0.30 ppm Propionate: 0.30 ppm Putrescine: <5 ppm Histamine: <5 ppm Cadaverine: 41.00 ppm Spermidine: 26.00 ppm Tyramine: <5 ppm Spermine: <5 ppm	(Torres-Pitarch et al., 2020b)
Fresh LF (ENZ +)	5.00	8.70	6.90	6.10	<3.00	Acetate: 29.70 ppm Butyrate: 0.40 ppm Propionate: 0.80 ppm Putrescine: 6.00 ppm Histamine: <5.00 ppm Cadaverine: 89.00 ppm	

						Spermidine: 8.00 ppm Tyramine: <5.00 ppm Spermine: <5.00 ppm	
SLC [e] (ENZ −)	5.70	8.90	6.40	6.60	<3.00	Acetate: 31.90 ppm Butyrate: 0.80 ppm Propionate: 0.40 ppm Putrescine: <5.00 ppm Histamine: <5.00 ppm Cadaverine: 46.00 ppm Spermidine: 13.00 ppm Tyramine: <5.00 ppm Spermine: <5.00 ppm	
SLC (ENZ +)	5.30	9.00	6.70	6.50	<3.00	Acetate: 28.60 ppm Butyrate: 0.30 ppm Propionate: 0.30 ppm Putrescine: 18.00 ppm Histamine: <5.00 ppm Cadaverine: 122.00 ppm Spermidine: 10.00 ppm Tyramine: <5.00 ppm Spermine: <5.00 ppm	
Fresh LF	5.98 ± 0.18	<6.90 ± 0.71	6.20 ± 0.59	5.00 ± 0.65	NM	Acetate: 2.30 ± 2.37 mmol/kg Lactic acid: 1.20 ± 2.31 mmol/kg	(Canibe & Jensen, 2003)

FLF	4.36 ± 0.17	9.40 ± 0.23	<3.20 ± 0.56	6.90 ± 0.69	NM	Acetate: 25.80 ± 6.32 mmol/kg Lactate: 168.60 ± 17.07 mmol/kg	
FLF	4.80	9.50 ± 0.34	6.70 ± 0.87	3.70 ± 0.95	NM	Acetate: 20.90 ± 6.21 mmol/kg Lactate: 91.20 ± 27.66 mmol/kg Butyric acid: 0.20 ± 0.07 mmol/kg Ethanol: 15.50 ± 1.31 mmol/kg Tyramine: <10.00 mg/kg DM Cadaverine: <10.00 mg/kg DM Putrescine: <10.00 mg/kg DM Histamine: <10.00 mg/kg DM	(Canibe et al., 2007b)
FLF (High feed intake group)	4.82	8.56	3.41	6.62	3.41	Acetate: 18.50 mmol/kg Lactate: 93.20 mmol/kg Ethanol: 18.90 mmol/kg Agmatine + Cadaverine + Histamine + Phenylethylamine + Putrescine + Spermidine + Tryptamine + Tyramine: 552 mg/kg DM	(Canibe et al., 2010)
FLF (Low feed intake group)	4.70	8.45	3.26	6.14	3.45	Acetate: 17.70 mmol/kg Lactate: 90.50 mmol/kg Ethanol: 19.30 mmol/kg Agmatine + Cadaverine + Histamine + Phenylethylamine + Putrescine + Spermidine + Tryptamine + Tyramine: 528 mg/kg DM	

FLF [*Lactobacillus plantarum*]	4.10	NM	<DL	NM	NM	*L. plantarum*: 9.40 ± 0.26 log CFU/g *Salmonella* spp.: ND Lactate: 261.00 ± 20.00 mmol/L Acetate: 25.00 ± 13.00 mmol/L Butyrate: 2.30 ± 1.50 mmol/L Propionate and Ethanol: <DL	(Van Winsen et al., 2001)
FLF (Diet 3) [f] [*Lactococcus lactis subsp. cremoris 303*]	4.14 ± 0.28	9.12 ± 0.23	1.33 ± 0.58 *	5.60 ± 0.26	3.84 ± 0.66	NM	(Lawlor et al., 2002)
FLF (Diet 4)	4.17 ± 0.32	9.39 ± 0.13	1.49 ± 0.56 *	5.60 ± 0.26	2.73 ± 0.60		
FLF (Diet 5)	4.10 ± 0.20	9.24 ± 0.03	< 1.00 *	7.07 ± 0.26	3.42 ± 0.57		
ALF [g] (Diet 3)	4.07 ± 0.17	5.44 ± 0.67	2.28 ± 1.15 *	4.2 ± 0.46	2.71 ± 0.63		
ALF (Diet 4)	4.09 ± 0.20	5.94 ± 1.30	1.55 ± 0.65 *	4.54 ± 0.84	2.32 ± 0.58		
ALF (Diet 5)	3.94 ± 0.28	6.39	<1.00 *	<1.00	5.40		
Dry feed (control)	4.80	9.50 ± 0.34	6.70 ± 0.87	3.70 ± 0.95	NM	Acetate: 20.9 ± 6.21 mmol/kg Lactate: 91.2 ± 27.66 mmol/kg Ethanol: 15.5 ± 1.31 mmol/kg Butyrate: 0.2 ± 0.07 mmol/kg Formic acid: <DL	(Canibe et al., 2007b)

ALF (Boliflor® FA 2300S)	5.60	6.00 ± 0.63	3.30 ± 0.00	<3.00 ± 0.00	NM	Acetate: 4.9 ± 0.33 mmol/kg Lactate: <DL Ethanol: 1.7 ± 0.28 mmol/kg Butyrate: 0.4 ± 0.05 mmol/kg Formic acid: 34.2 ± 0.55 mmol/kg
ALF (Formic acid)	5.30	<4.00 ± 0.00	5.20 ± 0.92	<3.30 ± 0.43	NM	Acetate: 4.6 ± 0.16 mmol/kg Lactate: <DL Ethanol: 0.4 ± 0.32 mmol/kg Butyrate: <DL Formic acid: 41.3 ± 0.67 mmol/kg

[a] FLC = Fermented liquid cereal: only the cereal fraction of the diet is fermented, with the remaining dietary ingredients added prior to feeding. [b] FLF = Fermented liquid feed: all dietary ingredients are fermented for a specific period of time prior to feeding. [c] Fresh LF = Fresh liquid feed: all dietary ingredients are mixed with water and fed out immediately. [d] ENZ = Xylanase and β-glucanase enzyme complex (Rovabio Excel AP, Adisseo France SAS, Antony, France). +/- indicates whether the diets were supplemented (+) or not (−) with ENZ. [e] SLC = Soaked liquid cereal: cereal fraction of the diet soaked in water for 3 h prior to mixing with balancer fraction (soybean meal, synthetic amino acids, minerals and vitamins) immediately prior to feeding. [f] Diets 3, 4 and 5 refer to different diets as per Table 1 in Lawlor et al. (2002). [g] ALF = Acidified liquid feed. [h] Either *Enterobacteriaceae* or coliform counts are reported. Coliform counts are denoted with an asterisk, counts without an asterisk represent *Enterobacteriaceae* counts. < DL = Less than detection limit. NM = Not measured. The less than symbol (<) denotes that some observations from which the mean was calculated had values less than the detection level of 3 $\log_{10}$ CFU/g or had no colonies detected, in which case the detection limit was used as the value to calculate the mean.

The use of a homofermentative LAB inoculant is preferable in order to maximise lactic acid production for pathogen inhibition and to minimise the production of undesirable metabolites from heterofermentative LAB. *Pediococcus pentosaceus*, a homofermentative LAB was present in the starter culture used in the study by Olstorpe et al. (2010) but was only detected when the diet was inoculated daily, suggesting that conditions were not suitable for the strain to grow and produce lactic acid and that it was outcompeted by resident microbiota. This indicates that strains already adapted to LF conditions should be isolated and used as starter cultures (Olstorpe et al., 2010).

Missotten et al. (2009) screened a bank of LAB isolated from FLF and the porcine GIT for their ability to produce good quality FLF (acetic acid < 40 mmol/L, lactic acid > 150 mmol/L, pH < 4.5). Three strains of *Lactobacillus*: *Lactobacillus johnsonii*, *Lactobacillus salivarius* and *L. plantarum* were found to be very effective in the laboratory, meeting the organic acid and pH requirements and also exhibiting antimicrobial activity against *Salmonella* spp. (Missotten et al., 2009). Another strategy is to use a probiotic starter culture; for example, in two separate experiments, Missotten et al. (2015b) produced FLF with a commercial probiotic *Pediococcus acidilactici* (Bactocell®), with daily backslopping. However, at the end of both 28-day trials, *Lactobacillus* spp., not the probiotic *P. acidilactici*, dominated the fermentation, when the LF was either inoculated with the probiotic on days 0–2 and 24–26 or when the probiotic was added to the dry feed (Missotten et al., 2015b). It is possible that the probiotic may have failed to dominate because the diet was fermented continuously with daily backslopping; therefore, batch fermentation may be a more suitable approach (Brooks, 2008). Nonetheless, fermenting LF with probiotic starter cultures may be an effective means of simultaneously improving the microbial quality of feed while delivering probiotics to the gut to improve pig health (Kenny et al., 2011; Missotten et al., 2015a).

Although backslopping is a commonly used fermentation strategy, in practice, many farms feeding LF backslop unintentionally due to the impracticality of sanitising the liquid feeding system before a new batch of feed (Plumed-Ferrer & Von Wright, 2009) (see Section 1.21). Moran et al. (2006) found that for wheat fermentation, there was no advantage to increasing the backslopping proportion above 20 % (up to 42 % was trialled), in agreement with the findings of Dujardin et al. (2014). The 20 % backslopping treatment reduced coliforms to the greatest extent and yielded the highest lactate concentrations and the lowest pH. The authors also agreed with the consensus that high lactic acid concentrations and low pH are the key factors in coliform exclusion in LF.

However, they noted that the time of exposure to these conditions is critical for coliform exclusion suggesting a minimum period of 24 h with a pH < 4.0, coupled with high lactic acid concentrations (Moran et al., 2006), while Dujardin et al. (2014) reported that coliforms were still not eliminated after 48 h under similar experimental conditions.

Fermentation of the cereal component of the complete diet (typically with LAB inoculants), followed by mixing with the remaining feed components prior to feeding has been used as a strategy, primarily to minimise the loss of synthetic amino acids due to microbial decarboxylation during the fermentation process (Brooks, 2008; Canibe et al., 2007a). Some other benefits of this strategy include a more rapid reduction in pH due to the lower buffering capacity of cereals compared to the whole diet (Canibe et al., 2007a; O'Meara et al., 2020c). A practical advantage is that a fermented cereal can be used as a component in multiple diets, as opposed to fermenting multiple whole diets separately, reducing the need for multiple fermentation tanks (Brooks, 2008; Moran et al., 2006). Torres-Pitarch et al. (2020a) compared fresh LF and LF in which the cereal fraction was fermented (fermented liquid cereal; FLC), both with and without carbohydrase supplementation. The cereal fraction was inoculated with a *L. plantarum*-*P. acidilactici* starter culture and fermented without adding or removing feed for an initial 52 h. This produced a fermented cereal fraction with desirable microbial characteristics: a pH of 3.7, LAB counts of 9.2 $\log_{10}$ CFU/g, undetectable *Enterobacteriaceae* and yeast counts of 6.8 $\log_{10}$ CFU/g. The use of high-throughput 16S ribosomal ribonucleic acid (rRNA) gene amplicon sequencing revealed the complete bacterial profile of a diet containing a fermented cereal fraction for the first time. Interestingly, both inoculated bacterial strains, although not specifically tracked during the fermentation, did not appear to predominate after the initial fermentation period, as *P. acidilactici* was undetectable after 52 h and *L. plantarum* was at a relative abundance of < 2 %. This was likely because the starter culture was a silage inoculant, highlighting the importance of selecting feed-specific starter cultures, as outlined above.

Pantoea and *Pseudomonas*, two potentially pathogenic bacterial genera, predominated in the dry cereal and at the start of the fermentation period. After the initial 52 h fermentation, LAB dominated the fermented cereal, with *Lactobacillus*, *Leuconostoc* and *Lactococcus* the most prevalent genera, in order of decreasing abundance. After the initial fermentation period, backslopping was performed and on day 10, *Lactobacillus* was still the predominant genus in both fermentation tanks (FLC and enzyme-supplemented FLC). However, by day 51, *Pediococcus parvulus* and several species of *Lactobacillus*

dominated the FLC, but only *Lactobacillus* dominated the enzyme-supplemented FLC. The inability of *P. parvulus* to metabolise xylose (a sugar released by the xylanase present in the enzyme complex) was suggested to be the reason that *Pediococcus* did not dominate the enzyme-supplemented FLC (Torres-Pitarch et al., 2020a). Similar microbial profiles were observed in the mixing tanks, with a lower proportion of *Firmicutes* and a higher proportion of *Proteobacteria* in the fresh LF compared to the fermented cereal diets. At day 10, the mixing tanks of the enzyme-supplemented diets were dominated by *Lactobacillus* while the diets without enzyme supplementation were dominated by *Lactobacillus* and *Leuconostoc*. The microbial composition in the unsupplemented diets shifted at day 51 in that *Lactobacillus* and *Pediococcus* became the dominant genera, while *Lactobacillus* was still the dominant genus in the enzyme-supplemented diets, but at a higher relative abundance. However, the composition of the diets in the troughs, as opposed to the tanks, is most relevant as this is what the pigs consume. Interestingly, these showed the least differences among treatments, indicating that the fresh diets had undergone spontaneous fermentation, thereby becoming similar in microbial composition to the fermented cereal diets (Torres-Pitarch et al., 2020a) (Table 1.7). As mentioned earlier, fermenting the cereal fraction of the diet is a strategy to minimise decarboxylation of synthetic amino acids. In fresh LF, lysine losses of > 35 % have been reported between the mixing tank and residual feed in troughs (O'Meara et al., 2020a). Lysine losses from the mixing tank to the troughs were < 9 % for the FLC diets, compared to 12 % for the fresh LF diets in the study by Torres-Pitarch et al. (2020a), in agreement with other studies that also found lysine losses to be higher in FLF compared to FLC diets (Canibe et al., 2007a; O'Meara et al., 2020c). Canibe et al. (2007a) and Torres-Pitarch et al. (2020a) also detected lower levels of putrescine and cadaverine when the cereal fraction alone was fermented, with comparable concentrations across studies (Table 1.7), indicating that cereal fraction fermentation is a suitable strategy for minimising amino acid loss in LF.

In another study, Torres-Pitarch et al. (2020b) investigated the microbiota of LF where the cereal component of the diet (with or without a carbohydrase complex) was soaked for 3 h prior to mixing with the remaining dietary components immediately before feeding. Like the previous study, *Pseudomonas* and *Pantoea* were the predominant genera in the mixing tank, while in the troughs there was a shift to *Firmicutes*, primarily LAB including *Lactobacillus*, *Leuconostoc*, *Weisseilla* and *Lactococcus*, hence the high LAB counts obtained from the culture-dependent data (Table 1.7). This provides further evidence of spontaneous fermentation in residual LF remaining in short *ad libitum* troughs. There were less obvious differences in the microbiota between treatments;

however, *Lactobacillus* was more abundant in all soaked diets, indicating that the enzyme complex released carbohydrates suitable for LAB fermentation during the enzyme soaking period. Additionally, similar to fermenting the cereal fraction of the diet alone, soaking the cereal fraction of the diet prior to mixing with the remainder of the diet resulted in lysine losses of < 4 % from the mixing tank to the troughs, while reductions of 12 % were observed for the fresh LF diets, owing to the occurrence of spontaneous fermentation in the troughs (Torres-Pitarch et al., 2020b).

Overall, the literature suggests that uncontrolled spontaneous fermentation of fresh LF contributes to decreased microbial and nutritional quality, with a need to implement strategies to improve feed quality. However, with deliberate fermentation, careful consideration should be given to the choice of microbial inoculant. The strain should be a homofermentative LAB isolated from feed and shown to be capable of dominating the fermentation and producing sufficient lactic acid. The inclusion of a suitable yeast strain that can dominate without contributing to off-flavours and significant energy loss in the diet should also be pursued. Cereal fraction fermentation or soaking also appear to be suitable strategies to improve the microbial and nutritional quality of LF, particularly in terms of minimising the loss of synthetic lysine. Nonetheless, analysis of the feed alone is not sufficient to justify use of these strategies. Section 1.22 will discuss how these strategies impact the pig gut microbiota, growth and FE.

1.21.3 Acidified liquid feed

One of the key benefits of fermenting LF is the increased production of organic acids from LAB fermentation, mainly lactic acid. Therefore, direct addition of organic acids to fresh LF is an alternative to intentional or spontaneous fermentation. As discussed, fermentation of LF can result in undesirable effects including decarboxylation of added synthetic amino acids, which has, at least in part, been attributed to *E. coli* and other members of the *Enterobacteriaceae* family (Niven et al., 2006). Rapid acidification of LF may therefore minimise free amino acid loss as *Enterobacteriaceae* are at their highest levels during the first phase of fermentation, when the pH is high and levels of LAB (and thus lactic acid concentrations) are low (Brooks, 2008). Therefore, adding acid to LF during preparation should rapidly decrease the pH and inhibit the proliferation of enterobacteria. This approach has a similar aim to that of fermenting the cereal fraction of the diet prior to addition of synthetic amino acids, as discussed in the previous section.

Geary et al. (1999) assessed the microbial profile of a fermented liquid weaner diet that was either supplemented with lactic acid or inoculated with *P. acidilactici* at the start of each new batch of feed. For the first 4 days of the experiment, coliforms increased in the *P. acidilactici*-inoculated FLF (pH 4.5) but then declined to become undetectable, while in the lactic acid-supplemented diet (pH 4) coliforms were eliminated after only 2 days. However, considering that both treatments were effective at inhibiting coliform growth, albeit at different rates, and there were no differences in the growth or FE of the pigs fed these diets, the authors suggested that fermentation with *P. acidilactici* is more practical. This is because to achieve the same drop in pH the cost of the inoculant was significantly lower compared to that of lactic acid. Lawlor et al. (2002) also used lactic acid to acidify a liquid diet for weaned pigs. However, it should be noted that they prepared fresh diets daily, while Geary et al. (1999) added lactic acid to each new batch of feed with residual feed from the previous batch remaining in the tank i.e., the diet was FLF supplemented with lactic acid. This difference in feed preparation was reflected by Lawlor et al. (2002) reporting LAB counts ~2 $\log_{10}$ CFU/g lower than Geary et al. (1999), along with lower yeast counts. However, Lawlor et al. (2002) also reported higher coliform counts compared to the aforementioned study, which may have been a consequence of the lower LAB population.

Canibe et al. (2007b) performed an *in vitro* study to investigate acidification of a liquid grower diet with either formic acid or a commercial acid blend (Boliflor® FA 2300S). The diets were fermented for 2 days with no feed or water removed or added, and thereafter the diets were backslopped with 10 % retained after 48, 55, 72, 79, and 96 h of fermentation. The control diet showed a typical LAB fermentation pattern; after ~24 h LAB counts were ~8 $\log_{10}$ CFU/g, at which point the lactic acid concentration increased, the pH decreased and *Enterobacteriaceae* began to decline, although counts were still relatively high after day 2. In contrast, in the acidified diets, *Enterobacteriaceae* began to decline immediately due to the bactericidal effect of the acids already present, and LAB populations were much lower (Canibe et al., 2007b). The authors also measured amino acid losses throughout fermentation. For all diets, at between 96 and 108 h of fermentation, losses were 26–34 %, 31–38 %, and 31–42 % for free lysine, threonine, and methionine, respectively. Dietary acidification prior to fermentation was effective in reducing *Enterobacteriaceae* counts, albeit not to the desired extent, which likely contributed to this amino acid degradation. Unexpectedly, however, levels of putrescine, cadaverine, tyramine and histamine were below the detection limit of 10 mg/kg DM. The pH of the acidified diets rose during the first 48 h of fermentation, to ~5.5 in the case of

Boliflor® FA 2300S-supplemented feed. This may have been a factor in reducing the antibacterial activity of the organic acids as they are more effective at a lower pH i.e., the lower the external pH, the more undissociated acid can cross the cell membrane (depending on the pK_a of the individual acids) and reduce intracellular pH, ultimately leading to cell death (Bearson et al., 1997). Therefore, it is advisable to add acid to the diet to achieve a pH of 4 as performed by Lawlor et al. (2002) and Geary et al. (1999) with lactic acid. Further research is required to identify the microbes in LF that are responsible for amino acid degradation and to investigate the contribution of LAB (Barbieri et al., 2019; Tavaria et al., 2002).

Benzoic acid is another organic acid that has been used to control fermentation. It is used as a food and feed preservative, owing to its antibacterial and antifungal properties (Mao et al., 2019; Tugnoli et al., 2020). O'Meara et al. (2020b) examined the microbial quality of LF supplemented with benzoic acid (VevoVitall®) at inclusion levels of 0, 2.5, 5 and 10 kg/t. As in other studies, spontaneous fermentation of the control diet was observed in the troughs, as evidenced by reduced pH and higher LAB counts and ethanol concentrations compared to the mixing tank. Inclusion of 10 kg/t (1 %) benzoic acid, however, appeared to control spontaneous fermentation to some extent, as evidenced by lower LAB counts and a higher pH in the troughs, compared to the control. However, yeast and *Enterobacteriaceae* counts were not affected by treatment, which like the previous study by Canibe et al. (2007b) may be due to an insufficient decrease in feed pH, albeit they did see reductions in *Enterobacteriaceae*. Interestingly, in the O'Meara et al. (2020b) study, the benzoic acid used did, however, appear to be more effective than the formic acid used by Canibe et al. (2007b) in preventing amino acid degradation, as the highest amino acid losses occurred in the control diet. In order to achieve a pH of 4 in the diet (so as to reduce *Enterobacteriaceae* effectively), the inclusion rate of benzoic acid would need to be 64.3 kg/t which would likely negatively affect growth and FE (van der Peet-Schwering et al., 1999) in addition to being too costly. However, considering that many organic acids are either more effective for pH reduction or bactericidal activity, depending on their pK_a value and the pH of the feed, it is typically recommended to use a blend of acids in order to maximise both effects (Nguyen et al., 2020). Although there appears to be some benefits associated with the addition of organic acids to LF, controlled microbial acidification with homofermentative strains may be a more appropriate and less expensive strategy to improve LF microbial quality. The impact on gut microbiota, growth and FE of ALF-fed pigs will be discussed in Section 1.22.

1.21.4 Other disadvantages of liquid feed

Despite the higher DM intake of pigs fed LF compared to dry feed, feed conversion efficiency (FCE) is usually poorer, primarily due to feed wastage, especially with short trough/*ad libitum* liquid feeding (L'Anson et al., 2012; Russell et al., 1996). Issues with feed palatability and thus feed refusals can also occur with LF, usually due to the formation of acetic acid and ethanol resulting from yeast blooms (Brooks et al., 2001; Moran, 2001). The DM content of LF is limited by the capacity of the liquid feeding system to pump the mixture through the pipelines (Sol et al., 2019). Older liquid feeding systems with less efficient pumps and more bends in the piping may require the use of excessive water:feed ratios to reduce the viscosity of LF mixtures. This results in reduced growth and poorer FE due to reduced feed intake (which is limited by the intake capacity of the pigs) as well as energy being expended to excrete excess water (Sol et al., 2019). Additionally, this results in increased manure production and therefore higher manure transport and storage costs due to a more dilute nutrient content (Brooks et al., 2001; Russell et al., 1996). The use of liquid co-products also requires on-farm storage, which incurs an extra cost. There is also the risk of spoilage during storage, with the potential for deterioration of nutritional quality and energy loss, thereby requiring a high level of quality control and management when formulating diets (de Lange & Zhu, 2012; Scholten et al., 2001).

1.22 Liquid feed system sanitisation and impact on the microbial profile of liquid feed

As discussed in Section 1.15, mixing of dry feed with water and/or co-products should lead to the development of a stable feed microbiota with desirable characteristics. However, malfermentation can occur resulting in undesirable metabolites, palatability issues, and loss of synthetic amino acids and energy (Brooks et al., 2001; Canibe et al., 2007b; Missotten et al., 2010; Niven et al., 2006). This could suggest that greater attention should be paid to the sanitisation of liquid feeding systems to prevent carryover between feeds. However, it remains unclear whether routine cleaning and disinfection of liquid feeding systems is beneficial or detrimental to the quality of LF, and clear guidelines for farmers are lacking. Frequent cleaning of liquid feeding systems is generally not recommended, unless analysis of the LF has revealed that it is of poor microbial quality, in which case the system should be completely cleaned between batches of pigs using acids and/or bases (Brooks et al., 2001; de Lange & Zhu, 2012; Fisker & Jørgensen, 2010; Meat and Livestock Commission, 2003). Sanitisation practices used for liquid feeding

systems on commercial farms are highly variable. For example, O'Meara et al. (2020a) surveyed cleaning practices on eight commercial pig farms in Ireland and found that mixing tanks were either not cleaned at all or were washed only with water, with the frequency of cleaning ranging from weekly to after every batch of pigs (Table 1.8).

Table 1.8: Liquid feed system cleaning and sanitisation regimes and dietary co-product inclusion for finisher feed on eight Irish, and 27 Swiss pig farms.

References	(O'Meara et al., 2020a)								(Heller et al., 2017)	
	Unit A	Unit B	Unit C	Unit D	Unit E	Unit F	Unit G	Unit H	Antibiotic (+) farms (n = 13) [A]	Antibiotic (–) farms (n = 14)
Cleaning frequency MT *	2×/month [†]	Never	Never	4×/day	1×/month	Never	1×/week	1×/10weeks	N/A	N/A
Cleaning agent MT	Water	N/A [‡]	N/A	Formic acid [§]	Water	N/A	Water	Water	N/A	N/A
Cleaning frequency pipes	2×/month	Never	Never	4×/day [¶]	1×/month	Never	Never	4 to 5×/day	1×/6–10 days (n = 4) 1×/3 months (n = 13)	1×/week (n = 2) 1×/3 months (n = 5) 1×/6–12 months (n = 3) Never (n = 4)
Cleaning agent pipes	Water	N/A	N/A	Formic acid **	Water	N/A	N/A	Air [††]	Organic acid (n = 4) [B] Caustic soda and/or sodium hypochlorite (n = 8) Never (n = 1)	Organic acid (n = 4) Caustic soda and/or sodium hypochlorite (n = 4) Other (n = 2) Never (n = 4)

Cleaning frequency troughs	Never	Never	Never	1×/12wks	2-3x/year ‡‡	Never	3×/year ‡‡	1×/10wks	N/A	N/A
Cleaning agent troughs	N/A	N/A	N/A	Water	Water and lime §§	N/A	Water and detergent ¶¶	Water and disinfectant ***	N/A	N/A
Co-product inclusion	Pot ale syrup (14 %) and Liquid whey (21 %)	N/A	N/A	N/A	N/A	Pot ale syrup (10 %)	Pot ale syrup (15 %)	N/A	Whey (*n* = 3) Acidified whey (*n* = 2 of 3)	Whey (*n* = 5) Acidified whey (*n* = 2 of 5)

* MT: Mix tank; † Twice per month; ‡ N/A: Not performed; § 85 % Formic acid (Water Technology Limited, Cork, Ireland) at a 1 % inclusion rate with water; ¶ 4x/day represents once after each feed daily; ** 85 % Formic acid (Water Technology Limited, Cork, Ireland) at a 1 % inclusion rate with water and mix sits in the feed pipes between feeds; †† Hydro Air liquid feeding system; ‡‡ Except during the winter months; §§ Water followed by lime; ¶¶ Water followed by detergent (Top Foam™, MS Schippers, Bladel, The Netherlands); *** Water followed by disinfectant (Hyperox, Du Pont, Sudbury, United Kingdom). A Antibiotic (+) farms were administering in-feed antibiotics to finisher pigs. Antibiotic (−) farms had not administered in-feed antibiotics for at least 2 years. B Cleaning agents for pipes for Heller et al. (2017) were either added to water for circuit pipeline cleaning or flushing after cleaning.

In the Irish survey, cleaning of troughs varied from once or twice a year to after each batch of pigs. One farm used a detergent for cleaning troughs, one used lime after washing troughs with water, and only one farm used a disinfectant (O'Meara et al., 2020a). Despite the highly variable cleaning practices used on Irish farms, O'Meara et al. (2020a) found that these practices had no significant impact on microbial counts or pH of the LF, although a larger scale study is warranted.

The surface of the mixing tanks, feed lines and troughs of liquid feeding systems are coated with biofilms (Heller et al., 2017). These are complex structures composed of a mucous-like extracellular polymeric matrix secreted by the enclosed microbes, which protects them from the environment (Boe-Hansen et al., 2003; Brooks et al., 2001; de Lange & Zhu, 2012) (Figure 1.9). In fact, recently, Heller et al. (2017) found that administration of antibiotics via liquid feeding systems selects for antibiotic resistant bacteria within these biofilms that may potentially confer resistance to the feed microbiota and the pig gut microbiome. In a similar manner, biofilms in the pipelines may act as an inoculum for feed passing through the pipes and, therefore, the microbial community within the biofilm will likely affect the composition of the LF microbial community i.e., biofilms may act as a reservoir of LAB, yeast or potential pathogens. Biofilms are difficult to remove and are highly resistant to cleaning and, therefore, if the microbial composition of biofilms in the liquid feeding system is unfavourable, this may warrant intensive cleaning (Puligundla & Mok, 2017). Conversely, if the pipeline is dominated by potentially beneficial LAB biofilms, cleaning may have a detrimental impact on feed quality. However, most likely due to the difficulty associated with sampling inside feedlines and mixing tanks, to our knowledge no studies to date have examined the impact of microbial biofilms on feed quality in LF systems.

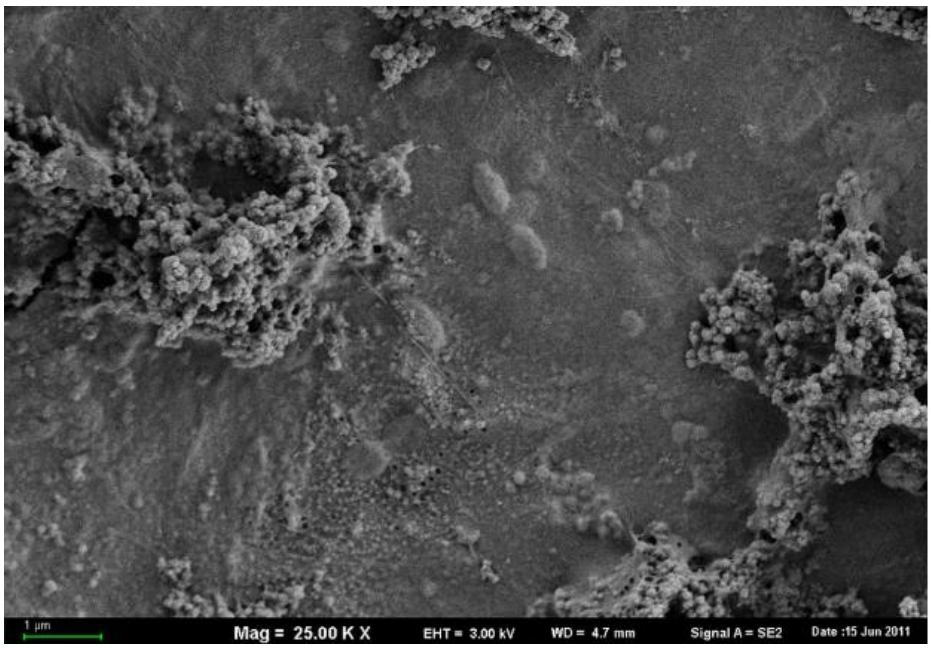

Figure 1.9: Scanning electron micrograph (×25,000 magnification) of bacterial cells in a biofilm (immersed in the extracellular polymeric substance matrix) on the surface of a high-density polyethylene pipe in a model drinking water distribution system [from Rożej et al. (2015)], distributed under the terms of the Creative Commons CC BY license).

Relatively few studies have explored the impact of cleaning on the microbiological quality of LF. The SEGES Danish Pig Research Centre reported that it took several days following cleaning and/or disinfection of liquid feeding systems for LAB fermentation to become re-established in the feed, allowing for the proliferation of coliforms in the days following cleaning, likely contributing to diarrhoea in the herds they studied (Hansen, 1987). They also recommended the addition of 2 % formic acid to LF to control coliforms after cleaning. In addition, they highlighted that drop pipes in particular are of concern in terms of mould growth and mycotoxin contamination, and may require weekly high-pressure cleaning (Hansen, 1987). However, in a study conducted by Royer et al. (2004) on nine finisher farms in France, four of which were termed 'problematic', with a high mortality rate associated with gastrointestinal issues, no clear association between cleaning and disinfection of the feeding systems and pig mortality was made. The authors suggested that the origin of the problem was likely multi-factorial but was possibly related to the inconsistent impact of the cleaning/disinfection regime on the microbial quality of the LF (Royer et al., 2004).

Royer et al. (2005) also performed a study to investigate the extent of microbial contamination in different parts of a liquid feeding system. They sampled 'contact water' that was passed through the system before and after an extensive cleaning and disinfection regime, to simulate the microbial load that a fresh batch of feed would acquire on passing

through. Microbial counts, ATP readings (an indicator of hygiene) and pH measurements of contact water sampled before, immediately after, and 14 days after cleaning, at different points of the liquid feeding systems are presented in Table 1.9. They found that the microbial load increased from the mixing tank to the drop pipes. This is in agreement with O'Meara et al. (2020a), who showed that LAB and yeast counts increased in LF from the mixing tank to fresh feed in the troughs, with further increases in residual feed in the trough just prior to the next feed-out, coupled with decreased pH and higher organic acid concentrations, indicating the occurrence of spontaneous fermentation (O'Meara et al., 2020a). Hence, the drop pipes were identified as a concern (Royer et al., 2005), as was the case in the SEGES study outlined above (Hansen, 1987). The general trend found by Royer et al. (2005) was that the cleaning and disinfection protocol resulted in a 2–3 $\log_{10}$ reduction in the bacterial load, but that within two weeks it had returned to pre-cleaning levels (Table 1.9). This possibly explains why O'Meara et al. (2020a) found no significant differences between farms that used different cleaning protocols. From this, Royer et al. (2005) suggested that cleaning of the mixing tank alone will likely have little impact unless other parts of the system i.e., the drop pipes and troughs are also cleaned. They noted, however, that this extent of cleaning and disinfection may not be justifiable due to a lack of evidence linking LF system hygiene with a significant risk to pig health. However, recently another French group established a relationship between a decrease in the LAB:*Enterobacteriaceae* ratio (i.e., a deterioration of LF quality) and the occurrence of digestive problems including diarrhoea, torsion and oedema in liquid-fed finisher pigs across 49 farms (Brunon et al., 2020).

Table 1.9: Microbiological counts (log_{10} CFU/g), ATP readings (relative light units) and pH (± SD) of contact water sampled at different locations on nine pig farms before, immediately after and 14 days after extensive cleaning of the liquid feeding systems [adapted from Royer et al. (2005)].

Microbial Groups and Other Parameters	Group		Time of Sampling [b]			Sampling Location [c]		
	Pb + [a] (*n* = 36)	Pb – (*n* = 45)	Before (*n* = 27)	After (*n* = 27)	D14 (*n* = 27)	Mixing Tank (*n* = 27)	Main Circuit (*n* = 27)	Drop Pipes (*n* = 27)
pH (*n* = 81)	7.35 ± 0.46	7.18 ± 0.62	7.05 ± 0.59	7.66 ± 0.42	7.07 ± 0.42	7.31 ± 0.46	7.34 ± 0.48	7.13 ± 0.69
ATP (*n* = 81)	3.54 ± 0.73	3.67 ± 0.86	4.03 ± 0.76	3.23 ± 0.54	3.55 ± 0.88	2.90 ± 0.61	3.60 ± 0.48	4.31 ± 0.61
Total bacteria (*n* = 79)	4.79 ± 1.63	4.70 ± 2.18	6.04 ± 1.60	3.06 ± 1.61	5.19 ± 1.35	3.56 ± 1.67	4.75 ± 1.90	5.86 ± 1.61
Lactic acid bacteria (*n* = 79)	4.16 ± 1.82	4.00 ± 2.28	5.28 ± 1.68	2.29 ± 1.67	4.71 ± 1.60	2.91 ± 1.80	4.03 ± 1.90	5.30 ± 1.89
Coliforms (*n* = 80)	2.13 ± 1.27	2.19 ± 1.31	2.70 ± 1.32	1.24 ± 0.85	2.55 ± 1.22	1.57 ± 1.07	2.09 ± 1.17	2.83 ± 1.30

[a] Pb + and Pb - groups are four and five problematic and non-problematic farms in southwestern France, respectively. Pb + refers to a mortality rate associated with gastrointestinal issues > 4 % during the finisher stage, while Pb – farms did not have issues with mortality. [b] Time of sampling: values before cleaning (Before), immediately after cleaning (After), and 14 days after cleaning (D14) for Pb + and Pb - farms. [c] Sampling location: samples of rinse water taken from the mixing tank where the diets are prepared (mixing tank), from lines of the main circuit (main circuit), and from drop pipes above the troughs (drop pipes). Values are means of all sampling times (before, immediately after, and 14 days after cleaning for Pb + and Pb - farms).

The design of liquid feeding systems on individual farms must also be taken into account when considering sanitisation protocols. For example, in some feeding systems, particularly older ones, residual feed is allowed to sit in the feed lines between feeds (described as 'residue-containing LF' in Section 1.16). Brunon et al. (2020) reported higher yeast and mould counts when residual LF remained stagnant in the feeding circuit compared to when pipes were rinsed with water and returned to a rinse tank, as is the case with 'residue-free' LF. It should be noted that for feeding systems where feed remains in the pipes between feeds, organic acids are often added to the feed to control fermentation in the pipes. Royer et al. (2005) reported that in the case of rinse water remaining in the feeding circuit for several hours, natural acidification occurred but was not sufficient to inhibit coliforms. Nonetheless, coliform counts in the rinse water remaining in the pipes were reduced 14 days post-cleaning and disinfection (Royer et al., 2005), potentially due to disruption of biofilms. An alternative to rinse water is the use of high-pressure air to clean feed lines.

Residue-free liquid feeding can result in lower concentrations of organic acids and biogenic amines in LF (Jørgensen et al., 2011). Fisker & Jørgensen (2010) suggested that extensive cleaning and disinfection of liquid feeding systems and regular weekly cleaning may have an effect similar to that of residue-free liquid feeding but by killing and/or inhibiting (rather than preventing proliferation of) undesirable microbes that produce fermentation products that can affect the pigs' appetite and health. They investigated four groups of lactating sows receiving LF and experiencing issues with appetite, diarrhoea, low weaning weight and piglet mortality. Details of the cleaning and disinfection procedure used can be found in Fisker & Jørgensen (2010) but, briefly, it involved filling, soaking, recirculating and rinsing the entire system with detergent and hot water (alkaline solution) followed by the same procedure with disinfectant (acidic solution). Liquid feed was sampled at 2-week intervals for 6 weeks before and 6 weeks after the cleaning and disinfection regime. No significant differences were observed in the pH, microbial counts and organic acid/biogenic amine concentrations between pre- and post-cleaning feed samples (Table 1.10). Therefore, the quality of the feed was unlikely to have been the cause of the sow health issues. However, the authors noted that the majority of the samples taken before cleaning were within the normal microbiological ranges for LF and this may explain why there was no significant difference in the quality of the feed following cleaning of the feeding system. They recommended that the entire system should be emptied and thoroughly cleaned if the *Enterobacteriaceae* count exceeds 7 $\log_{10}$ CFU/g in the feed. However, where *Enterobacteriaceae* counts are between 4 and 7 $\log_{10}$ CFU/g or if the mould counts are above 3 $\log_{10}$ CFU/g, the focus of cleaning should be on the mixing tank and supply pipes, not on emptying and cleaning the whole system (Fisker & Jørgensen, 2010)

Table 1.10: Microbiological counts and organic acid and biogenic amine concentrations in liquid feed sampled from valves mid-way along the circuit of four farms before and after cleaning and disinfection of the entire liquid feeding system [adapted from Fisker & Jørgensen (2010)].

		Microbial counts (log_{10} CFU/g) and pH					
Farm No.		pH	Enterobacteria	Lactic Acid Bacteria	Yeast	Mould	*Clostridium perfringens*
1	Before [a]	4.6	<3.5 [c]	9.1	7.0	<3.1	<2.2
	After	4.5	<3.5	9.0	6.6	<3.0	<2.4
2	Before	4.8	<3.7	8.9	6.6	<3.0	<2.0
	After	5.0	4.0	9.0	6.7	<3.0	<2.0
3	Before	4.9	5.4	9.1	6.0	<3.0	<2.0
	After	5.0	4.9	9.1	6.5	<3.0	<2.0
4	Before	5.1	4.9	8.9	6.5	<3.8	<2.0
	After	4.9	<3.2	9.0	6.6	<3.0	<2.0

		Organic acids and ethanol (mmol/kg)						
		Formic Acid	Acetic Acid	Propionic Acid	Lactic Acid	Succinic Acid	Butyric Acid	Ethanol
1	Before	21.1	15.3	3.9	81.5	1.5	ND	21.0
	After	20.8	12.2	1.4	81.7	ND	2.7	16.7
2	Before	9.1	18.4	ND	103.7	ND	0.6	22.4
	After	4.2	14.5	ND	83.1	ND	0.6	15.3

3	Before	2.8	12.6	4.0	88.4	1.2	2.3	6.4	
	After	ND [b]	13.8	1.1	81.7	1.1	1.4	13.0	
4	Before	ND	10.8	0.5	56.2	0.5	4.4	8.7	
	After	5.3	14.2	1.6	82.6	ND	1.2	14.4	
		Biogenic Amines [d] (mg/kg dry matter)							
		Phe	Cad	His	Put	Spd	Spr	Try	Tyr
1	Before	1	729	5	115	54	19	118	4
	After	1	983	2	172	61	17	136	3
2	Before	1	564	35	127	48	17	64	4
	After	1	811	44	129	50	20	10	4
3	Before	1	126	30	44	47	20	13	4
	After	1	267	44	64	47	20	40	4
4	Before	1	582	23	90	46	17	6	2
	After	1	471	20	67	43	14	18	3

[a] Before = Mean of values from liquid feed sampled at 2-week intervals for 6 weeks before the cleaning and disinfection regime. After = Mean of values from liquid feed sampled at 2-week intervals for 6 weeks after the cleaning and disinfection regime. For each farm, three samples were taken before (for farm 1, four samples were taken before cleaning) and three samples after the liquid feeding system had been cleaned and disinfected. All samples were taken during a normal feeding from a valve in the middle of the feeding circuit. [b] ND = Not detected. [c] The less than symbol (<) denotes that one or more observations from which the mean was calculated had values less than the detection limit (3 $\log_{10}$ CFU/g for enterobacteria and mould; 2 $\log_{10}$ CFU/g for *Clostridium perfringens*). [d] Phe = phenylethylamine; Cad = cadaverine; His = histamine; Put = putrescine; Spd = spermidine; Spr = spermine; Try = tryptamine; Tyr = tyramine.

In summary, studies investigating the impact of feed system sanitisation on the microbial quality of LF and/or the hygiene of feeding systems have yielded conflicting results, with more research required. However, in general, it does appear that intensive cleaning and disinfection of the entire system can improve the microbial quality of feed temporarily, but a quick return to pre-cleaning values occurs, with some studies showing no effect at all. In addition, immediately following cleaning, as at the beginning of fermentation, LAB counts are low, the pH is high and coliform blooms can occur in the LF (Hansen, 1987; Heller et al., 2017), which may be mitigated by the use of microbial inoculants immediately after cleaning and/or by acidification of feed with organic acids. Farms should be assessed on an individual basis before implementation of any cleaning protocol. First of all, if the microbial quality of the feed is in question due to poor growth, appetite or health of the pigs, a review of the liquid feeding system should be carried out to eliminate any obvious issues.

The type of liquid feeding system must also be considered e.g., a residue-containing liquid feeding system where feed or rinse water is sitting in the pipes between feeds, may benefit from regular intensive cleaning. The microbial quality of feed prior to cleaning must also be assessed before implementing an intensive cleaning protocol, as these cleaning regimes are costly as well as being time- and labour-intensive. Therefore, if microbial quality parameters are within the normal range, there is likely no benefit to cleaning the feed system. Further research is warranted to investigate alternative cleaning protocols in addition to the composition of biofilms in liquid feeding systems and their influence on feed microbial quality and the harbouring of antibiotic resistance genes. Access to biofilms in feedlines is challenging; however, it may be possible to remove a small section of piping from the feeding circuit for analysis. Alternatively, studies on water distribution systems have used ‘test-plugs’ or disks which are mounted inside pipes and can be removed for analysis (Boe-Hansen et al., 2003). The literature also indicates that the drop pipes and troughs should be key areas for cleaning; firstly, due to the inflow of air to the drop pipes, promoting fungal growth, and secondly, if stale feed remains in the troughs it may reduce the quality of freshly delivered feed, even if the mixing tank and pipelines have been cleaned.

1.23 Impact of liquid feed on the pig gut microbiome and influence on pig growth performance

Thus far we have discussed the microbiology of LF and ways to optimise microbial quality with the aim of improving gut health and growth of liquid-fed pigs. This section will discuss how LF and the aforementioned strategies to optimise feed microbial quality impact the gut microbiome of pigs, and their growth and FE. Research on the link between the pig gut microbiome, growth and FE has been growing in recent years, with some evidence of an association between taxa including *Lactobacillus* and *Ruminococcus*, for example, and growth and FE. However, there are conflicting reports and it can be difficult to determine cause and effect (Gardiner et al., 2020; McCormack et al., 2017, 2019; Si et al., 2020; Yang et al., 2017). Nonetheless, liquid feeding may be an effective means of targeting these beneficial microbes. It is well established that one of the key benefits of feeding a fermented liquid diet to pigs, in terms of gut microbial ecology, is a reduction in pathogen numbers along the GIT, provided that the pH and lactic acid concentration in the LF are at the desired levels (Brooks, 2003; Missotten et al., 2015a; Van Winsen et al., 2001). Many of the studies that have examined the impact of different liquid feeding strategies on the GIT of pigs have focused on key microbial groups via traditional culturing and molecular methods. However, an increasing number of studies are reporting high-throughput sequencing data, which is important in determining the full microbial profile of the GIT of liquid-fed pigs and the impact of liquid feeding on these microbial communities.

1.23.1 Suckling and weaned pigs

Many liquid feeding studies have focused on suckling and weaned piglets. Liquid feeding of weaned pigs promotes higher feed intake, helping to avoid the post-weaning lag in growth normally observed after weaning, as well as facilitating the transition from sow's milk to solid feed (Jiang et al., 2000; Missotten et al., 2015a). Additionally, during weaning, the ability of pigs to produce sufficient gastric acid is under-developed and, therefore, they depend on the fermentation of lactose to lactate in order to maintain a low pH in the stomach (Brooks, 2008; Lawlor et al., 2020). The stomach is the first line of defence against pathogens and therefore, the provision of LF with a low pH enhances this barrier effect. For these reasons, the benefits of LF tend to be more pronounced in suckling and weaned pigs compared to grow-finishers (Missotten et al., 2010, 2015a). Liquid feeding, however, is often associated with poorer FE compared to dry feeding as it can lead to increased feed wastage and sedimentation of solids in the trough (Han et al.,

2006; L'Anson et al., 2012; Plumed-Ferrer & Von Wright, 2009; Russell et al., 1996). Improvements in trough design can however, minimise wastage and improve FCR (Russell et al., 1996).

Tables 1.11 and 1.12 summarise the impact of various types of liquid feeding on the gut microbiota of suckling and weaned pigs, and the growth performance of piglets fed liquid diets, respectively. Mikkelsen & Jensen (1998) fed naturally fermented FLF or fresh LF to weaners for a period of 4 weeks and found no differences in growth or FE between treatments, although average daily gain (ADG) and FCR were marginally improved for fresh LF. However, gastric pH and coliforms along the entire GIT were lower in pigs fed FLF, while yeast counts were higher. Lawlor et al. (2002) performed a series of experiments in pigs from weaning to slaughter, comparing growth performance after feeding fresh LF, lactic acid-ALF, FLF (*Lactococcus lactis*) or dry pellets for a period of 27 days, after which pigs were given dry pellets to 35 kg and a liquid finisher diet to slaughter. In the first two experiments, ADG and FE were poorer for fresh LF-fed pigs from weaning to 27 days post-weaning.

Table 1.11: The impact of feeding fresh, fermented, cereal fraction only fermented, soaked and enzyme-supplemented liquid feed on the gut microbiota of suckling, weaned and grow-finishing pigs.

Details of Pigs, Initial Age/Weight If Applicable (No. of Pigs)	Type of Liquid Feed	Methodology	GI Section/Sample Type (No. of Samples)	Differences in Gut Microbial Taxa/Diversity (and pH if Applicable) between Treatments	Reference
Suckling and weaned pigs					
28 days/7.9 ± 1.0 kg (*n* = 30)	FLF [a] (Bactocell® added to water and feed)	Plate counts	Faeces (*n* = 3)	NSD [j] between coliform and *Lactobacillus* counts between pigs fed FLF or dry diet	(Missotten et al., 2015b)
27 days/6.3 ± 1.2 kg (*n* = 45)	FLF (Bactocell® already added to dry diet before mixing)			NSD between coliform and *Lactobacillus* counts between pigs fed FLF, dry diet or dry diet with Bactocell®, except ↓ *Lactobacillus* spp. in FLF vs. dry diet with Bactocell® (day 8 of the trial)	
24 days/6.98 ± 0.15 kg (*n* = 360)	Fresh LF [b]	qPCR [h]	Caecum (*n* = 6)	↑ total bacteria vs. dry feed	(Jiang et al., 2019)
28 days (*n* = 25)	FLF (*Lactobacillus plantarum LQ80*)	16S rRNA gene cloning and sequencing	Ileum (*n* = 3)	↓ *Sarcina* vs. dry feed	(Tajima et al., 2010)
			Caecum (*n* = 3)	↑ *Dorea*, *Lachnospiraceae Incertae Sedis* vs. dry feed ↓ *Lactobacillus* vs. dry feed	
28 ± 1 days/8 ± 1.1 kg	FLF	Plate counts	Stomach (*n* = 8)	↑ LAB [k] (20 °C) vs. dry, ↓ yeast (37 °C) vs. FLC, ↓ yeast (20 °C) vs. FLC, ↑ yeast (20 °C) vs. dry	(Canibe et al., 2007a)

(*n* = 120)	FLC [c]		Caudal small intestine (*n* = 8)	↓ LAB (37 °C), ↑ LAB (20 °C), ↑ yeast (20 °C) vs. dry, ↓ yeast (37 °C) vs. FLC	
			Caecum (*n* = 8)	↓ yeast (37 °C) vs. FLC and dry	
			Mid-colon (*n* = 8)	↑ LAB (37 °C), ↑ LAB (20 °C) vs. dry, ↓ yeast (37 °C) vs. FLC	
			Stomach (*n* = 8)	↑ LAB (20 °C) vs. dry, ↑ yeast (37 °C and 20 °C) vs. FLF and dry	
			Caudal small intestine (*n* = 8)	↑ LAB (20 °C), ↑ yeast (20 °C) vs. dry, ↑ yeast (37 °C) vs. FLF and dry	
			Caecum (*n* = 8)	↑ yeast (37 °C), ↑ yeast (20 °C) vs. FLF	
			Mid-colon (*n* = 8)	↑ yeast (37 °C) vs. FLF, ↓ LAB (37 °C) vs. dry	
28 days/7.9 ± 1.1 (*n* = 20)	Fresh LF	Plate counts	Stomach (*n* = 10)	↑ pH vs. FLF	(Mikkelsen & Jensen, 1998)
			Small intestine (*n* = 10)	↓ pH vs. FLF	
			Entire tract (*n* = 10)	↓ yeast, ↑ coliforms vs. FLF	

34.3 ± 2.2 days/9.1 ± 1.7 kg (*n* = 48)	Fresh LF vs. fresh LF (+ WWDG [d]) vs. dry	Plate counts and 16S rRNA gene sequencing of individual isolates	Faeces (*n* = 4)	NSD in diversity of coliforms or lactobacilli between diets. Similar composition of lactobacilli on day 1. On day 36, pigs fed fresh LF (+WWDG) were dominated by obligate heterofermentative lactobacilli (74 %) vs. 35 % and 49 % for dry and fresh LF, respectively	(Pedersen et al., 2005)
Farrowing sows (*n* = 18) and their piglets	FLF (*Lactobacillus plantarum*)	Plate counts	Faeces (NR)	↓ coliforms in sows fed FLF vs. fresh LF and dry (7 days after parturition) ↓ LAB in sows fed fresh LF and dry after farrowing ↑ LAB in piglets (7 days old) when sows were fed FLF and fresh LF vs. dry ↓ coliforms in piglets (7 days old) when sows were fed FLF vs. fresh LF and dry	(Demecková et al., 2002)
7 days/~2.8 kg (*n* = 110)	FLF (*Bacillus subtilis*) vs. dry pellets (*Bacillus subtilis*)	Pyrosequencing of V1–V3 region of the 16S rRNA gene	Jejunum (*n* = 6)	↓ Observed OTUs [l], Chao1 and Shannon diversity in FLF vs. dry pellets ↓ *Streptococcus*, *Clostridium sensu stricto*, *Bacteroides* and *Flavobacterium* in FLF vs. dry pellets	(He et al., 2017)
			Colon (*n* = 6)	↑ Observed OTUs, Chao1 in FLF vs. dry pellets ↑ *Pseudobutyrivibrio*, *Lachnospiraceae unclassified*, *Erysipelotrichaceae unclassified*, *Ruminococcus*, *Clostridiales unclassified* and *Lachnospiraceae uncultured* in FLF vs. dry pellets	
10 weeks (*n* = 48)	FLF (*Lactobacillus plantarum*) vs. dry feed in *Salmonella*-challenged pigs	Plate counts	Stomach (*n* = 10)	↓ pH, ↑ lactobacilli, ↓ *Enterobacteriaceae*	(Van Winsen et al., 2001)
			Ileum (*n* = 10)	↓ *Lactobacillus plantarum*, ↓ *Enterobacteriaceae*	
			Caecum	↓ *Enterobacteriaceae*	

			(*n* = 10)		
			Colon (*n* = 10)	↓ *Enterobacteriaceae*	
			Rectum (*n* = 10)	↑ pH, ↓ *Enterobacteriaceae*	
Grow-finisher pigs					
31 ± 3.5 kg (*n* = 60)	FLF vs. fresh LF vs. dry	Plate counts	Stomach (*n* = 5)	↓ pH, ↓ total anaerobes, ↑ LAB (20°C), ↓ enterobacteria, ↑ yeast (20°C and 37°C) for FLF vs. fresh LF and dry	(Canibe & Jensen, 2003)
			Mid-small intestine (*n* = 5)	↓ pH for fresh LF vs. FLF and dry	
			Distal small intestine (*n* = 5)	↓ pH for fresh LF vs. dry, ↑ LAB (20 °C), ↓ enterobacteria for FLF, ↑ yeast (20 °C) vs. fresh LF and dry	
			Caecum (*n* = 5)	↓ LAB (37 °C) in FLF vs. fresh LF, ↓ enterobacteria in FLF vs. fresh LF and dry	
			Mid-colon (*n* = 5)	↓ total anaerobes, ↓ LAB (37 °C), ↓ enterobacteria for FLF, ↑ yeast (20 °C) vs. fresh LF and dry	
			Distal colon (*n* = 5)	↓ pH for fresh LF vs. FLF	
16 weeks/54.3 ± 6.3 kg (*n* = 32)	FLF (*Lactobacillus salivarius*) vs. FLF (Bactocell®) vs. FLF (Stabisil™) vs.	Plate counts	Faeces (*n* = 8)	↓ coliforms for FLF (Stabisil™) vs. other treatments, NSD in LAB counts between treatments, ↑ LAB:coliform ratio of FLF (*Lactobacillus salivarius*) and FLF (Stabisil™) vs. fresh LF and FLF (Bactocell®)	(Brooks et al., 2005)

	fresh LF				
33.4 ± 0.88kg (*n* = 392)	Fresh LF vs. fresh LF + ENZ [e] vs SLC [f] vs SLC + ENZ	250 bp [i] paired-end Illumina amplicon sequencing of V3-V4 region of the 16S rRNA gene	Ileum (*n* = 6)	↑ *Cellulolysiticum* (negatively correlated with ADG [m]) in SLC + ENZ vs. SLC ↑ *Leuconostoc mesenteroides*, *Lactococcus* and *Lactococcus raffinolactis* (taxa negatively correlated with carcass weight) in SLC + ENZ vs. fresh LF + ENZ	(Torres-Pitarch et al., 2020b)
			Caecum (*n* = 6)	↓ *Megasphaera elsdenni* (positively correlated with carcass weight and butyrate) in fresh LF + ENZ vs. fresh LF ↑ *Prevotellaceae NK3B31* sp., *Oscillibacter* sp. and *Rikenellaceaceae RC9* (negatively correlated with growth parameters and butyrate) in fresh LF + ENZ vs. fresh LF ↓ *Selenomonas* (positively correlated with ADG) in SLC + ENZ vs. SLC ↑ *Escherichia*/*Shigella*/*Brenneria* (negatively correlated with ADG) in SLC + ENZ vs. fresh LF + ENZ ↑ *Prevotellaceae NKB31 group* sp. and *Clostridium saudiense*/*disporicum* (negatively correlated with carcass weight, ADG, and butyrate) in SLC + ENZ vs. SLC ↓ *Roseburia faecis*/*intestinalis*/*hominis* (positively correlated with ADG and butyrate) in soaked diets vs. fresh diets ↑ *Rikenellaceae RC9* (positively correlated with butyrate concentration) in soaked diets vs. fresh diets	
40.6 ± 0.50 kg (*n* = 252)	Fresh LF vs fresh LF + ENZ vs FLC (Sweetsile®) vs	250 bp paired-end Illumina amplicon sequencing of V3-V4	Ileum (*n* = 6)	↑ *Pediococcus* in FLC (17 % relative abundance vs. 1–3 % for other treatments ↑ *Lactobacillus kisonensis* in fresh LF + ENZ, FLC and FLC + ENZ (positively correlated with ADG)	(Torres-Pitarch et al., 2020a)

	FLC + ENZ (Sweetsile®)	region of the 16S rRNA gene		↓ *Megasphaera* (negatively correlated with carcass weight) in fresh LF + ENZ ↑ *Bifidobacterium* (negatively correlated with ADG) and *Howardella* (positively correlated with butyrate and negatively correlated with ADG) in fresh LF + ENZ ↑ *Streptococcus* (negatively correlated with ADG) in FLC + ENZ ↓ *Megasphaera*, *Bifidobacterium*, *Streptococcus*, *Howardella* and *Streptococcus pasteurianus*/*alactolyticus*/*macedonicus* (negatively correlated with either carcass weight or growth) in fermented cereal diets	
			Caecum (n = 6)	↑ pH for FLC + ENZ vs. other treatments ↑ *Roseburia faecis* in LF + ENZ and FLC + ENZ (positively correlated with ADG)	
61 ± 2 days/20.8 ± 2.06 kg (n = 20)	FLF (*Lactobacillus plantarum*, *Pediococcus pentosaceus* and *Lactococcus lactis*) vs. fresh LF	Amplicon sequencing of the V4 region of the 16S rRNA gene	Small intestine (n = 20)	↓ pH, ↓ Shannon diversity in pigs fed FLF vs. fresh LF	(Bunte et al., 2020)
			Colon (n = 20)	↑ pH, Numeric ↑ in Shannon diversity in FLF vs. fresh LF (NSD)	
			Faeces (n = 20)	↑ Observed species, Chao1 and Shannon diversity in FLF vs. fresh LF	
	PFLF [g] (with non-fermented coarse cereals) vs. fresh LF		Small intestine (n = 20)	↑ *Lactobacillus*, ↑ *Bifidobacterium*, ↓ pH, ↓ Observed species, Chao1 and Shannon diversity in PFLF vs. fresh LF, ↑ *Leuconostoc* in fresh LF vs. PFLF	

	Colon (*n* = 20)	↑ *Lactobacillus*, ↑ *Bifidobacterium*, numeric ↑ in Shannon diversity in PFLF vs. fresh LF (NSD), ↑ *Leuconostoc* in fresh LF vs. PFLF
	Faeces (*n* = 20)	↑ *Lactobacillus*, ↑ *Bifidobacterium*, ↑ Shannon diversity in PFLF vs. fresh LF

[a] FLF = Fermented liquid feed: all dietary ingredients are fermented for a specific period of time prior to feeding. [b] Fresh LF = Fresh liquid feed: all dietary ingredients are mixed with water and fed out immediately. [c] FLC = Fermented liquid cereal: only the cereal fraction of the diet is fermented, with the remaining dietary ingredients added prior to feeding. [d] WWDG = Wet wheat distillers' grain. [e] ENZ = Xylanase and β-glucanase enzyme complex (Rovabio Excel AP, Adisseo France SAS, Antony, France); +/- indicates whether diets were supplemented (+) or not (-) with ENZ. [f] SLC = Soaked liquid cereal: cereal fraction of the diet soaked in water for 3 h prior to mixing with balancer fraction (soybean meal, synthetic amino acids, minerals and vitamins) immediately prior to feeding. [g] PFLF = Partially fermented liquid feed: rapeseed extracted meal and part of the rye (60 % of the whole diet) were fermented while the remaining cereal components were not fermented. [h] qPCR = Quantitative polymerase chain reaction. [i] bp = Base pairs. [j] NSD = No statistical difference. [k] LAB = Lactic acid bacteria. [l] OTUs = Operational taxonomic units. [m] ADG = Average daily gain.

Table 1.12: Growth performance, feed intake, feed efficiency and carcass quality of suckling, weaned and grow-finishing pigs fed fresh, fermented, cereal fraction only fermented, soaked and enzyme-supplemented liquid feed.

Initial Age/Weight of Pigs (Number of Pigs)	Type of Liquid Feed	Water:Feed Ratio	Performance [i]	Notes	Reference
Suckling and weaned pigs					
24 days/6.98 ± 0.15 kg (*n* = 360)	Fresh LF [a]	4:1	↑ BW, ↑ ADFI, ↑ ADG, same G:F vs. dry meal	Piglets weaned at 24 ± 1 days fed fresh liquid feed for 7 days	(Jiang et al., 2019)
32 days/19.3 kg (*n* = 280)	Fresh LF	2.15–2.23:1	Similar ADG, FCE and carcass weight vs. dry feed	Piglets were weaned at 32 days with access to dry creep feed from 10 days of age until the trial began. Water:feed ratio of fresh LF was 2.15: 1 (up to 70 kg LW) and 2.23: 1 (from 70–115 kg LW)	(Zoric et al., 2015)
26 days/8.4 kg (*n* = 192)	Fresh LF	2:1	↑ DMI, ↓ G:F vs. dry pellets (d 0 to slaughter)	Length of each experiment was 27 days. After day 27 pigs were given dry pellets to 35 kg and liquid finisher diet (3:1 water:meal) to slaughter (95 kg)	(Lawlor et al., 2002)
26 days/8.4 kg (*n* = 150)	Fresh LF		↓ LW and carcass weight, ↓ G:F, ↑ lean meat % vs. dry pellets (d 0 to slaughter)		
26 days vs. 7.7 kg (*n* = 112)	Fresh LF		NSD in performance vs. dry pellets and ALF (d 0 to slaughter)		
26 days vs. 8.0 kg (*n* = 112)	FLF [b] (*Lactococcus lactis subsp. cremoris 303*)		↓ carcass weight, ↓ DMI vs. dry pelleted feed (d 0 to slaughter)		

22.56 ± 2.55 days (n = 48)	Fresh LF	2.5:1	↑ ADFI, ↑ ADG, ↓ FCR vs. dry pellets	Pigs were fed the treatments for 28 days in the two feeding trials	(Russell et al., 1996)
22.56 ± 2.55 days (n = 98)	Fresh LF		↑ ADFI, ↑ ADG, ↓ FCR vs. dry pellets		
28 days vs. 7.9 ± 1.0 kg (n = 30)	FLF (Bactocell® added to water and feed)	2.5:1	↓ BWG during week 4 of the trial vs. dry feed	FLF was produced with Bactocell® with 50 % daily backslopping. The experimental period was 4 weeks. The diet in the second trial contained 10 g/kg sepiolite to improve homogeneity of the liquid feed and avoid sedimentation	(Missotten et al., 2015b)
27 days vs. 6.3 ± 1.2 kg (n = 45)	FLF (Bactocell® already added to dry diet before mixing)		↑ DM intake, ↑ BWG, ↑ F:G vs. dry feed and dry feed with Bactocell®		
28 ± 1 days vs. 8 ± 1.1 kg (n = 120)	FLF	2.5:1	↓ ADG, ↓ ADFI, ↓ G:F vs. dry meal (d 1 to 42)	FLF and FLC were fermented at 20°C with 50 % backslopping 3 times daily. The experimental period began after 5 days of fermentation and lasted for a period of 6 weeks	(Canibe et al., 2007a)
	FLC [c]		↓ ADG, ↓ ADFI, ↓ G:F vs. dry meal (d 1 to 42)		
28 days vs. 7.9 ± 1.1 (n = 20)	Fresh LF	2.75:1	Numerical ↑ ADG and FCR vs. FLF (NSD)	FLF was fermented for 8 h with 50 % backslopping. The experimental period lasted for a period of 4 weeks	(Mikkelsen & Jensen, 1998)
21.0 ± 2.8 days vs. 5.7 ± 0.7 kg (n = 72)	Fresh LF-10 d	3: 1	↑ ADG, ↑ ADFI vs. dry feed (up to d 40)	Fresh LF-10 d was fed for 10 days followed by dry feed for 30 days vs. control (dry feed for 40 days)	(Han et al., 2006)
	Fresh LF-20 d		↑ ADFI vs. dry feed, ↓ FCE vs. dry feed and Fresh LF-10 d (up to d 40)	Fresh LF-20 d was fed for 20 days followed by dry feed for 20 days vs. control (dry feed for 40 days)	

28 days vs. 7.3 ± 0.3 kg (*n* = 136)	FLF	2.5:1	↑ BW, ↑ ADG, ↑ ADFI, ↓ FCR vs. dry feeding	FLF was mixed and then soaked for 15 h before feeding. No backslopping was performed with a new batch prepared for feeding the next day. The experimental period was 26 days	(L'Anson et al., 2012)
24 ± 4 days vs. 7 ± 1 kg (*n* = 48)	FLF (Bactocell®)	2.5:1	NSD in ADFI, ADG, FCR vs. control (on d 28) vs. control (but numerically improved for FLF)	Control diet was lactic acid-supplemented FLF. The experimental period lasted 28 days	(Geary et al., 1999)
28 days vs. 7.4 ± 0.4 kg (*n* = 72)	SLF [d]	2.5:1	↑ ADFI, ↑ ADG, ↑ FCR vs. dry feed	Feed was soaked for 1 h before feeding. Soaking for 24 h did not improve performance and supplementation with 300 ppm xylanase reduced feed intake. The experimental period lasted 26 days	(L'Anson et al., 2013)
Grow-finisher pigs					
31 ± 3.5 kg (*n* = 60)	Fresh LF FLF	2.5:1	↑ ADG vs. FLF, ↑ ADFI vs. FLF and dry feed ↓ ADG vs. fresh LF and dry feed, ↓ ADFI vs. fresh LF	FLF was fermented at 20°C for 4 days before the trial with 50 % backslopping at each feeding. G:F was similar for all diets. Performance parameters were recorded fortnightly up to final BW of 101 ± 4.0 kg	(Canibe & Jensen, 2003)
49.2 ± 0.68 kg (*n* = 64)	Fresh LF	3:1	↑ Final LW, ↑ ADG, ↑ Lean tissue growth rate vs. dry pellets	Trial period was 6 weeks of unrestricted feeding	(Hurst et al., 2008)
47.1 ± 1.55 kg (*n* = 64)	Fresh LF	3:1	↑ Final LW, ↑ ADG, ↑ ADFI, ↑ FCR vs. dry pellets	Trial period was 6 weeks of restricted feeding (5 % to 10 % below *ad libitum* intake).	
16 weeks vs. 54.3 ± 6.3 kg (*n* = 32)	Fresh LF FLF	2.5:1	NSD between treatments for ADG or FCR	FLF was inoculated with *Lactobacillus salivarius*, Bactocell™ or Stabisil™ and fermented for 24 h at 30 °C	(Brooks et al., 2005)

	(*Lactobacillus salivarius*)				
	FLF (Bactocell®)				
	FLF (Stabisil™)				
9-11 weeks vs. 18.3–29.6 kg (n=122)	Fresh LF	1.5, 2.5, 4:1	↑ ADG, ↑ FCR vs. dry diet	Treatments of fresh liquid feed with water:feed ratios of 1.5, 2.5, 4:1 were compared to dry feeding, where 2.5:1 resulted in better ADG and FCR	(Braude & Rowell, 1967)
32.7 kg (*n* = 432)	Fresh LF (meal)	2.5:1	↑ BW, ↑ ADG, ↓ G:F vs. dry and wet/dry meal	Two experiments were carried out with batches of 216 pigs. Both experimental periods lasted 64 days from 32.7 to 100 kg	(O'Meara et al., 2020d)
	Fresh LF (pellets)		↑ BW, ↑ ADG vs. dry pellets, ↓ G:F vs. dry and wet/dry pellets		
29.8 ± 0.92 (*n* = 216)	Fresh LF	2.5:1	↑ ADG vs. FLF, similar FCE to wet/dry (lowest)	The experimental period lasted 68 days prior to slaughter (29.8 ± 0.92 kg to 102.3 ± 0.76 kg). For FLF the whole diet was fermented prior to feeding, while for FLC only the cereal component of the diet was fermented and then mixed with the balancer and water prior to feeding	(O'Meara et al., 2020c)
	FLF (Sweetsile®)		↓ BW, ↓ ADG, ↓ FCE vs. fresh LF, FLC and wet/dry, ↑ ADFI vs. wet/dry		
	FLC (Sweetsile®)		↑ ADFI vs. wet/dry, ↑ ADG, ↑ FCE vs. FLF		
85.3 ± 1.69 kg (*n* = 160)	Fresh LF	2.5:1	↑ BW, ↑ ADG vs. wet/dry	The experimental period lasted 26 days prior to slaughter (85.3 ± 1.69 kg to 117.5 ± 0.72 kg). Diets were prepared as above	
	FLF (Sweetsile®)		↓ BW, ↓ ADG vs. FLC, ↓ ADFI vs. FLC and wet/dry, ↓ FCR vs. wet/dry		
	FLC (Sweetsile®)		↑ BW, ↑ ADFI vs. FLF and wet/dry, ↑ ADG vs. fresh LF, FLC and wet/dry		

33.4 ± 0.88kg (*n* = 392)	Fresh LF (ENZ [e] −) Fresh LF (ENZ +) SLC [f] (ENZ −) SLC (ENZ +)	2.5:1	Interaction between ENZ and soaking for ADG (d 21), ↑ LW (0.8 kg) for SLC vs. fresh LF (d 21), ↑ fat depth and lower lean meat % for ENZ + vs. ENZ (d 70), ↑ ADG for SLC (ENZ +) vs. fresh LF (ENZ +) at d 21	For fresh LF, the whole diet (including ENZ for ENZ +) was mixed with water and fed after 5 min mixing. For SLC diets, the cereal component of the diet (including ENZ for ENZ +) was mixed with water and agitated for 3 h, mixed with the balancer fraction for 5 min and fed out. The experimental period lasted 71 days	(Torres-Pitarch et al., 2020b)
40.6 ± 0.50 kg (*n* = 252)	Fresh LF (ENZ −) Fresh LF (ENZ +) FLC (Sweetsile®, ENZ -) FLC (Sweetsile®, ENZ +)	2.5:1	Interaction between FLC and ENZ for LW (d 28 and d 55), ↑ LW for FLC (ENZ −), ↑ ADG for FLC (4.1 %) vs. fresh LF, ↑ FCR for ENZ + diets (3.8 % lower), ↑ carcass weight (2.0 kg) and ↓ (1.0 %) lean meat % for FLC diets (d 55)	The experimental period lasted 55 days. Fresh LF diets were prepared as above. For FLC diets the cereal component of the diet (including ENZ for ENZ +) was mixed with water, inoculated with Sweetsile®, and fermented for 52 h, with daily backslopping. The balancer fraction was added to the fermented cereal and mixed for 5 min before feed out	(Torres-Pitarch et al., 2020a)
61 ± 2 days vs. 20.8 ± 2.06 kg (*n* = 20)	FLF (*Lactobacillus plantarum*, *Pediococcus pentosaceus* and *Lactococcus lactis*) vs. fresh LF PFLF [g] (with non-fermented course cereals) vs. fresh LF	NR [h]	Numerical ↑ ADFI in FLF (NSD), Numerical ↓ FCR in FLF (NSD)	Fresh LF was mixed with water immediately before feeding, while for FLF the whole diet was fermented with *Lactobacillus plantarum*, *Pediococcus pentosaceus* and *Lactococcus lactis* for 24 h. The experimental period lasted 28 days For PFLF, rapeseed extracted meal and part of the rye (60 % of the whole diet) were fermented as above while the remaining cereal components were not fermented. Phytases and the mineral supplement were added after fermentation. Fresh LF was prepared as above	(Bunte et al., 2020)

		but with phytases added. The experimental period lasted 28 days

↑ denotes an improvement in a parameter compared to the specified treatment group; ↓ denotes poorer performance in the parameter compared to the specified treatment group. [a] Fresh LF = Fresh liquid feed: all dietary ingredients are mixed with water and fed out immediately. [b] FLF = Fermented liquid feed: all dietary ingredients are fermented for a specific period of time prior to feeding. [c] FLC = Fermented liquid cereal: only the cereal fraction of the diet is fermented, with the remaining dietary ingredients added prior to feeding. [d] SLF = Soaked liquid feed: The whole diet was soaked for 1 h before feeding. [e] ENZ = Xylanase and β-glucanase enzyme complex (Rovabio Excel AP, Adisseo France SAS, Antony, France); +/− indicates whether diets were supplemented (+) or not (−) with ENZ. [f] SLC = Soaked liquid cereal: cereal fraction of the diet soaked in water for 3 h prior to mixing with balancer fraction (soybean meal, synthetic amino acids, minerals and vitamins) immediately prior to feeding. [g] PFLF = Partially fermented liquid feed: rapeseed extracted meal and part of the rye (60 % of the whole diet) were fermented while the remaining cereal components were not fermented. [h] NR: Not reported. [i] Performance parameters: BW: Body weight; ADFI: Average daily feed intake; ADG: Average daily gain; G:F: Gain to feed ratio; FCE: Feed conversion efficiency; DMI: Dry matter intake; LW: live weight; Lean meat %: Lean meat percentage; NSD: No statistical difference; ALF: Acidified liquid feed; FCR: Feed conversion ratio; BWG: Body weight gain; F:G: Feed to gain ratio.

During the third and fourth experiments, pigs fed ALF had the highest ADG, while the dry pellet-fed pigs had the best FE. The authors suggested that feeding ALF may be beneficial for the first 27 days post-weaning; however, the benefit was not sustained beyond that period. On the other hand, Geary et al. (1999) reported no growth benefit in pigs fed lactic acid-ALF for 28 days post-weaning. Nonetheless, other studies have shown much more promising results for liquid-fed weaned pigs (Han et al., 2006; L'Anson et al., 2012, 2013; Missotten et al., 2015b; Russell et al., 1996). Despite the improved growth rates often observed with liquid feeding, FCR is generally poorer than with dry feed, but there are exceptions, for example, a study by Missotten et al. (2015b) where sepiolite was added to a diet fermented with Bactocell® to help prevent sedimentation of solids. Additionally, L'Anson et al. (2013) found that soaking feed (i.e., mixing the dry feed with water and allowing it to steep for 1 h prior to feeding) improved feed intake, ADG and FCR compared to dry feeding weaners.

van Winsen et al. (2001) investigated the impact of *L. plantarum*-FLF on selected microbial groups of 10-week-old pigs challenged with *Salmonella* and found that FLF reduced *Enterobacteriaceae* counts along the entirety of the GIT, with lower pH and higher *Lactobacillus* counts in the stomach. Similar findings were reported by Demecková et al. (2002) who fed either *L. plantarum*-FLF, fresh LF or dry pellets to sows 2 weeks before farrowing, and for 3 weeks after. They found that coliform counts were lower, and LAB counts were higher in the faeces of sows fed FLF. Interestingly, coliforms were also lower in the faeces of piglets born to the sows fed FLF, while LAB counts were higher in piglets from sows fed either of the liquid diets compared to dry feed. Unfortunately, the growth of these piglets was not measured during the experimental period. The sow diet, microbiota and the microbes present in the environment (including the sow's faeces) are known to be key influences on microbial colonisation of the piglet GIT (Fouhse et al., 2016; Guevarra et al., 2019); therefore, liquid feeding a diet of desirable microbial quality to sows prior to farrowing may be an effective means of promoting gut health of the offspring. Tajima et al. (2010) fed weaned piglets *L. plantarum*-FLF and reported that the abundance of lactobacilli in the caecum decreased to ~30 %; however, a number of beneficial butyrate-producing genera within the *Firmicutes* phylum including *Coprococcus*, *Roseburia* and *Faecalibacterium* increased in abundance and therefore improved caecal bacterial diversity.

Canibe et al. (2007a) fed weaners (28 days old) FLF or FLC (LF where the cereal fraction alone was fermented) and found no difference between feed intake or FE between treatments but ADG was increased for the pigs fed FLC 2 and 6 weeks post-weaning. Yeast counts along the GIT of the pigs fed the FLC were consistently higher than when the whole diet was fermented. Additionally, terminal restriction fragment length polymorphism (TRFLP) analysis of digesta samples found fragments most likely representing *L. fermentum* to be dominant in the gut of pigs fed the FLF. This same fragment was also detected more frequently in the diet itself, and therefore it likely originated from the FLF. Similar to this, Pedersen et al. (2005) found that heterofermentative lactobacilli increased in the faeces of weaned piglets fed fresh LF with wet wheat distillers' grain compared to dry-fed and fresh LF-fed piglets, likely due to higher proportions of these strains in the wet wheat distillers' grain. It should be noted that in the Canibe et al. (2007a) study, comparisons were not possible for dry-fed pigs because the fermented liquid diets were fed restrictively to avoid further fermentation in the troughs, while dry feed was fed *ad libitum*. The authors suggested that the restricted feeding of the liquid diets may have limited the growth potential of these pigs (Canibe et al., 2007a). In the Pedersen et al. (2005) study, the pigs fed dry pelleted diets had improved growth and FE compared to those fed liquid diets at 5 weeks post-weaning. The LF with wet wheat distillers' grain reduced diarrhoea compared to the dry-fed and fresh LF-fed piglets during the same period (Pedersen et al., 2005).

Although growth performance was not investigated, He et al. (2017) studied the impact of feeding a liquid diet fermented with a probiotic *Bacillus subtilis* strain to 7-day-old piglets for 25 days compared to feeding the same probiotic in a dry pelleted diet. Cruz Ramos et al. (2000) identified lactate, acetate, and 2,3-butanediol to be the main products of *B. subtilis* fermentation. Additionally, *B. subtilis* can secrete a range of extracellular carbohydrases and proteases to release simple sugars, organic acids and amino acids (He et al., 2016). High-throughput 16S rRNA gene sequencing revealed that the pigs fed the *B. subtilis*-FLF had lower bacterial diversity but higher fungal diversity along the GIT. The former may be a result of the influx of *Lactobacillus* into the GIT of the pigs fed *B. subtilis*-FLF, while the latter may be a result of the introduction of LF-associated fungal taxa not normally resident in the GIT. Whether these fungi colonise the gut or are only temporarily present is a matter of debate. The authors reported that during suckling and early post-weaning, *B. subtilis*-FLF promoted the growth of LAB; however, diarrhoea

incidence was higher in this group, potentially due to the decreased bacterial diversity (He et al., 2017). In the jejunum of pigs fed the *B. subtilis*-FLF the relative abundance of *Streptococcus*, *Clostridium sensu stricto*, *Bacteroides* and *Flavobacterium* was decreased, while in the colon, *Pseudobutyrivibrio*, *Lachnospiraceae*, *Erysipelotrichaceae*, *Ruminococcus* and *Clostridiales* were at higher relative abundances compared to pigs fed the pelleted diet. Elevated levels of primary and secondary bile acids were also found in the colon of pigs fed the *B. subtilis*-FLF and it was suggested that this was a result of an increase in bile acid-metabolising bacteria, which include some members of *Lachnospiraceae*, *Erysipelotrichaceae*, *Ruminococcus* and *Clostridiales* which were found in the colon. This may have contributed to increased diarrhoea in these piglets (He et al., 2017; McJunkin et al., 1981). It is broadly known that bile acids are important for solubilising lipids and promoting their digestion and absorption (Burrin et al., 2013). These results indicate that further studies are required to better understand the effects of LF on intestinal microbiota composition and its potentially positive or negative health/production effects.

1.23.2 Grow-finishing pigs

Tables 1.11 and 1.12 summarise studies that investigated the impact of various types of liquid feeding on the gut microbiota of grow-finisher pigs, and the growth performance of pigs fed liquid diets, respectively. DNA sequence-based information on the microbial profile of the GIT of liquid-fed grow-finishers is still rather limited, although there are more data available compared to liquid-fed piglets. O'Meara et al. (2020d) investigated the optimal feed delivery method, as well as the optimal feed form for grow-finishers. They found that liquid-fed meal increased body weight and ADG, but worsened FE compared to feeding dry meal or wet/dry feeding meal (O'Meara et al., 2020d). Similar results were found for pigs fed a liquid pelleted diet compared to those fed dry and wet/dry-fed pelleted diets. Additionally, they found no advantage to liquid feeding a pelleted diet compared to a meal diet. The poorer FE of liquid-fed pigs was likely due to feed wastage as reported by others (Han et al., 2006; L'Anson et al., 2012; Russell et al., 1996). Additionally, the liquid diets in the O'Meara et al. (2020d) study were offered *ad libitum*, and considering that restricted feeding can improve FE compared to *ad libitum* feeding, this approach could mitigate against the negative effect on FE found in the study. However, restricted feeding will likely result in poorer growth as occurred in the study with weaned pigs by Canibe et al. (2007a). Zoric et al. (2015) found no differences

between ADG, FE or carcass weight in pigs fed fresh LF compared to dry feed up until slaughter.

Canibe & Jensen (2003) reported higher feed intake and ADG for growing pigs fed fresh LF compared to naturally fermented FLF and dry feed. However, similar to what has been observed in piglets fed FLF, the gastric pH and levels of enterobacteria along the GIT of growing pigs fed FLF decreased compared to those fed fresh LF, while lactic acid concentrations were also numerically higher in FLF-fed pigs. Brooks et al. (2005) compared fresh LF to three FLF diets that were inoculated with either *L. salivarius* (FLF-SAL), *P. acidilactici* (FLF-BAC), or a mixture of *P. acidilactici*, *P. pentosaceus*, *L. lactis*, and *L. plantarum* (FLF-STAB) to finisher pigs. There were no differences in ADG or FCR between treatments; however, coliform counts in the faeces of pigs fed the FLF-SAL diet were lower than in those fed fresh LF. Additionally, although LAB counts did not differ between treatments, the ratio of LAB:coliforms was higher in FLF-SAL and FLF-STAB compared to fresh LF.

Hurst et al. (2008) fed grow-finishers fresh LF or lactic acid-ALF at different water:feed ratios using either restricted or *ad libitum* feeding compared to dry feeding. *Ad libitum* feeding of fresh LF (water:feed ratio of 3:1) improved ADG and lean tissue growth rate compared to *ad libitum* dry feeding. Both the fresh LF and lactic acid-ALF, when offered on a restricted basis, resulted in improved ADG, lower feed intake, and hence improved FE, compared to restricted dry feeding (Hurst et al., 2008). Braude & Rowell (1967) also reported improvements in FE and ADG of growing pigs fed fresh LF, but at a water:feed ratio of 2.5:1. Benzoic acid has also been investigated as a means of improving growth in liquid-fed grow-finishers. Although 1% benzoic acid appeared to control spontaneous fermentation and minimise synthetic amino acid loss in LF (O'Meara et al., 2020b; Vils et al., 2018), O'Meara et al. (2020b) found no impact on growth, FE or carcass quality as a result of dietary benzoic acid inclusion. However, management of the liquid feeding system was excellent compared to other studies and therefore, feed wastage was minimal even with *ad libitum* feeding, resulting in exceptionally good growth rates and FE for all treatments. The authors noted that this likely made it difficult to observe a significant improvement in these parameters in benzoic-acid supplemented pigs.

In another study by O'Meara et al. (2020c) fresh LF, FLF, LF with FLC and wet/dry feeding were compared in grow-finishers. In the first experiment, pigs fed the FLF

(inoculated with Sweetsile® which contains *L. plantarum* and *P. acidilactici*) had lower ADG and FE, and were lighter at slaughter than pigs on the other treatments, while feed intake was higher in pigs fed the fermented diets. Carcass quality measures were also poorer for the FLF-fed pigs. At slaughter, during the second experiment, pigs fed FLC weighed more than FLF- and wet/dry-fed pigs with similar weights to those fed fresh LF, while FE in wet/dry fed pigs was better than for FLF-fed pigs. Overall, fermenting the whole diet (FLF) resulted in poorer growth and FE, likely due to a loss of dietary energy as well as amino acid decarboxylation (O'Meara et al., 2020c).

Although O'Meara et al. (2020d) did not investigate the gut microbiota of liquid-fed pigs in the aforementioned studies, Torres-Pitarch et al. (2020a, 2020b) examined the intestinal microbiota as well as the growth performance of grow-finishers fed fresh LF, FLF, or LF where the cereal fraction alone was either fermented i.e., FLC (Torres-Pitarch et al., 2020a) or soaked (Torres-Pitarch et al., 2020b), with or without enzyme supplementation. Fermenting the diet increased ADG and total tract nutrient digestibility, while supplementation with a xylanase and β-glucanase complex improved FE as well as ileal and total tract nutrient digestibility (Torres-Pitarch et al., 2020a). The gut microbiota composition of the pigs fed these diets provided an insight into why these improvements may have occurred. Beneficial bacteria correlated with improved growth, including *Lactobacillus kisonensis* and *Roseburia faecis*, were more abundant in the ileum and caecum of the enzyme-supplemented pigs, while fermentation of the cereal component of the diet decreased the abundance of gut bacteria including *Megasphaera* and *Streptococcus*, which were correlated with poorer growth. Considering that the inoculum strains did not dominate the feed, it is not surprising that they were not found in the gut of the pigs. However, interestingly, *P. parvulus* which was found to be dominant in the un-supplemented diets, was more abundant in the ileal digesta of pigs fed the unsupplemented-FLC diet.

Bunte et al. (2020) fermented 60 % of the whole diet, which was then supplemented with 40 % non-fermented coarse cereals (referred to as partially fermented LF; PFLF). This was compared with fresh LF in one experiment and in a separate experiment fresh LF was compared to the fully fermented diet (FLF). There were no effects of either partial or whole diet fermentation on feed intake, body weight or FE, although both of the fermented diets marginally improved feed intake and FE compared to the fresh LF. The FLF, however, decreased pH and bacterial alpha diversity in the small intestine, but

increased alpha diversity in the faeces (Bunte et al., 2020). The PFLF had a similar impact on bacterial alpha diversity compared to fresh LF (both diets in this experiment had phytase added) in that alpha diversity was decreased in the small intestine and increased further down the GIT. Additionally, principal coordinate analysis (PCoA) of Bray–Curtis distances, in both experiments, demonstrated that the faecal microbiota of pigs fed the same diets clustered together and had higher dissimilarity to pigs fed the other diets. This was supported by permutational multivariate ANOVA of Bray–Curtis distances, which found that 23.7 % of the variability in microbial composition was attributed to the diet. Beneficial taxa assigned to *Lactobacillus* and *Bifidobacterium* were at higher relative abundances in the GIT of the pigs fed the PFLF. The authors suggested that supplementation of the diet with non-fermented coarse cereals (large particle size) may have promoted proliferation of these bacteria due to the presence of undigested carbohydrates in the large intestine that were then available for fermentation. As discussed in Section 1.20.1, coarse meal can also increase the viscosity and slow the passage of the GI content, facilitating the proliferation of LAB (Bunte et al., 2020).

The study that focused on cereal fraction soaking by Torres-Pitarch et al. (2020b) was designed in a similar fashion to the fermentation experiment discussed earlier (Torres-Pitarch et al., 2020a), except that the cereal fraction of the diets were soaked in water for 3 h instead of being fermented. Pigs fed the enzyme-supplemented soaked diet had higher ADG than pigs fed the enzyme-supplemented fresh LF (between day 0 and 21 of the experiment) with no other effects on ADG reported throughout the trial. Enzyme supplementation increased total tract nutrient digestibility; however, growth and FE were not improved. In contrast to the previous fermentation study by Torres-Pitarch et al. (2020a), cereal soaking and enzyme-supplementation appeared to reduce the relative abundance of taxa that were positively correlated with good performance parameters, while those that were negatively correlated with desirable traits/physiological measures were more abundant (Table 1.11). This may explain the limited improvement in growth and the lack of improvement in FE in pigs fed these diets. The intention of soaking the diet for 3 h was to improve nutrient digestibility by increasing the time the cereals are exposed to the enzyme complex. However, during the soaking period the feed was likely in the first phase of fermentation and, therefore, the less favourable gut microbiota and lack of growth and FE improvements may have been related to the prevalence of less favourable bacteria in the feed during this period. It should also be noted that these

correlations should be interpreted with caution as they are merely associations between taxa and physiological data. Indeed, one of the biggest drawbacks of culture-independent methods, particularly using marker genes such as the 16S rRNA gene, is that taxa that have been associated with improved growth and FE often may not have been cultured previously, and therefore isolation from the pig GIT is necessary to validate any *in vivo* claims by identifying the mechanisms by which these microbes may improve growth and/or FE. Equally, although compositional studies of the microbiota provide useful information, studies on the functionality of the microbiota using functional metagenomics and metabolomics, would provide greater insight as to how liquid feeding alters the functionality of the gut microbiome as opposed to identifying changes in the predominant taxa alone (Gardiner et al., 2020).

1.24 Conclusions

The negative impacts of spontaneous fermentation in fresh LF are evident from the literature, affecting both the microbial and nutritional quality of feed, resulting in poorer pig health and growth performance, which ultimately leads to economic losses for pig producers. However, if managed correctly, liquid feeding can be an effective strategy for improving pig growth and reducing feed costs, particularly if farmers have access to liquid co-products. Also, if finisher accommodation is limited on-farm, faster growth rates to reach a target slaughter weight can be achieved with liquid feeding. The strategies to improve the microbial and nutritional quality of LF discussed throughout this review provide opportunities to select for a desirable feed and gut microbiome to maintain the nutritional quality of the feed and to maximise the health and growth of liquid-fed pigs. However, there are some conflicting reports regarding the precise microbial taxa that play roles in improving growth and FE. The increasing availability and decreasing costs of shotgun sequencing and other omics technologies will enable us to gain useful insights into the functionality of the LF microbiome and the liquid-fed pig gut microbiome as opposed to relying on compositional data alone. However, even compositional culture-independent data from LF and liquid-fed pigs are limited, as mentioned earlier, particularly regarding the mycobiome.

There is also scope for improving LF microbial and nutritional quality through more practical approaches associated with cleaning and sanitisation of liquid feeding systems, which may limit spontaneous fermentation and its associated negative effects. Although the available data indicate that extensive cleaning may have short-lived benefits or no

benefit at all, there is an opportunity to improve microbial quality by maintaining liquid feed system hygiene after cleaning. This may involve more frequent cleaning of the system after a less frequent intensive cleaning to limit a reversion to poor hygiene conditions, potentially with the use of organic acid blends. However, this will depend on the health status and performance of the pigs and the pre-cleaning microbial quality of the feed on a particular farm. Finally, the FE of liquid-fed pigs can be improved by better management of liquid feeding systems in order to reduce feed wastage; however, further improvements in FE can also be achieved using the aforementioned strategies to minimise the negative effects of uncontrolled fermentation, which include losses of amino acids and energy in the diet that could otherwise be utilised by pigs.

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1.26 Rationale and objectives of the research

The preceding literature review has highlighted the fact that a number of studies have investigated the microbial quality of liquid feed and strategies to improve it. However, it has also identified gaps in the area. For example, very few on-farm surveys of liquid feed quality have been conducted on commercial pig units, especially so on farms where liquid feed is not deliberately fermented. Moreover, the majority of studies to date have used culture-based methods to investigate a limited number of microbial groups within feed samples, with the focus often limited to bacteria as opposed to a more holistic cataloguing of the resident microbiome to include fungal and archaeal communities, for example. Similarly, many of the studies that have investigated the impact of liquid feed on the pig gut microbiome are also culture-based or use molecular methods that are now outdated. This highlights the need for the development and optimisation of suitable next-generation-sequencing workflows to profile the microbiome of liquid feed and the gut microbiome of liquid-fed pigs. Additional studies are also required to investigate the impact of different feed forms (meal versus pellets) and feed delivery methods (dry, liquid and wet/dry feeding) on the feed and pig gut microbiome, and whether differences in the microbiome may be responsible for any variances observed in growth, feed efficiency and/or carcass quality parameters in pigs. Furthermore, the limited number of studies in the area indicate that cleaning of liquid feeding systems may have short-lived benefits or none at all in terms of both system hygiene and feed quality. Consequently, there is an opportunity to improve liquid feed quality by developing an effective sanitisation programme that maintains liquid feeding system hygiene after cleaning.

Therefore, the objectives and hypotheses of this thesis are:

- To develop an optimal methodology for simultaneously extracting bacterial and fungal DNA from liquid feed and pig faecal samples for 16S rRNA gene and ITS2 library preparation and amplicon sequencing. The hypothesis was that by increasing the homogenisation (bead-beating) duration the recovery of DNA from difficult-to-lyse microbes in faeces and liquid feed would be improved by minimising lysis bias.

- To profile the bacteriome and mycobiome of liquid feed from the finisher section of commercial pig production units, using high-throughput 16S rRNA and ITS2

amplicon sequencing, and to determine biogenic amine concentrations as an indication of the level of spontaneous fermentation. The hypothesis was that genera of lactic acid bacteria and yeast would predominate in liquid feed as a result of spontaneous fermentation, and that relatively high concentrations of biogenic amines would be present due to amino acid decarboxylation.

- To investigate the impact of feeding dry, liquid or wet/dry feed, in meal or pelleted form, on both the feed bacteriome and the faecal and gut bacteriome of grow-finisher pigs and to correlate findings with previously obtained pig growth, feed efficiency and carcass quality data. The hypothesis was that spontaneous fermentation in liquid diets is linked with poorer feed efficiency in liquid-fed pigs due to proliferation of lactic acid bacteria in feed and the subsequent increased abundance of lactic acid bacteria within the gut bacteriome of liquid-fed pigs.

- To develop and implement an intensive sanitisation programme in a grow-finisher liquid feeding system in order to maintain system hygiene and improve the microbiological and nutritional quality of liquid feed. The hypothesis was that implementing an intensive sanitisation programme and maintaining hygiene post-cleaning would reduce spontaneous fermentation in liquid feed and therefore reduce energy and amino acid losses and the production of undesirable microbial metabolites in liquid feed.

- To investigate the bacteriome and mycobiome of liquid feed and liquid feeding system surfaces, before and after implementation of the intensive sanitisation programme. The hypothesis was that a DNA-sequence based approach would reveal specific bacterial and fungal taxa associated with the pre- and post-cleaning microbial communities in feed and on the feeding system surfaces.

Chapter 2: Optimisation of a bead-beating procedure for simultaneous extraction of bacterial and fungal DNA from pig faeces and liquid feed for 16S and ITS2 rDNA amplicon sequencing

Adapted from: Cullen, J.T., Lawlor, P.G., Cormican, P., Crispie. F., & Gardiner, G.E. (2022). Optimisation of a bead-beating procedure for simultaneous extraction of bacterial and fungal DNA from pig faeces and liquid feed for 16S and ITS2 rDNA amplicon sequencing. *Animal – open space*. https://doi.org/10.1016/j.anopes.2022.100012

2.1 Abstract

Efficient cell lysis is critical for extraction of DNA from difficult-to-lyse microorganisms such as Gram-positive bacteria and filamentous fungi. A bead-beating step is usually included in DNA extraction protocols to improve cell lysis. However, there is no consensus on the duration of bead-beating that is necessary for complete lysis of the microbial communities present in complex microbial ecosystems, but which will still maintain the integrity of DNA released from easy-to-lyse microbes. Another consideration is that most protocols are tailored to one particular target group of microbes, typically either bacteria or fungi, in a given sample matrix. In this study, we investigated the impact of five bead-beating durations (0, 3, 10, 15 and 20 minutes) during DNA extraction with the QIAamp® Fast DNA Stool Mini Kit, on the bacterial and fungal communities of pig faeces and liquid feed, with the objective of determining a suitable 'catch-all' method. Both sample types were subjected to the bead-beating durations in triplicate, followed by 16S (bacterial) and ITS2 (fungal) rDNA amplicon sequencing. The performance of the different bead-beating durations was assessed based on the quantity of total DNA extracted, alpha and beta-diversity analyses of the resultant microbial communities and differential abundance of bacterial and fungal taxa. Our results suggest that 20 minutes of bead-beating was most appropriate for maximising the lysis of difficult-to-lyse bacteria and fungi in both pig faeces and liquid feed, while minimising the negative impact on easier-to-lyse microbes. Total DNA yield increased with bead-beating duration for both sample types; however, the yield from faeces decreased after 20 minutes of bead-beating. Despite this, DESeq2 analysis indicated that changes in the differential abundances of the dominant taxa at this point were limited, which was supported by the Shannon diversity results. Maximising the bead-beating duration appeared to be necessary in order to obtain a representative profile of the Gram-positive bacteria, particularly in liquid feed, and of the filamentous fungi present in both sample types. However, considering the small sample size, along with the reliance on differential as opposed to absolute abundances to validate increases or decreases in taxa, a larger-scale study is necessary to verify the findings of the present study. Finally, although 20 minutes of bead-beating was suitable for amplicon sequencing, potential shearing of DNA due to excessive homogenisation could be problematic for metagenomic and transcriptomic analysis.

2.2 Implications

Considering the variability of bead-beating duration and intensity used in sequence-based microbiome studies, this study highlights the impact of bead-beating on the DNA recovery from difficult-to-lyse bacterial and fungal taxa present in pig faeces and liquid feed. Although additional studies are warranted, our results will inform the optimisation and standardisation of DNA extraction procedures so as to obtain a representative profile of liquid feed and porcine gut microbiota in future studies. By demonstrating the practicality of using a single DNA extract for simultaneous bacterial and fungal amplicon sequencing, the findings could also improve the cost- and time-effectiveness of microbiome studies.

2.3 Introduction

Bias can be introduced via a number of sources during sequencing workflows: from DNA extraction to library preparation, sequencing and bioinformatic analysis. During DNA extraction, efficient lysis of the microbial cell wall is critical for obtaining a representative yield of good quality DNA from both easy- and difficult-to-lyse microbes. Gram-positive bacteria in particular pose a challenge for complete lysis due to their thick cell walls, while fungal cell walls are more complex and can also be very difficult to lyse. Cell lysis methods include mechanical, chemical and enzymatic disruption of the microbial cell wall, and different methods are often combined (Frau et al., 2019; Islam et al., 2017). Although mechanical disruption methods such as bead-beating (BB) can enhance nucleic acid yield, excessive mechanical lysis may also shear microbial DNA into smaller fragments, which may impact success in subsequent downstream applications.

The most appropriate duration of BB necessary for complete lysis of the microbial communities present in complex microbial ecosystems, while also preserving the DNA integrity for downstream applications, remains open to debate. Most protocols are tailored to one particular target group of microbes, typically either bacteria or fungi, in a given sample matrix, although some studies have investigated procedures for simultaneous lysis of both bacteria and fungi (Fiedorová et al., 2019; Pérez-Brocal et al., 2020). This study aims to find the most appropriate BB duration, used in conjunction with the QIAamp® Fast DNA Stool Mini kit (Qiagen, Helden, Germany), to simultaneously extract both bacterial and fungal DNA from two different sample types: pig faeces and liquid feed. The performance of five different BB durations, often used across sequence-based

microbiome studies, was assessed on the basis of the quantity of total DNA extracted, alpha and beta-diversity estimates of the resultant microbial communities and the differential abundance of bacterial and fungal taxa across BB durations following 16S and ITS2 rDNA amplicon sequencing.

2.4 Material and methods

2.4.1 Experimental design

This study investigated the impact of four BB durations, compared to no BB, on the simultaneous extraction of bacterial and fungal DNA from both pig faeces and liquid feed. A faecal sample from an individual finisher pig as well as a single liquid feed sample were used throughout this study in order to avoid variability between individual pigs or different liquid feed batches to accommodate comparison of the BB durations. Differences between BB durations were evaluated based on the quantity of total DNA extracted, microbial diversity estimates and whether bacterial and fungal taxa were differentially abundant between BB durations following 16S and ITS2 rDNA amplicon sequencing. A gut microbiome mock community standard was also used to investigate the potential lysis bias of each BB duration.

2.4.2 Sample collection and storage

Freshly voided faeces was sampled from an individual finisher pig (~ 120 kg live weight) on the day prior to slaughter. A wheat, barley and soya bean-based liquid pig diet, prepared and fed using a liquid feeding system (HydroMix, BigDutchman, Vechta, Germany) was sampled from a trough in the finisher section. Both samples were immediately put on ice and stored at -80°C until DNA extraction.

2.4.3 Bead-beating procedures and DNA extraction

Four different BB durations selected from the literature are detailed in Table 2.1 (BB3, BB10, BB15 and BB20). They were compared to no BB (BB0) using the QIAamp® Fast DNA Stool Mini kit, following the 'Isolation of DNA from Stool for Pathogen Detection' protocol as per McCormack et al. (2019) with modifications to the following steps:

Steps 5-7: 540 µL of supernatant was added to 37.5 µL of proteinase K, followed by 500 µL of buffer AL for protein digestion.

Step 9: 500 µL of ethanol was added for DNA precipitation.

Step 10: 750 µL of lysate was applied to the spin column, followed by centrifugation for 2 min at 10,000 x g.

Step 11: Centrifugation performed at 10,000 x g for 2 min.

Step 13: Centrifugation performed at full speed for 5 min.

Step 14: 30 µL of Buffer ATE was applied to the membrane, incubated at room temperature for 5 min and centrifuged for 2 min at 10,000 x g to increase DNA yield.

The sample (0.25 g) was added to a 2-mL screw-cap tube containing 0.25 g of sterile zirconia beads (0.125 g of 0.1 mm and 0.125 g of 1.0 mm, a single bead of 2.5 mm; Stratech Scientific, Ely, UK). InhibitEX® buffer was then added (Step 2) and the relevant BB duration was used. All BB was performed using a Mini-Beadbeater-24 (BioSpec Products, Bartlesville, OK, U.S.A.) at 3,000 rpm and samples were stored on ice between BB cycles. Triplicate DNA extractions were performed on the faecal and liquid feed samples for each BB duration, along with negative controls (tubes containing only the beads and no sample). As a positive control, a mock community standard, ZymoBIOMICS® Gut Microbiome Standard (Zymo Research, Irvine, CA, U.S.A.) was also extracted in triplicate for each respective BB duration.

2.4.4 Library preparation and amplicon sequencing

2.4.4.1. *16S rDNA*

Bacterial communities were profiled via amplicon sequencing of the V3-V4 hypervariable region of the 16S rRNA gene on the Illumina MiSeq platform, according to the Illumina 16S Metagenomic Sequencing Library Preparation Guide, with some modifications. The procedure was performed as described by Fouhy et al. (2015), except that 25 ng of input DNA was used (or 5 µL of neat extracts for the mock community standard and negative controls) for the initial amplicon PCR, and 30 cycles were used instead of 25. The cleaned indexed PCR products were quantified on a Qubit® 3.0 Fluorometer using the Qubit® dsDNA HS Assay Kit (Bio-Sciences, Dublin, Ireland) and were pooled in an eqimolar fashion.

2.4.4.2. *ITS2 rDNA*

Fungal profiling was carried out by amplifying the nuclear ribosomal ITS2 region with the following locus-specific primers (in bold) containing Illumina overhang adapters at the 5' ends: forward primer ITS3 (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**GCATCGATGAAGAACGCAGC**-3') and reverse primer ITS4 (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**TCCTCCGCTTATTGATATGC**-3') (White et al., 1990). Each reaction contained either 50 ng or 100 ng of DNA template for liquid feed and faeces, respectively (amounts of the mock community and control extracts were as described for the 16S rDNA protocol), 1 µL forward primer (7.5 µM), 1 µL reverse primer (7.5 µM), 5 µL 5x HiFi buffer, 0.75 µL 1 mM dNTPs and 0.5 µL 1U/µL KAPA HiFi HotStart DNA polymerase (KAPA HiFi HotStart + dNTPs kit; Roche Diagnostics, West Sussex, U.K.) and nuclease-free PCR grade water in a final reaction volume of 25 µL. PCR parameters were as follows: 95 °C x 3 min, 35 cycles of 98 °C x 30 s, 65 °C x 30 s, 72 °C x 30 s, then 72 °C x 5 min and held at 4 °C.

PCR products were visualised and cleaned as described above for the 16S rDNA protocol and 5 µL of the cleaned PCR product was dual indexed via a limited cycle PCR (using the same parameters as the ITS2 amplicon PCR except that the number of amplification cycles was reduced from 35 to 8). The index PCR reactions contained 5 µL cleaned PCR product, 5 µL index 1 primer (N7xx), 5 µL index 2 primer (S5xx), 10 µL 5x HiFi buffer, 1.5 µL 1 mM dNTPs and 1 µL 1U/µL KAPA HiFi HotStart DNA polymerase (KAPA HiFi HotStart + dNTPs kit; Roche Diagnostics, West Sussex, U.K.) and nuclease-free PCR grade water in a final reaction volume of 50 µL. Indexed ITS2 PCR products were visualised, cleaned, quantified and pooled as described above. The final library was quantified by qPCR, diluted, denatured and sequenced using a 2 x 300 cycle V3 kit in the Teagasc sequencing facility as described by Fouhy et al. (2015) in accordance with standard Illumina sequencing protocols.

2.4.5 Bioinformatics and statistical analysis

Demultiplexed paired-end 16S and ITS2 rDNA sequences (available at: https://www.ebi.ac.uk/ena/browser/view/PRJEB49004) were imported (in Casava 1.8 demultiplexed paired-end format) into QIIME2 v.2020.8.0 (Bolyen et al., 2019), which was installed on a virtual machine (VirtualBox 6.0). Forward and reverse reads were

quality assessed using the ‘qiime demux summarize’ command, FastQC v.0.11.5 and MultiQC v.1.9. The 16S and ITS2 primers were removed from reads using the cutadapt plugin (Martin, 2011). The QIIME2 DADA2 (Callahan et al., 2016) plugin was used for filtering and dereplication, chimera removal, merging paired-end reads and to infer amplicon sequence variants (ASVs) in each sample after truncating reads to remove low quality bases. Read 1 and read 2 of the 16S rDNA sequences were truncated at 267 and 183 bp, respectively, while ITS2 sequences were truncated at 266 and 187 bp, respectively. For bacterial sequences, taxonomy was assigned to each ASV using a Naive Bayes classifier trained on 16S V3-V4 sequences from the SILVA database (Version 138) with the ‘q2-feature-classifier’ plugin, while taxonomy was assigned to fungal ASVs using a Naive Bayes classifier trained on full-length ITS sequences from the UNITE v.8.3 database (Kõljalg et al., 2013).

QIIME artefacts (taxonomy, ASV, metadata and phylogenetic tree) were imported into R v.4.0.2 as a phyloseq (McMurdie & Holmes, 2013) object with the qza_to_phyloseq() function in the qiime2r package (Bisanz, 2018). Contaminant bacterial and fungal ASVs identified using the decontam package (Davis et al., 2018) were removed prior to further analysis. For the 16S rDNA sequences, one liquid feed sample (BB3) replicate with a low number of reads compared to the other two replicates (n = 11,006) was excluded from the analysis, while one liquid feed sample (BB10) was excluded from the ITS analysis for the same reason (n = 9,507 reads). Alpha-diversity indices (Shannon diversity) and beta-diversity (Bray–Curtis), based on unrarefied sequences, were calculated using the phyloseq package (McMurdie & Holmes, 2013) in R. Shannon diversity was subsequently plotted using the alpha_boxplot function in the ‘amplicon’ package in R which performs an analysis of variance (ANOVA) followed by Tukey’s honestly significant difference (HSD) test (Liu et al., 2021), while PCoA plots were plotted using the ggplot2 package (Wickham, 2016). Differential abundance testing was performed using the DESeq2 package (Love et al., 2014) in R. Bacterial and fungal genera that were present at < 1 % mean relative abundance for each BB duration across either faecal or liquid feed samples were filtered from the unrarefied read counts prior to DESeq2 analysis. Log2 fold changes between BB durations with an adjusted p-value < 0.05 were considered significant.

2.5 Results

2.5.1 Impact of bead-beating on DNA yield

Figure 2.1 shows the concentrations of total DNA extracted from faeces, liquid feed and the mock community standard. DNA concentrations were highest for faeces. All samples, except for one BB0 replicate, had > 100 ng/µL total DNA. After 3 min BB, the mean total faecal DNA concentration increased substantially from ~100 ng/µL without BB to ~350 ng/µL, with a similar yield after 10 min and a further increase after BB15. There was a notable drop in the total DNA yield with BB20; however, the concentration was still higher compared to no BB. It should be noted that the total DNA concentration is not fully representative of microbial DNA as this figure will also include any host or plant DNA extracted.

The total DNA yields for liquid feed were lower compared to those obtained from faeces. Interestingly, BB did not appear to have an impact on the DNA yield until after 15 min. At BB15, the DNA concentration increased from ~35 ng/µL (for BB0, BB3 and BB10) to ~50 ng/µL, and further increased to ~60 ng/µL at BB20, albeit these increases were not as large as those observed in the pig faeces. The DNA yields from the mock community are much lower than the faecal and liquid feed samples because it contains only a mixture of 18 bacterial strains, 2 fungal strains, and 1 archaeal strain. Although yields were variable between BB durations, the most notable difference is that all samples processed with BB resulted in a higher total DNA yield compared to no BB, indicating improved lysis efficiency of the mock community with BB.

2.5.2 Alpha-diversity estimation

The Shannon diversity of the pig faecal and liquid feed microbiota and mycobiota are displayed in Figure 2.2. The microbial communities in liquid feed were much less diverse compared to those in the faeces. In faeces, the bacterial Shannon diversity decreased after BB10 ($p < 0.05$) but remained similar thereafter up to 20 min of BB ($p > 0.05$). Conversely, the diversity of fungi in the faeces increased after 3 min of BB compared to the samples with no BB ($p < 0.05$), with additional numerical increases up to BB20. Alpha-diversity of the bacterial and fungal communities in liquid feed were almost the inverse of those in faeces. Bacterial Shannon diversity was the same for BB0 and BB3 but was higher after BB10 ($p < 0.05$), although there were no further increases after 15

and 20 min. Despite the samples that were bead beaten for 10 min appearing to be outliers in the data, the fungal diversity in liquid feed showed a general decrease with increased BB, with BB15 and BB20 having lower diversity than samples that were not bead beaten ($p < 0.05$).

2.5.3 Beta-diversity

The bacterial and fungal beta-diversity was assessed using Principal coordinate analyses (PCoA) based on Bray-Curtis distances at the genus level to evaluate the between-sample dissimilarities for faeces and liquid feed (Figure 2.3). For bacterial beta-diversity in faeces, the samples generally clustered by BB duration, except for BB15 where the triplicate extracts were more dissimilar. The faecal samples that were bead beaten for 10 min or more clustered away from BB0 and BB3, indicating differences in the faecal microbiota composition at BB10 and beyond, which was supported by the decreased bacterial alpha-diversity observed in faeces after BB10. A similar trend was observed for the bacterial diversity of liquid feed, where after 10 min of BB, samples generally clustered together, indicating similar bacterial compositions for BB10, BB15 and BB20. In contrast to faeces, the liquid feed bacteriome differed between BB0 and BB3, indicating that even 3 min of BB had a dramatic effect on bacterial composition of the liquid feed.

The impact of BB on the fungal communities in faeces is apparent from Fig. 2.3 where all BB durations clustered distinctly away from the samples with no BB. Although the other BB durations were distinctly different from BB0, they also displayed distinct clustering from each other, suggesting that the fungal communities in faeces differed at each of the BB durations. The mycobiota in liquid feed showed a similar situation, although there was more inter-replicate variability between these samples. Nonetheless, the fungal composition of faeces and liquid feed appeared to be highly influenced by the different durations of BB.

2.5.4 Differential abundance analysis

In order to investigate whether specific bacterial or fungal taxa in pig faeces and liquid feed were differentially abundant between the different BB durations, differential abundance testing was performed using DESeq2. In order to focus on the microbes that predominated in the samples, only the taxa present at > 1 % mean relative abundance at

each BB duration in either faecal or liquid feed samples were analysed. Log2 fold changes (adjusted *p*-value < 0.05) between BB durations in faeces and liquid feed for the most abundant bacterial and fungal genera are presented in Tables 2.2 and 2.3, respectively.

The results for the differentially abundant bacterial genera in faeces were variable, with a similar number of tested taxa being differentially increased and decreased between the different BB durations. It was noted however, that fewer taxa were impacted by the increased BB time at BB20, compared to the other durations. This may suggest that at BB20 the faecal bacteriome is sufficiently lysed, and importantly, moving to BB20 did not show a large degree of decreased differential abundance of taxa, as might be expected due to the excessive heat generation and potential shearing of DNA. For liquid feed, which was dominated by only 5 genera, BB3 decreased the abundance of *Lactobacillus* and *Weissella* compared to BB0 (adjusted *p*-value < 0.05), while *Pediococcus* was enriched at BB10 compared to BB3 (adjusted *p*-value < 0.05).

A number of fungal genera were enriched at BB3 compared to BB0, which included the difficult-to-lyse moulds *Mucor* and *Monascus* (Scharf et al., 2020). However, differential abundance data must be interpreted with caution. For example, the results show that *Debaryomyces* had a log2 fold change of -26.30. This suggests that BB had a negative impact on the abundance of *Debaryomyces*; however, the large fold change was because this genus was present in the BB0 samples, but not in the BB3 faecal samples. However, *Debaryomyces* was detected again in the BB10, 15 and 20 samples, with increasing mean relative abundance, and therefore it is difficult to say based on these data that BB was responsible for the initial decrease in differential abundance.

Another surprising result was that *Malassezia*, a genus of difficult-to-lyse yeast (Diaz et al., 2017), appeared to be less abundant after BB20 compared to BB15. The relative abundance data supports this as the relative abundance of *Malassezia* from BB0 to BB15 decreased from 2.8 % to 0.1 %, and the genus was not present after BB20 (data not shown). Despite this, inferring changes in abundance based on relative abundance data can be problematic as the decreased relative abundance of *Malassezia* may be a result of an increase in the absolute abundance of a different fungal taxon with increasing BB (Morton et al., 2019). For this reason, it may be more appropriate to quantify absolute abundances of taxa of interest using real-time PCR to determine the impact of BB on their

lysis efficiency. However, this method is also subject to bias based on the amount of DNA extracted and the amount of input DNA in the reaction.

2.6 Author's points of view

Based on the aim of this study, which was to optimise a 'catch-all' workflow incorporating BB for optimal bacterial and fungal DNA recovery from both pig faeces and liquid feed, we recommend that 20 min BB (BB20) be used for pig faeces and liquid feed samples. Following an increase in total DNA yield from faeces with increasing BB time, the decrease in yield after BB20 indicated that a loss of DNA from easy-to-lyse microbes may have occurred. The fragile nature of the mammalian cell membrane compared to that of bacteria and fungi may also, at least in part, be responsible for the lower yield from BB20, as host DNA that was released early on may have been degraded, possibly in addition to DNA from easier-to-lyse microbes.

Despite this, DESeq2 analysis suggested that there were few changes in the differential abundances of the dominant taxa between BB15 and BB20. This was supported by the similar bacterial alpha-diversity in BB10 compared to BB20 for faeces. Despite a decrease in bacterial alpha-diversity after BB10, increasing the BB time is justified to increase the alpha-diversity of fungi in faeces, which increased with more BB. Additionally, beta-diversity analysis indicated a distinct microbiota and mycobiota associated with each BB duration and although differential abundance analysis suggested that one yeast genus was under-represented at BB20, we conclude that BB20 maximises the lysis of difficult-to-lyse microbes and does not appear to negatively impact easier-to-lyse microorganisms in faeces to a large extent.

Total DNA recovery from liquid feed was optimal at BB20; however, similar to the situation with host DNA in the faeces, it is also possible that plant DNA released from the cereal components of the liquid feed by excessive BB contributed to the increased total DNA yields. Nonetheless, the liquid feed samples were dominated by Gram-positive lactic acid bacteria, which are more difficult to lyse. Therefore, considering that species richness was quite low for bacteria in liquid feed, the increased Shannon diversity may be explained by increased evenness in the samples i.e. with BB, a greater proportion of DNA was released from some previously under-represented difficult-to-lyse bacteria. Despite a lower fungal alpha-diversity in liquid feed with increased BB, several taxa appeared to benefit from increased BB. A decrease in the evenness of fungi may have

been responsible for decreased Shannon diversity in liquid feed. While the yeast communities may have been sufficiently lysed, increased lysis of filamentous fungi, which are more difficult to lyse than yeast, may have influenced this.

For these reasons, we propose that maximising the BB duration at 20 min generates a more accurate representation of the true bacterial and fungal communities in pig faeces and liquid feed. This method development work will inform the optimisation and standardisation of DNA extraction procedures to help minimise lysis bias in future liquid feed and pig gut microbiota studies. By demonstrating the practicality of using a single DNA extract for simultaneous bacterial and fungal amplicon sequencing, the findings could also improve the cost- and time-effectiveness of microbiome studies. Nonetheless, the limitations to this study, which include a small sample size and a lack of absolute abundance data to validate increases or decreases in abundance, justify a larger-scale study which would complement the dataset from this study and provide further insights into lysis bias within these communities. Finally, although 20 minutes of bead-beating was suitable for amplicon sequencing, potential shearing of DNA due to excessive homogenisation could be problematic for metagenomic and transcriptomic analysis.

In conclusion, to optimise simultaneous bacterial and fungal DNA extraction from pig faeces and liquid feed with the QIAamp® Fast DNA Stool Mini kit, our results suggest that BB for 20 min is the most appropriate duration to minimise lysis bias of Gram-positive bacteria and filamentous fungi. However, a larger-scale study is necessary to validate these findings considering the small number of samples used in this study, in addition to the fact that we performed differential abundance testing as opposed to quantification of absolute abundances of relevant taxa.

2.7 Tables and figures

Table 2.1: Details of the bead-beating durations implemented during DNA extraction from pig faeces, liquid feed and a mock community standard using the QIAamp® Fast DNA Stool Mini Kit.

Bead-beating duration	Bead-beating time, frequency and rest	Total time required (min)	Description/Reference
BB0	0 mins	0	Control (no bead-beating)
BB3	1 min (3 cycles); 1 min rest between	5	Scanlan and Marchesi (2008); Huseyin et al. (2017)
BB10	3 min (2 cycles) + 4 min (1 cycle); 1 min rest between	12	McCormack et al. (2019); Torres-Pitarch et al. (2020)
BB15	5 min (3 cycles); 5 min rest between	25	ZymoBIOMICS® Gut Microbiome Standard (Catalog No. D6331) Protocol (Zymo Research Corporation., 2021)
BB20	5 min (4 cycles); 5 min rest between	35	ZymoBIOMICS® Gut Microbiome Standard (Catalog No. D6331) Protocol (Zymo Research Corporation., 2021)

Table 2.2: Differential abundance (log2 fold changes)[1] of bacterial genera, present at > 1 % mean relative abundance across each bead-beating duration[2] for pig faecal or liquid feed samples[3].

	Log2 fold changes at the genus level between different bead-beating durations			
Genus	BB3 vs BB0	BB10 vs BB3	BB15 vs BB10	BB20 vs BB15
	Faeces			
Prevotella	-0.18	-	0.12	-
Clostridium sensu stricto 1	0.63	-	-0.70	-
Prevotellaceae NK3B31 group	0.21	2.61	-	-
Muribaculaceae	-[4]	0.62	-0.23	-
Lactobacillus	-	2.00	-0.48	-
Treponema	-	-1.03	0.66	-0.24
Alloprevotella	-	0.76	-0.41	-
Rikenellaceae RC9 gut group	0.45	-	-0.19	-
Phascolarctobacterium	-0.67	1.51	-0.59	-
Oscillospiraceae UCG-005	-	-	-	0.29
Oscillospiraceae UCG-010	-0.23	-0.54	-	-0.36
Anaerovibrio	-0.45	-0.45	0.35	-
Terrisporobacter	0.59	1.63	-0.50	-
Christensenellaceae R-7 group	-	0.68	-0.50	-
Clostridia vadinBB60 group	-0.61	-1.70	0.88	-0.44
Streptococcus	0.43	2.02	-0.54	0.40
Blautia	-0.37	-0.52	0.68	-
Succinivibrio	-	-1.10	1.05	-
Clostridia UCG-014	-	-0.23	-	-
WCHB1-41	-0.56	-1.68	0.98	-
Oscillospiraceae UCG-002	-0.22	-	-0.24	-0.30
Faecalibacterium	-0.36	-0.36	0.38	-

Oscillospiraceae NK4A214 group	-	-0.46	0.24	-
Campylobacter	-	-1.48	1.08	-0.39
Ruminococcus	-0.21	-0.39	0.34	-
Subdoligranulum	-	0.28	-	0.39
Prevotellaceae UCG-003	0.47	0.34	-0.34	-
Gastranaerophilales	-0.49	-1.55	0.80	-
Megasphaera	1.13	2.42	-0.75	0.34
Eubacterium coprostanoligenes group	-	0.52	-0.36	-
Parabacteroides	-	0.25	-0.40	-
		Liquid feed		
Lactobacillus	-0.81	-1.18	-	-
Weissella	-0.61	-0.50	-	-
Pediococcus	-	0.40	-	-

[1] The adjusted *p*-value cut-off for Log2 fold changes was set at 0.05.

[2] Bead-beating durations during DNA extraction: no bead-beating (BB0); 3 min bead-beating (BB3); 10 min bead-beating (BB10); 15 min bead-beating (BB15); 20 min bead-beating (BB20).

[3] For DESeq2 analysis of each respective sample type, each bead-beating duration represented triplicate samples ($n = 3$), except that one liquid feed sample (BB3) was excluded from the analysis ($n = 2$).

[4] Dash (–) indicates that the log fold change of the genus between the two bead-beating durations was not statistically significant (adjusted *p*-value > 0.05).

Table 2.3: Differential abundance (log2 fold changes)[1] of fungal genera, present at > 1 % mean relative abundance across each bead-beating duration[2] for pig faecal or liquid feed samples[3].

	Log2 fold changes at the genus level between different bead-beating durations			
Genus	BB3 vs BB0	BB10 vs BB3	BB15 vs BB10	BB20 vs BB15
			Faeces	
Mucor	6.43	-	-	-
Peniophora	-11.87	-	-	-
Trichosporon	22.29	-	-	-
Debaryomyces	-26.30	22.16	-	-
Kazachstania	-2.08	-1.26	-	-
Gamsia	4.09	-	-	-
Scopulariopsis	4.22	-	-	-
Monascus	11.04	-	-	-
Pichia	-[4]	-1.43	-	-
Cladosporium	-	-2.90	-	-
Alternaria	-	-1.99	-	-
Malassezia	-	-	-	-22.20
			Liquid feed	
Pichia	-	2.23	-1.18	-
Kazachstania	1.97	2.50	-1.25	-
Saccharomyces	-	2.33	-1.63	0.58
Fusarium	-	-0.56	0.59	-
Gibberella	-	-0.81	-	-
Monographella	-	-0.76	0.67	-

[1] The adjusted *p*-value cut-off for Log2 fold changes was set at 0.05.

[2] Bead-beating durations during DNA extraction: no bead-beating (BB0); 3 min bead-beating (BB3); 10 min bead-beating (BB10); 15 min bead-beating (BB15); 20 min bead-beating (BB20).

[3] For DESeq2 analysis of each respective sample type, each bead-beating duration represented triplicate samples (n = 3), except that one liquid feed sample (BB10) was excluded from the analysis (n = 2).
[4] Dash (–) indicates that the log fold change of the genus between the two bead-beating durations was not statistically significant (adjusted p-value > 0.05).

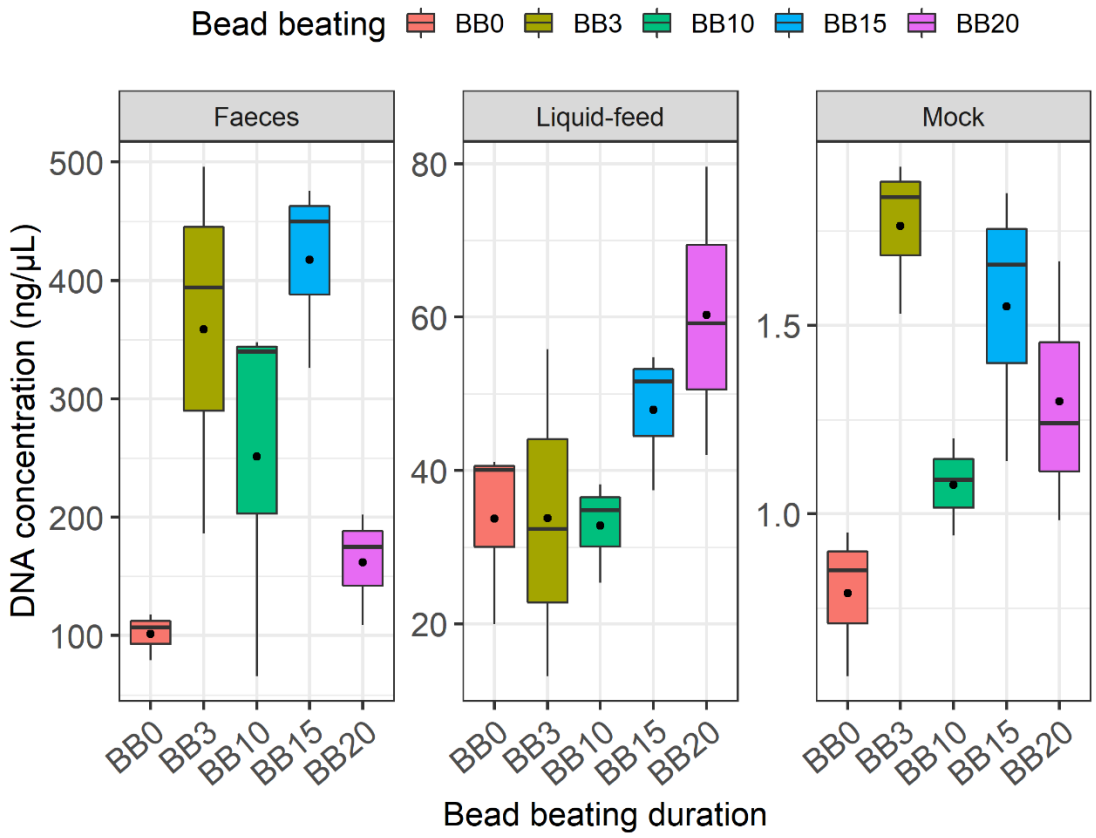

Figure 2.1: Boxplots displaying total DNA concentration (ng/µL; y-axis) from pig faeces, liquid feed and a mock community after DNA extraction using five different bead-beating durations [no bead-beating (BB0); 3 min bead-beating (BB3); 10 min bead-beating (BB10); 15 min bead-beating (BB15); 20 min bead-beating (BB20); x-axis]. For each respective sample type, each bead-beating duration represents triplicate DNA extractions (n = 3). The black dot in each box represents the mean DNA concentration for each bead-beating duration.

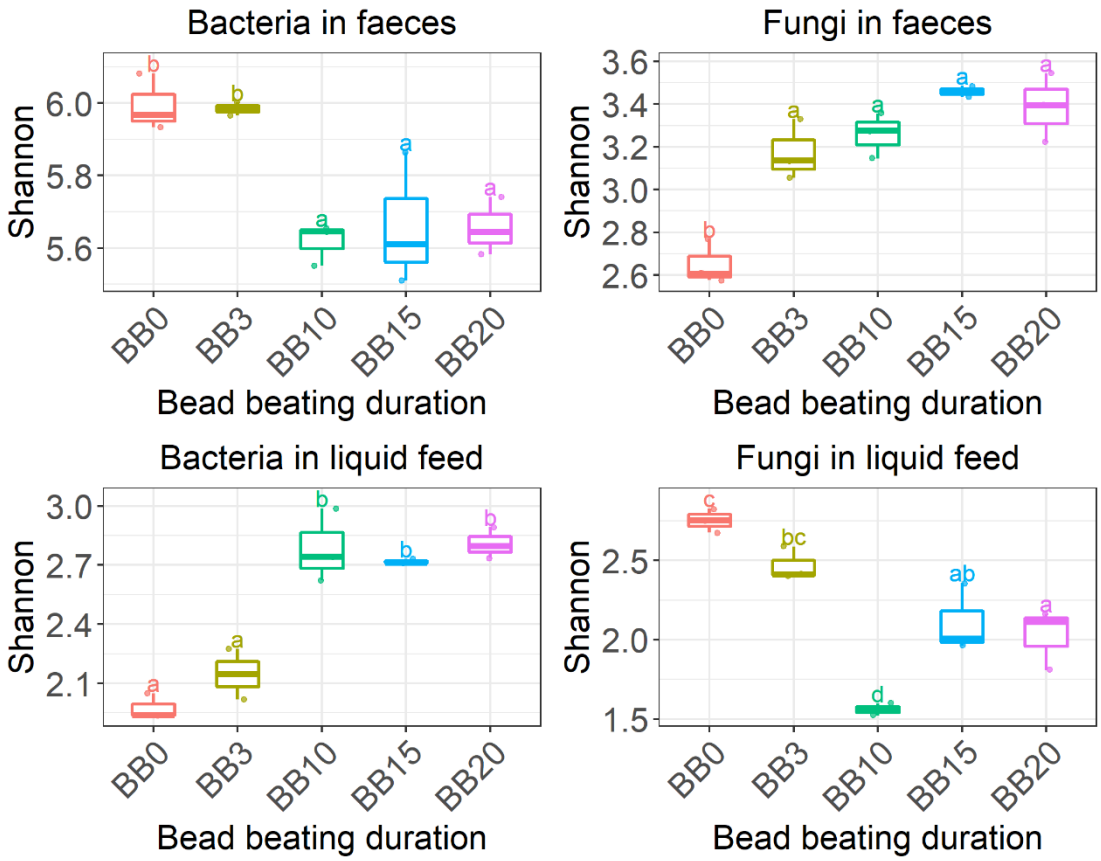

Figure 2.2: Boxplots displaying mean bacterial and fungal Shannon diversity estimates (y-axis) for pig faeces and liquid feed after DNA extraction using five different bead-beating durations [no bead-beating (BB0); 3 min bead-beating (BB3); 10 min bead-beating (BB10); 15 min bead-beating (BB15); 20 min bead-beating (BB20); x-axis]. For each respective plot, each bead-beating duration represents the mean Shannon diversity of triplicate samples (n = 3), except that one liquid feed sample (BB3) was excluded from the bacterial alpha-diversity analysis (n = 2) and one liquid feed sample (BB10) was excluded from the fungal alpha-diversity analysis (n = 2). a-d: For each respective plot, bead-beating durations that do not share a common letter are significantly different ($p < 0.05$).

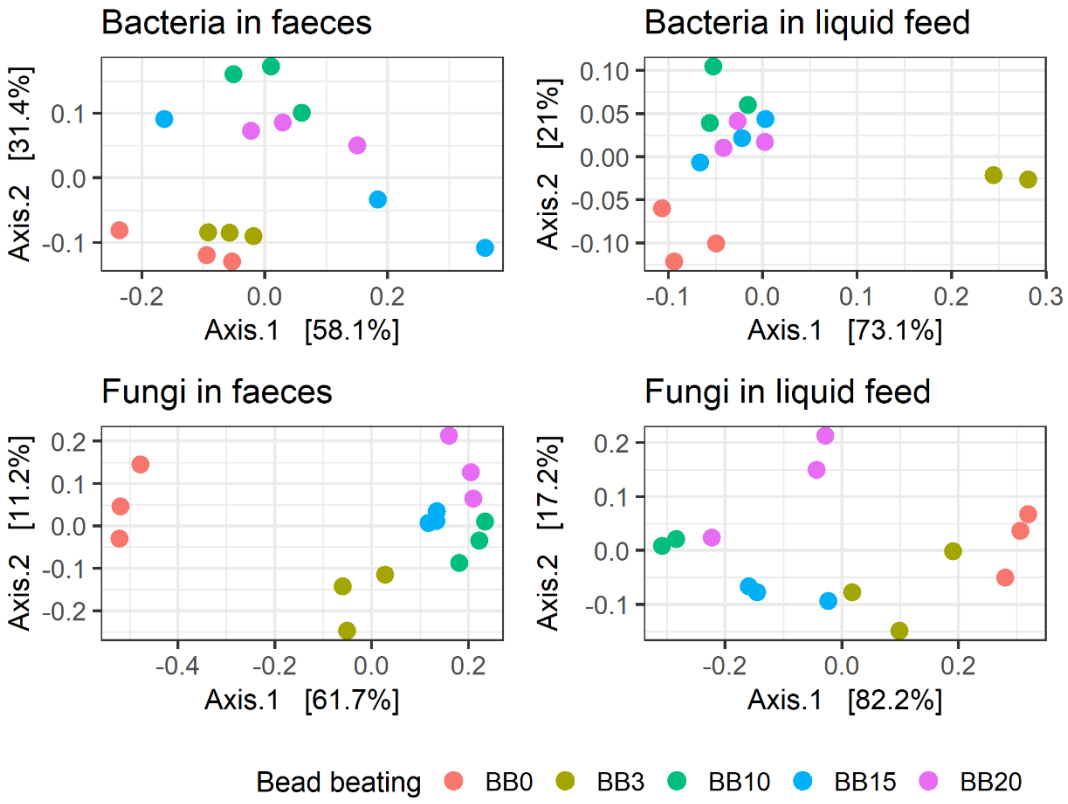

Figure 2.3: Beta-diversity of bacterial and fungal communities in pig faeces and liquid feed after DNA extraction using five different bead-beating durations [no bead-beating (BB0); 3 min bead-beating (BB3); 10 min bead-beating (BB10); 15 min bead-beating (BB15); 20 min bead-beating (BB20)]. Principal coordinate analyses (PCoA) plots display beta-diversity based on Bray-Curtis distances at the genus level. For each respective plot, each bead-beating duration (coloured) represents triplicate samples (n = 3), except that one liquid feed sample (BB3) was excluded from the bacterial beta-diversity analysis (n = 2) and one liquid feed sample (BB10) was excluded from the fungal beta-diversity analysis (n = 2).

2.8 References

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Chapter 3: Bacteriome and mycobiome profiling of liquid feed for finisher pigs on commercial pig production farms

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3.1 Abstract

Liquid-fed pigs typically have poorer feed efficiency compared to those fed dry feed. This may, in part, be due to spontaneous fermentation in liquid feed which reduces feed nutritional quality. The aim of this study was to assess the quality of liquid feed for grow-finisher pigs across a number of commercial pig farms by profiling the bacteriome and mycobiome of samples and determining biogenic amine concentrations. Amplicon sequencing of liquid feed samples revealed that bacterial and fungal community structures were influenced by the farm of origin and the sampling location (mixing tank and troughs) on a given farm. Decreases in alpha-diversity of liquid feed between the mixing tank and the troughs corresponded with increased relative abundances of bacteria, particularly *Lactobacillus*, *Weissella* and *Leuconostoc*, as well as yeasts, including *Kazachstania* and *Dipodascus*, indicative of spontaneous fermentation. The concentration of biogenic amines, resulting from amino acid loss from the feed, which likely plays a role in poorer FE, also increased between the mixing tank and the troughs. The highest concentrations of biogenic amines in the feed were found on the farm that had the highest lactic acid bacteria (LAB) and yeast counts. This highlights the unexplored role that LAB and yeast may play in amino acid decarboxylation and biogenic amine formation in liquid feed. Factors including the use of liquid co-products in diets also impacted the liquid feed microbiome.

3.2 Introduction

Considering that ~70 % of pigs in Ireland are fed liquid feed as opposed to dry feed (Lawlor & O'Meara, 2018), it is important that both the microbial and nutritional quality of liquid feed are optimised to improve growth and feed efficiency (FE). The need to optimise liquid feed quality stems primarily from the observation of unintentional 'spontaneous' fermentation occurring in fresh liquid feed, which can have negative impacts. For example, amino acid and gross energy losses from the diet have been attributed to spontaneous fermentation in fresh (not deliberately fermented) liquid feed (Canibe & Jensen, 2003; O'Meara et al., 2020a). This may, at least in part, explain the poorer feed conversion efficiency (FCE) observed in liquid-fed pigs, especially with *ad libitum* liquid feeding where residual feed remains in the trough for relatively long periods of time (L'Anson et al., 2012; Russell et al., 1996).

Additionally, microbial decarboxylation of amino acids can lead to the production of biogenic amines, which may impact feed palatability and potentially pig health (Brooks et al., 2001; Canibe & Jensen, 2003; Missotten et al., 2010). A survey of French finisher farms feeding liquid feed found that concentrations of biogenic amines in liquid feed may be of concern, particularly cadaverine, which is a product of lysine decarboxylation (Le Treut, 2012; Özogul & Özogul, 2019). Another potential negative impact of feed fermentation is decreased lipid absorption and energy harvest in pigs due to overgrowth of bile salt hydrolase-producing bacteria (e.g. strains of *Lactobacillus* and *Clostridium*) in the feed and hence potentially in the intestine (He et al., 2017; Geng & Lin, 2016). Although the microbial communities of liquid feed have been quite well-documented, studies to date have almost exclusively been culture-based. Therefore, the objectives of this study were: (1) to profile the bacteriome and (for the first time) mycobiome of liquid feed from the finisher section of commercial pig farms, using high-throughput 16S rRNA and ITS2 amplicon sequencing, respectively; and (2) to determine biogenic amine concentrations as an indication of the level of spontaneous fermentation occurring and to assess feed safety.

3.3 Materials and Methods

3.3.1 Sample collection

Sampling of liquid feed was performed by O'Meara et al. (2020a) in the finisher section of eight commercial pig farms in Ireland. Details of liquid feeding practices

on each surveyed farm are provided in Table S3.1. A detailed description of the sampling procedures is provided in the O'Meara et al. publication. Briefly, liquid feed was sampled from the mixing tanks and feed troughs on seven pig farms (Farms A to G) on one sampling occasion, while one farm (Farm H, a research farm) was sampled on seven different occasions. Seven liquid feed samples were collected from three sampling locations on each farm. Firstly, freshly prepared feed was collected from the mixing tank after agitation (n = 1), samples of fresh liquid feed were then collected immediately after delivery to different troughs (n = 3), and lastly, samples of residual liquid feed, remaining in the different troughs, were taken just before the next feed-out (n = 3). On Farm D, the troughs did not contain residual feed and therefore, only mixing tank and fresh trough samples were taken on this farm (O'Meara et al., 2020a). In addition, on four of the seven sampling occasions on Farm H, only two fresh and residual trough samples could be collected, in addition to the one mixing tank sample. Liquid feed samples for bacteriome and mycobiome analysis were transferred aseptically into 1.5 mL sterile Eppendorf tubes on-farm. Samples were transported to the laboratory on dry ice, snap-frozen in liquid nitrogen upon arrival and stored at -80 °C until DNA extraction. Liquid feed sub-samples (~20 g) from the three sampling locations of 6 of the farms (Farms B, C, E, F, G and H) were also collected and stored at -20 °C for biogenic amine analysis by O'Meara et al. (2020a) as described in their study for lactate, ethanol and volatile fatty acid analysis.

3.3.2 Bacteriome and mycobiome analysis of liquid feed samples

3.3.2.1. *DNA extraction*

DNA extraction from liquid feed samples was performed using the QIAamp® Fast DNA Stool Mini kit, following the 'Isolation of DNA from Stool for Pathogen Detection' protocol. Previously described modifications to this procedure were followed, which included a 20 minute bead-beating step for simultaneous extraction of bacterial and fungal DNA as optimised in Chapter 2.

3.3.2.2. *Library preparation and amplicon sequencing*

Bacterial communities were profiled via amplicon sequencing of the V3-V4 hypervariable region of the 16S rRNA gene on the Illumina MiSeq platform, according to the Illumina 16S Metagenomic Sequencing Library Preparation Guide, with some modifications, as described in Chapter 2. Fungal profiling was performed by

amplifying the nuclear ribosomal ITS2 region with the ITS3 and ITS4 primer set from White et al. (1990). Each PCR reaction contained 50 ng of DNA template extracted from feed samples. The reaction volume, components and PCR conditions were the same as those described in Chapter 2. The 16S and ITS2 PCR products were quality checked and purified as described in Chapter 2. Final libraries were quantified by qPCR, diluted, denatured and sequenced using 2 x 250 cycle V2 kits in the Teagasc sequencing facility as described by Fouhy et al. (2015) in accordance with standard Illumina sequencing protocols.

3.3.3 Bioinformatics and statistical analysis

Raw FASTQ files for this study have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under project accession number PRJEB72728 (https://www.ebi.ac.uk/ena/browser/view/PRJEB72728). Demultiplexed paired-end 16S and ITS2 rDNA sequences were imported (in Casava 1.8 demultiplexed paired-end format) into QIIME2 v.2020.8.0 (Bolyen et al., 2019). Sequence quality assessment and initial pre-processing including primer trimming, filtering, dereplication, chimera removal, and merging of paired-end reads were performed in QIIME2 as described in Chapter 2. Taxonomic assignment for bacterial and fungal ASVs was performed using classifiers previously trained in Chapter 2 on sequences from the SILVA (Quast et al., 2013) and UNITE (Kõljalg et al., 2013) databases, respectively.

QIIME artefacts (taxonomy, ASV table, metadata and phylogenetic tree) were imported into R (version 4.2.1) as a phyloseq (McMurdie & Holmes, 2013) object with the qza_to_phyloseq function in the qiime2r package (Bisanz, 2018). Contaminant bacterial and fungal ASVs, identified using the 'prevalence' method in the decontam package (Davis et al., 2018), were removed prior to further analysis. Further pre-processing included removal of ASVs that were not assigned to the kingdoms *Bacteria* and *Fungi*, for each respective dataset, and removal of ASVs that phylum-level taxonomy was not assigned to. Finally, the filter_taxa function in phyloseq was used to remove ASVs that were not observed more than 3 times in at least 20 % of the samples, for each respective dataset.

Alpha-diversity (Observed ASVs, Pielou's evenness and Shannon diversity) and beta-diversity, based on unrarefied filtered sequences, were calculated using the phyloseq

package. Differences in alpha-diversity metrics were analysed using a linear mixed-effects model using the lmer function in the lme4 package (Bates et al., 2015), with farm as a random effect. Statistical significance between sampling locations was tested using the ANOVA function in the car package, followed by pairwise comparisons using Tukey's HSD test with the emmeans package (Lenth, 2020). Alpha-diversity was plotted using the ggpubr package (Kassambara, 2020). Beta-diversity was measured using non-metric multidimensional scaling (NMDS) of Bray-Curtis dissimilarity distances, and was plotted using the ggplot2 package (Wickham, 2016). Permutational multivariate analysis of variance (PERMANOVA) with 10,000 permutations was performed to test for differences between samples using the adonis2 function in the vegan package (Oksanen et al., 2020).

The ancombc2 function in the ANCOMBC package (Lin & Peddada, 2020) was used to identify differentially abundant bacterial and fungal genera between sampling locations. Differential abundance was expressed as log-fold changes, which represent the difference in bias-corrected abundances between groups. Default ancombc2 settings were used unless otherwise specified. In order to avoid spurious results, 'prv_cut' was set to 0.65 and 0.60 for the bacterial and fungal datasets, respectively. Therefore, genera that were present in less than 65 and 60 % of samples for the bacterial and fungal datasets, respectively, were removed prior to analysis. Pairwise comparisons between sampling locations were tested using the pairwise directional test, with farm included in the model as a random effect using the 'rand_formula' option. Genera with an adjusted *p*-value of ≤ 0.05 were considered differentially abundant and log-fold changes of pairwise comparisons were plotted using ggplot2 (Wickham, 2016).

3.3.4 Extraction, derivatisation and HPLC analysis of biogenic amines

Extraction of biogenic amines from the liquid feed samples taken from the mixing tank and troughs was performed based on the method of Yoon et al. (2015) with modifications. Liquid feed samples, which had been stored at -20 °C, were defrosted at room temperature and ~20 g was homogenised for 3 minutes using a DI 25 Basic homogeniser (IKA, Königswinter, Germany) at 13,500 rpm. Briefly, 5 g of liquid feed homogenate was weighed and 3 mL of 2 % trichloroacetic acid (TCA) (Sigma-Aldrich, Wicklow, Ireland) was added, followed by homogenisation for 30 seconds at

13,500 rpm. Samples were reacted at 4 °C for 2 hours with shaking, followed by centrifugation (4,000 rpm) at 4 °C for 20 minutes. The supernatant was added to a 10 mL volumetric flask. The residue was then re-extracted with another 3 mL of 2 % TCA, following the same procedure, except that the second extract was reacted at 4 °C for 1 hour. Both supernatants were then pooled and diluted to a final volume of 10 mL with 2 % TCA and stored at -20 °C until derivatisation and HPLC analysis.

Benzoyl chloride derivatisation of biogenic amines and HPLC analysis were performed as described by O'Sullivan et al. (2015), with minor modifications, as follows. A Waters® Alliance 2695 HPLC System equipped with a 2487 Dual Absorbance Detector (Waters Chromatography, Dublin, Ireland) was employed with an injection volume of 10 µL used for samples and standards. Biogenic amines were quantified using calibration curves generated from 5 standard solution mixtures containing histamine (2.5 - 50 µg/mL), putrescine (1.25 - 5 µg/mL), cadaverine (1.25 - 25 µg/mL), tyramine (2.5 - 50 µg/mL) and tryptamine (2.5 - 50 µg/mL) (Sigma-Aldrich) (Table S3.2).

3.4 Results

3.4.1 Diversity of bacteria and fungi in liquid feed

The alpha-diversity of the bacterial and fungal communities in the liquid feed samples from the mixing tank and in the fresh and residual liquid feed samples from the troughs across the commercial pig farms is summarised in Figure 3.1 (a) and (b), respectively. The number of observed bacterial ASVs did not differ between sampling locations ($p > 0.05$), indicating that bacterial species richness (the number of species present) was similar. However, Pielou's evenness (the distribution of abundances, with high evenness indicating that all species are present in similar proportions) and Shannon diversity (a measure of both richness and evenness, with greater weighting given to richness) both decreased between the mixing tank and residual trough samples ($p \leq 0.001$) and between the fresh and residual trough samples (Pielou's evenness: $p \leq 0.001$; Shannon diversity: $p \leq 0.01$). In addition, evenness also decreased between the mixing tank and fresh trough feed ($p \leq 0.01$). The number of observed ASVs assigned to fungi decreased from both the mixing tank and the fresh trough feed to the residual trough feed ($p \leq 0.001$), indicating that species richness decreased across the sampling locations. Pielou's evenness and Shannon diversity of the fungal communities also

decreased from the mixing tank (Pielou's evenness: $p \leq 0.01$, Shannon diversity: ≤ 0.001) and fresh trough feed (Pielou's evenness and Shannon diversity: $p \leq 0.001$) to the residual trough feed.

Figures S3.1-S3.6 show the bacterial and fungal alpha-diversity of the liquid feed sampled on each of the farms at each of the sampling locations. The liquid feed sampled from Farm H (research unit) had numerically higher bacterial alpha-diversity values compared to the commercial pig farms. In order to determine if the high microbial diversity on Farm H was the driver of the differences observed across all farms, differences in bacterial and fungal alpha-diversity between sampling locations on the commercial farms only (Farms A-G) was investigated and the data are shown in Figure S3.7. The results were generally in agreement with the alpha-diversity results from when the Farm H sample data were included in the analysis, with significant decreases in alpha-diversity observed between when the feed was mixed to when it was collected from the troughs. One notable difference when the Farm H data were excluded was that the observed bacterial ASVs increased between the fresh and residual feed ($p \leq 0.05$), whereas no differences were found between sampling locations when Farm H data were included.

Non-metric multidimensional scaling plots of Bray-Curtis dissimilarities indicated significant differences in microbial community structure between sampling locations. Clustering was observed for both the bacteriome and mycobiome at each sampling location [Figure 3.2 (a) and (b)], respectively. The bacteriome in the mixing tank feed was more similar to that of the fresh liquid feed, while the residual samples were more dissimilar. The mycobiome was less dissimilar between sampling locations compared to the bacteriome, and the residual feed had greater variability between samples, as indicated by greater distances between points. Samples from the same farm generally clustered together, with the mycobiome being generally more similar between farms, compared to the bacteriome, as indicated by a greater degree of clustering [Figure 3.2 (c) and (d)]. In addition, as observed across the entire dataset, within-farm samples also clustered based on sampling location. Notably, the majority of samples from the different sampling occasions on Farm H (research farm) formed a distinct cluster away from the commercial farms with respect to both bacterial and fungal communities. For this reason, NMDS plots of Bray-Curtis dissimilarities between sampling location and farm were also prepared without data from the Farm H samples (Figure S3.8). These

showed that the bacteriome and mycobiome of liquid feed from the commercial farms still formed distinct clusters based on sampling location and farm.

Permutational analysis of variance revealed that the variable farm explained ~58 % ($p < 0.001$) of the variation in bacterial community structure, while sampling location (mixing tank vs fresh vs residual trough samples) was responsible for ~17 % ($p < 0.001$). The variation of the fungal community structure was also influenced by farm, which explained ~61 % ($p < 0.001$) of variation, with sampling location accounting for ~9 % ($p < 0.001$) of variation. Due to the distinct clustering of the Farm H samples, PERMANOVA was also performed without the Farm H sample data in order to assess whether the farm variable effect was mainly driven by Farm H. This analysis confirmed that on the 7 commercial farms, bacterial and fungal community structure were still significantly influenced by both farm (~67 % and ~66 % of variation, respectively; $p < 0.001$) and sampling location (~12 % and ~8 % of variation, respectively; $p < 0.001$).

3.4.2 Comparison of bacterial and fungal taxa in liquid feed between sampling locations

The relative abundances of bacterial and fungal taxa > 1 % RA in liquid feed, on average, across all farms at the phylum and genus-level are available in Tables S3.3 and S3.4. *Firmicutes, Proteobacteria, Bacteroidetes* and *Actinobacteriota* were the dominant bacterial phyla detected in the liquid feed (Table S3.3). The mean relative abundance (RA) of *Firmicutes* across all farms, increased from the mixing tank feed (43.6 %) to the fresh feed (70.5 %), and further increased in the residual feed (89.9 %). Conversely, *Proteobacteria*, *Bacteroidetes* and *Actinobacteriota* decreased from the mixing tank feed (31.8, 10.7 and 6.5 %, respectively) to the fresh feed (18.5, 7.6 and 2.7 %, respectively) down to 4.6, 4.6 and 0.5 % mean RA in the residual feed (Table S3.3).

At the genus-level, the dominant liquid feed bacteriome consisted of lactic acid bacteria (LAB) i.e. *Lactobacillus*, *Weissella* and *Leuconostoc* (Figure 3.3). The mean RA of the most dominant genus, *Lactobacillus*, across all farms, increased from 26.3 % in the mixing tank, up to 44.6 % and 48.5 % in the fresh and residual troughs, respectively. Similar trends were observed for *Weissella* and *Leuconostoc;* their RA increased from the mixing tank (2.9 and 6.3 %, respectively), to the fresh feed (9.4

and 8.5 %, respectively), with maximum abundances in the residual feed (16.9 and 10.4 %, respectively). The opposite trend was observed for most Gram-negative bacterial genera. For example, *Pantoea*, *Pseudomonas* and *Sphingomonas* were present in the mixing tank feed at 10.9, 9.1 and 6.3 % mean RA but decreased to 0.5, 0.1 and 0.3 % in the residual feed, respectively (Figure 3.3).

At the phylum-level, the mycobiome of liquid feed was dominated mainly by *Ascomycota* and *Basidiomycota* (Table S3.3). The former increased from the mixing tank feed (75.7 %) to the fresh feed (79.6 %), up to a mean RA of 87.5 % in the residual feed across all farms. The latter decreased in mean RA between the mixing tank feed (24.2 %), the fresh feed (20.4 %), and the residual feed (12.4 %), while *Mucoromycota* remained below 0.1 % mean RA at all sampling locations, across all farms. At the genus-level, a number of known cereal pathogens including the genera *Alternaria* (also a mycotoxin producer), *Neoascochyta* and *Microdochium* dominated the liquid feed. These genera were most prevalent in the mixing tank (15.8, 14.3 and 13.9 % RA, respectively) and although they decreased somewhat in the residual trough-sampled feed (12.7, 12.0 and 10.0 %, respectively), they remained a substantial component of the residual feed mycobiome. This was also the case for *Cladosporium*, which showed a similar decrease in RA from the mixing tank feed (11.8 % RA) to the residual feed in the troughs (6.1% RA). Yeasts, including *Kazachstania* and *Dipodascus* increased in RA between the mixing tank (1.5 and 2.3 %), fresh (8.6 and 4.4 %) and residual (15.9 and 13.7 %) trough-sampled liquid feed, while others such as *Vishniacozyma* and *Sporobolomyces* decreased (Figure 3.3).

3.4.3 Differentially abundant bacterial and fungal genera between sampling locations

In order to assess which bacterial and fungal genera were significantly differentially abundant between the different sampling locations ANCOMBC was used (Figure 3.4). The results were largely in agreement with the compositional data (Figure 3.2). In terms of the bacteriome [Figure 3.4 (a)] of the residual trough-sampled feed, compared to that of the mixing tank, the LAB *Weissella* and *Lactobacillus* were enriched, along with genera including *Terrisporobacter, Clostridium sensu stricto 1* and *Aerococcus* ($p < 0.05$). The majority of these genera also increased in differential abundance

between the fresh and residual feed, while *Acetobacter* was enriched in the fresh trough-sampled feed compared to the mixing tank.

Many of the bacterial genera outlined above whose RA decreased in the troughs compared to the mixing tank, including *Pseudomonas*, *Sphingomonas* and *Pantoea*, also exhibited log-fold decreases ($p < 0.05$). The majority of the fungi [Figure 3.4 (b)] found to be differentially abundant between the sampling locations were yeasts; all of these were enriched between the mixing tank and residual feed, except *Bullera* which decreased ($p < 0.05$). *Wickerhamiella, Diutina* and *Apiotrichum* had the highest log-fold increases between the mixing tank and residual trough-sampled feed ($p < 0.05$). Two genera of filamentous basidiomycetes were differentially abundant ($p < 0.05$); *Itersonilia* decreased in the residual feed compared to the mixing tank and fresh trough-sampled feed, while *Alternaria* increased.

3.4.4 Comparison of bacterial and fungal taxa in liquid feed between farms

There was considerable farm-to-farm variation in the bacteriome found at each sampling location (Figure 3.5). *Lactobacillus* was the predominant bacterial genus detected in the mixing tank feed on the majority of farms (A, B, D, E, G, H3 and H5), ranging from 13.2 to 89.8 % RA, and was highest on Farm B. On the other hand, *Pantoea* was the most abundant genus in the mixing tank on Farms C, H1, H2, H4 and H6, ranging from 9.8 to 17.8 % RA. Lastly, *Sphingomonas* (19.6 % RA) and *Chryseobacterium* (14 % RA) were the predominant bacterial taxa in the mixing tank feed on Farms F and H7, respectively. In the fresh liquid feed sampled from the troughs, *Lactobacillus* became the predominant genus on all farms (23.8 - 95.1 % RA), except on Farm C, where it was *Weissella* (33.8 %). *Lactobacillus* remained predominant in the residual trough-sampled feed on most farms. However, *Weissella* still had the highest RA on Farm C, and also became the most abundant genus in the residual feed on Farm H7. In addition, as mentioned earlier, it was noted that the samples from Farm H, which is a research farm, had higher bacterial alpha-diversity compared to the commercial farms (Figures S3.1-S3.3). This is supported by the data in Figure 3.4, which show that ~20 % of the total bacterial reads in the Farm H mixing tank samples were from genera other than the 25 most abundant, indicating that the Farm H feed had a more diverse bacteriome, compared to the commercial farms. Other

results of note, include the high RA of some bacterial taxa in the fresh trough feed on Farm H, e.g. *Acetobacter* but only on two sampling occasions (H4 and H5) and *Pediococcus* on one occasion (H1). This highlights the variation across sampling time points that is evident for some taxa on this farm. Finally, *Clostridium sensu stricto 1* was notably more prevalent on Farm F, with a RA of 19.6 % in the residual trough feed, while its RA was < 5 % on all other farms.

The mycobiome of the mixing tank feed also varied across farms (Figure 3.6). *Neoascochyta* was the most abundant genus in the mixing tank feed of farms A, C, D and F (14.8 - 24.1 % RA). *Alternaria* predominated on Farms B, E, H4 and H5 (14.2-38.2 % RA), while the yeast *Saccharomyces* predominated on Farm G (16.7 % RA). In fact, the only farms on which *Saccharomyces* was detected in the mixing tank were Farms G and F. As with the bacteriome, the most abundant genus in the mixing tank feed varied across time points on Farm H, with *Microdochium* most abundant in the H1, H2 and H3 samples (25.4 - 27.4 % RA), and *Cladosporium* predominating in H6 (15.2 % RA) and H7 (13.8 % RA).

Two yeast genera became dominant in the fresh trough-sampled feed on some farms; *Kazachstania* was most abundant on Farms A, B and E (ranging from 31.7 to 39.5 % RA), while *Dipodascus* predominated in the H4 (19.3 %) and H5 (20.3 % RA) samples. Meanwhile, cereal-associated filamentous fungi predominated in the fresh liquid feed on the remaining farms; *Alternaria* on Farms C, H6 and H7 (13.6 to 16.8 % RA); *Microdochium* on Farms G, H1, H2 and H3 (18.1 to 28.2 % RA); and *Neoascochyta* on Farms D (21.8 % RA) and F (21.3 % RA).

Between the fresh and residual trough-sampled liquid feed, the predominant genera remained the same on the majority of the farms/samples from the same farm (Farms A, B, C, E, F, H2, H3, H4, H5 and H7). Some of the farms where yeasts were already predominant in the fresh feed showed further increases in the residual feed. *Kazachstania* increased up to 55.7 % RA on Farm E, while its RA in the residual feed on Farm B reached 91 %. Similarly, *Dipodascus* increased dramatically in RA between the fresh and residual liquid feed in the H4 (from 19.3 to 70.7 % RA) and H5 (from 20.3 to 60.2 % RA) samples. *Neoascochyta* became the most abundant genus in the residual feed on Farm G (17.9 % RA), while *Dipodascus* predominated in the H1

(20 % RA) and H6 (24.6 % RA) samples. Interestingly, as for the mixing tank samples, *Saccharomyces* was only detected at > 1 % RA in the troughs on Farms F and G.

3.4.5 Biogenic amine concentrations in liquid feed sampled from the mixing tanks and troughs

The concentrations of cadaverine and putrescine detected in the liquid feed sampled from the mixing tank and troughs are shown in Figure 3.7. Concentrations were generally low across farms, except for Farms B and G which had relatively high levels compared to the others. Although histamine and tyramine were quantified for each farm, concentrations were below the detection limit on all farms. Additionally, the only samples in which tryptamine was found above the detection limit were the residual trough samples on Farm B (6.1 ppm; $n = 2$). Concentrations of putrescine and cadaverine, however, were notably higher compared to the other amines, particularly in the residual trough-sampled feed. Although concentrations of putrescine in the mixing tank samples from Farms G and B were low, they were marginally higher for Farm B. In both the fresh and residual trough-sampled feed, however, putrescine and cadaverine levels were highest on Farm B, with levels of putrescine and cadaverine at ~17 and ~84 ppm in the residual trough samples, respectively. Farms C, E, F and H, all had relatively low levels of all biogenic amines, although Farm E had cadaverine concentrations of ~3 ppm in fresh trough samples, which increased to just below 7 ppm in the residual samples.

3.5 Discussion

This study profiled the bacterial and fungal communities, and the biogenic amine concentrations, of liquid feed for grow-finisher pigs on a selection of commercial pig farms. Despite its widespread adoption, there are a number of disadvantages of liquid feeding, most notably the poorer FCE observed in liquid-fed pigs (Han et al., 2006; L'Anson et al., 2012; O'Meara et al., 2020b; Russell et al., 1996). One potential reason for this is the unintentional spontaneous fermentation observed in liquid feed which can lead to the depletion of dietary energy and amino acids, as well as the production of undesirable concentrations of ethanol and acetic acid (Brooks, 2008; Canibe & Jensen, 2003; Missotten et al., 2010; Niven et al., 2006; O'Meara et al., 2020a; Plumed-Ferrer et al., 2004; Plumed-Ferrer & Von Wright, 2009; Russell et al., 1996). Liquid feed provides a source of fermentable carbohydrate for LAB and yeast, and

hence, the occurrence of LAB and yeast fermentation in liquid feed is well-documented (Brooks, 2003; Canibe & Jensen, 2003; Geary et al., 1999; Plumed-Ferrer & Von Wright, 2009; Russell et al., 1996). However, many studies to date have focused on the microbial communities of deliberately fermented liquid feed, (fermented either via native feed microbiome or LAB inoculants), while the farms in the current study all practiced 'fresh' liquid feeding, i.e. the feed was not deliberately fermented. Additionally, since much of the research investigating the liquid feed microbiome has used only culture-based methods, this study is fundamental in establishing the complete bacterial and fungal profile of fresh liquid feed using culture-independent methods, and in determining the extent of biogenic amine production in liquid feed on commercial pig farms.

Decreases in the alpha-diversity of the bacterial and fungal communities in the feed were found between the mixing tank, and delivery to the troughs. However, the greatest decreases in alpha-diversity were observed in the feed that resided in the troughs until just before the next meal was fed. Although this was the case for both the bacteriome and mycobiome, the decreased alpha-diversity appears to have been influenced differently in each case. Since the bacterial species richness (Observed ASVs) did not decrease between sampling locations, it is likely that the decrease in evenness (Pielou's evenness), i.e. the distribution of abundances, was the main driver of decreased bacterial diversity in the residual feed. This is most likely a result of the proliferation and predominance of fermentative LAB. On the other hand, fungal richness (Observed ASVs) was lower in the residual feed compared to the mixing tank and the fresh trough-sampled liquid feed, indicating that a number of fungal taxa were no longer present in the residual feed. Fungal diversity was also impacted by changes in abundance of certain taxa, as evidenced by a decrease in evenness in the residual feed, compared to the mixing tank. The loss in fungal diversity can be attributed to fermentation by yeast such as *Kazachstania, Dipodascus* and *Diutina* which were highly abundant in the residual feed across several farms. These differences in the bacterial and fungal diversity in liquid feed between sampling locations indicate underlying changes in the functions of the bacteriome and mycobiome, although this study focuses primarily on community composition.

Beta-diversity analysis revealed distinct clustering of the bacteriome and mycobiome at each sampling location. This is in agreement with the fact that sampling location

was found to be a significant determinant of bacterial and fungal composition. However, microbial community structure was influenced to a greater extent by the farm of origin, which explained ~60 % of the variance between samples. This is perhaps unsurprising as each surveyed farm had different diet composition, liquid feeding systems and feeding practices (O'Meara et al., 2020a).

The decreased microbial diversity in the feed after mixing is likely a result of spontaneous fermentation by LAB and yeast, as reported by O'Meara et al. (2020a) on these farms. This finding was evidenced in the same samples as those analysed in the present study by decreased pH and increased LAB and yeast counts in fresh and residual trough-sampled feed compared with the mixing tank, as well as amino acid and gross energy losses (O'Meara et al., 2020a). The compositional data from the present study backs this up, as it shows that the LAB *Lactobacillus, Weissella* and *Leuconostoc* predominated in the liquid feed, with *Lactobacillus and Weissella* increasing sequentially in differential abundance from the mixing tank to the freshly delivered feed in the troughs to the residual feed sampled just before the next feed-out. A concurrent decrease in the RA of Gram-negative bacteria including *Pantoea* and *Pseudomonas* also occurred. This is in line with the second phase of feed fermentation where low pH and increasing levels of lactic acid, and other metabolites, produced by LAB fermentation, inhibit the proliferation of pathogenic and spoilage bacteria (Brooks, 2008; Dujardin et al., 2014; Russell et al., 1996). Hence, this bacterial shift could be considered beneficial. It should be noted, however, that although it was not possible in this study, species-level assignment is necessary to determine whether LAB are homofermentative or heterofermentative. The former is desirable as lactic acid is produced as the sole fermentation product, while the latter results in the production of lactic acid, CO_2, ethanol and/or acetic acid (Bintsis, 2018).

The mixing tank feed had a similar bacterial composition at the phylum level compared to that previously reported for fresh liquid feed. Torres-Pitarch et al. (2020a) reported 39.9 % RA of *Firmicutes* in fresh liquid feed (compared to 43.6 % on average across all farms in this study), and 33.2 % *Proteobacteria* (38.1 % in this study). It should be noted, however, that in the current study there were substantial differences between farms; *Firmicutes* was the predominant phylum in the mixing tank on Farms C, F and all of the Farm H sampling occasions except H5, with *Proteobacteria* predominating on the rest of the farms. At the genus level, *Lactobacillus*, *Pantoea* and

Pseudomonas were the most abundant bacteria in the mixing tanks, on average, across the surveyed farms. However, considering that the mixing tank feed had the most diverse bacteriome of all sampling locations, the aforementioned genera only accounted for ~50 % of the bacterial composition, on average, across the farms, with relatively high prevalence of several other genera that later became less abundant in the troughs during fermentation.

Despite the differences in the mixing tank bacteriome between farms, once the feed was delivered to the troughs, LAB (primarily *Lactobacillus* but also *Weissella* and *Leuconostoc*) predominated across all farms. Variability of spontaneous fermentation, however, was also evident on a number of farms. For example, *Acetobacter,* which was well below 1 % RA in the mixing tank samples from H4 and H5, increased in RA to reach 18.9 and 24.8 % RA, respectively, in the fresh trough-sampled feed. However, in the troughs, LAB fermentation appeared to outcompete that of *Acetobacter,* which fell to 6.1 and 4.3 % RA in the H4 and H5 residual feed samples respectively. The residual feed from Farm F was also notable because *Clostridium sensu stricto 1* occurred at a RA of ~20 %. Clostridial fermentation is undesirable in silage, for example, potentially resulting in excessive butyric acid, ammonia and biogenic amine production, leading to feed palatability issues and health concerns (Li et al., 2020). However, biogenic amines were not detected in the feed from Farm F.

Although the reason for the high proportion of *Clostridium* on Farm F is unclear, several factors may have contributed. For example, the pot ale syrup included in the diet may have been of poor microbiological quality. Additionally, this was one of the farms that performed no cleaning of the liquid feeding system and it operated a short trough system which can allow residual feed to remain in the troughs for longer, facilitating more feed fermentation (O'Meara et al., 2020a). Torres-Pitarch et al. (2020) found no differences in bacterial composition at the phylum or genus level in residual trough-sampled 'fresh' liquid feed or deliberately fermented feed. Our study, however, demonstrates that although LAB predominate in residual trough-sampled 'fresh' liquid feed following spontaneous fermentation, there are dramatic between-farm differences in the genera that predominate and in their RA. The differences between farms appear to be determined by the initial microbiome of the feed. However, once the feed is mixed, a similar pattern occurs; the mixing tank and freshly

sampled trough liquid feed remain relatively similar in composition, while the residual feed in the troughs differs substantially.

Despite only analysing a relatively small number of samples for biogenic amines on 6 of the 8 farms surveyed by O'Meara et al. (2020a) the present study revealed the formation of biogenic amines in 'fresh' liquid feed on commercial pig farms. The only farms on which appreciable concentrations of biogenic amines were detected were Farms B and G, providing further evidence of uncontrolled spontaneous fermentation in fresh liquid feed, particularly so when residual feed remains in the troughs between feeds. It was not surprising to find biogenic amines in liquid feed on these farms as O'Meara et al. (unpublished) measured a 35.6 % loss of lysine between the feed sampled in the mixing tank and the feed sampled in residual troughs, on average, on the 8 farms surveyed. Farm B, which had the highest levels of putrescine and cadaverine in this study, had a 34.8 % loss of lysine between the feed in the mixing tank and the residual liquid feed (O'Meara et al., unpublished).

The concentrations of putrescine and cadaverine in residual trough-sampled feed on Farm B were ~17 and ~84 ppm, respectively (on a fresh matter basis). Although not directly comparable due to being expressed on a dry matter basis, the average putrescine and cadaverine concentrations in troughs were 7 and 57 ppm in a survey of French finisher pig liquid feed (Le Treut, 2012). The putrescine concentrations found in the current study were also similar to those observed in residual liquid feed sampled from troughs by Torres-Pitarch et al. (2020) who detected 27.8 ppm (on a fresh matter basis). However, the concentrations of cadaverine were higher on Farm B in the current study [~84 ppm versus 18.5 ppm detected by Torres-Pitarch et al. (2020)]. The latter most likely explains the high degree of lysine degradation previously found in liquid feed on Farm B. However, it should be noted that there was substantial variation between the residual trough samples collected on Farm B (as indicated by the large standard deviation), likely resulting from varying degrees of fermentation in individual troughs. In contrast to Farm B, biogenic amine concentrations on Farm G were lower than in the aforementioned studies. The detection of substantial concentrations of biogenic amines in liquid feed can be used as an indicator of spontaneous fermentation and amino acid decarboxylation. There may also be implications for pig health; however, no data are available regarding acceptable concentrations of biogenic amines in liquid feed. Histamine, which was not detected

in this study, is considered the biogenic amine of greatest health concern, with a 50 mg/kg limit in food set by the US Food and Drug Administration (Ruiz-Capillas & Herrero, 2019). The European Food Safety Authority reported that there is insufficient information available to determine concentrations of putrescine and cadaverine associated with adverse health effects (EFSA., 2011); however, concentrations above 440.75 and 255.45 mg/kg, respectively, have been shown to be toxic to intestinal cells *in vitro* (del Rio et al., 2019). Additionally, these amines are known to enhance the toxicity of histamine (Ruiz-Capillas & Herrero, 2019; Tabanelli, 2020). Therefore, it is unlikely that the concentrations observed in this study are of concern. However, the maximum concentrations of putrescine and cadaverine reported by Le Treut (2012) were 310 and 1,182 ppm, respectively, highlighting that biogenic amine formation in liquid can reach levels of concern.

It is worth noting that Le Treut (2012) found that biogenic amine concentrations were much higher for farms using liquid co-products or pre-fermented high moisture corn in their diets, with putrescine and cadaverine concentrations of 101-141 and 211-616 ppm (on a dry matter basis), respectively, found on these farms. Interestingly, two of the farms surveyed for biogenic amines in the current study, Farms F and G, included pot ale syrup in their diets, which may have contributed to the higher concentrations of biogenic amines on Farm G compared to the other farms. Although there is limited research regarding the microbiology of pot ale syrup, it has a low pH and is dominated by yeast and lactobacilli (White et al., 2020). Farm B, however, did not use liquid co-products in their diet; nonetheless, LAB and yeast counts in residual trough-sampled liquid feed were highest on Farm B (O'Meara et al., unpublished). This helps to explain the higher concentrations of biogenic amines found on this farm, particularly in the residual trough samples. Niven et al. (2006) demonstrated *in vitro* that *E. coli* was primarily responsible for lysine metabolism, and thus cadaverine formation, in liquid feed. However, numerous LAB strains including members of *Lactobacillus, Leuconostoc* and *Pediococcus* are well-known to produce biogenic amines under acid stress conditions in fermented foods (Barbieri et al., 2019). Furthermore, there is evidence of decarboxylation of amino acids by yeasts (Nielsen, 2019; Siesto et al., 2022). Despite this, research into the role of LAB and yeast in amino acid metabolism and resultant biogenic amine production in liquid feed is lacking. Although not investigated in this study, another potential issue with LAB proliferation in liquid feed

that warrants further investigation is bile salt hydrolase activity of LAB strains. Overgrowth of bile salt hydrolase-positive bacteria in liquid feed and hence potentially in the small intestine of liquid-fed pigs can result in poorer lipid absorption and energy harvest in these animals (He et al., 2017; Geng & Lin, 2016).

Information regarding the fungal communities in fresh liquid feed is relatively scarce, with the majority of studies to-date focusing primarily on culturing yeasts from deliberately fermented liquid feed (Canibe et al., 2007, 2010; Gori et al., 2011; Mikkelsen & Jensen, 1998; Olstorpe et al., 2010; Plumed-Ferrer & von Wright, 2011), hence the value of the current study. For example, while O'Meara et al. (2020a) reported that mould counts were similar across the same mixing tank and trough samples analysed in the current study, the culture-independent methods used here identified the prevalence of certain moulds in fresh liquid feed and variations between sampling locations. For example, some of the moulds most commonly associated with mycotoxin production, including *Alternaria*, *Cladosporium*, *Aspergillus* and *Fusarium* were detected on all surveyed farms (Bryden, 2012; Holanda & Kim, 2021). *Alternaria* and *Cladosporium* in particular, were highly abundant on many farms, most especially in the mixing tank, with their RA generally decreasing in the troughs. Farm B, another farm that performed no cleaning of the liquid feeding system (O'Meara et al., 2020a), had a particularly high prevalence of moulds; the mixing tank feed contained *Alternaria*, *Cladosporium*, *Aspergillus* and *Fusarium* at 38.2, 10.7, 2.7 and 4.4 % RA, respectively. However, between the mixing tank feed and the fresh feed in the troughs, the yeast *Kazachstania* had increased to reach 31.7 % RA, and subsequently almost completely dominated the residual feed (91 % RA). Yeast fermentation on Farm B was evidenced by an ethanol concentration of 24.8 mmol/kg in the residual trough-sampled feed (O'Meara et al., unpublished), compared to an average of 15.8 mmol/kg across all farms (O'Meara et al., 2020a).

The predominance of *Kazachstania* in the residual trough-sampled feed on Farm B was likely due to its already high RA in the mixing tank feed (10.6 %). *Kazachstania* was also present in the mixing tank feed of Farm A at high RA (8.6 %). However, it was not as abundant in the residual feed compared to Farm B, likely due to competition from other yeasts including *Diutina* which was present at 17 % RA in the residual trough-sampled feed on Farm A. Yeast-dominated fermentation is generally considered undesirable in liquid feed because excessive ethanol production may

reduce feed palatability, and result in dry matter and energy losses from the diet (Brooks, 2008; Brooks et al., 2001; Missotten et al., 2015). Previous culture-based analysis of the samples from this study by O'Meara et al. (2020a) provided evidence of yeast fermentation across all farms, with yeast counts and ethanol concentrations increasing between the mixing tank and the troughs. In general, in the present study, *Kazachstania* and *Dipodascus* were the two most dominant yeasts that proliferated in the feed across all farms, with the highest RA found in the residual feed, again indicative of yeast fermentation. *Kazachstania* has previously been identified in deliberately fermented liquid feed (Gori et al., 2011; Plumed-Ferrer & von Wright, 2011); however, to our knowledge *Dipodascus* has not been previously reported in liquid feed.

O'Meara et al. (2020a) also reported that liquid feed sampled from the mixing tank and fresh from the troughs on farms that incorporated liquid co-products into their diets had a lower pH. Interestingly, the farms that included pot ale syrup, a co-product from whiskey production, in their diets (Farms A, F and G) were the only farms where *Saccharomyces* was detected in the feed. Although the species was not identified, *Saccharomyces cerevisiae* is used in whiskey production and therefore, pot ale syrup is likely the source of these yeasts (Walker & Hill, 2016). Furthermore, Farm A, which used liquid whey, a by-product of cheese-making, had the highest RA of *Diutina* in the residual feed. This yeast genus (formerly classified within the genus *Candida*) contains a number of species which are associated with dairy products, including cheese (Cogan et al., 2014; O'Brien et al., 2018). This highlights the impact that the initial microbial composition of the diet has on the subsequent progression of the bacteriome and mycobiome in the troughs.

3.6 Conclusion

This study supports the current evidence that spontaneous fermentation occurs in 'fresh' liquid feed on commercial pig farms and provides insight into the bacterial and, for the first time, the fungal populations of liquid feed using high-throughput amplicon sequencing. The bacterial and fungal community structures in liquid feed were influenced, not only by the sampling location on a given farm (i.e. mixing tank versus troughs), but in fact more so by the particular farm from which the feed was sampled. This highlights the unpredictable nature and the between-farm variability of spontaneous fermentation. The inclusion of liquid co-products in diets also influenced

the microbial community in the liquid feed considerably, with yeasts associated with co-products from whiskey production and cheese-making proliferating in liquid feed in the troughs. The decreases in alpha-diversity of liquid feed between the mixing tank and the troughs corresponded with increased RA of bacteria, particularly *Lactobacillus, Weissella* and *Leuconostoc*, as well as yeasts including *Kazachstania* and *Dipodascus*. The concentration of biogenic amines also increased between the liquid feed in the mixing tank and the troughs, as a result of amino acid decarboxylation, particularly so on certain farms. Biogenic amines can be toxic to humans and animals. Although there are no data on acceptable concentrations in liquid feed, the levels observed in the current study are likely not of concern based on *in vitro* cytotoxicity data and food regulatory guidance. Nonetheless, the concomitant amino acid losses likely play a role in the poorer FE previously observed in liquid-fed pigs. Targeted interventions such as dietary acidification and improved feeding system hygiene could potentially limit spontaneous fermentation in fresh liquid feed and additional research is needed in these areas.

3.7 Tables and figures

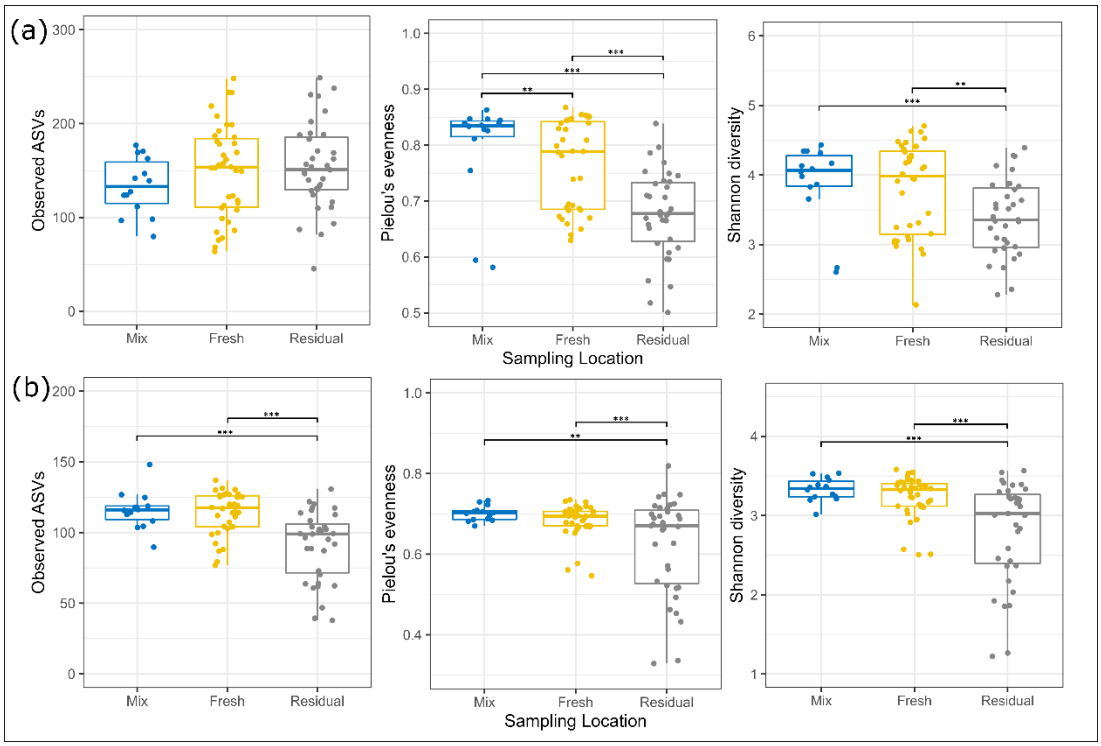

Figure 3.1: Boxplots displaying observed amplicon sequence variants (ASVs), Pielou's evenness and Shannon diversity of bacterial (a) and fungal (b) communities in liquid feed samples from the mixing tank and in fresh and residual liquid feed samples from troughs on eight commercial pig farms. ** $p \leq 0.01$, *** $p \leq 0.001$. Mixing tank (Mix): $n = 14$, Fresh trough (Fresh): $n = 38$, Residual trough (Residual): $n = 35$.

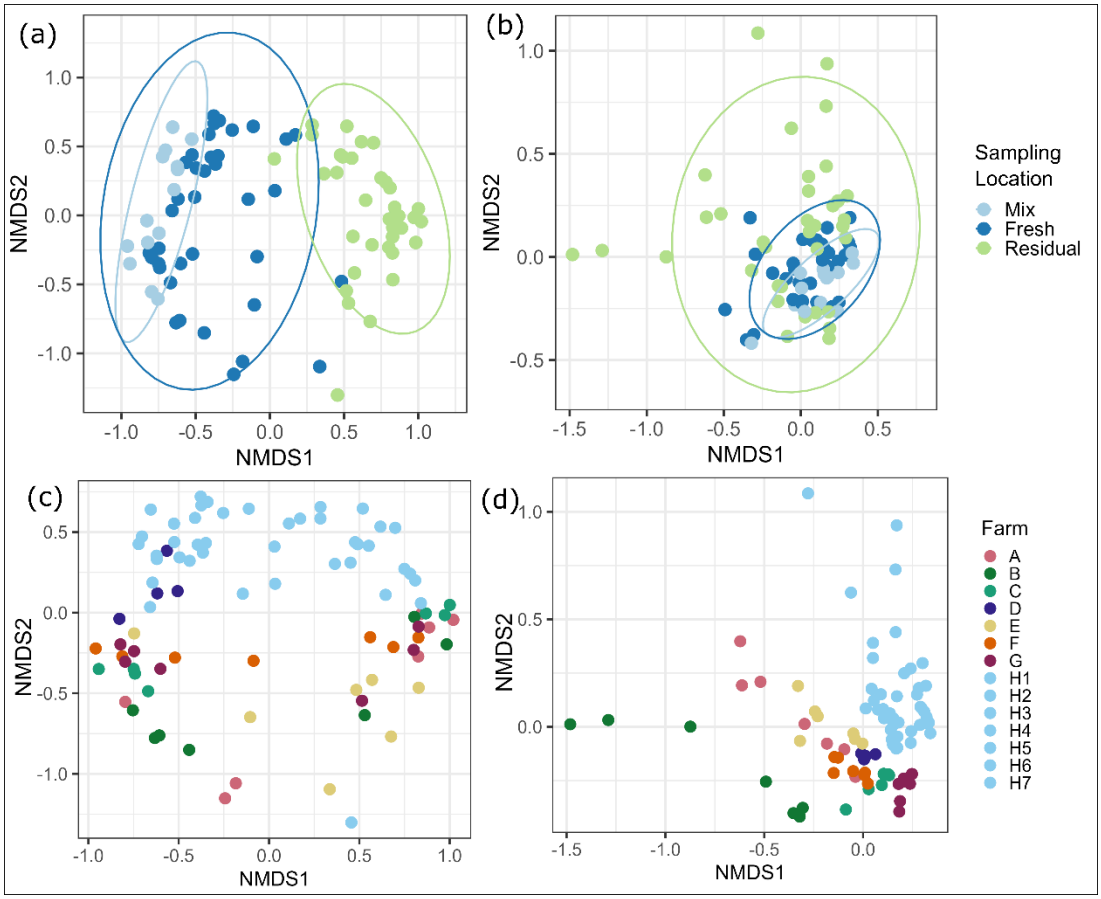

Figure 3.2: Non-metric multidimensional scaling (NMDS) plots based on Bray-Curtis dissimilarity in liquid feed collected at each respective sampling location; Mixing tank (Mix; *n* = 1/farm), liquid feed sampled immediately after delivery to the troughs (Fresh; *n* = 3/farm), liquid feed sampled prior to the next feed (Residual; *n* = 3/farm) on eight commercial pig farms for bacterial (a) and fungal (b) communities, and between farms for bacterial (c) and fungal (d) communities.

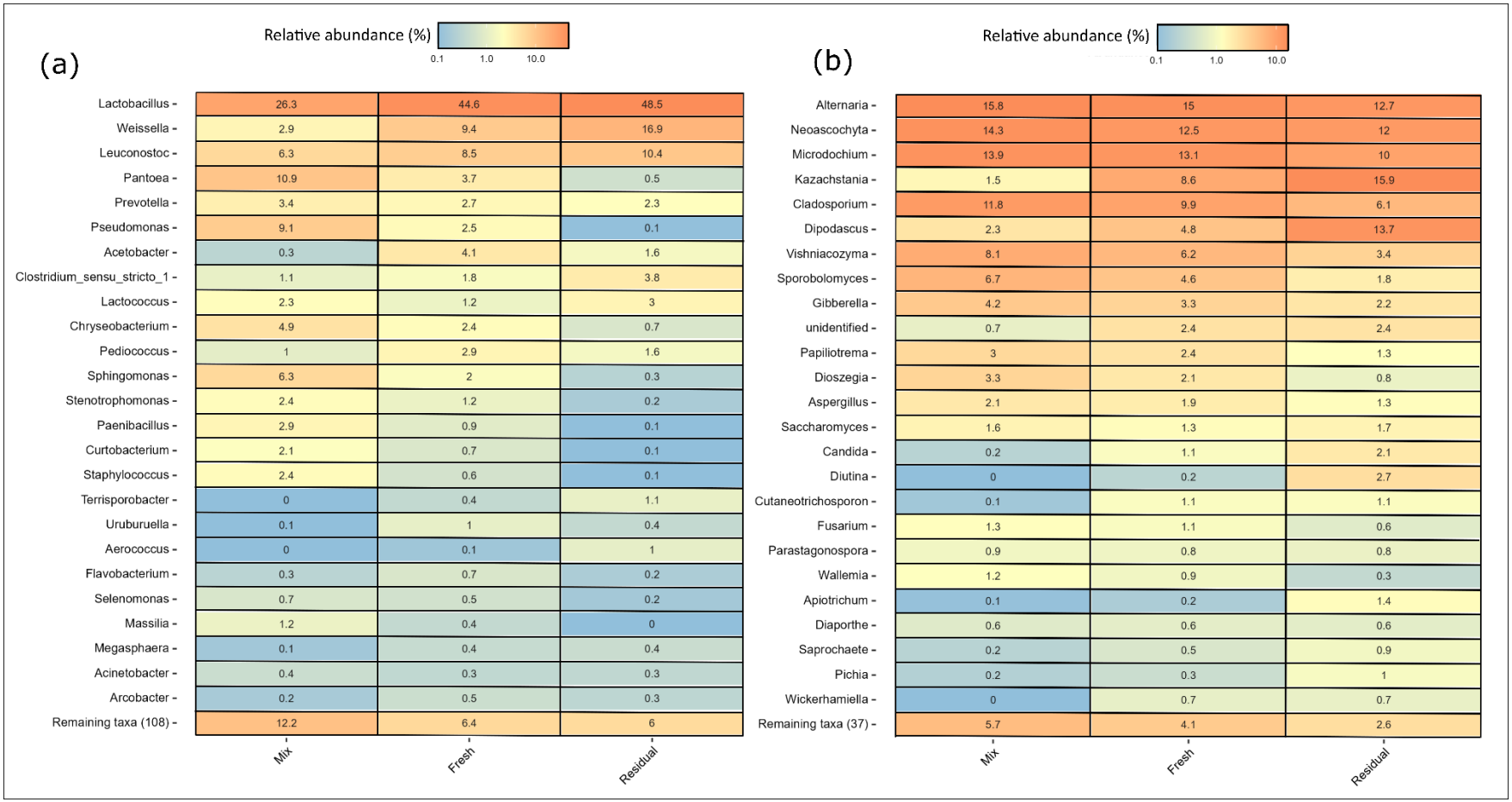

Figure 3.3: Heatmap displaying mean relative abundance (%) of the 25 most abundant bacterial (a) and fungal (b) genera in liquid feed between sampling locations. Data are the mean of values from all farms for each sampling location; mixing tank (Mix): *n* = 14; fresh feed from the trough (Fresh): *n* = 38, residual feed from the troughs (Residual): *n* = 35.

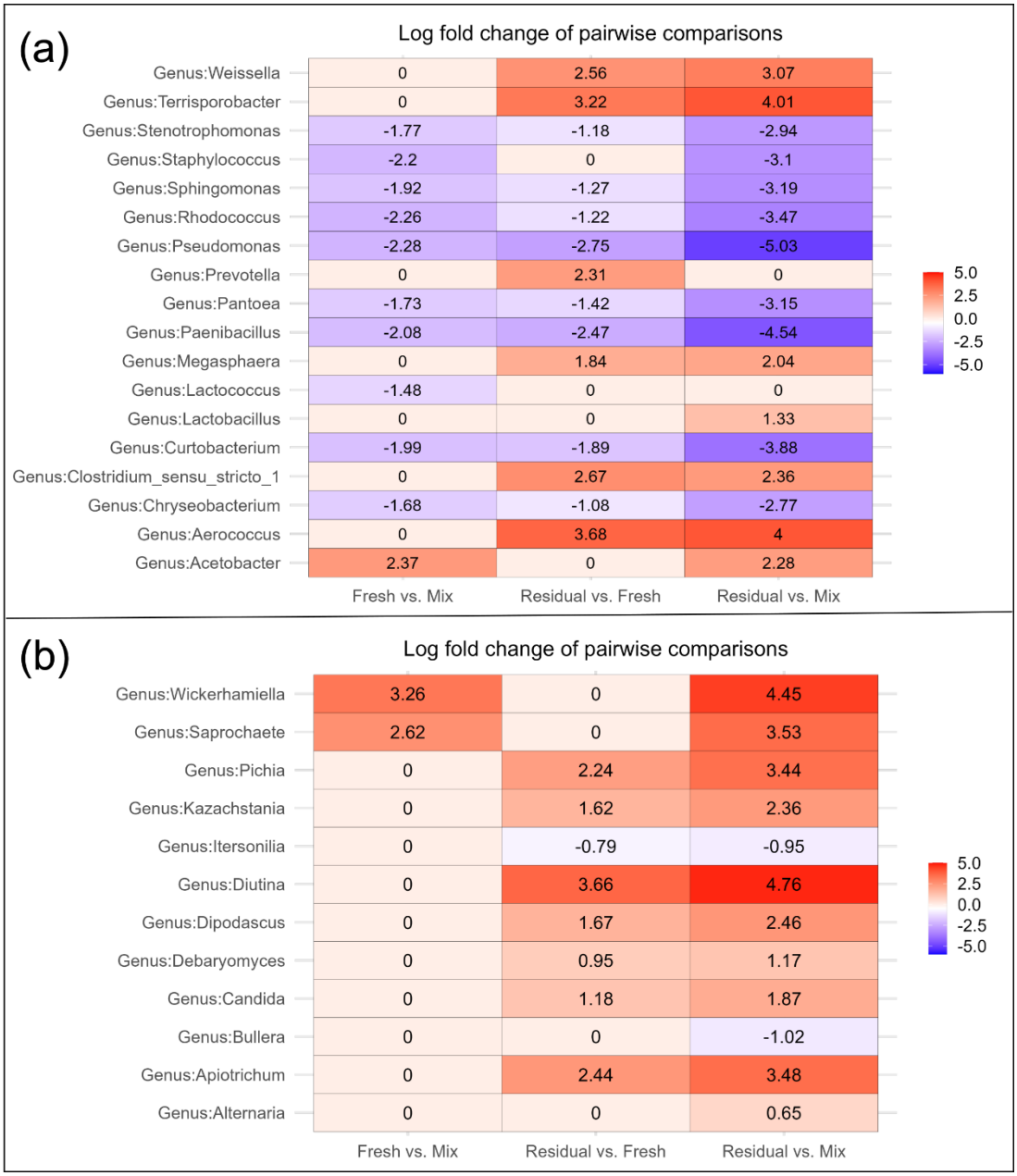

Figure 3.4: Heatmaps of log-fold changes of differentially abundant bacterial (a) and fungal (b) genera after pairwise comparisons between different liquid feed sampling locations. Log-fold changes of taxa between sampling locations with an adjusted *p*-value ≤ 0.05 were considered significant.

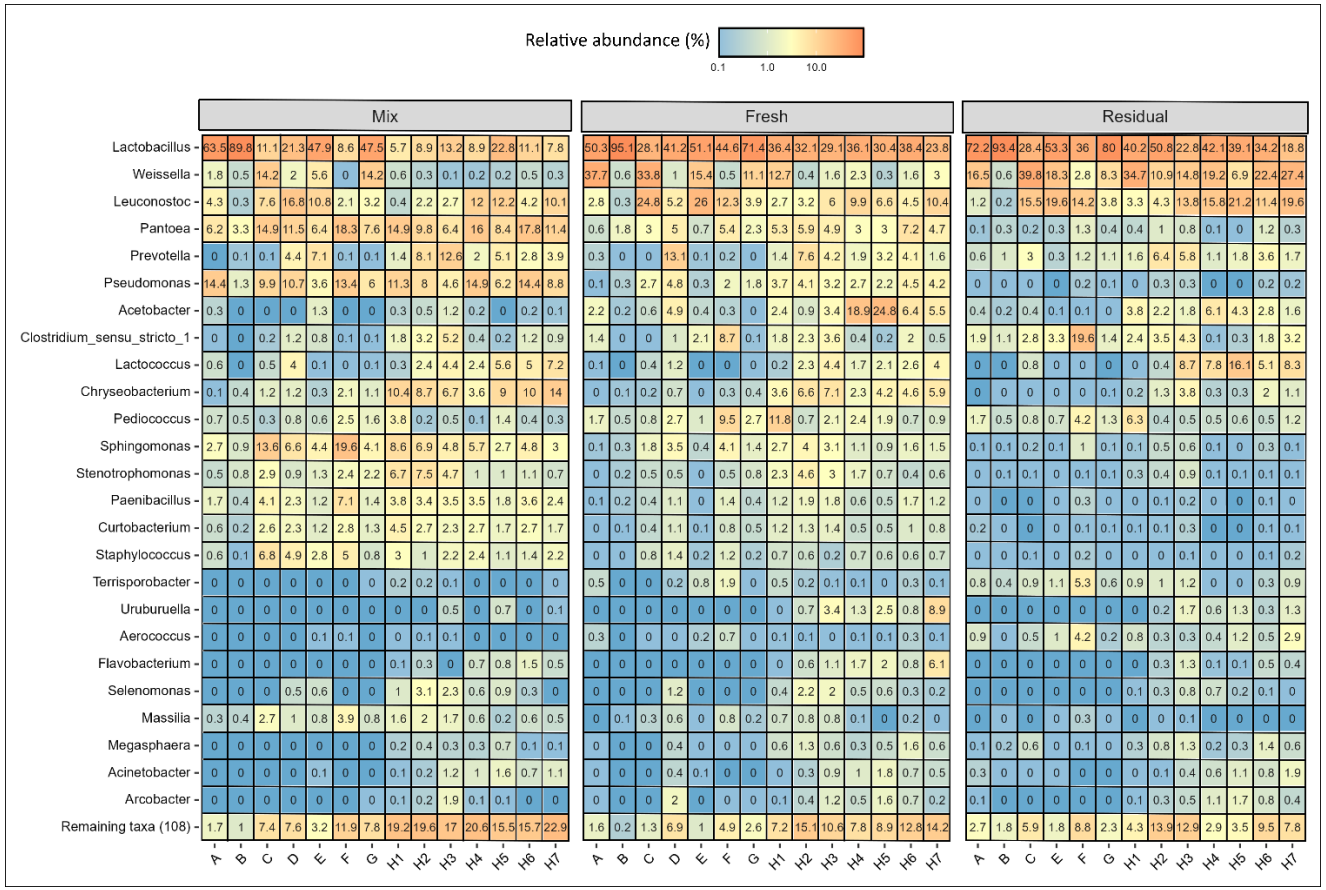

Figure 3.5: Heatmap displaying relative abundance (%) of the 25 most abundant bacterial genera in liquid feed sampled on each farm from the mixing tank (Mix: *n* = 1/farm), and from the troughs, immediately after delivery (Fresh: *n* = 3/farm*) and just before the next feed-out (Residual: *n* = 3/farm*). *No residual trough samples were collected on Farm D. On four of the seven sampling occasions on Farm H, only two fresh and residual trough samples were collected.

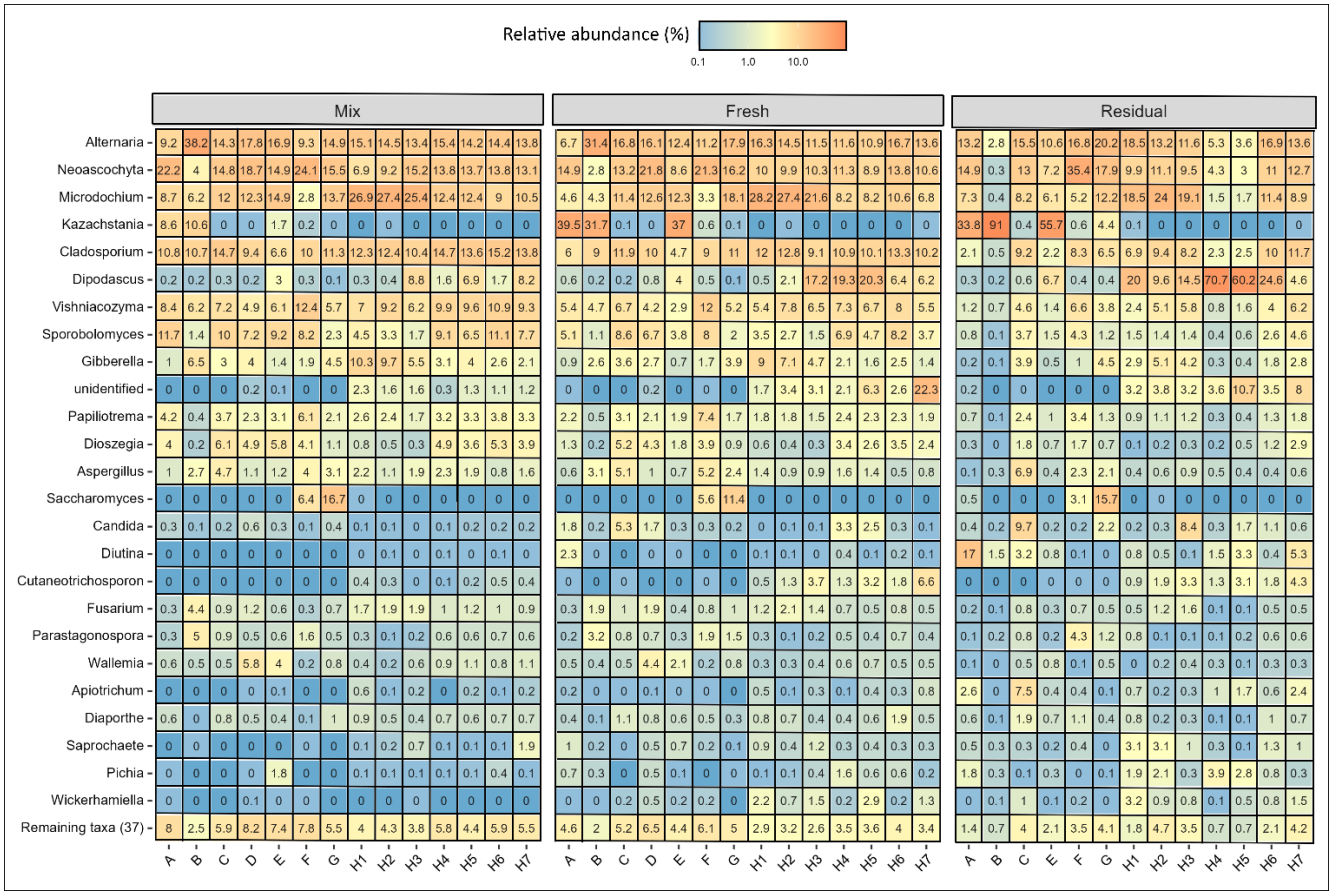

Figure 3.6: Heatmap displaying relative abundance (%) of the top 25 most abundant fungal genera in liquid feed on each farm in the mixing tank (Mix: *n* = 1/farm), and from the troughs, immediately after delivery (Fresh: *n* = 3/farm*) and just before the next feed-out (Residual: *n* = 3/farm*). *No residual trough samples were collected on Farm D. On four of the seven sampling occasions on Farm H, only two fresh and residual trough samples were collected.

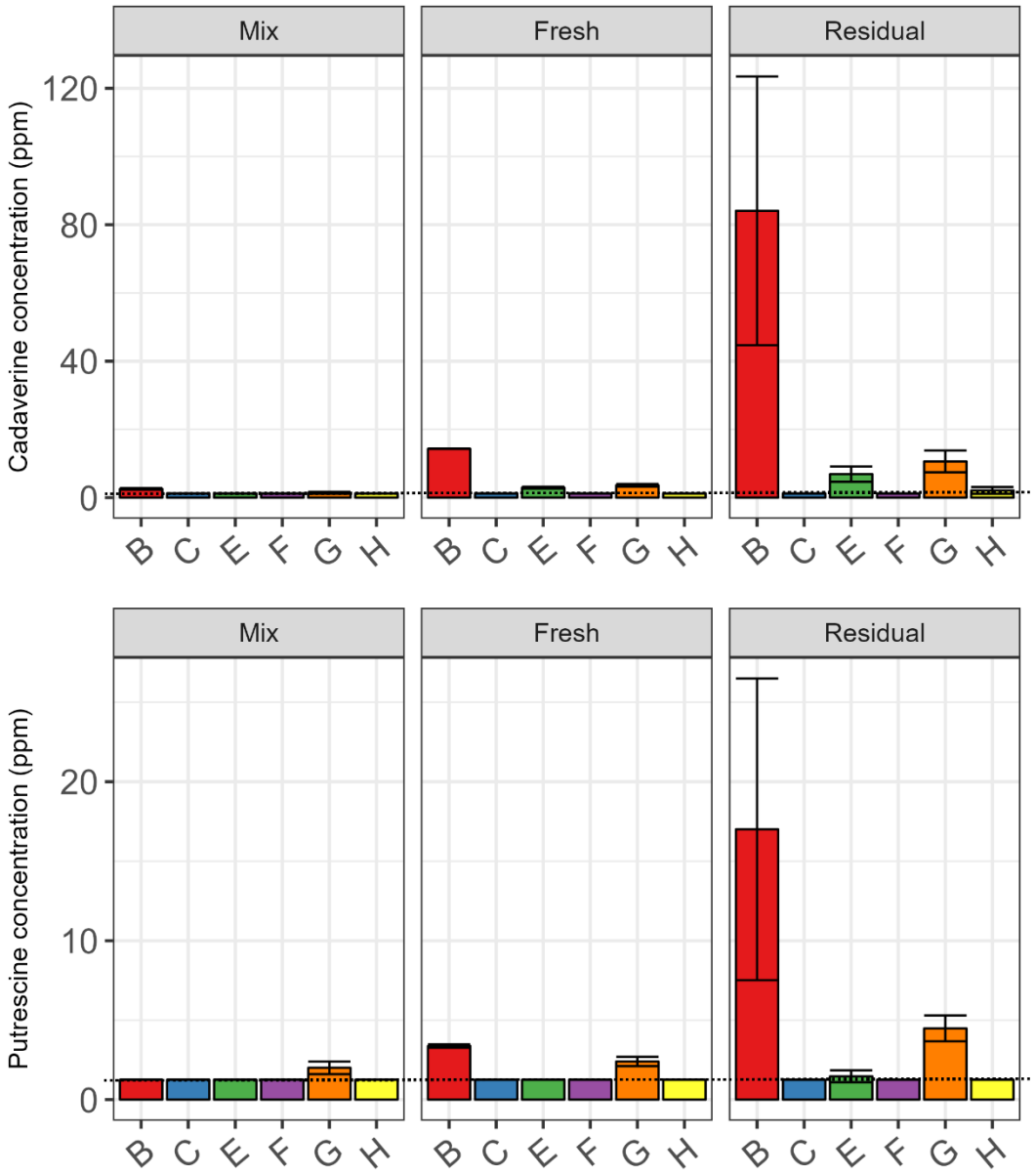

Figure 3.7: Biogenic amine concentrations in liquid feed sampled from mixing tanks (Mix) and troughs (Fresh and Residual) on six commercial pig production farms. Biogenic amine concentrations are presented as ppm, on a fresh matter basis i.e. in the liquid sample. The limit of detection (LOD; dotted horizontal line on plots) for putrescine and cadaverine was 1.25 ppm and for tryptamine, histamine and tyramine it was 2.5 ppm; values below the LOD were recorded as being at the LOD. For the mixing tank, values are the mean of duplicate extractions of one sample (n = 1) and for fresh and residual trough-sampled feed, values are the mean of duplicate extractions of two samples (n = 2) except for the fresh trough samples for Farm B where values are the mean of duplicate extracts from 1 sample (n = 1). Standard deviations of duplicate extractions per sample are indicated by error bars.

3.8 Supplementary Information

Table S 3.1: Summary of survey results on liquid feeding practices used in the finisher section on eight commercial pig production units (O'Meara et al. 2020a).

	Unit*							
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**[†]
Model of feed system	Funki	Funki	Funki	Funki	Datamix	BD[‡]	BD	BD
Trough type	Long	Long	Long[§]	Long	Short	Short	Short	Short
Age of feed system (yrs[¶])	4	10	0.167	10	3	20	10	4
Feed splits (feeds/day)	5	4	4	4	10	12	4	4 or 5
Water to meal ratio (FM[**])	2.1:1	2.4:1	3.5:1	3:1	2.6:1	3.4:1	3.1:1	2.5:1
Volume in mix tank (kg)	2,632	1529	11,000	1,500	3,000	3,000	11,000	~150
Agitation time (min)	23	20	10	2[††]	8 to 10	6[††]	30	5 to 20
Time for feed-out (min)	45	10	20	15	120	5	30	60
Pigs fed per trough	50	24	28	26	56	45	64	6
Pens on feed circuit	56	36	300	14	24	17	23	36
Pump method	Hydraulic	Hydraulic	Hydraulic	Hydraulic	Hydraulic	Hydraulic	Hydraulic	Pneumatic
Co-product inclusion	LW[‡‡], PAS[§§]					PAS	PAS	

*All units were home milling, feeding at night-time (i.e. feeding between 18:00 and 06:00) and feeding equal percentages of the daily feed allowance at each feed time.
†Unit H: Research unit where feeds/day, volume in mixing tank, agitation time and time for feed-out varied depending on experimental criteria. This unit was investigated on seven occasions; whereas all other units were investigated on one occasion.
‡BigDutchman.
§Although this unit had a long trough feeding system, a large volume of feed was mixed and fed continuously over a period of time.
¶Years.

**On a fresh matter basis.
††Units D and F both had a satellite tank. Feed goes to the satellite tank after the mixing tank prior to being fed out. On unit D feed resided there for a very short period prior to feed out so the 2 min agitation is in the mixing tank. On unit F, agitation time was 3 min in the mixing tank and 3 min in the satellite tank.
‡‡Liquid whey.
§§Pot-ale syrup.

Table S 3.2: Concentration of individual biogenic amines (µg/mL) in standard mixes used for HPLC quantification of biogenic amines in liquid feed.

Mixture No.	Histamine	Putrescine	Cadaverine	Tyramine	Tryptamine
1	2.5	1.25	1.25	2.5	2.5
2	5	2.5	2.5	5	5
3	10	5	5	10	10
4	25	12.5	12.5	25	25
5	50	25	25	50	50

Table S 3.3: Mean relative abundance (RA, %) of bacterial and fungal phyla > 1 % RA in liquid feed samples from the mixing tank and in fresh and residual liquid feed samples from troughs across all eight commercial pig farms. Mixing tank (Mix): *n* = 14, Fresh trough (Fresh): *n* = 38, Residual trough (Residual): *n* = 35.

Sampling location[1]	Kingdom	Phylum	Mean RA
Residual	Bacteria	*Firmicutes*	89.88
Fresh	Bacteria	*Firmicutes*	70.50
Mix	Bacteria	*Firmicutes*	43.61
Mix	Bacteria	*Proteobacteria*	38.11
Fresh	Bacteria	*Proteobacteria*	18.46
Mix	Bacteria	*Bacteroidota*	10.72
Fresh	Bacteria	*Bacteroidota*	7.58
Mix	Bacteria	*Actinobacteriota*	6.54
Residual	Bacteria	*Proteobacteria*	4.60
Residual	Bacteria	*Bacteroidota*	4.59
Fresh	Bacteria	*Actinobacteriota*	2.66
Mix	Fungi	*Ascomycota*	75.75
Fresh	Fungi	*Ascomycota*	79.56
Residual	Fungi	*Ascomycota*	87.52
Mix	Fungi	*Basidiomycota*	24.19
Fresh	Fungi	*Basidiomycota*	20.38
Residual	Fungi	*Basidiomycota*	12.41

Table S 3.4: Mean relative abundance (RA, %) of bacterial and fungal genera > 1 % RA in liquid feed samples from the mixing tank and in fresh and residual liquid feed samples from troughs across all eight commercial pig farms. Mixing tank (Mix): *n* = 14, Fresh trough (Fresh): *n* = 38, Residual trough (Residual): *n* = 35.

Sampling location	Kingdom	Genus	Mean RA
Residual	Bacteria	*Lactobacillus*	48.55
Fresh	Bacteria	*Lactobacillus*	44.63
Mix	Bacteria	*Lactobacillus*	26.29
Residual	Bacteria	*Weissella*	16.91
Mix	Bacteria	*Pantoea*	10.90
Residual	Bacteria	*Leuconostoc*	10.40
Fresh	Bacteria	*Weissella*	9.44
Mix	Bacteria	*Pseudomonas*	9.10
Fresh	Bacteria	*Leuconostoc*	8.54
Mix	Bacteria	*Leuconostoc*	6.34
Mix	Bacteria	*Sphingomonas*	6.32
Mix	Bacteria	*Chryseobacterium*	4.91
Fresh	Bacteria	*Acetobacter*	4.13
Residual	Bacteria	*Clostridium sensu stricto 1*	3.78
Fresh	Bacteria	*Pantoea*	3.70

Mix	Bacteria	*Prevotella*	3.42
Residual	Bacteria	*Lactococcus*	2.99
Fresh	Bacteria	*Pediococcus*	2.94
Mix	Bacteria	*Weissella*	2.89
Mix	Bacteria	*Paenibacillus*	2.87
Fresh	Bacteria	*Prevotella*	2.70
Fresh	Bacteria	*Pseudomonas*	2.53
Mix	Bacteria	*Staphylococcus*	2.45
Mix	Bacteria	*Stenotrophomonas*	2.40
Fresh	Bacteria	*Chryseobacterium*	2.40
Mix	Bacteria	*Lactococcus*	2.33
Residual	Bacteria	*Prevotella*	2.27
Mix	Bacteria	*Curtobacterium*	2.09
Fresh	Bacteria	*Sphingomonas*	1.96
Fresh	Bacteria	*Clostridium sensu stricto 1*	1.82
Residual	Bacteria	*Acetobacter*	1.62
Residual	Bacteria	*Pediococcus*	1.57
Fresh	Bacteria	*Lactococcus*	1.23
Mix	Bacteria	*Massilia*	1.23
Fresh	Bacteria	*Stenotrophomonas*	1.18
Residual	Bacteria	*Terrisporobacter*	1.12
Mix	Bacteria	*Clostridium sensu stricto 1*	1.08
Fresh	Bacteria	*Uruburuella*	1.03
Residual	Bacteria	*Aerococcus*	1.00
Residual	Fungi	*Kazachstania*	15.94
Mix	Fungi	*Alternaria*	15.83
Fresh	Fungi	*Alternaria*	14.99
Mix	Fungi	*Neoascochyta*	14.27
Mix	Fungi	*Microdochium*	13.90
Residual	Fungi	*Dipodascus*	13.67
Fresh	Fungi	*Microdochium*	13.13
Residual	Fungi	*Alternaria*	12.75
Fresh	Fungi	*Neoascochyta*	12.53
Residual	Fungi	*Neoascochyta*	11.99
Mix	Fungi	*Cladosporium*	11.84
Residual	Fungi	*Microdochium*	9.99
Fresh	Fungi	*Cladosporium*	9.89
Fresh	Fungi	*Kazachstania*	8.60
Mix	Fungi	*Vishniacozyma*	8.09
Mix	Fungi	*Sporobolomyces*	6.70
Fresh	Fungi	*Vishniacozyma*	6.22
Residual	Fungi	*Cladosporium*	6.09
Fresh	Fungi	*Dipodascus*	4.81
Fresh	Fungi	*Sporobolomyces*	4.63

Mix	Fungi	*Gibberella*	4.25
Residual	Fungi	*Vishniacozyma*	3.43
Fresh	Fungi	*Gibberella*	3.32
Mix	Fungi	*Dioszegia*	3.27
Mix	Fungi	*Papiliotrema*	3.01
Residual	Fungi	*Diutina*	2.66
Fresh	Fungi	*Papiliotrema*	2.37
Mix	Fungi	*Dipodascus*	2.30
Residual	Fungi	*Gibberella*	2.22
Fresh	Fungi	*Dioszegia*	2.12
Mix	Fungi	*Aspergillus*	2.11
Residual	Fungi	*Candida*	2.06
Fresh	Fungi	*Aspergillus*	1.92
Residual	Fungi	*Sporobolomyces*	1.84
Residual	Fungi	*Saccharomyces*	1.65
Mix	Fungi	*Saccharomyces*	1.65
Mix	Fungi	*Kazachstania*	1.52
Residual	Fungi	*Apiotrichum*	1.36
Fresh	Fungi	*Saccharomyces*	1.35
Residual	Fungi	*Aspergillus*	1.31
Mix	Fungi	*Fusarium*	1.29
Residual	Fungi	*Papiliotrema*	1.25
Mix	Fungi	*Wallemia*	1.24
Fresh	Fungi	*unidentified*	1.22
Residual	Fungi	*unidentified*	1.18
Residual	Fungi	*Cutaneotrichosporon*	1.13
Fresh	Fungi	*Cutaneotrichosporon*	1.11
Fresh	Fungi	*Candida*	1.10
Fresh	Fungi	*Fusarium*	1.09
Residual	Fungi	*Pichia*	1.03

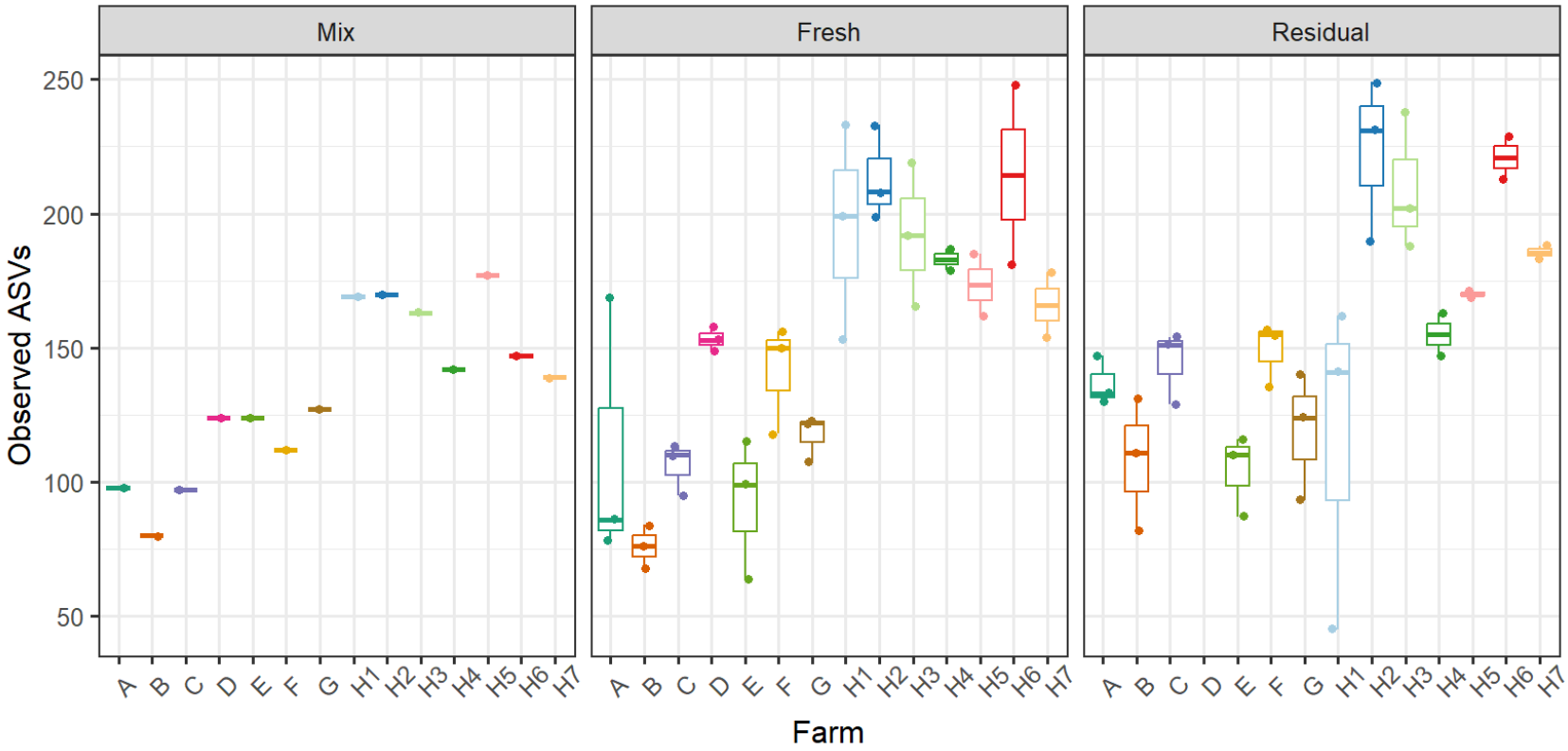

Figure S 3.1: Boxplots displaying observed amplicon sequence variants (ASVs) for bacterial taxa in liquid feed collected from each farm (A-H) at each respective sampling location; Mixing tank (Mix; *n* = 1), liquid feed sampled immediately after delivery to the troughs (Fresh; *n* = 3), liquid feed sampled prior to the next feed (Residual; *n* = 3). No Residual samples were collected on Farm D. H1-H7 indicate the seven occasions on which Farm H was sampled; on sampling occasions H4-H7 only two Fresh and two Residual samples were collected.

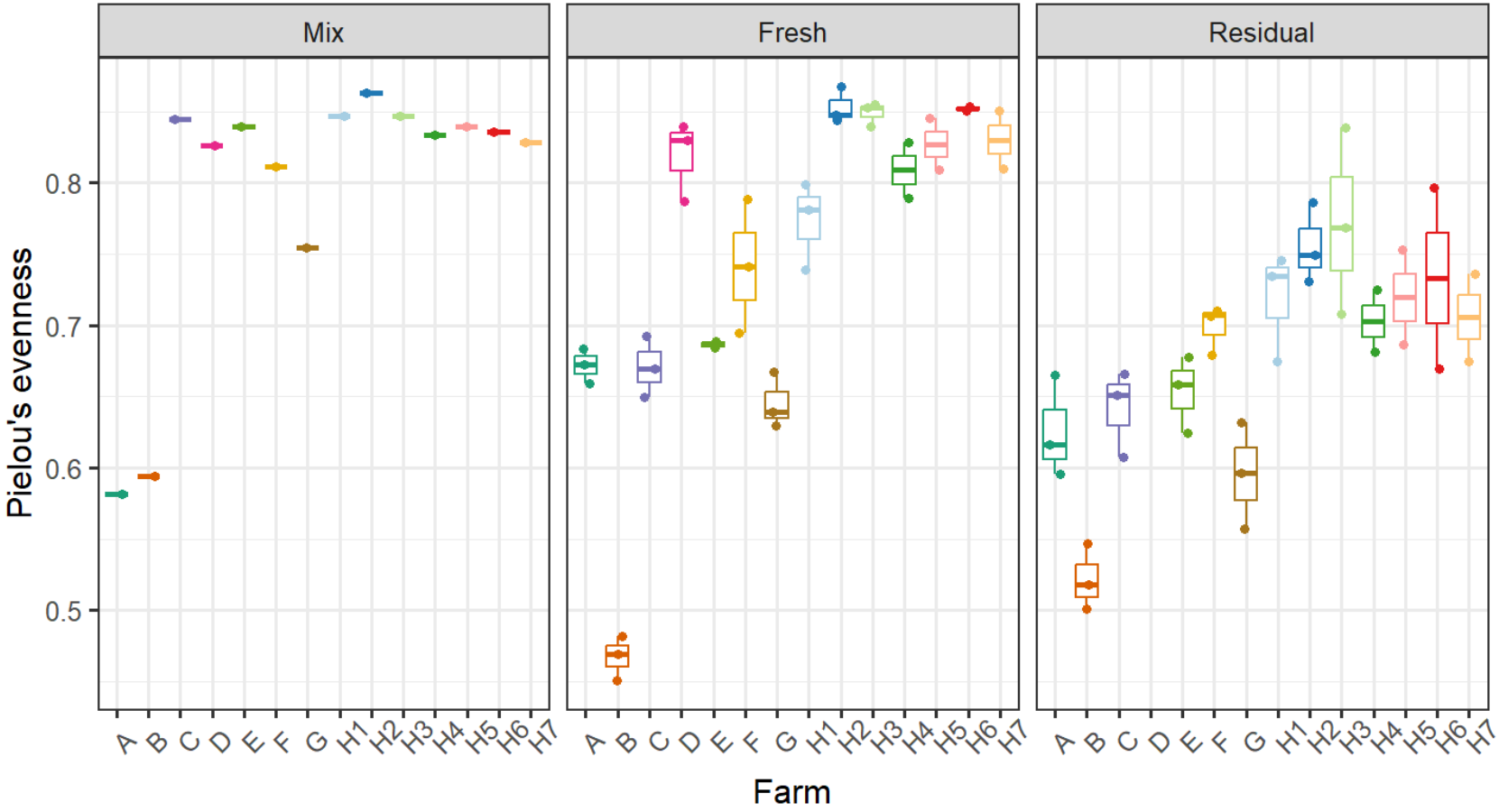

Figure S 3.2: Boxplots displaying Pielou's evenness of bacterial taxa in liquid feed collected from each farm (A-H) at each respective sampling location; Mixing tank (Mix; *n* = 1), liquid feed sampled immediately after delivery to the troughs (Fresh; *n* = 3), liquid feed sampled prior to the next feed (Residual; *n* = 3). No Residual samples were collected on Farm D. H1-H7 indicate the seven occasions on which Farm H was sampled; on sampling occasions H4-H7 only two Fresh and two Residual samples were collected.

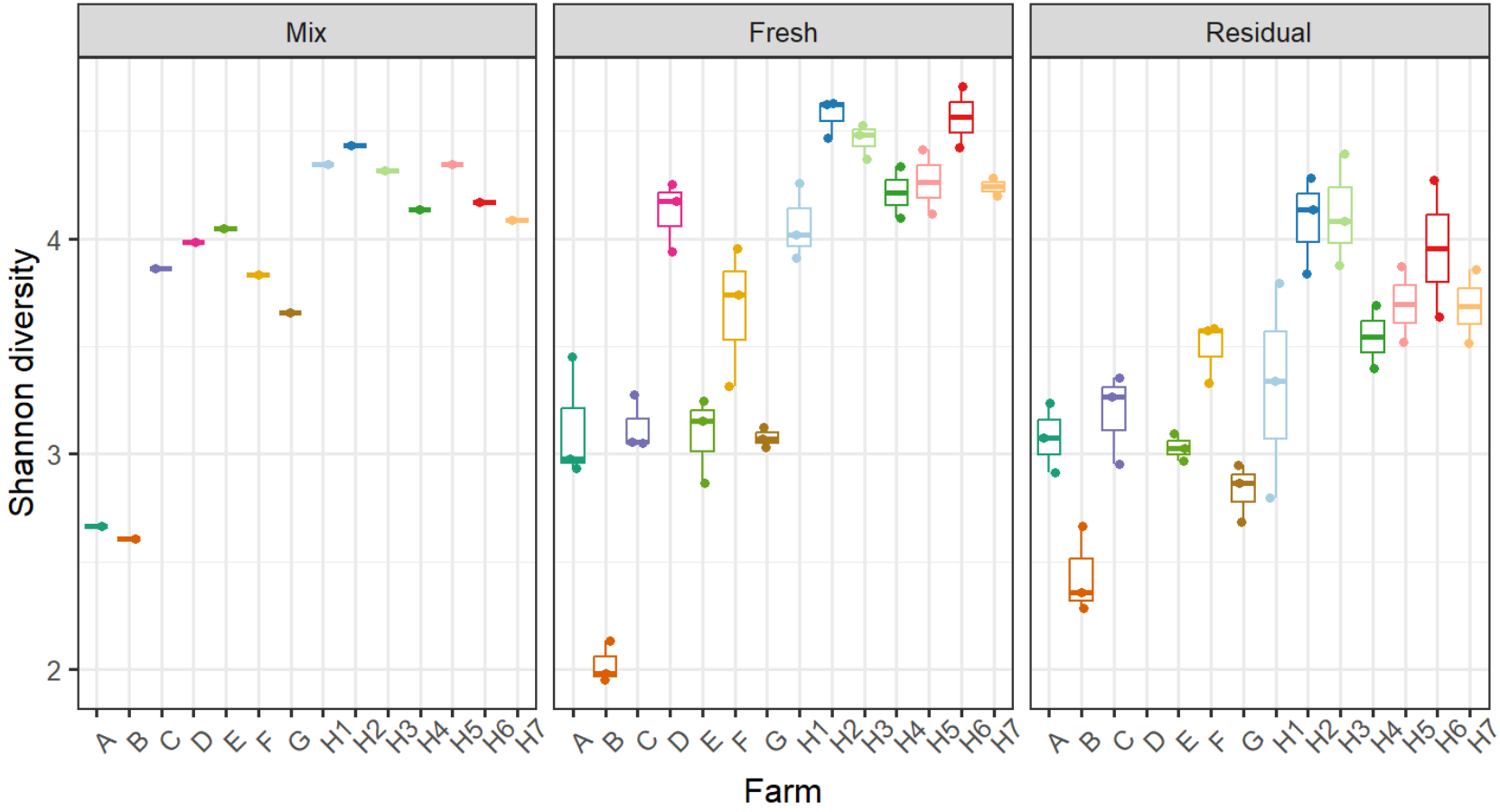

Figure S 3.3: Boxplots displaying Shannon diversity of bacterial taxa in liquid feed collected from each farm (A-H) at each respective sampling location; Mixing tank (Mix; *n* = 1), liquid feed sampled immediately after delivery to the troughs (Fresh; *n* = 3), liquid feed sampled prior to the next feed (Residual; *n* = 3). No Residual samples were collected on farm D. H1-H7 indicate the seven occasions on which Farm H was sampled; on sampling occasions H4-H7 only two Fresh and two Residual samples were collected.

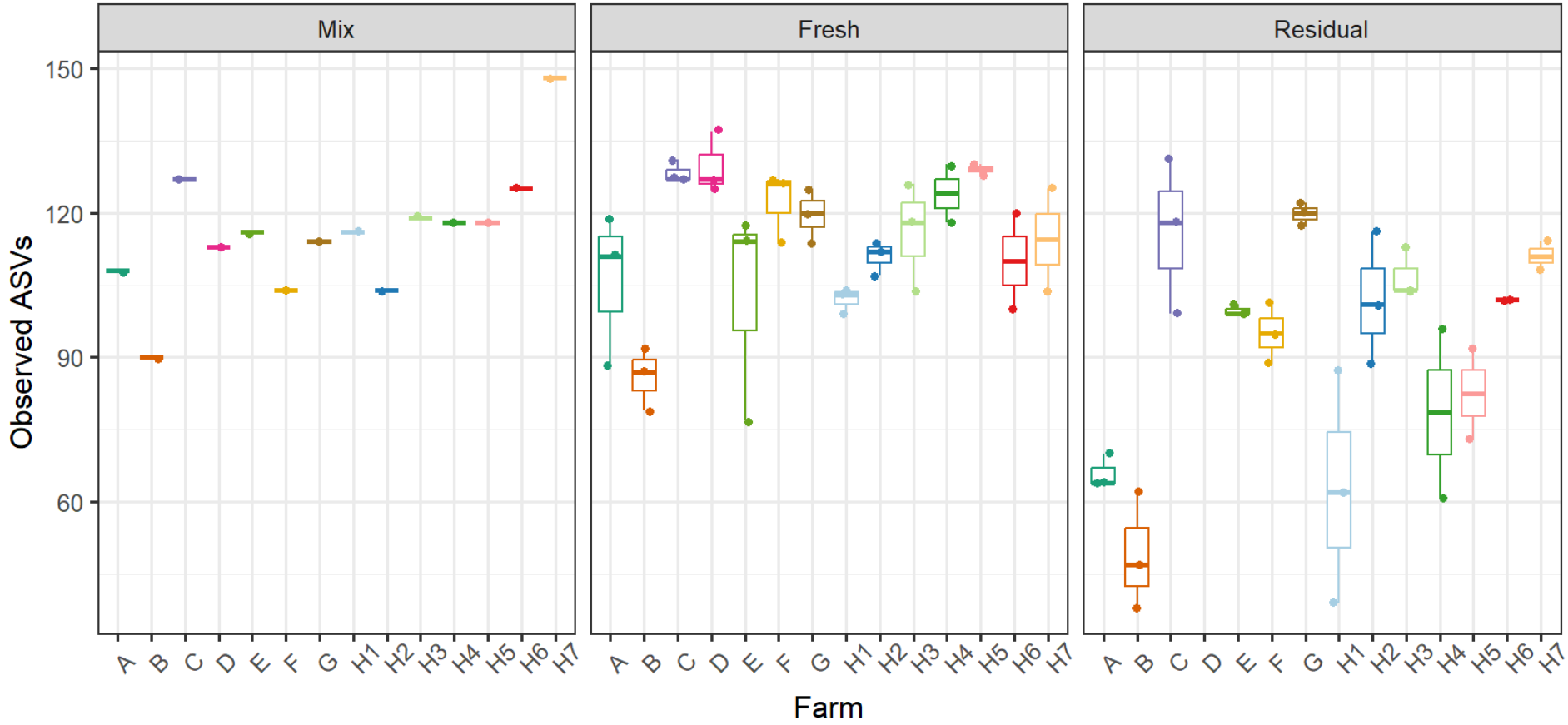

Figure S 3.4: Boxplots displaying observed amplicon sequence variants (ASVs) for fungal taxa in liquid feed collected from each farm (A-H) at each respective sampling location; Mixing tank (Mix; *n* = 1), liquid feed sampled immediately after delivery to the troughs (Fresh; *n* = 3), liquid feed sampled prior to the next feed (Residual; *n* = 3). No Residual samples were collected on Farm D. H1-H7 indicate the seven occasions on which Farm H was sampled; on sampling occasions H4-H7 only two Fresh and two Residual samples were collected.

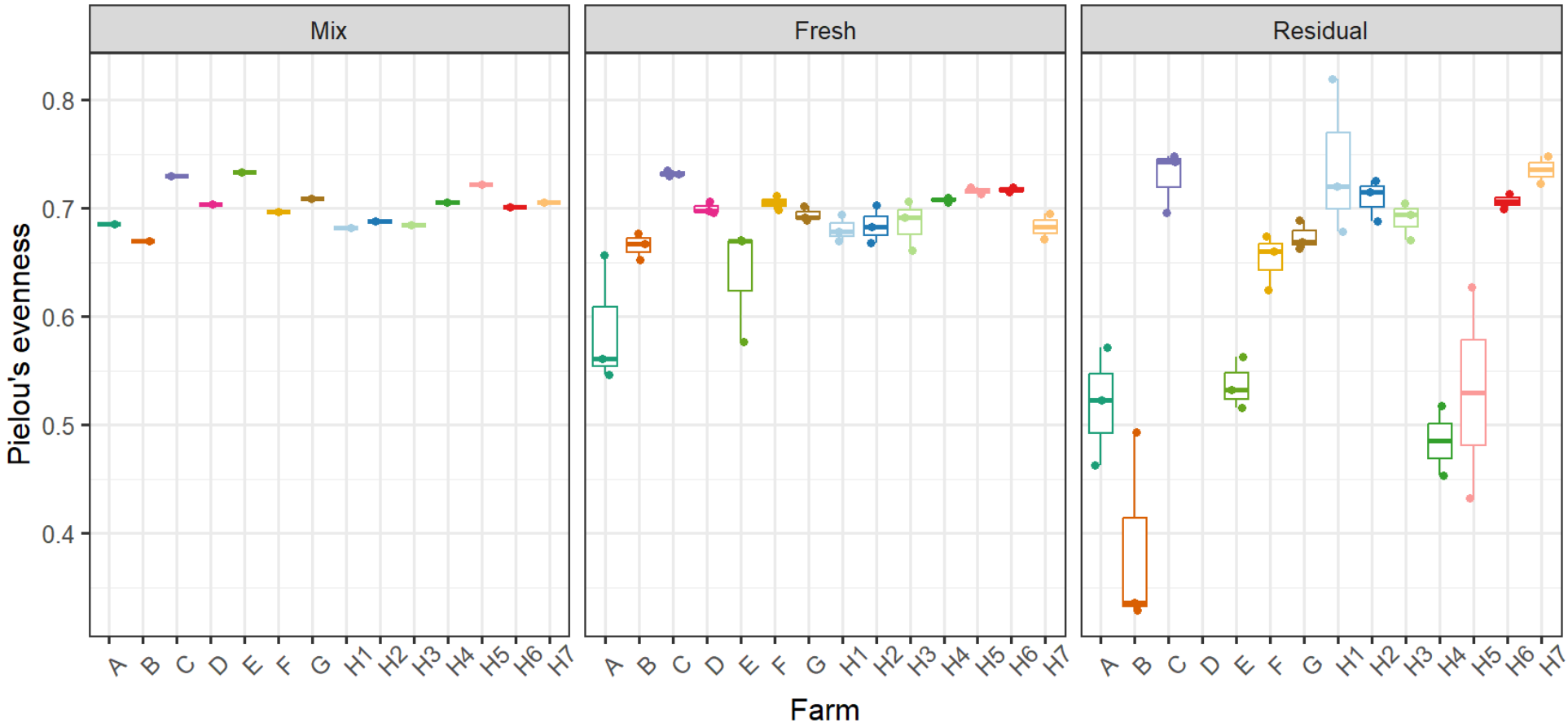

Figure S 3.5: Boxplots displaying Pielou's evenness of fungal taxa in liquid feed collected from each farm (A-H) at each respective sampling location; Mixing tank (Mix; *n* = 1), liquid feed sampled immediately after delivery to the troughs (Fresh; *n* = 3), liquid feed sampled prior to the next feed (Residual; *n* = 3). No Residual samples were collected on Farm D. H1-H7 indicate the seven occasions on which Farm H was sampled; on sampling occasions H4-H7 only two Fresh and two Residual samples were collected.

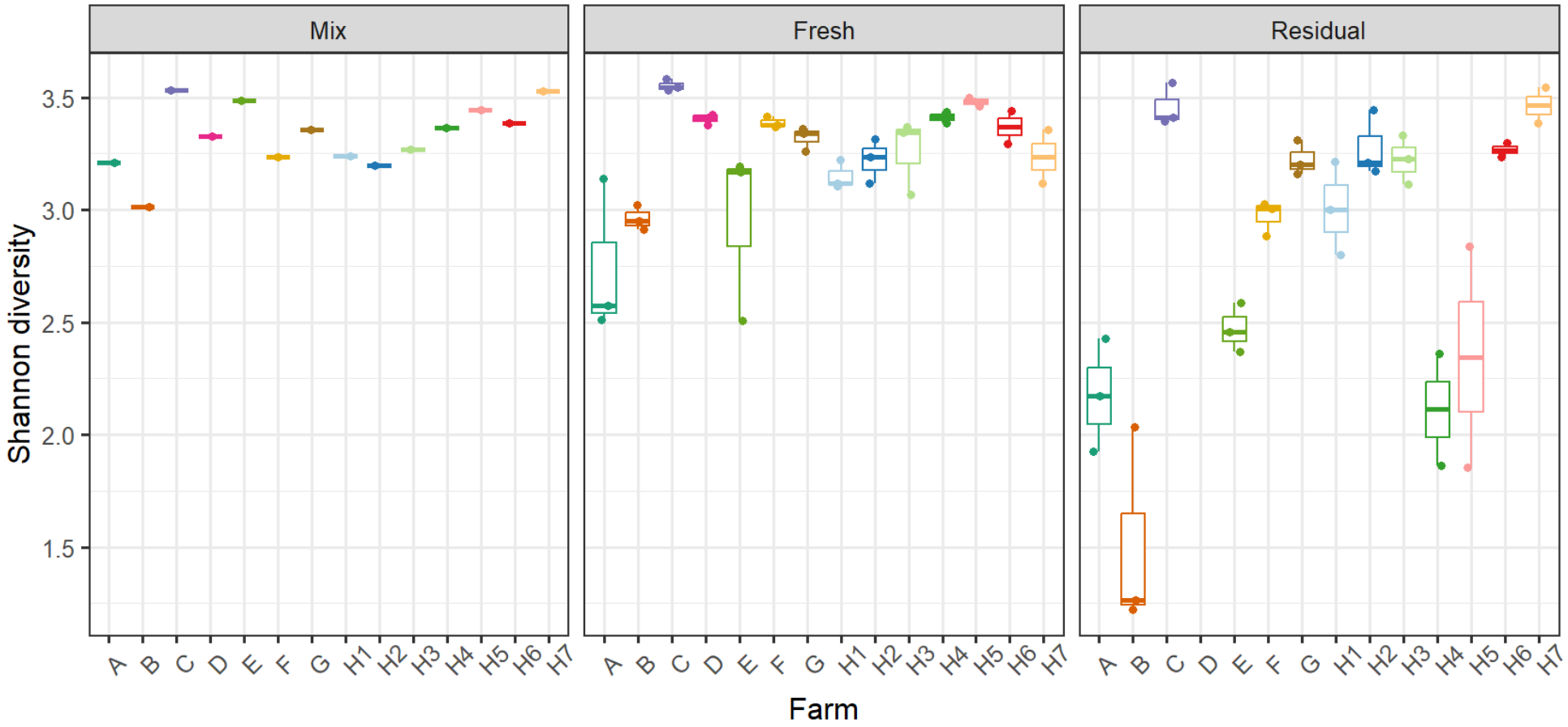

Figure S 3.6: Boxplots displaying Shannon diversity of fungal taxa in liquid feed collected from each farm (A-H) at each respective sampling location; Mixing tank (Mix; *n* = 1), liquid feed sampled immediately after delivery to the troughs (Fresh; *n* = 3), liquid feed sampled prior to the next feed (Residual; *n* = 3). No Residual samples were collected on Farm D. H1-H7 indicate the seven occasions on which Farm H was sampled; on sampling occasions H4-H7 only two Fresh and two Residual samples were collected.

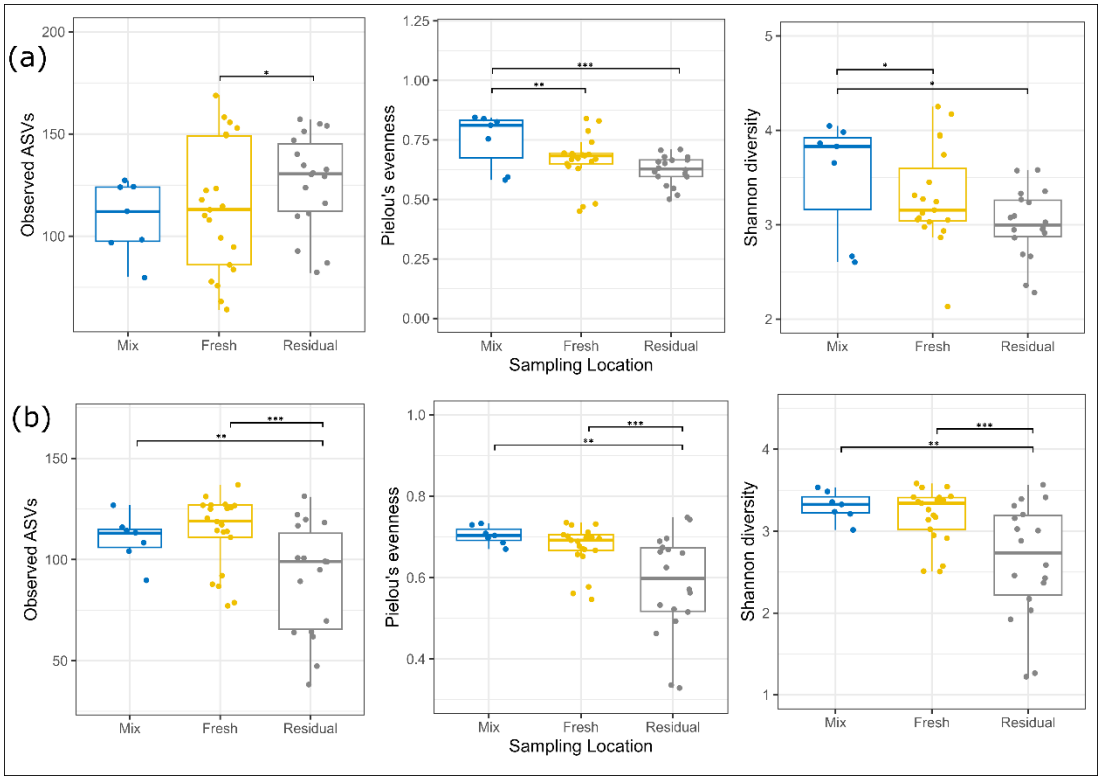

Figure S 3.7: Boxplots displaying observed amplicon sequence variants (ASVs), Pielou's evenness and Shannon diversity of bacterial (a) and fungal (b) communities in liquid feed samples from the mixing tank and in fresh and residual liquid feed samples from troughs on seven commercial pig production units; Mixing tank (Mix; n = 7), Fresh trough (Fresh; n = 21), Residual trough (Residual; n = 18). Data from Farm H has been omitted. No Residual samples were collected on Farm D. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

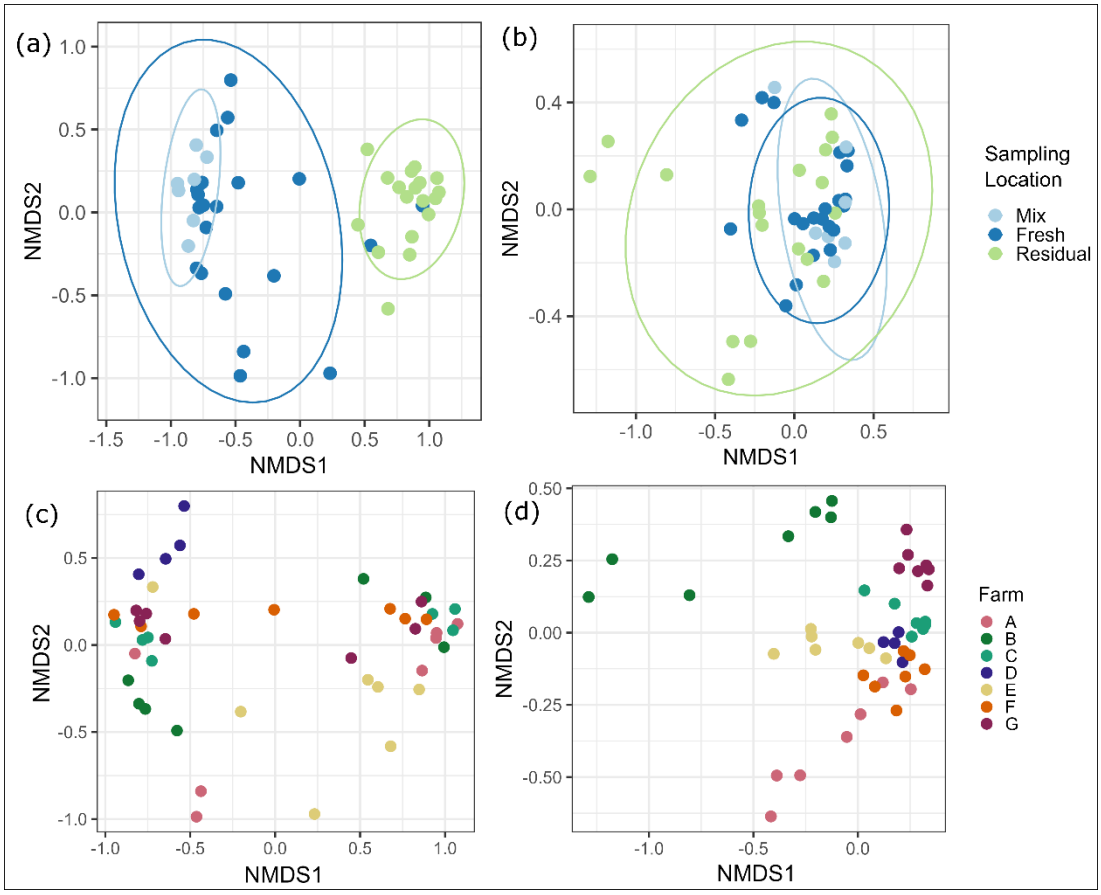

Figure S 3.8: Non-metric multidimensional scaling (NMDS) plots based on Bray-Curtis dissimilarity in liquid feed collected at each respective sampling location; Mixing tank (Mix; *n* = 1), liquid feed sampled immediately after delivery to the troughs (Fresh; *n* = 3), liquid feed sampled prior to the next feed (Residual; *n* = 3) on seven commercial pig units (data from Farm H has been omitted) for bacterial (a) and fungal (b) communities, and between farms for bacterial (c) and fungal (d) communities (excluding Farm H).

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Chapter 4: Profiling the bacteriome of a diet fed in meal or pelleted form, delivered as dry, wet/dry or liquid feed and its impact on the faecal and intestinal bacteriome of grow-finisher pigs

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4.1 Abstract

The aim of this study was to profile the intestinal and faecal bacteriome of grow-finisher pigs provided with the same diet in meal or pelleted form when delivered as dry, wet/dry or liquid feed and to investigate whether the differentially abundant bacterial taxa found are correlated with the growth, feed efficiency and/or carcass quality of these pigs. The study involved 216 Danavil Duroc x (Large White x Landrace) pigs penned in same sex (entire males or females) pen groups of 6 pigs of similar weight (average starting weight of ~33.3 kg). Pen groups were blocked by sex and weight before being randomly assigned to 1 of 6 dietary treatments in a completely randomised block design: (1) Dry meal diet; (2) Dry pelleted diet; (3) Liquid meal diet; (4) Liquid pelleted diet; (5) Wet/dry meal diet; and (6) Wet/dry pelleted diet. The diets were fed on an *ad-libitum* basis for 64 days. The experiment was a 2 x 3 factorial arrangement, with two factors for feed form (meal and pellets) and three factors for feed delivery (dry, liquid and wet/dry feeding). Bacterial richness was lower in the pelleted diet, compared to meal (p = 0.02). Several LAB including *Weissella*, *Leuconostoc* and *Lactococcus* were more abundant in the residual-trough sampled feed compared to the mixing tank and fresh trough-sampled feed. The ileal bacteriome of meal-fed pigs was more diverse (p < 0.01) compared to pellet-fed pigs, with increased relative abundances of *Megasphaera*, *Mitsuokella* and *Prevotella* (p < 0.001), while *Streptococcus* (p = 0.005) and *Escherichia-Shigella* (p = 0.009) were more abundant in pellet-fed pigs. *Prevotella* (p < 0.01) and *Streptococcus* (p < 0.01) were enriched in the caecal digesta and faeces of pigs fed pelleted diets. *Lactobacillus* was enriched in the caecal digesta and faeces of pigs fed liquid meal ($p \leq 0.05$), most likely due to its predominance in the liquid meal diet consumed. Liquid meal, liquid pellet and wet/dry pellet-fed pigs had the highest average daily gain (ADG) (p < 0.001), while average daily feed intake (ADFI) was highest in liquid meal and liquid pellet-fed pigs (p < 0.001). Feed conversion efficiency (FCE) was lower (improved) in the dry pellet-fed pigs compared to liquid-fed pigs and was similar in pigs fed meal or pellets via dry or wet/dry feeding (p < 0.001). *Leuconostoc*, although lowly abundant in general, was most abundant in the ileal digesta and faeces of liquid-fed pigs, and was associated with increased ADFI and poorer FCE across treatment groups ($p \leq 0.05$). Additionally, decreased abundance of *Turicibacter* was associated with increased ADFI in the liquid meal-fed pigs ($p \leq 0.05$), which may, in part, explain the

poorer FCE of liquid-fed pigs. This study associated the poorer feed efficiency of liquid-fed pigs with increased ileal and faecal abundance of *Leuconostoc*, a LAB associated with spontaneous fermentation in liquid feed.

4.2 Introduction

Pig diets are often fed in pellet form as opposed to in meal form when dry feeding or wet/dry feeding. Pelleting the diet increases nutrient digestibility and improves feed efficiency, while liquid feed is exclusively prepared using meal feed (Vukmirović et al., 2017). A recent study by O'Meara et al. (2020c), from which the samples for this study were obtained, investigated the effect of feeding the same diet in meal or pelleted form when delivered as dry, wet/dry or liquid feed on the growth and feed efficiency of grow-finisher pigs. It found that feed efficiency was optimal when dry or wet/dry feeding a pelleted diet, while growth was maximised by liquid meal feeding. Since wet/dry pellet feeding achieved comparable growth rates to that of liquid feeding while optimising feed efficiency, this was recommended as the optimal feeding strategy for grow-finisher pigs. Nonetheless, on-farm feed delivery choice depends on the requirements of the producer. As reviewed in Chapter 1 (Part 2), dry and wet/dry feeding results in a higher margin over feed than liquid feeding, largely because of the improvement in feed efficiency achieved. However, if maximising growth in pigs to achieve target slaughter weight as quickly as possible is the priority (e.g. where facilities are highly stocked), as with many finisher units, then liquid feeding is as cost-effective as dry and wet/dry feeding, due to the increased growth rate observed with liquid feeding. Furthermore, the ability to reduce the cost of diets by incorporating liquid co-products, when available, in the diet also makes liquid feeding an attractive option to some.

The impact of the intestinal microbiome on growth and feed efficiency in pigs has been studied extensively (Gardiner et al., 2020; McCormack et al., 2017; Si et al., 2020; Tan et al., 2017). However, to our knowledge, this is the first study to profile the gut bacteriome of grow-finisher pigs provided with the same diet in meal or pelleted form when delivered as dry, wet/dry or liquid feed. There are potentially beneficial microbiological differences between feed forms and the methods by which they are delivered to the pig which could impact positively on the pig gut microbiome. Although O'Meara et al. (2020c) conducted a culture-based assessment of these diets, the aim of this study was to determine the complete bacterial profile of the dietary treatments using high-throughput amplicon sequencing. In addition, we investigated whether the dietary treatments modulate the gut and faecal bacteriome of the pigs and

whether particular bacterial taxa are correlated with improved growth, feed efficiency and/or carcass quality in these pigs.

4.3 Materials and Methods

4.3.1 Study design, animal management, diet preparation and feeding

Experimental design, animal management, feed preparation and feeding were previously described by O'Meara et al. (2020c). Briefly, the cohort of pigs investigated here was one of the batches from the aforementioned study, comprising 216 pigs [Danavil Duroc x (Large White x Landrace); average starting weight of ~33.3 kg] formed into 36 same sex (entire male or female) pen groups of 6 pigs of similar weight (n = 6 pen replicates per treatment). Pen groups were blocked by sex and weight before being randomly assigned to one of six dietary treatments in a completely randomised block design: (1) Dry meal diet; (2) Dry pelleted diet; (3) Liquid meal diet; (4) Liquid pelleted diet; (5) Wet/dry meal diet; and (6) Wet/dry pelleted diet. The diets were milled using a hammer mill through a 3 mm screen and were fed on an *ad-libitum* basis for 64 days. The experiment was a 2 x 3 factorial arrangement, with two factors for feed form (meal and pellets) and three factors for feed delivery (dry, liquid and wet/dry feeding). Average daily gain (ADG), average daily feed intake (ADFI) and feed conversion efficiency (FCE) were calculated as described by O'Meara et al. (2020c) for the entire experimental period for each treatment group. At slaughter, carcass cold weight, lean meat yield and kill-out yield were determined as described by O'Meara et al. (2020c).

4.3.2 Feed, faecal and gut digesta sampling

Feed samples from each of the six treatments were collected on day (D)27 of the experiment for bacteriome analysis. One feed sample was collected from the feed silos used to store the dry diets in advance of liquid feeding (one silo for meal, one silo for pellets), feed bags (meal and pellets; from which the dry and wet/dry feeders were filled) and from the liquid feed mixing tanks, while freshly delivered and residual liquid feed samples from two troughs per liquid feeding treatment were also sampled. Residual feed is defined as uneaten feed that remained in the troughs until just prior to the next feeding. All feed samples were transferred aseptically into 1.5 mL sterile

Eppendorf tubes and were immediately snap-frozen in liquid nitrogen and stored at -80 °C until DNA extraction.

Pen groups were given a 2-week adjustment period prior to starting the experiment where all pigs were fed the experimental diet in meal form via dry, liquid or wet/dry feeding as per their treatment groups so that they were acclimatised to the feed delivery system and the new accommodation. Baseline faecal samples were collected by O'Meara et al. (2020c) 12 days prior to commencement of the experiment, i.e. 2 days after being introduced to the new feed delivery method. Faecal samples were also collected on D28 and D63, the latter being the day before slaughter. At each time point, faecal samples were collected from two pigs selected at random from each of 4 pens per treatment group (n = 8 pigs per treatment). On D64, at an average of ~101 kg live-weight, the pigs were slaughtered by CO_2 stunning followed by exsanguination (O'Meara et al., 2020c). At slaughter, digesta samples were collected from the terminal ileum (1.5 m proximal to the ileocaecal valve) and the blind end of the caecum. The number of digesta samples obtained from both the ileum and caecum for each treatment group were as follows: Dry meal (n = 7), Dry pellets (n = 7), Liquid meal (n = 8), Liquid pellets (n = 9), Wet/dry meal (n = 8) and Wet/dry pellets (n = 6). Duplicate aliquots of ileal and caecal digesta were aseptically transferred to 1.5 mL Eppendorf tubes and were immediately snap-frozen in liquid nitrogen. Samples were transported on dry ice and were stored at -80 °C until DNA extraction.

4.3.3 DNA extraction, library preparation and amplicon sequencing

DNA extractions from feed, faecal and gut digesta samples were performed using the QIAamp® Fast DNA Stool Mini kit, following the 'Isolation of DNA from Stool for Pathogen Detection' protocol as described in Chapter 2 with a 20-minute bead-beating step. Bacterial communities were profiled via amplicon sequencing of the V3-V4 hypervariable region of the 16S rRNA gene on the Illumina MiSeq platform, according to the Illumina 16S Metagenomic Sequencing Library Preparation Guide, with some modifications, as described in Chapter 2. Each PCR reaction contained 25 ng of DNA template and the reaction volume, components and PCR conditions were the same as those described in Chapter 2. The 16S PCR products were quality checked and purified as described previously in Chapter 2. Final libraries were quantified by qPCR, diluted, denatured and sequenced using 2 x 300 cycle V2 kits in the Teagasc sequencing

facility as described by Fouhy et al. (2015) in accordance with standard Illumina sequencing protocols.

4.3.4 Bioinformatics and statistical analysis

Demultiplexed paired-end 16S rDNA sequences were imported (in Casava 1.8 demultiplexed paired-end format) into QIIME2 v.2020.8.0 (Bolyen et al., 2019). Sequence quality assessment and initial pre-processing including primer trimming, filtering, dereplication, chimera removal, and merging of paired-end reads were performed in QIIME2 as previously described in Chapter 2. Samples from separate sequencing runs were pre-processed separately in QIIME2. The resultant feature tables and representative sequences were then merged using the 'qiime feature-table merge' and 'qiime feature-table merge-seqs' commands. Taxonomic assignment was performed on the merged representative sequences using a classifier trained on 16S rRNA gene sequences from the SILVA database (Quast et al., 2013). Where possible, species level taxonomic assignment of ASVs was performed using BLASTN (version 2.15.0+) against the nucleotide collection of the U.S. National Centre for Biotechnology Information (NCBI).

QIIME artefacts (taxonomy, ASV table, metadata and phylogenetic tree) were imported into R (version 4.2.1) as a phyloseq (McMurdie & Holmes, 2013) object with the qza_to_phyloseq function in the qiime2r package (Bisanz, 2018). Contaminant ASVs, identified using the 'prevalence' method in the decontam package (Davis et al., 2018), were removed prior to further analysis. Further pre-processing included removal of ASVs that were not assigned to the kingdom *Bacteria*, and removal of ASVs that phylum-level taxonomy was not assigned to. Finally, the filter_taxa function in phyloseq was used to remove ASVs that were not observed more than 3 times in at least 1 % of the samples.

Alpha-diversity (Observed ASVs, Pielou's evenness and Shannon diversity) and beta-diversity, based on unrarefied filtered sequences, were calculated using the phyloseq package. Differences in alpha-diversity metrics for faecal and gut digesta samples were analysed using a linear mixed-effects model using the lmer function in the lme4 package (Bates et al., 2015), with pen as a random effect. Statistical significance between time points, feed forms, delivery methods and their interactions were tested

using the Anova function in the car package, followed by pairwise comparisons using Tukey's HSD test with the emmeans package (Lenth, 2020). Differences in alpha-diversity for feed samples were tested using the same method, except differences between feed forms and sampling locations were tested separately, with the other variable considered a random effect in the model. Alpha-diversity was plotted using the ggpubr package (Kassambara, 2020). Beta-diversity was measured using non-metric multidimensional scaling (NMDS) of Bray-Curtis dissimilarity distances, and was plotted using the ggplot2 package (Wickham, 2016). Permutational multivariate analysis of variance (PERMANOVA) with 999 permutations was performed to test for differences between samples using the adonis2 function in the vegan package (Oksanen et al., 2020).

Bacterial relative abundance (RA) data was visualised via heatmaps using the Ampviz2 (Andersen et al., 2018) package. Differentially abundant bacterial genera across feed forms, delivery methods and time points were identified using the linear discriminant analysis (LDA) effect size (LEfSe) method in the microbiomeMarker package, based on normalised RA data (Cao et al., 2022; Segata et al., 2011). A non-parametric Kruskal-Wallis rank-sum test (cut-off: $p < 0.01$) was used for the identification of significantly different genera across groups. Linear discriminant analysis scores were used to estimate the effect sizes for differentially abundant genera, with an LDA score ($\log_{10}$) of 4.0 used as the cut-off value to avoid spurious results. DESeq2 (Love et al., 2014) was implemented in the microeco package (Chi Liu et al., 2021) to perform multiple pairwise comparisons between treatment groups to test for differentially abundant bacterial genera. The phyloseq object was converted to a microtable object using the file2meco package (Chi Liu et al., 2022). For each sample type and faecal sampling time point, the microtable objects were subset to retain only ASVs that had a mean RA of at least 0.01 % in at least 10 % of samples. The trans_diff class was used to perform differential abundance testing between treatment groups at the genus level using DESeq2 with default settings.

Pearson correlations between differentially abundant bacterial genera were performed against growth rate, feed intake, feed efficiency and carcass quality parameters in R using the microeco. The trans_diff class was used to perform differential abundance testing across treatment groups at the genus level using LefSe with default settings. A

trans_env object containing the growth, feed intake, feed efficiency and carcass quality data was generated and correlated with the genus level differential abundance data using the cal_cor function, using false discovery rate multiple testing correction. The trans_distance class was also utilised to generate a Bray-Curtis distance matrix and correlations between Bray-Curtis distances and growth, feed intake, feed efficiency and carcass quality data were tested using the Mantel test. It should be noted that since lower FCE values indicate an improvement in feed efficiency, negative correlations between the RA of a genus and FCE values indicate an improvement in feed efficiency.

Growth parameters (ADG, ADFI and FCE) from all pigs in the experiment and carcass parameters (carcass cold weight, lean meat yield, kill-out yield, carcass ADG and carcass FCE) from pigs sampled at slaughter were analysed in the Statistical Analysis Systems (SAS) software package version 9.4 (SAS Institute Inc., Cary, North Carolina, United States) using the linear mixed models procedure (PROC MIXED). Treatment, sex and their interaction were included in the model as fixed effects, while block was included as a random effect and average initial pig weight was included as a covariate in the model. Pen was the experimental unit in the case of growth parameters, while pig was the experimental unit in the case of carcass parameters.

4.4 Results

4.4.1 Bacterial composition and diversity of dietary treatments according to feed form and sampling location

Regarding the alpha-diversity of the feed, the number of Observed ASVs was lower in the pelleted diet compared to that of the meal diet, irrespective of sampling location [Figure S4.1 (a); $p = 0.02$], while Shannon diversity and Pielou's evenness were unaffected, i.e. the increased richness was driven mainly by lowly abundant ASVs. When alpha-diversity was compared between sampling location, irrespective of feed form, the alpha-diversity of the dry feed used for the liquid treatments (silo) and for the dry and wet/dry treatments (bagged) were similar [Figure S4.1 (b)]. However, the number of observed ASVs ($p = 0.001$) and Shannon diversity ($p = 0.004$) increased in the liquid feed collected from the mixing tank compared to the dry feed from the silo. The number of observed ASVs further increased between the mixing tank feed and

the residual liquid feed sampled from the trough (p = 0.02). The only differences in Pielou's evenness were in that of the residual trough-sampled feed, which was lower compared to the mixing tank (p = 0.05) and fresh trough-sampled feed (p = 0.02). Finally, Bray-Curtis beta-diversity analysis indicated a distinct feed bacteriome at each sampling location (Figure S4.2).

Although the number of feed samples collected was insufficient to facilitate differential abundance analysis, the RAs of bacterial genera in the dry meal and pelleted diets were similar (Figure 4.1). The dry diets were collected from bags used for the dry and wet/dry treatments and from the silo used for the liquid treatments. *Pantoea* (25.6-43.8 % RA), *Pseudomonas* (21.8-25.8 % RA) and *Sphingomonas* (7.3-12.1 % RA) were the most abundant genera in the dry feed, irrespective of feed form or sampling location. Perhaps the most notable difference was the high RA of *Pediococcus* in the pelleted feed collected from the silo (10.4 % RA); *Pediococcus* was not detected in the other dry feed samples, and subsequently was only detected in the pelleted liquid feed, albeit at < 1 % RA.

The composition of the liquid feed collected from the mixing tanks was similar, irrespective of feed form, with some variation in the RA of the predominant genera. For example, in the liquid meal, the RA of *Pantoea* and *Pseudomonas* was approximately twice that of the pelleted diet. In the fresh trough-sampled liquid feed, *Lactobacillus* increased in RA in both the meal (32.8 % RA) and pelleted (19.9 % RA) diets. Some notable differences between the meal and pelleted diets in the fresh trough-sampled feed included a higher RA of *Flavobacterium* (7.8 vs. 1.7 % RA) and *Uruburuella* (7.4 vs. 0.8 % RA) in the pelleted diet compared to the meal, respectively. A number of genera of lactic acid bacteria (LAB) including *Weissella*, *Leuconostoc* and *Lactococcus* increased in RA in the residual-trough sampled feed compared to the mixing tank and fresh trough-sampled feed. The greatest RA of these genera was observed in the liquid diet made up from pelleted feed, while *Lactobacillus* remained more abundant in the residual feed of the liquid meal (27.6 vs. 16.2 % RA).

4.4.2 Impact of feed form and delivery method on diversity of the faecal and gut bacteriome

The number of observed ASVs in the ileal digesta was higher in liquid-fed pigs compared to those fed wet/dry feed ($p < 0.05$) and tended to be higher than in those fed dry feed ($p = 0.08$) [Figure 4.2 (a)], while there were no differences in Shannon diversity or Pielou's evenness. Regarding feed form, both Shannon diversity and Pielou's evenness were higher in the ileal digesta of pigs fed meal compared to pellets, irrespective of feed delivery method [Figure 4.2 (b); $p < 0.01$], while the number of observed ASVs was not impacted. There were no alpha-diversity differences between feed form or delivery methods in the caecal digesta of the pigs ($p > 0.05$; Figure S4.3).

During the 2-week adaptation period, 10 days prior to the beginning of the experiment, all pigs were fed their respective dry, liquid or wet/dry diets in meal form. As such, there were no effects of feed form (whether the diet was fed as meal or pellets) on the faecal alpha-diversity of the pigs during this baseline period. The number of observed ASVs was similar between delivery methods ($p > 0.05$). Shannon diversity tended ($p = 0.06$) to be lower, while Pielou's evenness was lower in the faeces of pigs fed liquid feed compared to those fed wet/dry feed at baseline [Figure 4.3 (a); $p < 0.05$]. On D28 and 63 of the experiment, feed delivery method had no effect on faecal alpha diversity. Feed form also had no significant effects on faecal alpha diversity on D28 or 63; however, the number of observed ASVs ($p = 0.06$) and Shannon diversity ($p = 0.09$) tended to be lower in the faeces of pigs fed pelleted feed compared to those fed meal on D28 [Figure 4.3 (b)]. When the faecal alpha-diversity of the pigs was examined over time, irrespective of feed form or delivery method, all diversity metrics increased between baseline, D28 and D63 of the experiment (Figure S4.4; $p < 0.001$), except that the number of observed ASVs was similar between D28 and 63.

Beta-diversity of the ileal digesta was not impacted by feed delivery method but was influenced by whether the diet had been fed in meal or pelleted form [Figure 4.4 (a); $R^2 = 0.11$, $p = 0.001$]. Feed form had the same effect in the caecal digesta [Figure 4.4 (b); $R^2 = 0.11$, $p = 0.001$); however, delivery method was also found to influence the beta-diversity of the caecal digesta ($R^2 = 0.07$, $p = 0.010$). Prior to commencement of the experiment, at baseline, the beta-diversity estimates of the faecal bacteriome were

influenced by the feed delivery method, which explained 7 % of the variation in the bacterial community structure [Figure S4.5 (b); $R^2 = 0.07$, $p = 0.006$).

Both feed form and delivery method had a significant effect on the beta-diversity of the faecal bacteriome of pigs fed the experimental diets on D28 and 63 [Figure 4.4 (c) and (d)]. On D28, feed form had the greatest impact on beta-diversity, explaining 18 % of the variation in the bacterial community structure ($R^2 = 0.18$, $p = 0.001$), while feed delivery method explained 7 % of variation ($R^2 = 0.07$, $p = 0.013$). By D63 of the experiment, both feed form and delivery method impacted the bacterial community structure to a similar extent, both explaining ~9 % of variation ($R^2 = 0.09$, $p = 0.001$ and $R^2 = 0.09$, $p = 0.002$, respectively). When all faecal samples were analysed together, sampling time point was found to explain 22 % of variance in the bacterial community structure [Figure S4.5 (a); $R^2 = 0.22$, $p = 0.001$], while the feed form and delivery method accounted for 3 % ($R^2 = 0.03$, $p = 0.001$) and 2 % ($R^2 = 0.02$, $p = 0.006$), respectively.

4.4.3 Differential abundance of bacterial genera in the intestinal and faecal bacteriome of pigs fed dry, liquid or wet/dry feed in meal or pelleted form

The RA of the 15 most abundant bacterial genera in the intestinal and faecal bacteriome of pigs fed the experimental diets are presented by treatment group in Figures 4.5 and 4.6, respectively. The data for mean RA of bacterial genera in the ileal, caecal and faecal samples at each time point by feed form, delivery method and treatment are available in Tables S4.1-S4.3, in addition to between treatment group differential abundance analysis results (Table S4.4). *Megasphaera*, *Mitsuokella* and *Prevotella* were all differentially abundant in the ileal digesta of meal-fed pigs compared to those fed pellets [Figure 4.7 (a); $p < 0.001$]. *Megasphaera*, *Mitsuokella* and *Prevotella* were present at 6.1, 6.1 and 3.6 % RA in the ileal digesta of meal-fed pigs, while they only accounted for 0.1, 0.07 and 0.5 % RA in those fed pelleted diets (Table S4.2). *Megasphaera* and *Mitsuokella* were differentially abundant in the ileal digesta of dry meal, liquid meal and wet/dry meal-fed pigs compared to those fed the pelleted treatments (Table S4.4; $p \leq 0.05$), confirming that the increased abundance of these genera was driven by feed form. *Prevotella* followed the same pattern, in that pigs fed meal via dry and liquid feeding had a greater *Prevotella* abundance in their

ileal digesta compared to the pelleted treatments; however, this was not the case for wet/dry feeding (Table S4.4; $p \leq 0.05$). Across feed forms, *Streptococcus* ($p = 0.005$) and *Escherichia-Shigella* ($p = 0.009$) were both differentially abundant in the ileal digesta of pellet-fed pigs [Figure 4.7 (a)]. The RA of *Streptococcus* and *Escherichia-Shigell*a was 29.5 and 8.3 % in pellet-fed pigs, respectively, compared to 15.5 and 4.1 % in the ileal digesta of meal-fed pigs (Table S4.2). By treatment, *Strepotococcus* was more abundant in the ileal digesta of dry pellet and wet/dry pellet-fed pigs, compared to those fed liquid meal (Table S4.4; $p \leq 0.05$). *Escherichia-Shigella* was more abundant in the ileal digesta of dry pellet-fed pigs compared to liquid meal, liquid pellet and wet/dry meal-fed pigs (Table S4.4; $p \leq 0.05$).

As with the ileal digesta, there were no genera in the caecal digesta differentially abundant according to delivery method. However, when feed forms were compared, both *Prevotella* ($p = 0.008$) and *Streptococcus* ($p = 0.006$) were enriched in the caecum of pigs fed pelleted diets [Figure 4.7 (b)]. When the bacteriome of the caecal digesta was compared across treatments, *Clostridium sensu stricto 1* was more abundant in dry meal-fed pigs compared to liquid meal-, liquid pellet- and dry pellet-fed pigs (Table S4.4; $p \leq 0.05$). The RA of *Clostridium sensu stricto 1* in the caecum of dry meal-fed pigs was 17.5 % compared to 9.3-14.5 % in the other treatment groups (Table S4.1). Additionally, in the caecal digesta, *Lactobacillus* was more abundant in pigs fed liquid meal compared to all other treatments (12.8 versus 1.5-3.8 %), except for liquid pellets (Table S4.4; $p \leq 0.05$).

Results for the faecal bacteriome at baseline, indicate that *Faecalibacterium* was differentially abundant between delivery methods ($p = 0.003$) [Figure S4.6 (b)], with a mean RA of 3.9 % in the faeces of liquid-fed pigs compared to 1.4 and 1.8 % RA for dry and wet/dry-fed pigs, respectively (Table S4.1). With respect to feed form, on D28, *Prevotella, Streptococcus* and *Dialister* were enriched in the faeces of pigs fed pellets (Figure 4.7 (c); $p < 0.001$), irrespective of delivery method. The RA of *Prevotella* was 19.0 % in the faeces of pellet-fed pigs, compared to 12.0 % in meal-fed pigs (Table S4.2). However, when assessed by delivery method across both feed forms, *Prevotella* was not differentially abundant, with 15.2, 15.6 and 15.8 % RA found in the faeces of dry, liquid and wet/dry-fed pigs, respectively (Table S4.3).

In addition to being differentially abundant in the faeces of pellet-fed pigs compared to meal-fed pigs (8.5 % vs. 3.9 % RA), *Streptococcus* was also differentially abundant between feed delivery methods across feed forms, with the highest RA observed in wet/dry (8.4 %) compared to dry (6.6 %) and liquid-fed pigs (3.5 %) [Figure S4.6 and Table S4.3 (b); $p = 0.003$]. Conversely, *Lactobacillus* was differentially abundant in meal-fed pigs compared to those fed pellets, with a RA of 10.3 % compared to 4.5 % [Figure 4.7 (c); $p < 0.001$]. However, *Lactobacillus* was also differentially abundant between delivery methods across feed forms, with the greatest RA observed in liquid-fed pigs (10.7 %), compared to those fed dry (6.9 %) and wet/dry feed (4.6 %) [Figure S4.6 (b) and Table S4.3; $p = 0.003$]. Between treatments, *Lactobacillus* was more abundant in liquid meal-fed pigs compared to pigs fed dry, liquid and wet/dry pellets, as well as being enriched in dry meal-fed pigs compared to those fed wet/dry pellets (Table S4.4; $p \leq 0.05$). *Prevotellaceae NK3B31 group* ($p < 0.001$) and *Rikenellaceae RC9 gut group* ($p = 0.002$) were also differentially abundant between feed forms with both having greater RA in meal-fed pigs (7.2 and 4.0 % RA, respectively), compared to those fed pellets (4.2 and 2.7 % RA, respectively) [Figure 4.7 (c) and Table S4.2].

On D63, *Clostridium sensu stricto 1* was enriched in the faeces of pigs fed meal [Figure 4.7 (d); $p = 0.003$], while no genera were differentially abundant according to delivery method on D63. By treatment, *Clostridium sensu stricto 1* was more abundant in wet/dry meal-fed pigs compared to dry-, liquid-, wet/dry pellet-, and liquid meal-fed pigs (Table S4.4; $p \leq 0.05$). As on D28, *Prevotella* was enriched in pellet-fed pigs, compared to those fed meal ($p < 0.001$) and *Lactobacillus* was more abundant in the liquid meal-fed pigs compared to all other treatment groups on D63 (Table S4.4; $p \leq 0.05$). Lastly, differentially abundant bacterial genera in the faeces were also assessed across the different time points of the experiment. Several genera that had the greatest RA at baseline including *Lactobacillus, Streptococcus* and *Megasphaera* were differentially abundant in the faeces across time points [Figure S4.6 (a); ($p < 0.001$)]. *Treponema, Christensenellenaceae R-7 group* and *Rikenellaceae RC9 gut group*, which had higher RAs on D63 were also differentially abundant across time points [Figure S4.6 (a); $p < 0.001$].

4.4.4 Growth, feed intake, feed efficiency and carcass quality data

Liquid-fed pigs had the highest ADG over the entire experimental period, with values of 1166 and 1160 g/day achieved for the liquid meal- and liquid pellet-fed pigs, respectively ($p < 0.001$); however, wet/dry pellet-fed pigs also had similar ADG (1115 g/day) (Table 4.1). The ADFI of liquid meal- and liquid pellet-fed pigs (2713 and 2848 g/day, respectively) was higher than the other treatment groups; however, FCE was poorest in the liquid pellet-fed pigs (2.48; $p < 0.001$). Feed conversion efficiency was superior in the dry pellet-fed pigs (2.07) compared to the liquid meal (2.33) and liquid pellet-fed pigs (2.48; $p < 0.001$). Although the FCE of the dry pellet-fed pigs was optimal, it was similar to that of the pigs fed dry meal and to those fed meal or pellets via wet/dry feeding.

Carcass ADG was higher in the liquid-fed pigs, with growth rates of 938 and 958 g/day obtained for liquid meal- and liquid pellet-fed pigs, respectively (Table 4.2; $p < 0.001$). Carcass FCE was optimal in the dry pellet-fed pigs (2.67); however, only the carcass FCE of liquid-pellet-fed pigs was poorer (3.07; $p = 0.02$). There were no differences in lean meat yields between treatments, although it was numerically highest (58.3 %) in dry and wet/dry meal-fed pigs. The cold carcass weight of liquid meal- and liquid pellet-fed pigs was higher than that of dry and wet/dry meal-fed pigs and was similar to that of dry and wet/dry pellet-fed pigs ($p < 0.001$). The kill-out yield was similar between all treatment groups, except for dry meal-fed pigs (75.9 %; $p = 0.01$), with the highest numerical kill-out yields in the liquid meal and pellet-fed pigs (78.8 and 78.8 %, respectively).

4.4.5 Correlation of growth rate, feed intake and feed efficiency with beta diversity and differentially abundant bacterial genera in the faecal and gut bacteriome

Faecal beta-diversity, as measured by Bray-Curtis distances, of pigs fed the liquid meal treatment, was positively correlated with ADG ($r = 0.46$, $p = 0.006$), ADFI ($r = 0.51$, $p = 0.006$) and FCE ($r = 0.28$, $p = 0.037$) on D28 of the experiment (Table 4.3). These correlations indicate that increased dissimilarity of the faecal microbial composition between liquid meal-fed pigs correlates with increased dissimilarity of growth rate, feed intake and feed efficiency between these pigs. Positive associations were also found between beta-diversity and ADG ($r = 0.67$, $p = 0.003$) and ADFI ($r =$

0.58, p = 0.012) on D63 of the experiment in liquid meal-fed pigs. The only other correlation on D28 was a positive correlation between beta-diversity and FCE in wet/dry pellet-fed pigs (r = 0.59, p = 0.012). On D63, ADG and ADFI were positively correlated with faecal beta-diversity of pigs fed dry pellets (ADG: r = 0.65, p = 0.003; ADFI: r = 0.66, p = 0.006) and wet/dry meal (ADG: r = 0.57, p = 0.003; ADFI: r = 0.54, p = 0.006). Finally, on D63, there was a positive correlation between ADG and faecal beta-diversity of liquid pellet-fed pigs (r = 0.43, p = 0.030).

Next, correlation analyses were performed in order to investigate whether particular bacterial genera, that were enriched in the faeces or gut digesta between experimental treatment groups, were associated with ADG, ADFI and FCE. It should be noted that since a lower FCE value indicates an improvement in feed efficiency, negative correlations between the RA of a genus and FCE values indicate an improvement in feed efficiency. Conversely, a positive correlation between the RA of a genus and FCE indicates a dis-improvement in feed efficiency. In the faeces on D28, *Pseudoramibacter* was the only differentially abundant genus that was positively correlated with ADG [Figure 4.8 (a); r = 0.41, p = 0.05]. *Romboutsia* (r = 0.53, p = 0.004), *Leuconostoc* (r = 0.46, p = 0.01) and *Weissella* (r = 0.47, p = 0.01) in the faeces on D28 were all positively associated with ADFI. The same three genera, *Romboutsia* (r = 0.73, p < 0.001), *Leuconostoc* (r = 0.51, p = 0.005) and *Weissella* (r = 0.48, p = 0.01) also had positive correlations with FCE, in addition to *Turicibacter* (r = 0.56, p = 0.002), meaning that they were associated with higher (i.e. poorer) feed efficiency. Conversely, *Catenibacterium* was negatively correlated with FCE in the faeces on D28, i.e. it was associated with improved feed efficiency. The only significant correlation that was found between individual treatment groups on D28, was that *Syntropococcus* in the faeces was negatively associated with ADFI of the dry pellet-fed pigs [Figure 4.8 (d), r = -0.97, p = 0.03].

On D63, *Butyrivibrio* was associated with poorer feed efficiency, i.e. it had the greatest positive correlation with FCE [Figure 4.8 (b), r = 0.55, p = 0.006] and was the only genus positively correlated with ADFI (r = 0.46, p = 0.03). *Leuconostoc* (r = 0.47, p = 0.03) was again associated with poorer feed efficiency in the faeces on D63, in addition to *Pygmaiobacter* (r = 0.48, p = 0.03). In the ileal digesta, *Leuconostoc* was positively correlated with ADFI (r = 0.49, p = 0.01) and associated with poorer feed

efficiency (r = 0.50, p = 0.01), while *Paracoccus* in the ileal digesta was also associated with poorer feed efficiency (r = 0.54, p = 0.008). No significant correlations with ADG, ADFI or FCE were found in either the ileal or caecal digesta between individual treatment groups.

There were a number of bacterial genera in the faeces that were correlated with production metrics on D63. Perhaps most notably, several genera were negatively correlated with ADG and ADFI in the dry pellet-fed pigs [Figure 4.8 (e)]. These genera included *Acidaminococcus* (ADG: r = -0.99, p = 0.001; ADFI: r = -0.97, p = 0.008), *Dialister* (ADG: r = -0.99, p = 0.001; ADFI: r = -0.94, p = 0.02) and *Megasphaera* (ADG: r = -0.99, p = 0.001; ADFI: r = -0.92, p = 0.05). *Oribacterium* was also negatively correlated with ADG (r = -0.95, p = 0.02) and ADFI (r = -0.96, p = 0.01) of dry pellet-fed pigs on D63, with *Holdemanella* also negatively associated with ADFI (r = -0.95, p = 0.02). *Turicibacter,* which was associated with poorer feed efficiency (positively correlated with FCE) on D28, was positively correlated with ADFI in the liquid meal-fed pigs on D63, along with *Mycoplasma* (r = 0.97, p = 0.007) and *Bacteroidales RF16 group* (r = 0.94, p = 0.03). *Solobacterium* (r = 0.94, p = 0.02) and *Clostridium methylpentosum group* (r = 0.93, p = 0.03) were both positively associated with ADG in the faeces of pigs fed wet/dry meal on D63, while *Solobacterium* was negatively associated with ADG in the faeces of dry pellet-fed pigs (r = -0.96, p = 0.02). *Catenibacterium,* which was associated with improved feed efficiency (negatively correlated with FCE) in the faeces on D28, was negatively correlated with ADG in dry pellet-fed pigs on D63 (r = 0.93, p = 0.03), while *Fusicatenibacter* was associated with improved feed efficiency in dry meal-fed pigs (r = 0.94, p = 0.03). Finally, *Prevotella* was found to be negatively correlated with ADG in the faeces of liquid meal-fed pigs on D63 (r = 0.93, p = 0.03).

4.5 Discussion

This study investigated the feed bacteriome of the same diet in meal or pelleted form delivered as dry, wet/dry or liquid feed and the impact of feeding these diets on the intestinal and faecal bacteriome of grow-finisher pigs, using high-throughput amplicon sequencing. The objective was to determine the composition and diversity of the feed itself, profile the intestinal and faecal bacteriome of pigs fed these diets and to investigate whether certain bacterial taxa were associated with improved

growth, feed efficiency or carcass quality parameters. Generally, the composition of the dry meal and pellets was similar, although *Pediococcus* was present at a higher RA in the one pelleted diet sample collected from the silo. Alpha-diversity analysis showed that the number of observed bacterial ASVs was lower in the pelleted, compared to the meal feed. Similarly, the study from which these samples were sourced (O'Meara et al., 2020c), performed culture-based analysis across a total of 6 time points and found that LAB, *Enterobacteriaceae*, yeast, and mould counts were lower in the pelleted compared to the meal diet (O'Meara et al., 2020c). Although not a measure of decreased abundance of specific taxa, the lower number of observed ASVs found in the pelleted diet in the current study indicates lower species richness. Both the findings here, and those of the previous related study, are in line with several other studies (Burns et al., 2015; Canibe et al., 2005; Mikkelsen et al., 2004), with the lower microbial load and number of different species in pelleted diets likely resulting from the high temperature and pressure used in the pelleting process.

Overall, there was a greater impact of the dietary treatments on alpha-diversity in the ileum, compared to the caecum and faeces. The ileal digesta was more diverse in pigs fed meal compared to pellets, in line with the higher species richness found in the meal diet, while liquid-fed pigs had a more diverse ileal bacteriome compared to those fed dry or wet/dry feed. The caecal alpha-diversity was not impacted by treatment; however, the faecal alpha-diversity tended to be higher in pigs fed meal, with no differences between dry, liquid or wet/dry-fed pigs. At baseline, when the pigs were all fed meal via dry, liquid or wet/dry feeding (albeit for only 2 days), the community evenness was lower in the faeces of liquid-fed pigs, meaning that fewer bacterial taxa dominated the community, relative to the dry and wet/dry-fed pigs. This may have been influenced by the decrease in evenness that was observed in the residual trough-sampled liquid feed, where several LAB including *Lactobacillus*, *Weissella* and *Leuconostoc* became predominant. Although, not considered statistically differentially abundant at baseline, *Lactobacillus* was present at a higher RA in the faeces of liquid-fed pigs (10.7 % RA) compared to those fed dry (7.2 % RA) or wet/dry feed (6.1 % RA).

The growth of pigs from the cohort used for sampling in the current study (n = 6 pens of pigs per treatment) was in line with that reported by O'Meara et al. (2020c), where

growth data from two batches of grow-finisher pigs fed the same experimental diets (n = 12 pens of pigs per treatment) was reported. The pigs fed the liquid treatments had the highest ADG and ADFI. Feed efficiency was optimal in the dry pellet-fed pigs, but it was similar in the pigs fed dry meal and wet/dry meal or pellets. Feeding liquid pellets maximised carcass ADG, carcass weight and kill-out yield; however, carcass FCE of liquid pellet-fed pigs was the poorest compared to all other treatments. Liquid feeding meal and wet/dry feeding pellets produced comparable carcass ADG, FCE, carcass weight and kill-out yield. However, liquid feeding meal optimises carcass ADG, while feeding wet/dry pellets optimises feed efficiency. Therefore, assessment of the requirements of the pig producer and the facilities available on their unit will likely dictate using one over the other.

The faecal beta-diversity of several treatment groups were associated with pig growth performance metrics. For example, the faecal beta-diversity of the pigs fed the liquid meal diet was positively correlated with ADG, ADFI and FCE. Optimal FCE was observed in the dry pellet-fed pigs, with a positive correlation between faecal beta-diversity and ADG and ADFI, while beta-diversity was positively correlated with FCE in the wet/dry pellet-fed group. The positive correlation of faecal beta-diversity with measures of growth, feed intake and feed efficiency here indicate that the dissimilarity of the microbial community composition between samples was related to the dissimilarity between the respective performance metrics of the pigs in that group, i.e. indicating a relationship between the two. However, more specific associations between performance metrics and individual bacterial genera that were differentially abundant will be discussed later.

Overall, the differential abundance of genera between treatment groups was influenced more by feed form than by delivery method. Pellet-fed pigs had a greater abundance of *Streptococcus* and *Escherichia-Shigella* in their ileal digesta compared to those fed meal, while *Streptococcus* and *Prevotella* were more abundant in the caecal digesta of pigs fed pellets. The enrichment of *Streptococcus* and *Escherichia-Shigella* in the ileal digesta of pellet-fed pigs is interesting as both genera contain species that are potentially pathogenic to pigs. This finding is in line with previous studies where feeding meal to pigs has been shown to facilitate lactic acid production in the stomach and small intestine due to increased viscosity, and therefore a slower

digesta passage rate, aiding in acidification of the gastrointestinal contents and thus promoting pathogen exclusion (Canibe et al., 2005; Mikkelsen et al., 2004; Vukmirović et al., 2017). Although there are limitations to using short-read amplicon data for species level classification (Martínez-Porchas et al., 2016), the most abundant *Streptococcus* ASV in the ileal and caecal digesta was tentatively identified as *Streptococcus gallolyticus*. Quan et al. (2019) identified *S. gallolyticus* subsp. *gallolyticus* as a potential candidate for improving feed efficiency in pigs. However, Sitthicharoenchai et al. (2022) recently reported *S. gallolyticus* as an emerging pathogen in pigs, responsible for ~8.6 % of bacterial valvular endocarditis cases. The most abundant *Escherichia-Shigella* ASV detected in the ileal and caecal digesta was classified as *Escherichia coli*; however, it is difficult to deduce whether the higher abundance of *E. coli* in pellet-fed pigs is of concern without serotype-specific information, as *E. coli* that inhabit the gastrointestinal tract can be commensal or potentially pathogenic (Abraham et al., 2012).

Megasphaera and *Mitsuokella,* two lactate-utilising bacteria, were enriched in the ileal digesta of meal-fed pigs. Their enrichment may have been facilitated by the aforementioned increase in lactate production in the small intestine when meal is fed to pigs. *Megasphaera* has previously been positively correlated with carcass weight and butyrate concentration in the caecum of grow-finisher pigs (Torres-Pitarch et al., 2020b). *Mitsuokella* has previously been found at a higher RA in the ileum of more feed efficient pigs, while a lower faecal RA was associated with improved feed efficiency (McCormack et al., 2019). *Lactobacillus* was also consistently more abundant in the caecal digesta and faeces of meal-fed pigs, particularly so in pigs fed meal via liquid feeding. This is in line with the high RA of *Lactobacillus* observed in the liquid feed collected from the troughs i.e. 32.8 % RA in the fresh liquid meal, compared to < 1 % in the dry meal or pellets. *Lactobacillus, Megasphaera* and *Mitsuokella* have previously been shown to be enriched in the stomach of pigs fed a coarse non-pelleted diet (Mikkelsen et al., 2007). *Lactobacillus* is often used as a probiotic (Dowarah et al., 2017), and as reviewed by Gardiner et al. (2020) is consistently more abundant in the caecal and faecal bacteriome of highly feed efficient pigs. It should be noted, however, that several LAB including some *Lactobacillus* species are associated with increased bile salt hydrolase activity in the intestine (Geng & Lin, 2016). Overgrowth of these bacteria can lead to poorer lipid metabolism and

energy harvest by pigs and as such, this could be a potential driver of the poorer feed efficiency of liquid-fed pigs (He et al. 2017). *Clostridium sensu stricto 1* was another genus that was enriched in the caecal digesta and faeces after feeding meal, compared to pellets. This genus was also previously reported to be more abundant in the caecum and faeces of more feed efficient pigs (He et al., 2019; McCormack et al., 2017; Quan et al., 2018). Additionally, *Clostridium methylpentosum group*, which has been associated with fibre degradation (Gensheng Liu et al., 2021), was found to be positively correlated with ADG in the faeces of wet/dry meal-fed pigs in the present study.

As in the caecal digesta, *Prevotella* and *Streptococcus* were also consistently more abundant in the faecal bacteriome of pellet-fed pigs. There have been conflicting reports on associations between *Streptococcus* and feed efficiency (McCormack et al., 2017, 2019; Quan et al., 2018; Yang et al., 2017). However, in this study, no correlation was found between *Streptococcus* and growth, feed efficiency or carcass quality, although *Streptococcus* was more abundant in the bacteriome of pellet-fed pigs, which were generally more feed-efficient. *Prevotella* in the faeces was found to be negatively correlated with ADG of pigs fed liquid meal, despite this genus being known for fermenting plant-derived polysaccharides in the pig gut, and thus enhancing energy harvest (Amat et al., 2020). Previous studies have associated a *Prevotella*-dominant enterotype with increased feed intake (Yang et al., 2018) and body weight (Mach et al., 2015). However, several studies have also associated *Prevotella* with poorer feed efficiency in pigs (Quan et al., 2020; Tan et al., 2017; Yang et al., 2017). *Dialister* was also more abundant in the faeces of pellet-fed pigs and was found to be negatively correlated with ADG and ADFI in the dry pellet-fed pigs, which had the best feed efficiency of all treatment groups. Despite the negative association between *Dialister* and ADG and ADFI, the ratio of feed intake to growth rate is what determines feed efficiency. As such, although it was not statistically significant in the current study, *Dialister* may play a role in improving feed efficiency, supported by previous studies where it was found to be more abundant in the ileum (McCormack et al., 2019) and caecum (Quan et al., 2018) of more feed efficient animals.

Despite the higher growth rate and feed intake of liquid-fed pigs in this study, poorer feed efficiency was observed in this cohort, which was likely due, at least in part, to

increased feed wastage, as previously reported (Han et al., 2006; L'Anson et al., 2012; Russell et al., 1996). The study from which the samples for the current study were obtained reported that although steps were taken to minimise feed wastage, it was still possible for pigs to remove feed from the troughs (O'Meara et al., 2020c). Additionally, losses of energy and amino acids due to spontaneous fermentation in liquid feed may also contribute to poorer feed efficiency in liquid-fed pigs (Canibe & Jensen, 2003; O'Meara et al., 2020a, 2020c, 2020b; Torres-Pitarch et al., 2020a, 2020b). Moreover, some bacterial genera that were enriched in the intestinal digesta and faeces of liquid-fed pigs were associated with poorer feed efficiency in the current study. *Leuconostoc,* a genus of LAB that was also more abundant in the faeces and ileal digesta of pigs fed liquid diets, was associated with higher ADFI and poorer feed efficiency. Although *Leuconostoc* was quite lowly abundant (< 1 % RA), its RA was highest in the ileal digesta of liquid pellet-fed pigs, and it was absent in pigs fed dry meal or pellets. The association with poorer feed efficiency may be explained by the higher feed intake associated with increased *Leuconostoc* abundance without a corresponding increase in ADG. *Leuconostoc* has previously been found to be more abundant in the small intestine and colon of pigs fed non-fermented liquid feed (the liquid diet in the current study was also non-fermented) compared to fermented liquid feed (Bunte et al., 2020). In line with the current study, to our knowledge, the only correlation between *Leuconostoc* and pig growth metrics to date was reported by Torres-Pitarch et al. (2020b), where increased ileal RA of *Leuconostoc mesenteroides* in pigs fed liquid feed was negatively correlated with carcass weight. This finding supports the association of *Leuconostoc* with the poorer feed efficiency observed in this study, as reduced weight combined with increased ADFI will result in poorer feed efficiency. Another genus that showed a similar result to *Leuconostoc* was *Turicibacter* in the faeces on D28, which was associated with poorer feed efficiency across all treatments. Decreased abundance of *Turicibacter* was associated with increased ADFI in the liquid meal-fed pigs, which again may explain the poorer feed efficiency i.e. as a result of increased feed intake. In agreement with the current study, *Turicibacter* has been reported as more abundant in more feed efficient pigs (McCormack et al., 2019) and has been positively correlated with body weight (Wang et al., 2019).

4.6 Conclusion

Several LAB including *Weissella*, *Leuconostoc* and *Lactococcus* were more abundant in the residual-trough sampled feed compared to the mixing tank and fresh trough-sampled feed. The greatest RA of these genera was observed in the liquid diet prepared with pelleted feed, while *Lactobacillus* remained more abundant in the residual feed of the liquid meal diet. In fact, the pigs fed the liquid meal diet had a greater abundance of *Lactobacillus* in their caecal digesta and faeces; however, this was not associated with growth or feed efficiency in this study. Bacterial richness was higher in the meal diet, potentially explaining why the ileal digesta of pigs fed meal was more diverse compared to pellets. Another LAB associated with spontaneous fermentation in liquid feed, *Leuconostoc*, was enriched in the ileal digesta and faeces of liquid-fed pigs. *Leuconostoc* was correlated with higher feed intake and poorer feed efficiency in liquid-fed pigs, while decreased faecal abundance of *Turicibacter* was also correlated with increased feed intake, implicating these microbes in influencing feed efficiency. However, further research is required to investigate the mechanisms by which this may occur. The current study also confirms previous findings, showing that liquid feeding meal and wet/dry feeding pellets to grow-finisher pigs produce comparable carcass gain, feed efficiency and kill-out yield. However, liquid feeding meal optimises carcass gain, while feeding wet/dry pellets optimises feed efficiency. Therefore, the feeding method used for grow-finisher pigs on a given pig unit will depend on the requirements of the producer. The poorer feed efficiency of liquid-fed pigs observed in the current study has previously been attributed to increased feed wastage. However, deterioration of the nutritional quality of liquid feed resulting from spontaneous fermentation likely also contributes to poorer feed efficiency in liquid-fed pigs. Furthermore, this study associated the poorer feed efficiency of liquid-fed pigs with increased ileal and faecal abundance of *Leuconostoc*, with liquid feed being the likely source. Therefore, strategies to reduce the occurrence of spontaneous fermentation in liquid feed to maintain the nutritional quality of the diet may help further improve the feed efficiency of liquid-fed pigs.

4.7 Tables and figures

Table 4.1: Average daily gain (ADG), average daily feed intake (ADFI) and feed conversion efficiency (FCE; ADFI/ADG) of grow-finisher pigs fed meal or pellets via dry, liquid or wet/dry feeding pigs over the entire 76-day experimental period, on a pen basis (least square means ± SEM[1]; *n* = 6 pens/treatment).

Treatment	**ADG (g/day)**	**ADFI (g/day)**	**FCE**
Dry meal	1033^{c}	2280^{b}	2.22^{bc}
Dry pellets	1072^{bc}	2232^{b}	2.07^{c}
Liquid meal	1166^{a}	2713^{a}	2.33^{ab}
Liquid pellets	1160^{a}	2848^{a}	2.48^{a}
Wet/dry meal	1036^{bc}	2340^{b}	2.26^{bc}
Wet/dry pellets	1115^{ab}	2424^{b}	2.17^{bc}
SEM	18.7	56.1	0.042
p-value	< 0.001	< 0.001	< 0.001

[1] SEM: Standard error of the mean.
^{a,b,c} Within each column, values that do not share a common superscript are significantly different ($p < 0.05$).

Table 4.2: Carcass characteristics of grow-finisher pigs fed meal or pellets via dry, liquid or wet/dry feeding pigs at slaughter on an individual pig basis[1] (least square means ± SEM[2]).

Treatment	ADG[3] (g/day)	FCE[4]	Cold weight (kg)	Kill-out yield (%)	Lean meat yield (%)
Dry meal	840[b]	2.94[ab]	76.1[b]	75.9[b]	58.3
Dry pellets	896[ab]	2.67[b]	79.8[ab]	77.9[ab]	58.0
Liquid meal	938[a]	3.00[ab]	82.4[a]	78.8[a]	56.6
Liquid pellets	958[a]	3.07[a]	83.7[a]	78.9[a]	57.2
Wet/dry meal	844[b]	2.84[ab]	76.4[b]	76.6[ab]	58.3
Wet/dry pellets	914[ab]	2.75[ab]	80.9[ab]	77.2[ab]	57.0
SEM	21.1	0.087	1.35	0.69	0.79
p-value	< 0.001	0.02	< 0.001	0.01	0.53

[1] Dry meal and dry pellets: $n = 7$; Liquid meal $n = 8$, Liquid pellets: $n = 9$; Wet/dry meal: $n = 8$; Wet/dry pellets: $n = 6$.
[2] SEM: Standard error of the mean.
[a,b] Within each column, values that do not share a common superscript are significantly different ($p < 0.05$).

Table 4.3: Mantel test correlation of faecal beta-diversity (Bray-Curtis distances) with average daily gain (ADG), average daily feed intake (ADFI) and feed conversion efficiency (FCE) of grow-finisher pigs fed dry, liquid or wet/dry feed in meal or pelleted form (n = 8 per treatment) on day (D)28 and D63.

Treatment group	**Time point**	**Variable**	**Correlation coefficient (r)**	**Adjusted p - value**
Liquid meal	D28	ADG	0.46	0.006
Liquid meal	D28	ADFI	0.51	0.006
Liquid meal	D28	FCE	0.28	0.037
Wet/dry pellets	D28	FCE	0.59	0.012
Dry pellets	D63	ADG	0.65	0.003
Dry pellets	D63	ADFI	0.66	0.006
Liquid meal	D63	ADG	0.67	0.003
Liquid meal	D63	ADFI	0.58	0.012
Liquid pellets	D63	ADG	0.43	0.030
Wet/dry meal	D63	ADG	0.57	0.003
Wet/dry meal	D63	ADFI	0.54	0.006

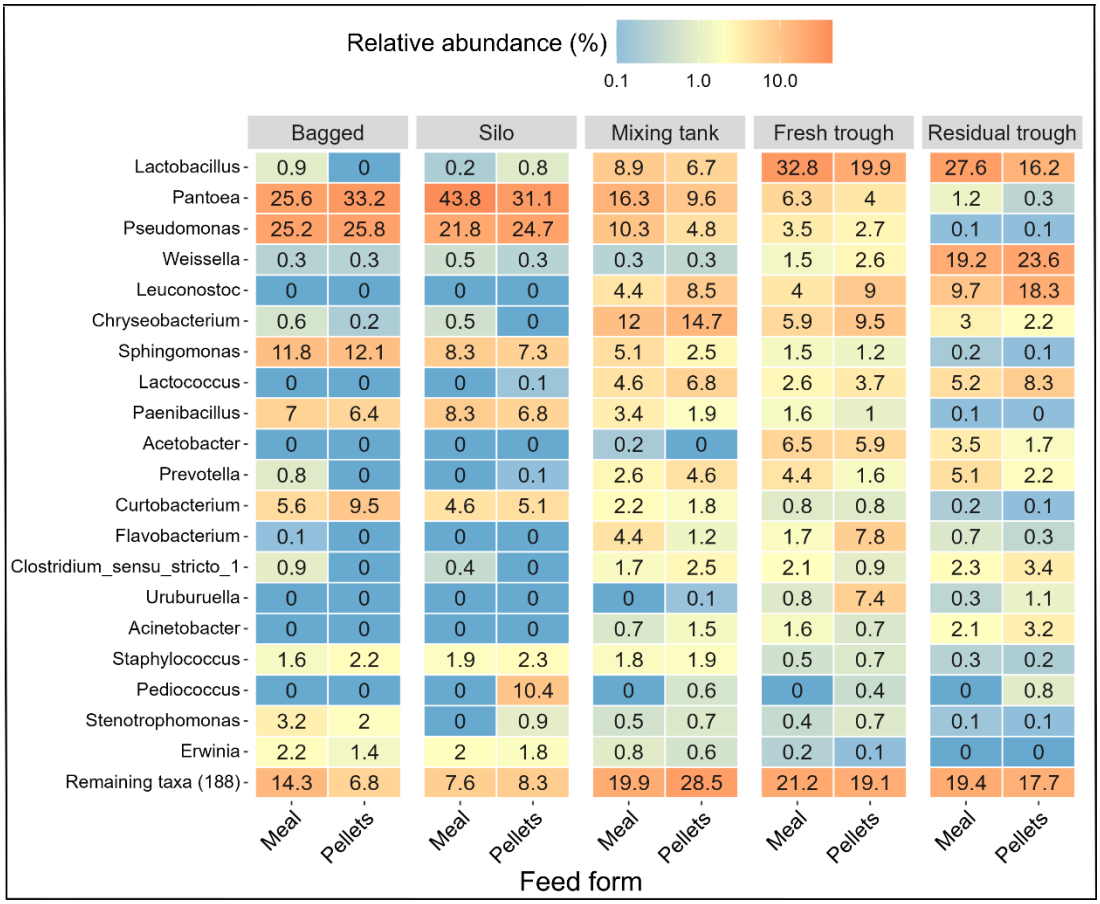

Figure 4.1: Heatmap displaying the mean relative abundance (%) of the 20 most abundant bacterial genera by feed form (meal or pellets) at each sampling location. Bagged feed (n = 2) was used for the dry and wet/dry diets. Feed collected from the silo (n = 2) was used to prepare the liquid diets. The mixing tank (n = 2), fresh (n = 4) and residual trough (n = 4) samples were collected for the liquid feeding treatments.

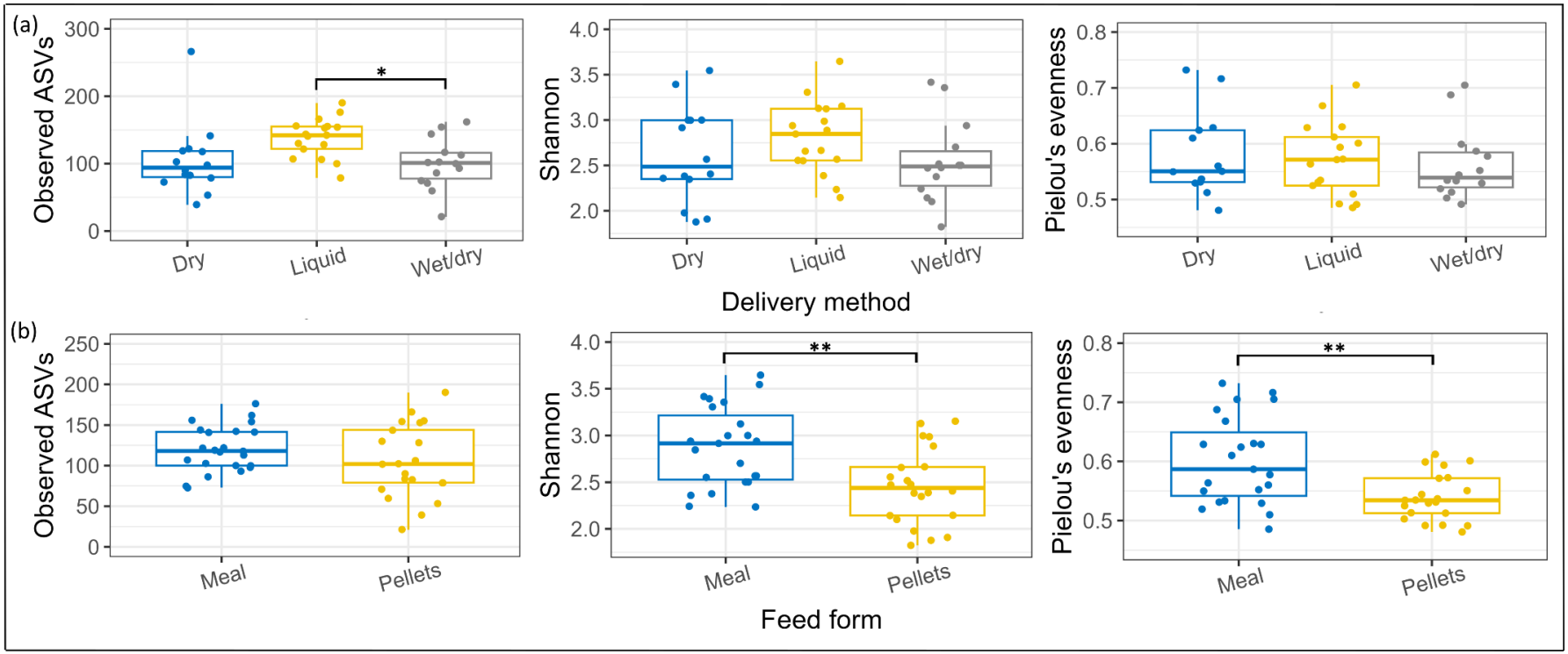

Figure 4.2: Boxplots displaying alpha-diversity [Observed amplicon sequence variants (ASVs), Shannon diversity and Pielou's evenness] of the bacteriome in the ileal digesta of grow-finisher pigs fed dry, liquid or wet/dry feed in meal or pelleted form. **(a)** Ileal alpha-diversity by delivery method (dry; n = 14, liquid; n = 17, or wet/dry; n =14), averaged across feed forms. **(b)** Ileal alpha-diversity by feed form (meal; n = 23, or pellets; n = 22), averaged across delivery methods. * $p < 0.05$, ** $p < 0.01$.

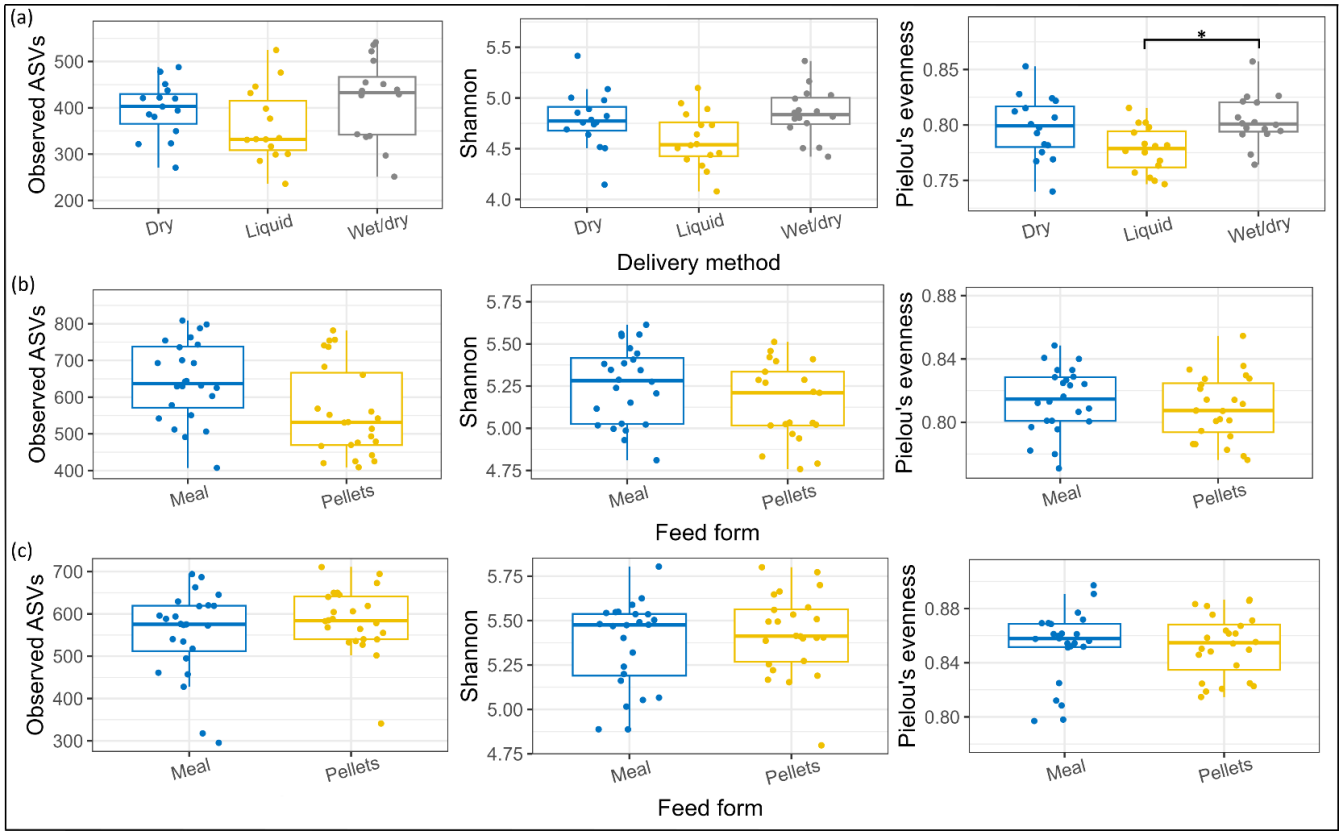

Figure 4.3: Boxplots displaying alpha-diversity [Observed amplicon sequence variants (ASVs), Shannon diversity and Pielou's evenness] of the faecal bacteriome of grow-finisher pigs fed dry, liquid or wet/dry feed in meal or pelleted form. **(a)** Alpha-diversity by delivery method (dry; n = 16, liquid; n = 16, or wet/dry; n = 16) at baseline. All diets were fed in meal form during this period. **(b)** Alpha-diversity by feed form (meal; n = 24, or pellets; n = 24, averaged across delivery methods) on D28. **(c)** Alpha-diversity by feed form (meal; n = 24, or pellets; n = 24, averaged across delivery methods) on D63. * $p < 0.05$.

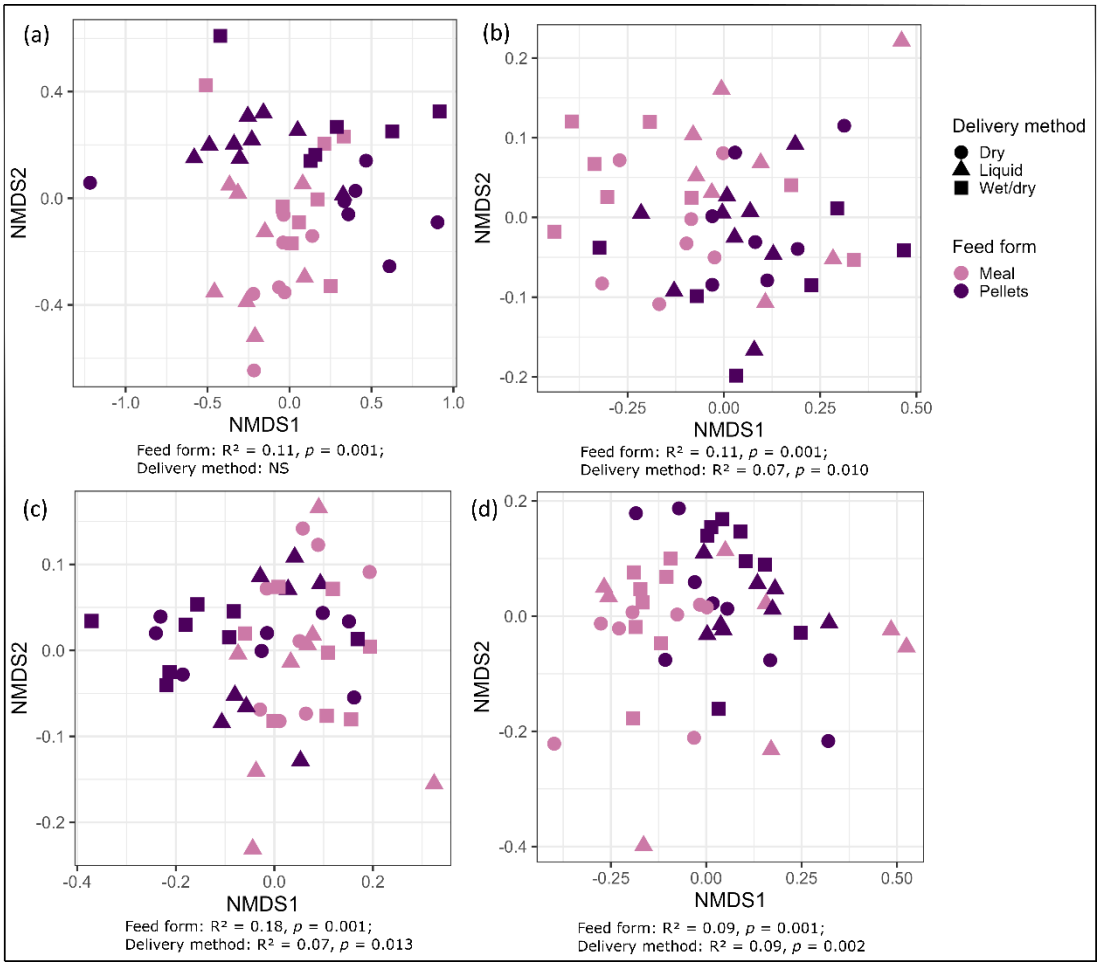

Figure 4.4: Non-metric multidimensional scaling (NMDS) plots based on Bray-Curtis dissimilarity in the **(a)** ileal (*n* = 45) and **(b)** caecal (*n* = 45) digesta, and in the faeces (*n* = 48) of grow-finisher pigs on **(c)** day (D)28 and **(d)** D63. Samples are labelled by feed form (meal or pellets) and delivery method (dry, liquid or wet/dry feeding) and permutational analysis of variance (PERMANOVA) results for the intestinal digesta and faecal samples are presented below each respective plot.

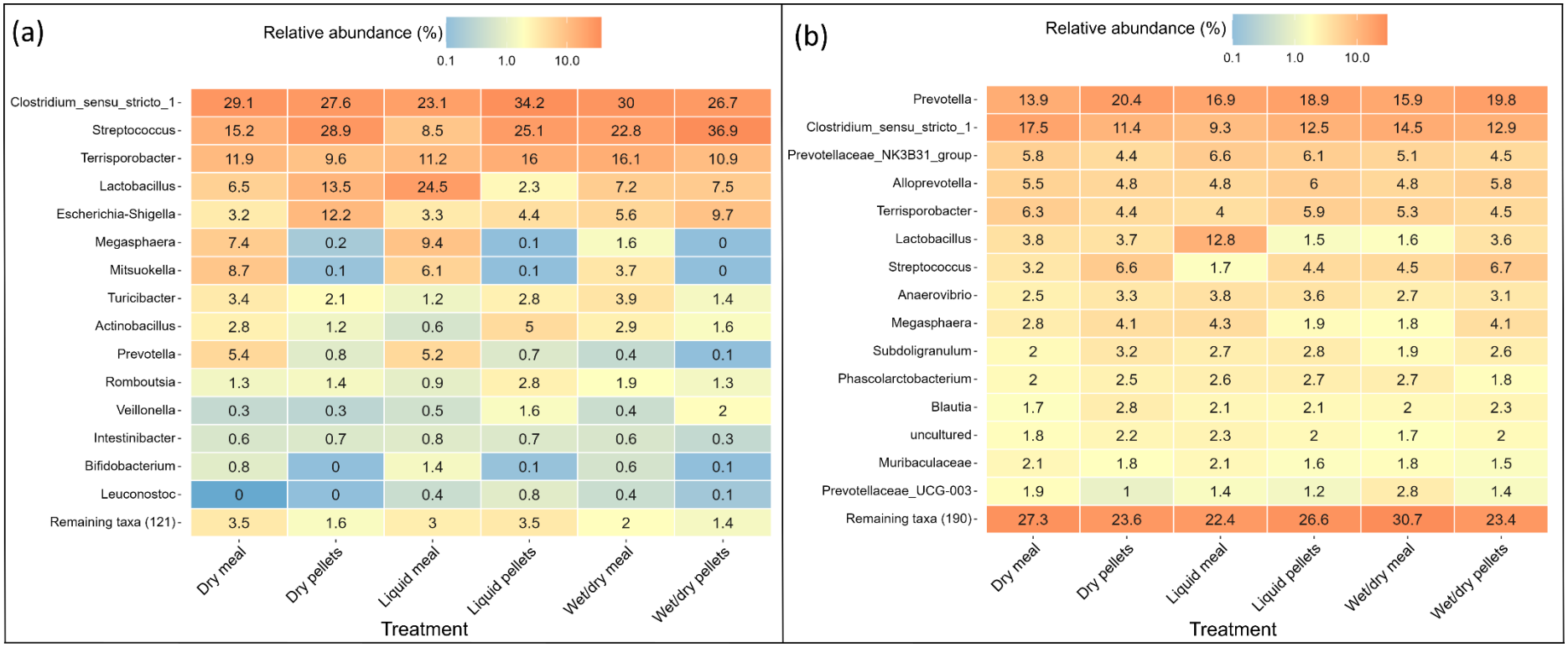

Figure 4.5: Heatmap displaying the mean relative abundance (%) of the 15 most abundant bacterial genera by treatment group in the (a) ileal and (b) caecal digesta of grow-finisher pigs fed dry, liquid or wet/dry feed in meal or pelleted form. For each treatment group in the ileal and caecal digesta: Dry meal (n = 7), Dry pellets (n = 7), Liquid meal (n = 8), Liquid pellets (n = 9), Wet/dry meal (n = 8), Wet/dry pellets (n = 6).

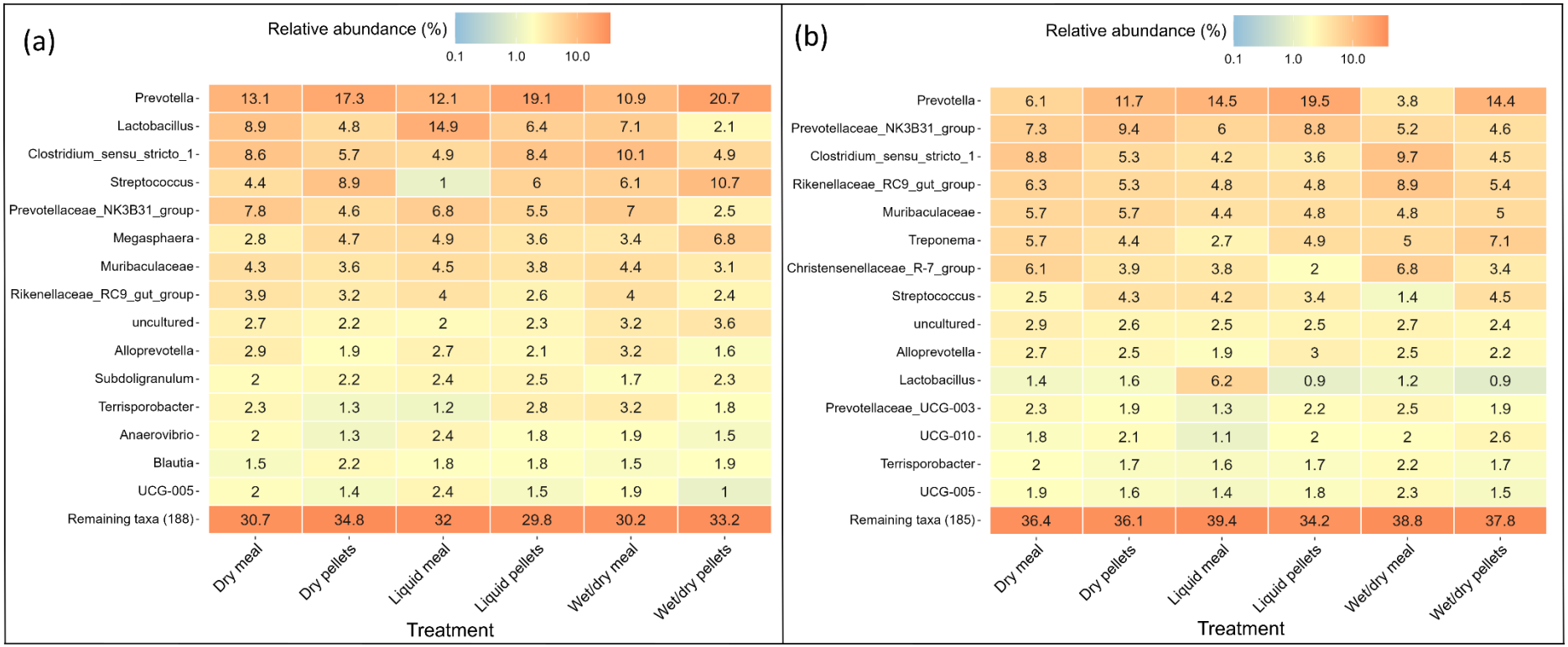

Figure 4.6: Heatmap displaying the mean relative abundance (%) of the 15 most abundant bacterial genera by treatment group in the faeces of grow-finisher pigs fed dry, liquid or wet/dry feed in meal or pelleted form on (a) day (D) 28 and (b) D63. In the faeces at each time point, n = 8 per treatment group. For each treatment group in the ileal and caecal digesta: Dry meal (n = 7), Dry pellets (n = 7), Liquid meal (n = 8), Liquid pellets (n = 9), Wet/dry meal (n = 8), Wet/dry pellets (n = 6).

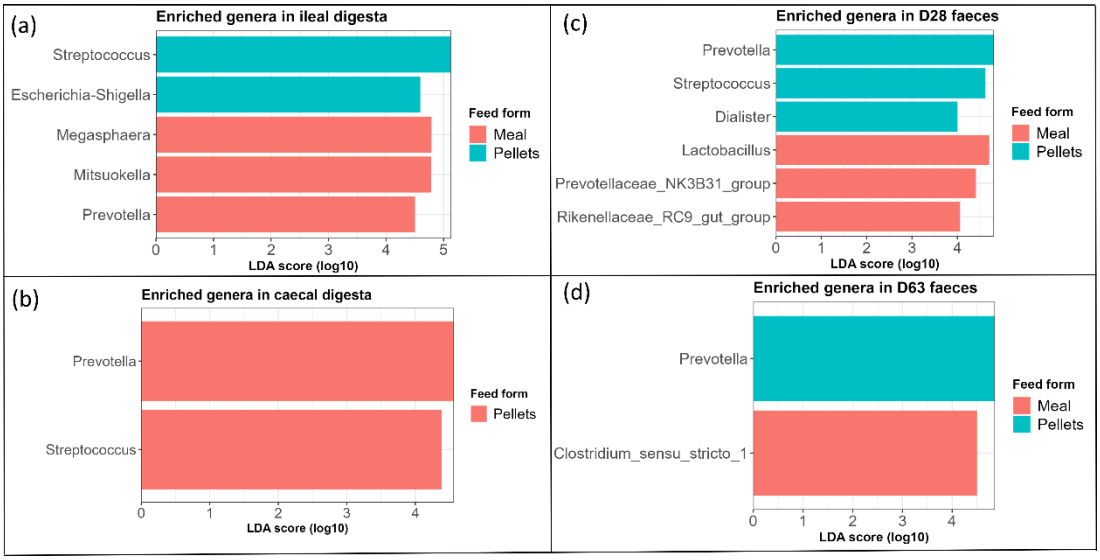

Figure 4.7: Differentially abundant bacterial genera in the **(a)** ileal and **(b)** caecal digesta, and in the faeces on **(c)** day (D) 28 and **(d)** D63 of grow-finisher pigs fed dry, liquid or wet/dry feed in meal (ileal and caecal digesta: n = 23; faeces: n = 24) or pelleted (ileal and caecal digesta: n = 22; faeces: n = 24) form. Differential abundances between feed forms were identified by linear discriminant analysis (LDA) with effect size (LefSe). Only genera with an LDA score ($\log_{10}$) > 4.0 are shown. The colour of the bars for each respective plot denotes the treatment group in which the differentially abundant genera had the highest relative abundance (%).

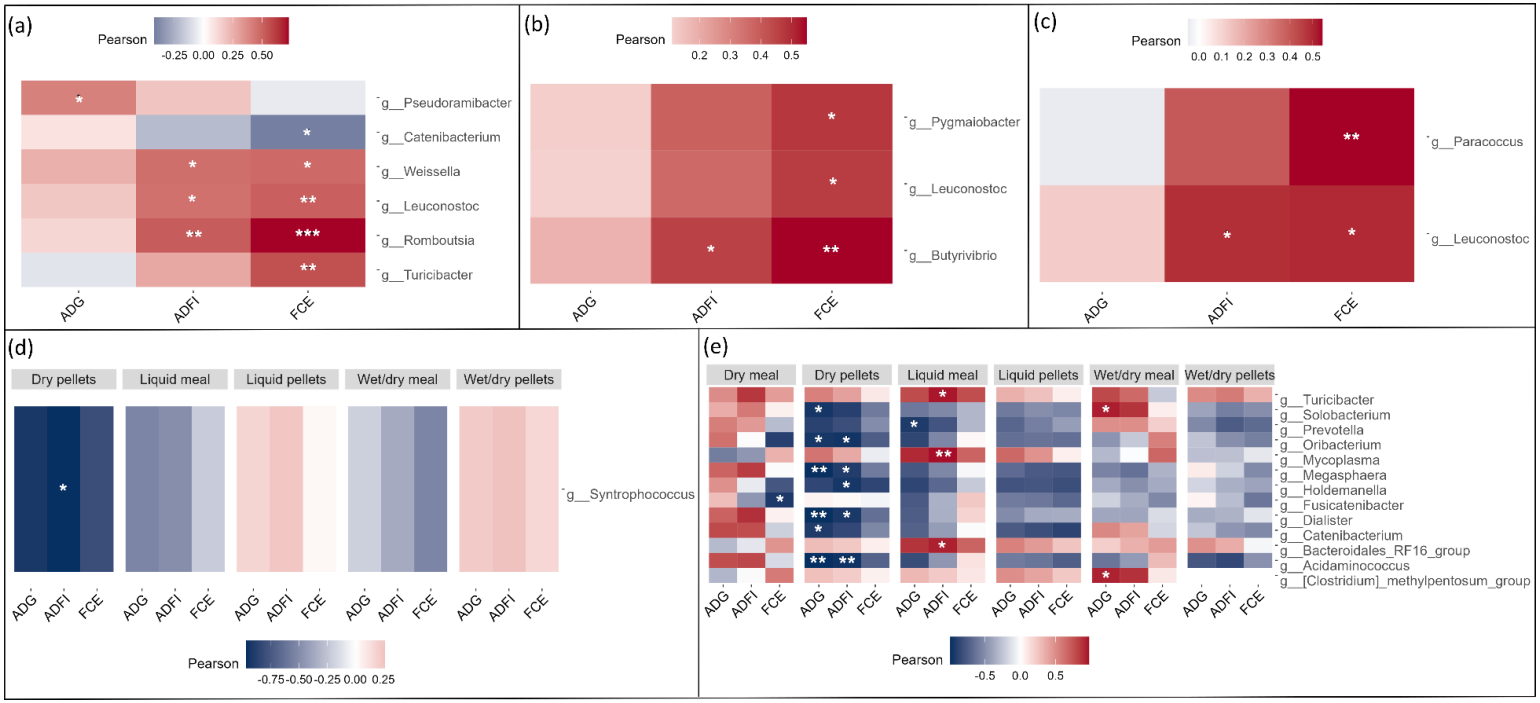

Figure 4.8: Pearson correlation plots of differentially abundant bacterial genera with average daily gain (ADG), average daily feed intake (ADFI) and feed conversion efficiency (FCE) in the faeces and gut digesta of pigs fed dry, liquid or wet/dry feed in meal or pelleted form. Correlations were performed between genera that were differentially abundant across all treatment groups, with ADG, ADFI and FCE in the faeces on **(a)** day (D)28; $n = 48$, **(b)** in the faeces on D63; $n = 48$, and **(c)** in the ileal digesta; $n = 45$. Correlations were also performed between genera that were differentially abundant in each treatment group with ADG, ADFI and FCE in the faeces on **(d)** D28; $n = 8$ per treatment, and **(e)** D63; $n = 8$ per treatment. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. False discovery rate multiple testing correction was performed. Red indicates a positive correlation, white indicates no correlation and blue indicates a negative correlation. Note: Lower FCE values indicate an improvement in feed efficiency, therefore, negative correlations between the differentially abundant bacterial genera and FCE values indicate improved feed efficiency.

4.8 Supplementary information

Table S 4.1: Bacterial genera ≥ 1 % mean relative abundance (RA, %) by treatment in the intestinal digesta and faeces of grow-finisher pigs fed the experimental diets.

Genus	Sample type	Treatment	Mean RA
Streptococcus	Ileum	Wet/dry pellets	36.90
Clostridium sensu stricto 1	Ileum	Liquid pellets	34.20
Clostridium sensu stricto 1	Ileum	Wet/dry meal	30.00
Clostridium sensu stricto 1	Ileum	Dry meal	29.10
Streptococcus	Ileum	Dry pellets	28.90
Clostridium sensu stricto 1	Ileum	Dry pellets	27.60
Clostridium sensu stricto 1	Ileum	Wet/dry pellets	26.70
Streptococcus	Ileum	Liquid pellets	25.10
Lactobacillus	Ileum	Liquid meal	24.50
Clostridium sensu stricto 1	Ileum	Liquid meal	23.10
Streptococcus	Ileum	Wet/dry meal	22.80
Terrisporobacter	Ileum	Wet/dry meal	16.10
Terrisporobacter	Ileum	Liquid pellets	16.00
Streptococcus	Ileum	Dry meal	15.20
Lactobacillus	Ileum	Dry pellets	13.50
Escherichia-Shigella	Ileum	Dry pellets	12.20
Terrisporobacter	Ileum	Dry meal	11.90
Terrisporobacter	Ileum	Liquid meal	11.20
Terrisporobacter	Ileum	Wet/dry pellets	10.90
Escherichia-Shigella	Ileum	Wet/dry pellets	9.70
Terrisporobacter	Ileum	Dry pellets	9.60
Megasphaera	Ileum	Liquid meal	9.40
Mitsuokella	Ileum	Dry meal	8.70
Streptococcus	Ileum	Liquid meal	8.50
Lactobacillus	Ileum	Wet/dry pellets	7.50
Megasphaera	Ileum	Dry meal	7.40
Lactobacillus	Ileum	Wet/dry meal	7.20
Lactobacillus	Ileum	Dry meal	6.50
Mitsuokella	Ileum	Liquid meal	6.10
Escherichia-Shigella	Ileum	Wet/dry meal	5.60
Prevotella	Ileum	Dry meal	5.40
Prevotella	Ileum	Liquid meal	5.20
Actinobacillus	Ileum	Liquid pellets	5.00
Escherichia-Shigella	Ileum	Liquid pellets	4.40
Turicibacter	Ileum	Wet/dry meal	3.90
Mitsuokella	Ileum	Wet/dry meal	3.70
Turicibacter	Ileum	Dry meal	3.40
Escherichia-Shigella	Ileum	Liquid meal	3.30

Escherichia-Shigella	Ileum	Dry meal	3.20
Actinobacillus	Ileum	Wet/dry meal	2.90
Romboutsia	Ileum	Liquid pellets	2.80
Turicibacter	Ileum	Liquid pellets	2.80
Actinobacillus	Ileum	Dry meal	2.80
Lactobacillus	Ileum	Liquid pellets	2.30
Turicibacter	Ileum	Dry pellets	2.10
Veillonella	Ileum	Wet/dry pellets	2.00
Romboutsia	Ileum	Wet/dry meal	1.90
Actinobacillus	Ileum	Wet/dry pellets	1.60
Veillonella	Ileum	Liquid pellets	1.60
Megasphaera	Ileum	Wet/dry meal	1.60
Turicibacter	Ileum	Wet/dry pellets	1.40
Romboutsia	Ileum	Dry pellets	1.40
Bifidobacterium	Ileum	Liquid meal	1.40
Romboutsia	Ileum	Wet/dry pellets	1.30
Romboutsia	Ileum	Dry meal	1.30
Actinobacillus	Ileum	Dry pellets	1.20
Turicibacter	Ileum	Liquid meal	1.20
Prevotella	Caecum	Dry pellets	20.36
Prevotella	Caecum	Wet/dry pellets	19.78
Prevotella	Caecum	Liquid pellets	18.93
Clostridium sensu stricto 1	Caecum	Dry meal	17.53
Prevotella	Caecum	Liquid meal	16.95
Prevotella	Caecum	Wet/dry meal	15.94
Clostridium sensu stricto 1	Caecum	Wet/dry meal	14.48
Prevotella	Caecum	Dry meal	13.90
Clostridium sensu stricto 1	Caecum	Wet/dry pellets	12.86
Lactobacillus	Caecum	Liquid meal	12.82
Clostridium sensu stricto 1	Caecum	Liquid pellets	12.46
Clostridium sensu stricto 1	Caecum	Dry pellets	11.42
Clostridium sensu stricto 1	Caecum	Liquid meal	9.34
Streptococcus	Caecum	Wet/dry pellets	6.68
Streptococcus	Caecum	Dry pellets	6.64
Prevotellaceae NK3B31 group	Caecum	Liquid meal	6.57
Terrisporobacter	Caecum	Dry meal	6.25
Prevotellaceae NK3B31 group	Caecum	Liquid pellets	6.11
Alloprevotella	Caecum	Liquid pellets	6.05
Terrisporobacter	Caecum	Liquid pellets	5.91
Prevotellaceae NK3B31 group	Caecum	Dry meal	5.79
Alloprevotella	Caecum	Wet/dry pellets	5.78
Alloprevotella	Caecum	Dry meal	5.45
Terrisporobacter	Caecum	Wet/dry meal	5.32
Prevotellaceae NK3B31 group	Caecum	Wet/dry meal	5.09

Alloprevotella	Caecum	Dry pellets	4.82
Alloprevotella	Caecum	Liquid meal	4.80
Alloprevotella	Caecum	Wet/dry meal	4.79
Streptococcus	Caecum	Wet/dry meal	4.55
Prevotellaceae NK3B31 group	Caecum	Wet/dry pellets	4.53
Terrisporobacter	Caecum	Wet/dry pellets	4.51
Streptococcus	Caecum	Liquid pellets	4.45
Terrisporobacter	Caecum	Dry pellets	4.43
Prevotellaceae NK3B31 group	Caecum	Dry pellets	4.37
Megasphaera	Caecum	Liquid meal	4.34
Megasphaera	Caecum	Wet/dry pellets	4.07
Megasphaera	Caecum	Dry pellets	4.05
Terrisporobacter	Caecum	Liquid meal	3.99
Lactobacillus	Caecum	Dry meal	3.79
Anaerovibrio	Caecum	Liquid meal	3.78
Lactobacillus	Caecum	Dry pellets	3.71
Anaerovibrio	Caecum	Liquid pellets	3.60
Lactobacillus	Caecum	Wet/dry pellets	3.58
Anaerovibrio	Caecum	Dry pellets	3.26
Streptococcus	Caecum	Dry meal	3.20
Subdoligranulum	Caecum	Dry pellets	3.20
Anaerovibrio	Caecum	Wet/dry pellets	3.11
Prevotellaceae UCG-003	Caecum	Wet/dry meal	2.84
Subdoligranulum	Caecum	Liquid pellets	2.81
Megasphaera	Caecum	Dry meal	2.80
Blautia	Caecum	Dry pellets	2.78
Anaerovibrio	Caecum	Wet/dry meal	2.73
Phascolarctobacterium	Caecum	Liquid pellets	2.73
Subdoligranulum	Caecum	Liquid meal	2.72
Phascolarctobacterium	Caecum	Wet/dry meal	2.67
Phascolarctobacterium	Caecum	Liquid meal	2.64
Subdoligranulum	Caecum	Wet/dry pellets	2.61
Anaerovibrio	Caecum	Dry meal	2.54
Phascolarctobacterium	Caecum	Dry pellets	2.46
Mitsuokella	Caecum	Dry meal	2.31
Blautia	Caecum	Wet/dry pellets	2.26
Rikenellaceae RC9 gut group	Caecum	Wet/dry meal	2.24
Blautia	Caecum	Liquid meal	2.14
Muribaculaceae	Caecum	Dry meal	2.14
Muribaculaceae	Caecum	Liquid meal	2.08
Blautia	Caecum	Liquid pellets	2.05
Blautia	Caecum	Wet/dry meal	2.03
Phascolarctobacterium	Caecum	Dry meal	2.01
Subdoligranulum	Caecum	Dry meal	1.98

Megasphaera	Caecum	Liquid pellets	1.94
Subdoligranulum	Caecum	Wet/dry meal	1.94
Escherichia-Shigella	Caecum	Dry pellets	1.94
Prevotellaceae UCG-003	Caecum	Dry meal	1.85
Phascolarctobacterium	Caecum	Wet/dry pellets	1.84
Muribaculaceae	Caecum	Wet/dry meal	1.83
Faecalibacterium	Caecum	Liquid meal	1.81
Rikenellaceae RC9 gut group	Caecum	Dry meal	1.80
Megasphaera	Caecum	Wet/dry meal	1.78
Faecalibacterium	Caecum	Dry pellets	1.78
Muribaculaceae	Caecum	Dry pellets	1.76
Campylobacter	Caecum	Dry meal	1.75
Streptococcus	Caecum	Liquid meal	1.66
Blautia	Caecum	Dry meal	1.65
Lactobacillus	Caecum	Wet/dry meal	1.65
Muribaculaceae	Caecum	Liquid pellets	1.64
Roseburia	Caecum	Liquid meal	1.61
Roseburia	Caecum	Dry pellets	1.57
Campylobacter	Caecum	Liquid pellets	1.55
Muribaculaceae	Caecum	Wet/dry pellets	1.54
UCG-005	Caecum	Dry meal	1.53
Roseburia	Caecum	Liquid pellets	1.51
Faecalibacterium	Caecum	Liquid pellets	1.50
Lactobacillus	Caecum	Liquid pellets	1.46
Prevotellaceae UCG-003	Caecum	Liquid meal	1.45
UCG-005	Caecum	Wet/dry meal	1.43
Turicibacter	Caecum	Dry meal	1.42
Prevotellaceae UCG-003	Caecum	Wet/dry pellets	1.42
Faecalibacterium	Caecum	Wet/dry pellets	1.41
Actinobacillus	Caecum	Dry meal	1.41
Campylobacter	Caecum	Wet/dry meal	1.40
Rikenellaceae RC9 gut group	Caecum	Liquid meal	1.38
Turicibacter	Caecum	Wet/dry meal	1.36
Rikenellaceae RC9 gut group	Caecum	Liquid pellets	1.35
Rikenellaceae RC9 gut group	Caecum	Dry pellets	1.34
Rikenellaceae RC9 gut group	Caecum	Wet/dry pellets	1.34
UCG-005	Caecum	Liquid meal	1.32
Faecalibacterium	Caecum	Wet/dry meal	1.32
Treponema	Caecum	Wet/dry meal	1.27
UCG-005	Caecum	Liquid pellets	1.27
Agathobacter	Caecum	Liquid meal	1.23
Prevotellaceae UCG-003	Caecum	Liquid pellets	1.23
Gastranaerophilales	Caecum	Liquid pellets	1.18
Mitsuokella	Caecum	Wet/dry pellets	1.17

Agathobacter	Caecum	Dry pellets	1.16
Turicibacter	Caecum	Liquid pellets	1.15
Agathobacter	Caecum	Liquid pellets	1.14
Roseburia	Caecum	Wet/dry meal	1.13
Dialister	Caecum	Wet/dry pellets	1.11
Escherichia-Shigella	Caecum	Liquid pellets	1.09
Mitsuokella	Caecum	Wet/dry meal	1.01
Agathobacter	Caecum	Wet/dry meal	1.01
Prevotella	Baseline faeces	Dry meal	14.25
Prevotella	Baseline faeces	Wet/dry meal	14.09
Prevotella	Baseline faeces	Wet/dry pellets	13.95
Prevotella	Baseline faeces	Dry pellets	13.66
Prevotella	Baseline faeces	Liquid pellets	13.40
Prevotella	Baseline faeces	Liquid meal	11.99
Lactobacillus	Baseline faeces	Liquid pellets	11.77
Streptococcus	Baseline faeces	Liquid meal	11.23
Lactobacillus	Baseline faeces	Liquid meal	9.64
Streptococcus	Baseline faeces	Liquid pellets	9.16
Streptococcus	Baseline faeces	Dry pellets	9.06
Streptococcus	Baseline faeces	Wet/dry pellets	8.85
Streptococcus	Baseline faeces	Dry meal	8.63
Streptococcus	Baseline faeces	Wet/dry meal	8.25
Lactobacillus	Baseline faeces	Dry pellets	7.87
Lactobacillus	Baseline faeces	Wet/dry pellets	7.86
Clostridium sensu stricto 1	Baseline faeces	Dry meal	7.74
Megasphaera	Baseline faeces	Liquid pellets	6.95
Clostridium sensu stricto 1	Baseline faeces	Wet/dry pellets	6.89
Clostridium sensu stricto 1	Baseline faeces	Wet/dry meal	6.88
Lactobacillus	Baseline faeces	Dry meal	6.61
Megasphaera	Baseline faeces	Liquid meal	6.25
Megasphaera	Baseline faeces	Wet/dry pellets	5.60
Megasphaera	Baseline faeces	Wet/dry meal	5.51
Clostridium sensu stricto 1	Baseline faeces	Liquid pellets	5.50
Megasphaera	Baseline faeces	Dry pellets	5.47
Prevotellaceae NK3B31 group	Baseline faeces	Dry pellets	5.21
Prevotellaceae NK3B31 group	Baseline faeces	Liquid meal	5.18
Muribaculaceae	Baseline faeces	Dry meal	4.87
Prevotellaceae NK3B31 group	Baseline faeces	Dry meal	4.87
Megasphaera	Baseline faeces	Dry meal	4.76
Prevotellaceae NK3B31 group	Baseline faeces	Wet/dry meal	4.70
Subdoligranulum	Baseline faeces	Liquid pellets	4.47
Faecalibacterium	Baseline faeces	Liquid pellets	4.40
Muribaculaceae	Baseline faeces	Dry pellets	4.28
Lactobacillus	Baseline faeces	Wet/dry meal	4.25

Blautia	Baseline faeces	Wet/dry pellets	4.25
Muribaculaceae	Baseline faeces	Wet/dry pellets	4.22
Blautia	Baseline faeces	Liquid pellets	4.22
Anaerovibrio	Baseline faeces	Dry meal	4.18
Clostridium sensu stricto 1	Baseline faeces	Liquid meal	4.17
Muribaculaceae	Baseline faeces	Wet/dry meal	4.08
Clostridium sensu stricto 1	Baseline faeces	Dry pellets	4.03
Blautia	Baseline faeces	Wet/dry meal	3.85
Prevotellaceae NK3B31 group	Baseline faeces	Wet/dry pellets	3.75
Anaerovibrio	Baseline faeces	Wet/dry meal	3.62
Blautia	Baseline faeces	Dry pellets	3.54
Blautia	Baseline faeces	Liquid meal	3.54
Muribaculaceae	Baseline faeces	Liquid meal	3.44
Phascolarctobacterium	Baseline faeces	Liquid pellets	3.34
Faecalibacterium	Baseline faeces	Liquid meal	3.32
Phascolarctobacterium	Baseline faeces	Wet/dry pellets	3.13
Phascolarctobacterium	Baseline faeces	Dry pellets	3.12
Rikenellaceae RC9 gut group	Baseline faeces	Dry meal	2.96
Muribaculaceae	Baseline faeces	Liquid pellets	2.91
Rikenellaceae RC9 gut group	Baseline faeces	Wet/dry meal	2.87
Blautia	Baseline faeces	Dry meal	2.84
Rikenellaceae RC9 gut group	Baseline faeces	Wet/dry pellets	2.80
Anaerovibrio	Baseline faeces	Liquid meal	2.80
Rikenellaceae RC9 gut group	Baseline faeces	Liquid meal	2.69
Phascolarctobacterium	Baseline faeces	Wet/dry meal	2.63
Rikenellaceae RC9 gut group	Baseline faeces	Dry pellets	2.54
Subdoligranulum	Baseline faeces	Liquid meal	2.52
Subdoligranulum	Baseline faeces	Wet/dry pellets	2.47
Prevotellaceae NK3B31 group	Baseline faeces	Liquid pellets	2.35
Subdoligranulum	Baseline faeces	Dry pellets	2.32
Subdoligranulum	Baseline faeces	Wet/dry meal	2.24
Phascolarctobacterium	Baseline faeces	Dry meal	2.19
Phascolarctobacterium	Baseline faeces	Liquid meal	2.17
Roseburia	Baseline faeces	Dry pellets	2.11
Succinivibrio	Baseline faeces	Liquid pellets	2.07
Roseburia	Baseline faeces	Dry meal	2.05
Roseburia	Baseline faeces	Liquid pellets	2.00
Alloprevotella	Baseline faeces	Wet/dry pellets	1.99
Rikenellaceae RC9 gut group	Baseline faeces	Liquid pellets	1.96
Faecalibacterium	Baseline faeces	Wet/dry meal	1.90
Subdoligranulum	Baseline faeces	Dry meal	1.89
Terrisporobacter	Baseline faeces	Wet/dry pellets	1.88
Catenibacterium	Baseline faeces	Liquid pellets	1.84
Prevotellaceae UCG-003	Baseline faeces	Wet/dry pellets	1.82

Faecalibacterium	Baseline faeces	Wet/dry pellets	1.78
Anaerovibrio	Baseline faeces	Wet/dry pellets	1.76
Anaerovibrio	Baseline faeces	Dry pellets	1.76
Ruminococcus	Baseline faeces	Wet/dry pellets	1.75
Ruminococcus	Baseline faeces	Dry meal	1.74
Ruminococcus	Baseline faeces	Wet/dry meal	1.72
Roseburia	Baseline faeces	Wet/dry meal	1.68
Mitsuokella	Baseline faeces	Liquid meal	1.67
Succinivibrio	Baseline faeces	Dry pellets	1.67
Alloprevotella	Baseline faeces	Wet/dry meal	1.62
Alloprevotella	Baseline faeces	Liquid meal	1.58
Succinivibrio	Baseline faeces	Wet/dry meal	1.58
Agathobacter	Baseline faeces	Liquid meal	1.53
Faecalibacterium	Baseline faeces	Dry pellets	1.53
Treponema	Baseline faeces	Dry pellets	1.51
Prevotellaceae UCG-003	Baseline faeces	Wet/dry meal	1.51
Alloprevotella	Baseline faeces	Liquid pellets	1.47
Alloprevotella	Baseline faeces	Dry pellets	1.46
Agathobacter	Baseline faeces	Dry meal	1.45
Ruminococcus	Baseline faeces	Dry pellets	1.43
Ruminococcus	Baseline faeces	Liquid meal	1.43
Terrisporobacter	Baseline faeces	Dry meal	1.42
Ruminococcus	Baseline faeces	Liquid pellets	1.38
Roseburia	Baseline faeces	Liquid meal	1.36
Prevotellaceae UCG-003	Baseline faeces	Dry pellets	1.35
Terrisporobacter	Baseline faeces	Wet/dry meal	1.28
Agathobacter	Baseline faeces	Wet/dry meal	1.27
Faecalibacterium	Baseline faeces	Dry meal	1.27
Dialister	Baseline faeces	Liquid meal	1.27
Acidaminococcus	Baseline faeces	Liquid meal	1.26
Agathobacter	Baseline faeces	Liquid pellets	1.22
Terrisporobacter	Baseline faeces	Liquid pellets	1.21
Acidaminococcus	Baseline faeces	Liquid pellets	1.19
Roseburia	Baseline faeces	Wet/dry pellets	1.18
Catenibacterium	Baseline faeces	Dry pellets	1.14
Escherichia-Shigella	Baseline faeces	Wet/dry pellets	1.13
Alloprevotella	Baseline faeces	Dry meal	1.10
UCG-005	Baseline faeces	Wet/dry pellets	1.10
Catenibacterium	Baseline faeces	Liquid meal	1.09
Agathobacter	Baseline faeces	Dry pellets	1.09
UCG-002	Baseline faeces	Dry meal	1.07
Catenibacterium	Baseline faeces	Wet/dry pellets	1.04
Escherichia-Shigella	Baseline faeces	Dry pellets	1.04
Selenomonas	Baseline faeces	Dry pellets	1.04

Mitsuokella	Baseline faeces	Dry pellets	1.03
Prevotellaceae UCG-003	Baseline faeces	Dry meal	1.02
UCG-005	Baseline faeces	Wet/dry meal	1.02
Escherichia-Shigella	Baseline faeces	Liquid pellets	1.01
Acidaminococcus	Baseline faeces	Wet/dry pellets	1.01
Anaerovibrio	Baseline faeces	Liquid pellets	1.01
Christensenellaceae R-7 group	Baseline faeces	Wet/dry pellets	1.00
Prevotella	D28 faeces	Wet/dry pellets	20.67
Prevotella	D28 faeces	Liquid pellets	19.13
Prevotella	D28 faeces	Dry pellets	17.31
Lactobacillus	D28 faeces	Liquid meal	14.88
Prevotella	D28 faeces	Dry meal	13.11
Prevotella	D28 faeces	Liquid meal	12.10
Prevotella	D28 faeces	Wet/dry meal	10.89
Streptococcus	D28 faeces	Wet/dry pellets	10.67
Clostridium sensu stricto 1	D28 faeces	Wet/dry meal	10.14
Lactobacillus	D28 faeces	Dry meal	8.92
Streptococcus	D28 faeces	Dry pellets	8.86
Clostridium sensu stricto 1	D28 faeces	Dry meal	8.60
Clostridium sensu stricto 1	D28 faeces	Liquid pellets	8.43
Prevotellaceae NK3B31 group	D28 faeces	Dry meal	7.75
Lactobacillus	D28 faeces	Wet/dry meal	7.08
Prevotellaceae NK3B31 group	D28 faeces	Wet/dry meal	7.04
Prevotellaceae NK3B31 group	D28 faeces	Liquid meal	6.81
Megasphaera	D28 faeces	Wet/dry pellets	6.79
Lactobacillus	D28 faeces	Liquid pellets	6.44
Streptococcus	D28 faeces	Wet/dry meal	6.12
Streptococcus	D28 faeces	Liquid pellets	5.99
Clostridium sensu stricto 1	D28 faeces	Dry pellets	5.72
Prevotellaceae NK3B31 group	D28 faeces	Liquid pellets	5.49
Megasphaera	D28 faeces	Liquid meal	4.93
Clostridium sensu stricto 1	D28 faeces	Liquid meal	4.91
Clostridium sensu stricto 1	D28 faeces	Wet/dry pellets	4.86
Lactobacillus	D28 faeces	Dry pellets	4.83
Megasphaera	D28 faeces	Dry pellets	4.66
Prevotellaceae NK3B31 group	D28 faeces	Dry pellets	4.56
Muribaculaceae	D28 faeces	Liquid meal	4.49
Muribaculaceae	D28 faeces	Wet/dry meal	4.43
Streptococcus	D28 faeces	Dry meal	4.43
Muribaculaceae	D28 faeces	Dry meal	4.26
Rikenellaceae RC9 gut group	D28 faeces	Wet/dry meal	4.05
Rikenellaceae RC9 gut group	D28 faeces	Liquid meal	3.97
Rikenellaceae RC9 gut group	D28 faeces	Dry meal	3.91
Muribaculaceae	D28 faeces	Liquid pellets	3.81

Muribaculaceae	D28 faeces	Dry pellets	3.61
Megasphaera	D28 faeces	Liquid pellets	3.59
Megasphaera	D28 faeces	Wet/dry meal	3.42
Alloprevotella	D28 faeces	Wet/dry meal	3.19
Rikenellaceae RC9 gut group	D28 faeces	Dry pellets	3.16
Terrisporobacter	D28 faeces	Wet/dry meal	3.15
Muribaculaceae	D28 faeces	Wet/dry pellets	3.07
Alloprevotella	D28 faeces	Dry meal	2.95
Terrisporobacter	D28 faeces	Liquid pellets	2.80
Megasphaera	D28 faeces	Dry meal	2.80
Treponema	D28 faeces	Liquid meal	2.69
Alloprevotella	D28 faeces	Liquid meal	2.65
Rikenellaceae RC9 gut group	D28 faeces	Liquid pellets	2.58
Prevotellaceae NK3B31 group	D28 faeces	Wet/dry pellets	2.48
Subdoligranulum	D28 faeces	Liquid pellets	2.47
UCG-005	D28 faeces	Liquid meal	2.45
Rikenellaceae RC9 gut group	D28 faeces	Wet/dry pellets	2.44
Anaerovibrio	D28 faeces	Liquid meal	2.40
Subdoligranulum	D28 faeces	Liquid meal	2.37
Terrisporobacter	D28 faeces	Dry meal	2.30
Subdoligranulum	D28 faeces	Wet/dry pellets	2.28
Treponema	D28 faeces	Dry meal	2.21
Subdoligranulum	D28 faeces	Dry pellets	2.17
Blautia	D28 faeces	Dry pellets	2.16
Lactobacillus	D28 faeces	Wet/dry pellets	2.12
Alloprevotella	D28 faeces	Liquid pellets	2.11
Dialister	D28 faeces	Wet/dry pellets	2.10
Acidaminococcus	D28 faeces	Wet/dry pellets	2.09
Christensenellaceae R-7 group	D28 faeces	Liquid meal	2.07
Faecalibacterium	D28 faeces	Wet/dry pellets	2.06
Subdoligranulum	D28 faeces	Dry meal	2.04
Anaerovibrio	D28 faeces	Dry meal	2.01
Phascolarctobacterium	D28 faeces	Liquid meal	2.01
UCG-005	D28 faeces	Dry meal	1.96
Phascolarctobacterium	D28 faeces	Wet/dry meal	1.95
Anaerovibrio	D28 faeces	Wet/dry meal	1.94
Alloprevotella	D28 faeces	Dry pellets	1.93
UCG-005	D28 faeces	Wet/dry meal	1.90
Phascolarctobacterium	D28 faeces	Dry meal	1.86
Blautia	D28 faeces	Wet/dry pellets	1.86
Blautia	D28 faeces	Liquid pellets	1.80
Terrisporobacter	D28 faeces	Wet/dry pellets	1.79
Anaerovibrio	D28 faeces	Liquid pellets	1.77
Blautia	D28 faeces	Liquid meal	1.76

Subdoligranulum	D28 faeces	Wet/dry meal	1.74
Roseburia	D28 faeces	Wet/dry pellets	1.73
Roseburia	D28 faeces	Liquid meal	1.73
Prevotellaceae UCG-003	D28 faeces	Liquid pellets	1.72
Christensenellaceae R-7 group	D28 faeces	Wet/dry meal	1.69
Ruminococcus	D28 faeces	Dry pellets	1.68
Alloprevotella	D28 faeces	Wet/dry pellets	1.64
Phascolarctobacterium	D28 faeces	Liquid pellets	1.63
Christensenellaceae R-7 group	D28 faeces	Dry meal	1.61
Faecalibacterium	D28 faeces	Dry pellets	1.57
Treponema	D28 faeces	Dry pellets	1.56
Prevotellaceae UCG-003	D28 faeces	Wet/dry meal	1.55
Blautia	D28 faeces	Wet/dry meal	1.54
Christensenellaceae R-7 group	D28 faeces	Dry pellets	1.54
Blautia	D28 faeces	Dry meal	1.53
Prevotellaceae UCG-003	D28 faeces	Dry meal	1.52
Phascolarctobacterium	D28 faeces	Dry pellets	1.48
UCG-005	D28 faeces	Liquid pellets	1.46
Anaerovibrio	D28 faeces	Wet/dry pellets	1.46
Treponema	D28 faeces	Wet/dry meal	1.44
UCG-005	D28 faeces	Dry pellets	1.40
Ruminococcus	D28 faeces	Wet/dry pellets	1.38
Ruminococcus	D28 faeces	Wet/dry meal	1.36
Ruminococcus	D28 faeces	Dry meal	1.35
Treponema	D28 faeces	Liquid pellets	1.35
Prevotellaceae UCG-003	D28 faeces	Liquid meal	1.34
Ruminococcus	D28 faeces	Liquid pellets	1.33
Prevotellaceae UCG-003	D28 faeces	Dry pellets	1.32
Terrisporobacter	D28 faeces	Dry pellets	1.32
Anaerovibrio	D28 faeces	Dry pellets	1.25
Roseburia	D28 faeces	Dry meal	1.24
Mitsuokella	D28 faeces	Wet/dry pellets	1.22
Ruminococcus	D28 faeces	Liquid meal	1.22
Phascolarctobacterium	D28 faeces	Wet/dry pellets	1.20
Terrisporobacter	D28 faeces	Liquid meal	1.18
Roseburia	D28 faeces	Dry pellets	1.18
Roseburia	D28 faeces	Liquid pellets	1.10
Faecalibacterium	D28 faeces	Liquid pellets	1.06
Dialister	D28 faeces	Dry pellets	1.05
Coprococcus	D28 faeces	Liquid meal	1.04
Roseburia	D28 faeces	Wet/dry meal	1.03
UCG-010	D28 faeces	Liquid pellets	1.03
Christensenellaceae R-7 group	D28 faeces	Liquid pellets	1.02
UCG-005	D28 faeces	Wet/dry pellets	1.01

Streptococcus	D28 faeces	Liquid meal	1.01
Faecalibacterium	D28 faeces	Liquid meal	1.00
Prevotella	D63 faeces	Liquid pellets	19.48
Prevotella	D63 faeces	Liquid meal	14.52
Prevotella	D63 faeces	Wet/dry pellets	14.40
Prevotella	D63 faeces	Dry pellets	11.68
Clostridium sensu stricto 1	D63 faeces	Wet/dry meal	9.71
Prevotellaceae NK3B31 group	D63 faeces	Dry pellets	9.42
Rikenellaceae RC9 gut group	D63 faeces	Wet/dry meal	8.91
Clostridium sensu stricto 1	D63 faeces	Dry meal	8.78
Prevotellaceae NK3B31 group	D63 faeces	Liquid pellets	8.77
Prevotellaceae NK3B31 group	D63 faeces	Dry meal	7.34
Treponema	D63 faeces	Wet/dry pellets	7.09
Christensenellaceae R-7 group	D63 faeces	Wet/dry meal	6.78
Rikenellaceae RC9 gut group	D63 faeces	Dry meal	6.33
Lactobacillus	D63 faeces	Liquid meal	6.23
Christensenellaceae R-7 group	D63 faeces	Dry meal	6.13
Prevotella	D63 faeces	Dry meal	6.09
Prevotellaceae NK3B31 group	D63 faeces	Liquid meal	6.00
Muribaculaceae	D63 faeces	Dry meal	5.71
Treponema	D63 faeces	Dry meal	5.70
Muribaculaceae	D63 faeces	Dry pellets	5.66
Rikenellaceae RC9 gut group	D63 faeces	Wet/dry pellets	5.44
Clostridium sensu stricto 1	D63 faeces	Dry pellets	5.34
Rikenellaceae RC9 gut group	D63 faeces	Dry pellets	5.33
Prevotellaceae NK3B31 group	D63 faeces	Wet/dry meal	5.23
Treponema	D63 faeces	Wet/dry meal	5.04
Muribaculaceae	D63 faeces	Wet/dry pellets	5.01
Treponema	D63 faeces	Liquid pellets	4.86
Rikenellaceae RC9 gut group	D63 faeces	Liquid pellets	4.83
Muribaculaceae	D63 faeces	Liquid pellets	4.80
Muribaculaceae	D63 faeces	Wet/dry meal	4.76
Rikenellaceae RC9 gut group	D63 faeces	Liquid meal	4.76
Prevotellaceae NK3B31 group	D63 faeces	Wet/dry pellets	4.58
Streptococcus	D63 faeces	Wet/dry pellets	4.51
Clostridium sensu stricto 1	D63 faeces	Wet/dry pellets	4.50
Muribaculaceae	D63 faeces	Liquid meal	4.41
Treponema	D63 faeces	Dry pellets	4.40
Streptococcus	D63 faeces	Dry pellets	4.26
Streptococcus	D63 faeces	Liquid meal	4.24
Clostridium sensu stricto 1	D63 faeces	Liquid meal	4.17
Megasphaera	D63 faeces	Liquid meal	4.06
Christensenellaceae R-7 group	D63 faeces	Dry pellets	3.85
Christensenellaceae R-7 group	D63 faeces	Liquid meal	3.79

Prevotella	D63 faeces	Wet/dry meal	3.76
Clostridium sensu stricto 1	D63 faeces	Liquid pellets	3.55
Christensenellaceae R-7 group	D63 faeces	Wet/dry pellets	3.43
Streptococcus	D63 faeces	Liquid pellets	3.36
Alloprevotella	D63 faeces	Liquid pellets	3.03
Prevotellaceae UCG-001	D63 faeces	Wet/dry meal	2.88
Alloprevotella	D63 faeces	Dry meal	2.69
Treponema	D63 faeces	Liquid meal	2.69
UCG-010	D63 faeces	Wet/dry pellets	2.60
Bacteroidales RF16 group	D63 faeces	Dry meal	2.56
Prevotellaceae UCG-001	D63 faeces	Wet/dry pellets	2.55
Alloprevotella	D63 faeces	Wet/dry meal	2.54
Prevotellaceae UCG-003	D63 faeces	Wet/dry meal	2.51
Alloprevotella	D63 faeces	Dry pellets	2.48
Streptococcus	D63 faeces	Dry meal	2.46
UCG-005	D63 faeces	Wet/dry meal	2.34
Prevotellaceae UCG-003	D63 faeces	Dry meal	2.32
Alloprevotella	D63 faeces	Wet/dry pellets	2.24
Parabacteroides	D63 faeces	Wet/dry meal	2.24
Terrisporobacter	D63 faeces	Wet/dry meal	2.22
Prevotellaceae UCG-003	D63 faeces	Liquid pellets	2.17
UCG-010	D63 faeces	Dry pellets	2.14
UCG-010	D63 faeces	Wet/dry meal	2.03
Phascolarctobacterium	D63 faeces	Liquid pellets	2.02
UCG-010	D63 faeces	Liquid pellets	2.00
Terrisporobacter	D63 faeces	Dry meal	1.99
Christensenellaceae R-7 group	D63 faeces	Liquid pellets	1.95
UCG-005	D63 faeces	Dry meal	1.93
Prevotellaceae UCG-003	D63 faeces	Dry pellets	1.91
dgA-11 gut group	D63 faeces	Wet/dry pellets	1.90
Anaerovibrio	D63 faeces	Liquid meal	1.89
Prevotellaceae UCG-003	D63 faeces	Wet/dry pellets	1.88
Alloprevotella	D63 faeces	Liquid meal	1.85
Megasphaera	D63 faeces	Dry pellets	1.79
UCG-010	D63 faeces	Dry meal	1.78
UCG-005	D63 faeces	Liquid pellets	1.78
Terrisporobacter	D63 faeces	Liquid pellets	1.71
Terrisporobacter	D63 faeces	Dry pellets	1.68
Parabacteroides	D63 faeces	Dry pellets	1.68
UCG-002	D63 faeces	Dry meal	1.67
Terrisporobacter	D63 faeces	Wet/dry pellets	1.66
Clostridia vadinBB60 group	D63 faeces	Wet/dry pellets	1.65
UCG-005	D63 faeces	Dry pellets	1.65
Phascolarctobacterium	D63 faeces	Dry pellets	1.64

UCG-002	D63 faeces	Wet/dry meal	1.63
Clostridia vadinBB60 group	D63 faeces	Dry pellets	1.62
Terrisporobacter	D63 faeces	Liquid meal	1.59
Lactobacillus	D63 faeces	Dry pellets	1.55
UCG-005	D63 faeces	Wet/dry pellets	1.53
Bacteroidales RF16 group	D63 faeces	Dry pellets	1.53
Phascolarctobacterium	D63 faeces	Liquid meal	1.53
UCG-002	D63 faeces	Wet/dry pellets	1.48
Phascolarctobacterium	D63 faeces	Dry meal	1.47
Bacteroidales RF16 group	D63 faeces	Wet/dry meal	1.46
Ruminococcus	D63 faeces	Wet/dry pellets	1.45
UCG-005	D63 faeces	Liquid meal	1.45
Clostridia vadinBB60 group	D63 faeces	Wet/dry meal	1.42
Streptococcus	D63 faeces	Wet/dry meal	1.42
Lachnospiraceae XPB1014 group	D63 faeces	Wet/dry meal	1.40
Phascolarctobacterium	D63 faeces	Wet/dry meal	1.39
Parabacteroides	D63 faeces	Dry meal	1.39
Lactobacillus	D63 faeces	Dry meal	1.39
Phascolarctobacterium	D63 faeces	Wet/dry pellets	1.38
p-2534-18B5 gut group	D63 faeces	Liquid meal	1.37
Fibrobacter	D63 faeces	Wet/dry pellets	1.36
Prevotellaceae UCG-001	D63 faeces	Dry pellets	1.32
UCG-002	D63 faeces	Liquid pellets	1.32
NK4A214 group	D63 faeces	Wet/dry meal	1.31
Ruminococcus	D63 faeces	Liquid meal	1.31
Clostridia vadinBB60 group	D63 faeces	Dry meal	1.29
Megasphaera	D63 faeces	Wet/dry pellets	1.29
Prevotellaceae UCG-003	D63 faeces	Liquid meal	1.28
Anaerovibrio	D63 faeces	Liquid pellets	1.27
UCG-002	D63 faeces	Dry pellets	1.25
Lactobacillus	D63 faeces	Wet/dry meal	1.24
WCHB1-41	D63 faeces	Wet/dry pellets	1.22
Prevotellaceae UCG-001	D63 faeces	Liquid pellets	1.20
Eubacterium coprostanoligenes group	D63 faeces	Wet/dry pellets	1.18
p-2534-18B5 gut group	D63 faeces	Dry meal	1.16
p-2534-18B5 gut group	D63 faeces	Wet/dry meal	1.15
Clostridia UCG-014	D63 faeces	Wet/dry meal	1.15
Anaerovibrio	D63 faeces	Dry meal	1.15
Ruminococcus	D63 faeces	Dry meal	1.14
WCHB1-41	D63 faeces	Wet/dry meal	1.13
dgA-11 gut group	D63 faeces	Dry meal	1.11
Ruminococcus	D63 faeces	Liquid pellets	1.09
Prevotellaceae UCG-001	D63 faeces	Liquid meal	1.09

UCG-010	D63 faeces	Liquid meal	1.09
Subdoligranulum	D63 faeces	Liquid pellets	1.09
Anaerovibrio	D63 faeces	Dry pellets	1.08
Eubacterium ruminantium group	D63 faeces	Liquid meal	1.08
WCHB1-41	D63 faeces	Dry pellets	1.06
Parabacteroides	D63 faeces	Wet/dry pellets	1.06
Parabacteroides	D63 faeces	Liquid pellets	1.06
Family XIII AD3011 group	D63 faeces	Wet/dry meal	1.06
Ruminococcus	D63 faeces	Dry pellets	1.06
Clostridia vadinBB60 group	D63 faeces	Liquid meal	1.05
Sphaerochaeta	D63 faeces	Dry pellets	1.04
Eubacterium coprostanoligenes group	D63 faeces	Wet/dry meal	1.04
Prevotellaceae UCG-001	D63 faeces	Dry meal	1.03
p-1088-a5 gut group	D63 faeces	Wet/dry meal	1.02
Bacteroides	D63 faeces	Wet/dry meal	1.02
Megasphaera	D63 faeces	Liquid pellets	1.01
Eubacterium coprostanoligenes group	D63 faeces	Dry meal	1.00
NK4A214 group	D63 faeces	Dry meal	1.00
Eubacterium coprostanoligenes group	D63 faeces	Dry pellets	1.00

Table S 4.2: Bacterial genera ≥ 1 % mean relative abundance (RA, %) by feed form in the intestinal digesta and faeces of grow-finisher pigs fed the experimental diets.

Genus	Sample	Feed Form	Mean RA
Clostridium sensu stricto 1	Ileum	Pellets	30.02
Streptococcus	Ileum	Pellets	29.52
Clostridium sensu stricto 1	Ileum	Meal	27.36
Streptococcus	Ileum	Meal	15.52
Terrisporobacter	Ileum	Meal	13.12
Lactobacillus	Ileum	Meal	12.98
Terrisporobacter	Ileum	Pellets	12.55
Escherichia-Shigella	Ileum	Pellets	8.32
Lactobacillus	Ileum	Pellets	7.25
Megasphaera	Ileum	Meal	6.06
Mitsuokella	Ileum	Meal	6.05
Escherichia-Shigella	Ileum	Meal	4.07
Prevotella	Ileum	Meal	3.60
Actinobacillus	Ileum	Pellets	2.86
Turicibacter	Ileum	Meal	2.79
Turicibacter	Ileum	Pellets	2.18
Actinobacillus	Ileum	Meal	2.04
Romboutsia	Ileum	Pellets	1.97
Romboutsia	Ileum	Meal	1.35
Veillonella	Ileum	Pellets	1.29
Prevotella	Caecum	Pellets	19.62
Prevotella	Caecum	Meal	15.67
Clostridium sensu stricto 1	Caecum	Meal	13.62
Clostridium sensu stricto 1	Caecum	Pellets	12.24
Lactobacillus	Caecum	Meal	6.19
Prevotellaceae NK3B31 group	Caecum	Meal	5.82
Streptococcus	Caecum	Pellets	5.75
Alloprevotella	Caecum	Pellets	5.59
Terrisporobacter	Caecum	Meal	5.14
Prevotellaceae NK3B31 group	Caecum	Pellets	5.13
Terrisporobacter	Caecum	Pellets	5.05
Alloprevotella	Caecum	Meal	4.99
Anaerovibrio	Caecum	Pellets	3.36
Megasphaera	Caecum	Pellets	3.19
Streptococcus	Caecum	Meal	3.13
Anaerovibrio	Caecum	Meal	3.03
Megasphaera	Caecum	Meal	2.98
Subdoligranulum	Caecum	Pellets	2.88
Lactobacillus	Caecum	Pellets	2.76
Phascolarctobacterium	Caecum	Meal	2.46

Phascolarctobacterium	Caecum	Pellets	2.40
Blautia	Caecum	Pellets	2.34
Subdoligranulum	Caecum	Meal	2.22
Prevotellaceae UCG-003	Caecum	Meal	2.06
Muribaculaceae	Caecum	Meal	2.01
Blautia	Caecum	Meal	1.95
Rikenellaceae RC9 gut group	Caecum	Meal	1.81
Muribaculaceae	Caecum	Pellets	1.65
Faecalibacterium	Caecum	Pellets	1.56
UCG-005	Caecum	Meal	1.42
Mitsuokella	Caecum	Meal	1.38
Roseburia	Caecum	Pellets	1.37
Faecalibacterium	Caecum	Meal	1.37
Rikenellaceae RC9 gut group	Caecum	Pellets	1.35
Escherichia-Shigella	Caecum	Pellets	1.32
Prevotellaceae UCG-003	Caecum	Pellets	1.20
Roseburia	Caecum	Meal	1.17
Campylobacter	Caecum	Meal	1.12
Agathobacter	Caecum	Pellets	1.09
UCG-005	Caecum	Pellets	1.08
Turicibacter	Caecum	Meal	1.04
Prevotella	D28 faeces	Pellets	19.04
Prevotella	D28 faeces	Meal	12.03
Lactobacillus	D28 faeces	Meal	10.29
Streptococcus	D28 faeces	Pellets	8.51
Clostridium sensu stricto 1	D28 faeces	Meal	7.88
Prevotellaceae NK3B31 group	D28 faeces	Meal	7.20
Clostridium sensu stricto 1	D28 faeces	Pellets	6.34
Megasphaera	D28 faeces	Pellets	5.01
Lactobacillus	D28 faeces	Pellets	4.47
Muribaculaceae	D28 faeces	Meal	4.40
Prevotellaceae NK3B31 group	D28 faeces	Pellets	4.18
Rikenellaceae RC9 gut group	D28 faeces	Meal	3.98
Streptococcus	D28 faeces	Meal	3.85
Megasphaera	D28 faeces	Meal	3.72
Muribaculaceae	D28 faeces	Pellets	3.50
Alloprevotella	D28 faeces	Meal	2.93
Rikenellaceae RC9 gut group	D28 faeces	Pellets	2.73
Subdoligranulum	D28 faeces	Pellets	2.31
Terrisporobacter	D28 faeces	Meal	2.21
Anaerovibrio	D28 faeces	Meal	2.12
Treponema	D28 faeces	Meal	2.11
UCG-005	D28 faeces	Meal	2.10
Subdoligranulum	D28 faeces	Meal	2.05

Terrisporobacter	D28 faeces	Pellets	1.97
Blautia	D28 faeces	Pellets	1.94
Phascolarctobacterium	D28 faeces	Meal	1.94
Alloprevotella	D28 faeces	Pellets	1.89
Christensenellaceae R-7 group	D28 faeces	Meal	1.79
Blautia	D28 faeces	Meal	1.61
Faecalibacterium	D28 faeces	Pellets	1.56
Anaerovibrio	D28 faeces	Pellets	1.49
Prevotellaceae UCG-003	D28 faeces	Meal	1.47
Ruminococcus	D28 faeces	Pellets	1.46
Phascolarctobacterium	D28 faeces	Pellets	1.44
Dialister	D28 faeces	Pellets	1.35
Roseburia	D28 faeces	Pellets	1.34
Roseburia	D28 faeces	Meal	1.33
Ruminococcus	D28 faeces	Meal	1.31
UCG-005	D28 faeces	Pellets	1.29
Prevotellaceae UCG-003	D28 faeces	Pellets	1.28
Treponema	D28 faeces	Pellets	1.25
Acidaminococcus	D28 faeces	Pellets	1.12
Christensenellaceae R-7 group	D28 faeces	Pellets	1.09
Prevotella	D63 faeces	Pellets	15.19
Prevotella	D63 faeces	Meal	8.12
Prevotellaceae NK3B31 group	D63 faeces	Pellets	7.59
Clostridium sensu stricto 1	D63 faeces	Meal	7.55
Rikenellaceae RC9 gut group	D63 faeces	Meal	6.67
Prevotellaceae NK3B31 group	D63 faeces	Meal	6.19
Christensenellaceae R-7 group	D63 faeces	Meal	5.57
Treponema	D63 faeces	Pellets	5.45
Rikenellaceae RC9 gut group	D63 faeces	Pellets	5.20
Muribaculaceae	D63 faeces	Pellets	5.16
Muribaculaceae	D63 faeces	Meal	4.96
Treponema	D63 faeces	Meal	4.48
Clostridium sensu stricto 1	D63 faeces	Pellets	4.47
Streptococcus	D63 faeces	Pellets	4.04
Christensenellaceae R-7 group	D63 faeces	Pellets	3.08
Lactobacillus	D63 faeces	Meal	2.95
Streptococcus	D63 faeces	Meal	2.71
Alloprevotella	D63 faeces	Pellets	2.58
Alloprevotella	D63 faeces	Meal	2.36
UCG-010	D63 faeces	Pellets	2.25
Prevotellaceae UCG-003	D63 faeces	Meal	2.04
Prevotellaceae UCG-003	D63 faeces	Pellets	1.99
Terrisporobacter	D63 faeces	Meal	1.94
UCG-005	D63 faeces	Meal	1.90

Prevotellaceae UCG-001	D63 faeces	Pellets	1.69
Terrisporobacter	D63 faeces	Pellets	1.68
Phascolarctobacterium	D63 faeces	Pellets	1.68
Prevotellaceae UCG-001	D63 faeces	Meal	1.67
UCG-005	D63 faeces	Pellets	1.65
UCG-010	D63 faeces	Meal	1.63
Parabacteroides	D63 faeces	Meal	1.49
Bacteroidales RF16 group	D63 faeces	Meal	1.48
Megasphaera	D63 faeces	Meal	1.47
Phascolarctobacterium	D63 faeces	Meal	1.47
UCG-002	D63 faeces	Meal	1.42
Clostridia vadinBB60 group	D63 faeces	Pellets	1.41
Megasphaera	D63 faeces	Pellets	1.36
UCG-002	D63 faeces	Pellets	1.35
Parabacteroides	D63 faeces	Pellets	1.27
Clostridia vadinBB60 group	D63 faeces	Meal	1.25
p-2534-18B5 gut group	D63 faeces	Meal	1.22
Ruminococcus	D63 faeces	Pellets	1.20
dgA-11 gut group	D63 faeces	Pellets	1.17
Ruminococcus	D63 faeces	Meal	1.14
Lactobacillus	D63 faeces	Pellets	1.12
Anaerovibrio	D63 faeces	Meal	1.10
Anaerovibrio	D63 faeces	Pellets	1.07
Lachnospiraceae XPB1014 group	D63 faeces	Meal	1.02
WCHB1-41	D63 faeces	Pellets	1.01
Eubacterium coprostanoligenes group	D63 faeces	Pellets	1.00

Table S 4.3: Bacterial genera ≥ 1 % mean relative abundance (RA, %) across delivery methods in the intestinal digesta and faeces of grow-finisher pigs fed the experimental diets.

Genus	Sample type	Delivery Method	Mean RA
Clostridium sensu stricto 1	Ileum	Liquid	28.98
Streptococcus	Ileum	Wet/dry	28.84
Clostridium sensu stricto 1	Ileum	Wet/dry	28.59
Clostridium sensu stricto 1	Ileum	Dry	28.34
Streptococcus	Ileum	Dry	22.02
Streptococcus	Ileum	Liquid	17.3
Terrisporobacter	Ileum	Wet/dry	13.89
Terrisporobacter	Ileum	Liquid	13.73
Lactobacillus	Ileum	Liquid	12.73
Terrisporobacter	Ileum	Dry	10.72
Lactobacillus	Ileum	Dry	9.99
Escherichia-Shigella	Ileum	Dry	7.7
Escherichia-Shigella	Ileum	Wet/dry	7.33
Lactobacillus	Ileum	Wet/dry	7.29
Megasphaera	Ileum	Liquid	4.45
Mitsuokella	Ileum	Dry	4.39
Escherichia-Shigella	Ileum	Liquid	3.9
Megasphaera	Ileum	Dry	3.79
Prevotella	Ileum	Dry	3.1
Mitsuokella	Ileum	Liquid	2.9
Actinobacillus	Ileum	Liquid	2.9
Turicibacter	Ileum	Wet/dry	2.85
Prevotella	Ileum	Liquid	2.82
Turicibacter	Ileum	Dry	2.72
Actinobacillus	Ileum	Wet/dry	2.34
Mitsuokella	Ileum	Wet/dry	2.13
Turicibacter	Ileum	Liquid	2.01
Actinobacillus	Ileum	Dry	1.98
Romboutsia	Ileum	Liquid	1.9
Romboutsia	Ileum	Wet/dry	1.65
Romboutsia	Ileum	Dry	1.35
Veillonella	Ileum	Wet/dry	1.1
Veillonella	Ileum	Liquid	1.07
Prevotella	Caecum	Liquid	17.99
Prevotella	Caecum	Wet/dry	17.59
Prevotella	Caecum	Dry	17.13
Clostridium sensu stricto 1	Caecum	Dry	14.47
Clostridium sensu stricto 1	Caecum	Wet/dry	13.78
Clostridium sensu stricto 1	Caecum	Liquid	11.00

Lactobacillus	Caecum	Liquid	6.81
Prevotellaceae NK3B31 group	Caecum	Liquid	6.33
Streptococcus	Caecum	Wet/dry	5.46
Alloprevotella	Caecum	Liquid	5.46
Terrisporobacter	Caecum	Dry	5.34
Alloprevotella	Caecum	Wet/dry	5.21
Alloprevotella	Caecum	Dry	5.14
Prevotellaceae NK3B31 group	Caecum	Dry	5.08
Terrisporobacter	Caecum	Liquid	5.00
Terrisporobacter	Caecum	Wet/dry	4.97
Streptococcus	Caecum	Dry	4.92
Prevotellaceae NK3B31 group	Caecum	Wet/dry	4.85
Lactobacillus	Caecum	Dry	3.75
Anaerovibrio	Caecum	Liquid	3.68
Megasphaera	Caecum	Dry	3.42
Streptococcus	Caecum	Liquid	3.14
Megasphaera	Caecum	Liquid	3.07
Anaerovibrio	Caecum	Dry	2.90
Anaerovibrio	Caecum	Wet/dry	2.89
Subdoligranulum	Caecum	Liquid	2.77
Megasphaera	Caecum	Wet/dry	2.76
Phascolarctobacterium	Caecum	Liquid	2.68
Subdoligranulum	Caecum	Dry	2.59
Lactobacillus	Caecum	Wet/dry	2.48
Phascolarctobacterium	Caecum	Wet/dry	2.32
Phascolarctobacterium	Caecum	Dry	2.23
Prevotellaceae UCG-003	Caecum	Wet/dry	2.23
Subdoligranulum	Caecum	Wet/dry	2.23
Blautia	Caecum	Dry	2.22
Blautia	Caecum	Wet/dry	2.13
Blautia	Caecum	Liquid	2.09
Muribaculaceae	Caecum	Dry	1.95
Rikenellaceae RC9 gut group	Caecum	Wet/dry	1.85
Muribaculaceae	Caecum	Liquid	1.85
Muribaculaceae	Caecum	Wet/dry	1.70
Faecalibacterium	Caecum	Liquid	1.64
Mitsuokella	Caecum	Dry	1.60
Rikenellaceae RC9 gut group	Caecum	Dry	1.57
Roseburia	Caecum	Liquid	1.56
Prevotellaceae UCG-003	Caecum	Dry	1.41
Rikenellaceae RC9 gut group	Caecum	Liquid	1.37
Faecalibacterium	Caecum	Wet/dry	1.36
Faecalibacterium	Caecum	Dry	1.35
Prevotellaceae UCG-003	Caecum	Liquid	1.33

UCG-005	Caecum	Liquid	1.29
UCG-005	Caecum	Dry	1.25
UCG-005	Caecum	Wet/dry	1.22
Escherichia-Shigella	Caecum	Dry	1.18
Agathobacter	Caecum	Liquid	1.18
Roseburia	Caecum	Dry	1.14
Mitsuokella	Caecum	Wet/dry	1.08
Roseburia	Caecum	Wet/dry	1.05
Gastranaerophilales	Caecum	Liquid	1.03
Turicibacter	Caecum	Wet/dry	1.01
Prevotella	Baseline faeces	Wet/dry	14.02
Prevotella	Baseline faeces	Dry	13.96
Prevotella	Baseline faeces	Liquid	12.7
Lactobacillus	Baseline faeces	Liquid	10.7
Streptococcus	Baseline faeces	Liquid	10.19
Streptococcus	Baseline faeces	Dry	8.84
Streptococcus	Baseline faeces	Wet/dry	8.55
Lactobacillus	Baseline faeces	Dry	7.24
Clostridium sensu stricto 1	Baseline faeces	Wet/dry	6.88
Megasphaera	Baseline faeces	Liquid	6.6
Lactobacillus	Baseline faeces	Wet/dry	6.06
Clostridium sensu stricto 1	Baseline faeces	Dry	5.88
Megasphaera	Baseline faeces	Wet/dry	5.55
Megasphaera	Baseline faeces	Dry	5.12
Prevotellaceae NK3B31 group	Baseline faeces	Dry	5.04
Clostridium sensu stricto 1	Baseline faeces	Liquid	4.84
Muribaculaceae	Baseline faeces	Dry	4.58
Prevotellaceae NK3B31 group	Baseline faeces	Wet/dry	4.23
Muribaculaceae	Baseline faeces	Wet/dry	4.15
Blautia	Baseline faeces	Wet/dry	4.05
Blautia	Baseline faeces	Liquid	3.88
Faecalibacterium	Baseline faeces	Liquid	3.86
Prevotellaceae NK3B31 group	Baseline faeces	Liquid	3.76
Subdoligranulum	Baseline faeces	Liquid	3.49
Blautia	Baseline faeces	Dry	3.19
Muribaculaceae	Baseline faeces	Liquid	3.18
Anaerovibrio	Baseline faeces	Dry	2.97
Phascolarctobacterium	Baseline faeces	Wet/dry	2.88
Rikenellaceae RC9 gut group	Baseline faeces	Wet/dry	2.83
Phascolarctobacterium	Baseline faeces	Liquid	2.76
Rikenellaceae RC9 gut group	Baseline faeces	Dry	2.75
Anaerovibrio	Baseline faeces	Wet/dry	2.69
Phascolarctobacterium	Baseline faeces	Dry	2.66
Subdoligranulum	Baseline faeces	Wet/dry	2.36

Rikenellaceae RC9 gut group	Baseline faeces	Liquid	2.33
Subdoligranulum	Baseline faeces	Dry	2.1
Roseburia	Baseline faeces	Dry	2.08
Anaerovibrio	Baseline faeces	Liquid	1.9
Faecalibacterium	Baseline faeces	Wet/dry	1.84
Alloprevotella	Baseline faeces	Wet/dry	1.8
Ruminococcus	Baseline faeces	Wet/dry	1.73
Roseburia	Baseline faeces	Liquid	1.68
Prevotellaceae UCG-003	Baseline faeces	Wet/dry	1.66
Ruminococcus	Baseline faeces	Dry	1.59
Terrisporobacter	Baseline faeces	Wet/dry	1.58
Alloprevotella	Baseline faeces	Liquid	1.53
Catenibacterium	Baseline faeces	Liquid	1.47
Roseburia	Baseline faeces	Wet/dry	1.43
Ruminococcus	Baseline faeces	Liquid	1.4
Faecalibacterium	Baseline faeces	Dry	1.4
Agathobacter	Baseline faeces	Liquid	1.38
Succinivibrio	Baseline faeces	Liquid	1.36
Alloprevotella	Baseline faeces	Dry	1.28
Agathobacter	Baseline faeces	Dry	1.27
Mitsuokella	Baseline faeces	Liquid	1.26
Acidaminococcus	Baseline faeces	Liquid	1.22
Succinivibrio	Baseline faeces	Dry	1.22
Prevotellaceae UCG-003	Baseline faeces	Dry	1.19
Terrisporobacter	Baseline faeces	Dry	1.14
Terrisporobacter	Baseline faeces	Liquid	1.1
Escherichia-Shigella	Baseline faeces	Wet/dry	1.06
UCG-005	Baseline faeces	Wet/dry	1.06
Treponema	Baseline faeces	Dry	1.05
Agathobacter	Baseline faeces	Wet/dry	1.03
Prevotella	D28 faeces	Wet/dry	15.78
Prevotella	D28 faeces	Liquid	15.62
Prevotella	D28 faeces	Dry	15.21
Lactobacillus	D28 faeces	Liquid	10.66
Streptococcus	D28 faeces	Wet/dry	8.39
Clostridium sensu stricto 1	D28 faeces	Wet/dry	7.50
Clostridium sensu stricto 1	D28 faeces	Dry	7.16
Lactobacillus	D28 faeces	Dry	6.87
Clostridium sensu stricto 1	D28 faeces	Liquid	6.67
Streptococcus	D28 faeces	Dry	6.64
Prevotellaceae NK3B31 group	D28 faeces	Dry	6.16
Prevotellaceae NK3B31 group	D28 faeces	Liquid	6.15
Megasphaera	D28 faeces	Wet/dry	5.11
Prevotellaceae NK3B31 group	D28 faeces	Wet/dry	4.76

Lactobacillus	D28 faeces	Wet/dry	4.60
Megasphaera	D28 faeces	Liquid	4.26
Muribaculaceae	D28 faeces	Liquid	4.15
Muribaculaceae	D28 faeces	Dry	3.94
Muribaculaceae	D28 faeces	Wet/dry	3.75
Megasphaera	D28 faeces	Dry	3.73
Rikenellaceae RC9 gut group	D28 faeces	Dry	3.54
Streptococcus	D28 faeces	Liquid	3.50
Rikenellaceae RC9 gut group	D28 faeces	Liquid	3.27
Rikenellaceae RC9 gut group	D28 faeces	Wet/dry	3.24
Terrisporobacter	D28 faeces	Wet/dry	2.47
Alloprevotella	D28 faeces	Dry	2.44
Subdoligranulum	D28 faeces	Liquid	2.42
Alloprevotella	D28 faeces	Wet/dry	2.42
Alloprevotella	D28 faeces	Liquid	2.38
Subdoligranulum	D28 faeces	Dry	2.11
Anaerovibrio	D28 faeces	Liquid	2.09
Treponema	D28 faeces	Liquid	2.02
Subdoligranulum	D28 faeces	Wet/dry	2.01
Terrisporobacter	D28 faeces	Liquid	1.99
UCG-005	D28 faeces	Liquid	1.96
Treponema	D28 faeces	Dry	1.89
Blautia	D28 faeces	Dry	1.85
Phascolarctobacterium	D28 faeces	Liquid	1.82
Terrisporobacter	D28 faeces	Dry	1.81
Blautia	D28 faeces	Liquid	1.78
Blautia	D28 faeces	Wet/dry	1.70
Anaerovibrio	D28 faeces	Wet/dry	1.70
UCG-005	D28 faeces	Dry	1.68
Phascolarctobacterium	D28 faeces	Dry	1.67
Anaerovibrio	D28 faeces	Dry	1.63
Christensenellaceae R-7 group	D28 faeces	Dry	1.57
Phascolarctobacterium	D28 faeces	Wet/dry	1.57
Christensenellaceae R-7 group	D28 faeces	Liquid	1.55
Prevotellaceae UCG-003	D28 faeces	Liquid	1.53
Ruminococcus	D28 faeces	Dry	1.51
UCG-005	D28 faeces	Wet/dry	1.45
Prevotellaceae UCG-003	D28 faeces	Dry	1.42
Roseburia	D28 faeces	Liquid	1.41
Faecalibacterium	D28 faeces	Wet/dry	1.39
Roseburia	D28 faeces	Wet/dry	1.38
Ruminococcus	D28 faeces	Wet/dry	1.37
Ruminococcus	D28 faeces	Liquid	1.27
Faecalibacterium	D28 faeces	Dry	1.23

Acidaminococcus	D28 faeces	Wet/dry	1.22
Roseburia	D28 faeces	Dry	1.21
Christensenellaceae R-7 group	D28 faeces	Wet/dry	1.19
Prevotellaceae UCG-003	D28 faeces	Wet/dry	1.17
Dialister	D28 faeces	Wet/dry	1.15
Treponema	D28 faeces	Wet/dry	1.14
Faecalibacterium	D28 faeces	Liquid	1.03
Prevotella	D63 faeces	Liquid	17.00
Prevotella	D63 faeces	Wet/dry	9.08
Prevotella	D63 faeces	Dry	8.88
Prevotellaceae NK3B31 group	D63 faeces	Dry	8.38
Prevotellaceae NK3B31 group	D63 faeces	Liquid	7.39
Rikenellaceae RC9 gut group	D63 faeces	Wet/dry	7.18
Clostridium sensu stricto 1	D63 faeces	Wet/dry	7.11
Clostridium sensu stricto 1	D63 faeces	Dry	7.06
Treponema	D63 faeces	Wet/dry	6.07
Rikenellaceae RC9 gut group	D63 faeces	Dry	5.83
Muribaculaceae	D63 faeces	Dry	5.69
Christensenellaceae R-7 group	D63 faeces	Wet/dry	5.11
Treponema	D63 faeces	Dry	5.05
Christensenellaceae R-7 group	D63 faeces	Dry	4.99
Prevotellaceae NK3B31 group	D63 faeces	Wet/dry	4.90
Muribaculaceae	D63 faeces	Wet/dry	4.89
Rikenellaceae RC9 gut group	D63 faeces	Liquid	4.79
Muribaculaceae	D63 faeces	Liquid	4.60
Clostridium sensu stricto 1	D63 faeces	Liquid	3.86
Streptococcus	D63 faeces	Liquid	3.80
Treponema	D63 faeces	Liquid	3.77
Lactobacillus	D63 faeces	Liquid	3.59
Streptococcus	D63 faeces	Dry	3.36
Streptococcus	D63 faeces	Wet/dry	2.97
Christensenellaceae R-7 group	D63 faeces	Liquid	2.87
Prevotellaceae UCG-001	D63 faeces	Wet/dry	2.72
Alloprevotella	D63 faeces	Dry	2.58
Megasphaera	D63 faeces	Liquid	2.54
Alloprevotella	D63 faeces	Liquid	2.44
Alloprevotella	D63 faeces	Wet/dry	2.39
UCG-010	D63 faeces	Wet/dry	2.31
Prevotellaceae UCG-003	D63 faeces	Wet/dry	2.20
Prevotellaceae UCG-003	D63 faeces	Dry	2.11
Bacteroidales RF16 group	D63 faeces	Dry	2.05
UCG-010	D63 faeces	Dry	1.96
Terrisporobacter	D63 faeces	Wet/dry	1.94
UCG-005	D63 faeces	Wet/dry	1.94

Terrisporobacter	D63 faeces	Dry	1.84
UCG-005	D63 faeces	Dry	1.79
Phascolarctobacterium	D63 faeces	Liquid	1.77
Prevotellaceae UCG-003	D63 faeces	Liquid	1.73
Parabacteroides	D63 faeces	Wet/dry	1.65
Terrisporobacter	D63 faeces	Liquid	1.65
UCG-005	D63 faeces	Liquid	1.61
Anaerovibrio	D63 faeces	Liquid	1.58
Phascolarctobacterium	D63 faeces	Dry	1.56
UCG-002	D63 faeces	Wet/dry	1.56
UCG-010	D63 faeces	Liquid	1.54
Clostridia vadinBB60 group	D63 faeces	Wet/dry	1.54
Parabacteroides	D63 faeces	Dry	1.53
Lactobacillus	D63 faeces	Dry	1.47
UCG-002	D63 faeces	Dry	1.46
Clostridia vadinBB60 group	D63 faeces	Dry	1.46
dgA-11 gut group	D63 faeces	Wet/dry	1.41
Phascolarctobacterium	D63 faeces	Wet/dry	1.39
Ruminococcus	D63 faeces	Wet/dry	1.21
Ruminococcus	D63 faeces	Liquid	1.20
WCHB1-41	D63 faeces	Wet/dry	1.18
Prevotellaceae UCG-001	D63 faeces	Dry	1.17
Prevotellaceae UCG-001	D63 faeces	Liquid	1.15
UCG-002	D63 faeces	Liquid	1.13
Fibrobacter	D63 faeces	Wet/dry	1.12
Anaerovibrio	D63 faeces	Dry	1.12
p-2534-18B5 gut group	D63 faeces	Liquid	1.11
Eubacterium coprostanoligenes group	D63 faeces	Wet/dry	1.11
Ruminococcus	D63 faeces	Dry	1.10
NK4A214 group	D63 faeces	Wet/dry	1.08
Lactobacillus	D63 faeces	Wet/dry	1.05
p-2534-18B5 gut group	D63 faeces	Wet/dry	1.04
WCHB1-41	D63 faeces	Dry	1.03
Subdoligranulum	D63 faeces	Liquid	1.02
Megasphaera	D63 faeces	Dry	1.00
Eubacterium coprostanoligenes group	D63 faeces	Dry	1.00
Clostridia vadinBB60 group	D63 faeces	Liquid	1.00

Table S 4.4: Differentially abundant bacterial genera between treatment groups in the intestinal digesta and faeces of grow-finisher pigs fed the experimental diets, following pairwise comparisons in DESeq2.

Treatment comparison[1]	Sample type	Genus	Base mean	Log2-fold-change	lfcSE[2]	Stat	Adjusted *p*-value[3]	Enriched group
DM - DP	Ileal digesta	*Mitsuokella*	7245.05	8.56	1.50	5.71	8.61E-08	DM
DM - DP	Ileal digesta	*Megasphaera*	8018.46	7.98	1.50	5.32	6.92E-07	DM
DM - DP	Ileal digesta	*Prevotella*	4611.63	6.02	1.42	4.26	7.53E-05	DM
DM - LP	Ileal digesta	*Lactobacillus*	18643.69	4.61	1.09	4.23	7.33E-05	DM
DM - LP	Ileal digesta	*Mitsuokella*	7245.05	9.85	1.41	6.97	1.80E-11	DM
DM - LP	Ileal digesta	*Megasphaera*	8018.46	9.45	1.41	6.68	1.22E-10	DM
DM - LP	Ileal digesta	*Prevotella*	4611.63	6.84	1.33	5.13	1.26E-06	DM
DM - WDM	Ileal digesta	*Mitsuokella*	7245.05	3.70	1.45	2.55	3.62E-02	DM
DM - WDM	Ileal digesta	*Megasphaera*	8018.46	4.91	1.45	3.38	3.38E-03	DM
DM - WDM	Ileal digesta	*Prevotella*	4611.63	6.41	1.37	4.68	1.94E-05	DM
DM - WDP	Ileal digesta	*Lactobacillus*	18643.69	3.09	1.20	2.56	2.22E-02	WDP
DM - WDP	Ileal digesta	*Mitsuokella*	7245.05	11.94	1.58	7.56	3.82E-13	DM
DM - WDP	Ileal digesta	*Megasphaera*	8018.46	10.36	1.57	6.61	2.24E-10	DM
DM - WDP	Ileal digesta	*Prevotella*	4611.63	9.08	1.48	6.14	4.22E-09	DM
DP - LM	Ileal digesta	*Escherichia-Shigella*	4857.56	2.17	0.70	3.09	5.83E-03	DP
DP - LM	Ileal digesta	*Mitsuokella*	7245.05	-6.83	1.45	-4.71	9.74E-06	LM
DP - LM	Ileal digesta	*Megasphaera*	8018.46	-7.07	1.45	-4.87	5.17E-06	LM
DP - LM	Ileal digesta	*Prevotella*	4611.63	-4.85	1.37	-3.54	1.35E-03	LM
DP - LP	Ileal digesta	*Lactobacillus*	18643.69	4.95	1.09	4.54	4.45E-05	DP
DP - LP	Ileal digesta	*Escherichia-Shigella*	4857.56	2.50	0.68	3.66	1.70E-03	DP
DP - WDM	Ileal digesta	*Lactobacillus*	18643.69	2.91	1.12	2.60	2.45E-02	DP

DP - WDM	Ileal digesta	*Escherichia-Shigella*	4857.56	2.16	0.70	3.08	6.93E-03	DP
DP - WDM	Ileal digesta	*Mitsuokella*	7245.05	-4.87	1.45	-3.35	2.87E-03	WDM
DP - WDP	Ileal digesta	*Lactobacillus*	18643.69	3.42	1.20	2.84	3.02E-02	DP
LM - LP	Ileal digesta	*Lactobacillus*	18643.69	4.97	1.05	4.72	1.38E-05	LM
LM - LP	Ileal digesta	*Mitsuokella*	7245.05	8.12	1.36	5.96	2.33E-08	LM
LM - LP	Ileal digesta	*Megasphaera*	8018.46	8.54	1.36	6.27	4.32E-09	LM
LM - LP	Ileal digesta	*Prevotella*	4611.63	5.66	1.29	4.40	5.54E-05	LM
LM - WDM	Ileal digesta	*Lactobacillus*	18643.69	2.93	1.08	2.70	3.04E-02	LM
LM - WDM	Ileal digesta	*Megasphaera*	8018.46	4.00	1.40	2.86	2.24E-02	LM
LM - WDM	Ileal digesta	*Prevotella*	4611.63	5.24	1.32	3.96	5.12E-04	LM
LM - WDP	Ileal digesta	*Lactobacillus*	18643.69	3.44	1.17	2.94	9.08E-03	LM
LM - WDP	Ileal digesta	*Mitsuokella*	7245.05	10.21	1.54	6.65	2.70E-10	LM
LM - WDP	Ileal digesta	*Megasphaera*	8018.46	9.46	1.52	6.21	4.03E-09	LM
LM - WDP	Ileal digesta	*Prevotella*	4611.63	7.91	1.44	5.51	2.42E-07	LM
LP - WDM	Ileal digesta	*Mitsuokella*	7245.05	-6.16	1.36	-4.52	4.85E-05	WDM
LP - WDM	Ileal digesta	*Megasphaera*	8018.46	-4.54	1.36	-3.33	5.08E-03	WDM
WDM - WDP	Ileal digesta	*Mitsuokella*	7245.05	8.25	1.54	5.37	6.09E-07	WDM
WDM - WDP	Ileal digesta	*Megasphaera*	8018.46	5.46	1.52	3.58	1.76E-03	WDM
DM - DP	Caecal digesta	*Clostridium sensu stricto 1*	10794.55	0.80	0.23	3.53	7.14E-03	DM
DM - LM	Caecal digesta	*Clostridium sensu stricto 1*	10794.55	0.99	0.22	4.51	2.24E-04	DM
DM - LM	Caecal digesta	*Lactobacillus*	3890.41	-1.86	0.66	-2.82	4.38E-02	LM
DM - LP	Caecal digesta	*Clostridium sensu stricto 1*	10794.55	0.75	0.21	3.51	1.02E-02	DM
DM - LP	Caecal digesta	*Mitsuokella*	941.82	2.19	0.69	3.19	2.41E-02	DM
DP - LM	Caecal digesta	*Streptococcus*	3813.57	2.07	0.55	3.79	2.27E-03	DP
DP - LM	Caecal digesta	*Lactobacillus*	3890.41	-2.26	0.66	-3.41	8.80E-03	LM

DP - LM	Caecal digesta	*Escherichia-Shigella*	766.55	3.46	0.78	4.42	1.64E-04	DP
LM - LP	Caecal digesta	*Lactobacillus*	3890.41	3.48	0.62	5.61	5.63E-07	LM
LM - LP	Caecal digesta	*Escherichia-Shigella*	766.55	-2.49	0.73	-3.39	1.04E-02	LP
LM - WDM	Caecal digesta	*Clostridium sensu stricto 1*	10794.55	-0.73	0.21	-3.43	1.04E-02	WDM
LM - WDM	Caecal digesta	*Streptococcus*	3813.57	-1.64	0.53	-3.11	2.49E-02	WDM
LM - WDM	Caecal digesta	*Lactobacillus*	3890.41	3.23	0.64	5.05	1.98E-05	LM
LM - WDM	Caecal digesta	*Escherichia-Shigella*	766.55	-2.68	0.75	-3.55	7.35E-03	WDM
LM - WDP	Caecal digesta	*Streptococcus*	3813.57	-2.18	0.57	-3.83	2.21E-03	WDP
LM - WDP	Caecal digesta	*Escherichia-Shigella*	766.55	-2.55	0.82	-3.12	2.44E-02	WDP
DM - DP	D28 faeces	*Streptococcus*	3065.63	-1.17	0.35	-3.33	1.26E-02	DP
DM - LM	D28 faeces	*Streptococcus*	3065.63	1.97	0.35	5.60	3.15E-06	DM
DM - WDP	D28 faeces	*Prevotella*	7562.87	-1.03	0.28	-3.66	1.90E-03	WDP
DM - WDP	D28 faeces	*Lactobacillus*	3588.67	1.55	0.44	3.54	2.83E-03	DM
DM - WDP	D28 faeces	*Streptococcus*	3065.63	-1.57	0.35	-4.46	1.07E-04	WDP
DM - WDP	D28 faeces	*Megasphaera*	2201.20	-1.69	0.43	-3.91	7.68E-04	WDP
DM - WDP	D28 faeces	*Mitsuokella*	229.45	-2.55	0.77	-3.32	5.44E-03	WDP
DP - LM	D28 faeces	*Lactobacillus*	3588.67	-1.76	0.44	-4.02	1.68E-03	LM
DP - LM	D28 faeces	*Streptococcus*	3065.63	3.15	0.35	8.92	3.40E-17	DP
DP - LP	D28 faeces	*Escherichia-Shigella*	24.28	4.45	1.18	3.77	1.15E-02	DP
DP - WDM	D28 faeces	*Prevotella*	7562.87	0.82	0.28	2.91	4.26E-02	DP
DP - WDM	D28 faeces	*Clostridium sensu stricto 1*	3319.02	-0.81	0.29	-2.83	4.82E-02	WDM
LM - LP	D28 faeces	*Lactobacillus*	3588.67	1.33	0.44	3.03	3.09E-02	LM
LM - LP	D28 faeces	*Streptococcus*	3065.63	-2.45	0.35	-6.95	2.16E-10	LP
LM - LP	D28 faeces	*Escherichia-Shigella*	24.28	3.67	1.18	3.11	2.86E-02	LM
LM - WDM	D28 faeces	*Clostridium sensu stricto 1*	3319.02	-0.92	0.29	-3.21	3.79E-02	WDM

LM - WDM	D28 faeces	*Streptococcus*	3065.63	-2.45	0.35	-6.95	5.15E-10	WDM
LM - WDP	D28 faeces	*Prevotella*	7562.87	-0.97	0.28	-3.44	4.05E-03	WDP
LM - WDP	D28 faeces	*Lactobacillus*	3588.67	2.51	0.44	5.73	3.35E-07	LM
LM - WDP	D28 faeces	*Streptococcus*	3065.63	-3.54	0.35	-10.05	6.32E-22	WDP
LP - WDM	D28 faeces	*Prevotella*	7562.87	0.87	0.28	3.08	2.96E-02	LP
LP - WDP	D28 faeces	*Streptococcus*	3065.63	-1.09	0.35	-3.11	2.07E-02	WDP
LP - WDP	D28 faeces	*Megasphaera*	2201.20	-1.22	0.43	-2.83	4.22E-02	WDP
LP - WDP	D28 faeces	*Mitsuokella*	229.45	-2.55	0.77	-3.32	1.59E-02	WDP
LP - WDP	D28 faeces	*Escherichia-Shigella*	24.28	-3.91	1.18	-3.32	1.59E-02	WDP
WDM - WDP	D28 faeces	*Prevotella*	7562.87	-1.25	0.28	-4.42	1.42E-04	WDP
WDM - WDP	D28 faeces	*Lactobacillus*	3588.67	1.28	0.44	2.92	1.70E-02	WDM
WDM - WDP	D28 faeces	*Clostridium sensu stricto 1*	3319.02	0.86	0.29	3.01	1.39E-02	WDM
WDM - WDP	D28 faeces	*Streptococcus*	3065.63	-1.09	0.35	-3.10	1.10E-02	WDP
WDM - WDP	D28 faeces	*Megasphaera*	2201.20	-1.30	0.43	-3.02	1.39E-02	WDP
WDM - WDP	D28 faeces	*Mitsuokella*	229.45	-2.90	0.77	-3.77	1.29E-03	WDP
DM - DP	D63 faeces	*Megasphaera*	737.78	-3.70	0.86	-4.28	6.34E-04	DP
DM - LM	D63 faeces	*Prevotella*	4665.16	-2.08	0.47	-4.40	2.33E-04	LM
DM - LM	D63 faeces	*Lactobacillus*	906.76	-3.09	0.67	-4.62	1.17E-04	LM
DM - LM	D63 faeces	*Megasphaera*	737.78	-5.23	0.86	-6.06	1.07E-07	LM
DM - LM	D63 faeces	*Mitsuokella*	73.05	-7.05	1.62	-4.35	2.51E-04	LM
DM - LP	D63 faeces	*Prevotella*	4665.16	-1.89	0.47	-4.01	1.33E-03	LP
DM - LP	D63 faeces	*Clostridium sensu stricto 1*	2011.12	1.12	0.30	3.70	3.68E-03	DM
DM - LP	D63 faeces	*Megasphaera*	737.78	-2.55	0.86	-2.96	2.49E-02	LP
DM - LP	D63 faeces	*Mitsuokella*	73.05	-4.94	1.62	-3.04	2.15E-02	LP
DM - WDP	D63 faeces	*Prevotella*	4665.16	-1.57	0.47	-3.32	1.51E-02	WDP

DM - WDP	D63 faeces	*Megasphaera*	737.78	-2.89	0.86	-3.35	1.51E-02	WDP
DM - WDP	D63 faeces	*Mitsuokella*	73.05	-4.56	1.62	-2.81	4.25E-02	WDP
DP - LM	D63 faeces	*Lactobacillus*	906.76	-2.71	0.67	-4.05	1.90E-03	LM
DP - WDM	D63 faeces	*Prevotella*	4665.16	2.05	0.47	4.35	4.06E-04	DP
DP - WDM	D63 faeces	*Clostridium sensu stricto 1*	2011.12	-0.80	0.30	-2.65	4.93E-02	WDM
DP - WDM	D63 faeces	*Streptococcus*	1441.57	2.22	0.72	3.08	2.22E-02	DP
DP - WDM	D63 faeces	*Megasphaera*	737.78	4.48	0.87	5.18	1.13E-05	DP
DP - WDM	D63 faeces	*Escherichia-Shigella*	42.59	2.29	0.84	2.75	4.02E-02	DP
LM - LP	D63 faeces	*Lactobacillus*	906.76	3.36	0.67	5.03	2.95E-05	LM
LM - LP	D63 faeces	*Megasphaera*	737.78	2.68	0.86	3.10	3.55E-02	LM
LM - WDM	D63 faeces	*Prevotella*	4665.16	2.84	0.47	6.03	6.40E-08	LM
LM - WDM	D63 faeces	*Clostridium sensu stricto 1*	2011.12	-0.77	0.30	-2.53	3.83E-02	WDM
LM - WDM	D63 faeces	*Streptococcus*	1441.57	2.53	0.72	3.51	2.53E-03	LM
LM - WDM	D63 faeces	*Lactobacillus*	906.76	3.18	0.67	4.75	3.78E-05	LM
LM - WDM	D63 faeces	*Megasphaera*	737.78	6.02	0.87	6.95	2.78E-10	LM
LM - WDM	D63 faeces	*Mitsuokella*	73.05	5.10	1.60	3.20	6.39E-03	LM
LM - WDM	D63 faeces	*Escherichia-Shigella*	42.59	2.01	0.84	2.40	4.93E-02	LM
LM - WDP	D63 faeces	*Lactobacillus*	906.76	3.66	0.67	5.47	3.37E-06	LM
LP - WDM	D63 faeces	*Prevotella*	4665.16	2.66	0.47	5.63	9.05E-07	LP
LP - WDM	D63 faeces	*Clostridium sensu stricto 1*	2011.12	-1.24	0.30	-4.10	5.68E-04	WDM
LP - WDM	D63 faeces	*Megasphaera*	737.78	3.34	0.87	3.86	1.25E-03	LP
WDM - WDP	D63 faeces	*Prevotella*	4665.16	-2.33	0.47	-4.95	2.82E-05	WDP
WDM - WDP	D63 faeces	*Clostridium sensu stricto 1*	2011.12	0.85	0.30	2.81	3.09E-02	WDM
WDM - WDP	D63 faeces	*Streptococcus*	1441.57	-2.03	0.72	-2.81	3.09E-02	WDP
WDM - WDP	D63 faeces	*Megasphaera*	737.78	-3.68	0.87	-4.25	4.01E-04	WDP

[1] Treatment groups were abbreviated as follows: DM = Dry meal-fed pigs, DP = Dry pellet-fed pigs, LM = Liquid meal-fed pigs, LP = Liquid pellet-fed pigs, WDM = Wet/dry meal-fed pigs, WDP = Wet/dry pellet-fed pigs.
[2] lfcSE: Standard error of the log2 fold-change.
[3] Benjamini-Hochberg adjusted *p*-value.

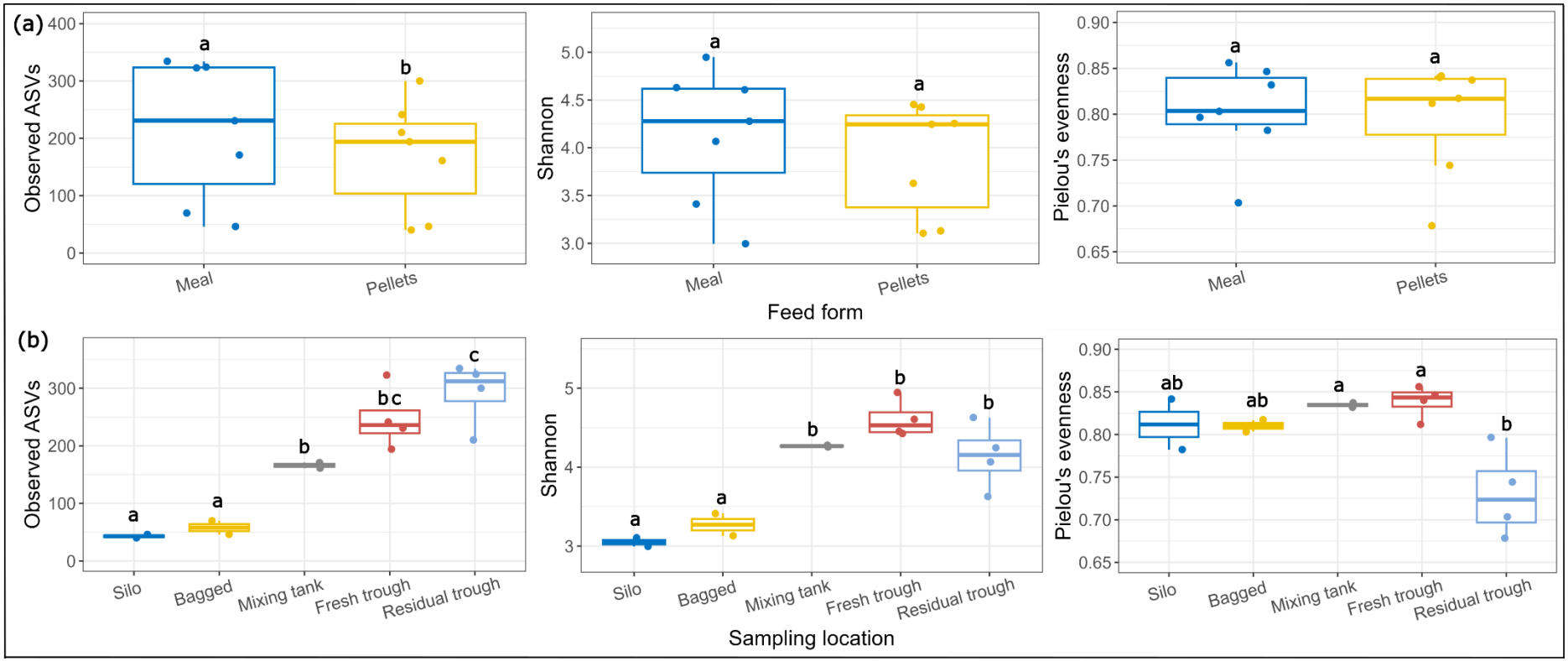

Figure S 4.1: Boxplots displaying alpha-diversity [Observed amplicon sequence variants (ASVs), Shannon diversity and Pielou's evenness] of the feed bacteriome of the experimental diets on day (D)27 of the experiment by **(a)** feed form (meal; n =7, or pellets; n = 7) and **(b)** sampling location. Dry feed collected from the silo (n = 2) was used to prepare the liquid diets while the bagged dry feed (n = 2) was used for the dry and wet/dry diets. The mixing tank (n = 2), fresh (n = 4) and residual trough (n = 4) samples were collected for the liquid feeding treatments. For each individual boxplot, feed forms or sampling locations that do not share a common letter are significantly different ($p \leq 0.05$).

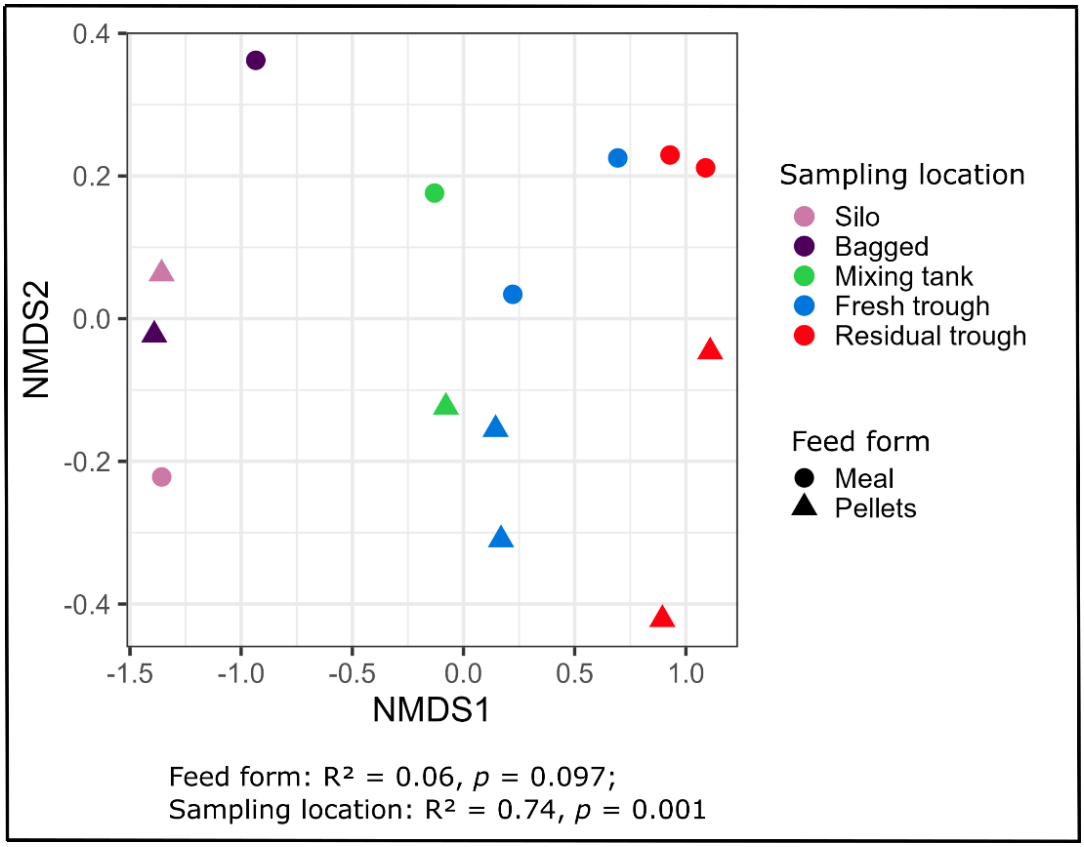

Figure S 4.2: Non-metric multidimensional scaling (NMDS) plots based on Bray-Curtis dissimilarity in feed collected on day (D)27 of the experiment by sampling location and feed form (meal or pellets). Feed collected from the silo (n = 2) was used to prepare the liquid diets while the bagged feed (n = 2) was used for the dry and wet/dry diets. The mixing tank (n = 2), fresh (n = 4) and residual trough (n = 4) samples were collected for the liquid feeding treatments. Permutational analysis of variance (PERMANOVA) results for the feed samples are presented below each respective plot.

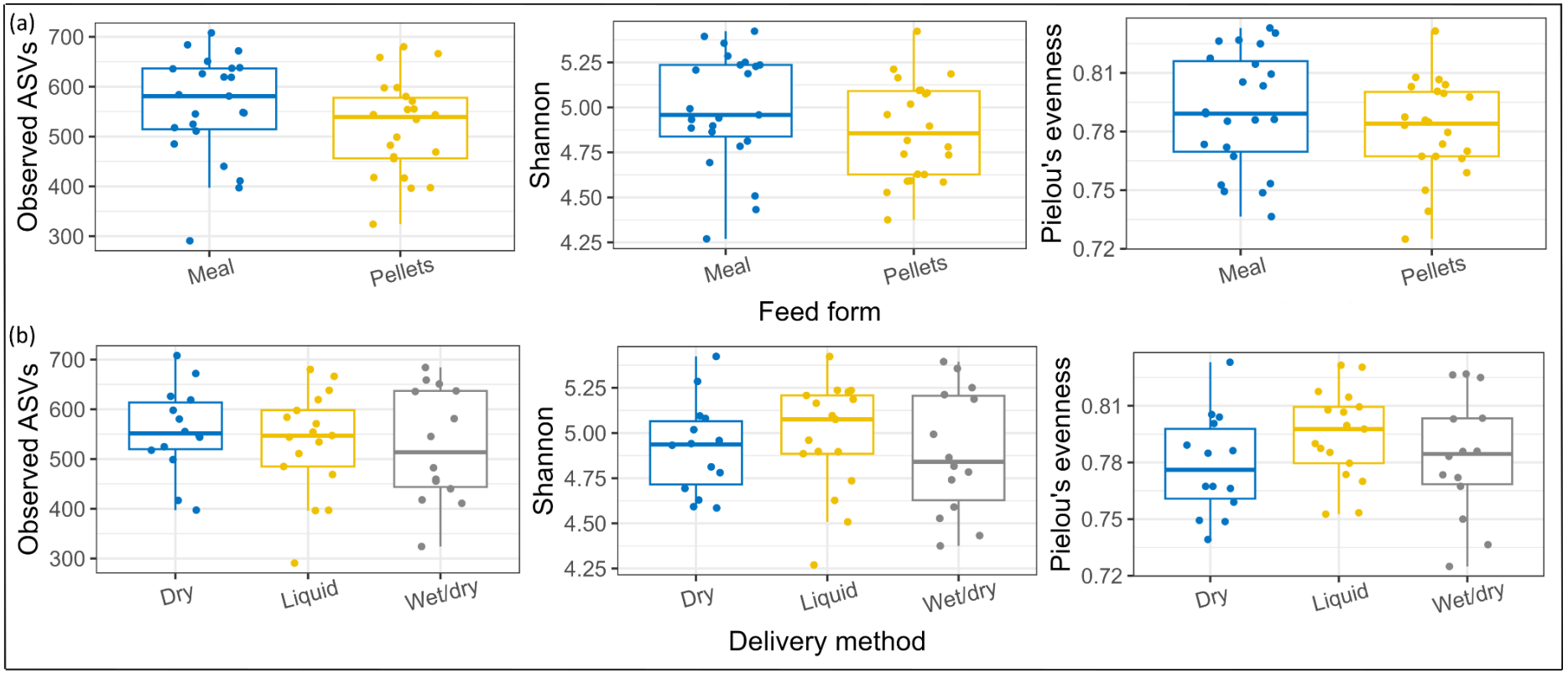

Figure S 4.3: Boxplots displaying alpha-diversity [Observed amplicon sequence variants (ASVs), Shannon diversity and Pielou's evenness] of the bacteriome in the caecal digesta of grow-finisher pigs fed dry, liquid or wet/dry feed in meal or pelleted form. **(a)** Alpha-diversity by feed form (meal; *n* = 23, or pellets; *n* = 22), averaged across delivery methods. **(b)** Alpha-diversity by delivery method (dry; *n* = 14, liquid; *n* = 17, or wet/dry; *n* =14), averaged across feed forms).

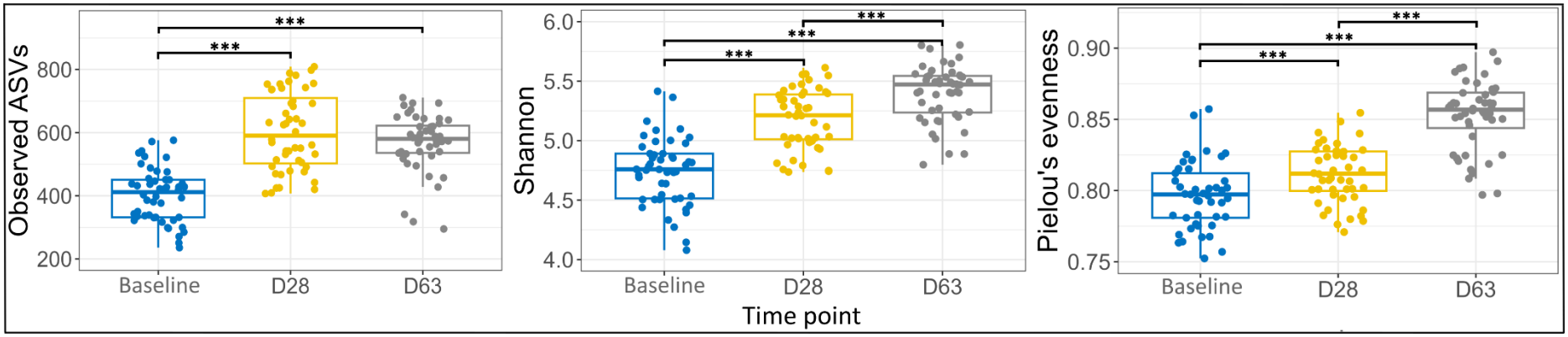

Figure S 4.4: Boxplots displaying alpha-diversity [Observed amplicon sequence variants (ASVs), Shannon diversity and Pielou's evenness] of the faecal bacteriome of grow-finisher pigs fed dry, liquid or wet/dry feed in meal or pelleted form at baseline, on day 28 (D28) and day 63 (D63) of the experiment. Data are averaged across feed forms and delivery methods for each time point, except at baseline where all diets were fed in meal form. *** $p < 0.001$.

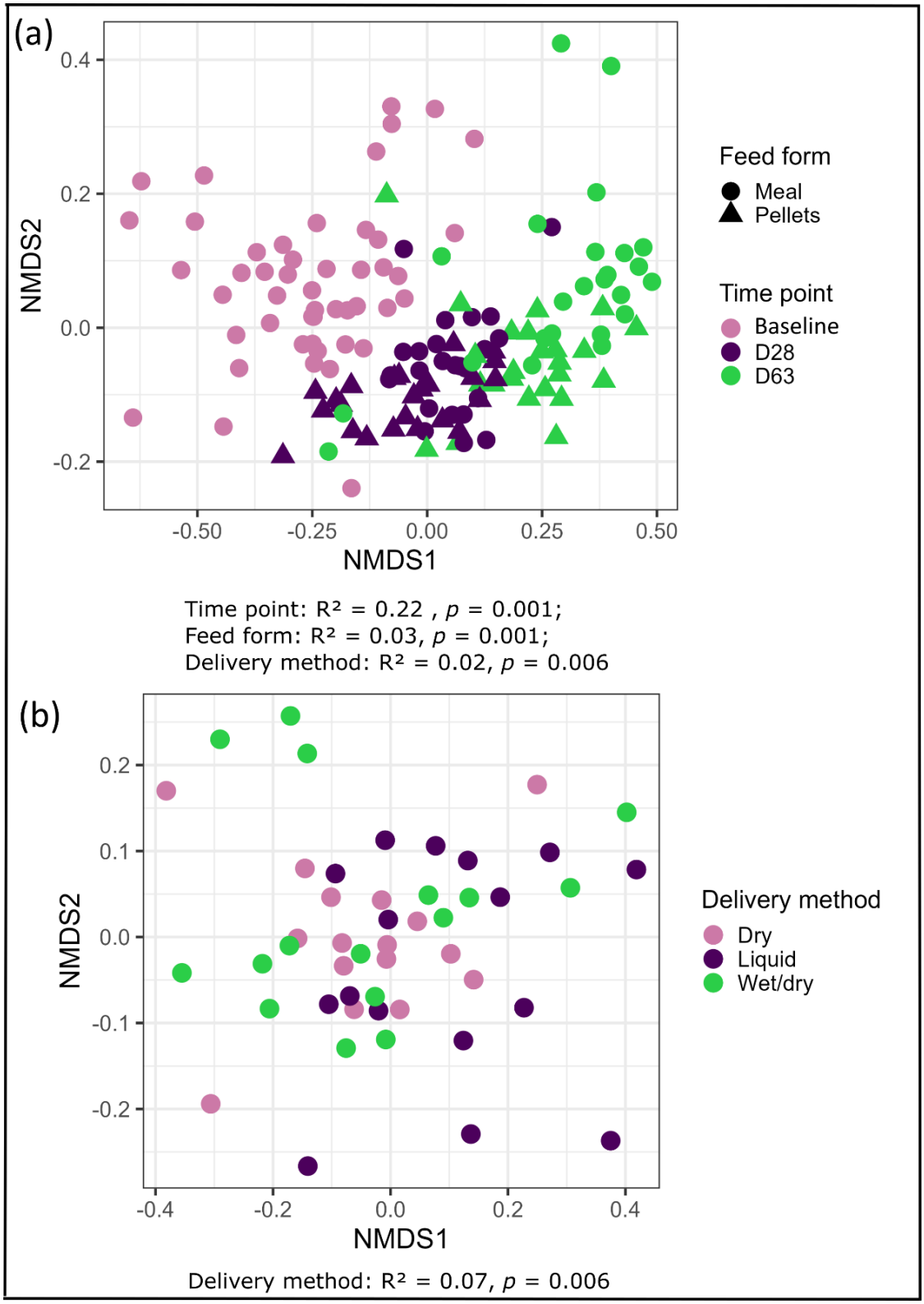

Figure S 4.5: Non-metric multidimensional scaling (NMDS) plots based on Bray-Curtis dissimilarity in the faeces of grow-finisher pigs **(a)** by feed form and time point at baseline (n = 48), on D28 (n = 48) and D63 (n = 48), and **(b)** by delivery method at baseline (n = 48) where all diets were fed in meal form. Permutational analysis of variance (PERMANOVA) results for the faecal samples are presented below each respective plot.

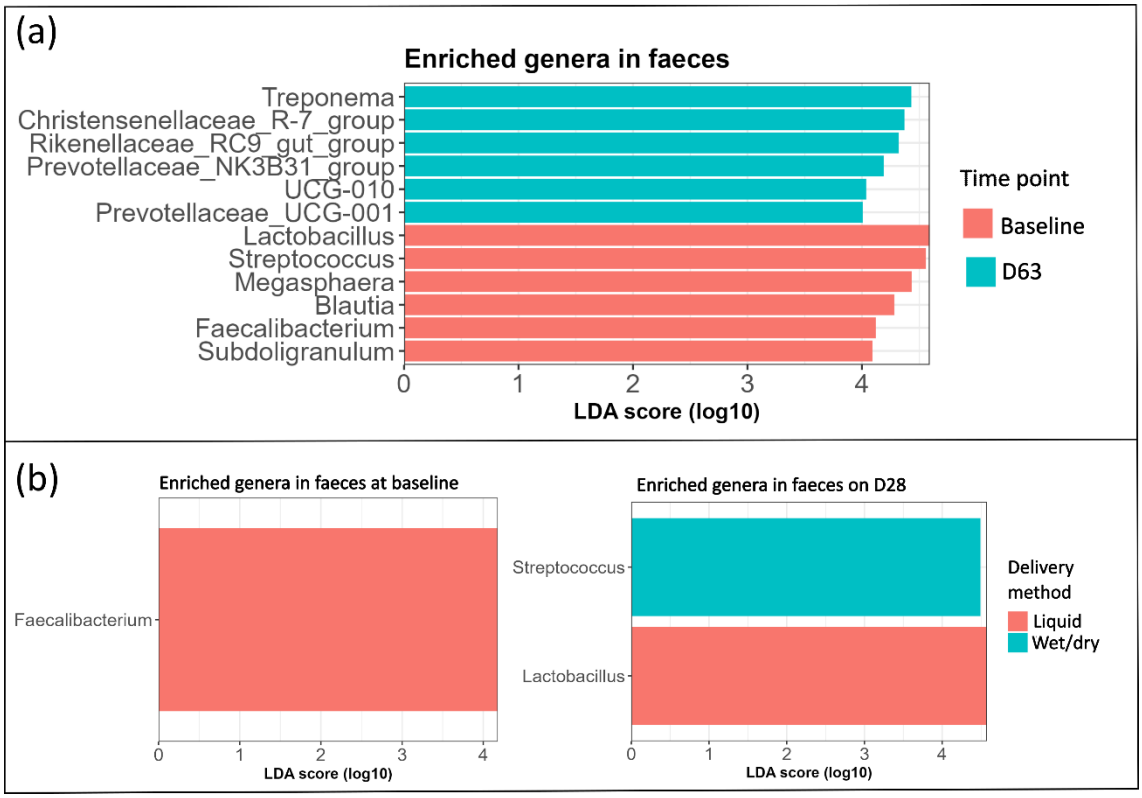

Figure S 4.6: Differentially abundant bacterial genera in the faeces of grow-finisher pigs fed dry, liquid or wet/dry feed in meal or pelleted form by **(a)** time point [baseline; *n* = 48, D28; *n* = 48, and D63; *n* = 48], and **(b)** between delivery methods (dry; *n* =16, liquid; *n* =16, or wet/dry; *n* = 16) at baseline and on D28. Note that at baseline all diets were fed in meal form. Differential abundances between time points and delivery methods were identified by linear discriminant analysis (LDA) with effect size (LEfSe). Only genera with an LDA score (log_{10}) > 4.0 are shown. The colour of the bars for each respective plot represents the treatment group in which the differentially abundant genera had the highest relative abundance (%).

4.9 References

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Chapter 5: Optimising the hygiene of a liquid feeding system to improve the quality of liquid feed for pigs

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5.1 Abstract

Poor feeding system hygiene may contribute to uncontrolled spontaneous fermentation in liquid pig feed and its associated undesirable effects. This study aimed to determine the effects of an intensive sanitisation programme in a grow-finisher liquid feeding system by monitoring microbiological and physico-chemical parameters of liquid feed and microbial colonisation of the feeding system surfaces. The sanitisation programme involved a combination of physical and chemical cleaning between batches of grow-finisher pigs, combined with nightly rinsing of the system with an organic acid blend. Improved hygiene of the internal surfaces of the mixing tank and feed pipeline, particularly until week 5 post-cleaning (PC), was evidenced by reduced counts of lactic acid bacteria, total aerobes, *Enterobacteriaceae*, yeasts and moulds and decreased adenosine triphosphate concentrations. *Enterobacteriaceae* and moulds remained undetectable on pipeline surfaces for 10 weeks. Scanning electron microscopy of the feed pipelines confirmed these findings. Conversely, the impact on liquid feed microbiology was minimal and short-lived. However, acetic acid, ethanol and biogenic amine concentrations decreased in the feed PC and no gross energy losses were observed. Therefore, by controlling surface microbial communities on liquid feeding systems via implementation of the sanitisation programme developed in the current study, on-farm liquid feed quality should be improved.

5.2 Introduction

The frequency of cleaning of liquid feeding systems on pig farms ranges from no cleaning at all, to only cleaning between batches of pigs, with no standard guidelines available for producers (Heller et al., 2017; O'Meara et al., 2020a). Lack of cleaning can lead to a build-up of feed residues and biofilms within the system. Biofilms are surface-associated microbial communities that secrete polymeric substances extracellularly, facilitating the formation of a protective matrix, and known for their resistance to antimicrobials and disinfectants (Donlan, 2001). Although fresh liquid feed has a high initial microbial load, feed residues and biofilms likely seed the fresh liquid feed with microbes as it passes through the system, potentially accelerating uncontrolled spontaneous fermentation of feed. As seen in Chapter 3, spontaneous fermentation is common in liquid feed on commercial pig farms. It can lead to the proliferation of undesirable bacteria and fungi, with a subsequent loss of dietary energy and amino acids and the concomitant production of undesirable microbial

metabolites, such as biogenic amines and excessive acetic acid and ethanol (Brooks et al., 2001; O'Meara et al., 2020a). This may be responsible, at least in part, for the poorer feed conversion efficiency (FCE) of liquid-fed pigs, as found in Chapter 4 where the feed efficiency of liquid meal-fed grow-finisher pigs was 0.26 of an FCE unit poorer than dry pellet-fed pigs. A disimprovement of 0.20 of an FCE unit equates to an increase in feed cost of ~€5.10 per pig, based on a 5-year average finisher feed price (Lawlor & Viard, 2021; O'Meara et al., 2020b). Therefore, improving the microbial and nutritional quality of liquid feed is particularly pertinent to improve feed efficiency and reduce feed costs. This is an issue particularly with short-trough *ad-libitum* liquid feeding, the FCE disparity between liquid- and dry-fed pigs may not be as large with restricted long-trough liquid feeding.

There are conflicting reports on the impact of liquid feeding system cleaning practices on the microbiological quality of liquid feed. Fisker & Jørgensen (2010) reported no significant differences in pH, organic acid and biogenic amine concentrations, or in counts of *Enterobacteriaceae,* lactic acid bacteria (LAB), yeasts, moulds or *Clostridium perfringens* in liquid feed sampled after cleaning and disinfection of the feeding system. Hansen (1987) reported that disruption to the feed microbiota after cleaning, allowed coliforms to proliferate in the days following cleaning, similar to the undesirable microbial growth that occurs during the first phase of feed fermentation (Brooks, 2008). It has also been reported that total mesophilic bacteria, LAB and coliform counts in 'contact water' that simulated liquid feed passing through the feeding system, decreased by 2-3 $\log_{10}$ CFU/mL following cleaning of liquid feeding systems (Royer et al., 2004b, 2005). It should be noted that the feeding systems involved in these studies used 'residue-containing' liquid feeding systems, i.e. where feed or water sits in the pipelines between feeds. In these systems, water or residual feed from the feed pipeline is recirculated to the mixing tank containing the next batch of feed. With all of these practices, there is more time for feed fermentation to occur and the residual feed/water to act as an inoculum for the next batch of feed, thereby potentially accelerating feed fermentation. Royer et al. (2005) highlighted the importance of minimising stagnant residues in liquid feeding system pipes between feeds.

To our knowledge, this study is the first to implement a sanitisation program in a 'residue-free' liquid feeding system that uses high-pressure air to ensure that no feed residue remains in the pipelines between feeds. The objective was to determine the effects of implementation of an intensive sanitisation regime in a grow-finisher liquid feeding system on microbial counts and adenosine triphosphate (ATP) concentrations on the mixing tank and pipeline surfaces, and for the first time, to use scanning electron microscopy (SEM) to examine the internal pipe surfaces. The impact on liquid feed microbial counts, pH, temperature, gross energy (GE), biogenic amines and organic acid concentrations were also measured. The hypothesis was that by implementing an intensive sanitisation programme, energy and amino acid losses from the liquid diet and the production of undesirable microbial metabolites would be reduced as a consequence of reducing undesirable microbial growth.

5.3 Materials and Methods

5.3.1 Ethical approval

Ethical approval for this study was granted by the Teagasc Animal Ethics Committee (approval no. TAEC2020-271). The experiment was conducted in accordance with the legislation for commercial pig production set out in the European communities (welfare of farmed animals) regulations 2010 and in Irish legislation (SI no. 311/2010).

5.3.2 Sanitisation of liquid feeding system

A two-step sanitisation programme was implemented on the automated liquid feeding system (HydroMix, BigDutchman, Vechta, Germany) in the grow-finisher section of the research pig unit at Teagasc, Moorepark, Fermoy, Co. Cork. Sanitisation was performed during the routine unit cleaning that is normally carried out between batches of pigs. A schematic diagram outlining the sanitisation programme and sampling time points is shown in Figure 5.1.

5.3.2.1. *Initial intensive cleaning*

The mixing tank lid was removed, scrubbed clean and rinsed with water (~5 min). Both the wash balls and the exhaust pipe were removed from the 500 L mixing tank and were scrubbed clean and rinsed with water (~10 min). An air gun was used to

remove feed and debris from the external surfaces of the mixing tank (~10 min). The area where dry feed enters the mixing tank was also scrubbed to remove feed debris (~5 min). Next, the inside of the mixing tank was scrubbed with a long-handled brush to remove feed debris from the tank (30 min) and was power washed for ~7.5 min (40.6 L of water). The agitator fins were cleaned by scrubbing, followed by power washing for ~5 min with the agitators turned on (~27.5 L water). An additional 100 L of water was brought into the mixing tank via the two wash balls to rinse the residual debris from the tank (with agitation) and was dispensed out through the feedlines to the troughs.

For the alkali wash, 450 L of water was brought into the mixing tank and Avalksan Gold Standard Chlorine Free (Carbon Chemicals Group Ltd., Ringaskiddy, Co. Cork, Ireland) which contains 24 % caustic, surfactants and wetting agents, was added at a 0.9 % inclusion rate (4.05 L). Nine recirculation cycles were performed in 2-hour intervals. The mixing tank agitator was turned on for the duration of the recirculation cycles. The recirculation time (the time taken for the alkali wash to leave the mixing tank, pass through the main feedline and return to the mixing tank) was 10 min. Therefore, the contact time of the alkali wash with the main feedline was 1.5 hours.

5.3.2.2. *Acid wash and set up of nightly acid rinse*

After the last overnight recirculation cycle, the alkali wash was dispensed out to each trough to ensure that the down pipes were rinsed with the alkali. Approximately 100 L of water was brought into the mixing tank and recirculated through the main line to remove residual alkali, which was dispensed out to the troughs. To ensure complete rinsing of the tank and feedlines, ~460 L of water was brought into the mixing tank. This water was dispensed out to each trough, as with the alkali wash, to ensure that the down pipes were rinsed. A pump was set up to dose a feed-grade acid blend [Interpronutri Plus BE; Interchem (Ireland) Ltd., Co. Dublin, Ireland] containing 60 % formic acid, 15 % propionic acid and 2.5 % benzoic acid, into the mixing tank. An initial 155 L acid wash (140 % of main feedline capacity plus an additional 100 L) containing 0.6 % Interpronutri Plus BE (930 mL) was set up and 4-hourly recirculation cycles were performed (10 min recirculation time). The acid wash was dispensed out to each trough and the troughs were emptied and washed with water.

Finally, a 55 L nightly maintenance acid rinse (140 % of main feedline capacity) containing 0.85 % Interpronutri Plus BE (472 mL) was set up to occur overnight for the duration of the experiment. Six hourly recirculation cycles (10 min recirculation time) were performed for each nightly acid rinse. The acid rinse was allowed to sit in the main feedline for 2 hours until the first feed of each day when it was returned to the mixing tank to form part of the liquid portion of the first feed of the day. Therefore, overnight there was a total of 3 hours of acid contact time in the main feedline. From day (d)1 post-cleaning (PC), a new batch of pigs was introduced to their pens where they were liquid-fed from the sanitised system for a 76-day grow-finisher period, with sampling performed up to week (wk) 10 PC, as outlined in Figure 5.1 and detailed below.

5.3.3 Diet preparation and feeding

The experimental diet was manufactured in meal form at the Teagasc Moorepark feed mill. All ingredients were milled through a 3 mm screen before incorporation in the diet. The ingredient composition and calculated chemical composition at formulation of the diet is given in Table S5.1. Pigs were provided with as close to *ad libitum* access to liquid feed as possible, with 6 sensor checks per day, and care taken to minimise feed wastage. Additional water was available from one drinking bowl per pen (DRIK-O-MAT, Egebjerg International A/S, Egebjerg, Denmark). Feeding was based on a feeding curve that supplied 18 MJ digestible energy (DE) per pig per day at the start of the experiment, increasing up to 50 MJ DE per pig per day at the end of the experiment.

Liquid feed was prepared in the mixing tank of the automated liquid feeding system 6 times per day at a water to feed ratio of 3:1 on a dry matter (DM) basis (equivalent to ~2.5:1 on a fresh matter basis). In the mixing tank, the liquid feed was agitated for 10 min with a 6-fin agitator prior to feed-out. The feed was delivered from the mixing tank to the troughs via the feedline using high-pressure air. At each feeding, electronic sensors in each trough (100 x 32.5 x 21 cm) ensured that when feed was above the sensor in the trough, feed was not dispensed to that particular trough. When the feed was below the sensor, feed was dispensed.

5.3.4 Animal housing, management and records

On d1 PC, 180 Danavil Duroc × (Landrace × Large White) female and entire male pigs (35.0 kg ± 4.90 SD) were introduced to the grow-finisher house. Pigs were penned in groups of 5 across 36 pens (dimensions: 2.37 m x 2.36 m) with concrete slatted floors. The air temperature was maintained at 20-22 °C by a mechanical ventilation system with fan speed and air inlets regulated by a Steinen PCS 8100 climate controller (Steinen BV, Nederwert, The Netherlands). All veterinary treatments were recorded including pig ID, symptoms, medication, dosage and duration of treatment.

Pen-group weights were recorded at the start (d1 PC), and at the end (d76 PC) of the experiment prior to sale. Data on the quantity of DM delivered to each pen for the period between weighing days was exported from the liquid feeding system computer. Average daily gain (ADG), average daily feed intake (ADFI), and FCE were calculated as an average for the pen for the entire experimental period. Feed conversion efficiency was calculated as ADFI/ADG. Pigs removed from the experiment were accounted for when calculating ADG and ADFI.

5.3.5 Sample collection

Baseline samples were collected 12 days prior to the start of the sanitisation programme, after which samples were collected at d1 and d3 PC and wk1 PC, followed by weekly sampling up to wk10 PC (Figure 5.1). At each of the 13 sampling time points, surface swabs from the mixing tank and inside the feed pipeline were collected for microbiological, ATP and SEM analyses. Feed samples were also collected at each time point from the mixing tank and troughs for microbiological and physicochemical analyses (after cleaning, feed samples were collected from the first feed of each day, which contained the acidified rinsings). Dry feed and water samples were also collected on three sampling days (wk6, wk9 and wk10 PC) for microbiological analysis.

5.3.5.1. *Feed pipeline internal surface*

Samples from the internal surface of the feed pipeline were collected by removing and replacing ~15 cm sections of the polyvinyl chloride (PVC) feed pipe. On each sampling day, the exterior surface of the pipe section to be removed was cleaned with ethanol wipes. The direction of flow within the pipe and the top and bottom sections

were labelled. The joiners connecting the sections were unscrewed and the entire pipe section (15 cm in length; 32 mm diameter) was removed into a sterile plastic bag, sealed and immediately stored on ice. Throughout the experiment, pipe sections were removed sequentially from along the pipeline, moving back towards the mixing tank each time, with an existing pipe section replaced with a new section after sampling. On the sampling days where microbiology and SEM analyses were performed, two separate pipe sections were removed from the sampling location, as described above. Prior to analysis, PVC pipe cutters, sterilised by ethanol flaming, were used to aseptically cut each pipe section into separate ~5 cm long sections; one for microbiological swabbing and one for ATP swabbing. The pipe sections used for SEM were cut into separate sections in the same way.

A sterile cell scraper (Fisher Scientific, Loughborough, Leicestershire, UK) was used to scrape a 50 cm^2 area around the entire circumference of the inside of the pipe for 30 seconds. The head of the scraper was added to a sterile stomacher bag. Then, a sterile sponge swab pre-soaked with neutralising buffer (Sponge-stick; 3M, Saint Paul, MN, USA) was used to swab the same 50 cm^2 area of the internal surface of the pipe and added to the same stomacher bag. A separate 5 cm section of the pipe was used for ATP swabbing, where a 50 cm^2 area was swabbed with an UltraSnap™ Surface ATP Test (Hygiena, Watford, UK). Swabs were read immediately using the EnSURE® Touch luminometer (Hygiena) according to the manufacturer's instructions and results were presented as relative light units (RLU)/cm^2.

5.3.5.2. *Mixing tank internal surface*

Samples from the internal surface of the mixing tank were collected using sponge swabs pre-soaked with neutralising buffer (Sponge-stick; 3M). The mixing tank surface was swabbed within a 10 x 10 cm template, which was cleaned with ethanol prior to each use. On each sampling day, two separate sponge swabs were obtained from two different sides of the mixing tank, pooled (200 cm^2 swabbed), placed on ice and transported to the laboratory immediately for microbiological analysis. A different section of the mixing tank surface was sampled on each sampling day in order to avoid re-swabbing the same area. Adenosine triphosphate swabs were also collected from the mixing tank surface, as described for the feed pipe surface, except that a 100 cm^2 area was sampled with a template, as outlined above. Adenosine triphosphate swabs

were also taken from a different section of the mixing tank surface on each sampling day.

5.3.5.3. *Feed and water*

On each sampling day, liquid feed samples were collected from the first feed of the day (i.e. the feed prepared using the acidified rinsings) as follows: from the mixing tank (n = 1) using a sterile stainless-steel sampler which was lowered into the mixing tank after 10 min of feed agitation; fresh liquid feed (n = 3 troughs) as it was dispensed into the troughs; and residual liquid feed (n = 3 troughs) from the troughs after ~2.5 hours i.e. just prior to delivery of the next feed. For all liquid feed sampling, ~ 500 g of liquid feed was collected from each sampling location into sterile 500 mL containers and transported on ice to the laboratory for same-day analysis. For microbiological analysis, 5 mL aliquots of the three samples of fresh trough-sampled liquid feed, were pooled prior to analysis and the same was done for the residual trough-sampled feed. Aliquots (50 mL) of feed sampled from the mixing tank and each sample of fresh and residual trough-sampled feed were also sub-sampled and stored at -20 °C for analysis of volatile fatty acids (VFAs), lactic acid, ethanol and biogenic amines.

On five sampling days (baseline and d1, wk1, wk5 and wk10 PC), an additional ~250 mL of liquid feed was collected in foil trays from each sampling location (mixing tank and individual troughs) and transported on ice to the laboratory where it was stored at -20 °C for subsequent DM and GE analyses. Samples of the dry diet were also collected from the silo on three sampling days (wk6, wk9 and wk10 PC) for microbiological analysis. Sub-samples of the dry feed were also stored at -20 °C for VFA, lactic acid, ethanol, biogenic amine, DM and GE analysis. Finally, water samples were collected on three sampling days (wk6, wk9 and wk10 PC) from a connection beside the mixing tank (n = 3). The water was allowed to flow for 5 min prior to sample collection into sterile 200 mL containers, which were stored on ice prior to same-day microbiological analysis.

5.3.6 SEM sample preparation and observation

The 5 cm pipe sections used for SEM, as well as an unused PVC pipe section used as a control, were gently rinsed by immersion in phosphate buffered saline (PBS) (Sigma-Aldrich, Wicklow, Ireland) to remove loose debris, followed by rinsing with

an additional 150 mL of PBS to further remove loosely-associated debris. The pipe was then sectioned into pieces of ~1 cm^2 representative of the bottom (3 for plan view and 1 side elevation view) sections of the pipe.

The pipe sections were prepared for SEM observation by performing an overnight fixation in 2.5 % glutaraldehyde (Sigma-Aldrich) in 100 mM sodium cacodylate (pH 7.3) solution (Sigma-Aldrich). The samples were then dehydrated in a graded ethanol series, starting at 40 % up to 100 % (v/v) with a 10 % per hour increase. The ethanol was then replaced by hexamethyldisilazane (HMDS) (Sigma-Aldrich) by immersing the samples in a 50:50 HMDS:Ethanol solution for 1 hour followed by 100 % HMDS for another hour. The HMDS was then left to evaporate completely prior to sputter coating.

The dehydrated pipe sections were attached to SEM stubs using Leit C conducting carbon cement (Agar Scientific, Stansted, UK) and sputter-coated with gold at 80 mA for 1 min using an Emitech K575X sputter coater (Quorum Technologies, Lewes, UK). Gold-coated samples were analysed using a Gemini field emission scanning electron microscope (Zeiss, Oberkochen, Germany) at an accelerating voltage of 2-3 kV and a working distance of 3-8 mm. Two detectors were used for imaging: an in-lens detector and a secondary electron detector.

5.3.7 Microbiological analysis

Total aerobic bacteria, LAB, *Enterobacteriaceae*, *Escherichia coli*, and yeasts and moulds were enumerated in feed samples and sponge swabs taken from the mixing tank and feed pipe surfaces, as follows. For feed samples, ~10 g of sample was homogenised as a 10-fold dilution in maximum recovery diluent (MRD) (Merck, Darmstadt, Germany) and further 10-fold serial dilutions were performed in MRD. Maximum recovery diluent (50 mL) was added to the stomacher bag containing both the scraper and sponge swab used to sample the pipe surfaces and also to the stomacher bag containing the sponge swab used to sample the mixing tank surfaces. The samples were homogenised in a stomacher for 2 min. This homogenate was considered to be the 10^0 dilution and was serially diluted 10-fold in MRD. Relevant dilutions were plated in duplicate as follows: (i) 1 mL was plated on Petrifilm™ Aerobic Count Plates (3M) and incubated at 37 °C for 48 hours for total aerobic bacteria; (ii) 1 mL was pour-

plated on de Man Rogosa & Sharpe agar (Merck), containing 50 U/mL nystatin (Sigma-Aldrich), overlaid and incubated at 30 °C for 72 hours for LAB; (iii) 1 mL was pour-plated on Violet Red Bile Dextrose agar (Merck), overlaid and incubated at 37 °C for 24 hours for *Enterobacteriaceae*; (iv) 1 mL was pour-plated on Chromocult® Tryptone Bile X-glucuronide agar (Merck) and incubated at 44 °C for 24 hours for *E. coli*; and (v) 0.1 mL was spread-plated on Dichloran Rose-Bengal Chloramphenicol agar (Merck) and incubated at 25 °C for 5 days for yeasts and moulds. Colonies were counted, and the mean of duplicate counts obtained. Mean counts were log-transformed and presented as $\log_{10}$ CFU/g for feed samples [CFU/g = (average no. of colonies) ÷ (volume of suspension plated, mL) x (dilution factor)] and $\log_{10}$ CFU/cm^2 for surfaces swabs [CFU/cm^2 = (average no. of colonies) x (volume of original suspension, mL) ÷ (total surface area swabbed, cm^2) x (dilution factor)](Teagasc, 2008). Counts that were below the limit of detection (LOD) were reported at the LOD.

Water samples were analysed as follows: (i) Coliforms and *E. coli* were enumerated using the most probable number (MPN)-based Colilert®-18/Quanti-Tray® (IDEXX, Westbrook, ME, USA) method (ISO 9308-2:2012); (ii) Enterococci were enumerated using the Enterolert®/Quanti-Tray® (IDEXX) method (ISO 7899-1:1998); (iii) Total aerobic bacteria were enumerated using 3M™ Petrifilm™ Aerobic Count plates, with one set incubated at 37 °C for 48 hours and another at 22 °C for 72 hours, which meets applicable criteria for routine quality control and microbiological performance (ISO 11133:2014). Colilert®-18 and Enterolert® results were expressed as MPN/100 mL and total aerobic counts were expressed as $\log_{10}$ CFU/mL of water.

5.3.8 Physico-chemical analysis of feed samples

At each sampling location, the pH and temperature of the feed samples were recorded immediately using a pH meter (Mettler Toledo, Greisensee, Switzerland). Lactic acid, VFAs, ethanol and biogenic amines were analysed in the feed by Alimetrics Research (Espoo, Finland). Lactic acid and VFAs were analysed as free acids, using pivalic acid (Sigma-Aldrich) as an internal standard. A 400 µL aliquot of sample and 2.4 mL of 1.0 mM pivalic acid solution were mixed, vigorously shaken for 5 min, and then centrifuged at 3,000 × g for 10 min. Then 800 µL of the supernatant and 400 µL of saturated oxalic acid solution were mixed, incubated at 4 °C for 60 min, and centrifuged at 18,000 × g for 10 min. The supernatant was analysed by gas

chromatography (Agilent 7890B GC-FID; Agilent, Santa Clara, CA, USA) using a glass column packed with 80/120 Carbopack B-DA/4 % Carbowax stationary phase (Sigma-Aldrich), helium as a carrier gas, and a flame ionisation detector. The acids quantified were acetic, propionic, butyric, valeric, isobutyric, 2-methylbutyric, isovaleric, and lactic acid.

For ethanol analysis, the samples were diluted in a 1:5 ratio with water and centrifuged. The supernatant was collected and analysed with gas chromatography (Agilent 7890B GC-FID; Agilent) equipped with a Supelco packed glass column (2 m x ¼ in x 2 mm, 80/120 Carbopack B-DA/4 % phase; Sigma-Aldrich). Biogenic amines (putrescine, cadaverine, histamine, tyramine, tryptamine, spermidine, spermine and 2-phenylethylamine) were derivatised with dansyl chloride. Resulting dansyl derivatives were analysed using high-performance liquid chromatography with a fluorescence detector (HPLC-FLD) Shimadzu Prominence (Shimadzu, Duisburg, Germany). Matrix-matched internal standard calibration with heptyl amine was used in quantitation.

Liquid and dry feed samples for DM and GE determination were dried in an oven at 55 °C for 72 hours and ground in a Christy Norris mill through a 2 mm screen. Gross energy was determined using an adiabatic bomb calorimeter (Parr Instruments, Moline, IL), and DM was determined according to the Association of Official Analytical Chemists method (AOAC.934.01)(AOAC, 2005).

5.3.9 Statistical analysis

The impact of the sanitisation programme on microbial counts was assessed at each sampling location separately; i.e. mixing tank swabs, feed pipe swabs, mixing tank feed, fresh trough-sampled feed and residual trough-sampled feed, respectively. Data from different sampling days were pooled as follows for each sampling location: baseline (n = 1); d1-wk1 PC period (comprising d1, d3 and wk1 PC; n = 3); wk2-wk4 PC period (comprising wks 2, 3 and 4 PC; n = 3); wk5-wk7 PC period (comprising wks 5, 6 and 7 PC; n = 3) and wk8-wk10 PC period (comprising wks 8, 9 and 10 PC; n = 3). Mean microbial counts and standard error of the mean for each microbial group were calculated and plotted as grouped bar plots in R (version 4.2.1) using the ggplot2 (Wickham, 2016) and ggpubr (Kassambara, 2022) packages. Mean concentrations of

ATP (RLU/cm^2) at each pooled time point were plotted for each sampling location along a secondary y-axis. To test for differences between sampling locations, microbial counts, pH and temperature data from the 12 PC sampling occasions were pooled by sampling location. Differences between sampling locations were tested using the Kruskal-Wallis rank sum test, followed by Dunn's test of multiple comparisons. Differences with $p < 0.05$ were considered statistically significant. Figures were edited using Inkscape (version 1.3). Data from the mixing tank and feed pipe surface swabs were pooled and simple regression analysis was performed using the PROC REG procedure in Statistical Analysis Systems (SAS) software package version 9.4 (SAS Institute Inc., Cary, North Carolina, United States) to predict the variation in indicator microbial counts (log_{10} CFU/cm^2) based on ATP luminometer readings (RLU/cm^2).

5.4 Results

5.4.1 Veterinary treatments and pig deaths

A total of four pigs were treated during the experimental period, due to either lameness or infection. Each pig received three consecutive days of treatment consisting of 3 mL Unicillin and 1 mL Loxicom. Three pigs died during the experimental period; one due to a broken leg (during wk4 PC; 46.6 kg), one due to a ruptured hernia (wk7 PC; 87.4 kg) and one due to a suspected heart attack (wk9 PC; 123 kg).

5.4.2 Body weight, feed intake, growth and feed efficiency

Based on pen group weights, the average body weight (BW) at the start of the experiment was 35.0 kg ± 5.0 SD, with an average BW of 127.4 kg ± 7.9 SD at the end of the experiment. The ADFI across all of the pens throughout the experiment was 2,854 g/day, with an ADG of 1,216 g/day and an average FCE of 2.35 (data available in Table S5.2).

5.4.3 Microbiology of the dry feed and water used for the preparation of liquid feed

The microbial counts in the dry diet that was used to prepare the liquid feed are presented in Figure S5.1. Bacterial counts were consistent across the three different batches of feed sampled, as indicated by the minimal standard deviation, with an

average total aerobic count of 6.7 $\log_{10}$ CFU/g detected. However, yeast and mould counts were slightly more variable, with the former ~1 $\log_{10}$ CFU/g higher in the dry feed collected at wk9 PC, compared to that sampled at wk10 PC. *Enterobacteriaceae* and yeasts were the most abundant of the microbial groups monitored, with mean counts of ~6 $\log_{10}$ and 5.5 $\log_{10}$ CFU/g of dry feed, respectively, while LAB and moulds were detected at ~3.5 $\log_{10}$ CFU/g on average. *Escherichia coli* was not detected in the dry feed.

Total aerobic counts from samples of the water used to prepare the liquid feed are presented in Figure S5.2. The microbiological quality of the water was considered safe for consumption with no enterococci, coliforms or *E. coli* detected (data not shown). The European Union (EU) Drinking Water Regulations 2023 (S.I. No. 99/2023) state that intestinal enterococci, *E. coli* or coliform bacteria are not acceptable in drinking water and that there should be no abnormal change in values for a 22 °C colony count. Less than 3 $\log_{10}$ CFU/mL for a total aerobic count at 22 °C or 37 °C is considered acceptable for drinking water for pigs by the UK Agriculture and Horticulture Development Board (AHDB, 2018). Despite the count at 22 °C marginally exceeding this threshold in one of the water samples, no coliforms were detected, and therefore this level of total aerobes was not considered a concern.

5.4.4 Impact of the sanitisation programme on the microbiology of the liquid feeding system

The microbial counts and ATP concentrations on the mixing tank surface before and after cleaning are presented in Figure 5.2. The pre-cleaning (baseline) *E. coli* count was below the LOD and remained undetectable at each of the PC sampling time points. The *Enterobacteriaceae* count (2.3 $\log_{10}$ CFU/cm^2 before cleaning) fell below the LOD after cleaning, and remained so up to and including wk4 PC, returning close to baseline during the latter two PC periods. The LAB count on the mixing tank surface was 8.4 $\log_{10}$ CFU/cm^2 before cleaning. It decreased (to 2.9 $\log_{10}$ CFU/cm^2) in the d1-wk1 PC period and gradually increased back up to baseline levels during the experiment, even exceeding baseline levels slightly during the wk8-wk10 PC period.

The total aerobic count on the surface of the mixing tank (6.3 $\log_{10}$ CFU/cm^2 before cleaning) decreased by ~3.0 $\log_{10}$ CFU/cm^2 in the d1-wk1 PC period and remained

around this for the duration of the experiment. Yeast and mould counts on the mixing tank surface (5.1 and 4.9 $\log_{10}$ CFU/cm^2, respectively, before cleaning) both decreased below the LOD up to and including wk4 PC. During the wk5-wk7 PC period, both began to proliferate again on the mixing tank surface. As a result, the yeast count was slightly above the baseline level during the final (wk8-wk10) PC period, but moulds, although increased, remained lower than at baseline. The ATP concentrations decreased PC, and began to increase by wk4 PC, however, they did not return to baseline by wk10 PC, similar to the total aerobe and mould counts. The microbiology data are in line with visual inspection of the mixing tank surface; an improvement and gradual return to poor hygiene was observed after cleaning, with baseline and wk10 PC having a similar appearance (Figure S5.3).

The microbial counts and ATP concentrations on the internal surface of the feed pipe before and after cleaning are presented in Figure 5.3. As was the case with the mixing tank surface, *E. coli* was undetectable in the feed pipe, both before and after cleaning. *Enterobacteriaceae* counts were higher in the feed pipe before cleaning compared to the mixing tank surface (3.4 $\log_{10}$ CFU/cm^2); however, *Enterobacteriaceae* were undetectable in the feed pipe PC and remained so for the duration of the experiment, unlike on the mixing tank surface. As on the mixing tank surface, LAB were also present in the feed pipe at high levels before cleaning (7.9 $\log_{10}$ CFU/cm^2) and there was a substantial reduction in counts PC, decreasing below the LOD on d3 PC. Counts increased again after wk1 PC but stabilised at ~5 $\log_{10}$ CFU/cm^2 for the rest of the experiment, remaining lower than pre-cleaning levels thereafter. There was a gradual decrease in the total aerobic counts PC, with counts ~2 $\log_{10}$ CFU/cm^2 lower during the final (wk8-wk10 PC) period compared to baseline.

Yeasts, which were present on the feed pipe surface at 4.2 $\log_{10}$ CFU/cm^2 before cleaning, declined on d1 PC and thereafter remained undetectable until wk5 PC. From this point, counts remained quite stable for the duration of the experiment at ~1 $\log_{10}$ CFU/cm^2 below baseline counts. Moulds which were at a similar level to yeasts in the feed pipe decreased immediately PC but were still detectable on d1 PC, albeit at low levels. However, by d3 PC they fell below the LOD and were subsequently undetectable in the feed pipe for the duration of the experiment. The concentration of ATP detected in the feed pipe before cleaning was 37.9 RLU/cm^2. This decreased

immediately after cleaning, in line with the microbial counts, and remained low for the duration of the experiment. However, it did increase towards the end of the experiment, with the highest PC reading (10.1 RLU/cm^2) recorded during the wk5-wk7 PC period.

5.4.1 Relationship between ATP concentrations and microbial counts on liquid feeding system surfaces

Regression analysis determined that the ATP concentration on liquid feeding system surfaces was a moderate predictor of yeast counts ($R^2 = 0.58$; $p < 0.001$) and LAB counts ($R^2 = 0.53$; $p < 0.001$), while ATP concentration was a strong predictor of mould counts ($R^2 = 0.64$; $p < 0.001$). Concentrations of ATP were found to be a very weak predictor of *E. coli* counts ($R^2 = 0.14$; $p < 0.05$), while no relationship was found between ATP concentrations and total aerobic counts ($R^2 = -0.04$; $p > 0.05$).

5.4.2 Imaging of mixing tank and feed pipe surfaces pre- and post-cleaning

Figure 5.4 shows SEM images of the bottom inner surface of the feed pipe taken at baseline and at d1, wk5 and wk10 PC. The top row shows control images of an unused PVC pipe, displaying the absence of any microbial growth or biofilm. The baseline images show an array of microbial growth within the feed pipe before cleaning. Perhaps most notably, fungal hyphae were highly visible on the pipe surface, which was also colonised by bacterial cells (indicated by yellow arrow in Figure 5.4). The surface structure of the pipe seen in the control images is not visible in the baseline images. This is due to the presence of a biofilm coating the surface of the feed pipe, with both bacteria and fungi visibly embedded in the biofilm matrix, which is composed of extracellular polymeric substances (EPS).

On d1 PC, fungal hyphae were still visible on the pipe surface. However, imaging at higher magnification (5,000X) revealed that they were damaged, with ruptures in the hyphal structure visible, most likely as a result of the cleaning process (indicated by red arrow in Figure 5.4). At wk5 and wk10 PC there were no visible fungal hyphae observed on the feed pipe surface, indicating that from some point after d1 PC, up until wk10 PC, moulds were eliminated from the feed pipe. There were also substantially less bacterial cells observed at d1 PC and at wk5 PC, with evidence of

cell disruption; however, by wk10 PC some bacterial re-colonisation was again observed. Feed particles and feed-derived starch granules (indicated by pink arrow in Figure 5.4) and yeast cells (indicated by blue arrow in Figure 5.4) were also observed at wk10 PC. Despite this re-appearance of bacterial colonisation and feed residue on the feed pipe surface, visually, the internal surfaces of the feed pipe remained relatively clean up to wk10 PC (Figure S5.3).

5.4.3 Impact of the sanitisation programme on the microbiology of the liquid feed

The microbial counts, pH and temperature of liquid feed collected from the mixing tank are presented in Figure 5.5. In general, any changes in the mixing tank feed were more subtle compared to those found on the mixing tank surface. *Escherichia coli* was below the LOD in the mixing tank feed at baseline and for the duration of the experiment, as it was on the mixing tank surface. The *Enterobacteriaceae* count in the feed at baseline (4.91 $\log_{10}$ CFU/g) decreased marginally in the d1-wk1 PC period and thereafter, counts continued to increase gradually. Most notably, the LAB count decreased by ~2 $\log_{10}$ CFU/g between baseline and the d1-wk1 PC period; however, LAB began to increase again and returned to close to baseline levels between wk5 and wk10 PC. The total aerobe count also decreased initially after cleaning and followed a similar trend to LAB, with counts stabilising around baseline levels in the latter part of the experiment. Both yeasts and moulds had small initial decreases after cleaning; however, the yeast counts were slightly above the baseline level at the end of the experiment, while moulds remained consistently ~1 $\log_{10}$ CFU/g below baseline levels. The feed pH was lower at each PC period (4.75 - 5.48), compared to baseline (6.28). Finally, the temperature of the feed in the mixing tank gradually increased from 14.5 °C at baseline up to a mean temperature of 18.3 °C during the wk8-wk10 PC period.

The microbial counts, pH and temperature of fresh liquid feed collected from the troughs immediately after feed-out are presented in Figure 5.6. *Escherichia coli* was detectable at baseline and increased during the d1-wk1 PC period. However, counts declined thereafter and *E. coli* was below the LOD in the wk8-wk10 PC period. There was a similar increase in *Enterobacteriaceae* counts after cleaning, especially on d1 and d3 PC (data not shown), which may have been driven by *E. coli.* However, mean *Enterobacteriaceae* counts remained marginally above baseline levels from wk5 PC until the end of the experiment. There was no change in LAB counts across the time points and only a minimal reduction in total aerobe counts. Yeast counts in the fresh liquid feed increased (from 5.1 $\log_{10}$ CFU/g at baseline) during the d1-wk1 PC period, remaining at a similar level during the wk2-wk4 PC period and declining thereafter,

albeit remaining marginally above baseline levels. Moulds were present at 4.6 $\log_{10}$ CFU/g at baseline and generally declined after cleaning. The pH of the fresh liquid feed varied between 5.28 and 5.62 after cleaning, compared to 6.18 at baseline. Throughout the experiment, the temperature of the fresh liquid feed increased from 15.3 °C at baseline up to a mean temperature of 18.8 °C during the wk8-wk10 PC period.

The microbial counts, pH and temperature of the residual liquid feed collected from the troughs are presented in Figure 5.7. The *E. coli* count in the residual feed at baseline (3.6 $\log_{10}$ CFU/g) increased during the d1-wk1 PC period, but began to decrease afterwards, returning to just below baseline levels during the final PC period. Similarly, *Enterobacteriaceae* counts increased during the d1-wk1 PC period, declining thereafter to below baseline levels. Lactic acid bacteria and total aerobic counts remained relatively stable throughout the experiment, with only minimal fluctuations in counts. Yeast counts in the residual feed followed a similar pattern to the fresh feed where counts increased after cleaning compared to baseline. Moulds also followed a similar trend as in the fresh liquid feed, with a slight decline in counts after cleaning, albeit they increased again thereafter. The pH of the residual feed remained quite stable throughout the experiment. The same trend occurred in the residual troughs as with the mixing tank and fresh liquid feed, where the temperature of the feed increased from 16.3 °C at baseline to an average of 19.4 °C at the end of the experiment.

5.4.4 Impact of sampling location on the microbiology of the liquid feed

In order to investigate the influence of sampling location on the microbial counts, pH and temperature of the liquid feed, data from all 12 PC time points for each sampling location were averaged (Figure 5.8). The pH of the liquid feed did not differ between sampling locations ($p > 0.05$), while the temperature of the residual feed was higher compared to that of the mixing tank and the fresh trough-sampled liquid feed ($p < 0.05$). *Escherichia coli* counts were higher in the fresh trough-sampled liquid feed compared to the mixing tank ($p < 0.05$), with further increases in the residual trough-sampled feed compared to the mixing tank ($p < 0.001$) and fresh feed ($p < 0.01$). *Enterobacteriaceae* counts were higher in the residual feed compared to the mixing tank ($p < 0.01$), but no significant differences were found between the fresh feed and

the feed sampled at the other locations. The residual feed also had higher counts of total aerobes compared to the mixing tank ($p < 0.001$) and fresh liquid feed ($p < 0.01$). Lactic acid bacteria and yeasts both increased from the mixing tank to the fresh liquid feed ($p < 0.01$) and again in the residual feed compared to the mixing tank ($p < 0.001$) and fresh liquid feed ($p < 0.01$). Mould counts were higher in the residual feed compared to the mixing tank feed ($p < 0.01$).

5.4.5 Effect of the sanitisation programme on the gross energy (GE), ethanol, lactic acid, volatile fatty acid and biogenic amine content of liquid feed

The GE, ethanol, lactic acid and VFA content of liquid feed at baseline, d1 PC, wk5 PC and wk10 PC for each sampling location are presented in Table 5.1. The GE of the dry feed collected from the silo was 17.7 MJ/kg. There was very little variation from this GE value in the liquid feed, either by sampling location or time point, with the greatest variation being ~0.4 MJ/kg. At baseline, increasing concentrations of ethanol were observed between the mixing tank and the troughs, especially in the residual liquid feed. After cleaning, ethanol concentrations in the mixing tank and fresh liquid feed were < 0.2 mmol/kg. Despite a moderate ethanol concentration in the residual feed at baseline, < 0.2 mmol/kg was detected on d1 PC. Nonetheless, by wk5 PC, the concentration had begun to increase again, with further increases observed by wk10 PC, albeit the concentration remained lower than at baseline.

A low concentration of lactic acid was found in the dry feed; however, at baseline, lactic acid concentrations exceeded 20 mmol/kg liquid feed in the mixing tank and were substantially higher in the fresh trough-sampled feed, reaching up to 232.65 mmol/kg in the residual feed. Lactic acid concentrations in the mixing tank and fresh trough-sampled liquid feed were notably lower after cleaning. The lowest concentrations were observed at d1 PC in the troughs. However, as with ethanol, concentrations had increased by wk5 PC, especially in the residual feed in which they continued to increase up to wk10 PC (although not back to baseline levels). Large variations in lactic acid concentrations were observed in the residual trough samples.

The concentration of acetic acid in the dry feed was higher than ethanol or lactic acid concentrations, with levels of > 5 mmol/kg detected. As with lactic acid and ethanol, moderate concentrations of acetic acid were found in the liquid feed sampled from the

mixing tank, with higher concentrations found in the fresh and residual feed sampled from the troughs (> 22 and > 65 mmol/kg, respectively). Acetic acid concentrations decreased after cleaning; however, as with the other microbial metabolites above, there was a gradual increase from wk5 PC but they did not return to baseline concentrations.

Low concentrations of propionic acid were detected in the dry feed, with only slight increases between the mixing tank and the troughs. After cleaning, propionic acid concentrations increased at all sampling locations. However, this was expected because the liquid feed contained the maintenance acid rinse and the acid blend used for this rinse was composed of 15 % propionic acid. There was some variation in propionic acid concentrations at different sampling locations and time points, but they were generally between ~12 and 24 mmol/kg, with the lowest concentrations found in the trough-sampled feed. Butyric acid and isovaleric acid were not detected in the dry feed or in the feed sampled from the mixing tank and were only detected in the fresh trough samples at baseline, and then only at very low concentrations. Low concentrations were also detected in the residual trough-sampled liquid feed at all time points, except that isovaleric acid was not detected on d1 PC.

The concentrations of biogenic amines in residual feed collected from the troughs at baseline, d1 PC, wk5 PC and wk10 PC are presented in Table 5.2. In general, cadaverine was found at the highest concentration, followed by spermidine, tryptamine and putrescine. All biogenic amines detected were at their highest concentration at baseline, except for spermidine. However, both spermidine and spermine concentrations only varied minimally before and after cleaning. The most notable observation was that all amines, except for spermine and spermidine, were at their lowest concentration on d1 PC, with concentrations increasing after wk5 PC. Concentrations had returned closer to baseline by wk10 PC; however, all of the aforementioned biogenic amines remained below baseline concentrations until the end of the experiment.

5.5 Discussion

In this study an intensive sanitisation program improved the hygiene of a liquid feeding system. This was evidenced by the fact that counts of LAB, total aerobes, *Enterobacteriaceae*, yeasts and moulds were reduced on the mixing tank and feed

pipeline surfaces, in particular until wk5 PC, while *Enterobacteriaceae* and moulds were undetectable in the pipes for up to 10 weeks. One novel aspect of this study involved the use of SEM to image the internal surfaces of the feed pipeline. This confirmed the eradication of biofilm-associated fungal mycelia and the microbial load reductions within the feed pipes. The microbiological and SEM data were also backed up by ATP readings from the mixing tank and feed pipe surfaces. In fact, ATP luminometer readings were found to be a moderate predictor of yeast and LAB counts and a strong predictor of mould counts on liquid feeding system surfaces. This method may serve as a convenient and labour-saving means of monitoring feeding system hygiene on-farm, as has been shown to be the case for hygiene monitoring of pen surfaces (Heinemann et al., 2020; Yi et al., 2020).

Previous studies have implemented sanitisation programmes in liquid feeding systems, combining physical cleaning with alkali and/or acid washing. These studies have only monitored feed or 'contact water' that has been passed through the system, as opposed to the internal surfaces of the feeding system and have found either no impact on or only temporary reductions in microbial counts, which generally recovered within 2 weeks of cleaning (Fisker & Jørgensen, 2010; Royer et al., 2004a). The initial cleaning steps employed in the current study were similar to that of the aforementioned studies. However, to our knowledge, this study was the first to implement a subsequent nightly acid rinse to maintain the improved hygiene of the system and to recirculate the acid rinse to form part of the liquid feed. The addition of this nightly rinse with an organic acid blend, which was developed in consultation with the feed industry, was likely the main contributor to the longevity of the improved system hygiene. This may have been due to disruption of biofilm re-formation in the feed pipe, which can occur within days or weeks, at least in water distribution system pipeliness (Geng & Lin, 2016). Another difference in the sanitisation programme compared to previous studies was that the alkali and acid washes were flushed through the downpipes to maximise cleaning of these surfaces. This is important as the downpipes are difficult to clean and have been previously emphasised as a source of feed contamination (Royer et al., 2004a).

Changes in the microbial load of the liquid feed PC were less pronounced compared to those observed within the liquid feeding system. The most noticeable changes occurred in the mixing tank. At baseline, all of the microbial counts in the mixing tank

feed were within the ranges recommended for 'residue-free' or non-fermented liquid feed (i.e., feed that is not deliberately fermented, as in the current study), except for moulds which were just above the 3-4 $\log_{10}$ CFU/g recommended range (Vils et al., 2018). Both lactic and acetic acids were also in excess of the recommended range of 0-10 mmol/kg, with values of ~23 and 17 mmol/kg obtained, respectively. Thereafter, LAB counts decreased by 2.2 $\log_{10}$ CFU/g during the d1-wk1 PC period, with small decreases observed in the other groups monitored. However, these decreases were short-lived, with counts of all microbial groups (except *E. coli*, which was not detected) increasing again during the wk2-wk4 PC period. The pH of the mixing tank feed also decreased from 6.28 to 5.11 during the initial PC period, which was maintained thereafter. However, this was probably not due to microbial growth, as LAB, which are the main acid-producing microbes in the feed, were reduced at this time. It was, therefore, most likely due to use of the acid rinse to prepare the first feed of the day.

Furthermore, even after improving the hygiene of the liquid feeding system via implementation of the sanitisation programme, spontaneous fermentation still occurred in the liquid feed. This type of fermentation is a common occurrence in liquid feed which is not deliberately fermented, as found in Chapter 3 and several other studies (Canibe & Jensen, 2003; O'Meara et al., 2020a, 2020c, 2020b; Torres-Pitarch et al., 2020a, 2020b). In the present study it was evidenced by increases in the LAB, yeast and *E. coli* counts from when the feed was mixed, to when it was delivered to the troughs. Increases in the counts of all of the microbial groups monitored were also observed in the residual compared to the fresh trough-sampled feed. This was likely aided by the higher temperature of the residual feed, compared to that of the feed in the mixing tank and the fresh feed in the troughs. This was likely caused in part by the longer duration spent in the troughs, as well as by heat generated during fermentation. Additionally, the higher *E. coli* counts in the fresh and residual liquid feed PC were likely due to faecal contamination in the troughs, particularly so when the pigs were lighter at the beginning of the experiment and were observed stepping into the troughs. This also explains why counts were below the LOD at the end of the experiment when the pigs were nearing slaughter weight.

Chemical analysis of the liquid feed also revealed evidence of spontaneous fermentation, especially at baseline. Spontaneous fermentation is known to result in elevated levels of ethanol, acetic acid and biogenic amines, which can negatively impact liquid feed palatability, while biogenic amines are also toxic at high concentrations (Canibe et al., 2010; de Lange & Zhu, 2012; del Rio et al., 2024; Missotten et al., 2015). Increasing levels of lactic acid were observed between the mixing tank and the fresh feed sampled from the troughs, with the greatest concentrations found in the residual trough-sampled feed. Although the liquid feed in the current study is considered non-fermented, the organic acid profile of the feed in the troughs was more similar to standard values for fermented liquid feed (Vils et al., 2018). Lactic acid concentrations in the fresh and residual trough-sampled feed, for example, at 66.15 and 232.65 mmol/kg, respectively, were hugely in excess of the 0-10 mmol/kg recommended for residue-free liquid feed (Vils et al., 2018). However, these concentrations dropped dramatically (to 1.38 and 7.83 mmol/kg, respectively), immediately after sanitisation of the liquid feeding system. This indicates control of spontaneous fermentation of the feed, despite the fact that only marginal decreases in LAB were observed in the feed collected from the troughs immediately PC compared to baseline, as outlined above. However, by wk5 PC, lactic acid concentrations had begun to increase again, although they did not return to baseline levels.

Another organic acid of interest in liquid feed is acetic acid; it is produced by yeast during fermentation as well as by heterofermentative LAB (Rudbäck, 2013). As with lactic acid, the acetic acid concentration in the residual trough-sampled feed pre-cleaning (65 mmol/kg) was more similar to standard values for fermented liquid feed, being well in excess of the recommended value of < 40 mmol/kg for residue-free liquid feed (Vils et al., 2018). High levels of acetic acid are suggested to decrease the palatability of liquid feed and may therefore affect feed intake (Brooks et al., 2001; Canibe et al., 2007; Missotten et al., 2015), although Rudbäck (2013) found that feed intake and growth rate of piglets were not affected at concentrations of up to 150 mmol/L (Rudbäck, 2013). As with lactic acid, acetic acid concentrations in the feed immediately after sanitisation of the liquid feeding system were more than half that of baseline concentrations and remained below baseline levels until the end of the experiment.

Ethanol is another fermentation end-product associated with palatability issues that is undesirable at high concentrations in liquid feed (Brooks et al., 2001; Missotten et al., 2015; Van Winsen et al., 2001). Ethanol concentrations at baseline, even in the mixing tank feed (3 mmol/kg), exceeded the recommendation for residue-free liquid feed (0-0.5 mmol/kg) (Vils et al., 2018). Concentrations increased more than 8-fold in the residual trough-sampled feed, indicating undesirable levels of spontaneous yeast fermentation. Ethanol concentrations in the feed were also reduced as a result of cleaning of the feeding system, with < 0.2 mmol/kg detected in the mixing tank and fresh trough-sampled feed PC, for the duration of the experiment, indicating control of spontaneous fermentation. This was despite the fact that there was only a small reduction in yeast counts immediately PC and only in the mixing tank. In the residual trough-sampled feed, the ethanol concentration was the same as in the mixing tank and fresh trough-sampled feed immediately PC, and although concentrations increased throughout the experiment, they were still only half that of baseline levels at wk10 PC. Therefore, sanitisation of the liquid feeding system also controlled fermentation in the troughs, which is the location at which the greatest amount of feed fermentation occurs.

Overall, the low levels of ethanol and acetic acid found in the liquid feed PC indicated that yeast fermentation was disrupted by the sanitisation protocol, in a similar fashion to lactic acid fermentation. This stabilisation of the chemical quality of the liquid feed was maintained throughout the experiment, with levels generally remaining well below baseline at wk10 PC. This was also supported by the consistent GE content of the feed, irrespective of sampling time point or location, as excessive yeast fermentation is known to contribute to GE losses in liquid feed (Brooks, 2008; Keller et al., 2020; O'Meara et al., 2020a). However, since the sanitisation programme was multi-faceted, it is difficult to determine which specific steps were most effective in improving system hygiene and liquid feed quality. The disruption to yeast and LAB fermentation was likely a result of several factors, including the initial physical and chemical cleaning of the feeding system, the maintenance acid rinse of the feed pipeline, and/or the addition of the acid blend to the liquid feed. Future work could include sampling system surfaces and feed after each of the individual steps in order to identify which are most effective. Plumed-Ferrer & von Wright (2011) previously reported that the addition of formic acid (which accounts for 60 % of the acid blend

used in the current study) to either intentionally or spontaneously fermented liquid feed stabilised the growth of some yeast species (*Kazachstania exigua*) and reduced the growth of others (*Debaromyces hansenii* and *Pichia deserticola*). They also found a slight reduction in LAB counts in spontaneously fermented liquid feed after addition of 2 g/L formic acid.

It is possible that the combination of acids used in the maintenance acid rinse in the current study induced acid stress in the yeast and LAB, thereby hindering microbial fermentation temporarily (Guan & Liu, 2020). This is because LAB and yeast counts in the feed only dropped temporarily and only in the mixing tank, but fermentation end-products, albeit increased throughout the experiment, never returned to pre-cleaning levels. It is also possible that the yeasts and LAB associated with the liquid feeding system surfaces were in the main responsible for the organic acid and ethanol content in the liquid feed. The reductions in both LAB and yeast counts observed on the mixing tank and feed pipe surfaces immediately after cleaning coincided with the decreased organic acid and ethanol production in the feed. Similarly, the subsequent re-colonisation of the liquid feeding system by these microbes after wk4 PC coincided with increasing organic acid concentrations at wk5 PC, which nonetheless remained lower than baseline at the end of the experiment.

The concentration of several biogenic amines in the residual liquid feed collected from the troughs also decreased immediately PC compared to baseline. Although concentrations had begun to increase again by wk5 PC, even by wk10 PC, biogenic amine concentrations were still substantially lower than baseline levels; concentrations of putrescine, cadaverine, histamine, tyramine and tryptamine were approximately half of the baseline concentrations. This suggests that amino acid losses in the feed were lower after sanitisation of the liquid feeding system. However, amino acid data are not available to support this. Overall, the findings of the current study are contrary to those of Fisker & Jørgensen (2010), who reported no reductions in acetic acid, lactic acid, ethanol or biogenic amines in liquid feed after cleaning of the feeding system. However, ‘residual’ liquid feeding was practiced on the farms in the latter study where feed remained in the pipelines after feeding, thereby providing a greater opportunity for fermentation to occur.

In the current study, it was not possible to compare the growth and FCE of pigs fed from the sanitised system to a control group. However, the sanitisation programme certainly did not hinder growth performance, as both growth rates and FCE were excellent throughout the experiment and were comparable to those obtained in liquid feeding studies performed in the same grow-finisher house previously, including Chapter 4 (O'Meara et al., 2020d, 2020c). Assuming that the observed improvement in liquid feed quality improved FCE by a very conservative estimate of 0.05 of FCE unit, a cost benefit analysis of implementing this sanitisation programme indicates that it results in an increased margin of between €0.87 and €1.20 per pig (Table S5.3). Therefore, future research should investigate the growth performance and FCE of pigs fed from sanitised versus non-sanitised systems. Also, since only one replicate of this experiment was performed, additional studies should be performed in order to ensure that the findings are repeatable. Obtaining the complete microbial profile of the bacterial and fungal communities of the liquid feeding system and the feed itself using next-generation sequencing would also be of interest in order to further explore the impact on the microbiology of both. Finally, it may be possible to increase the longevity of the improved system hygiene, thereby potentially further improving feed quality; for example, by increasing the concentration of the acid blend in the liquid feed, and/or using a fogger in the mixing tank to better distribute the acid and alkali to the internal surfaces of the mixing tank.

5.6 Conclusion

This study involved implementation of an intensive sanitisation program in a 'residue-free' liquid feeding system which involved physical and chemical cleaning, as well as nightly acid rinsing and use of the acid rinse to prepare the first feed of the day. The main novelty of this study lies in the fact that, in addition to the feed itself, for the first time, microbial biofilms and feed residue on the internal surfaces of the feed pipelines were monitored pre- and post-sanitisation. Using this approach, it was found that the sanitisation programme dramatically improved the hygiene of the internal surfaces of the feeding system, especially for the initial 5-week period. This was evidenced by reduced microbial counts and decreases in ATP concentrations post-sanitisation, with SEM confirming these findings for the feed pipelines. Although only subtle impacts on the microbiology of the liquid feed were observed PC, and microbial counts were consistent with the occurrence of spontaneous fermentation, even after sanitisation, no

GE losses were found. Furthermore, considerable decreases in acetic acid, ethanol and biogenic amine concentrations were found in the feed post-sanitisation and these undesirable microbial metabolites remained well below pre-cleaning levels up to 10 weeks after program implementation. The concentrations of these metabolites in liquid feed coincided with changes in LAB and yeast counts on the liquid feeding system surfaces, implicating these surface microbial communities as one of the main factors contributing to chemical quality of the feed. Therefore, by controlling these surface microbial communities via implementation of the feeding system sanitisation programme developed and tested in the current study, on-farm liquid feed quality should be improved. Finally, based on a very conservative estimated improvement in FCE, implementing the sanitisation programme in liquid feeding systems for grow-finisher pigs should be cost beneficial for pig producers.

5.7 Tables and figures

Table 5.1: Gross energy (MJ/kg ± SD[1]) and concentrations of ethanol, lactic acid and volatile fatty acids (mmol/kg ± SD[1] on a dry matter basis) in feed collected at different sampling locations before and after liquid feeding system cleaning.

	Silo[2]	**Mixing tank[3]**				**Fresh trough[4]**				**Residual trough[5]**			
	Baseline	Baseline	d1 PC	wk5 PC	wk10 PC	Baseline	d1 PC	wk5 PC	wk10 PC	Baseline	d1 PC	wk5 PC	wk10 PC
Gross energy	17.7	17.8	18.1	17.6	17.5	17.9 ± 0.05	18.0 ± 0.09	17.5 ± 0.20	17.3 ± 0.18	17.9	18.0 ± 0.17	17.4 ± 0.16	17.7 ± 0.06
Ethanol	< 0.2	3.05	< 0.2	< 0.2	< 0.2	5.87 ± 1.46	< 0.2	< 0.2	< 0.2	25.52	< 0.2	2.90 ± 0.94	12.44 ± 8.83
Lactic acid	0.69	22.74	7.46	2.68	8.50	66.15 ± 21.12	1.38 ± 1.09	16.42 ± 11.38	16.99 ± 7.79	232.65	7.83 ± 4.03	50.75 ± 4.85	112.22 ± 77.24
Acetic acid	5.66	17.07	8.68	9.19	10.31	22.75 ± 1.88	9.29 ± 0.22	10.63 ± 0.53	11.00 ± 0.76	65.36	10.76 ± 1.31	20.84 ± 3.13	44.08 ± 22.33
Propionic acid	0.25	1.03	21.40	23.80	12.92	1.22 ± 0.09	20.78 ± 1.31	16.79 ± 12.26	12.52 ± 0.24	2.70	16.61 ± 0.67	21.33 ± 2.68	12.01 ± 1.17
Butyric acid	nd[6]	nd	nd	nd	nd	0.43 ± 0.05	nd	nd	nd	1.18	0.55 ± 0.06	0.74[7]	1.00 ± 1.01
Isovaleric acid	nd	nd	nd	nd	nd	< 0.1	nd	nd	nd	< 0.1	nd	< 0.1	< 0.1

[1] SD: Standard deviation. [2] Dry feed collected from the silo (n = 1). [3] Liquid feed collected from the mixing tank (n = 1). [4] Fresh liquid feed collected from the troughs immediately after delivery to the troughs (n = 3). [5] Residual liquid feed collected from the troughs just prior to the next feed (n = 3, except at baseline where n = 1). [6] nd: Not detected. [7] Butyric acid was detected in only one of three residual trough samples. Note: Valeric acid, 2-methylbutyric acid and isobutyric acid were not detected in any samples.

Table 5.2: Concentration of biogenic amines (mg/kg on a dry matter basis) in residual liquid feed collected from troughs before and after liquid feeding system cleaning.

Amine	Time point			
	Baseline	d1 PC	wk5 PC	wk10 PC
Putrescine	43.37	17.95 ± 2.17	21.76 ± 0.76	25.54 ± 12.32
Cadaverine	196.53	18.59 ± 10.36	35.68 ± 5.74	70.32 ± 73.61
Histamine	9.37	3.41 ± 1.24	6.46 ± 5.21	5.73 ± 3.77
Tyramine	7.20	1.86 ± 0.91	2.31 ± 1.72	3.50 ± 3.46
Tryptamine	53.79	10.90 ± 1.38	12.90 ± 2.91	20.33 ± 7.73
Spermidine	50.67	52.34 ± 1.70	52.75± 7.75	48.02 ± 1.05
Spermine	24.10	23.13 ± 0.83	23.62 ± 4.12	22.79 ± 0.36

Note: 2-phenylethylamine was not detected at any time point.

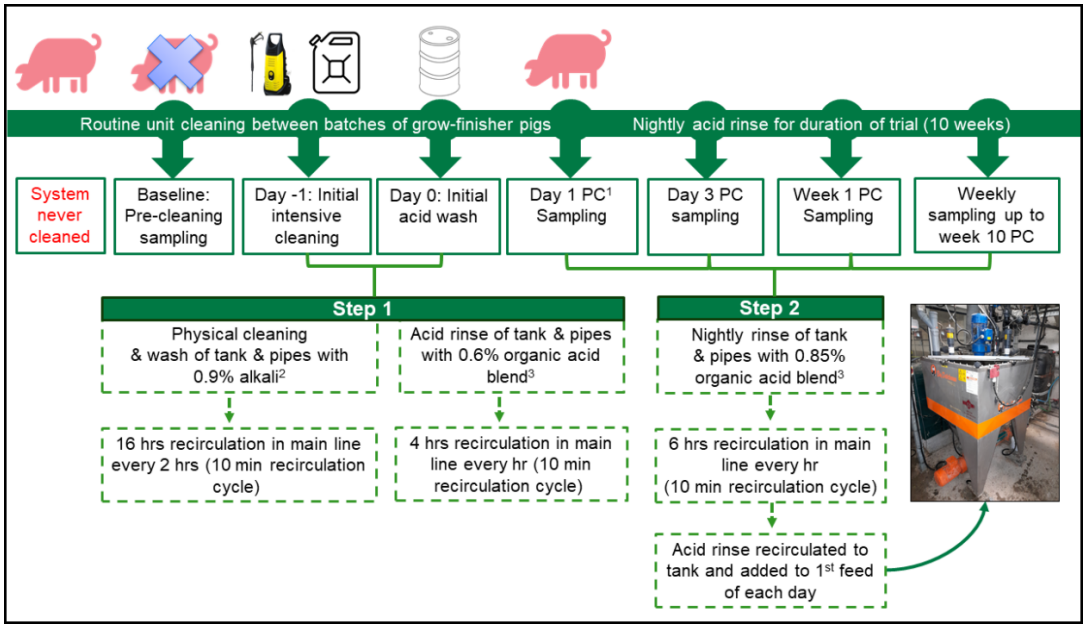

Figure 5.1: Schematic diagram outlining details of the two-step liquid feeding system sanitisation programme used and sampling time points. [1]PC = post-cleaning. [2]Avalksan Gold Standard CF Chlorine Free. [3]Interpronutri Plus BE (60 % formic acid, 15 % propionic acid, 2.5 % benzoic acid).

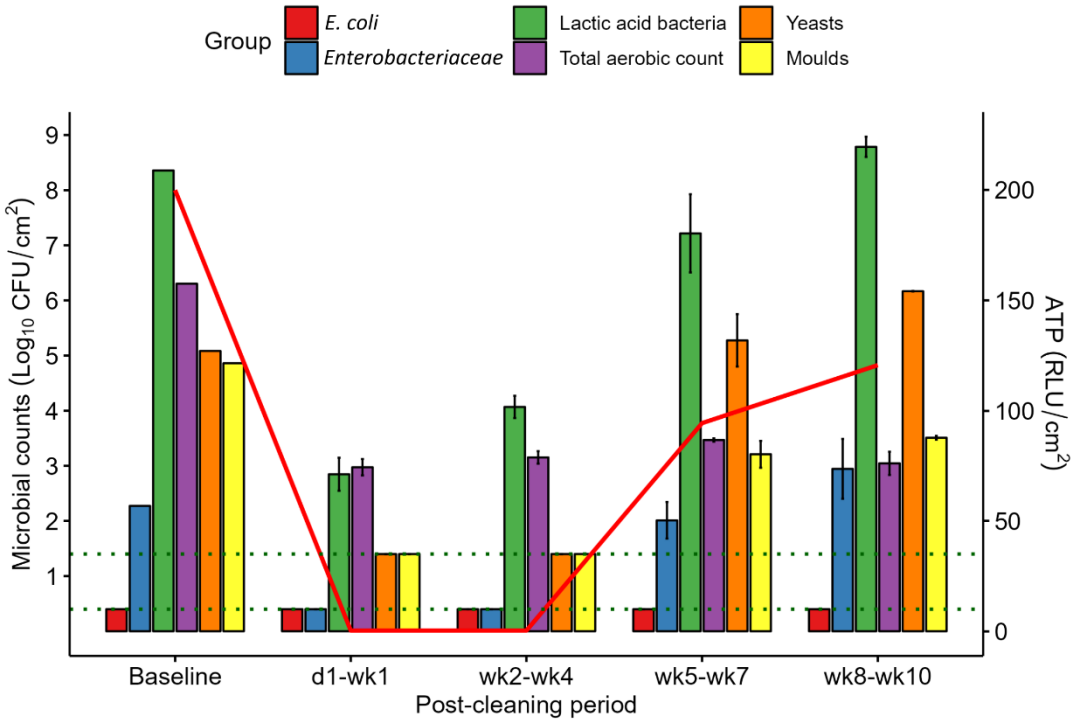

Figure 5.2: Mean microbial counts ($\log_{10}$ CFU/cm^2 ± SE) on the mixing tank surface before cleaning (Baseline; n = 1) and at each post-cleaning period (n = 3). Horizontal dotted lines represent the limit of detection (LOD) for different microbial groups: LOD for *Escherichia coli* (*E. coli*), *Enterobacteriaceae*, lactic acid bacteria and total aerobic count = 0.4 $\log_{10}$ CFU/cm^2; LOD for yeasts and moulds = 1.4 $\log_{10}$ CFU/cm^2. Counts below the LOD are reported at the LOD. The solid red line indicates mean ATP concentrations (RLU/cm^2).

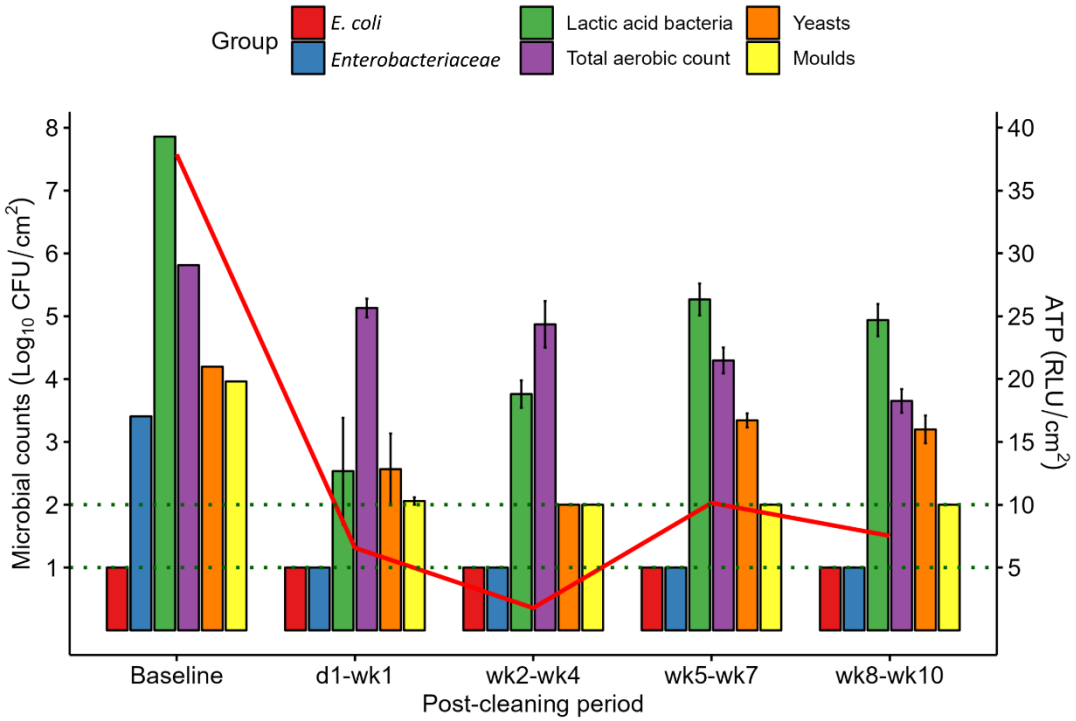

Figure 5.3: Mean microbial counts (log_{10} CFU/cm^2 ± SE) on internal feed pipe surfaces before cleaning (baseline; *n* = 1) and at each post-cleaning period (*n* = 3). Horizontal dotted lines represent the limit of detection (LOD) for different microbial groups: LOD for *Escherichia coli* (*E. coli*), *Enterobacteriaceae*, lactic acid bacteria and total aerobic count = 1 log_{10} CFU/cm^2; LOD for yeasts and moulds = 2 log_{10} CFU/cm^2. Counts below the LOD are reported at the LOD. The solid red line indicates mean ATP concentrations (RLU/cm^2).

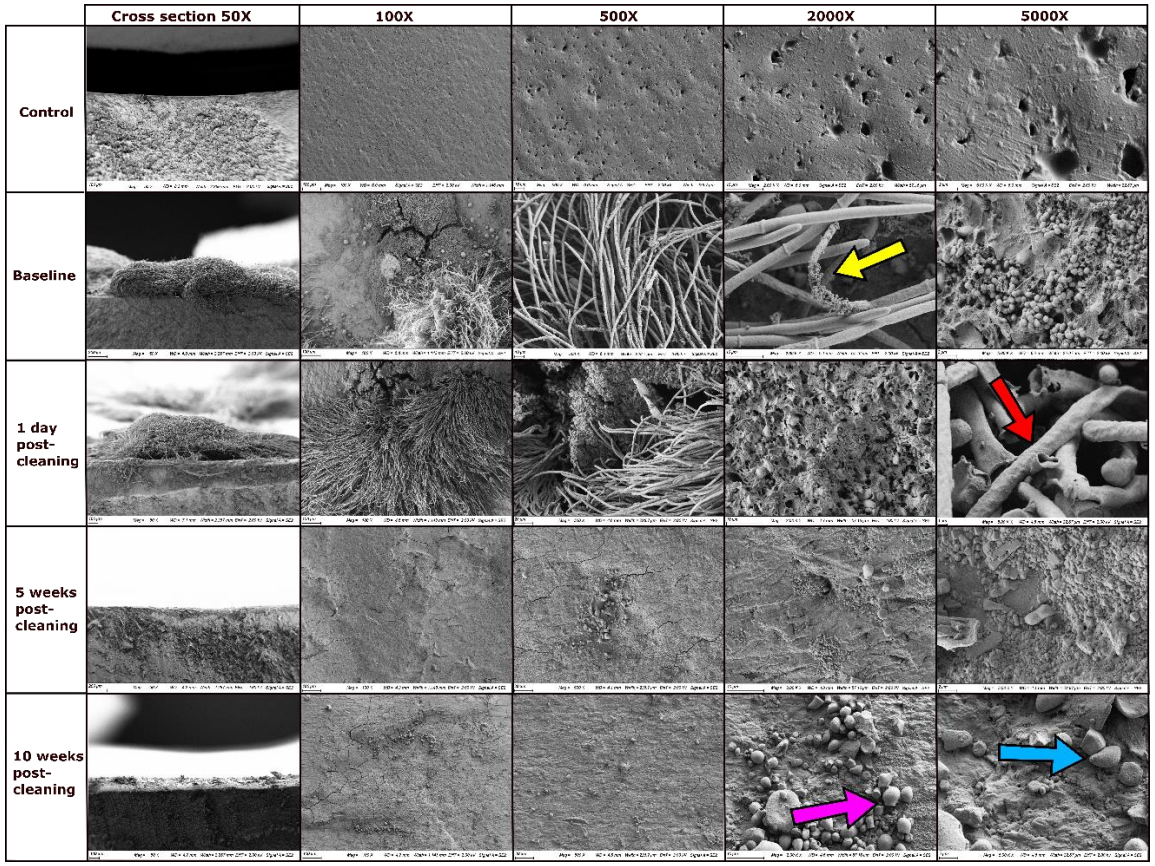

Figure 5.4: Panel of scanning electron microscopy images of the bottom of feed pipe sections, removed from a liquid feeding system before and after cleaning. The images are organised by magnification (horizontally) and time point (vertically). The top row labelled 'control' contains images of an unused PVC pipe section with no microbial growth or biofilm present. Yellow arrow indicates fungal hyphae colonised by bacterial cells. Red arrow indicates damage in the hyphal structure. Pink arrow indicates feed particles and feed-derived starch granules. Blue arrow indicates yeast cells.

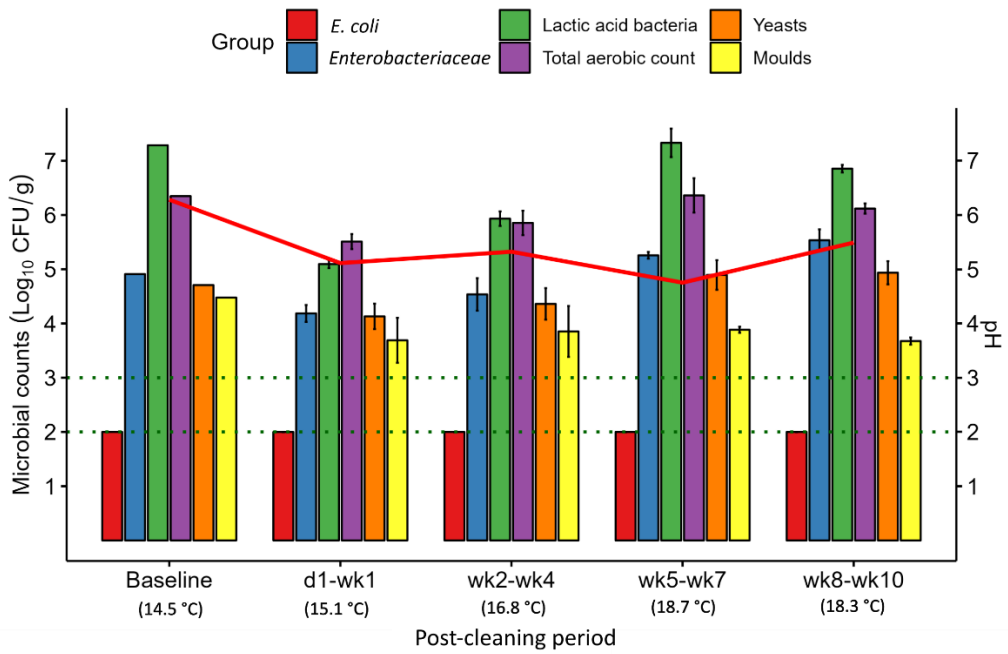

Figure 5.5: Mean microbial counts (log_{10} CFU/g ± SE) in liquid feed collected from the mixing tank before cleaning (baseline; *n* = 1) and at each post-cleaning period (*n* = 3). Mean liquid feed temperatures at each post-cleaning period are presented in parentheses below the x-axis labels. Horizontal dotted lines represent the limit of detection (LOD) for different microbial groups: LOD for *Escherichia coli* (*E. coli*), *Enterobacteriaceae*, lactic acid bacteria and total aerobic count = 2 log_{10} CFU/g; LOD for yeasts and moulds = 3 log_{10} CFU/g. Counts below the LOD are reported at the LOD. The mean feed pH is indicated by the solid red line.

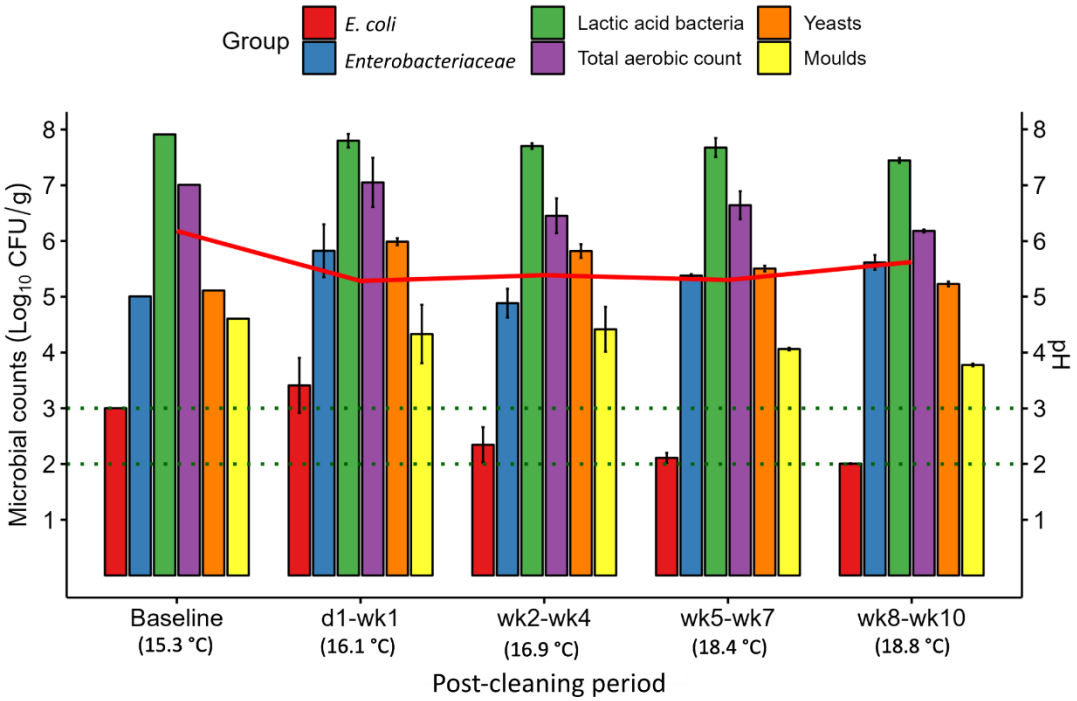

Figure 5.6: Mean microbial counts ($\log_{10}$ CFU/g ± SE) of fresh liquid feed collected from the troughs before cleaning (baseline; n = 1) and at each post-cleaning period (n = 3). Mean liquid feed temperatures at each post-cleaning period are presented in parentheses below the x-axis labels. Horizontal dotted lines represent the limit of detection (LOD) for different microbial groups: LOD for *Escherichia coli* (*E. coli*), *Enterobacteriaceae*, lactic acid bacteria and total aerobic count = 2 $\log_{10}$ CFU/g; LOD for yeasts and moulds = 3 $\log_{10}$ CFU/g. Counts below the LOD are reported at the LOD. The mean feed pH is indicated by the solid red line.

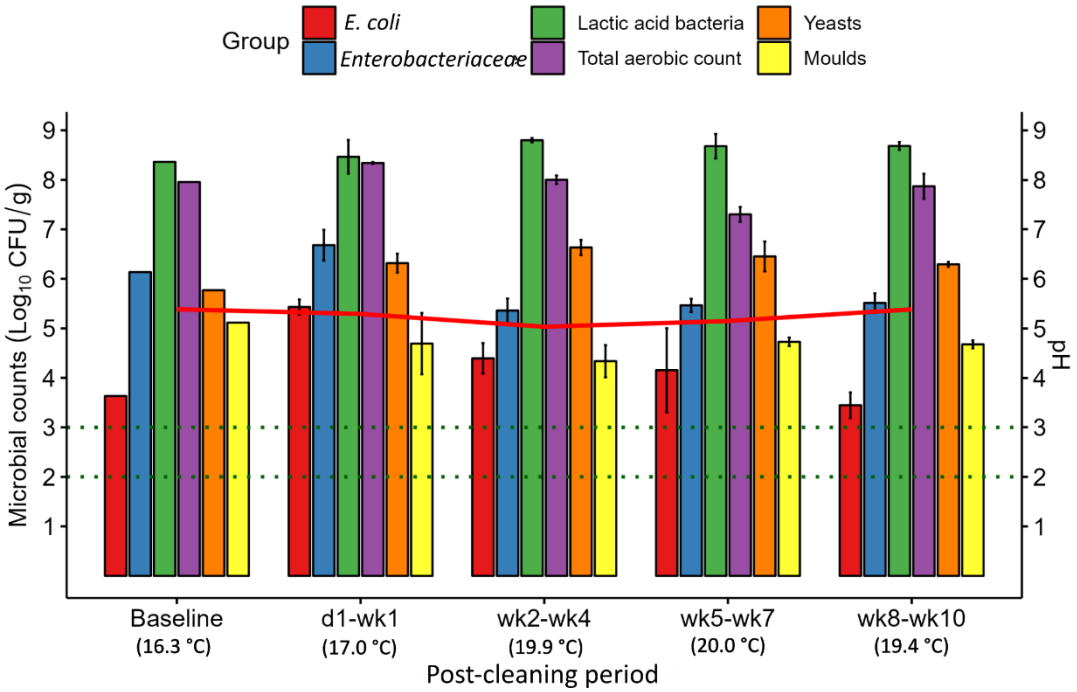

Figure 5.7: Mean microbial counts ($\log_{10}$ CFU/g ± SE) of residual liquid feed collected from the troughs before cleaning (baseline; n = 1) and at each post-cleaning period (n = 3). Mean liquid feed temperatures at each post-cleaning period are presented in parentheses below the x-axis labels. Horizontal dotted lines represent the limit of detection (LOD) for different microbial groups: LOD for *Escherichia coli* (*E. coli*), *Enterobacteriaceae*, lactic acid bacteria and total aerobic count = 2 $\log_{10}$ CFU/g; LOD for yeasts and moulds = 3 $\log_{10}$ CFU/g. Counts below the LOD are reported at the LOD. The mean feed pH is indicated by the solid red line.

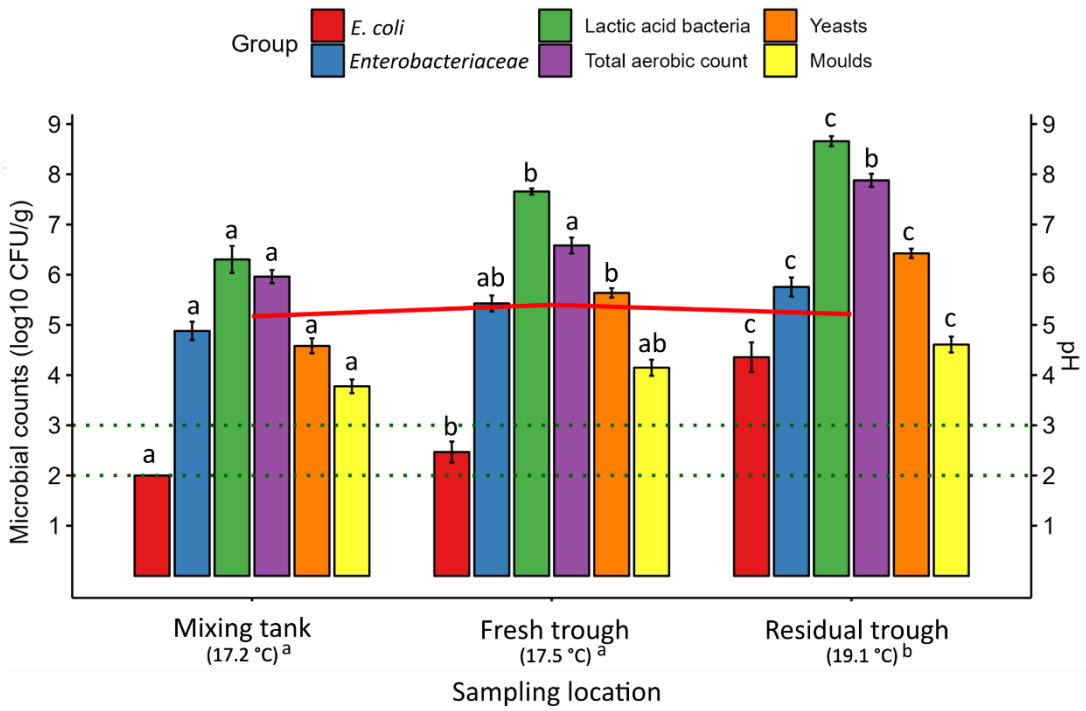

Figure 5.8: Mean microbial counts (log_{10} CFU/g ± SE) of liquid feed samples pooled by sampling location for each time point (n = 12). Mean liquid feed temperatures at each sampling location are presented in parentheses below the x-axis labels. Horizontal dotted lines represent the limit of detection (LOD) for different microbial groups: LOD for *Escherichia coli* (*E. coli*), *Enterobacteriaceae*, lactic acid bacteria and total aerobic count = 2 log_{10} CFU/g; LOD for yeasts and moulds = 3 log_{10} CFU/g. Mean feed pH is indicated by the solid red line. Bars of the same colour (microbial group) and temperature values that do not share a common letter/superscript are significantly different ($p < 0.05$).

5.8 Supplementary Information

Table S 5.1: Ingredient composition and calculated chemical composition of the diet on an as-fed basis (g/kg unless otherwise stated).

Ingredient composition	
Wheat	400.0
Soya bean meal	186.9
Barley	380.7
Soya oil	7.1
Lysine HCl	3.9
DL-Methionine	1.3
L-Threonine	2.0
L-Tryptophan	0.2
Limestone flour	11.0
Mono DiCalcium Phosphate	0.8
Salt	3.0
Vitamin and mineral premix[1]	1.0
Celite	2.0
Phytase[2]	0.1
Chemical composition	
Dry matter	874.4
Protein	170.0
Fat	25.3
Ash	38.2
Fibre	28.9
Net energy (MJ/kg)	9.8
Total lysine	11.0

[1] Cu from copper sulphate, 15 mg; Fe from ferrous sulphate monohydrate, 24 mg; Mn from manganese oxide, 31 mg; Zn from zinc oxide, 80 mg; I from potassium iodate, 0.3 mg; Se from sodium selenite, 0.2 mg; vitamin A as retinyl acetate, 0.7 mg; vitamin D3 as cholecalciferol, 12.5 µg; vitamin E as DL-alpha-tocopheryl acetate, 40 mg; vitamin K, 4 mg; vitamin B12, 15 µg; riboflavin, 2 mg; nicotinic acid, 12 mg; pantothenic acid, 10 mg; vitamin B1, 2 mg; vitamin B6, 3 mg.

[2] The diet contained 500 phytase units (FYT) per kg of feed from Ronozyme HiPhos (DSM, Belfast, UK).

Table S 5.2: Pen group weights and average pig weights in each pen of grow-finisher pigs at the beginning (day 1 post-cleaning; d1 PC) and the end of the experiment (day 76 post-cleaning; d76 PC). Average total gain, daily gain, daily feed intake and feed conversion efficiency are presented as an average for each pen.

Pen No.	No. pigs	Pen group weight (kg) on d1 PC	Average weight/pig (kg) on d1 PC	Pen group weight (kg) on d76 PC	Average weight/pig (kg) on d76 PC	Average total gain (g)	Average daily gain (g/day)	Average daily feed intake (g/day)	Feed conversion efficiency
1	5	134.0	26.8	595.4	119.1	92280	1214	2754	2.27
2	5	146.0	29.2	596.2	119.2	90040	1185	2536	2.14
3	5	126.0	25.2	623.2	124.6	99440	1308	2923	2.23
4	5	128.0	25.6	573.2	114.6	89040	1172	2397	2.05
5	5	160.0	32	600.0	120.0	88000	1158	2878	2.49
6	5	142.0	28.4	621.4	124.3	95880	1262	2283	1.81
7	5	170.0	34	690.0	138.0	104000	1368	3774	2.76
8	5	196.0	39.2	666.0	133.2	94000	1237	2705	2.19
9	5	176.0	35.2	674.0	134.8	99600	1311	2740	2.09
10	5	150.0	30	580.8	116.2	86160	1134	2891	2.55
11	5	184.0	36.8	617.8	123.6	86760	1142	2912	2.55
12	5	178.0	35.6	545.4	136.4	100750	1326	2087	1.57
13	5	176.0	35.2	605.4	121.1	85880	1130	3089	2.73
14	5	148.0	29.6	607.4	121.5	91880	1209	2415	2.00
15	5	232.0	46.4	734.6	146.9	100520	1323	3277	2.48
16	5	206.0	41.2	530.0	132.5	91300	1201	2898	2.41
17	5	186.0	37.2	648.2	129.6	92440	1216	3010	2.47
18	5	204.0	40.8	650.0	130.0	89200	1174	3125	2.66
19	5	164.0	32.8	636.2	127.2	94440	1243	2962	2.38

20	5	196.0	39.2	681.6	136.3	97120	1278	2832	2.22
21	5	204.0	40.8	686.4	137.3	96480	1269	3134	2.47
22	5	164.0	32.8	619.0	123.8	91000	1197	2405	2.01
23	5	164.0	32.8	563.8	112.8	79960	1052	2657	2.53
24	5	196.0	39.2	683.0	136.6	97400	1282	2944	2.30
25	5	172.0	34.4	567.2	113.4	79040	1040	2692	2.59
26	5	214.0	42.8	621.8	124.4	81560	1073	3202	2.98
27	5	192.0	38.4	674.6	134.9	96520	1270	3109	2.45
28	5	182.0	36.4	623.8	124.8	88360	1163	2552	2.19
29	5	164.0	32.8	673.0	134.6	101800	1339	3027	2.26
30	5	168.0	33.6	633.2	126.6	93040	1224	2968	2.42
31	5	154.0	30.8	630.8	126.2	95360	1255	2820	2.25
32	5	156.0	31.2	601.4	120.3	89080	1172	2985	2.55
33	5	194.0	38.8	665.2	133.0	94240	1240	3202	2.58
34	5	182.0	36.4	640.4	128.1	91680	1206	2895	2.40
35	5	186.0	37.2	660.0	132.0	94800	1247	2958	2.37
36	5	200.0	40.0	511.8	128.0	87950	1157	2710	2.34

Table S 5.3: Cost benefit summary for liquid feeding system sanitisation programme for first five years.[1]

	Number of pigs per batch		
	1,000	5,000	10,000
Feed saving/year (€/year)	5,390	26,948	53,896
Total cost of cleaning (€/year)	420	2,100	4,200
Increased labour cost (€/year)	401	401	401
Repayments/year (5-year loan; €)	1,077	1,077	1,077
Margin over increased cost (€/year)	3,491	23,369	48,217
Increased margin per pig (€/pig)	0.87	1.16	1.20

[1]Cost benefit analysis was based on assumed 0.05-unit improvement in feed conversion efficiency between 30-115 kg live weight. Feed savings were calculated based on the 5-year average finisher feed price up to 2022 (Teagasc Pig Feed and Price Monitor). Figures are based on four batches of pigs per year (each batch including 10 days turnaround during which the sanitisation programme is performed).

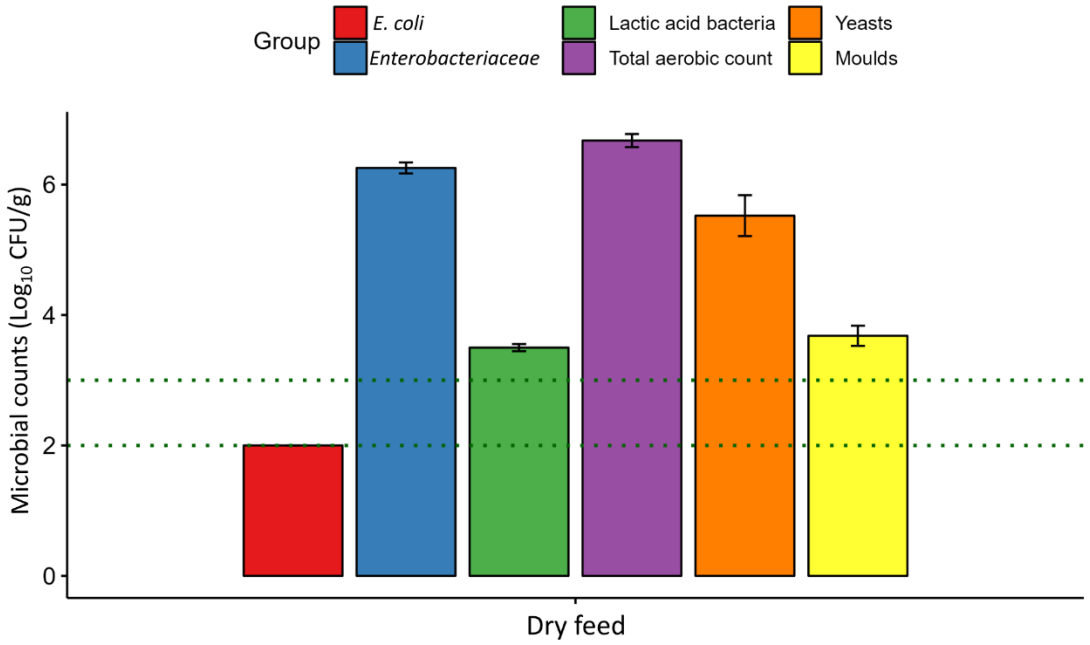

Figure S 5.1: Mean microbial counts (log_{10} CFU/g ± SE) in dry feed samples collected from the silo (*n* = 3). Horizontal dotted lines represent the limit of detection (LOD) for different microbial groups: LOD for *E. coli*, *Enterobacteriaceae*, lactic acid bacteria and total aerobic count = 2 log_{10} CFU/g; LOD for yeasts and moulds = 3 log_{10} CFU/g. Counts below the LOD are reported at the LOD.

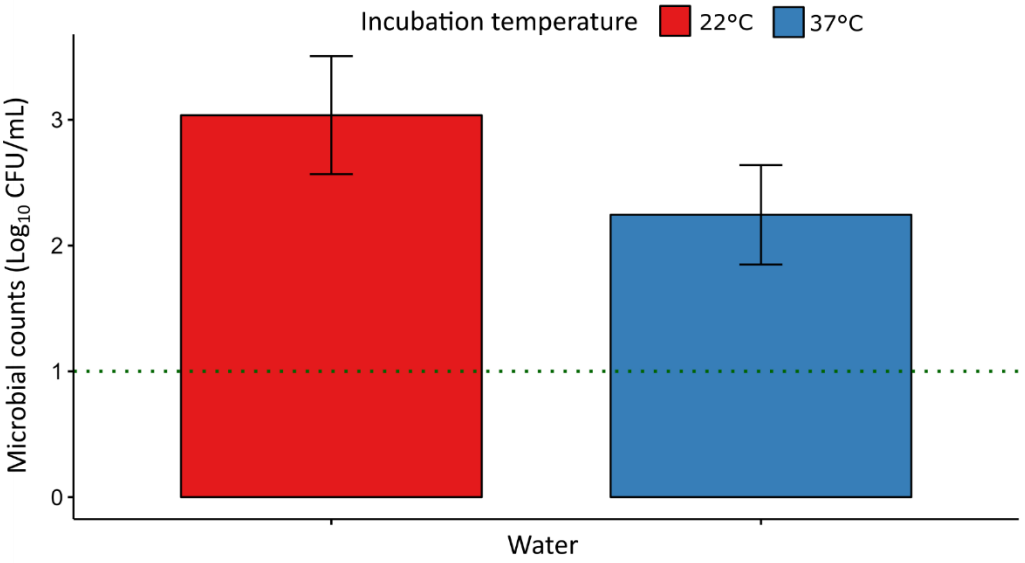

Figure S 5.2: Mean total aerobic counts (log_{10} CFU/mL ± SE) in water samples collected from a connection beside the mixing tank obtained at 22°C (*n* = 2) and 37°C (*n* = 3). The horizontal dotted line represents the limit of detection (LOD) for total aerobic counts (1 log_{10} CFU/mL).

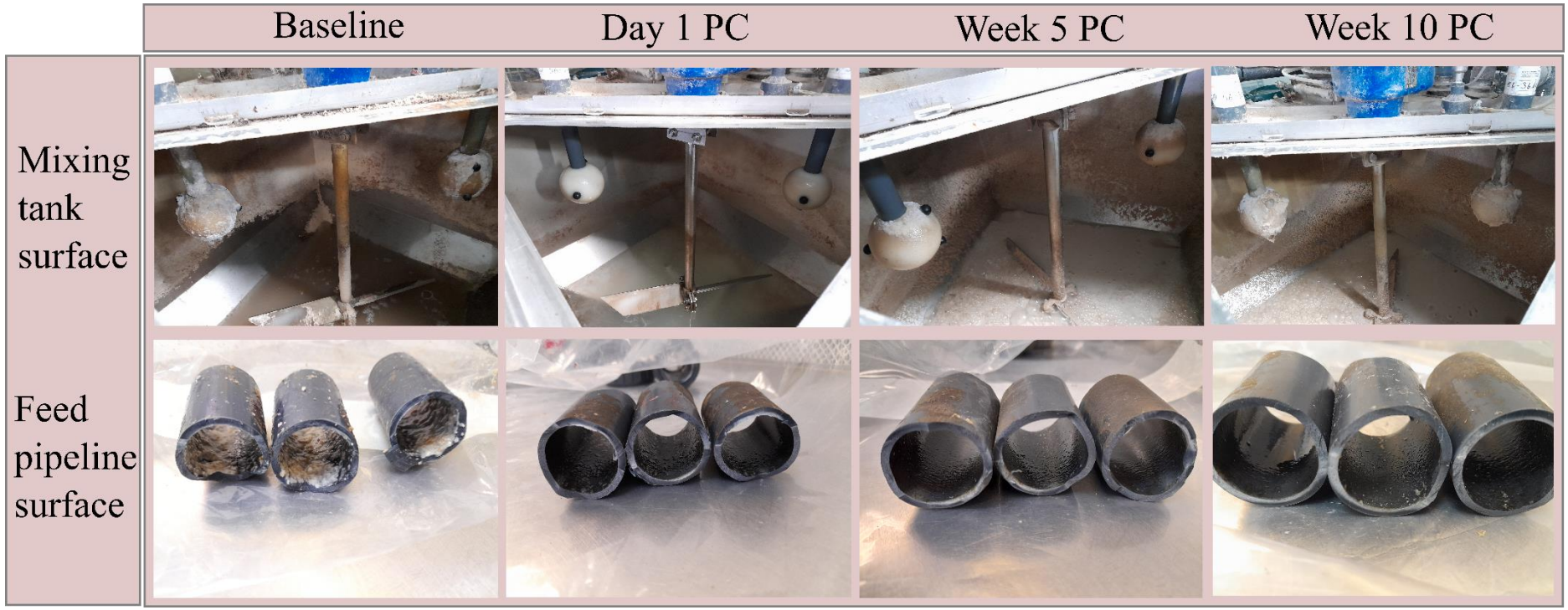

Figure S 5.3: Images of both the mixing tank and feed pipeline surfaces before (baseline) and after implementation of the sanitisation programme on day 1, week 5 and week 10 post-cleaning (PC).

5.9 References

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Chapter 6: Investigating the impact of liquid feeding system sanitisation on the bacteriome and mycobiome of liquid feed and liquid feeding systems for pigs

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6.1 Abstract

The aim of this study was to investigate the effect of implementing an intensive liquid feeding system sanitisation programme on the bacteriome and mycobiome of the liquid feed and the feeding system surfaces. The results confirm previous findings that the resident bacterial and fungal biofilms on the internal surfaces of the feed pipeline were disrupted post-cleaning (PC). Before cleaning, the feed pipeline biofilm was dominated by a low diversity microbial community, comprised mainly of *Lactobacillus, Leuconostoc and Chryseobacterium* and the yeast *Apiotrichum*. Microbial diversity increased on the pipeline surface PC, but community composition and abundance were highly unstable throughout the 10-week PC period and never returned to baseline values. This was likely due to the nightly acid rinse circulated through the feed pipeline preventing re-colonisation. The cleaning programme also impacted the profile of the bacterial and fungal communities in the liquid feed. For example, *Weissella* and the yeast *Kazachstania* both became predominant in the trough-sampled liquid feed PC. However, despite the cleaning, spontaneous fermentation was still observed in the feed, as evidenced by decreased abundance of *Pantoea* and *Pseudomonas* between the mixing tank and troughs, with a concurrent increase in *Lactobacillus*. Nonetheless, previous analysis revealed only minimal losses in gross energy in the liquid feed. Additionally, organic acid, ethanol and biogenic amine concentrations were lower PC, indicating that spontaneous fermentation was reduced, thereby improving the chemical quality of the liquid feed. Overall, although microbes began to re-colonise the feeding system PC, hygiene was still improved at the end of the 10-week grow-finisher period, compared to baseline, as evidenced by disruption of surface microbial communities. Therefore, we recommend intensive cleaning of liquid feeding systems between batches of grow-finisher pigs.

6.2 Introduction

A two-step sanitisation programme was implemented in an on-farm liquid feeding system between batches of grow-finisher pigs in Chapter 5. The first step consisted of intensive physical cleaning of the mixing tank, followed by cleaning of the tank and pipelines with an alkaline detergent and rinsing of the tanks and pipes with a feed-grade organic acid blend. This aimed to disrupt microbial biofilms in the system, which may be responsible for poor hygiene and contribute to amino acid and energy losses in the feed. The second step involved daily rinsing with the same feed-grade organic acid blend, with the rinsings used to prepare the first feed of the day. This aim of this step was to suppress the proliferation of *Enterobacteriaceae* and undesirable fungi to maintain the hygiene of the system. This sanitisation programme proved effective in terms of suppressing the growth of *Enterobacteriaceae* and moulds and temporarily reducing lactic acid and yeast counts on the mixing tank and pipeline surfaces of the feeding system. However, it had little impact on the microbiology of the liquid feed itself. Of note, it did reduce acetic acid, ethanol and biogenic amine concentrations in the feed and no gross energy losses were observed post-cleaning (PC). This highlights the usefulness of sanitisation of liquid feeding systems for the improvement of feed quality.

However, one drawback of the work in Chapter 5, and other studies to-date in this area, is that microbiological analysis of the liquid feed and feeding systems is limited to monitoring a small number of microbial groups using culture-based methods. While scanning electron microscopy (SEM) images also obtained in Chapter 5 showed the first definitive proof of the diversity of bacteria and fungi present within the liquid feeding system pipeline, and the effectiveness of the sanitisation programme in removing them, these microbes cannot be identified. As a result, neither culture- nor microscopy-based methods provide a taxonomic profile of the entire microbial community within the feed or feeding system. Therefore, the objective of this chapter was to use DNA sequence-based methods to determine the complete bacteriome and mycobiome composition of the grow-finisher liquid feed and feeding system surfaces, before and after implementation of the intensive sanitisation programme.

6.3 Materials and Methods

6.3.1 Ethical approval

Ethical approval for this study was granted by the Teagasc Animal Ethics Committee (approval no. TAEC2020-271). The experiment was conducted in accordance with the legislation for commercial pig production set out in the European communities (welfare of farmed animals) regulations 2010 and in Irish legislation (SI no. 311/2010).

6.3.2 Sanitisation of the liquid feeding system

A two-step sanitisation programme was implemented on the automated liquid feeding system (HydroMix, BigDutchman, Vechta, Germany) in the grow-finisher section of the research unit at Teagasc, Moorepark, Fermoy, Co. Cork. Sanitisation was performed during the routine unit cleaning that is normally carried out between batches of pigs. The sanitisation programme is described in detail in Chapter 5. Briefly, it involved physical cleaning of the liquid feeding system, in addition to washing the mixing tank and feed pipelines with an alkali detergent (Avalksan Gold Standard Chlorine Free; Carbon Chemicals Group Ltd., Ringaskiddy, Co. Cork, Ireland) and an organic acid blend (60% formic acid, 15% propionic acid, 2.5% benzoic acid; Interpronutri Plus BE; Interchem Ireland Ltd., Co. Dublin, Ireland). The new batch of grow-finisher pigs then entered the house [on day (d)1 PC], after which the same organic acid blend was used to perform a nightly maintenance rinse of the system, which was recirculated into the first feed of each day for the duration of the experiment. The pigs were liquid-fed from the sanitised system for a 76-day grow-finisher period, with sampling performed up to week (wk) 10 PC, as detailed below.

6.3.3 Diet preparation and feeding and animal housing and management

The ingredient and calculated chemical composition of the experimental diet at formulation is described in Chapter 5. It was manufactured in meal form. Liquid feed was prepared in the mixing tank of the automated liquid feeding system 6 times per day at a water to feed ratio of 3:1 on a dry matter (DM) basis (equivalent to ~2.5:1 on a fresh matter basis). In the mixing tank, the liquid feed was agitated for 10 min prior to feed-out. The feed was delivered from the mixing tank to the troughs via the feedline

using high-pressure air. At each feeding, electronic sensors in each trough ensured that when feed was above the sensor in the trough, feed was not dispensed to that particular trough. When the feed was below the sensor, feed was dispensed.

Animal housing and management during the experiment were described in Chapter 5. Briefly, 180 Danavil Duroc × (Landrace × Large White) female and entire male pigs (35.0 kg ± 4.90 SD at the start of the experiment) were housed in the grow-finisher house, penned in groups of 5 pigs across 36 pens with concrete slatted floors.

6.3.4 Sample collection for bacteriome and mycobiome analysis

Baseline samples were collected at 12 days prior to implementation of the sanitisation programme, after which samples were collected at d1 and d3 PC and wk1 PC, followed by weekly sampling up to wk10 PC. At each of the 13 sampling time points, swabs from the internal surface of the feed pipeline, and liquid feed samples from the mixing tank and troughs were collected. Dry feed and water samples were also collected. A detailed description of the sampling is given below.

6.3.4.1. *Feed pipeline internal surfaces*

Samples from the internal surfaces of the feed pipeline were collected by removing and replacing ~15 cm sections of the polyvinyl chloride (PVC) feed pipeline. On each sampling day, the exterior surface of the pipe section to be removed was cleaned with ethanol wipes. The direction of flow within the pipe and the top and bottom sections were labelled. The joiners connecting the sections were unscrewed and the entire pipe section (length: 15 cm; diameter: 32 mm) was removed into a sterile plastic bag, sealed and immediately stored on ice. Throughout the experiment, pipe sections were removed sequentially from along the pipeline, moving back towards the mixing tank each time an existing pipe section was replaced with a new section after sampling.

A PVC pipe cutters, sterilised by ethanol and flaming, was then used to aseptically cut a ~5 cm long circular piece from the pipe section. A sterile cell scraper (Fisher Scientific, Loughborough, Leicestershire, UK) was used to scrape a 50 cm^2 area around the entire circumference of the inside of this circular sub-section of pipe for 30 seconds. The head of the scraper was added to a sterile stomacher bag. Then, a sterile sponge swab pre-soaked with neutralising buffer (Sponge-stick; 3M, Saint Paul, MN,

USA) was used to swab the same 50 cm^2 area of the inside surface of the pipe. Maximum recovery diluent (MRD, 50 mL; Merck, Darmstadt, Germany) was added to the stomacher bag containing the scraper and swab, which was then stomached for 2 min. Based on the method described by McHugh et al. (2021), 2 x 10 mL aliquots of the resulting homogenate were centrifuged at 4,500 × g for 20 min at 4 °C. The supernatant from each tube was discarded and each pellet was re-suspended in 500 µL phosphate buffered saline (PBS; Sigma-Aldrich, Wicklow, Ireland). These suspensions were then pooled and centrifuged at 13,000 × g for 2 min, and the supernatant discarded. The pellet was stored at −80 °C for DNA extraction and subsequent bacteriome and mycobiome analysis.

6.3.4.2. *Feed and water*

On each sampling day, liquid feed samples were collected from the mixing tank (*n* = 1) using a sterile stainless-steel sampler which was lowered into the mixing tank after 10 min of agitation. Fresh liquid feed (*n* = 2 troughs) was collected as it was dispensed into the troughs and residual liquid feed (*n* = 2 troughs) was collected from the troughs after ~2.5 hours just prior to the next feed. At each sampling location, ~500 g of liquid feed was collected into sterile 500 mL containers and transported on ice to the laboratory. Aliquots (50 mL) were sub-sampled and stored at -20 °C until DNA extraction. Samples of the dry diet were also collected from the silo (*n* = 2; wk6 and wk10 PC) and were stored at -20 °C until DNA extraction. Finally, a water sample was collected from a connection beside the mixing tank (*n* = 1; wk10 PC). The water was allowed to flow for 5 min prior to sample collection into sterile 200 mL containers. A total of 2 L of water was vacuum filtered through a sterile cellulose nitrate membrane filter (0.45 µm pore size). The filter was stored at -80 °C for subsequent DNA extraction. All DNA extractions were performed within 6 months of sample collection.

6.3.5 DNA extraction

DNA extraction from dry feed, liquid feed and pipe swabs was performed using the QIAamp® Fast DNA Stool Mini kit (Qiagen, Hilden, Germany) following the 'Isolation of DNA from Stool for Pathogen Detection' protocol. Previously described modifications to this procedure were followed, which included a 20 min bead-beating

step for simultaneous extraction of bacterial and fungal DNA as described in Chapter 2. Based on the method described by McHugh et al. (2021), pellets that were recovered from pipe swabs were re-suspended in 1 mL of PBS and 500 µL was removed and centrifuged at 12,000 × g for 2 min. The supernatant was discarded and 1 mL of InhibitEX buffer from the QIAamp® Fast DNA Stool Mini kit was added, and the procedure described above was followed.

For the water sample, DNA was extracted from the filter using the DNeasy PowerWater Kit (Qiagen) as per the manufacturer's instructions. An additional step to improve lysis of difficult-to-lyse microbes was performed which involved heating the sample at 65°C for 10 min after addition of solution PW1. Finally, to concentrate the DNA, the volume of solution EB was reduced from 100 µL to 30 µL for elution.

All DNA extracts were quantified on a Qubit® 3.0 Fluorometer using the Qubit® dsDNA HS Assay Kit (Bio-Sciences, Dublin, Ireland).

6.3.6 Library preparation and amplicon sequencing

Library preparation and sequencing were performed by Macrogen Inc. (Seoul, South Korea) after an initial DNA quantity check with the QuantiFluor® dsDNA System (Promega, Madison, WI, USA) using a Victor Nivo Multimode Microplate Reader (PerkinElmer, Waltham, MA, USA).

The 16S libraries were prepared according to the Illumina 16S Metagenomic Sequencing Library protocols to amplify the V3-V4 region of the 16S rRNA gene. The initial amplicon PCR mixture contained 5 ng of DNA, 5x reaction buffer, 1 mM dNTPs, 500 nM of each of the forward and reverse primers, and Herculase II fusion DNA polymerase (Agilent Technologies, Santa Clara, CA, USA). The conditions for the initial PCR were 3 min at 95 °C, followed by 25 cycles of 30 sec at 95 °C, 30 sec at 55 °C and 30 sec at 72 °C, followed by a 5 min final extension at 72 °C. The primer pair with Illumina adapter overhang sequences used for the initial PCR were as follows: V3-F: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3', V4-R: 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATC

TAATCC-3'. The initial PCR products were purified with AMPure beads (Agencourt Bioscience, Beverly, MA, USA). Following purification, an index PCR was performed with 2 µL of the initial PCR products for final library construction containing Nextera XT indices. The same PCR conditions were used as the initial PCR, except that the number of cycles was reduced to 10. The indexed PCR product was purified with AMPure beads and was quantified using qPCR according to the qPCR Quantification Protocol Guide (KAPA Library Quantification kits for Illumina Sequencing platforms) and library sizes were measured using the TapeStation D1000 ScreenTape (Agilent Technologies, Waldbronn, Germany). Paired-end (2 × 300 bp) sequencing was performed using the MiSeq™ platform (Illumina, San Diego, CA, USA).

The internal transcribed spacer 2 (ITS2) libraries were prepared according to the Illumina ITS Metagenomic Sequencing Library protocols to amplify the ITS3-ITS4 region. The procedure was the same as for 16S library preparation except that the initial amplicon PCR mixture contained 10 ng of DNA. The ITS2 primer pair with Illumina adapter overhang sequences used for the initial PCR were as follows: ITS3-F: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGCATCGATGAAGAACGCAGC-3', ITS4-R: 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTCCTCCGCTTATTGATATGC-3'.

6.3.7 Bioinformatics and statistical analysis

Demultiplexed paired-end 16S and ITS2 rDNA sequences were imported (in PairedEndFastqManifestPhred33V2 format) into QIIME2 v.2020.8.0 (Bolyen et al., 2019), which was installed on a virtual machine (VirtualBox 7.0). Forward and reverse reads were quality assessed as described in Chapter 2 using the 'qiime demux summarize' command, FastQC v.0.11.5 and MultiQC v.1.9. The 16S and ITS2 primers were removed from reads using the cutadapt plugin (Martin, 2011). The QIIME2 DADA2 (Callahan et al., 2016) plugin was used for filtering and dereplication, chimera removal, merging paired-end reads and to infer amplicon sequence variants (ASVs) in each sample after truncating reads to remove low quality

bases. For bacterial sequences, taxonomy was assigned to each ASV using a Naive Bayes classifier trained on 16S V3-V4 sequences from the SILVA database (Version 138) with the 'q2-feature-classifier' plugin, while taxonomy was assigned to fungal ASVs using a Naive Bayes classifier trained on full-length ITS sequences from the UNITE v.8.3 database (Kõljalg et al., 2013) as per Chapter 2.

QIIME artefacts (taxonomy, ASV table, metadata and phylogenetic tree) were imported into R studio (Rversion v.4.2.1) as a phyloseq (McMurdie & Holmes, 2013) object with the qza_to_phyloseq() function in the qiime2r package (Bisanz, 2018). Contaminant bacterial and fungal ASVs, identified using the 'prevalence' method in the decontam package (Davis et al., 2018), were removed prior to further analysis. Further pre-processing included removal of ASVs that were not assigned to the kingdoms Bacteria and Fungi, for each respective dataset, and removal of ASVs that phylum-level taxonomy was not assigned to. Finally, the filter_taxa function in phyloseq was used to remove ASVs that were not observed more than 3 times in at least 1% of the samples, for each respective dataset. After pre-processing, the bacterial dataset comprised 4,512 ASVs with an average of 24,061.0 high-quality reads per sample, while the fungal dataset comprised 1,043 ASVs with an average of 24,426.3 high-quality reads per sample.

For all analyses, data from different sampling time points were pooled as follows for each sampling location: baseline (n = 1); d1-wk1 PC period (comprising d1, d3 and wk1 PC; n = 3); wk2-wk4 PC period (comprising wks 2, 3 and 4 PC; n = 3); wk5-wk7 PC period (comprising wks 5, 6 and 7 PC; n = 3) and wk8-wk10 PC period (comprising wks 8, 9 and 10 PC; n = 3). Alpha-diversity (Observed ASVs and Pielou's evenness) and beta-diversity, based on unrarefied filtered sequences, were calculated using the phyloseq package. Differences in alpha-diversity metrics were analysed using a linear mixed-effects model using the lmer function in the lme4 package (Bates et al., 2015), accounting for random effects. Statistical significance was tested using the analysis of variance (ANOVA) function in the car package, followed by pairwise comparisons using Tukey's HSD test with the emmeans package (Lenth, 2020). Alpha-diversity was subsequently plotted using the ggpubr package (Kassambara, 2020). Beta-diversity was measured using non-metric multidimensional scaling (NMDS) of Bray-Curtis dissimilarity distances, and was plotted using the ggplot2 package (Wickham,

2016). Permutational multivariate ANOVA (PERMANOVA) with 10,000 permutations was performed to test for differences between samples using the adonis2 function in the vegan package (Oksanen et al., 2020).

The ancombc2 function in the analysis of compositions of microbiomes with bias correction (ANCOMBC) package (Lin & Peddada, 2020) was used to identify differentially abundant bacterial and fungal genera between time points and sampling locations for liquid feed. Default ancombc2 settings were used unless otherwise specified. In order to avoid spurious results, 'prv_cut' was set higher than the default of 0.1 (specified in results section) for the bacterial and fungal datasets, respectively. Pairwise comparisons between sampling locations were tested using the pairwise directional test, with PC period included in the model as a random effect using the 'rand_formula' option. For multiple comparisons between time points, Dunnett's test was used to identify differentially abundant taxa at each PC period compared to baseline. Genera with an adjusted *p*-value of ≤ 0.05 were considered differentially abundant and log fold changes (LFC) of pairwise comparisons were plotted using ggplot2 (Wickham, 2016). Differences in alpha-diversity metrics and PERMANOVA results with $p < 0.05$ were considered statistically significant.

6.4 Results

6.4.1 Diversity of the bacterial and fungal communities

6.4.1.1. *Feed pipeline*

Figure 6.1 shows the alpha-diversity of the bacterial and fungal communities detected on the internal feed pipeline surfaces at baseline and at each PC period. It was not possible to perform statistical analysis on the data due to the low number of samples; however, the numerical values before and after cleaning will be outlined. Both the bacterial [Figure 6.1 (A)] and fungal [Figure 6.1 (B)] community richness (Observed ASVs: the number of species present) was lowest at baseline. Bacterial richness increased during the d1-wk1 PC period, decreased during the wk2-wk4 PC period and subsequently increased, with the maximum number of Observed ASVs detected at the end of the experiment (wk10). Conversely, Pielou's evenness of the bacteriome [Figure 6.1 (C)] was highest at baseline, and decreased PC (high evenness indicates that all species are present in similar proportions, while low evenness indicates that

only one or a few species predominate). However, the data followed an inverted bell curve trend, with evenness returning close to that of baseline during the wk8-wk10 PC period.

The richness of the mycobiome [Figure 6.1 (B)] also increased after cleaning, but unlike bacterial richness, maximum species richness was detected during the wk2-wk4 PC period, with a slight decline thereafter. However, as with bacterial richness, the number of Observed fungal ASVs remained notably higher compared to baseline. Unlike the bacteriome, evenness of the mycobiome [Figure 6.1 (D)] on the feed pipeline surface was lowest at baseline, increased until the wk2-wk4 PC period and decreased during the wk5-wk7 PC period; however, at the end of the experiment, it was higher than at baseline.

6.4.1.2. *Liquid feed*

Differences in alpha-diversity of liquid feed between baseline and each PC period, as well as between each of the PC periods were investigated, irrespective of sampling location. Neither the richness nor evenness of bacterial [Figure S6.1 (A) and (C)] nor fungal [Figure S6.1 (B) and (D)] communities in liquid feed differed based on time point ($p > 0.05$). Although the average evenness was similar for each time point, a number of samples of residual trough-sampled feed had numerically lower bacterial and fungal evenness [Figure S6.1 (C) and (D)]. Therefore, differences between sampling location were investigated next. Bacterial richness was higher in the residual trough-sampled feed compared to both the mixing tank and the fresh trough-sampled feed [Figure 6.2 (A); $p < 0.05$]. Conversely, fungal community richness was lower in the residual feed sampled from the trough compared to feed sampled from the mixing tank and fresh trough-sampled liquid feed sampled [Figure 6.2 (B); $p < 0.01$]. Contrary to the results for bacterial and fungal richness, Pielou's evenness of the bacteriome and mycobiome was lower in the residual feed sampled from the trough compared to fresh liquid feed from the trough [Figure 6.2 (C); $p < 0.01$ and Figure 6.2 (D); $p < 0.001$]. Fungal community evenness was also lower in the residual trough feed compared to feed from the mixing tank [Figure 6.2 (D); $p < 0.01$].

Non-metric multidimensional scaling (NMDS) plots of Bray-Curtis dissimilarity distances between time points and sampling location for the bacterial and fungal

communities in the liquid feed are presented in Figure 6.3 (A) and (B), respectively. With regard to the beta-diversity of the bacteriome, Figure 6.3 (A) shows distinct clustering of the mixing tank feed at all time points, except baseline, suggesting that the bacteriome in the mixing tank feed changed after cleaning, but remained quite similar during each PC period. The clustering of the fresh trough-sampled liquid feed during the d1-wk1 PC period away from all other time points indicate that the greatest shift in bacterial community structure was immediately PC, which was also the case in the residual trough-sampled feed, where only the samples collected during the d1-wk1 PC period clustered distinctly. Permutational multivariate ANOVA testing revealed that both sampling location and PC period influenced the bacteriome composition. Sampling location accounted for ~30% ($p < 0.001$) of variance in bacterial community structure, while PC period had a lesser, but still significant contribution to community structure (~18%; $p < 0.001$).

Regarding the effect of sanitisation of the liquid feeding system, fungal beta-diversity followed a different trend compared to the bacteriome [Figure 6.3 (B)]. Two distinct clusters were observed; the first representing baseline up to the wk2-wk4 PC period, while the wk5-wk7 and the wk8-wk10 PC period samples formed their own distinct cluster. However, differences between sampling locations were similar to those observed for bacterial communities [Figure 6.3 (B)]. The mixing tank and fresh trough samples were more similar compared to the residual trough samples, with greater variability between residual trough samples. Permutational multivariate ANOVA demonstrated that both PC period and sampling location also influenced the mycobiome composition. As indicated by the clustering in Figure 6.3 (B), PC period had a greater influence, accounting for ~24 % of variance ($p < 0.001$), compared to location (~20 % of variance; $p < 0.001$).

6.4.2 Composition of the bacteriome and mycobiome

6.4.2.1. *Feed pipeline*

The mean read abundance (RA) of the top 20 bacterial genera detected on the internal surface of the feed pipeline at baseline and at each PC period are shown in Figure 6.4. At baseline, over half of the reads (53.1 %) detected in the feed pipeline were assigned to the genus *Lactobacillus*, while *Leuconostoc* was the next most abundant genus with

a RA of 23.8 %. Several other genera were detected at baseline, including *Bifidobacterium* and the lactic acid bacteria (LAB) *Pediococcus* and *Lactococcus.* However, these genera only accounted for ~5 % of the total reads in the feed pipeline. The other notable genus present in the feed pipeline at baseline was *Chryseobacterium* which was present at a RA of 14.6 %.

After cleaning, there was a dramatic shift in the bacteriome of the feed pipeline. *Lactobacillus* decreased to 5.7 % RA in the d1-wk1 PC period, with further decreases in the wk2-wk4 PC period. The other LAB also decreased in RA after cleaning, and all remained lower than baseline, except for *Pediococcus*, albeit it only accounted for 3.3 % of reads during the wk8-wk10 PC period. After wk4 PC, *Lactobacillus* increased in RA; however, even by wk8-wk10 PC, it was only around half of baseline RA. A number of genera that were present at < 1 % RA at baseline increased in RA during the d1-wk1 PC period; most notably, *Brachybacterium* (18.7 % RA), *Jeotgalicoccus* (15.9 % RA), *Staphylococcus* (9.5 % RA) and *Psychrobacter* (9 % RA). Other genera that were lowly abundant at baseline, and increased in RA PC, included *Corynebacterium, Aerococcus* and *Clostridium sensu stricto 1*. Despite some fluctuations in RA, the aforementioned genera that proliferated in the feed pipeline PC remained quite stable in RA for the duration of the experiment.

The mycobiome of the feed pipeline at the genus level at baseline and at each PC period is summarised in Figure 6.5. At baseline, the fungal community was dominated by the yeast *Apiotrichum* at 71.7 % RA, while 22.4 % of the reads were assigned to an ASV in the *Trichosporonaceae* family, of which *Apiotrichum* is a member. During the d1-wk1 PC period, the RA of the aforementioned taxa decreased to 20.2 and 16.9 % RA, respectively, while the RA of *Scopulariopsis* increased from 0.1 to 46.7 % RA. The fungal community detected in the feed pipeline during the wk2-wk4 PC period differed dramatically from both baseline and the d1-wk1 PC period, with increased RA of yeast such as *Kazachstania* (15.5 % RA) and *Wickerhamiella* (19.9 % RA), as well as moulds including *Aspergillus* (11.1 % RA) and *Penicillium* (9.7 % RA).

Yeast continued to dominate the pipeline mycobiome, with *Kazachstania* accounting for 62.4 % of the reads during the wk5-wk7 PC period, with a number of other genera increasing in RA during the wk8-wk10 PC period, including *Wickerhamiella*,

Wickerhamomyces, *Debaromyces* and *Cryptococcus*. *Aspergillus* remained at a mean RA of 7 % during the wk8-wk10 PC period; however, *Penicillium* was only present at 0.1 % RA.

6.4.2.2. *Liquid feed*

Figure 6.6 displays the mean RA of the top 15 bacterial genera at each sampling location at baseline and at each PC period. Similar to the feed pipeline at baseline, *Lactobacillus* (34 % RA), *Chryseobacterium* (20.1 % RA) and *Leuconostoc* (6.2 % RA) were the most abundant genera in the mixing tank feed at baseline; however, *Pantoea* was also present at a notable proportion (7.5 % RA). During the d1-wk1 PC period, the RA of *Lactobacillus*, *Chryseobacterium* and *Leuconostoc* declined considerably, followed by an increase in the RA of several taxa, most notably, *Pantoea* (28.2 % RA), *Stenotrophomonas* (8.3 % RA), *Sphingomonas* (11.6 % RA), *Pedobacter* (6 % RA) and *Pseudomonas* (7.6 % RA). During the wk2-wk4 PC period, the RA of *Lactobacillus* began to increase, reaching 19.5 % RA during the wk5-wk7 PC period. Other genera, including *Kosakonia*, a member of the *Enterobacteriaceae* family, became more abundant (8.8-12.5 % RA during the final two PC periods).

Despite the decreasing RA of *Lactobacillus* in the mixing tank feed over time, *Lactobacillus* remained dominant in the fresh trough-sampled liquid feed throughout the experiment, albeit its RA declined slightly over time. Some other notable differences in the fresh trough samples were increases in *Acinetobacter* and *Bacteroides* in the d1-wk1 PC period. In the residual trough samples, *Lactobacillus*, which was present at 26.7 % RA at baseline, remained predominant after cleaning (29.3-38.5 %RA) until wk5-wk7 PC. However, *Weissella* represented a much greater proportion of the reads in the residual feed after cleaning (6.4-39.5 % RA), compared to baseline (0.2 % RA). Conversely, there was a greater proportion of *Prevotella* (10.9 % RA) and *Bacteroides* (7.5 % RA) at baseline compared to after cleaning.

Differentially abundant bacterial taxa in liquid feed were investigated during each PC period compared to baseline, irrespective of sampling location. Bacterial taxa that were not present in more than 60 % of samples were filtered in order to avoid spurious results. It should be noted that many of the taxa that were significantly differentially abundant between time points were present at quite low RA. As shown in Figure S6.

2, the genus env.OPS 17 was less abundant during the d1-wk1, wk2-wk4 and wk5-wk7 PC periods compared to baseline with the greatest decrease during the d1-wk1 PC period (LFC: -4.07; $p < 0.05$). *Lactococcus* also decreased in differential abundance during the wk2-wk4 and wk8-wk10 PC periods, compared to baseline, with a LFC of -3.54 ($p < 0.05$) during the latter PC period. A number of bacterial taxa increased in differential abundance during the wk5-wk7 PC period compared to baseline, namely *Kosakonia, Sphingobacterium* and *Carnobacterium*, in order of decreasing LFC. *Kosakonia* (LFC: 3.5; $p < 0.05$) and *Rhodococcus* (LFC: 2.5; $p < 0.05$) were also more abundant during the wk8-wk10 PC period, while *Empedobacter* decreased in differential abundance during the same period (LFC: -3.76; $p < 0.05$) compared to baseline.

Differential abundance was also assessed according to sampling location, irrespective of sampling time point. Bacterial genera that increased in differential abundance in the fresh trough-sampled feed compared to the mixing tank included several LAB: *Pediococcus, Weissella, Streptococcus, Lactobacillus,* and *Leuconostoc*, in order of decreasing LFC (Figure S6.3). The majority of these genera increased further in the residual trough feed, with the greatest LFCs in the residual vs mixing tank observed for *Weissella, Clostridium sensu stricto 1* and *Streptococcus* with LFC values of 6.8, 6.37 and 6.25, respectively ($p < 0.05$). The only genera that decreased in differential abundance were *Erwinia, Pedobacter, Paenibacillus* and *Chryseobacterium* (LFC values of between -1.17 and -1.84 in the residual feed compared to the fresh and mixing tank feed; $p < 0.05$).

As shown in Figure 6.7, *Alternaria* was the most abundant member of the liquid feed mycobiome in the mixing tank at baseline and remained so after cleaning, except during the d1-wk1 PC period where *Blumeria* had a slightly higher RA (18.8 vs 14.3 %). *Blumeria* was also highly abundant at baseline and at the other PC periods. Other genera that constituted a large proportion of the fungal population in the mixing tank feed were *Monographella* and *Neoascochyta.* With regard to differences over time, the RA of *Neoascochyta* decreased during the wk5-wk7 and wk8-wk10 PC periods, while both *Gibberella* and *Fusarium* became more abundant during the same periods. Despite *Kazachstania* only being present at 2.4 % RA in the residual trough feed at

baseline, it constituted a much greater proportion of the residual liquid feed mycobiome PC (13.6-43.2 % RA).

Differentially abundant fungal taxa in liquid feed were investigated during each PC period compared to baseline, irrespective of sampling location. Fungal taxa that were not present in more than 80 % of samples were filtered in order to avoid spurious results. The LFC of fungal genera that were differentially abundant during each PC period, compared to baseline are displayed in Figure S6.4. *Entyloma* was the only fungal genus to increase in differential abundance during the d1-wk1 and wk2-wk4 PC periods; however, it did not comprise a large proportion of the liquid feed mycobiome. Of the top 15 fungal genera that had the highest mean RA in liquid feed (Figure 6.7), 6 were significantly differentially abundant with respect to time point. Of these, *Gibberella* and *Fusarium* increased in differential abundance during the wk5-wk7 PC period compared to baseline (LFC: 0.98 and 1.76, respectively; $p < 0.05$). The remaining highly abundant genera, *Neoascochyta, Diaporthe, Microdochium* and *Sporobolomyces* all decreased in differential abundance, most notably during the wk8-wk10 PC period. Interestingly, *Wickerhamomyces*, which increased in RA in the feed pipeline during the wk8-wk10 PC period (Figure 6.5), decreased in differential abundance in liquid feed, during the last two PC periods (wk5-wk7 and wk8-wk10), compared to baseline (LFC: -2.67 and -2.63, respectively; $p < 0.05$).

Fungal genera that were differentially abundant between sampling locations irrespective of PC period are displayed in Figure S6.5. *Apiotrichum* and *Kazachstania* both increased in differential abundance in the fresh trough-sampled liquid feed compared to the mixing tank (LFC: 3.07 and 2.41, respectively; $p < 0.05$). *Kazachstania* further increased in differential abundance in the residual trough compared to the fresh trough feed, along with *Pichia.* Both *Cutaneotrichsporon* and *Blumeria* also increased in differential abundance when residual feed was compared to the mixing tank feed (LFC: 1.20 and 0.86, respectively; $p < 0.05$). Overall, *Kazachstania, Pichia* and *Apiotrichum,* which were all amongst the top 15 most abundant fungal genera in the liquid feed, increased in differential abundance to the greatest extent in the residual feed, compared to the mixing tank, with LFC values of between 4.39 and 5.05 ($p < 0.05$).

6.4.2.3. *Dry feed and water used to prepare liquid feed*

The bacterial and fungal profiles of the dry feed and water used to prepare the liquid diet were assessed in order to investigate their influence on the feed pipeline and liquid feed microbiome. The most abundant bacterial genera in the dry feed were *Pantoea*, *Stenotrophomonas*, *Sphingomonas* and *Pseudomonas* [Figure S6.6 (A)], all of which comprised a relatively large proportion of the mixing tank feed. However, *Lactobacillus*, which dominated the mixing tank feed, was not present at a high RA in the dry feed. The RA of the other genera were quite variable between the different batches of dry feed sampled; however, the composition was consistent.

Similarly, the majority of the predominant fungal taxa in the mixing tank feed were also present in the dry feed [Figure S6.6 (B)]. The most abundant fungal genera in the dry feed were *Alternaria*, *Monographella*, *Blumeria*, *Cladosporium* and *Neoascochyta*; their RA was also quite variable between the batches of feed. However, as with *Lactobacillus*, the yeast *Kazachstania* was not highly abundant in the dry feed although it came to be the dominant fungal component of the residual trough samples. The bacterial and fungal diversity in the water sample was relatively low compared to that in the dry feed. Three bacterial genera accounted for > 80 % of the reads in the water samples, namely, in order of abundance, *Mycobacterium*, *Sphingomonas* and *Undibacterium*. The water mycobiome was dominated by *Cutaneotrichsporon* (45.9% RA), while 13.5 % of the reads were assigned to unidentified genera. Several other lowly abundant genera, which were also detected in the feed, were present in the water mycobiome including *Fusarium, Neoascochyta* and *Blumeria*. Two genera that were not present in the feed pipeline nor the liquid feed, had relatively high proportions in the water sample: *Tricladium* (8.4% RA) and *Bjerkandera* (7.4% RA).

6.5 Discussion

This study obtained, for the first time, the complete bacteriome and mycobiome profile of liquid feed and the liquid feeding system, before and after implementation of a sanitisation programme. Previous culture-based analyses in Chapter 5 showed that the sanitisation programme was effective in suppressing the growth of *Enterobacteriaceae* and moulds on the mixing tank and feed pipeline surfaces but had little impact on the liquid feed microbiology. However, culture-based analyses only

monitor a small number of microbial groups and hence the value in using DNA sequence-based methods here.

Considerable research has been performed to investigate microbial biofilms within water distribution pipes and their potential as a source of undesirable microbes in water (Liu et al., 2020; Rożej et al., 2015). However, little is known about the microbial biofilms within liquid feeding systems, which likely also influence the microbial composition of the feed as it passes through the system. Both bacterial and fungal richness on the feed pipeline surface was low at baseline, with *Lactobacillus*, *Leuconostoc* and *Chryseobacterium* representing 91.5 % of the bacterial reads and *Apiotrichum* and a *Trichosporonaceae* ASV representing 94.1 % of the fungi. This suggests that the feed pipeline was colonised by an established low-diversity microbial biofilm. The subsequent variability in the bacterial and fungal alpha-diversity PC suggests that this biofilm was disrupted by the sanitisation process, with the community structure left in a state of flux. Specifically, the RA of the resident baseline LAB and *Chryseobacterium* on the feed pipeline surface decreased dramatically PC, accompanied by increased RA of aerobic bacteria i.e. *Jeotgalicoccus* and *Brachybacterium*. Although LAB are generally regarded as desirable in liquid feed due to lactic acid production, *Leuconostoc* and some *Lactobacillus* species are heterofermentative, and can produce undesirable concentrations of metabolites including acetic acid and ethanol (Du Toit et al., 2001). Some LAB also produce biogenic amines, although this is strain-specific (Barbieri et al., 2019; Özogul & Özogul, 2019). *Chryseobacterium*, which has been associated with food spoilage and production of biogenic amines (Mwanza et al., 2022), and therefore is likely undesirable in feed, also decreased in RA in the mixing tank and fresh liquid feed PC. This coincided with its elimination from the feed pipeline; however, its RA increased again by wk2 PC, likely due to its presence in the dry feed (3.4-7.6% RA). Although a re-colonisation of *Lactobacillus* occurred on the feed pipeline surface from wk5 on, the RA of the aerobic bacteria that proliferated PC remained quite stable until the end of the experiment. These changes in community structure on the feed pipeline surface are supported by the culturing data in Chapter 5 that showed a substantial drop in LAB counts PC, with gradual increases during the wk2-wk4 PC period. However, the increased RA of aerobic bacterial taxa found in the current study is relative to the

actual decrease in LAB observed in the feed pipeline via culturing. Despite increases in the RA of these aerobic taxa, the culturing data showed that total aerobe counts declined PC. This demonstrates the benefits of relating molecular analyses to culture-based data.

A similar shift in the feed pipeline fungal community occurred PC, where the yeast *Apiotrichum,* for example, decreased dramatically in RA, especially during the first week PC, with a concomitant increase in the mould *Scopulariopsis*. Although *Scopulariopsis* is not a known mycotoxin producer, moulds are generally considered undesirable in feed due to spoilage and mycotoxin production (Menegat et al., 2019). However, the previous culturing data showed that yeasts were undetectable (< 100 CFU/cm^2) in the feed pipeline until wk5 PC, while moulds remained undetectable for the duration of the experiment (Chapter 5). Therefore, it should be noted that the actual abundance of fungi in the pipeline after cleaning was low. The yeast re-colonisation of the feed pipeline at wk5 PC found by culturing is reflected in the RA data, with *Kazachstania* and *Wickerhamiella* predominating. They remained the most abundant fungal taxa during the final PC period; however, community evenness increased during the same period, with a more diverse mycobiome observed. Nonetheless, contrary to findings for the bacteriome, no stabilisation of the fungal community occurred after cleaning, compared to baseline, where the feed pipeline was dominated by only a few fungal taxa. This may be because the nightly acid rinse of the feed pipeline prevented a new resident mycobiome from establishing.

Despite the fact that the yeast, *Apiotrichum*, was found to predominate the feed pipeline surface mycobiome at baseline, culturing in Chapter 5 detected moulds, and SEM imaging revealed extensive fungal hyphae. These hyphae were consistent with filamentous fungi such as *Penicillium*, *Aspergillus* or *Cladosporium*, albeit identification was not possible via microscopy. However, the DNA sequence-based methods employed in this chapter did not detect these moulds at baseline, despite previous optimisation of the method for the simultaneous extraction of bacterial and fungal DNA from liquid feed in Chapter 2. Therefore, the lack of DNA recovery from the feed pipeline samples may be due to the protection against physical and chemical disruption offered by biofilms (Sharma et al., 2019).

The bacterial and fungal alpha-diversity of liquid feed collected from the mixing tank and the troughs was not impacted by the sanitisation programme. However, bacterial richness increased in the residual feed in the trough compared to the mixing tank and fresh feed in the trough, while fungal richness decreased at the same locations. Community evenness decreased in the residual feed compared to the mixing tank and fresh liquid feed for both the bacteriome and mycobiome. These changes in alpha-diversity between sampling locations are in line with findings for liquid feed samples collected from commercial farms in Chapter 3. With regard to sampling location, *Clostridium sensu stricto 1*, *Weissella*, *Streptococcus, Prevotella*, *Corynebacterium, Lactobacillus, Bacteroides* and *Leuconostoc* all increased in differential abundance in the fresh trough-sampled feed compared to the mixing tank feed. The only genera that decreased were *Erwinia*, *Pedobacter* and *Chryseobacterium.* These results also support those found in the liquid feed survey in Chapter 3, where LAB in particular increased in abundance after the feed was mixed, with Gram-negative bacteria decreasing. This pattern of microbial succession is indicative of spontaneous fermentation, where decreasing pH and increasing concentrations of lactic acid and other LAB fermentation metabolites suppress pathogenic and spoilage-associated bacteria (Brooks, 2008; Dujardin et al., 2014; Russell et al., 1996). However, despite this pattern of spontaneous fermentation, chemical analyses in Chapter 5 showed no gross energy losses in the liquid feed, irrespective of sampling location or PC period. Furthermore, acetic acid, ethanol and biogenic amine concentrations in the feed were lower after sanitisation, which is a huge benefit in itself and also indicates that amino acid losses were less PC.

Beta-diversity analysis of the liquid feed also showed that the greatest differences between the bacteriome were driven by sampling location (explained ~30 % of variance in bacterial community structure versus ~18 % explained by PC period). This is in agreement with culturing data from Chapter 5, which showed increases in bacterial and fungal counts across sampling locations. Nonetheless, differences in bacterial community structure were evident PC, with baseline mixing tank and fresh trough samples clustering away from corresponding PC samples. This is in agreement with the RA data outlined below. Interestingly, beta-diversity analysis showed that sampling location also influenced the fungal communities (explained ~20 % of

variance). The residual trough samples in particular were more dissimilar from the other samples; however, PC period also had a strong effect on the fungal community (explained ~24 % of variance). Distinct clustering of samples taken from baseline up to wk4 PC was observed with a separate cluster for samples taken from wk5-wk10 PC. These clusters align with differences observed in the RA of certain fungal taxa between these time points, as outlined below.

Generally, bacteria in the liquid feed that were differentially abundant at each PC time point compared to baseline were lowly abundant, while those that differed between sampling location were more dominant. However, *Kosakonia*, which was also present in the dry feed, was more abundant in the mixing tank and fresh trough-sampled feed during the later PC periods compared to earlier. Although a member of *Enterobacteriaceae,* this genus is mainly known for its plant growth-promoting properties (Berger et al., 2017), with little known about its role in feed. Despite the low abundance of the sanitisation-related differentially abundant bacterial taxa, there were a number of interesting compositional changes in the liquid feed that were not considered significant by ANCOMBC. This is likely due to a high degree of variance during particular PC periods. This variance PC indicates that, as on the feed pipeline surface, the liquid feed bacteriome did not stabilise PC, with large variation in community structure up until the end of the experiment. Immediately after the sanitisation programme was implemented, the RA of *Lactobacillus, Chryseobacterium* and *Leuconostoc* in the mixing tank feed dropped, which corresponds to what occurred in the feed pipeline, suggesting that the bacteriome of the feed is influenced by the pipeline bacteriome. This was followed by an increase in the RA of several Gram-negative genera including *Pantoea*, *Pseudomonas* and *Sphingomonas,* all of which were found to be highly abundant in the dry feed. This is similar to previously reported Gram-negative (coliform) blooms in liquid feed after cleaning of the feeding system, which was attributed to decreased lactic acid fermentation (Brooks, 2008; Hansen, 1987). Similarly, here the proliferation of Gram-negative bacteria in the mixing tank feed PC was likely due to reduced lactic acid fermentation. This is supported by the reduced abundance of LAB in this study and the previously recorded decrease in LAB counts and lactic acid concentration in Chapter 5, albeit the pH decreased PC from addition of the acid rinse. *Pantoea* and

Pseudomonas have been associated with food spoilage and are therefore undesirable in feed (Grady et al., 2016; Ragaert et al., 2006; Stellato et al., 2017); however, their RA decreased in the feed sampled from the troughs compared to the mixing tank PC. This was likely due to the proliferation of LAB in the troughs, and the higher lactic acid concentrations found in the troughs compared to the mixing tank PC in Chapter 5.

The reduction in *Lactobacillus* in the mixing tank liquid feed PC is likely a direct result of the physical and chemical cleaning of the mixing tank, as in Chapter 5, the culturing data showed that LAB counts on the mixing tank surface pre-cleaning were $> 8 \log_{10} CFU/cm^2$. Similar to what occurred on the feed pipeline surface, the cleaning-associated drop in *Lactobacillus* in the mixing tank feed allowed other bacterial taxa to proliferate. Despite their low RA in the dry feed, and the drop in their RA in the mixing tank feed after cleaning, *Lactobacillus* quickly re-established itself PC to constitute a substantial proportion of the liquid feed bacteriome in the fresh and residual trough-sampled feed. However, PC, the RA of *Lactobacillus* remained just below baseline in the fresh trough-sampled feed, likely due to the nightly acid rinse. This may explain why another LAB, *Weissella* also increased in RA, particularly in the residual trough-sampled feed, potentially due to a competitive advantage, as *Lactobacillus* populations had not yet fully re-established. The survey of commercial pig units in Chapter 3 also showed that *Weissella* was present at a high RA in the residual feed on some, but not all of the farms.

Liquid feed, that has been fermented, whether spontaneously or intentionally, is known to have a high diversity of yeasts, including *Pichia, Kazachstania* and *Candida* (Gori et al., 2011; Olstorpe et al., 2010). *Kazachstania* increased in differential abundance in the fresh and residual feed, compared to the mixing tank; however, its RA was dramatically higher in the residual trough samples during the PC periods compared to baseline. Excessive yeast growth is generally considered undesirable in liquid feed, potentially resulting in foaming, reduced palatability, and nutrient and energy losses (Keller et al., 2020; Plumed-Ferrer & von Wright, 2011). However, *Kazachstania slooffiae,* a dominant member of the post-weaning piglet gut mycobiome, has been associated with improved gut health via symbiotic relationships with beneficial bacteria including *Lactobacillus* (Arfken et al., 2019; Summers et al.,

2021). Two moulds, *Gibberella* and *Fusarium*, both of which are undesirable in feed due to mycotoxin production (Burlakoti et al., 2008; Ji et al., 2019), also increased in differential abundance PC, but not until wk5. This is in line with the two distinct time point-associated clusters revealed by beta-diversity analysis of the mycobiome which showed that samples from the latter two PC periods clustered away from those taken at baseline and during the earlier PC periods. This may have been due to differences in the mycobiome of the dry feed used to prepare the liquid feed during these periods, as there were notable variances in the RA of fungi in the dry feed at the two time points on which feed was analysed.

The farm survey in Chapter 3 identified increases in differential abundance of *Saprochaete* and *Wickerhamiella* in fresh trough-sampled liquid feed compared to the mixing tank, with many other yeasts increasing in the residual trough feed including *Diutina, Apiotrichum, Pichia, Dipodascus* and *Kazachstania*. In the current study, both *Apiotrichum* and *Kazachstania* increased in differential abundance in the fresh trough-sampled feed compared to the mixing tank, with *Kazachstania* further increasing in the residual compared to the fresh trough-sampled feed, along with *Pichia*. These findings are indicative of spontaneous fermentation; however, chemical analysis of the liquid feed in Chapter 5, showed reduced concentrations of ethanol and acetic acid PC, which indicates a disruption to yeast fermentation as a result of cleaning. The use of organic acids, such as benzoic acid (present in the acid blend used in the current study) has been proposed as a means of controlling yeasts in liquid feed, without affecting lactic acid fermentation (Plumed-Ferrer & von Wright, 2011; Vils et al., 2018). When feed and water are mixed, the pH is generally ~6, which allows for proliferation of undesirable microbes, particularly *Enterobacteriaceae*. It is recommended that feed pH be reduced to between 3.5-4.5 via acidification or fermentation to prevent *Enterobacteriaceae* proliferation (Plumed-Ferrer et al., 2005; Rudbäck, 2013; Russell et al., 1996). In this study, the first batch of liquid feed each day was prepared using the maintenance acid rinse. Although the pH of the mixing tank feed containing the acid rinse decreased to 4.75-5.48 PC, compared to 6.28 at baseline, it did not decrease sufficiently (< pH 4) to prevent the proliferation of several Gram-negative bacteria including spoilage-associated *Pseudomonas* and *Pantoea* (Brooks, 2008). However, as discussed earlier, the lower concentration of lactic acid

in the mixing tank feed PC likely also contributed to this. Nonetheless, the sanitisation programme may have a greater impact on the feed microbiome if the acid blend was also included in the diet in order to achieve a pH of < 4.

6.6 Conclusion

Despite the culture-based data in Chapter 5 indicating minimal impact of liquid feeding system sanitisation on the microbiology of liquid feed, this study provided evidence of shifts in the bacteriome and mycobiome of liquid feed PC. It also supports the microscopy- and culture-based findings showing that the intensive liquid feeding system sanitisation programme disrupts the resident bacterial and fungal biofilms in the feed pipeline. This led to a more diverse but unstable microbial community on the pipeline surface and, to a lesser extent, in the feed itself. Furthermore, the pipeline and feed microbial communities did not return to those observed pre-cleaning. It is likely that either insufficient time elapsed during the experiment for their re-establishment, or that the nightly maintenance acid rinse prevented full re-colonisation. However, the microbial changes that usually occur in liquid feed between the mixing tank and troughs, that are indicative of spontaneous fermentation, followed the same pattern in this study, despite implementation of the sanitisation programme. Interestingly, decreases in the RA of LAB and *Chryseobacterium* in the feed pipeline PC were also reflected in the mixing tank feed, with increased abundances of *Pantoea* and *Pseudomonas*, while *Lactobacillus* again became predominant in the feed from the troughs. *Weissella* also became highly abundant in the trough-sampled feed compared to baseline, potentially as a result of *Lactobacillus* not having fully re-established yet. The yeast *Kazachstania* represented a large component of the residual trough-sampled feed mycobiome PC, while *Gibberella* and *Fusarium* also increased in RA during the latter PC periods. Nonetheless, the decreases in organic acid, ethanol and biogenic amine concentrations in the liquid feed sampled PC in Chapter 5 indicate improved feed quality as a result of sanitisation of the feeding system. Based on these findings, we recommend that this intensive sanitisation programme is implemented in liquid feeding systems during the grow-finisher stage every 10 weeks, between batches of pigs. Considering that microbial re-colonisation of feeding system surfaces occurs PC, albeit delayed by the sanitisation programme and the acid rinse, inoculating the liquid feed with a beneficial microbe such as a homofermentative LAB strain, in order to

control re-colonisation of the feeding system surfaces, may be a strategy worth exploring. Potential inoculants, however, should be amino acid decarboxylase- and bile salt hydrolase-negative to avoid biogenic amine production and deconjugation of bile salts, respectively.

6.7 Tables and figures

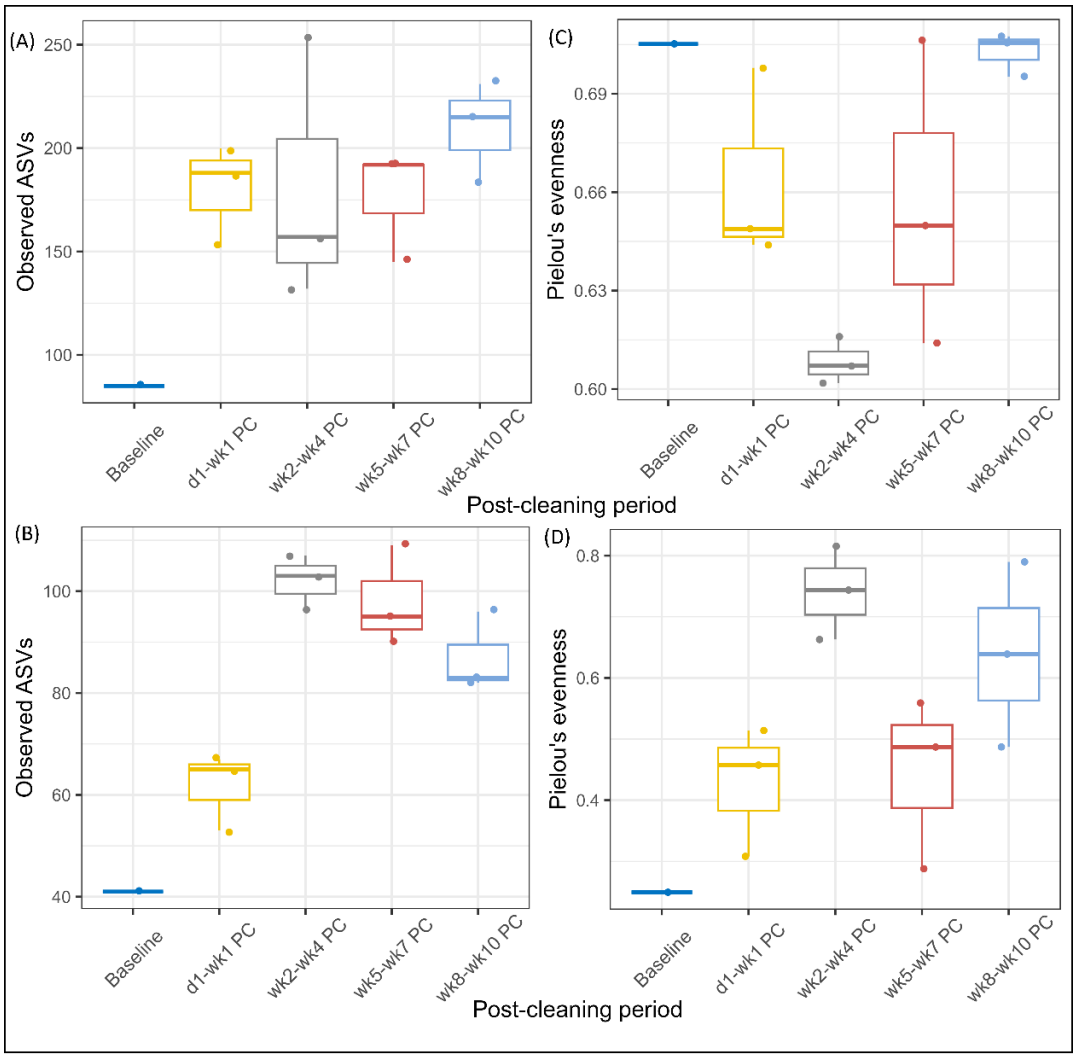

Figure 6.1: Boxplots displaying Observed amplicon sequence variants (ASVs) and Pielou's evenness of bacterial [(A) and (C)] and fungal [(B) and (D)] communities on the internal surface of the feed pipeline at baseline and at each post-cleaning (PC) period (n = 3 at each time point, except at baseline, where n = 1).

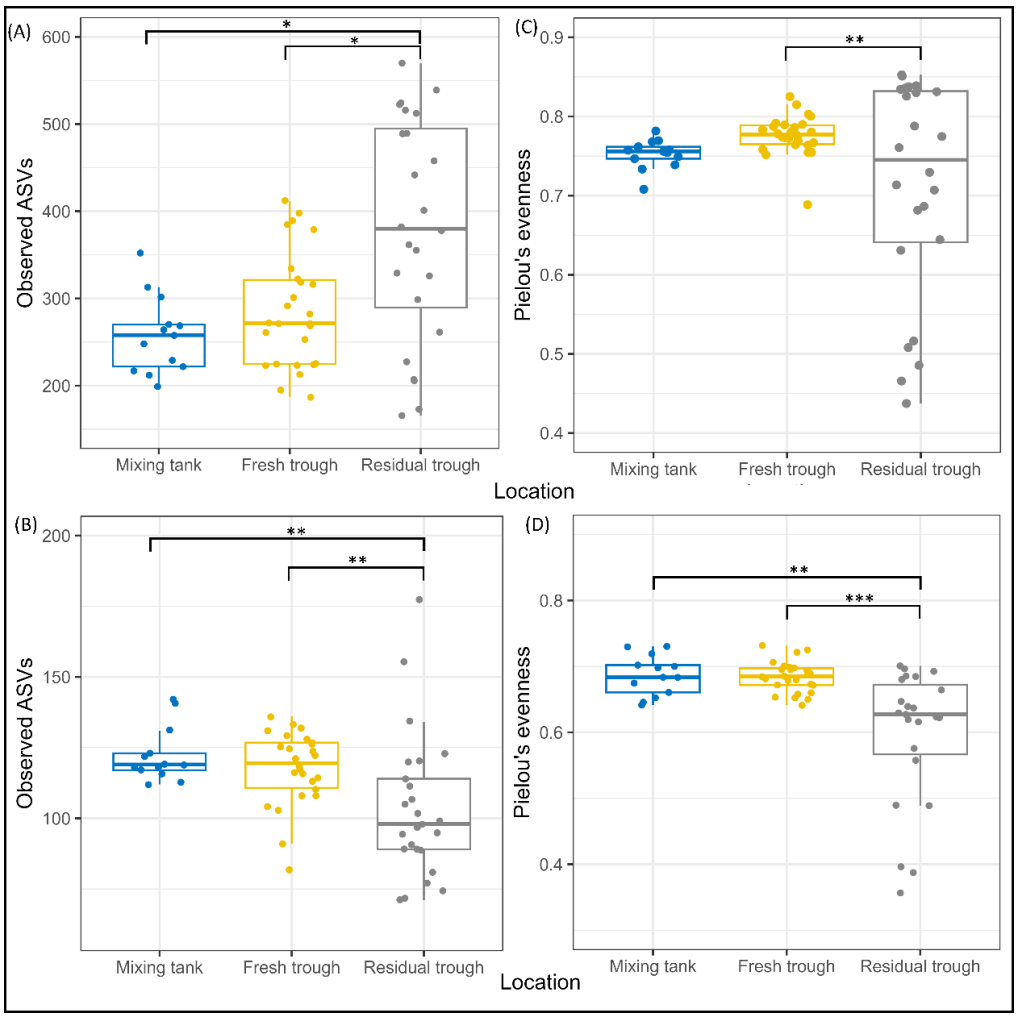

Figure 6.2: Boxplots displaying Observed amplicon sequence variants (ASVs) and Pielou's evenness of bacterial [(A) and (C)] and fungal [(B) and (D)] communities in liquid feed at each sampling location (Mixing tank: n = 13; Fresh trough: n = 26; Residual trough: n = 25). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

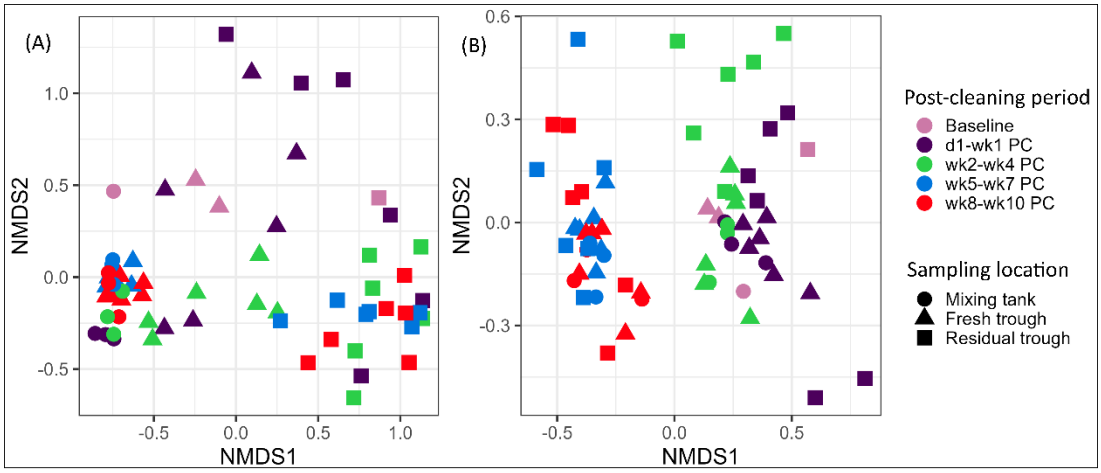

Figure 6.3: Non-metric multidimensional scaling (NMDS) plots based on Bray-Curtis dissimilarity between sampling location and post-cleaning (PC) period for bacterial (A) and fungal (B) communities in liquid feed.

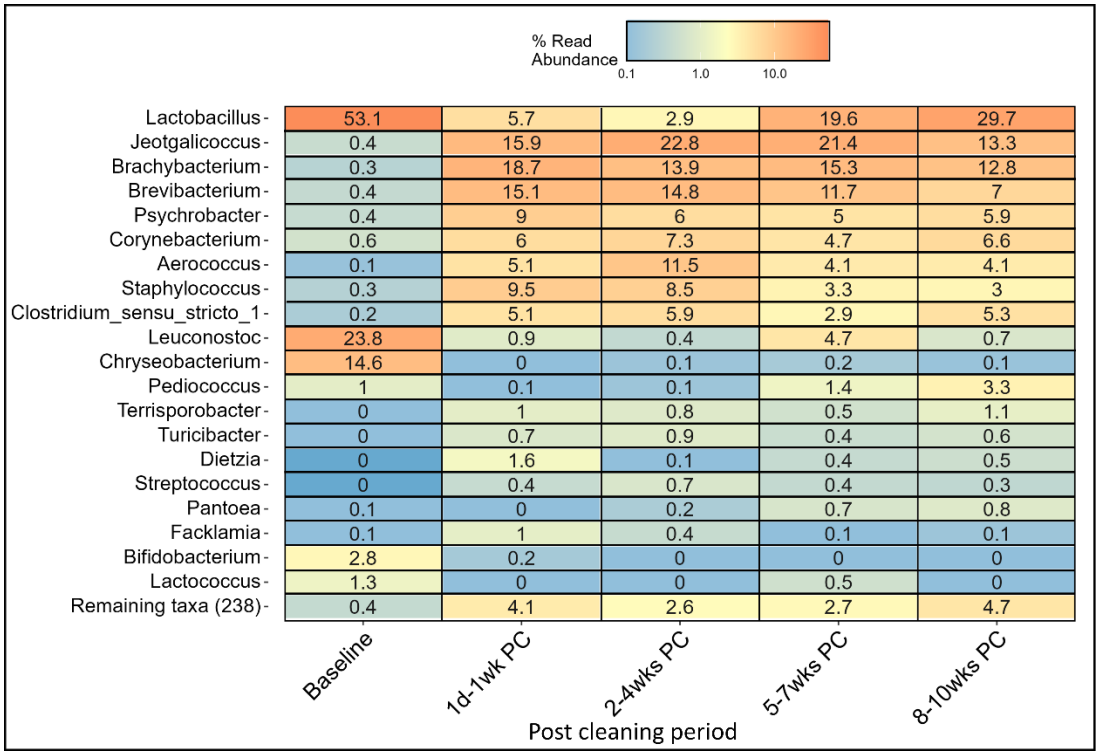

Figure 6.4: Mean percentage read abundance of the top 20 bacterial genera detected on the internal surface of the feed pipeline at baseline and at each post-cleaning (PC) period.

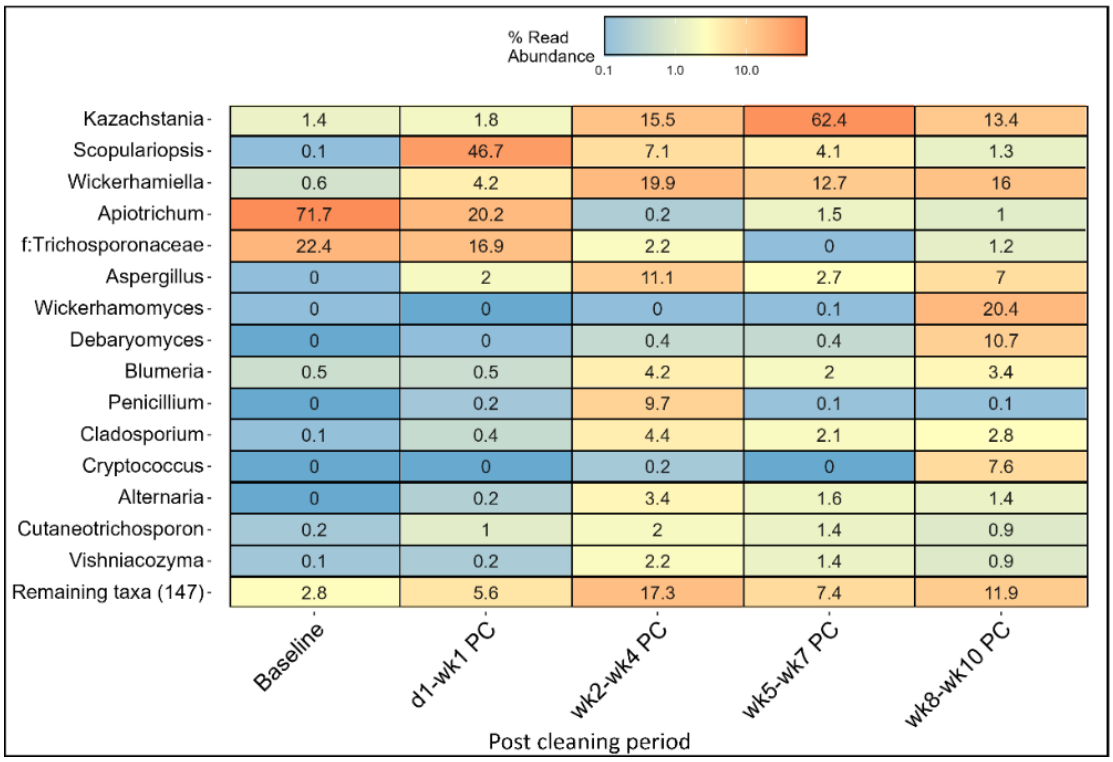

Figure 6.5: Mean percentage read abundance of the top 15 fungal genera detected on the internal surface of the feed pipeline at baseline and at each post-cleaning (PC) period.

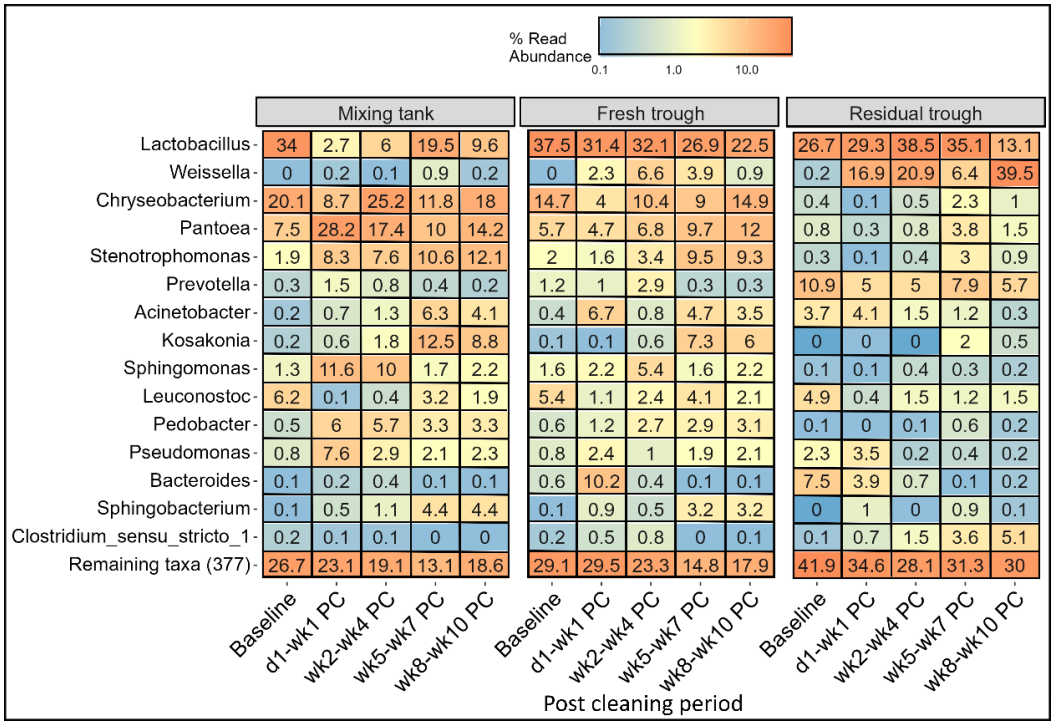

Figure 6.6: Mean percentage read abundance of the top 15 bacterial genera detected in liquid feed at baseline and at each post-cleaning (PC) period across different sampling locations.

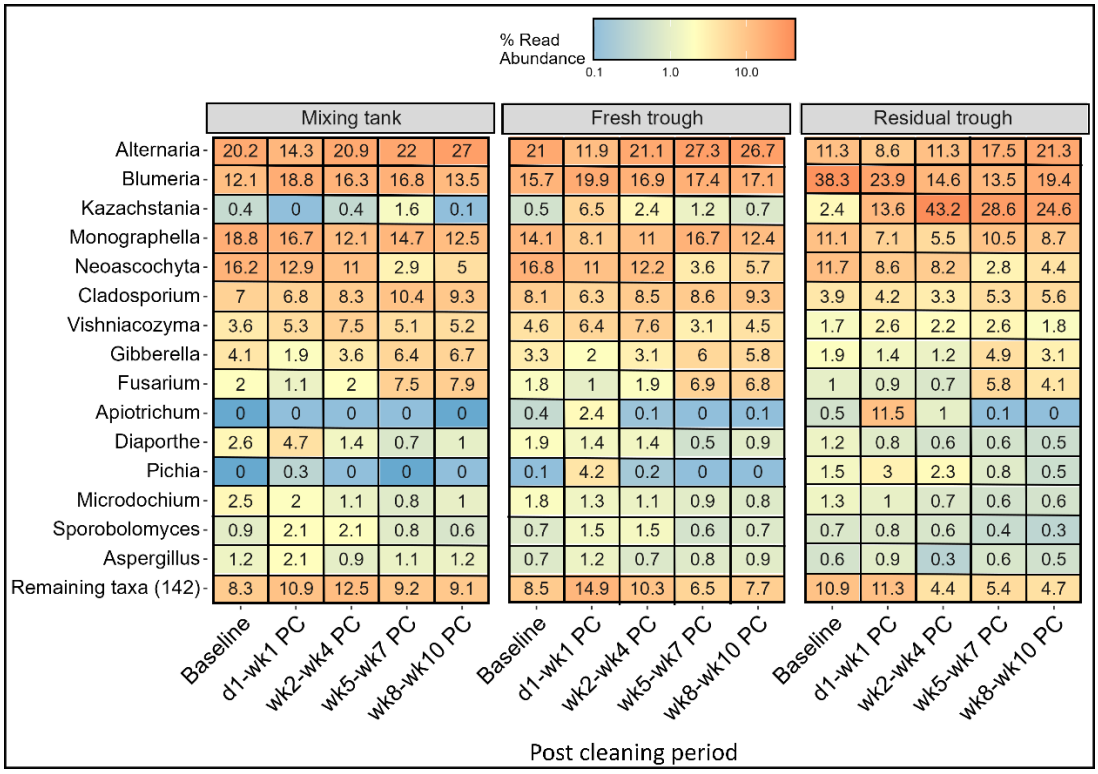

Figure 6.7: Mean percentage read abundance of the top 15 fungal genera detected in liquid feed at baseline and at each post-cleaning (PC) period across different sampling locations.

6.8 Supplementary information

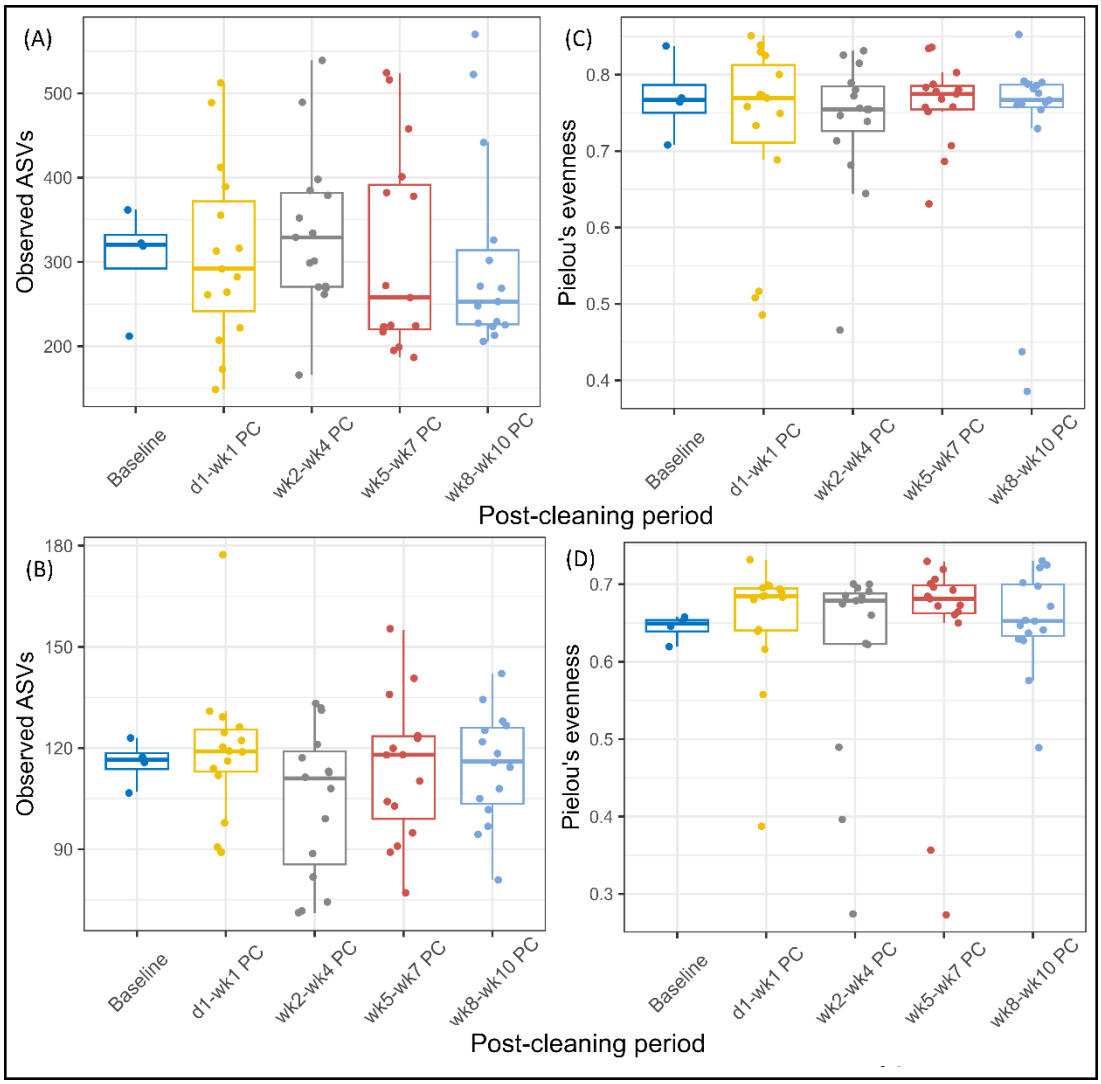

Figure S 6.1: Boxplots displaying Observed amplicon sequence variants (ASVs) and Pielou's evenness of bacterial [(A) and (C)] and fungal [(B) and (D)] communities in liquid feed at baseline and at each post-cleaning (PC) period (n = 15, except at baseline, where n = 4).

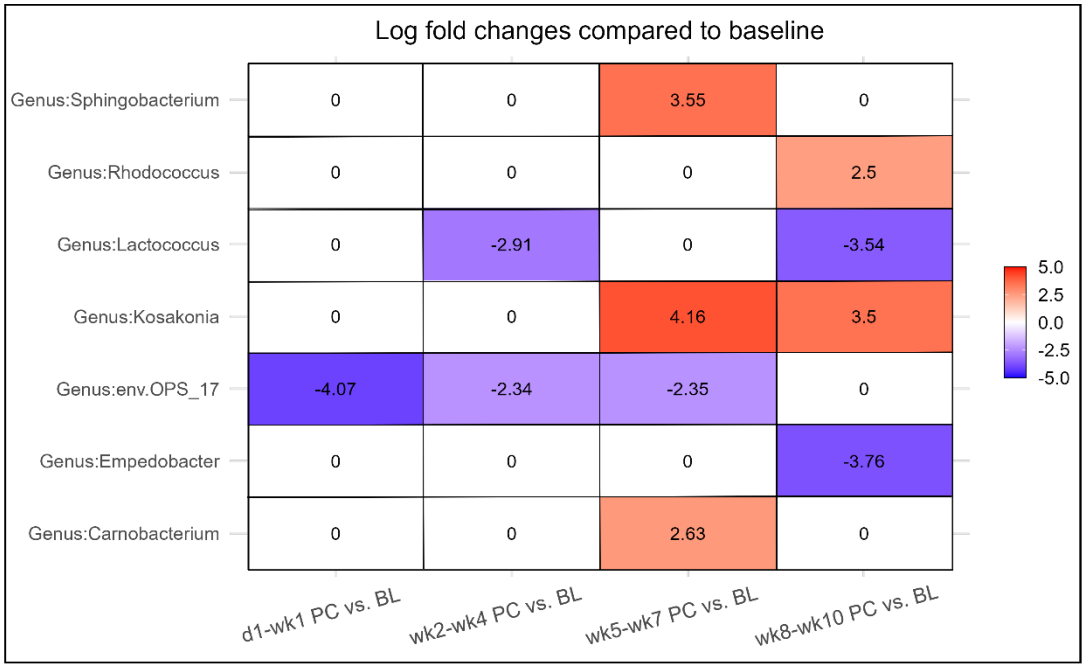

Figure S 6.2: Log-fold changes in bacterial differential abundance (genus level) in the liquid feed after pairwise comparisons between each post-cleaning (PC) period and baseline (Dunnett's test; 'prv_cut' = 0.6, alpha = 0.05).

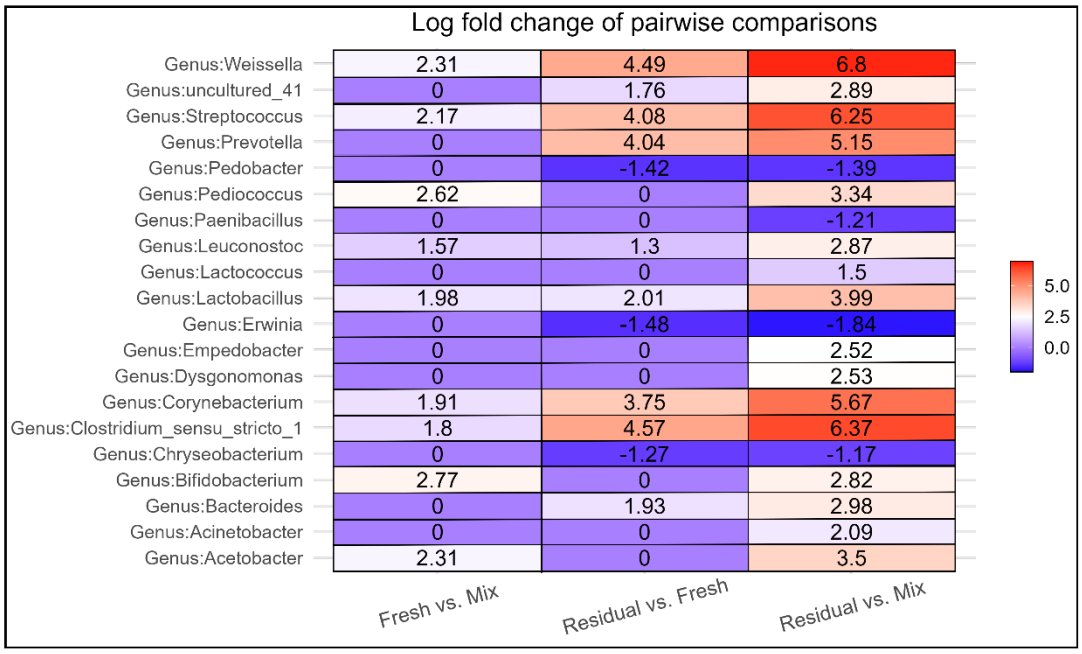

Figure S 6.3: Log-fold changes in differential abundance of bacterial genera in the liquid feed after pairwise comparisons between sampling locations with post-cleaning (PC) period as a random effect ('prv_cut' = 0.6; alpha = 0.05).

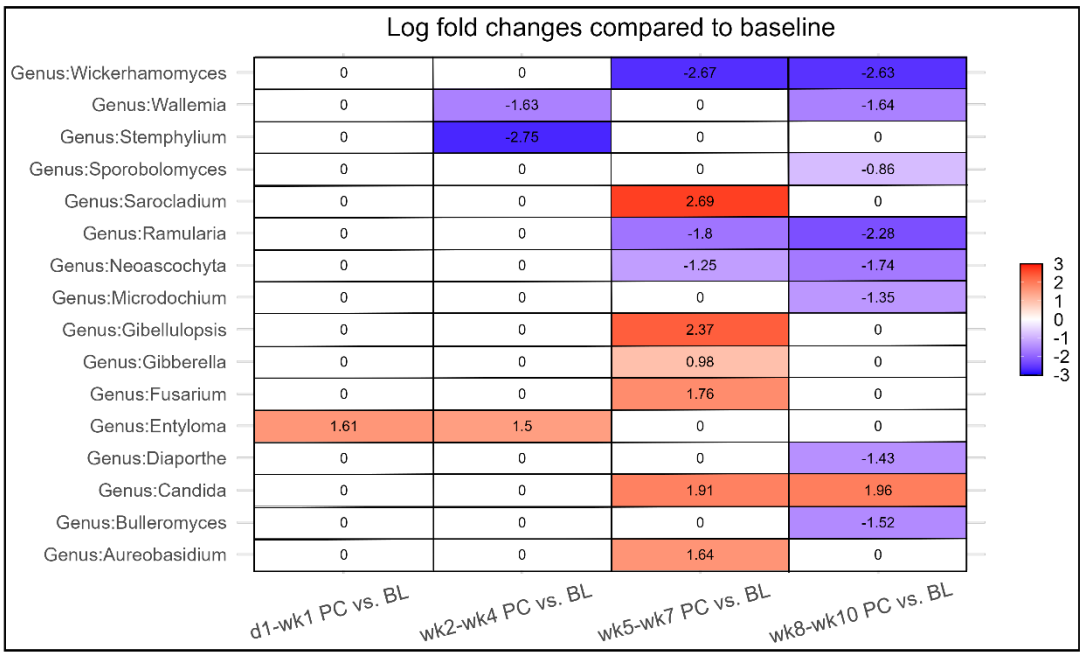

Figure S 6.4: Log-fold changes in fungal differential abundance (genus level) in the liquid feed after pairwise comparisons between each post-cleaning (PC) period and baseline (Dunnett's test; 'prv_cut' = 0.8, alpha = 0.05).

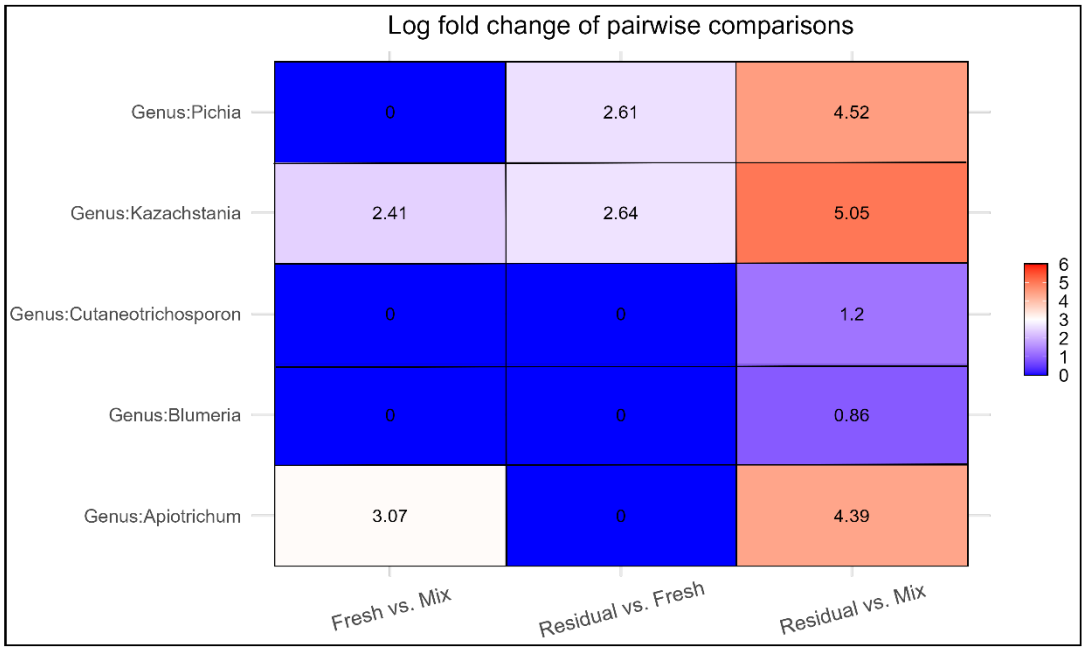

Figure S 6.5: Log fold changes of the differential abundance of fungal genera in the liquid feed after pairwise comparisons between sampling locations with post-cleaning (PC) period as a random effect ('prv_cut' = 0.6; alpha = 0.05).

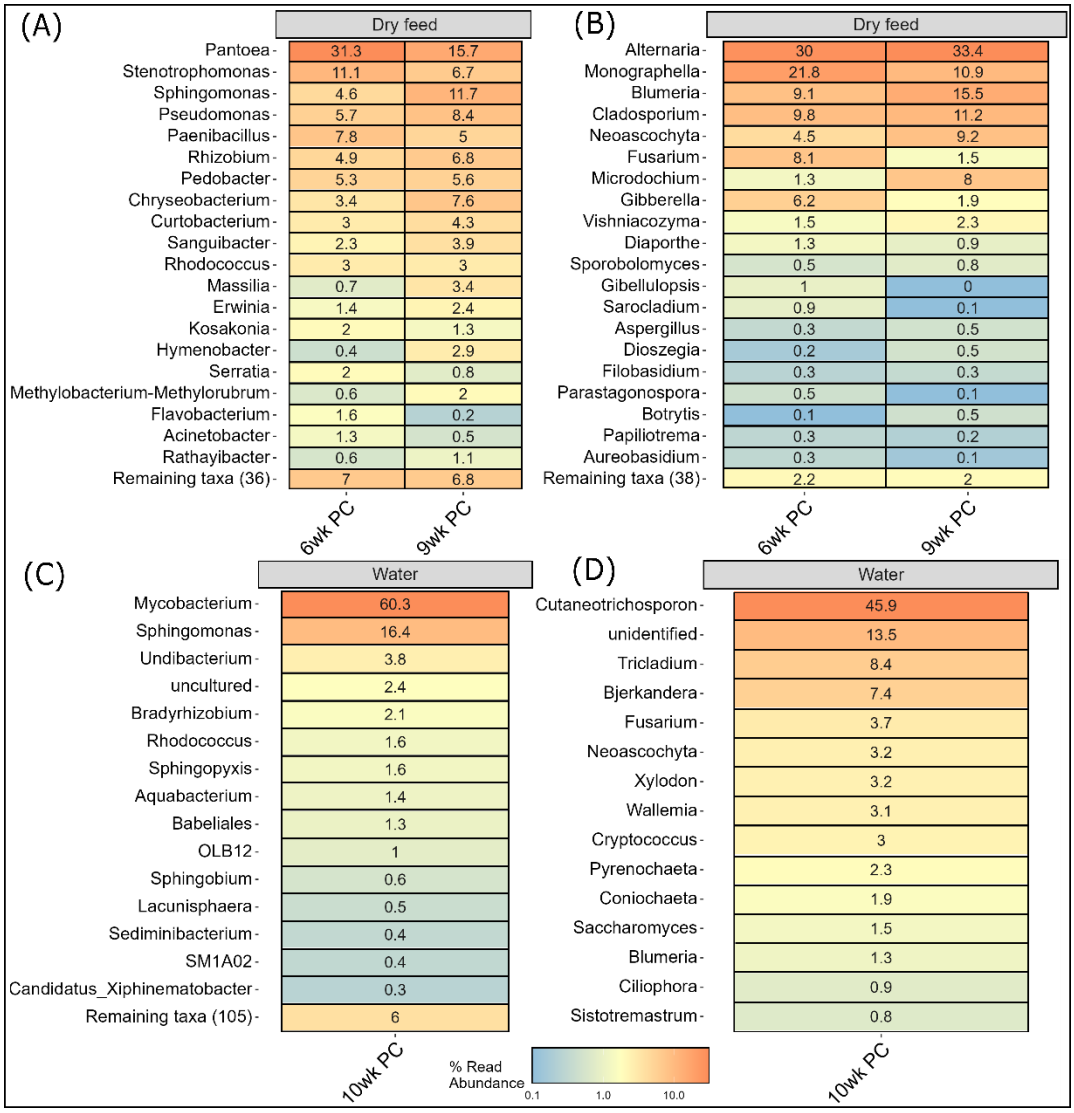

Figure S 6.6: Percentage read abundance of the top 15 bacterial and fungal genera detected in the dry feed (A and B, respectively) and water (C and D, respectively) used for preparation of the liquid feed. *Note that the full '*Rhizobium'* classification at the genus level is '*Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*'.

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Chapter 7: Overall discussion

Liquid feeding grow-finisher pigs is common in many parts of the world, particularly in Europe. Despite this, the feed efficiency of liquid-fed pigs can be up to 0.20 of a feed conversion efficiency (FCE) unit poorer compared to dry feeding (Lawlor & O'Meara, 2018; O'Meara et al., 2020c), which equates to an increase in feed cost of ~€5.10 per pig, based on a 5-year average finisher feed price (Lawlor & Viard, 2021). This poorer feed efficiency has been partly attributed to increased physical feed wastage at the trough (Han et al., 2006; L'Anson et al., 2012; Russell et al., 1996); however, unintentional feed fermentation likely also contributes. The occurrence of unintentional spontaneous (or uncontrolled) fermentation in liquid feed has been widely reported, particularly so with *ad libitum* short-trough feeding, where residual feed remains in the troughs for relatively long periods of time (Brooks et al., 2001; O'Meara et al., 2020b; Plumed-Ferrer & Von Wright, 2009). This uncontrolled fermentation can have negative impacts on feed quality, including amino acid and gross energy losses from the diet (Brooks et al., 2001; Canibe & Jensen, 2003; O'Meara et al., 2020a). This may, at least in part, explain the poorer FCE observed in liquid-fed pigs.

Gaps identified in the literature include the fact that, although several studies have investigated the quality of liquid feed and strategies to improve it, very few on-farm surveys of liquid feed quality have been conducted on commercial pig units, especially so on farms where liquid feed is not deliberately fermented. Additionally, many studies to date that have profiled microbial groups within liquid feed and/or investigated the impact of liquid feed on the pig gut microbiome, have used only culture-based methods to investigate a select few microbial groups. Data are also lacking regarding characterisation of the fungal communities in liquid feed, particularly using culture-independent methods. For this reason, there is a lack of standard next-generation-sequencing workflows available to profile the bacteriome and mycobiome of liquid feed and the gut microbiome of liquid-fed pigs. Moreover, additional studies are required to investigate the impact of different feed forms (meal versus pellets) and feed delivery methods (dry, liquid and wet/dry feeding) on the feed and pig gut microbiome, and to determine whether differences in the microbiome may be responsible for any variances observed in growth, feed efficiency and/or carcass characteristics in pigs. Lastly, the limited number of studies in the area indicate that cleaning of liquid feeding systems may have only short-lived benefits, or none at all, for system hygiene and liquid feed quality. Consequently, there is an opportunity to improve liquid feed quality by developing an

effective sanitisation programme that improves liquid feeding system hygiene and subsequently maintains the hygiene after cleaning.

Considering all of the above, the objectives and hypotheses of this thesis were to:

1. Develop an optimal methodology for simultaneously profiling the bacteriome and mycobiome of liquid feed and the gut microbiome of liquid-fed pigs. The hypothesis was that by increasing the homogenisation (bead-beating) duration the recovery of DNA from difficult-to-lyse microbes in faeces and liquid feed would be improved by minimising lysis bias.
2. Profile the liquid feed bacteriome and mycobiome and determine the concentration of biogenic amines in liquid feed on commercial pig production farms. The hypothesis was that genera of lactic acid bacteria and yeast would predominate in liquid feed as a result of spontaneous fermentation, and that relatively high concentrations of biogenic amines would be present due to amino acid decarboxylation.
3. Investigate the bacteriome of a diet in meal versus pelleted form, delivered as dry, liquid or wet/dry feed, and determine the intestinal and faecal bacteriome of grow-finisher pigs fed these diets to ascertain whether certain bacterial taxa are associated with pig growth, FCE and carcass quality. The hypothesis was that spontaneous fermentation in liquid diets is linked with poorer feed efficiency in liquid-fed pigs due to proliferation of lactic acid bacteria in feed and the subsequent increased abundance of lactic acid bacteria within the gut bacteriome of liquid-fed pigs.
4. Develop and implement an intensive sanitisation programme in a grow-finisher liquid feeding system in order to improve and maintain system hygiene and improve the microbiological, physico-chemical and nutritional quality of liquid feed. The hypothesis was that implementing an intensive sanitisation programme and maintaining hygiene post-cleaning would reduce spontaneous fermentation in liquid feed and therefore reduce energy and amino acid losses and the production of undesirable microbial metabolites in liquid feed.
5. Profile the bacteriome and mycobiome of liquid feed and liquid feeding system surfaces, before and after implementation of the intensive sanitisation programme. The hypothesis was that a DNA-sequence based approach would

reveal specific bacterial and fungal taxa associated with the pre- and post-cleaning microbial communities in feed and on the feeding system surfaces.

Due to the lack of standardised workflows for simultaneously profiling bacterial and fungal communities via high-throughput amplicon sequencing, Chapter 2 aimed to develop a 'catch-all' workflow that incorporated bead-beating to optimise bacterial and fungal DNA recovery from liquid feed and pig faeces. The results showed that a bead-beating duration of 20 minutes provided a more accurate representation of the bacterial and fungal communities in these samples, when combined with an optimised protocol of a commonly used, commercially available DNA extraction kit. This work will help to minimise lysis bias, particularly for Gram-positive bacteria and filamentous fungi in future liquid feed and pig gut microbiome studies. Although 20 minutes of bead-beating was suitable for amplicon sequencing, potential shearing of DNA due to excessive homogenisation could be problematic for metagenomic and transcriptomic analyses. In addition, the work shows that a single DNA extract can be used for simultaneous bacterial and fungal amplicon sequencing, and thus will improve the cost- and time-effectiveness of profiling the micro- and mycobiome in future studies by reducing the number of DNA extractions required.

The standardised workflow developed in Chapter 2 was subseqeuntly applied in Chapter 3 to profile the bacteriome and mycobiome of liquid feed for grow-finisher pigs from the finisher section of commercial pig farms. The concentration of biogenic amines was also determined as an indication of the level of spontaneous fermentation occurring. The results of this chapter supported the current evidence that spontaneous fermentation occurs in 'fresh' (i.e. not deliberately fermented) liquid feed on commercial pig farms. The bacterial and fungal community structures in the liquid feed were influenced by the location at which the samples were collected from on a given farm (i.e. in the mixing tank and troughs). The liquid feed micro- and mycobiome were influenced even more so more so by the particular farm from which the feed was sampled, highlighting the unpredictable nature and the between-farm variability of spontaneous fermentation. The inclusion of liquid co-products in diets also influenced the microbial community in the liquid feed considerably, with the yeasts *Diutina* (associated with liquid whey) and *Saccharomyces* (associated with pot ale syrup) most abundant in the feed on the farms using these co-products. The decreases in alpha-diversity of liquid feed between the mixing tank and the troughs on the farms in this study corresponded with increased relative abundances (RA)

of lactic acid bacteria (LAB) including *Lactobacillus, Weissella* and *Leuconostoc*, as well as yeasts including *Kazachstania* and *Dipodascus*. The concentration of biogenic amines also increased between the liquid feed in the mixing tank and the troughs, as a result of amino acid decarboxylation, particularly on two farms. However, the biogenic amine concentrations found in this study are likely not of concern for pig health based on human toxicity data, although there are no guidelines available on acceptable concentrations in liquid feed. Nonetheless, the amino acid losses associated with biogenic amine production on these farms likely play a role in the poorer FCE previously observed in liquid-fed pigs. In addition to the impact the feed microbiome has on the quality of liquid feed itself, the feed microbiome, may also impact the intestinal microbiome of liquid-fed pigs and subsequently influence their growth and feed efficiency.

As shown in Chapter 3, differences in dietary composition, due to the inclusion of co-products, can dramatically influence feed microbiology. There are also microbiological differences between the feed form (meal or pellets) used and the method by which they are delivered to pigs (dry, liquid and wet/dry feeding) (O'Meara et al., 2020c). As such, these factors may influence the growth and feed efficiency of pigs by modulating the intestinal microbiome. The objective of Chapter 4 was to determine the impact of these different feed forms and delivery methods on the feed bacteriome and on the profile of the intestinal and faecal bacteriome of pigs fed these diets. An additional objective was to investigate whether certain bacterial taxa were associated with growth, feed efficiency or carcass characteristics. In agreement with Chapter 3, bacteriome profiling of the diets used in this chapter showed increased RAs of the lactic acid bacteria (LAB) *Weissella*, *Leuconostoc* and *Lactococcus* in the residual-trough sampled feed compared to the mixing tank and fresh trough-sampled feed, consistent with spontaneous fermentation. The greatest RA of these genera was observed in the liquid pellet diet, while *Lactobacillus* was more abundant in the liquid meal diet. In fact, the pigs fed the liquid meal diet had a greater abundance of *Lactobacillus* in their caecal digesta and faeces; however, this was not associated with growth or feed efficiency in this study.

Another LAB associated with spontaneous fermentation in liquid feed, *Leuconostoc*, was enriched in the ileal digesta and faeces of liquid-fed pigs. *Leuconostoc* was correlated with higher feed intake and poorer feed efficiency in liquid-fed pigs, while decreased faecal abundance of *Turicibacter* was also correlated with increased feed intake, implicating these microbes in influencing feed efficiency. Increased ileal abundance of

Leuconostoc has previously been negatively correlated with carcass weight in liquid-fed pigs (Torres-Pitarch et al., 2020). Decreased faecal abundance of *Turicibacter* was also correlated with higher feed intake. This genus has been reported as more abundant in more feed efficient animals (McCormack et al., 2019) and has been positively correlated with body weight (Wang et al., 2019). These intake-associated microbes may be potential biomarkers of feed efficiency in pigs; however, further research is required to investigate the mechanisms by which they may influence feed intake. The abundance of the aforementioned fermentative bacteria in the intestinal and faecal bacteriome of liquid-fed pigs is likely influenced by their proliferation in liquid feed during spontaneous fermentation, which was observed in Chapters 3 and 4.

With regard to pig growth performance parameters, Chapter 4 confirmed that liquid feeding meal and wet/dry feeding pellets produce similar carcass gain, feed efficiency and kill-out yield; however, liquid feeding meal optimises carcass gain, while feeding wet/dry pellets optimises feed efficiency. Therefore, the feeding method used for grow-finisher pigs on a given pig unit will depend on the requirements of the producer. For example, liquid feeding may be preferable if maximising growth rates is the priority (e.g. where facilities are highly stocked), as with many finisher units. The higher growth rates achievable with liquid feeding also have environmental implications, as reducing the number of days to slaughter decreases the carbon footprint of pig production. Nonetheless, as shown in Chapter 4, the feed efficiency of liquid meal-fed pigs was 0.26 of an FCE unit poorer than dry pellet-fed pigs, in agreement with previous findings (O'Meara et al., 2020c). Therefore, improving feed efficiency of liquid-fed pigs would result in significant feed savings for producers already practicing liquid feeding. This study associated the poorer feed efficiency of liquid-fed pigs with increased ileal and faecal abundance of *Leuconostoc*, with liquid feed being the likely source. Therefore, practical strategies such as improving the hygiene of liquid feeding systems to reduce the occurrence of spontaneous fermentation in liquid feed, may help to improve feed efficiency of liquid-fed pigs.

A previous survey of the farms from Chapter 3 found that cleaning of mixing tanks and pipelines in liquid feeding systems was highly variable, with three farms performing no cleaning at all (O'Meara et al., 2020a). This lack of cleaning can lead to a build-up of feed residues and microbial biofilms within the system, which likely seed the liquid feed with microbes as it passes through the system, accelerating uncontrolled spontaneous feed

fermentation. For example, poor liquid feed quality was found on two of the farms (B and F) that performed no cleaning in Chapter 3. Farm B had a particularly high prevalence of yeasts and moulds and the highest concentration of biogenic amines, while Farm F had a high proportion of *Clostridium* in the fresh and residual trough-sampled feed. Therefore, the aim of Chapter 5 was to determine the effects of an intensive sanitisation programme in a grow-finisher liquid feeding system by monitoring microbiological and physico-chemical parameters of liquid feed and microbial colonisation of the feeding system surfaces. The sanitisation programme involved a combination of intensive physical and chemical cleaning between batches of grow-finisher pigs, combined with nightly rinsing of the system with an organic acid blend and use of the acid rinse to prepare the first feed of the day. The sanitisation programme dramatically improved the hygiene of internal surfaces of the feeding system, especially for the initial 5-week period, as evidenced by reduced counts of lactic acid bacteria, total aerobes, *Enterobacteriaceae*, yeasts and moulds and decreased adenosine triphosphate (ATP) concentrations on the mixing tank and feed pipeline internal surfaces. The ATP concentrations were in line with the microbial counts, and therefore may serve as a practical tool for on-farm monitoring of feeding system hygiene. *Enterobacteriaceae* and moulds remained undetectable on pipeline surfaces for 10 weeks, as confirmed by scanning electron microscopy of the feed pipeline surfaces. These findings are in contrast to those of Royer et al. (2004) who circulated 'contact water' through a liquid feeding system to assess the microbiological load of the system surfaces before and after cleaning. They found that ATP concentrations, and counts of total bacteria, lactic acid bacteria and coliforms decreased immediately after liquid feeding system cleaning but returned to pre-cleaning levels within two weeks, highlighting the effectiveness and longevity of the sanitisation programme used in Chapter 5.

However, the impact on liquid feed microbiology in the current study was minimal and short-lived. This was in agreement with Fisker & Jørgensen (2010) who reported no significant differences in counts of *Enterobacteriaceae*, LAB, yeasts, moulds or *Clostridium perfringens* in liquid feed sampled after cleaning and disinfection of the feeding system, compared to pre-cleaning. They also found no differences in pH, organic acid or biogenic amine concentrations; however, in Chapter 5, acetic acid, ethanol and biogenic amine concentrations decreased in the feed post-cleaning and did not return to baseline concentrations. Additionally, no gross energy losses were observed in the liquid

feed. The concentrations of these metabolites in liquid feed coincided with variations in LAB and yeast counts on the liquid feeding system surfaces, implicating these communities in contributing to the chemical composition of liquid feed. Therefore, by controlling surface microbial communities on liquid feeding systems via implementation of the sanitisation programme, on-farm liquid feed quality should be improved.

Chapter 6 built upon this work by profiling the bacteriome and mycobiome of the liquid feed and the feeding system surfaces before and after cleaning. The results confirmed that the resident bacterial and fungal biofilms on the internal surfaces of the feed pipeline were disrupted post-cleaning. Before cleaning, the feed pipeline biofilm was dominated by a low diversity community, mainly *Lactobacillus*, *Leuconostoc*, *Chryseobacterium* and the yeast *Alternaria*. Microbial diversity increased on the pipeline surface post-cleaning, but community composition and abundance were highly unstable throughout the experiment, likely due to the nightly acid rinse circulated through the feed pipeline, preventing re-colonisation. The cleaning programme and sampling location both impacted the profile of the bacterial and fungal communities in liquid feed. However, spontaneous fermentation was observed in the liquid feed (as in Chapter 3), despite the cleaning. This was characterised by decreases in the abundance of *Pantoea* and *Pseudomonas* between the mixing tank and troughs, with a concurrent increase in *Lactobacillus*. *Weissella* also became highly abundant in the trough-sampled feed after cleaning, which may have been a result of *Lactobacillus* populations not having fully re-established. The yeast *Kazachstania* represented a large component of the post-cleaning mycobiome in the residual trough-sampled feed, while *Gibberella* and *Fusarium* also increased in abundance during the latter PC periods. The latter two genera are mycotoxin producers (Burlakoti et al., 2008; Ji et al., 2019). Although mycotoxins were not measured as part of this work, it would be interesting to determine whether liquid feeding system sanitisation impacts mycotoxin concentrations.

Nonetheless, as outlined for Chapter 5 above, only minimal losses in gross energy in the liquid feed were found, irrespective of time point or sampling location. Additionally, the decreases in microbial metabolite concentrations outlined above (organic acid, ethanol and biogenic amines) indicated a reduction in uncontrolled spontaneous fermentation. Although microbes began to re-colonise the feeding system, hygiene was still improved at the end of the 10-week grow-finisher period, compared to pre-cleaning and microbial metabolites did not return to baseline concentrations. Therefore, intensive cleaning of

liquid feeding systems between batches of grow-finisher pigs is recommended. It may also be possible to prolong the impact of the improved feeding system hygiene and further improve the microbial and chemical quality of the feed itself. For example, increasing the concentration of the acid blend in the liquid feed, and/or using a mixing tank fogger to better distribute the alkali and acid to the internal surfaces of the mixing tank, could both be strategies worth exploring. Growth rate and feed efficiency during the experiment were on par with previous liquid feeding studies performed in the same grow-finisher house. However, the effect of feeding grow-finisher pigs from sanitised compared to non-sanitised liquid feeding systems on the pig microbiome, feed intake, growth and FCE remains to be investigated. A 5-year cost-benefit analysis of implementing this sanitisation programme between every batch of grow-finisher pigs showed that there is potential to increase margin over feed by €1.20 per pig, which represents a saving on feed costs of ~€54,000 per year. This cost-benefit analysis was based on a pig unit producing four batches of 10,000 pigs per year (30-115 kg live weight), assuming a 0.05-unit improvement in FCE. This improvement in FCE is conservative and considering that pigs fed liquid meal in Chapter 4 had a 0.26-unit poorer FCE compared to dry pellet-fed pigs, there is potential for significant feed savings for producers.

Overall, this thesis provides essential information regarding the role of spontaneous fermentation in deterioration of the microbial and nutritional quality of liquid feed, and its contribution to poorer feed efficiency in liquid-fed pigs. Moreover, it has shown that implementation of a liquid feeding system sanitisation programme is an effective and economically viable means of improving the quality of liquid feed for grow-finisher pigs.

Based on the findings of this thesis, future work should include:

1. Determining the ability of lactic acid bacteria and yeasts, isolated from liquid feed, to decarboxylate amino acids and produce biogenic amines *in vitro* to investigate their role in reducing the nutritional quality of liquid feed.
2. Investigating the impact of implementing an optimised version of the sanitisation programme, including the use of a fogger in the mixing tank, on a large sample of commercial pig farms in a pre- and post-cleaning study. This will help to determine whether the protocol is effective in different types of liquid feeding systems and on farms with poorer baseline hygiene conditions.

3. Addition of a suitable microbial inoculant to liquid feed/feeding system surfaces post-sanitisation to investigate whether controlled re-colonisation of the liquid feeding system with a desirable microbiome is possible, and whether liquid feed quality is impacted. The impact of liquid feeding system sanitisation on mycotoxin concentrations in liquid feed should also be assessed.
4. Using the feed-grade organic acid blend from Chapters 5 and 6, to directly acidify liquid feed during each feed preparation using different inclusion rates (up to 1 % inclusion in liquid feed) to assess whether there is an enhanced impact on the nutritional, microbiological and physico-chemical parameters of liquid feed, as opposed to addition of the acid rinse only.
5. Determining the impact of liquid feeding system sanitisation on the growth, feed efficiency, carcass characterisitics and intestinal microbiome of grow-finisher pigs fed from a sanitised versus non-sanitised liquid feeding system.

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Appendix

Research Dissemination

Peer-reviewed publications:

Cullen, J. T., Lawlor, P. G., Viard, F., Lourenco, A., Gómez-Mascaraque, L. G., O'Doherty, J. V., Cormican, P., Gardiner, G. E. (2024). Optimising the hygiene of a liquid feeding system to improve the quality of liquid feed for pigs. *Sci. Rep.* (accepted).

Rattigan, R., Lawlor, P.G., Cormican, P., Crespo-Piazuelo, D., **Cullen, J.**, Phelan, J.P., Ranjitkar, S., Crispie, F. and Gardiner, G.E. (2023). Maternal and/or post-weaning supplementation with *Bacillus altitudinis* spores modulates the microbial composition of colostrum, digesta and faeces in pigs. *Sci. Rep.*, 13, 8900. https://doi.org/10.1038/s41598-023-33175-2

Cullen, J. T., Lawlor, P.G., Cormican, P., Crispie. F. & Gardiner, G.E. (2022). Optimisation of a bead-beating procedure for simultaneous extraction of bacterial and fungal DNA from pig faeces and liquid feed for 16S and ITS2 rDNA amplicon sequencing. *Animal – Open Space*. 1(1), 100012. https://doi.org/10.1016/j.anopes.2022.100012

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Book chapters:

Cullen, J. T., Lawlor, P. G., & Gardiner, G. E. (2022). Microbiological services delivered by the pig gut microbiome. In M. Bailey & C. Stokes (Eds.), Understanding Gut Microbiomes as Targets for Improving Pig Gut Health. Burleigh Dodds Science Publishing Ltd., Cambridge, UK.

Conference abstracts and presentations:

Cullen, J.T., Viard, F., Lourenco, A., Gómez-Mascaraque, L.G., O'Doherty, J. V., Cormican, P., Gardiner, G.E. & Lawlor, P.G. (2023). Investigating the impact of sanitisation of the feeding system on the bacterial and fungal microbiota of liquid feed and liquid feeding systems for pigs. *Joint Pig Veterinary Society & Society of Feed Technologists 2023 Autumn Meeting*, 8th-9th November, 2023 **(oral presentation)**.

Cullen, J.T., Viard, F., Lourenco, A., Gómez-Mascaraque, L.G., O'Doherty, J. V., Cormican, P., Gardiner, G.E. & Lawlor, P.G. (2023). Optimising liquid feeding system hygiene to improve the microbiological quality of liquid feed for grow-finisher pigs, p. 361. *In Animal - Science Proceedings*. https://doi.org/10.1016/j.anscip.2023.01.480 (Proceedings of British Society of Animal Science Conference, Birmingham, UK, 28th - 30th March 2023) **(oral presentation)**.

Cullen, J.T., Viard, F., Lourenco, A., Gómez-Mascaraque, L.G., O'Doherty, J. V., Cormican, P., Gardiner, G.E. & Lawlor, P.G. (2022). Optimising liquid feeding system hygiene to improve the microbiological quality of liquid feed for grow-finisher pigs. *Agri Sparks*, South East Technological University, Cork Road Campus, Co. Waterford, 20th October, 2022 **(oral presentation)**.

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Posters presented:

Cullen, J. T., Lawlor, P.G., Cormican, P. and Gardiner, G.E. (2021). Optimisation of a bead-beating procedure for simultaneous extraction of bacterial and fungal DNA from pig faeces and liquid feed for 16S and ITS2 rDNA amplicon sequencing. *12th International Symposium on Gut Microbiology, 13th-15th October, 2021, Online*, page 93.

Farmer/industry open days:

Cullen, J.T., Lawlor, P.G., Cormican, P. and Gardiner, G.E. (2023). Cost implications of liquid feed management. In proceedings Teagasc Pig Open Day, Moorepark, Fermoy, Co. Cork and Ballyhaise Agricultural College, Ballyhaise, Co. Cavan, 22nd & 24th May 2024 **(poster & demonstration)**.

Cullen, J.T., Lawlor, P.G., and Gardiner, G.E. (2023). Liquid feeding system hygiene. In proceedings Teagasc Pig Open Day, Moorepark, Fermoy, Co. Cork and Ballyhaise Agricultural College, Ballyhaise, Co. Cavan, 9th & 11th May 2023 **(poster & demonstration)**.

Popular press:

Cullen, J.T., Viard, F., Gardiner, G.E. & Lawlor, P.G. Improving liquid feeding system hygiene to improve the feed efficiency of liquid-fed grow-finisher pigs. Teagasc Pig Development Department Newsletter, May 2023.

Awards:

- The 2023 British Society of Animal Science (BSAS) Industry Prize Award
- The 2022 Society of Feed Technologists' Edgar Pye Research Scholarship