IMPROVING ENTERIC HEALTH, UNDERSTANDING IMPACT ON GUT MICROBIOME AND WEANER PERFORMANCE THROUGH THE USE OF PROTEASE ENZYMES

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Final Report prepared for the
Australasian Pork Research Institute Limited
(APRIL)

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Executive Summary

Feed is the single largest cost of pig production, and its efficient use is a continuous challenge for nutritionists. Enzyme supplementation is one technique that has been used to improve nutrient digestibility. Phytase use to unlock plant bound phosphorus is near universal, and the use of non-starch polysaccharide enzymes such as xylanases and ß-glucanase to target grain and grain by-products continues to increase. However, the use of protease enzymes is still limited.

However, there is more to the use of enzymes than just feed utilization. The increased utilization of proteases is likely to be focused on their role within an integrated health program. The ability to reduce dietary protein levels can have a large impact on the level of undigested proteins in the hindgut, which are available for fermentation, producing many potentially toxic breakdown products such as amines, phenols and ammonia, as well as allowing the proliferation of many pathogenic bacteria.

This project sought to improve the intestinal health of the weaner pig, through reducing the substrates for microbial growth and resulting in an increase in the efficiency of growth through better utilization of feed. Whilst also seeking to understand the impact of production practices on the microbial community of the pig, how that community changes with diet ingredient digestibility, antibiotic use and diet transitions over the growing period. This research project initially involved two studies, one in weaner pigs and one in grower pigs, to look at the role of protease as part of an integrated health strategy. As a result of the outcomes observed, the project has resulted in four studies – two identical weaner studies where diet analysis suggested a repeat was necessary, a grower study as proposed, and another grow-finisher study looking at a higher rate of protease use offsetting a down-specification in amino acids.

The weaner studies were identical in format and examined a 2 x 2 factorial design with protein content (standard, 22.5% crude protein and low, 18.5% crude protein) and protease inclusion (0 or 150 ppm) as the factors, over a 28-day period. Low protein diets were fed for the first 14 days after weaning only.

The inclusion of 150 ppm of protease in diets had little impact on weaner growth performance, whilst those pigs that received the low protein diet in the first 14 days had reduced daily feed intake and poorer feed conversion that continued even when they had transitioned to the common standard diet after day 15. However, lower levels of plasma urea nitrogen, lower digesta ammonia-N concentration, lower VFA production and the reduced relative abundance of *Enterobacteriaceae* and increased *Prevotellaceae* observed in this study agree with previous studies, but the lack of challenge to the standard feeding treatment didn't allow the benefits of low protein diets to be fully expressed.

The first grower study examined a 2 x 2 factorial design with protease inclusion (0 or 125 ppm) with or without medication for seven consecutive days from day 8 to 15 of a 7-week experiment. The inclusion of 125 ppm of protease in the diets of grower pigs from 10 to 17 weeks of age had no impact on their growth performance. The introduction of medication resulted in a significantly improved feed conversion in the subsequent period; however, no improvement in growth rate was observed, but rather a reduction in feed intake.

The second grow-finisher study saw pigs offered one of three treatments;

- A positive control,
- A treatment diet formulated to the same nutrient specification as the positive control, where the contribution of crude protein and amino acids though the addition of 250 ppm protease was included in the formulation (equivalent to a 5% improvement in digestibility), and
- A negative control where the protease was not included in this treatment diet, resulting in a diet with lowered crude protein and amino acid content.

Growth performance was measured over a 56-day period, with pigs slaughtered at the end of the project. The inclusion of 250 ppm protease was able to maintain growth performance in a diet that was reduced in amino acid specification by 5%. The down-specified diet that included 250 ppm of protease was similar to the positive control in average daily feed intake, average daily gain and feed efficiency across the experimental period, whilst both of these treatments resulted in increased feed intake and average daily gain compared to the negative control across the total growth period. There was no significant difference in carcase characteristics between treatments.

The evidence generated in this project suggests that improvements in the intestinal health of the weaner pig were able to be achieved, whilst other factors such as differences in plasma urea nitrogen, digesta ammonia-N and total volatile fatty acid production were in line with previous studies that have shown the benefits of low protein diets when challenged.

The largest changes in microbiome in grower pigs were not a result of treatment, but sampling time, with the moving of the pigs to the research finisher facility and the sudden transition to a new shed and new diets resulting in disturbance of the relative abundance of the most common bacteria phyla *Bacteroides* and *Firmicutes*. Understanding the impact of change on the microbiome, and developing strategies to minimize this in commercial environments, is likely to result in decreased gut disturbance and reduce performance checks.

Finally, the project was able to show that the inclusion of a higher dose of protease in a diet fed to grow-finish pigs was able to positively influence their performance, adding another management tool to deal with increasingly scarce sources of protein.

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1. Introduction

With feed being the largest single cost in pig production, improving its utilization and the efficiency of its digestion is a continuous challenge for nutritionists. Dietary enzyme supplementation has been one technique applied to improve nutrient digestibility and utilisation efficacy, and subsequently reduce feed costs in the pig industry.

With grains and grain by-products being the major component of feed, much focus has been placed on increasing the digestibility of the non-starch polysaccharide (NSP) component (cellulose, arabinoxylan, ß-glucan, mannan, etc...) of the diet, with the ready adoption of NSP enzymes such as xylanase and ß-glucanase. Moreover, the use of phytases to increase the availability of phosphorus to the pig, and reduce the environmental impact of undigested phosphorus, is now considered normal practice (Dersjant-Li *et al.*, 2015). However, the use of protease enzymes within pig diets is still quite limited. The lesser use of protease enzymes is somewhat a consequence of the success of phytase and NSP enzymes. When formulating diets including these enzymes, an uplift is applied that accounts for the consequential increased availability, adding further uplifts for the use of protease enzymes means the economic impact becomes marginal.

However, there is more to the use of enzymes in pig feed than just improving feed utilisation. The increased utilisation of protease enzymes would appear to be focused on their role within an integrated health program, impacting on the nutrition, health, and environment of the pig. Whilst the nutritional benefits of increased digestibility of proteins/amino acids are self-evident, the ability to reduce dietary protein levels can have a big impact on the level of undigested proteins in the hindgut. Reducing the flow of undigested protein into the large intestine has been stated as 'the single most important nutritional factor for reducing scouring in pigs fed diets without antibiotic growth promoters' (Stein, 2007). These undigested proteins are a fermentation substrate that may produce many potential toxic breakdown products such as phenols, amines and ammonia, and allows for the proliferation of many bacterial species that have a great capacity to ferment undigested protein such as *E. coli*, *Klebsiella* spp, *Campylobacter* spp and *Clostridia* spp.

The typical approach to this has been using low crude protein diets that rely heavily on the use of synthetic amino acids to maintain the level of amino acids within the diet (Brewster *et al.*, 2015). However, this approach can add considerable cost to the most expensive diets used within pig production. An alternative approach to this is to utilise exogenous proteases to help improve the digestion of protein in the diet of weaner pigs. There is a wealth of evidence supporting the use of proteases within grow-finisher pigs with improvements in growth rates and feed efficiency (O'Doherty and Forde, 1999; McAlpine *et al.*, 2012), but less evidence exists for the benefits of protease use on the gut health of weaner pigs.

As Pluske *et al.* (2015) noted, any discussion pertaining to gut health cannot occur without reference to the microbiota that inhabit the gastrointestinal tract (GIT) of the pig. The GIT is a home to a complex and diverse microbial community that are intimately involved in 'communication' between enteric bacteria and its host influencing growth, health and disease. In recent years the exploration of the gut microbiota has become easier with the introduction of techniques like 16S rRNA profiling utilising next generation sequencing

approaches. Whilst these techniques allow us an insight into the microbiota, it is poorly understood how changes in diet influence the microbiota (Frese *et al.*, 2015).

This study aimed to bring these components together to understand the influence of protease as part of an integrated health strategy on the microbiome of the weaner pig, how it effects digestibility, its role in reducing the production of potential toxic breakdown products, reducing the growth of bacteria associated with disease and ultimately in how we can improve the performance of weaner pigs.

This study also sought to understand the impact of protease inclusion on the microbial community within the grower pig and how the community changes with this improvement in diet digestibility, as well as the influence that targeted antibiotic use has on the microbiome and performance.

2. Methodology

All studies in this project were approved by the CHM Alliance Pty Ltd Animal Ethics Committee (CHM PP 127/20 and CHM PP 145/21).

This research project initially involved two studies, one in weaner pigs and one in grower pigs, to look at the role of protease as part of an integrated health strategy. As a result of the outcomes observed, the project has resulted in four studies – two identical weaner studies where diet analysis suggested a repeat was necessary, a grower study as proposed, and another grow-finisher study looking at a higher rate of protease use offsetting a down-specification in amino acids.

Experiment 1a and b. Influence of protease and low crude protein diets on the microbial community, feed digestibility and utilisation, and growth performance of weaner pigs.

These studies were identical in format and examined a 2 x 2 factorial design with protein content (standard vs low) and protease inclusion (0 or 150 ppm) as the factors over a 28-day experimental period.

Pigs were fed either a standard weaner diet (14.7 MJ digestible energy (DE)/kg, 22.5 % crude protein (CP), 0.89 g standard ileal digestible lysine (SID Lys)/MJ DE) or a low protein diet (14.7 MJ DE/kg, 18.5 % CP, 0.87 g SID Lys/MJ DE), with both diets having 0 or 150 ppm protease (1.10 U/g derived from *Streptomyces fradiae*; Jefo Nutrition Inc., Saint-Hyacinthe, Quebec, Canada) (for diet formulations see Appendix 1). The low protein diets were fed for only the first 14 days after weaning, with treatments transitioning to the standard weaner diet, with or without protease, for the remainder of the 28-day experimental period.

Weaner pigs were received at weaning (~20 days of age), blocked by sex (female and male, 50:50 split), individually identified by eartag and allocated to pens (14 pigs per pen). Treatments were allocated as a randomised block, with the pen as the treatment. One-hundred and forty pigs were recruited into the study each week, for four weeks, resulting in 10 pens per treatment. Pens were weighed after allocation (d 0) and on a weekly basis thereafter (d 7, d 14, d 21 and d 28).

Feed was offered to the pigs via a multispace, round, transition pan feeder (TIGSA Transit plus, PGSaludables, Barcelona, Spain on an *ad libitum* basis, with the mass of feed delivered recorded, as well as the feed refusal at the end of the week to correspond with weigh events. Water was also available *ad libitum* and delivered through the combination of one nipple and one bowl drinker per pen and was also monitored weekly.

In Experiment 1a, at day 14 of the experiment, 24 pigs (6 per treatment from different pens) were identified and immobilized using a combination of zoletil/xylazil, a blood sample was obtained via jugular venepuncture, and the pig was euthanised using Lethabarb, with samples of ileal digesta and faeces from the mid-colon/rectum collected. A section of small intestine was placed into formalin, with similar measures taken at day 28 from another 24 pigs (6 per treatment from different pens). In Experiment 1b, blood samples and voided faeces were collected at day 14 and 28 in a similar design, with no euthanasia undertaken.

Blood samples were collected into lithium heparin tubes, centrifuged and plasma was suctioned off into multiple aliquots and frozen. Digesta was collected as two samples, with one being frozen for the assessment of digestibility and the other being stored 1:1 with TCA (Trichloroacetic acid, 10%) for the assessment of ammonium-N. Faeces were collected as three samples, with one being frozen for the assessment of digestibility, one frozen for microbiota assessment, and the third being stored 1:1 with TCA 10% for the assessment of ammonium-N (Heo *et al.,* 2009; Heo *et al.*, 2010).

Experiment 1b was instigated after a lack of growth performance differences were observed between the low protein and standard diets, where subsequent laboratory analysis of a sub-sampling of the treatment diets showed that crude protein levels of the standard diets were 20.7% and low protein diets were 18.1%, compared to a formulated level of 22.5% and 18.5% respectively. Batch sheets were checked at the mill and diets were mixed as formulated, with variation in protein concentration between formulation matrix values and actual ingredients being the potential cause of the issue.

Experiment 2. Influence of protease (and antibiotic) usage on the microbial community, feed digestibility and utilisation, and growth performance of grow-finisher pigs.

This study examined a 2 x 2 factorial design with protease inclusion (0 or 150 ppm) and medication (0 or 1 L per 1,000 L of drinking water of Tiamulin (Tiamulin fumarate 125 mg/mL, Alltech Lienert Australia Pty Ltd) as the factors over a 7-week experimental period.

Pigs were fed a standard grower diet (14.0 MJ digestible energy (DE)/kg, 19.9% crude protein (CP), 0.72 g standard ileal digestible lysine (SID Lys)/MJ DE, see Appendix 2) with either 0 or 125 ppm protease (1.10 U/g derived from *Streptomyces fradiae*; Jefo Nutrition Inc., Saint-Hyacinthe, Quebec, Canada). The diet was fed across the complete experimental period. Pigs were allowed to acclimate to pens and diets for the first 7 days, prior to the introduction of medication. Medication was offered in drinking water for 7 consecutive days from day 8 to 14 in an effort to modify the gut microbiota, with these pens returning to unmedicated drinking water from day 15 of the experiment.

Grower pigs entered the experiment at approximately 10 weeks of age, were blocked by sex (female and male 50:50 split), individually identified by eartag and allocated to pens (11 pigs per pen). Treatments were allocated as a randomised block, with the pen as the

treatment. One-hundred and thirty-two pigs were recruited into the study each week, for two weeks, resulting in 6 pens per treatment. Pens were weighed after allocation (d 0) at the end of the medication period (d 14) and at the end of the experiment (d 47).

Feed was offered to the pigs via a standard "penguin" type feeder on an *ad libitum* basis, with the mass of feed delivered recorded via a FeedLogic smart feeding system, with feed remaining in the feeder at day 14 and day 47 estimated to account for feed refusal. Water was also available *ad libitum* and delivered through three nipple drinkers per pen.

Voided composite faecal samples were collected at day 14 and day 47 from each pen. Faeces samples were divided into three, with one being frozen for the assessment of digestibility, one frozen for microbiota assessment, and the third being stored 1:1 with TCA 10% for the assessment of ammonium-N (Heo *et al.*, 2009; Heo *et al.*, 2010).

Experiment 3. The ability of protease to offset a down-specification of protein in grow-finisher pigs.

Grower pigs were offered one of three treatments across the experimental period. The positive control treatment consisted of a grower and a finisher diet.

The protease diets, containing 250 ppm protease (1.10 U/g derived from *Streptomyces fradiae*; Jefo Nutrition Inc., Saint-Hyacinthe, Quebec, Canada), were formulated to the same nutrient specification as the positive control. The protease was included as a raw material and was assigned a contribution of amino acids and crude protein (Table 1), as per the recommendations of the supplier, to account for the expected effects of protease in improving amino acid digestibility (for further information on the use of matrix values for exogenous enzymes please see Bedford and Cowieson, 2020).

The final treatment was a negative control, where the protease was not included in the diet formulated for the protease treatment, therefore having a lower level of digestible amino acids. The diet specifications can be found below (Table 2) and the formulations can be found in Appendix 3. The ratio of other amino acids to lysine were held constant in grower and finisher treatment diets.

Grower pigs entered the experiment at approximately 12 weeks of age, blocked by sex, individually identified by eartag and allocated to pens (11 pigs per pen). Treatments were allocated as a randomised block, with the pen as the treatment. One-hundred and thirty-two pigs were recruited into the study each week, for two weeks, resulting in 8 pens per treatment. Pens were weighed after allocation (d 0), when they transitioned from the grower to the finisher diets (d 35) and at the end of the experiment (d 56).

Table 1. Contribution (%) of crude protein and total amino acids of the inclusion of 250 ppm of protease to treatment diets, as provided by protease supplier (Jefo Nutrition Inc., Saint-Hyacinthe, Quebec, Canada).

	Grower		Finisher	
Nutrients	Matrix value	Contribution (%)	Matrix value	Contribution (%)
Crude protein	3520	0.88	3200	0.80
Lysine	256	0.064	233	0.058
Methionine	78	0.019	70	0.017
Methionine+Cysteine	151	0.038	137	0.034
Threonine	173	0.043	158	0.039
Tryptophan	51	0.013	47	0.012
Valine	166	0.041	151	0.038
Leucine	289	0.072	262	0.066
Isoleucine	166	0.041	151	0.038
Arginine	217	0.054	198	0.049

Table 2. Nutrient specifications of treatment diets fed to grower-finisher pigs in Experiment 3.

Treatment	Diet	Energy (MJ DE/kg)	Crude protein (%)	SID Lysine (g/MJ DE)	Met:Lys	M+C:Lys	Try:Lys	Thr:Lys
Positive control								
	Grower	14.0	18.78	0.68	0.30	0.62	0.18	0.64
	Finisher	13.6	17.31	0.62	0.30	0.67	0.18	0.67
Negative control								
	Grower	14.0	17.78	0.64	0.30	0.62	0.18	0.64
	Finisher	13.6	16.32	0.58	0.30	0.67	0.18	0.67
Protease 250 ppm								
	Grower	14.0	18.65	0.68	0.30	0.62	0.18	0.64
	Finisher	13.6	17.12	0.62	0.30	0.67	0.18	0.67

Feed was offered to the pigs via a standard "penguin" type feeder on an *ad libitum* basis, with the mass of feed delivered recorded via a FeedLogic smart feeding system, with feed remaining in the feeder at day 35 and day 56 estimated to account for feed refusal. Water was also available *ad libitum* and delivered through three nipple drinkers per pen.

Growth performance was measured over the 56-day period, with the first cut of pigs from each pen going to slaughter at this time (~20 weeks of age). Pigs were marketed on a set weight, rather than a set time basis, and they remained on their respective treatment diets until they reached market weight. Carcase weight (hot-standard carcase weight, HSCW) and backfat depth at the P2 site were recorded for all pigs at slaughter.

Sample Analysis

Collected samples were analysed at various research laboratories, that were best suited for the conduct of the different tests and availability due to numerous challenges associated with Covid-19 restrictions. Microbiome analysis was undertaken at NSW DPI EMAI, Menangle (Dr Alison Collins), utilizing 16S rRNA gene sequencing (full methodology is provided in Appendices 4 & 5). Volatile fatty acid levels were determined by gas-liquid chromatography at the WA Department of Primary Industries and Regional Development, South Perth. Ammonia-N, and apparent total tract and ileal amino acid digestibility, were determined at

Murdoch University using methodology as outlined in Heo *et al.* (2009) and Heo *et al.* (2010). Serum markers of metabolism were also conducted at Murdoch University via ELISA assays.

Statistical Analysis

Statistical analysis of growth performance and sample analysis was performed utilizing a general analysis of variance in GenStat 21st edition (VSN International Ltd, Hemel Hempstead, HP2 4TP, UK), with an unbalanced treatment structure utilized for sample analysis where needed. Sex differences were only significant in Experiment 3; however, there was a general lack of interaction between treatment and sex. Differences in removals were determined by Chi-square analysis, also in GenStat 21st edition. Statistically significant differences were determined at $P < 0.05$. Statistical analysis of the microbiome component of this study is outlined in detail in Appendices 4 & 5.

3. Outcomes

Experiment 1a and b. Influence of protease and low crude protein diets on the microbial community, feed digestibility and utilisation, and growth performance of weaner pigs.

The inclusion of 150 ppm of protease in diets had little impact on weaner performance in either Experiment 1a (Table 3) or Experiment 1b (Table 4). Protease treatment significantly improved the growth performance in week 3 of Experiment 1a, but had no other statistically significant impact on weight, feed intake, feed efficiency or removals.

However, those pigs that received the low protein diet in the first 14 days had reduced daily gain (Exp 1a, $P < 0.001$; 1b $P = 0.082$) and feed intake (1a $P = 0.015$; 1b $P = 0.030$) and poorer feed conversion (1a $P = 0.019$; 1b $P = 0.37$), that continued even when they had transitioned to the common standard diet after day 15. There was a larger impact seen in Experiment 1a (Table 3) compared to Experiment 1b (Table 4).

Plasma urea nitrogen (PUN) concentration at both day 14 and day 28 reflected the treatments with a significantly lower level of PUN observed in the low protein pigs when samples were taken at day 14 (Table 5), but no difference observed at day 28 when they had transitioned to the standard diet. The lower ileal digesta ammonia-N concentration at day 14 also reflected the impact of the low protein diet, although no difference was observed in faeces at either day 14 or 28 (Table 6). Calprotectin concentration was significantly lower at day 28 in those pigs that had received the 150-ppm protease treatment (Table 5), indicative of a generalised reduction in inflammation.

There was no significant difference observed in ileal or total tract dry matter digestibility between either of the main effects, nor any interactions observed (Table 6). However, it should be noted that the reported dry matter digestibility values are lower than reported in other literature (e.g., Kim *et al.*, 2005). Whilst both the low protein compared to the standard diets and the 150-ppm protease compared to no protease diet tended to show lower levels of amino acid digestibility, the only significant difference was for the aromatic amino acid tyrosine (Table 7). The values for apparent ileal amino acid digestibility within this experiment are also at the lower end of values reported in other literature (Engelsmann *et al.*, 2022; Kim *et al.*, 2009).

On an absolute basis of VFA production, those pigs receiving the low protein diets had a significantly lower concentration of acetate than the standard diets but produced more caproate (Table 8). However, when looking at VFA production from a proportional perspective, pigs receiving low protein diets produced more iso-butyrate, iso-valerate, valerate and caproate. When protease was included in the diet at 150 ppm there was less total VFA production (P = 0.046), primarily through less propionate production, which held on a proportional basis with significantly relatively less propionate and more acetate being produced by pigs receiving 150 ppm protease in their diets.

Full results of the microbiome analysis can be found in Appendix 4. However, the alpha diversity of bacteria within each treatment was not significantly different between treatments at day 14 or day 28 and showed a good coverage of bacterial sequences, while clustering of bacterial communities by treatment was not visible at either timepoint.

Differences were observed between treatments in the relative abundance of specific taxa. Pigs receiving low protein diets for 14 days had a reduced abundance of *Bacteroides* and a higher abundance of *Firmicutes*, such that the ratio of *Bacteroides:Firmicutes* in pigs fed standard diets was 2.44 (66% *Bacteroides* and 27% *Firmicutes*) compared with 1.84 in the low protein diets (59% *Bacteroides* and 32% *Firmicutes*), with the reduced abundance of *Bacteroides* in pigs on the low protein diets primarily due to a reduced level of *Prevotellaceace*.

After 28 days when all pigs were being fed standard diets, with or without their respective levels of protease, there was no difference in the relative abundance of the three major phyla (27% *Firmicutes*, 66% *Bacteroides*, 4% *Proteobacteria*). Within the *Firmicutes* phyla, the *Clostridiales* were the major order, and within this order the relative abundance differed between standard and low protein for the families *Veillonellaceace* (26 *vs* 33%), *Ruminococcaceae* (38 *vs* 35%) and *Lachnospiraceae* (27 *vs* 24%).

Table 3. Experiment 1a: Mean growth performance of weaner pigs fed standard or low protein diets, with or without 150 ppm protease. Low protein diets were fed for the first 14 days after weaning before they transitioned to their respective standard diets for the remainder of the experimental period.

	Diet				Protease				Treatment interactions					
	Standard	Low Protein	SED	*P* value	0 ppm	150 ppm	SED	*P* value	Standard 0 ppm	Standard 150 ppm	Low Protein 0 ppm	Low Protein 150 ppm	SED	*P* value
Weight, kg														
d 0	6.2	6.2	0.05	0.91	6.2	6.2	0.05	0.84	6.2	6.2	6.2	6.1	0.08	0.45
d 7	6.7	6.7	0.04	0.82	6.7	6.7	0.04	0.39	6.7	6.8	7.7	6.7	0.05	0.42
d 14	8.7[a]	8.5[b]	0.07	0.002	8.6	8.6	0.13	0.76	8.7	8.7	8.5	8.4	0.09	0.29
d 21	11.2[a]	10.7[b]	0.11	<0.001	10.8	11.0	0.11	0.14	11.1	11.3	10.6	10.7	0.16	0.46
d 28	14.4[a]	13.5[b]	0.18	<0.001	13.8	14.1	0.17	0.13	14.2	14.6	13.5	13.6	0.25	0.24
Average daily gain, kg/d														
d 0-7	0.081	0.079	0.005	0.82	0.078	0.082	0.005	0.39	0.076	0.085	0.079	0.080	0.007	0.42
d 8-14	0.280[a]	0.248[b]	0.008	<0.001	0.268	0.260	0.008	0.35	0.280	0.279	0.255	0.242	0.011	0.45
d 15-21	0.360[a]	0.314[b]	0.010	<0.001	0.324[a]	0.350[b]	0.010	0.009	0.346	0.374	0.301	0.326	0.014	0.86
d 22-28	0.455[a]	0.411[b]	0.019	0.031	0.426	0.440	0.019	0.47	0.439	0.471	0.413	0.409	0.027	0.55
Average daily feed intake, kg/d														
d 0-7	0.14	0.14	0.008	0.99	0.14	0.14	0.007	0.32	0.13	0.15	0.14	0.14	0.011	0.18
d 8-14	0.35	0.33	0.011	0.068	0.34	0.34	0.011	0.44	0.34	0.36	0.33	0.33	0.015	0.31
d 15-21	0.50[a]	0.47[b]	0.016	0.041	0.47	0.50	0.016	0.92	0.49	0.51	0.45	0.48	0.022	0.92
d 22-28	0.68[a]	0.64[b]	0.016	0.026	0.66	0.66	0.016	0.94	0.67	0.68	0.65	0.63	0.023	0.55
Feed conversion ratio, kg/kg														
d 0-7	1.83	1.87	0.083	0.59	1.84	1.86	0.082	0.87	1.79	1.87	1.90	1.84	0.117	0.42
d 8-14	1.25	1.33	0.042	0.066	1.23	1.33	0.041	0.100	1.22	1.29	1.30	1.37	0.059	0.99
d 15-21	1.40[a]	1.52[b]	0.052	0.024	1.49	1.43	0.051	0.29	1.42	1.38	1.55	1.49	0.073	0.90
d 22-28	1.51	1.62	0.082	0.19	1.58	1.55	0.080	0.64	1.57	1.45	1.60	1.64	0.115	0.33
Total growth performance, d 0-28														
ADG	0.294[a]	0.263[b]	0.006	<0.001	0.274	0.283	0.006	0.13	0.285	0.302	0.262	0.264	0.009	0.24
ADFI	0.41[a]	0.39[b]	0.008	0.015	0.39	0.41	0.008	0.097	0.40	0.42	0.39	0.39	0.011	0.19
FCR	1.40[a]	1.51[b]	0.041	0.019	1.45	1.46	0.040	0.96	1.41	1.40	1.50	1.51	0.058	0.85
Removals														
	3	1	$\chi^2(1)=1.01$, P=0.32		3	1	$\chi^2(1)=1.01$, P=0.32		2	1	1	0	$\chi^2(3)=2.01$, P=0.57	

[a,b]Means within a row, within effect, with different superscripts differ significantly (P < 0.05); SED, standard error difference of the means; ADG, average daily gain; ADFI, average daily feed intake; FCR, feed conversion ratio.

Table 4. Experiment 1b: Mean growth performance of weaner pigs fed standard or low protein diets, with or without 150 ppm protease. Low protein diets were fed for the first 14 days after weaning before they transitioned to their respective standard diets for the remainder of the experimental period.

	Diet				Protease				Treatment interactions					
	Standard	Low Protein	SED	*P* value	0 ppm	150 ppm	SED	*P* value	Standard 0 ppm	Standard 150 ppm	Low Protein 0 ppm	Low Protein 150 ppm	SED	*P* value
Weight, kg														
d 0	6.2	6.2	0.02	0.19	6.2	6.2	0.02	0.78	6.2	6.2	6.2	6.2	0.03	0.69
d 7	6.8	6.8	0.05	0.63	6.8	6.8	0.05	0.53	6.8	6.8	6.9	6.8	0.07	0.37
d 14	8.5	8.3	0.08	0.085	8.4	8.4	0.08	0.46	8.4	8.6	8.4	8.3	0.12	0.061
d 21	10.8[a]	10.5[b]	0.13	0.045	10.6	10.6	0.13	0.72	10.6	10.9	10.5	10.4	0.19	0.18
d 28	14.0	13.7	0.18	0.082	13.8	13.9	0.18	0.66	14.0	14.1	13.7	13.7	0.26	0.65
Average daily gain, kg/d														
d 0-7	0.079	0.083	0.008	0.63	0.083	0.079	0.008	0.53	0.078	0.080	0.089	0.077	0.011	0.37
d 8-14	0.242[a]	0.217[b]	0.007	0.002	0.223	0.237	0.007	0.078	0.227[a]	0.257[b]	0.219[a]	0.216[a]	0.011	0.040
d 15-21	0.325	0.307	0.010	0.067	0.317	0.315	0.010	0.83	0.325	0.326	0.310	0.304	0.014	0.73
d 22-28	0.462	0.467	0.014	0.73	0.469	0.461	0.014	0.61	0.473	0.464	0.452	0.471	0.020	0.31
Average daily feed intake, kg/d														
d 0-7	0.16	0.16	0.009	0.63	0.17	0.16	0.009	0.19	0.17	0.15	0.17	0.16	0.012	0.87
d 8-14	0.32	0.30	0.013	0.093	0.30	0.31	0.013	0.58	0.31	0.33	0.30	0.29	0.018	0.50
d 15-21	0.46[a]	0.44[b]	0.012	0.027	0.46	0.44	0.012	0.25	0.47	0.46	0.44	0.43	0.017	0.86
d 22-28	0.67	0.65	0.015	0.15	0.67	0.66	0.015	0.41	0.68	0.66	0.65	0.65	0.021	0.68
Feed conversion ratio, kg/kg														
d 0-7	2.21	2.18	0.201	0.86	2.18	2.20	0.200	0.92	2.27	2.15	2.10	2.25	0.282	0.49
d 8-14	1.32	1.36	0.046	0.48	1.36	1.32	0.046	0.33	1.37	1.28	1.36	1.36	0.065	0.29
d 15-21	1.44	1.44	0.024	0.82	1.46	1.42	0.024	0.090	1.47	1.41	1.45	1.42	0.033	0.43
d 22-28	1.45	1.42	0.038	0.49	1.46	1.41	0.038	0.26	1.45	1.44	1.46	1.38	0.054	0.38
Total growth performance, d 0-28														
ADG	0.279	0.267	0.007	0.082	0.271	0.274	0.007	0.066	0.276	0.282	0.267	0.267	0.009	0.65
ADFI	0.41[a]	0.38[b]	0.010	0.030	0.40	0.39	0.010	0.63	0.41	0.40	0.39	0.38	0.014	0.96
FCR	1.46	1.44	0.022	0.37	1.47	1.43	0.022	0.13	1.49	1.44	1.45	1.43	0.031	0.43
Removals														
	1	1	$\chi^2(1)=0.00$, P=1.00		0	2	$\chi^2(1)=2.01$, P=0.16		0	1	0	1	$\chi^2(3)=2.01$, P=0.57	

[a,b]Means within a row, within effect, with different superscripts differ significantly (P < 0.05); SED, standard error difference of the means; ADG, average daily gain; ADFI, average daily feed intake; FCR, feed conversion ratio.

Table 5. Experiment 1: Markers of metabolism of weaner pigs fed standard or low protein diets, with or without 150 ppm protease. Low protein diets were fed for the first 14 days after weaning before they transitioned to their respective standard diets for the remainder of the experimental period.

	Diet				Protease				Treatment interactions					
	Standard	Low Protein	SED	*P* value	0 ppm	150 ppm	SED	*P* value	Standard 0 ppm	Standard 150 ppm	Low Protein 0 ppm	Low Protein 150 ppm	SED	*P* value
Plasma urea nitrogen, mmol/L														
d 14	5.45[a]	3.80[b]	0.341	<0.001	4.49	4.69	0.341	0.56	5.27	5.62	3.77	3.83	0.483	0.68
d 28	4.85	4.46	0.567	0.58	4.46	4.71	0.567	0.61	3.83	5.50	4.86	4.20	0.813	0.057
Creatinine, μmol/L														
d 14	84.6	86.3	4.41	0.70	85.4	85.6	4.41	0.96	84.8	84.3	85.8	86.7	6.24	0.88
d 28	79.0	74.8	3.86	0.26	78.5	75.1	3.86	0.39	81.5	77.4	76.6	73.7	5.53	0.88
Haptoglobin, g/L														
d 14	0.70	0.74	0.179	0.66	0.77	0.68	0.179	0.66	0.76	0.62	0.74	0.74	0.254	0.83
d 28	0.64	0.71	0.189	0.63	0.58	0.75	0.189	0.34	0.34	0.84	0.73	0.70	0.271	0.19
Alkaline phosphatase, IU/L														
d 14	301.7	312.4	35.14	0.76	301.6	312.4	35.14	0.76	299.8	303.3	303.2	320.9	49.72	0.84
d 28	277.9	278.7	23.28	0.97	294.2	268.3	23.28	0.29	285.8	272.8	299.6	265.3	33.36	0.66
Calprotectin, mg/kg														
d 14	36.7	31.3	4.47	0.25	33.7	34.1	4.47	0.83	30.3[ab]	42.5[b]	36.8[ab]	26.3[a]	6.32	0.019
d 28	23.0	23.0	3.79	0.80	28.5[a]	19.2[b]	3.70	0.024	25.3	21.4	30.3	18.0	5.37	0.28

[a,b]Means within a row, within effect, with different superscripts differ significantly (P < 0.05); SED, standard error difference of the means.

Table 6. Experiment 1: Ammonia-N concentrations and dry matter (DM) digestibility of ileal digesta and faeces of weaner pigs fed standard or low protein diets, with or without 150 ppm protease. Low protein diets were fed for the first 14 days after weaning before they transitioned to their respective standard diets for the remainder of the experimental period.

	Diet				Protease				Treatment interactions					
	Standard	Low Protein	SED	*P* value	0 ppm	150 ppm	SED	*P* value	Standard 0 ppm	Standard 150 ppm	Low Protein 0 ppm	Low Protein 150 ppm	SED	*P* value
Ammonia-N concentrations, μg/g														
Digesta, d 14	62.2[a]	33.7[b]	7.95	0.002	50.9	47.0	7.95	0.63	64.8	59.1	34.6	32.8	11.28	0.81
Faecal, d 14	288.1	293.8	25.8	0.82	289.0	293.3	25.87	0.87	293.4	282.3	285.0	303.4	36.60	0.58
Faecal, d 28	385.6	422.9	42.9	0.41	426.4	397.6	43.82	0.53	382.5	387.3	455.6	404.5	62.51	0.54
Ileal DM digestibility, %														
d 14	36.8	24.5	7.87	0.14	32.2	28.8	7.87	0.66	36.9	36.8	27.9	21.4	11.14	0.69
d 28	42.1	33.2	14.8	0.58	37.1	38.3	14.89	0.94	48.9	35.3	25.3	41.2	21.06	0.38
Total tract DM digestibility, %														
d 14	67.6	69.2	1.93	0.42	69.0	67.9	1.93	0.58	68.8	66.5	69.2	69.3	2.73	0.54
d 28	62.6	65.2	4.29	0.57	65.2	62.7	4.29	0.59	64.0	61.3	66.4	64.0	6.06	0.97

[a,b]Means within a row, within effect, with different superscripts differ significantly (P < 0.05); SED, standard error difference of the means.

Table 7. Experiment 1: Apparent ileal amino acid digestibility of weaner pigs fed standard or low protein diets, with or without 150 ppm protease. Low protein diets were fed for the first 14 day after weaning before they transitioned to their respective standard diets for the remainder of the experimental period.

	Diet				Protease				Treatment interactions					
	Standard	Low Protein	SED	*P* value	0 ppm	150 ppm	SED	*P* value	Standard 0 ppm	Standard 150 ppm	Low Protein 0 ppm	Low Protein 150 ppm	SED	*P* value
Amino acid digestibility, %														
Alanine	40.7	29.5	9.73	0.32	40.0	302	9.73	0.32	49.5	31.0	29.6	29.6	13.76	0.36
Arginine	62.5	53.5	7.17	0.27	61.1	55.1	7.17	0.41	68.4	56.0	53.0	54.1	10.15	0.36
Aspartic acid	48.4	35.6	7.34	0.11	45.1	39.2	7.34	0.42	56.6	39.4	32.6	38.9	10.39	0.13
Cysteine	28.8	14.7	11.43	0.25	24.8	19.1	11.43	0.60	40.3	16.2	7.8	22.2	16.17	0.11
Glutamic acid	62.4	52.9	7.31	0.22	58.9	56.8	7.31	0.75	68.6	55.6	48.3	58.0	10.34	0.14
Histidine	56.4	44.4	7.46	0.18	55.7	45.1	7.46	0.18	58.9	53.6	52.3	35.8	10.55	0.46
Isoleucine	54.2	42.8	7.76	0.19	52.1	45.1	7.75	0.37	61.5	46.3	41.9	43.9	10.97	0.29
Leucine	54.1	47.8	8.50	0.54	54.8	47.1	8.50	0.37	61.8	45.6	47.1	48.7	12.02	0.31
Lysine	51.0	44.0	7.89	0.47	52.0	42.9	7.89	0.26	57.6	43.8	45.8	41.9	11.16	0.54
Methionine	47.4	33.4	8.65	0.15	44.6	36.5	8.65	0.36	52.7	41.5	35.7	31.0	12.25	0.72
Phenylalanine	54.9	48.0	8.37	0.51	56.5	46.2	8.37	0.23	64.4	44.5	47.9	48.2	11.84	0.24
Proline	39.1	10.2	13.56	0.055	29.7	20.5	13.56	0.50	46.9	30.5	10.8	9.5	19.19	0.59
Serine	47.8	35.0	9.45	0.22	45.0	38.1	9.45	0.47	55.4	39.4	33.5	36.7	13.37	0.32
Threonine	39.6	26.7	9.52	0.21	35.8	30.9	9.52	0.61	46.1	32.5	24.4	29.3	13.47	0.35
Tyrosine	52.8[a]	34.9[b]	8.06	0.043	46.3	42.1	8.06	0.60	60.3	44.6	30.8	39.4	11.41	0.15
Valine	42.5	35.0	9.48	0.48	41.9	35.7	9.48	0.51	50.3	33.9	32.6	37.6	13.41	0.28

[a,b]Means within a row, within effect, with different superscripts differ significantly ($P < 0.05$); SED, standard error difference of the means.

Table 8. Experiment 1: Absolute levels and relative proportions of volatile fatty acids produced by weaner pigs fed standard or low protein diets, with or without 150 ppm protease. Low protein diets were fed for the first 14 days after weaning before they transitioned to their respective standard diets for the remainder of the experimental period.

	Diet				Protease				Treatment interactions					
	Standard	Low Protein	SED	*P* value	0 ppm	150 ppm	SED	*P* value	Standard 0 ppm	Standard 150 ppm	Low Protein 0 ppm	Low Protein 150 ppm	SED	*P* value
Absolute levels, mM														
Acetate	63.4[a]	59.7[b]	1.75	0.049	62.6	60.5	1.75	0.25	64.2	62.6	61.0	58.5	2.48	0.82
Propionate	21.5	20.1	0.92	0.16	22.5[a]	19.1[b]	0.92	0.001	23.2	19.7	21.8	18.4	1.30	0.99
Iso-butyrate	1.71	1.91	0.136	0.15	1.88	1.74	0.136	0.30	1.79	1.62	1.97	1.85	0.192	0.82
Butyrate	14.4	13.3	1.20	0.39	14.6	13.1	1.20	0.24	14.9	13.8	14.2	12.4	1.70	0.79
Iso-valerate	2.47	2.90	0.246	0.097	2.80	2.58	0.246	0.39	2.61	2.34	2.98	2.82	0.347	0.81
Valerate	3.52	3.84	0.230	0.18	3.80	3.56	0.230	0.32	3.60	3.44	4.00	3.68	0.230	0.72
Caproate	0.51[a]	0.88[b]	0.100	0.002	0.63	0.76	0.100	0.22	0.36	0.67	0.91	0.85	0.142	0.083
TOTAL	107.5	102.7	3.52	0.19	108.8[a]	101.3[b]	3.52	0.046	110.7	104.2	106.9	98.5	4.98	0.80
Relative proportion, %														
Acetate	59.1	58.3	0.95	0.40	57.5[a]	59.9[b]	0.95	0.023	58.0	60.2	57.1	59.5	1.35	0.88
Propionate	19.9	19.5	0.48	0.44	20.7[a]	18.8[b]	0.48	<0.001	20.9	18.9	20.4	18.7	0.68	0.85
Iso-butyrate	1.6[a]	1.9[b]	0.11	0.023	1.7	1.7	0.11	0.91	1.6	1.5	1.8	1.9	0.16	0.52
Butyrate	13.3	12.9	0.87	0.63	13.4	12.8	0.87	0.50	13.5	13.1	13.3	12.5	1.23	0.77
Iso-valerate	2.3[a]	2.8[b]	0.21	0.020	2.6	2.6	0.21	0.93	2.4	2.2	2.8	2.9	0.29	0.56
Valerate	3.3[a]	3.7[b]	0.17	0.014	3.5	3.5	0.17	1.00	3.3	3.3	3.8	3.7	0.24	0.86
Caproate	0.5[a]	0.9[b]	0.10	<0.001	0.6	0.7	0.10	0.12	0.3	0.6	0.9	0.9	0.14	0.14

[a,b]Means within a row, within effect, with different superscripts differ significantly ($P < 0.05$); SED, standard error difference of the means.

Experiment 2. Influence of protease (and antibiotic) usage on the microbial community, feed digestibility and utilisation, and growth performance of grow-finisher pigs.

The inclusion of 125 ppm of protease into the diets of grower pigs from 10 to 17 weeks of age had no impact on their growth performance (Table 9), with no statistically significant differences in growth rate, feed intake or efficiency observed. Unlike the weaner study, the inclusion of 125 ppm protease tended to increase the ammonia-N concentration in digesta when measured at day 14, with this relationship weakening at the end of the experiment. No differences in faecal ammonia-N concentrations were observed.

The introduction of medication in the form of Tiamulin for 7 days from day 8 to 14 resulted in a significantly improved feed conversion in the subsequent period (day 15 to 47, Table 9). However, no improvement in growth rate was observed, just a significant reduction in feed intake. There were no significant interactions between treatments, although the combination of the inclusion of 125 ppm protease and medication with Tiamulin tended to have the greatest impact on reducing feed intake.

Total tract dry matter digestibility measured at day 14 showed significantly ($P<0.001$) higher digestibility in those pigs receiving the diet containing protease (Table 9); however, it needs to be noted that the reported dry matter digestibility values are well below those reported in other literature (e.g., Kim *et al.*, 2008). Amino acid digestibility results have not been included in this report as the data indicates that errors must have occurred in the sampling with many negative values for digestibility being evident.

Again, full results of the microbiome analysis can be found in Appendix 5. However, the alpha diversity of bacteria within each treatment was not significantly different between treatments at day 14 or day 47 and showed a good coverage of bacterial sequences, while clustering of bacterial communities by treatment was not visible by principal component analysis at either timepoint.

The ratio of *Bacteroides:Firmicutes* was observed to be different between pigs receiving the standard diet and that supplemented with 125 ppm protease (0.12 *vs* 0.056), indicating a halving of the abundance of *Bacteroides* and thus an increased abundance of *Firmicutes*. Of these *Firmicutes*, the class *Bacilli* was more abundant in protease-supplemented pigs (20% *vs* 6%), primarily associated with an increased relative abundance of the family *Lactobacillales* in protease-fed pigs (69% *vs* 59%).

Linear discriminate analysis (LEfSe) of the relative abundance of the bacterial taxa demonstrated that after 14 days on their respective diets, pigs on the standard diet were characterized by a Log_{10} 3-fold increase in *Enterococcaceae*, pigs on the protease-supplemented diet had a Log_{10} 4-fold increase in *Lactobacillaceae*, *Turicibacteriaceace* and *Mycoplasmatacea*. When medication was introduced, the treatments were further differentiated, with a Log_{10} 4-fold increase in *Prevotellaceae* in pigs not receiving protease diets, and a Log_{10} 3-fold increase in *Streptomycetaceae*, *Porphyromonadaceae* and *Fibrobacteriaceae*.

Whilst not directly comparable, it was observed that the ratio of *Bacteroides:Firmicutes* for these grower pigs at day 14 was 0.08 (7.5% *Bacteroides*, 92.5% *Firmicutes*) compared to the weaner pigs at 2.12 (62.5% *Bacteroides*, 29.5% *Firmicutes*). However, at day 47, the relative abundance of *Bacteroides:Firmicutes* had returned to 2.52 (63% *Bacteroides*, 25% *Firmicutes*). The most pronounced impact on the grower pigs' microbiomes were therefore not treatment but the effect of sampling time.

Table 9. Experiment 2: Growth performance, faecal dry matter (DM) digestibility and ammonia-N concentrations of grower pigs fed standard diets with 0 or 125 ppm protease, in the presence or absence of medication. Medication (Tiamulin) was delivered to pigs via drinking water for 7 consecutive days from day 8 to 14 of the experiment.

	Protease				Medication				Treatment interactions					
	0 ppm	125 ppm	SED	*P* value	Nil	Tiamulin	SED	*P* value	0 ppm, Nil	0 ppm, Tiamulin	125 ppm, Nil	125 ppm, Tiamulin	SED	*P* value
Weight, kg														
d 0	26.5	26.9	0.15	0.94	26.9[a]	26.2[b]	0.15	<0.001	26.9	26.2	26.9	26.2	0.21	0.85
d 14	36.7	36.8	0.35	0.67	36.5	37.1	0.50	0.22	36.2	37.2	36.8	36.9	0.57	0.19
d 47	69.2	68.4	0.73	0.28	68.2	69.5	1.07	0.21	68.3	70.2	68.8	68.8	1.21	0.50
Average daily gain, kg/d														
d 0-14	0.725	0.736	0.025	0.67	0.708	0.752	0.036	0.22	0.686	0.764	0.731	0.741	0.041	0.19
d 15-47	0.986	0.957	0.038	0.11	0.960	0.982	0.025	0.38	0.974	0.998	0.946	0.967	0.028	0.96
Average daily feed intake, kg/d														
d 0-14	1.21	1.23	0.038	0.65	1.24	1.20	0.055	0.45	1.21	1.21	1.27	1.18	0.063	0.29
d 15-47	2.03	2.03	0.038	0.99	2.12[a]	1.94[b]	0.056	0.006	2.09	1.97	2.15	1.91	0.063	0.13
Feed conversion ratio, kg/kg														
d 0-14	1.69	1.70	0.094	0.92	1.78	1.61	0.094	0.22	1.79	1.60	1.78	1.62	0.156	0.85
d 15-47	2.06	2.13	0.057	0.26	2.22[a]	1.97[b]	0.083	0.009	2.15	1.97	2.29	1.97	0.094	0.24
Total growth performance, d 0-28														
ADG	0.908	0.891	0.016	0.28	0.885	0.914	0.016	0.21	0.889	0.928	0.882	0.900	0.026	0.50
ADFI	1.77	1.77	0.027	0.95	1.83[a]	1.71[b]	0.027	0.006	1.81	1.73	1.86	1.68	0.045	0.079
FCR	1.95	1.99	0.045	0.37	2.08[a]	1.87[b]	0.065	0.006	2.03	1.87	2.12	1.87	0.074	0.40
Total tract DM digestibility, %														
d 14	35.4	53.1	3.63	<0.001	43.2	45.9	3.63	0.47	36.3	34.5	49.6	56.3	4.54	0.26
d 47	46.3	60.4	1.28	<0.001										
Ammonia-N concentrations, µg/g														
Digesta d 14	48.0	57.9	5.42	0.084	51.5	54.3	5.42	0.61	49.9	46.1	53.1	62.6	7.67	0.23
Digesta d 47	55.8	65.7	6.01	0.12	58.0	63.5	6.01	0.37	55.3	56.4	60.8	70.5	8.50	0.48
Faeces d 14	218.0	206.1	24.23	0.63	211.0	213.0	24.23	0.94	226.0	209.9	196.1	216.1	34.26	0.46
Faeces d 47	289.4	257.0	21.80	0.15	270.3	276.2	21.80	0.79	289.0	289.8	251.5	262.6	30.82	0.82

[a,b]Means within a row, within effect, with different superscripts differ significantly ($P < 0.05$); SED, standard error difference of the means; ADG, average daily gain; ADFI, average daily feed intake; FCR, feed conversion ratio.

Experiment 3. The ability of protease to offset a down-specification of protein and amino acids in grow-finisher pigs.

The inclusion of 250 ppm protease into grow-finisher diets was able to maintain growth performance in a diet that was reduced in amino acid specification by 5% (Table 10). The down-specified diet that included 250 ppm of protease was similar to the positive control in average daily feed intake, average daily gain or feed efficiency across the experimental period, whilst both of these treatments resulted in increased feed intake and average daily gain compared to the negative control across the total growth period. There was no significant difference in carcase characteristics between treatments.

Unlike previous studies, that did not extend into the latter finisher phase, there was a large sex effect seen in this study. This impact was seen greatest in the last three weeks of the growth component of the study which corresponded to the period after the second dose of Improvac was administered. This saw a 20% improvement in growth rate when comparing the immunocastrated males to the females, a 10% improvement in feed efficiency, and a reduction in the coefficient of variation of liveweight.

Table 10. Experiment 3: Growth performance of grower pigs fed standard diets (Positive Control) or diets formulated to account for the uplift delivered by the addition of protease, with 0 ppm (Negative Control) or 250 ppm protease (Protease 250 ppm) in both grower and finisher periods.

	Treatment					Sex				Interaction
	Positive Control	Negative Control	Protease 250 ppm	SED	*P* value	Female	Immuno	SED	*P* value	*P* value
Weight, kg										
d 0	33.7	33.4	33.4	0.43	0.78	33.9	33.2	0.35	0.051	0.79
d 35	65.9	64.1	65.4	0.98	0.19	65.6	64.7	0.80	0.28	0.97
d 56[1]	87.4	84.8	87.5	1.23	0.074	85.0[a]	88.1[b]	1.01	0.006	0.25
Weight CV, %										
d 0	15.3	14.3	13.6	1.72	0.63	14.9	13.9	1.41	0.50	0.64
d 35	12.1	14.8	12.7	1.41	0.16	13.9	12.5	1.15	0.22	0.98
d 56	10.7	13.3	12.1	1.13	0.11	13.0[a]	11.0[b]	0.92	0.045	0.64
Average daily gain, kg/d										
d 0-35	0.908	0.864	0.902	0.023	0.14	0.893	0.889	0.019	0.81	0.99
d 36-56	1.050	1.010	1.076	0.029	0.095	0.948[a]	1.143[b]	0.023	<0.001	0.017
Average daily feed intake, kg/d										
d 0-35	2.11	2.04	2.08	0.039	0.26	2.14[a]	2.02[b]	0.032	0.001	0.75
d 36-56	2.51[a]	2.33[b]	2.49[a]	0.070	0.037	2.33[a]	2.56[b]	0.057	<0.001	0.064
Feed conversion ratio, kg/kg										
d 0-35	2.32	2.37	2.31	0.033	0.19	2.39[a]	2.27[b]	0.027	<0.001	0.68
d 36-56	2.43	2.31	2.33	0.087	0.34	2.47[a]	2.25[b]	0.071	0.006	0.093
Total growth performance, d 0-56										
ADG, kg/d	0.959[a]	0.917[b]	0.966[a]	0.019	0.038	0.913[a]	0.982[b]	0.015	<0.001	0.24
ADFI, kg/d	2.25[b]	2.15[a]	2.23[ab]	0.039	0.037	2.20	2.21	0.032	0.77	0.14
FCR, kg/kg	2.36	2.34	2.31	0.038	0.47	2.42[a]	2.26[b]	0.031	<0.001	0.11
Carcase characteristics										
HSCW[2], kg	77.6	78.4	78.8	0.82	0.32	77.6	79.0	0.67	0.048	0.53
Back fat, mm	11.5	11.0	11.2	0.35	0.33	10.6[a]	11.9[b]	0.29	<0.001	0.14
Back fat – HSCW as covariate, mm	11.6	11.0	11.1	0.34	0.16	10.6[a]	11.8[b]	0.28	<0.001	0.15

[a,b]Means within a row, within effect, with different superscripts differ significantly (P < 0.05); [1]Pigs were marketed on a weight basis, with end weight being the average weight at time of first cut; [2]Pigs marketed on a weight rather than time basis, hence no difference in slaughter weight; SED, standard error difference of the means; ADG, average daily gain; ADFI, average daily feed intake; FCR, feed conversion ratio.

4. Application of Research

The weaner studies within this project showed that protease had little impact on performance at low inclusion rates on diets that are formulated with generally highly digestible sources of amino acids. When the inclusion rate was doubled in the second grow-finisher study and the matrix values associated with the uplift in nutrient availability were taken into account, the ability to maintain performance indicates that proteases likely have a role in profitable pork production, and that the levels used in the weaner studies were likely too low to illicit a response. The impacts when protease is included at appropriate rates may be even greater when access to ingredients becomes scarcer through either reduced availability or increased cost.

Whilst there was little performance impact, the reduction in calprotectin levels in weaner pigs consuming diets with 150 ppm protease suggests a reduction in inflammation in the gut after 28 days, with protease most likely reducing the amount of substrate available for the proliferation of pathogenic bacteria. Calprotectin is a marker for intestinal inflammation where elevated levels indicate white blood cells have leaked into the luminal content. To see this result when pigs are not being challenged is encouraging for the role of protease as part of an integrated gut health program.

These studies showed the lack of value of low protein diets in the absence of significant challenge, such as that associated with post-weaning diarrhoea because of *E. coli* infection (Heo *et al.*, 2009). The lower PUN results clearly indicated the impact of the consumption of the low protein diet, but when pigs were not challenged, the lack of protein in the diet resulted in poorer performance at the start which compounded throughout the experiment. The differences observed in PUN, Ammonia-N and VFA production associated with low proteins seen in this study agree with the findings of Heo *et al.* (2009), but the lack of challenge to the standard feeding treatment didn't allow the benefits of low protein diets to be expressed.

The reduced relative abundance of *Enterobacteriaceae* and increased abundance of *Prevotellaceae* in weaner pigs fed lower protein diets in this study agrees with previous studies, with a higher abundance of *Prevotellaceae* having been reported as a dominant feature of the faecal microbiota in healthy pigs as compared to pigs suffering diarrhoea after weaning (Dou *et al.*, 2017). It was evident that the low-protein diets in this study performed as they were expected, but in the absence of a challenge their full benefit could not be expressed.

Lower levels of protease (125 ppm) were not able to enhance the performance of grower pigs fed adequate diets. However, when higher levels of protease (250 ppm) were applied to a diet below specifications, performance was able to be restored. Whilst it was not able to be determined if the higher level of protease inclusion could enhance an adequate diet, the application of a protease to better utilize protein could allow for the inclusion of lower levels of protein when access to feedstuffs is challenging, at a lower cost per unit gain.

The largest impact on the microbiome of the grower pig was neither the antibiotic treatment nor the differing levels of protease, but rather simply the effect of sampling time. The ratio of *Bacteroidetes:Firmicutes* observed at day 14, was significantly altered from that at day 47, as well as that observed in the weaner study that was more reflective of the day 47 measure. Whilst there are known issues with freeze/thawing samples and a resultant higher increase in *Firmicutes*, all samples in this study were handled in a similar manner. It is

possible that the shift in food and housing that occurred to these pigs at day 0 of the experiment caused a major, but temporary shift, in their microbiome with similar findings being observed in tonsil microbiome communities (Pena Cortes *et al.*, 2018).

This study showed that determining the correct inclusion rate of protease is important to achieve the desired outcome of improved performance. There was some evidence of protease being able to modify protein metabolism such that a lower immune response was seen through a reduced level of calprotectin.

Outside of the specific treatment effects, the greatest impact on industry seen in this study is the marked difference in performance between female and immunocastrate pigs after they receive their second dose of Improvac. Immunocastrated males had a 20% improvement in growth rate, a 10% improvement in feed efficiency and a reduction in the coefficient of variation of liveweight, with other work also showing the cost of gilt production (Woodworth *et al.*, 2021). The delay of puberty in immunocastrated males and the development of oestrus in gilts and its associated increase in activity and riding behaviour may be one potential explanation for this divergence in growth performance.

5. Conclusion

This project sought to improve the intestinal health of the weaner and grow-finish pig, through reducing the substrates for microbial growth and resulting in an increase in the efficiency of growth through better utilization of feed. The study also sought to understand the impact of production practices on the microbial community of the pig, and how that community changes with diet ingredient digestibility, antibiotic use and diet transitions over the growing period.

Evidence generated suggests that improvements in the intestinal health of the weaner pig were able to be achieved, with a decreased level of calprotectin observed at the end of the weaner trial. Other factors such as differences in plasma urea nitrogen, digesta ammonia-N and total volatile fatty acid production were in general agreement with previous studies that have investigated the role of low protein diets in weaner pig production. However, as the weaner pigs in this study were not subjected to a post-weaning diarrhoea challenge, these apparent improvements in intestinal health did not result in an increase in the efficiency of growth.

The largest changes in microbiome in grow-finish pigs were not a result of treatment, but sampling time. It is likely that the moving of the pigs to the research finisher facility and the sudden transition to a new shed and new diets resulted in disturbance of the relative abundance of the most common bacteria phyla *Bacteroides* and *Firmicutes*, that transitioned from a ratio of 2.12 in established stable weaner pigs, to 0.08 in the grower pigs that moved sheds and feed and back to 2.52 when they had become totally accustomed to their new surrounds. Understanding the impact of change on the microbiome, and developing strategies to minimize this in commercial environments, is likely to result in decreased gut disturbance and reduce performance checks.

Finally, the project was able to show that the inclusion of a higher dose of protease in a diet fed to grow-finish pigs was able to positively influence their performance, adding another management tool to deal with increasingly scarce sources of protein.

6. Limitations/Risks

This research was conducted within a commercial research facility with exceptional management, a strict adherence to space allowances and a generally high level of health. Given the known data around the use of low protein diets in the control of PWD, the absence of a diarrhoeal challenge within this environment may not have allowed the full benefits of low protein diets to be expressed.

The protease inclusion rates used in this study are applicable to the mix of ingredients on offer and the environment in which the pig was housed. It may well be the case that different levels of protease are required to observe a response in different production environments.

7. Recommendations

As a result of the outcomes in this study the following recommendation has been made:

- The impact of protease on growth performance is likely to be dose dependent with higher inclusion rates of protease, for instance 250 ppm *c.f.* 125-150 ppm being required to see a positive growth response.

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Appendices

Appendix 1: Experiment 1a & b weaner diets

Protein… Protease…	Diet W1 Standard 0 ppm	Diet W2 Standard 150 ppm	Diet W3 Low 0 ppm	Diet W4 Low 150 ppm
Ingredients (%)				
Wheat	60.23	60.23	64.11	64.11
Biscuit meal			6.00	6.00
Full-fat soybean			1.00	1.00
Soybean meal	10.00	10.00		
Blood meal	1.80	1.80		
Meat meal	3.00	3.00	2.35	2.35
Fish meal	4.00	4.00	3.50	3.50
Skim milk powder	13.00	13.00	13.00	13.00
Whey powder	1.50	1.50	1.50	1.50
Hilyses	2.00	2.00	2.00	2.00
Canola oil	2.80	2.80	3.00	3.00
Salt	0.200	0.200	0.200	0.200
Zinc oxide	0.100	0.100	0.100	0.100
Betaine	0.100	0.100	0.100	0.100
DL-Methionine	0.120	0.120	0.225	0.225
Lysine HCl	0.310	0.310	0.650	0.650
L-Threonine	0.095	0.095	0.265	0.265
L-Tryptophan	0.025	0.025	0.080	0.080
L-Isoleucine			0.135	0.135
L-Valine			0.165	0.165
Xylanase	0.050	0.050	0.050	0.050
Phytase	0.0075	0.0075	0.0075	0.0075
Protease		0.015		0.015
Phytomolecule blend	0.020	0.020	0.020	0.020
Acidifier	0.400	0.400	0.400	0.400
Sweetener	0.030	0.030	0.030	0.030
Bentonite	0.015		0.015	
Vitamin/mineral premix	0.200	0.200	0.200	0.200
Analysis				
Energy, MJ DE/kg	14.7	14.7	14.7	14.7
SID Lysine, g/MJ DE	0.89	0.89	0.87	0.87
Protein (%)	22.5	22.5	18.5	18.5
Met:Lys	0.35	0.35	0.39	0.39
Met+Cys:Lys	0.57	0.57	0.57	0.57
Try:Lys	0.19	0.19	0.19	0.19
Thr:Lys	0.63	0.63	0.63	0.63

Appendix 2: Experiment 2 grower diets

Protease…	Diet G1 0 ppm	Diet G2 150 ppm
Ingredients (%)		
Sorghum	25.00	25.00
Wheat	35.89	35.89
Millrun	10.00	10.00
Canola meal	13.50	13.50
Soybean meal	5.75	5.75
Blood meal	2.00	2.00
Meat meal	3.50	3.50
Vegetable oil	2.90	2.90
Limestone	0.500	0.500
Salt	0.250	0.250
Choline chloride	0.010	0.010
DL-Methionine	0.040	0.040
Lysine HCl	0.400	0.400
Xylanase	0.050	0.050
Phytase	0.0075	0.0075
Protease		0.0125
Vitamin/mineral premix	0.200	0.200
Analysis		
Energy, MJ DE/kg	14.0	14.0
SID Lysine, g/MJ DE	0.72	0.72
Protein (%)	19.9	19.9

Appendix 3: Experiment 3 grower and finisher diets

	Grower			Finisher		
	Positive Control	Negative Control	Protease 250 ppm	Positive Control	Negative Control	Protease 250 ppm
Protease (ppm)…	0	0	250	0	0	250
Ingredients (%)						
Barley	3.00	7.00	7.00	19.00	25.25	25.25
Wheat	79.52	78.90	78.88	62.21	60.18	60.16
Canola meal	3.25			13.50	9.00	9.00
Soybean meal	6.00	6.00	6.00			
Blood meal	1.60	1.30	1.30			
Meat meal	4.30	4.40	4.40	2.70	3.00	3.00
Vegetable oil	1.00	1.00	1.00	1.00	1.00	1.00
Limestone	0.300	0.350	0.350	0.600	0.550	0.550
Salt	0.250	0.250	0.250	0.250	0.250	0.250
Choline chloride	0.030	0.045	0.045	0.005	0.020	0.020
DL-Methionine	0.055	0.055	0.055	0.015	0.020	0.020
Lysine HCl	0.400	0.400	0.400	0.420	0.420	0.420
L-Threonine	0.090	0.090	0.090	0.090	0.095	0.095
L-Tryptophan	0.003			0.005	0.005	0.005
Phytase	0.0075	0.0075	0.0075	0.0075	0.0075	0.0075
Protease			0.025			0.025
Vitamin/mineral premix	0.200	0.200	0.200	0.200	0.200	0.200
Analysis						
Energy, MJ DE/kg	14.0	14.0	14.0	13.6	13.6	13.6
SID Lysine, g/MJ DE	0.68	0.64	0.68	0.62	0.58	0.62
Protein (%)	18.78	17.78	18.65	17.31	16.32	17.12
Met:Lys	0.30	0.30	0.30	0.30	0.30	0.30
Met+Cys:Lys	0.62	0.63	0.62	0.67	0.67	0.66
Try:Lys	0.18	0.18	0.18	0.18	0.18	0.18
Thr:Lys	0.64	0.64	0.64	0.67	0.67	0.67

Appendix 4: Weaner microbiome analysis report.

Australasian Pork Research Institute Ltd
APRIL

A1-103: Improving enteric health, understanding impact on gut microbiome and weaner performance through the use of protease enzymes

Introduction

Feeding lower protein diets to weaner pigs challenged with enterotoxigenic *E. coli* reduces the incidence of post weaning diarrhoea (PWD), and also reduces measures of protein fermentation including plasma urea nitrogen and faecal ammonia nitrogen (Heo et al., 2009). Lower protein diets may aid in the control of PWD via a combination of factors including reduced intestinal inflammation, increased stability of the microbiome and reduced production of toxic metabolites through bacterial fermentation of undigested amino acids in the pig colon.

This project aimed to investigate the impact of lower protein diets and the addition of a protease on pigs' faecal microbiome. The hypothesis tested was that the addition of a protease and lower crude protein levels in weaner diets would, by reducing the transit of undigested amino acids into the colon and therefore reducing the bacterial fermentation of undigested amino acids into amines and ammonia, ultimately reducing blood urea. Ammonia production can alter the morphology and intermediary metabolism of intestinal cells, so is often considered an antinutritive factor. A large range of bacteria can decarboxylate or deaminate amino acids to short-chain fatty acids (SCFA), biogenic amines and ammonia including lactic acid bacteria, *Bifidobacterium, Bacteroides, Clostridium, Proteus, Klebsiella, Enterococcus, Hafnia, Vibrio, Pseudomonas, Veillonellaceae, Fusobacteria, Eubacteria, Acidaminococcus* and Peptococci, hence the study investigated the effect of diet on both microbiome diversity and changes in relative abundance of bacterial families.

Materials and Methods

EMAI Microbiome analysis (July/August 2021)

1. **Weaner pigs**, allocated to a 2 x 2 factorial design of treatments: two diets (Control v Low Protein; LP) +/- protease (Jefo) addition to the diet (150 g/kg of diet, added on-top).
2. Pooled pen faecal samples were collected at time 1 (14 days on diets after weaning) and time 2 (28 days on diets after weaning).

Table 1. Overview of treatment samples collected.

Time	Treatment	# replicates (pens)	Gender replicates
1	Control protein	6	3F + 3M
1	Control protein + protease	6	3F + 3M
1	Low protein	6	3F + 3M
1	Low protein + protease	6	3F + 3M
2	Control protein	4	2F + 2M
2	Control protein + protease	5	2F + 3M
2	Low Protein	5	4F + 1M
2	Low protein + protease	8	2F + 6M

Sample and analytical issues

- Different pens sampled for times 1 and 2, so times were analysed independently (not the same pigs).
- The number of pens per gender varied between treatments at time 2 (1-6), hence the gender effect could not be evaluated.
- The number of samples (pens) per treatment is 6 for time 1 and varies between 4 and 8 for time 2, and this is likely to be insufficient to show significant differences between samples.
- Therefore, only the effects of dietary protein and protease supplementation on the gut microbiome were evaluated.

The DNA was extracted from 64 pooled pen faecal samples (one per pen) using established techniques, then purified and quantified before submitting for amplification and sequencing of the V4 region of 16S rRNA gene (Illumina 454 pyrosequencing), able to detect all bacteria. All amplified DNA was sequenced in both directions, and then sequencing adapters were trimmed before sequence quality control was performed.

Ambiguous nucleotides were trimmed, chimeras removed, sequence reads below 5 were discarded and sequences with less than 100 reads were filtered out. Overlapping forward and reverse paired reads were merged to produce one high quality read. An OTU table was produced containing the abundance for the sequences that clustered with Operational Taxonomy Units (OTU) from the annotated reference data base. A phylogenetic tree of all OTU's was constructed using a maximum likelihood analysis based on multiple sequence alignment of the OTU's generated by MUSCLE. Alpha diversity analysis provided an estimate of the diversity of bacteria within a sample.

Beta diversity was measured by UniFrac, an analysis tool used to determine whether communities are significantly different by using phylogenetic data for each sample to cluster similar bacterial communities together and display the results with standard multivariate statistical techniques including principal coordinates analysis (PCoA). Beta diversity analysis estimated differences in species diversity between samples. The treatment and time factors were incorporated into the results to observe differences in relative abundance of bacterial species between treatments and over time.

Results

Plateauing of the rarefaction plots of phylogenetic diversity (alpha diversity) of all samples within each treatment at time 1 (14 days on diets after weaning) and time 2 (28 days on diets after weaning) indicates good coverage of bacterial sequences in the faecal samples (Figure 1). The alpha diversity of bacteria within each treatment was not significantly different between treatments at either time 1 or time 2 (Kruskal-Wallis P value ≥ 0.05, Figure 2).

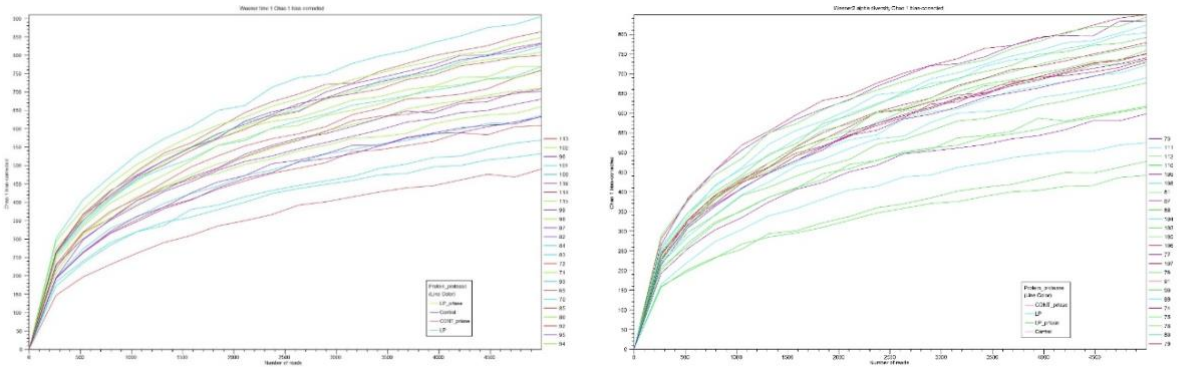

Figure 1. Phylogenetic diversity (alpha diversity Chao 1 bias corrected) within each of the 24 faecal samples from weaner pigs at time 1 (left panel) and time 2 (right panel).

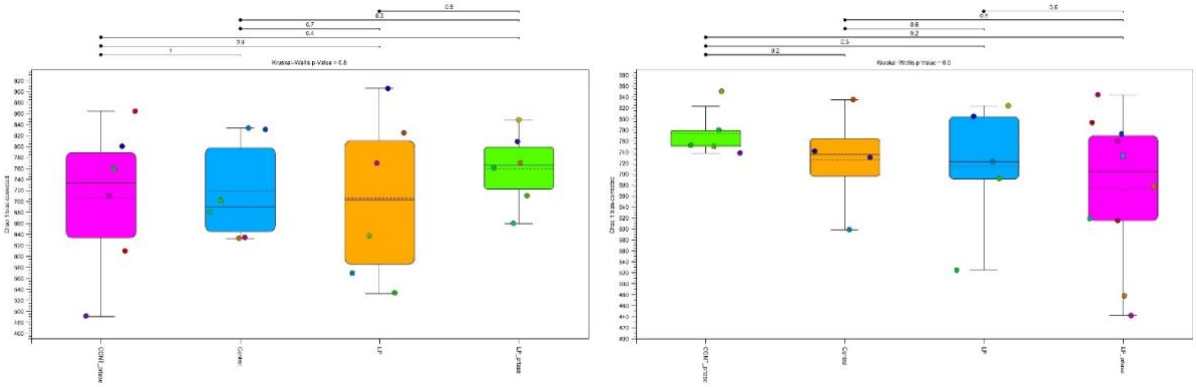

Figure 2. Boxplot of phylogenetic diversity in each treatment (Chao 1 alpha diversity) at time 1 (left panel) and time 2 (right panel), and significant differences in diversity within treatments (Kruskal-Wallis).

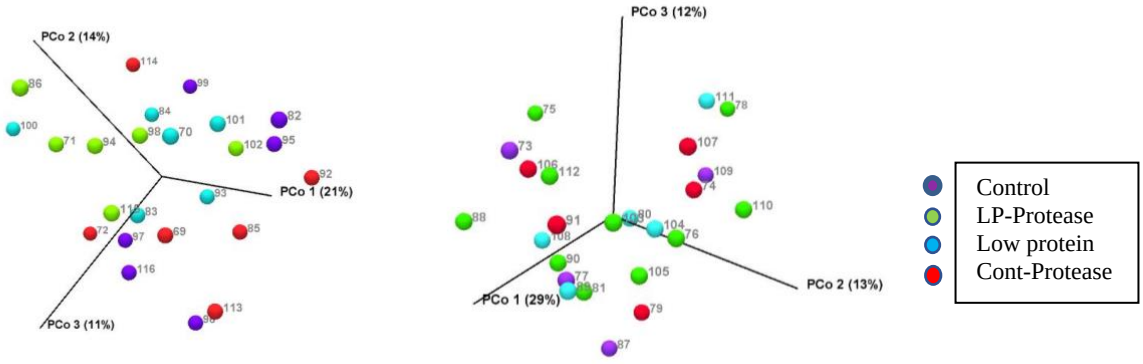

Figure 3. Principal components analysis of microbiome clustering of pens (numbered) within and between treatments (beta diversity) at time 1 (left panel) and 2 (right panel) (UniFrac D_0.5).

Clustering of bacterial communities by treatment was not visible at either time point (Figure 3) and no significant difference in microbial communities was observed across treatments by permutational multivariate analysis of variance (PERMANOVA), a non-parametric multivariate statistical test (Table 2). The null hypothesis tests whether the clustering (made up of centre points and dispersion) of bacterial communities were equivalent for all treatments. At time 1, analysis by PERMANOVA demonstrated that treatment had no significant effect on clustering of microbial communities ($P = 0.25$). Likewise, at time 2, no significant difference in microbial communities was observed between treatments ($P = 0.999$) and pair-wise comparisons showed that bacterial communities were not significantly different between any treatments ($P > 0.05$) (Table 2).

Table 2. Pair-wise comparisons (PERMANOVA) between treatments.

Time 1 (14 days after weaning)			**Time 2** (28 days after weaning)		
Group 1	Group 2	P value	Group 1	Group 2	P value
LP + protease	Cont	0.037	LP + protease	Cont	0.992
LP + protease	Cont + protease	0.017	LP + protease	Cont + protease	0.944
Cont	Cont + protease	0.95	Cont	Cont + protease	0.944
LP + protease	LP	0.342	LP + protease	LP	0.961
Cont	LP	0.608	Cont	LP	1.00
Cont + protease	LP	0.385	Cont + protease	LP	0.754

Differences in the relative abundance of specific taxa in bacterial communities between treatments can be visualised in stacked bar charts, at different classification levels. Pigs fed LP + protease diets for 14 days (time 1) showed reduced *Prevotellaceae* abundance (relative to total bacteria) compared with pigs fed Control protein (Cont), Control protein + protease (C-prtase) and LP diets, respectively (Figure 4). Interestingly, after 28 days, pigs fed the LP diets appeared to have a higher relative abundance of *Prevotellaceae*.

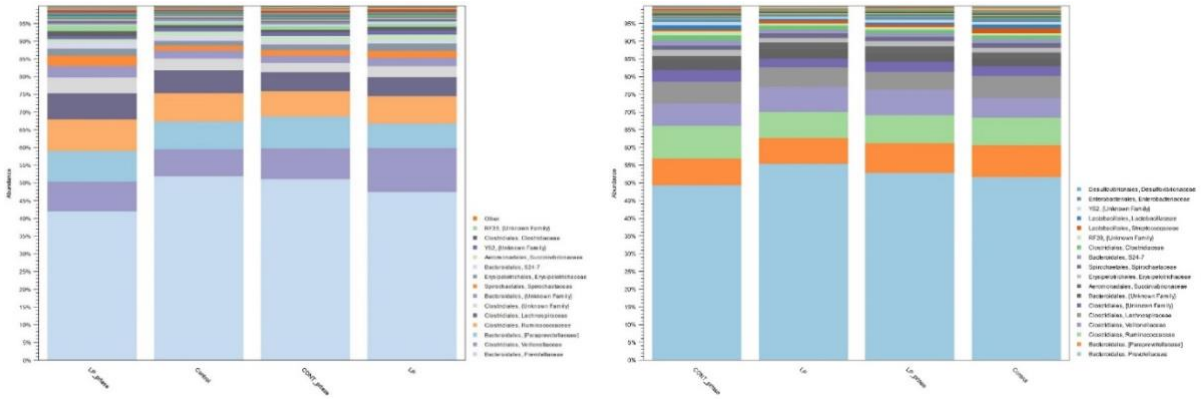

Figure 4. Stacked bar chart of relative abundance of bacterial groups at the order and Family level between all four treatments at time 1 (14 days on diets – left panel) and time 2 (28 days on diets – right panel).

Pigs fed LP diets for 14 days appeared to have a higher relative abundance of the *Firmicutes* phylum and a reduced abundance of *Bacteroides*, with no difference in the proportion of *Proteobacteria* (approximately 3-4% of the microbiome) between treatments (Figure 5). Low protein diets therefore altered the ratio of *Bacteroides:Firmicutes*, with higher ratios for weaner pigs on control protein diets (2.44, equivalent to 66% and 27% Bacteroides and Firmicutes, respectively) compared with LP diets (1.84, equivalent to 59% and 32%, respectively). The reduced relative abundance of *Bacteroides* in pigs on LP diets was primarily due to the reduced relative abundance of *Prevotellaceae*.

Prevotellaceae were the major family within *Bacteroides*, comprising 84%, 82%, 85% and 78% of the *Bacteroidales* order in pigs fed Control protein, Control + protease, LP, and LP + protease diets, respectively. *Clostridiales* were the major order within the phylum Firmicutes, primarily consisting of the families *Veillonellaceae, Ruminococcaceae* and *Lachnospiraceae*. The proportion of these three families was similar between pigs fed control, control + protease and LP + protease diets. However, pigs fed LP diets without protease had higher relative abundance of *Veillonellaceae* and a reduced abundance of *Ruminococcaceae*.

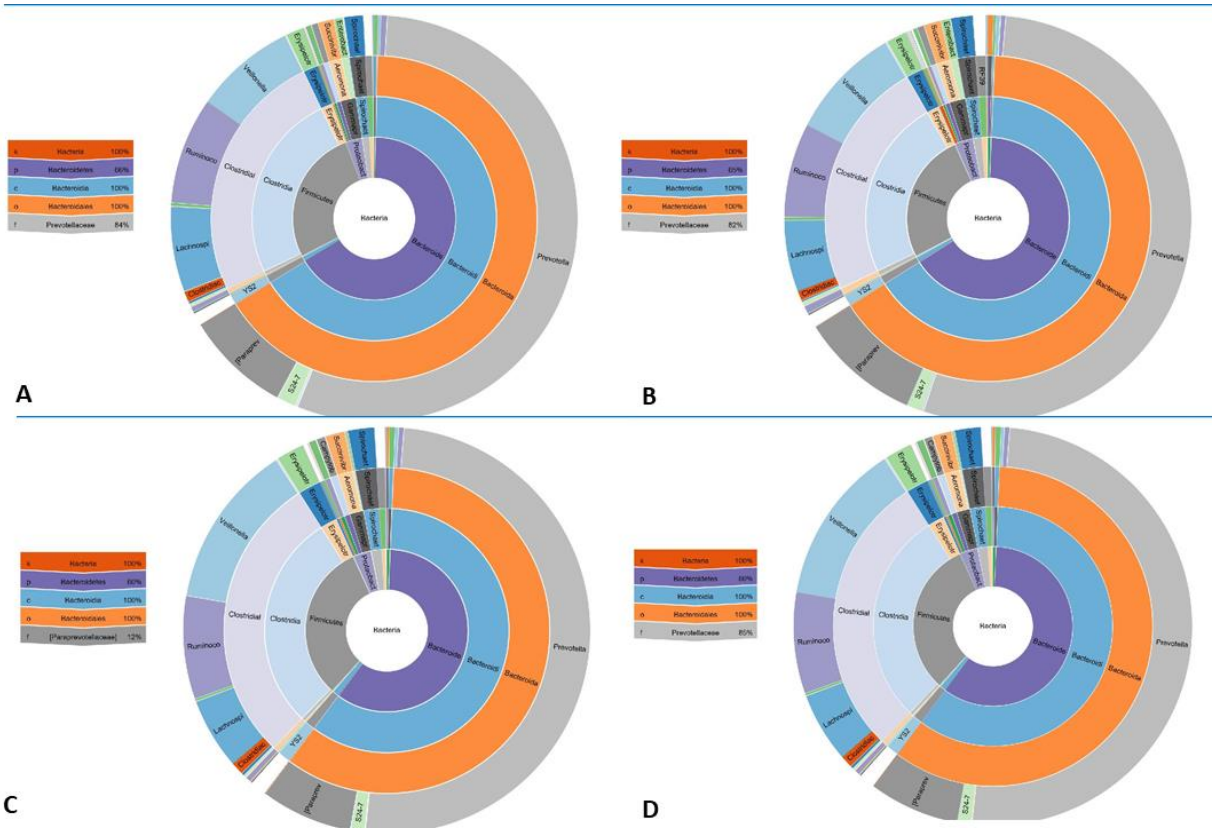

Figure 5. Relative abundance of bacterial families at time 1 (14 days on diet) for all treatments A) Control, B) Control + protease, C) Low protein and D) LP + protease.

Linear discriminate analysis (LEfSe) of the relative abundance of bacterial taxa demonstrated that pigs fed a LP diet for two weeks were characterised by more than a Log_{10} 4-fold increase in *Enterococcaceae* (Figure 6). Weaner pigs fed LP + protease diets were characterised by a Log_{10} 3.5-fold increase in *Eubacteriaceae, Oxalobacteriaceae, Methanobacteriaceae* and *Desulfivibrionaceae*. Pigs fed a Control protein + protease diet were characterised by a more than Log_{10} 4-fold increase in Tremblayales after 14 days on the diet.

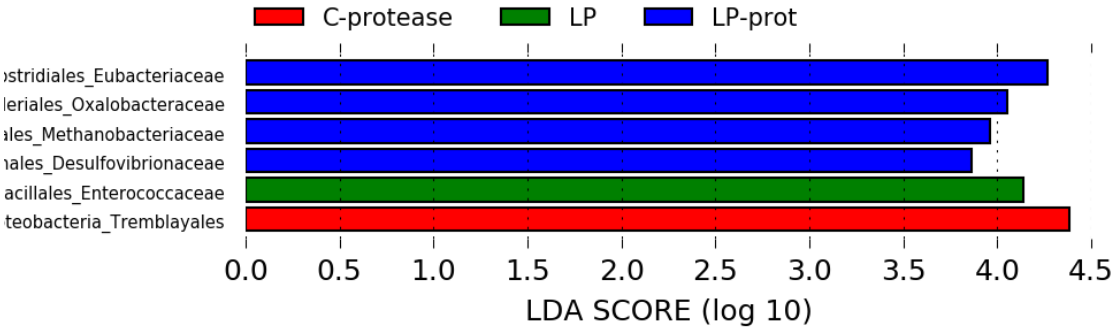

Figure 6. Linear discriminate analysis of the combination of bacterial taxa that characterises and separates pigs into treatment groups at time 1 (14 days on diets).

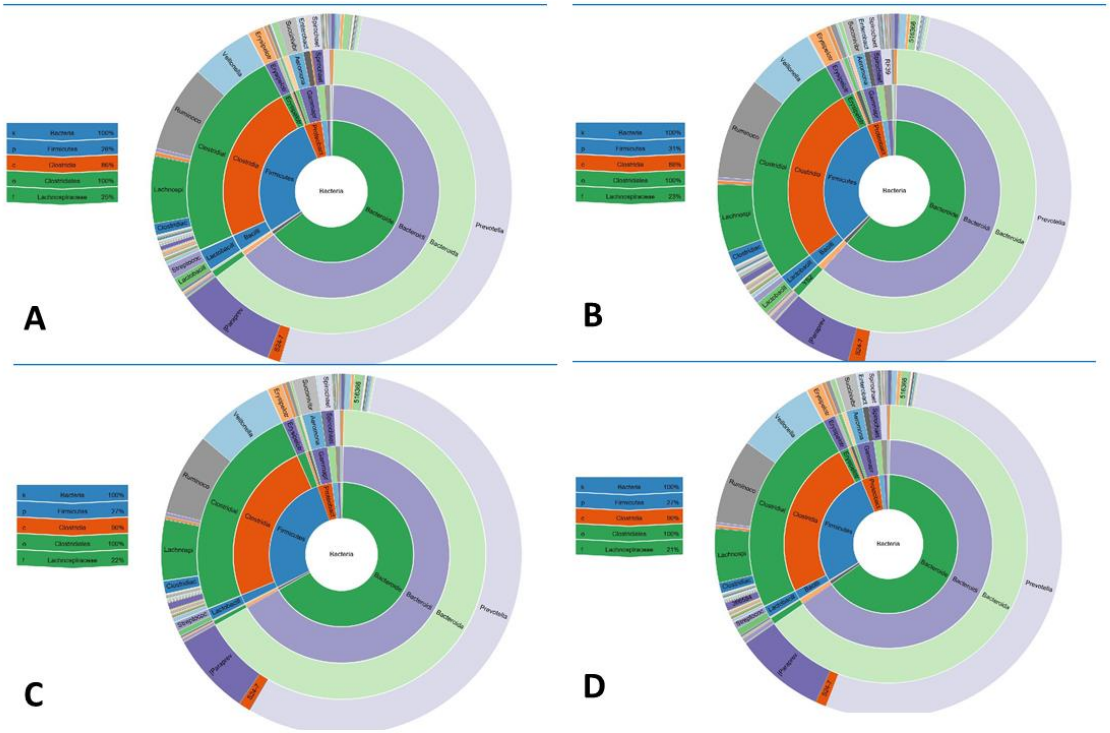

Figure 7. Relative abundance of bacterial families at time 2 (28 days on diet) for all treatments A) Control, B) Control + protease, C) Low protein and D) LP + protease.

The relative abundance of the three major phyla (27% Firmicutes, 66% Bacteroides and 4% Proteobacteria) were similar for pigs in all treatments fed their respective diets for 28 days (Figure 7). Prevotellaceae were the major family within Bacteroides, comprising 84% - 85% of the Bacteroidales order in pigs fed each of the four diets. Clostridiales were the major order within the phylum Firmicutes, primarily consisting of the families *Veillonellaceae, Ruminococcaceae* and *Lachnospiraceae*.

The proportion of each of these three families differed between pigs fed control protein and LP diets. Pigs fed LP diets had higher relative abundance of *Veilloneallaceae* (33%) and a reduced abundance of *Ruminococcaceae* (35%) and *Lachnospiraceae* (24%) relative to pigs fed control protein diets (26%, 38% and 27%, respectively) for 28 days. Characteristic differences in the relative abundance of bacterial taxa were less pronounced between treatments (Figure 8) at 28 days relative to 14 days on diets (Figure 6) using linear discriminate analysis. Pigs fed control protein diets were characterised by an increased relative abundance of the genus *Roseburia* within the *Lachnospiraceae* family. Pigs fed control protein diets plus protease were characterised by a reduction in the relative abundance of bacterial genus *Ruminococcus*.

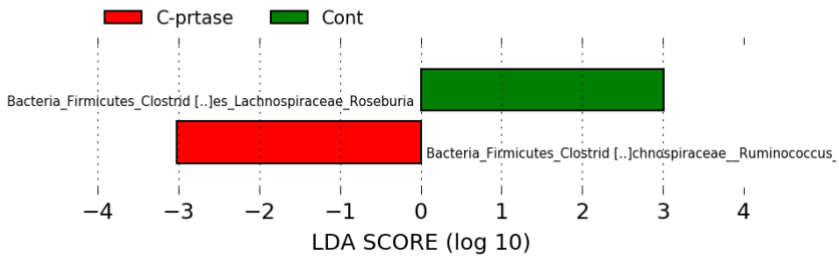

Figure 8. Linear discriminate analysis of the combination of bacterial taxa that characterises and separates pigs into treatment groups at time 2 (28 days on diets).

As discussed previously, the impact of diets on weaner pigs were separated into two analyses because only 25% of the pens sampled at time 1 were also sampled at time 2. Clustering analysis of bacterial communities by time (Figure 9) indicated significant differences between pigs on diets for 14 and 28

days, and PERMANOVA analysis of beta diversity at time 1 and 2 showed significant differences in microbial communities (P = 0.00003). If both time points were analysed together, treatment had no significant effect on bacterial community clustering or beta diversity (P = 0.849). *Prevotellaceae* were more abundant at time 2 and *Veillonellaceae* were more abundant at time 1 (Figure 9). Linear discriminate analysis demonstrated no differentially abundant taxa between treatment using data combined from both times.

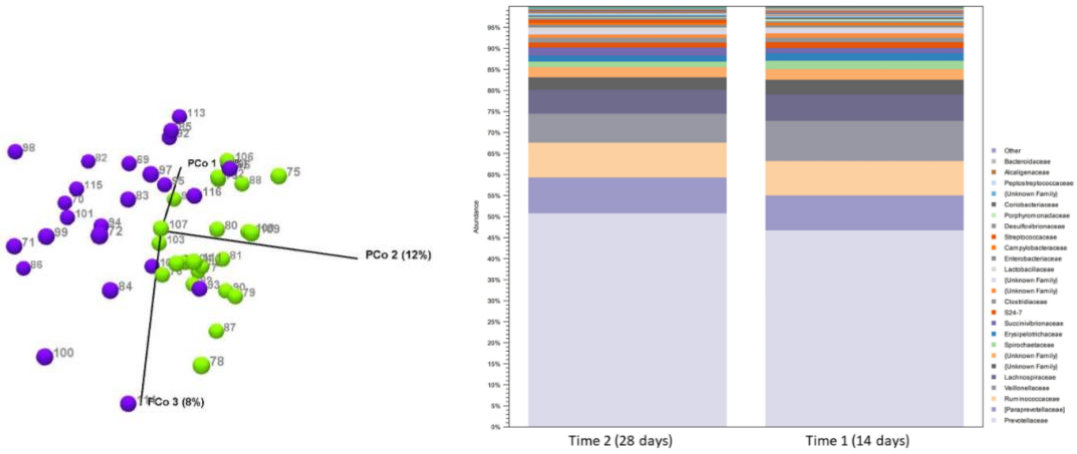

Figure 9. Left panel: Effect of time (purple = time 1; green = time 2) on clustering of weaner microbiomes (PCA). Right panel: Effect of time on relative abundance of bacterial taxa.

Discussion

Previous studies reported that feeding lower protein diets to weaner pigs challenged with enterotoxigenic *E. coli* reduced the incidence of PWD commensurate with reduced plasma urea nitrogen and faecal ammonia nitrogen, associated with bacterial fermentation of proteins (Heo et al., 2009). It was hypothesised that lower protein diets may aid in the control of PWD via a combination of mechanisms including reduced intestinal inflammation, increased stability of the microbiome and reduced production of toxic metabolites through bacterial fermentation of undigested amino acids in the pig colon. This study focussed mainly on microbiome stability and the production of toxic metabolites via bacterial fermentation.

The reduced relative abundance of *Enterobacteriaceae* and increased abundance of *Prevotellaceae* in weaners fed lower protein diets in this study is supported by published results demonstrating the benefits of lower protein diets on reducing numbers of enterotoxigenic *E. coli* and the incidence of PWD (Heo et al., 2009). A higher abundance of *Prevotellaceae* has been reported as a dominant feature of the faecal microbiota in healthy pigs as compared to diarrhoeic pigs after weaning (Dou et al., 2017). Non-diarrhoeic pigs after weaning were shown to have a continuous decrease in *Lactobacillus* and *Escherichia* and a gradual increase in *Prevotellaceae* with the transition to solid food after weaning (Frese et al., 2015; Yang et al., 2019). *Prevotellaceae* increase rapidly after weaning due to the availability of plant polysaccharides and can metabolise both proteins and carbohydrates to produce acetate (Duncan et al., 2004a; 2004b) and propionate (Ramirez-Farias et al., 2009; Scott et al., 2014) and provide energy for the pig.

The addition of a protease and lower protein diets was expected to reduce the bacterial fermentation of undigested amino acids into potentially toxic metabolites, like amines and ammonia produced by decarboxylation and deamination in the lower gastrointestinal tract. While a wide range of bacterial species are involved in protein fermentation, the major changes observed at both 14 and 28 days were in the relative abundance of *Veillonellaceae, Ruminococcaceae* and *Lachnospiraceae* with the phylum *Firmicutes*.

The reduced relative abundance of *Ruminococcaceae* in pigs fed lower protein diets agrees with previous reports in rats fed a combination of high fat and relatively low protein diets. Conversely, rats fed high fat and high protein diets showed an increased relative abundance of *Ruminococcaceae* and

Lactobacillus spp. relative to rats fed high fat and low protein diets, with these microbiome changes positively correlated with increasing SCFA production (Shi et al., 2020). High protein diets allow more undigested amino acids to transit to the colon where bacteria including *Ruminococcaceae* can ferment amino acids to SCFA and amines and are therefore expected to proliferate. We observed this in reverse with decreased abundance of *Ruminococcaceae* in pigs fed lower protein diets. The production of amines from amino acid fermentation is inhibited in many bacteria by the presence of fermentable carbohydrates as alternative substrates in the colon. However, this doesn't lead to the proliferation of bacteria that are only weakly able to ferment carbohydrates including *Veillonellaceae, Fusobacteria, Eubacteria, Acidaminococcus, Clostridia* and Peptococci (Macfarlane and Macfarlane, 1997). This inhibition of fermentation may explain the observed decreased relative abundance of *Veillonelllaceae* in pigs fed higher protein diets and the increased relative abundance of *Veillonellaceae* in pigs fed lower protein diets. High fat and high meat-protein diets in rats also led to inflammation in their colons, with increased pro-inflammatory cytokines IL-Iβ, TNF-α and IL-6 (Shi et al., 2020).

Although bacterial diversity between dietary treatments was not significantly different in this study, a larger number of replicates per treatment may have shown significant differences in bacterial diversity, as diet is a primary factor influencing intestinal microbiomes in many mammalian species (Barron Pastor, 2017; Cox et al., 2020; Frese et al., 2015). In rodents, high fat and high meat-protein diets led to reduced bacterial diversity within samples (alpha diversity) relative to rats on low fat and protein diets and significant differences in bacterial diversity between dietary treatments (Shi et al., 2020).

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Appendix 5: Grower microbiome analysis report.

A1-103: Improving enteric health, understanding impact on gut microbiome and weaner performance through the use of protease enzymes

Introduction

This project aimed to investigate the impact of the addition of a protease and the antibiotic tiamulin on the grower pig's faecal microbiome. The hypothesis examined was that the addition of a protease would improve the digestion of proteins and absorption of amino acids in the pigs' small intestine and would reduce the passage of undigested amino acids into the colon, where bacterial fermentation can lead to the production of short chain fatty acids (SCFA) as well as toxic metabolites including amines and ammonia. Ammonia production can alter the morphology and intermediary metabolism of intestinal cells, so is often considered an antinutritive factor. The study also hypothesised that the provision of a tiamulin pulse to grower pigs would reduce the abundance of pathogens that impact on gut health, including inflammation, digestion and absorption of nutrients, but may also have consequences on commensal bacteria in the gastrointestinal tract. The study investigated the effect of a protease and medication on both microbiome diversity and changes in the relative abundance of bacterial families.

Materials and Methods

EMAI Microbiome analysis (July/August 2021)

3. **Grower pigs**, allocated to a 2 x 2 factorial design of treatments: two diets (Control v Protease) (150 g/kg of diet, added on-top) for 47 days and two medications +/- tiamulin for the first 7 days.
4. Pooled pen samples were collected at time 1 (day 14) = 7 days acclimation + 7 days +/- medication and time 2 (day 47).

Table 1. Overview of treatment samples collected.

Time	Treatment	# replicates (pens)	Gender replicates
1	Control protein	6	3F + 3M
1	Control protein + Medication	6	3F + 3M
1	Protease	6	3F + 3M
1	Protease + Medication	6	3F + 3M
2	Control protein	3	3F
2	Control protein + Medication	3	3F
2	Protease	3	3M
2	Protease + Medication	3	3M

Sample and analytical issues

- It was not possible to investigate the gender effects because at time 2 there was only one gender for each treatment, i.e., 12 pens = 4 treatments x 3 pens/treatment x 1 gender.
- Samples from time 1 and 2 showed some overlap with pen numbers (same pigs), providing an opportunity to look at time effects.
- At time 2, only three samples per treatment were collected, which is likely to be insufficient to show significant effects of diets.

The DNA was extracted from 64 pooled pen faecal samples (one per pen) using established techniques, then purified and quantified before submitting for amplification and sequencing of the V4 region of 16S rRNA gene (Illumina 454 pyrosequencing), able to detect all bacteria. All amplified DNA was sequenced in both directions, and then sequencing adapters were trimmed before sequence quality control was performed. Ambiguous nucleotides were trimmed, chimeras removed, sequence reads below 5 were discarded and sequences with less than 100 reads were filtered out. Overlapping forward and reverse paired reads were merged to produce one high quality read. An OTU table was produced containing the abundance for the sequences that clustered with Operational Taxonomy Units (OTU) from the annotated reference data base. A phylogenetic tree of all OTU's was constructed using a maximum likelihood analysis based on multiple sequence alignment of the OTU's generated by MUSCLE. Alpha diversity analysis provided an estimate of the diversity of bacteria within a sample.

Beta diversity was measured by UniFrac, an analysis tool used to determine whether communities are significantly different by using phylogenetic data for each sample to cluster similar bacterial communities together and display the results with standard multivariate statistical techniques including principal coordinates analysis (PCoA). Beta diversity analysis estimated differences in species diversity between samples. The treatment and time factors were incorporated into the results to observe differences in relative abundance of bacterial species between treatments and over time.

Results

Plateauing of the rarefaction plots of phylogenetic diversity (alpha diversity) of all samples within each treatment at time 1 (14 days on diets) and time 2 (47 days on diets) indicates good coverage of bacterial sequences in the faecal samples (Figure 1). The phylogenetic diversity of bacteria within each treatment was not significantly different between treatments at either time 1 or 2 (Kruskal-Wallis P value ≥ 0.05, Figure 2).

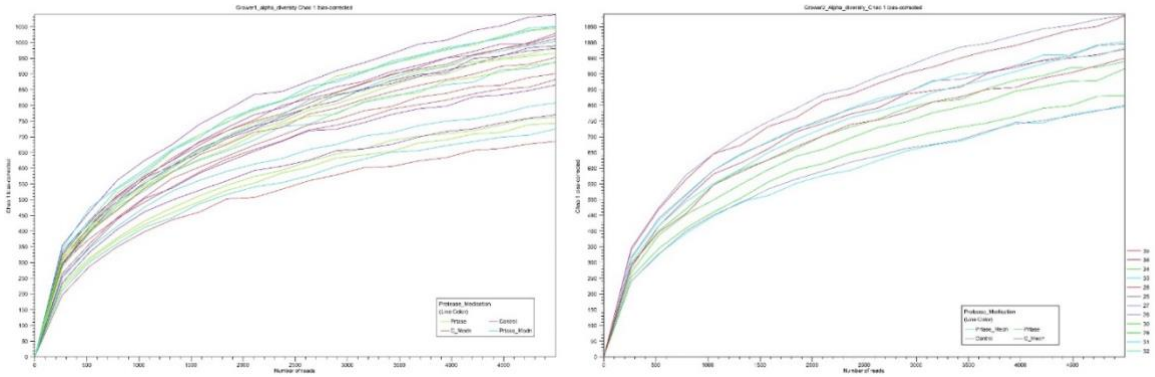

Figure 1. Phylogenetic diversity (alpha diversity Chao 1 bias corrected) within each faecal sample from grower pigs at time 1 (left panel) and time 2 (right panel).

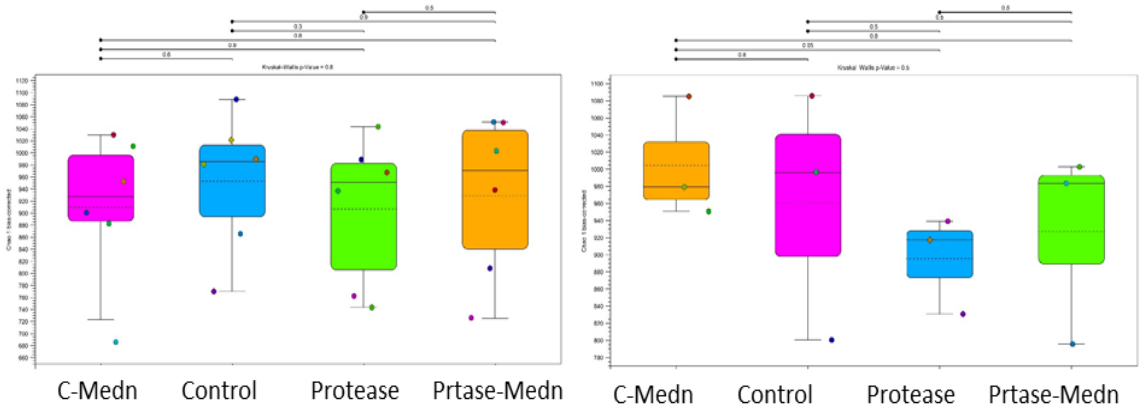

Figure 2. Boxplot of phylogenetic diversity (Chao 1 alpha diversity) at time 1 (left panel) and time 2 (right panel), and significant differences in diversity within treatments (Kruskal- Wallis).

Clustering of bacterial communities by treatment was not visible by principal component analysis (PCA) at either time point (Figure 3). However, significant differences in microbial communities were observed across treatments by permutational multivariate analysis of variance (PERMANOVA) at time 1 (P = 0.039). Pair-wise comparisons showed that microbial communities in pigs supplemented with protease were significantly different to Control growers (P = 0.024) and Control + Medicated growers (P = 0.022) (Table 2). The microbial community in pigs fed the Protease + medication diet was not significantly different to any other treatment.

In contrast, at time 2 (47 days on diets), no significant difference in microbial communities was observed between treatments (P = 0.448) and pair-wise comparisons showed that bacterial communities were not significantly different between any treatments (P > 0.05) (Table 2).

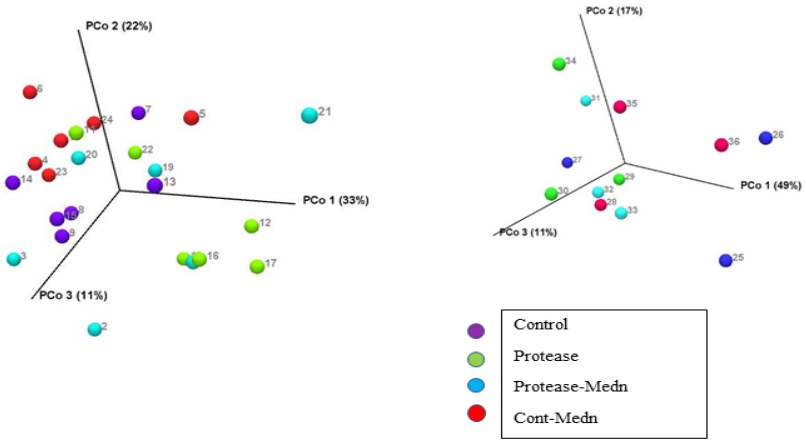

Figure 3. Principal components analysis of microbiome clustering of pens (numbered) within and between treatments (beta diversity) at time 1 (left panel) and 2 (right panel) (UniFrac D_0.5).

Table 2. Pair-wise comparisons (PERMANOVA) between treatments

Time 1 (14 days)			**Time 2** (47 days)		
Group 1	Group 2	P value	Group 1	Group 2	P value
Protease	Control	0.024	Protease	Control	1.0
Protease	Control + Medn	0.022	Protease	Control + Medn	1.0
Control	Control + Medn	0.160	Control	Control + Medn	1.0
Protease	Prtease + Medn	0.799	Protease	Prtease + Medn	1.0
Control	Prtease + Medn	0.420	Control	Prtease + Medn	1.0
Control + Medn	Prtease + Medn	0.141	Control + Medn	Prtease + Medn	1.0

Differences in the relative abundance of specific bacterial families from growers medicated with diets varying in protease and medication are shown in Figure 4. Pigs fed control protein diets showed increased *Prevotellaceae* abundance at 14 days (time 1), but reduced *Prevotellaceae* at 47 days (time 2) compared with grower pigs on diets supplemented with protease (Figure 4). The differences between treatments appeared more clearly at time 1, where pigs fed control protein diets had increased relative abundance of *Ruminococcaceae*, and reduced Lactobacillaceae compared with pigs on protease-supplemented diets. The addition of medication to control protein diets also reduced *Clostridiaceae* and increased *Lachnospiraceae* relative to all other treatments.

Taxonomic profiling of the bacterial abundance of growers' faecal microbiomes showed a marked reduction in the relative abundance of the phyla *Bacteroides* and a large increase in the proportion of the phyla *Firmicutes* relative to weaner pigs. The relative proportion of *Bacteroides* and *Firmicutes* in weaner pigs was 62.5% and 29.5% respectively, but this changed dramatically in grower pigs to 7.5% *Bacteroides* and 92.5% *Firmicutes*, indicating that age and diet have a significant impact of the faecal microbiome of pigs. The phyla *Proteobacteria* was almost absent from the grower pigs.

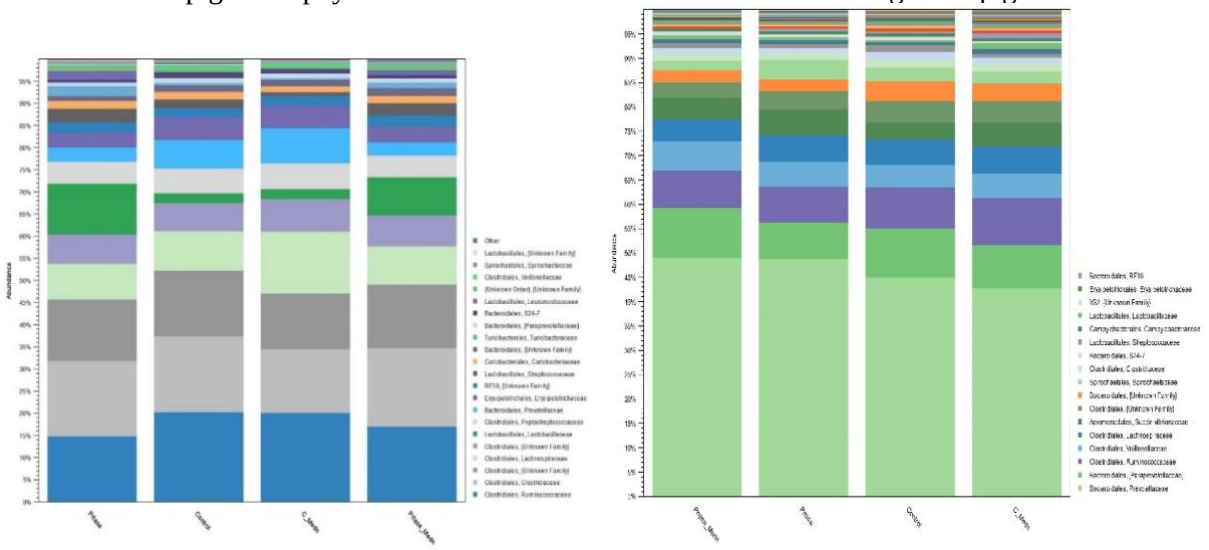

Figure 4. Stacked bar chart of relative abundance of bacterial groups at the order and family level between all four treatments at time 1 (14 days on diet – left panel) and time 2 (44 days on treatment – right panel).

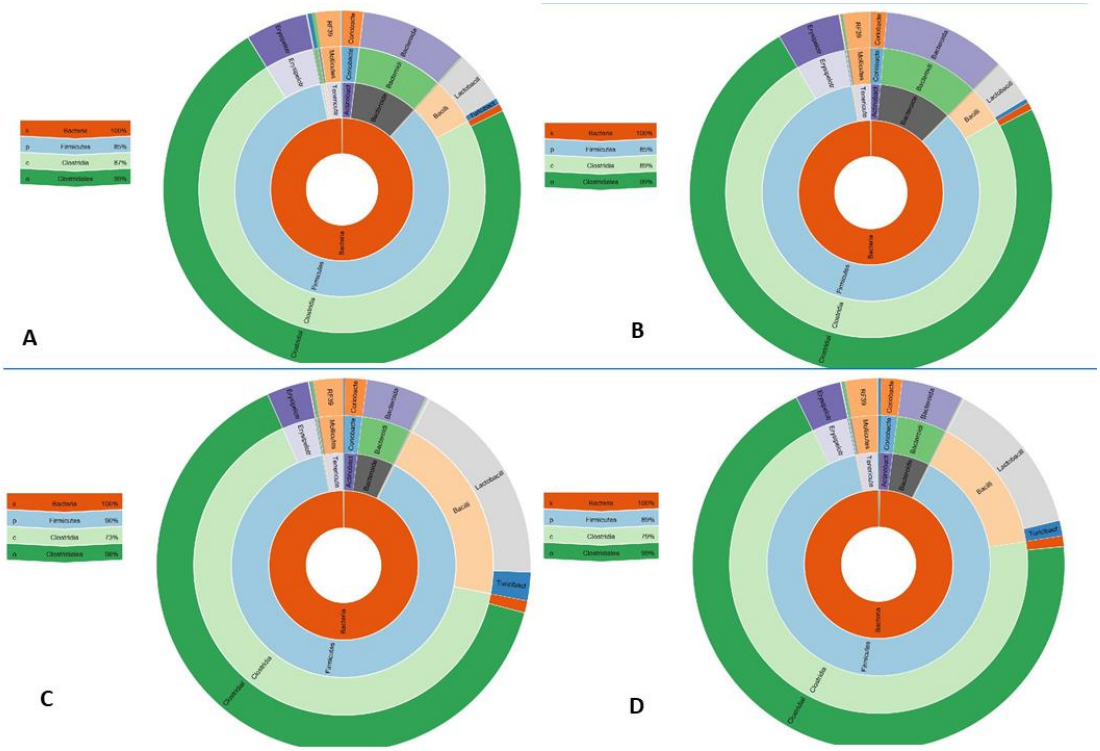

Figure 5. Relative abundance of bacterial families at time 1 (14 days on diet) for all treatments A) Control, B) Control + Medication, C) Protease and D) Protease + Medication.

The ratio of *Bacteroides:Firmicutes* between treatments did appear to vary with a ratio of 0.056 in grower pigs supplemented with protease and 0.12 in grower pigs on control protein diets, indicating that protease in grower pigs led to an increased abundance of the phyla *Firmicutes*. At time 1, the class *Bacilli*, within *Firmicutes*, were more abundant in protease-supplemented pigs (20% of all *Firmicutes*) compared to control protein pigs (6%). This difference was primarily due an increased relative abundance of the family *Lactobacillaceae* in protease-fed pigs (69% of the order *Lactobacillales*) compared to control protein-fed pigs (59% of the order *Lactobacillales*) (Figures 4 and 5).

Linear discriminate analysis (LEfSe) of the relative abundance of bacterial taxa demonstrated that grower pigs fed a protease-supplemented diet for 14 days were characterised by more than a Log_{10} 4-fold increase in *Lactobacillaceae* and *Turicibacteriaceae* and a Log_{10} 4-fold increase in *Mycoplasmataceae* relative to other treatments. Pigs fed control protein diets were characterised by a Log_{10} 3-fold increase in *Enterococcaceae*, while the microbiome of control pigs with medication was characterised by a Log_{10} 4-fold increase in *Prevotellaceae* (Figure 6). Grower pigs fed protease diets with medication were characterised by a Log_{10} 3-fold increase in *Streptomycetaceae, Porphyromonadaceae* and *Fibrobacteriaceae*.

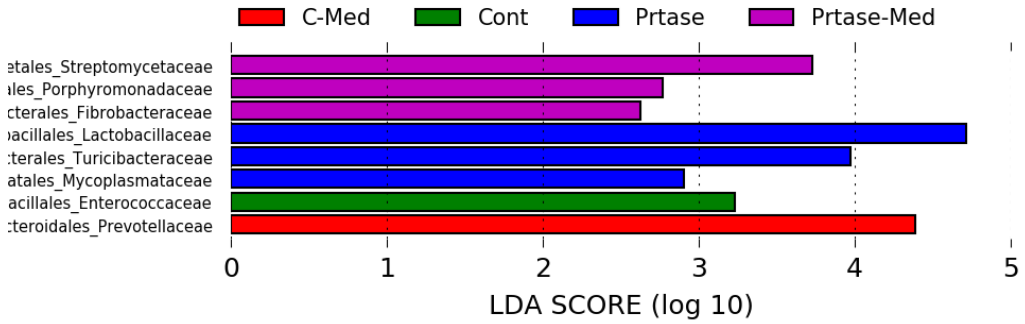

Figure 6. Linear discriminate analysis of the combination of bacterial taxa that characterises and separates pigs into treatment groups at time 1 (14 days on diet).

At time 2 (47 days on diets), minimal differences were observed between the relative abundance of the most common bacterial phyla, class and order between treatments (Figure 7). The relative proportion of phyla *Bacteroides* and *Firmicutes* returned to similar levels as previously observed in weaner pigs (63 ± 1% and 25 ± 1% respectively). The increased abundance of *Lactobacillaceae* observed in protease-fed grower pigs at time 1 disappeared (Figures 4 and 7). While the relative abundance of the order *Clostridiales* was similar between treatments, the relative abundance of *Ruminococcaceae* decreased (38% and 42%) and *Veillonellaceae* increased (23% and 28%) in protease-fed growers relative to pigs fed with control protein.

No differentially abundant bacterial families were found between treatments at time 2 (47 days on diet), which may have been due to the low number of pens per treatment.

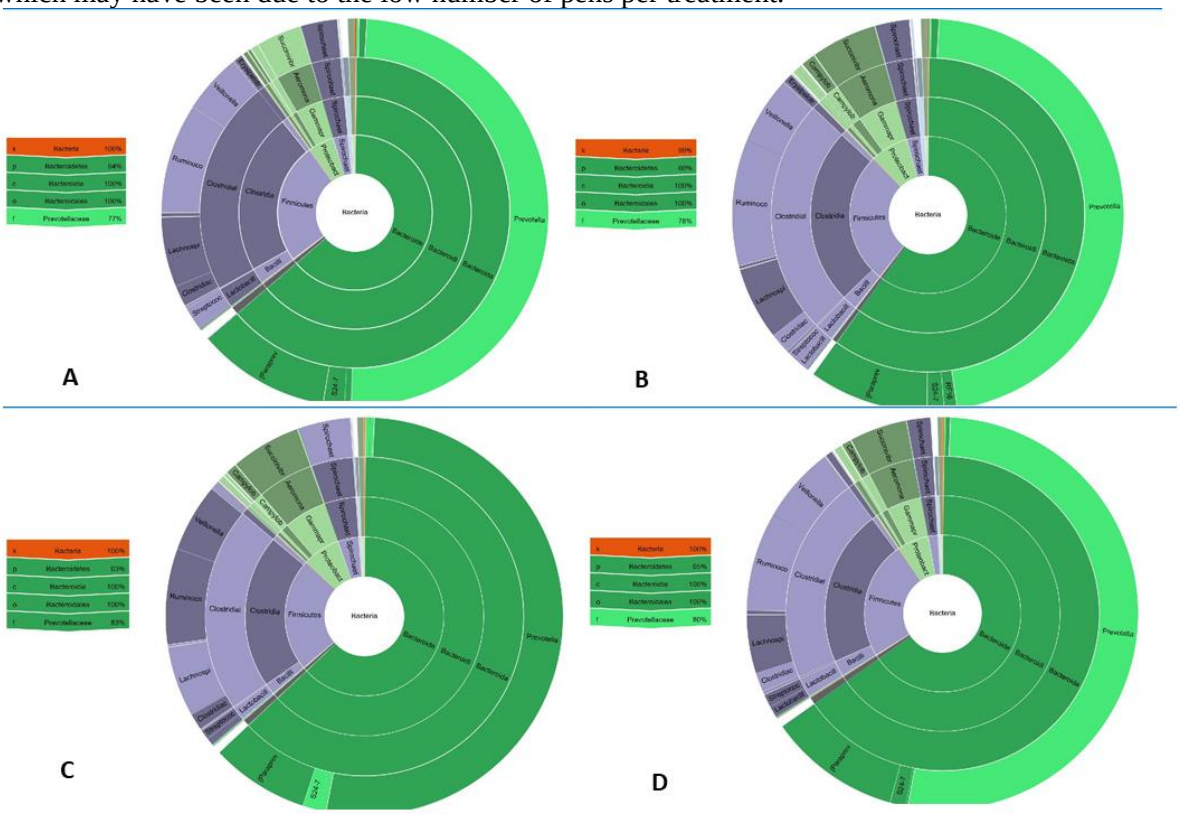

Figure 7. Relative abundance of bacterial families at time 2 (47 days on diet) for all treatments A) Control, B) Control + Medication, C) Protease and D) Protease + medication.

Impacts of time (age) on the microbiome

As discussed previously, the impact of diets on grower pigs was separated into two analyses based on time because only 50% of the pens sampled at time 1 were also sampled at time 2. Clustering of bacterial communities by time (Figure 8) indicated significant differences in beta diversity (microbial communities between time points) between 14 and 47 days, and PERMANOVA analysis of beta diversity at time 1 and 2 showed significant differences in microbial communities (P = 0.00001). If both time points were analysed together, treatment had no significant effect on bacterial community clustering or beta diversity (P = 0.836).

The largest difference between the two time points was in the relative abundance of the phyla Bacteroidetes and Firmicutes at 14 days (8% and 87%, respectively) and at 47 days (63% and 25%, respectively). This resulted in a very large increase in the relative abundance of *Prevotellaceae* from time 1 (5.7% of all bacteria) to time 2 (50% of all faecal bacteria). The phyla Spirochaetes and Proteobacteria were absent at time 1 but consisted of 3% and 7% of all bacteria at time 2, respectively. The class Bacilli made up 13% and 8% of the phylum Firmicutes and within that, the family *Lactobacillaceae* made up 7.75% and 10.34% of all Firmicutes at time 1 and 2, respectively. The *Ruminococcaceae* were more abundant at time 1 than time 2, making up 21% and 8.9% of all Firmicutes,

respectively. In contrast, *Veillonaceae* were less abundant at time 1 than time 2, comprising 0% and 5.5% of all Firmicutes, respectively.

Linear discriminate analysis of bacterial taxa at the 2 time points showed strong differentiation of the growers' microbiomes at time 1 and 2 (Figure 9 and 10).

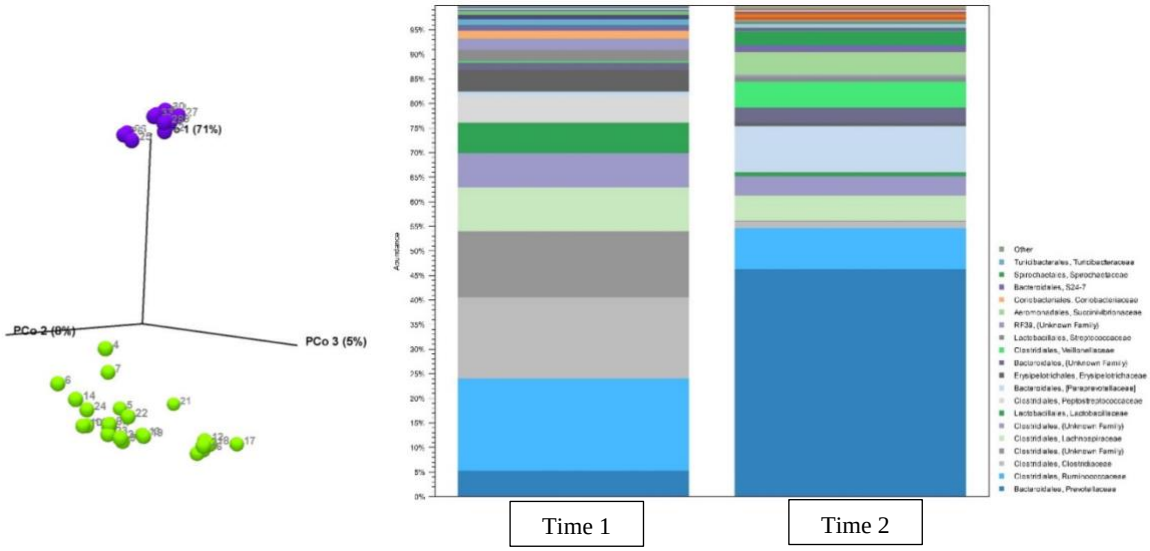

Figure 8. Left panel: Effect of time (green = time 1; purple = time 2) on clustering of grower microbiomes (PCA). Right panel: Effect of time on relative abundance of bacterial taxa.

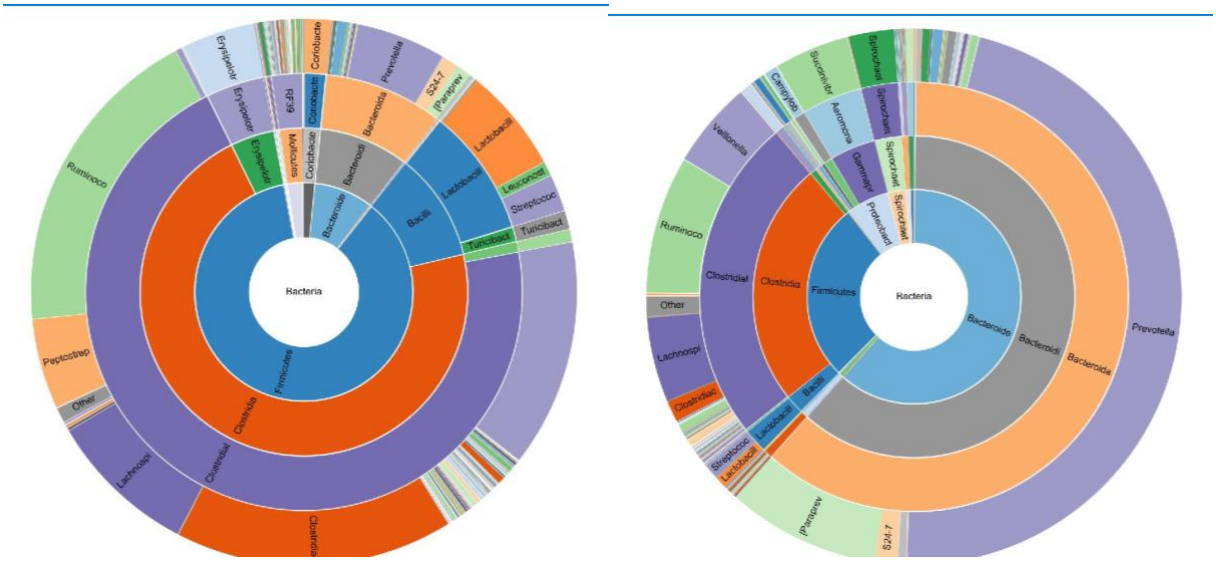

Figure 9. Relative abundance of bacterial families at time 2 (47 days on diet).

Figure 10. Linear discriminate analysis characterising the effect of time on the combination of bacterial taxa (time 1 = 14 days in red; time 2 – 58 days in green).

Discussion

The most pronounced impact on the grower pigs' microbiomes was not protease or antibiotic treatment, but the effect of sampling time. At day 14, the two main bacterial phyla found in pig faeces, Bacteroidetes and Firmicutes, were significantly altered from relative abundances reported previously and were markedly different from the relative abundances at day 47, with 40-60% Firmicutes and 30-40% *Bacteroidetes* (Ke et al., 2019; Wang et al., 2019; Zhao et al., 2015). In faeces from day 14, the relative proportion of *Bacteroidetes* reduced dramatically (7.5%) and the abundance of *Firmicutes* increased significantly (92.5%). Differences in the storage of faeces (frozen versus fresh) prior to DNA extraction are the most likely explanation for these dramatic differences in bacterial phyla between the two faecal sampling times. Higher relative abundances of *Firmicutes* have been reported from frozen and thawed faeces relative to fresh faeces, caused by the release of additional DNA from Gram positive *Firmicutes* after freeze/thawing (Bahl et al., 2012). Freezing of faeces can lead to 5.2-fold increases in the ratio of *Firmicutes* to *Bacteroidetes* in Qiagen stool DNA extraction systems.

Other factors such as the diet or age of pigs can also impact the relative abundance of both *Bacteroidetes* and *Firmicutes*. The abundance of *Firmicutes* increases with pig age, especially in the period around weaning, with the percentage of *Firmicutes* increasing from 73% of all bacteria in 1-month-old pigs to 92% of bacteria in 2-, 3- and 6-month-old pigs (Zhao et al., 2015). The abundance of *Bacteroidetes* varied from 5% of all bacteria at 1 month of age to 0.9%, 2.7% and 4.9% at 2, 3 and 6 months, respectively. The relative abundance of Proteobacteria (containing Enterobacteriaceae) is also affected by age, with significant reductions from 16% to 1.9% in pigs at 1 and 2 months of age, respectively.

Diet changes at weaning can also cause substantial microbiome alterations due to the change from milk to plant based solid feed. *Bacteroides* become less abundant as catabolism of milk sugar complexes is no longer important, whereas *Prevotellaceae* becomes more abundant to help digest plant polysaccharides (Frese et al. 2015). *Lactobacillaceae*, a member of the Firmicutes phylum, also becomes more abundant to aid digestion of disaccharides. *Ruminococcaceae, Lachnospiraceae* and *Veillonellaceae*, members of the *Clostridiales* order within the phylum *Firmicutes*, also become more abundant with the change to a plant-based diet.

The location where samples are collected also has a significant effect on the relative abundance of bacterial phyla. The pigs' small intestinal microbiome is characterised by 1% *Bacteroidetes*, 20% *Firmicutes* and 74% *Proteobacteria*, which is remarkably different to the characteristic microbiome of the caecum, ileum and faeces consisting of 6-8% *Bacteroidetes*, 75% Firmicutes and 13% *Proteobacteria* in the pig caecum and colon (Zhao et al, 2015). Antibiotics can also affect the microbiome, by reducing pathogen numbers and altering commensal numbers and diversity. However, as all treatments had increased *Firmicutes* and decreased *Bacteroidetes* at day 14, these changes are unlikely to be due to medication or the addition of protease to diets.

The effects of a protease and medication therefore need to be analysed independently of faecal sampling time. One week after medication commenced, medicated pigs had a reduced abundance of *Clostridiaceae*, but an increased abundance of *Lachnospiraceae* relative to all other treatments. Both medicated and control pigs also had an increased abundance of *Prevotellaceae* compared to all other treatments. A higher abundance of *Prevotellaceae* has been reported as a dominant feature of the faecal microbiota in healthy pigs as compared to diarrhoeic pigs after weaning (Dou et al., 2017). *Prevotellaceae* increase rapidly after weaning due to the availability of plant polysaccharides and can metabolise both proteins and carbohydrates to produce acetate (Duncan et al., 2004a; 2004b) and propionate (Ramirez-Farias et al., 2009; Scott et al., 2014), which provide energy for the pig.

One week after the protease treatment commenced, protease-supplemented pigs had an increased abundance of *Firmicutes*, specifically *Lactobacillaceae* relative to control pigs. *Lactobacillaceae* increase following weaning in response to dietary changes as they can digest plant polysaccharides as well as simple sugars in milk. However, *Lactobacillaceae* can ferment both carbohydrates and proteins in the colon depending on which substrate is more abundant. Pigs supplemented with dietary protease would be expected to have reduced amino acid substrates for bacterial fermentation, so an increased abundance of *Lactobacillaceae* may suggest the preferential fermentation of carbohydrates in the

absence of AA substrate. This contrasts with studies in rats, where high fat and low protein diets led to a decreased abundance of *Lactobacillus* spp. relative to rats fed high fat and high protein diets (Shi et al., 2020). Rats fed high protein and high fat diets also produced more skatole.

The impact of medication and protease supplementation were more difficult to see in pigs at day 47 relative to day 14. The increased abundance of *Lactobacillaceae* observed in protease-fed grower pigs disappeared. Within the order *Clostridiales*, protease fed growers showed small reductions in the relative abundance of *Ruminococcaceae* (38% and 42%) and increased abundance of *Veillonellaceae* (23% and 28%) relative to grower pigs fed with control protein diets, much as was observed in weaner pigs fed low protein diets.

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