The interaction between dietary crude protein and amino acid levels in weaner pigs in an antibiotic-free environment with or without *Bacillus amyloliquefaciens* supplementation

By

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DECLARATION

I, Natasha van Niekerk, declare that the dissertation: '**The interaction between dietary crude protein and amino acid levels in weaner pigs in an antibiotic-free environment with or without *Bacillus amyloliquefaciens* supplementation'** which I hereby submit for the degree MSc (Agric) Animal Nutrition at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

2020/07/07

NATASHA VAN NIEKERK **DATE**

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ABSTRACT

The interaction between dietary crude protein and amino acid levels in weaner pigs in an antibiotic-free environment with or without *Bacillus amyloliquefaciens* supplementation

Natasha van Niekerk

High crude protein (CP) diets cause a myriad of digestive problems in weaner pigs due to incomplete digestion. This is caused by an immature digestive system at weaning. These problems present themselves as increased diarrhoea and reduced growth performance in weaner pigs. Many of the negative consequences could in the past be rectified through the use of antibiotic growth promoters (AGPs). However, AGPs are becoming more closely scrutinised by the general public and government officials and it is therefore imperative that alternative feed additives and dietary strategies are explored so that weaner pig performance is not impaired in the face of AGP bans.

The effect of a 3% dietary CP reduction and 10% elevation of lysine, methionine, threonine, tryptophan and valine and/or *Bacillus amyloliquefaciens* was investigated in weaner pigs of 28 days old. The experiment was performed on 162 pigs with six dietary treatments; comprising of two CP levels (21% or 18% in the pre-starter diets and 18% or 15% in the starter diets), two amino acid levels (recommended amino acid level or elevated by 10%) and with or without the supplementation of a probiotic additive (*Bacillus amyloliquefaciens*). The total trial period was 42 days, divided into a pre-starter phase and starter phase, both of 21 days each. Growth performance and faecal score was measured for the entire trial period. In addition, after 21 days of the trial, 54 pigs were humanely euthanised to determine the gut health effects of the dietary treatments.

Crude protein level resulted in significant effects on body weight (BW), average daily gain (ADG) and gain to feed ratio (G: F) in both the pre-starter and starter phase. The pigs consuming the HP diets had increased ($p < 0.05$) BW at day 7, 14, 21, 28 and 35, and higher ADG ($p < 0.01$) between days 7-14 and 7-21. In addition, high CP diets resulted in increased ($p < 0.05$) G: F in the periods 7-14, 7-21, 21-42 and 7-42. This was due to the negative impact incurred as a result of deficiencies in certain essential AA and the standard ileal digestible (SID) Lys to CP ratio (SID Lys: CP) of 6.9% being exceeded when CP was reduced, and supplemental AA increased. The reduction in CP resulted in improved faecal scores. The

supplementation of the probiotic did not result in any significant effects on performance but did exert a positive synergistic effect with the low CP diet on gut health, as observed through improved faecal score.

Ileal villi height was significantly increased in high CP diets, possibly indicative of increased nutrient availability in the high CP diets. Crypt depth was also increased in the high CP diets, indicative of increased cell proliferation. There were no significant dietary effects on the pH and ammonia content of caecal digesta, or the presence and enumeration of *Salmonella* and *Escherichia coli* (*E. coli*) in ileal and caecal digesta. Interestingly, the supplementation of the probiotic increased the relative liver weights of the pre-starter pigs.

Results of the study suggest that in order for the strategy of reducing CP in weaner pig diets to be successful, no deficiencies in essential or non-essential amino acids should be present. In addition, low CP diets in conjunction with a probiotic can improve faecal scores of weaner pigs, providing an effective solution to replacing AGPs.

LIST OF ABBREVIATIONS

AA	Amino acids
ADFI	Average daily feed intake
ADG	Average daily gain
AGP	Antibiotic growth promoter
AOAC	The Association of Official Analytical Chemists
Arg	Arginine
BCAA	Branched chain amino acids
BW	Body weight
CD	Crypt depth
CFU	Colony- forming units
CP	Crude protein
CRF	Corticotropin releasing factor
CuSO4	Copper sulphate
Cys	Cysteine
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
EAA	Essential amino acids
ETEC	Enterotoxigenic *Escherichia coli*
FCR	Feed conversion ratio
FOS	Fructooligosaccharides
G: F	Gain to feed ratio
GIT	Gastrointestinal tract
Gln	Glutamine
Glu	Glutamate

GSH	Glutathione
HCl	Hydrochloric acid
Hcy	Homocysteine
His	Histidine
HPLC	High-performance liquid chromatography
HT	Heat stable
IDO	Indoleamine 2,3 dioxygenase
IGF	Insulin-like growth factor
Ile	Isoleucine
Isc	Short circuit current
ISO	International organization for standardization
L:C	*Lactobacillus* to coliform ratio
Leu	Leucine
LNAA	Large neutral amino acids
LT	Heat labile
Lys	Lysine
M +C	Methionine + Cysteine
ME	Metabolisable energy
Met	Methionine
N	Nitrogen
NE	Net energy
NEAA	Non-essential amino acids
NFE	Nitrogen free extract
NH_3	Ammonia
NH_3: NH_4	Ammonia to ammonium ratio
NH_4	Ammonium

NIRS	Near-infrared spectroscopy
NRC	National research council
Phe	Phenylalanine
Phe + Tyr	Phenylalanine + Tyrosine
Pro	Proline
PWD	Post-weaning diarrhoea
PWED	Post-weaning enteric disorders
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SID	Standard ileal digestible
Tau	Taurine
Thr	Threonine
TOS	Transgalactooligosaccharides
Trp	Tryptophan
Tyr	Tyrosine
UV	Ultraviolet
Val	Valine
VDLUFA	Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten
VFA	Volatile fatty acids
VH	Villi height
VH: CD	Villi height to crypt depth ratio
ZnO	Zinc oxide

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CHAPTER 1: INTRODUCTION

In commercial pig operations, weaning typically occurs at 3-4 weeks-of-age when piglets are between 5-10 kg body weight. This is known as the weaner/ pre-starter phase (Pluske, 2016). This is a drastic reduction in weaning age from natural weaning that would typically only occur at 12 weeks-of-age (Weary & Fraser, 1997). Weaning results in several stressors to the piglet, such as separation from the sow, a drastic change in diet, vaccination and the movement into a new environment (Moeser *et al*., 2017). The period directly after weaning also results in a drastic reduction in feed intake, and pre-weaning feed intake is only achieved approximately 14 days after weaning (Le Dividich & Sève, 2000). In order to avoid any further negative impact on growth performance for the remainder of the production cycle after this period of anorexia, highly nutrient-dense (particularly high CP) diets are fed to weaner pigs to mitigate any 'growth-check' during this period. In addition, a piglet's inherent ability for protein deposition during this period is very high, so it may appear that pigs would be able to utilise these nutrients for growth and muscle development more readily (Nyachoti & Jayaraman, 2016).

Full maturation of the digestive system is only complete at 12 weeks-of-age, and therefore, there may be incomplete digestion of dietary nutrients at the earlier weaning ages (Moeser *et al*., 2017). This may result in protein fermentation in the large intestine which produces ammonia and other toxic metabolites that serve as a substrate for the proliferation of pathogenic bacteria such as *E. coli* and *Salmonella spp.* (Wellock *et al*., 2008). The proliferation of pathogenic bacteria causes significant intestinal damage such as decreasing villi height and increasing crypt depths, which results in a further negative impact on the digestion and absorption of nutrients (Nyachoti & Jayaraman, 2016). This usually presents itself as diarrhoea in the newly-weaned pig (Montagne *et al*., 2004).

Feeding diets which are lower in dietary CP can help prevent intestinal damage and gut health imbalance. It is imperative that diets are correctly balanced when reducing the dietary CP so as to not limit any of the essential or non-essential amino acids (NEAA). As a rule of thumb, a SID Lys: CP value of 6.9% is maintained to allow enough nitrogen for the production of NEAA (Htoo 2017). There are potential benefits of increasing the dietary levels of the first five limiting amino acids (lysine, methionine, threonine, tryptophan and valine) above the levels currently used in South Africa. This is to firstly encourage the high growth potential of modern-day lean pigs (Htoo, 2013), and secondly, to potentially improve gut health due to the numerous beneficial roles the essential amino acids have on maintaining good gut integrity. Threonine plays an integral role in maintaining the intestinal barrier and producing mucins (Faure *et al*., 2007). Increased tryptophan supplementation in the diets of weaned pigs improves intestinal morphology by increasing the villi height to crypt depth ratio (VH: CD) (Koopmans *et al*., 2006). Lysine is crucial for carnitine synthesis, which is responsible for the transportation of long-chain fatty acids into the

mitochondria for β-oxidation (Htoo, 2013; Liao *et al*., 2015). Lastly, due to methionine's involvement in antioxidation within the intestine, it can prevent any mucosal epithelial damage from reactive oxygen species (Wang *et al*., 2009). An improvement in gut health could potentially be observed through improved intestinal histomorphology and reduced incidences of diarrhoea. Maintaining good gut health in weaner pigs is imperative as it ensures the optimal uptake of nutrients from the feed needed for growth and development. Also, the negative impact of poor gut health caused by a compromised gastrointestinal barrier causes increased occurrences of diarrhoea and subsequent poorer growth for long into the production cycle of pigs (Pohl *et al*., 2017).

Antibiotics have been extensively used in animal agriculture for their growth promotion properties which has resulted in the inevitable misuse of compounds that were developed for the treatment of disease (Dibner & Richards, 2005). This has resulted in the development of antibiotic resistant bacteria and increased public pressure to reduce this threat as much as possible (Liao & Nyachoti, 2017). The pursuit of suitable alternatives is at an all-time high and is an ongoing battle. One of the possible alternatives to replace antibiotics could be probiotics due to their bactericidal properties and other unique modes of action. Probiotics are live organisms, usually bacteria or yeasts, which are able to stabilise the gut microflora either through the colonisation of the gut by the probiotics themselves, or by stimulating the proliferation of beneficial bacteria such as *Lactobacillus* (Cho *et al*., 2011). The use of the *Bacillus* species of bacteria in animal diets as probiotics is increasing due to their robust and resilient nature under various conditions. *Bacillus* are spore-forming bacteria, which enables them to withstand high temperatures exerted during pelleting and ensures their survival during extended periods of storage (Liao & Nyachoti, 2017). Probiotics have demonstrated the ability to significantly improve ADG, average daily feed intake (ADFI) as well as feed conversion ratio (FCR) in pigs (Liao & Nyachoti, 2017). In addition, low CP diets and probiotics can act synergistically to improve growth performance and gut health in weaner pigs (Bhandari *et al*., 2010).

AIM AND OBJECTIVES

The aim of the study is to investigate how growth performance, organ weight and gut health of weaner pigs is affected when CP is reduced by 3% and amino acid levels are elevated by 10% in addition to a probiotic (*Bacillus amyloliquefaciens*) being added.

The following objectives were set for the trial:

- The effect of different dietary treatments (varying CP and AA levels, with or without the supplementation of the probiotic additive, *Bacillus amyloliquefaciens*) on growth performance was measured (ADG, ADFI, G: F)
- The effect of the different dietary treatments on organ weights (liver and kidneys) were measured
- The effect of the different dietary treatments on total gut health was measured, including faecal score, duodenal and ileal histomorphology, pH and ammonia level in caecal digesta, and the enumeration of *Salmonella spp.* and *E. coli* in ileal and caecal digesta

HYPOTHESES

The hypotheses tested in this study were:

H_{01}: Lowering CP by 3% will reduce pig performance, even when there are no deficiencies in standard ileal digestible amino acids and the diets are iso-energetic.

H_{A1}: Lowering CP by 3% will not reduce pig performance, even when there are no deficiencies in standard ileal digestible amino acids and the diets are iso-energetic.

H_{02}: Lowering CP by 3% will not improve pig gut health, even when there are no deficiencies in standard ileal digestible amino acids and the diets are iso-energetic.

H_{A2}: Lowering CP by 3% will improve pig gut health, even when there are no deficiencies in standard ileal digestible amino acids and the diets are iso-energetic.

H_{03}: Supplementing *Bacillus amyloliquefaciens* in addition to lowering CP by 3% will not improve pig performance and gut health, even when there are no deficiencies in standard ileal digestible amino acids and the diets are iso-energetic.

H_{A3}: Supplementing *Bacillus amyloliquefaciens* in addition to lowering CP by 3% will improve pig performance and gut health, even when there are no deficiencies in standard ileal digestible amino acids and the diets are iso-energetic.

H_{04}: Lowering CP by 3% and increasing the supplementation of lysine, methionine, threonine, tryptophan and valine by 10% will not improve pig performance and gut health, even when there are no deficiencies in standard ileal digestible amino acids and the diets are iso-energetic.

H_{A4}: Lowering CP by 3% and increasing the supplementation of lysine, methionine, threonine, tryptophan and valine by 10% will improve pig performance and gut health, even when there are no deficiencies in standard ileal digestible amino acids and the diets are iso-energetic.

H_{05}: Supplementing *Bacillus amyloliquefaciens* in addition to lowering CP by 3% and increasing the supplementation of lysine, methionine, threonine, tryptophan and valine by 10% will not improve pig performance and gut health, even when there are no deficiencies in standard ileal digestible amino acids and the diets are iso-energetic.

H_{A5}: Supplementing *Bacillus amyloliquefaciens* in addition to lowering CP by 3% and increasing the supplementation of lysine, methionine, threonine, tryptophan and valine by 10% will improve pig performance and gut health, even when there are no deficiencies in standard ileal digestible amino acids and the diets are iso-energetic.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

Diets for weaned piglets are usually high in crude protein because of their inherent ability for fast growth during this period, but also due to weaner pigs' limited capacity for feed consumption. Besides being costly, these high CP diets have their downfalls, as often the pigs cannot utilise the increased CP efficiently, resulting in excess nitrogen (N) being excreted into the environment. The levels of N intake from the diet can be reduced by decreasing the total dietary CP (Canh *et al*., 1998). Reducing dietary CP can pose a risk to optimal growth performance as all essential amino acids must be provided at the required levels with adequate N available for the production of other important nutrients, such as the non-essential amino acids (NEAA). If this balance is not achieved, this may have a negative impact on weaner performance (Htoo, 2017).

2.2 Growth, development and amino acid requirements of the young pig

The growth phases of commercial pigs are grouped according to body weight in order to accurately meet the nutrient requirements at specific body weight growth periods. The pre-starter/weaned phase is when pigs are between 5-10 kg body weight and this usually coincides with the period directly after weaning (Pluske, 2016). The starter phase is between 10-25 kg body weight, grower phase between 20-80 kg body weight and lastly, the finisher phase from 80-120 kg body weight. The grower phase can be further divided into three subgroups, grower 1 (20-40 kg), grower 2 (40-60 kg) and grower 3 (60-80 kg). Similarly, the finisher phase can be divided into two subgroups, finisher 1 (80-100 kg) and finisher 2 (100-120 kg) (Htoo, 2013).

Genetic breeding programs have aimed at producing a leaner and leaner pig over the last few decades and therefore by only following the amino acid recommendations by the national research council (NRC), this may lead to deficiencies, as the genetic progress may well be faster than the release of new amino acid recommendations from the NRC (Htoo, 2013). For this reason, there have been a vast number of studies exploring the optimal lysine (Lys) requirement of modern lean pigs over the last ten years. Researchers such as Gaines *et al*. (2003), Kendall *et al*. (2008) and Main *et al*. (2008) have found that Lys requirements may be as much as 20-30% higher than the recommendations by the NRC (1998) (Htoo, 2013).

Table 2.1 and Table 2.2 provide the current essential amino acid recommendations from AMINOPig® 1.0 (2011) for growing pigs. Even though other amino acids such as glutamine, glutamate and

arginine have important metabolic functions and are also crucial in sustaining gut health of pigs (Wang *et al.*, 2009); the focus in the following sections will be on the first five limiting amino acids in pigs (lysine, methionine, threonine, tryptophan and valine) and the importance of these amino acids in growth performance and gut health.

Table 2.1 Amino acid inclusion levels (in %) for growing pigs according to body weight (kg), as recommended by AMINOPig® 1.0 (2011)

Amino acid	**Pre-Starter** **5-10 kg**	**Starter** **10-20 kg**	**Grower 1** **20-40 kg**
SID* Lys	1.42	1.32	1.08
Met	0.47	0.43	0.37
Met +Cys	0.85	0.79	0.67
Thr	0.89	0.83	0.70
Trp	0.31	0.29	0.22
Ile	0.78	0.72	0.60
Val	0.97	0.89	0.74
Leu	1.42	1.32	1.08
Arg	0.60	0.55	0.43
Phe	0.85	0.80	0.65
Phe + Tyr	1.35	1.25	1.03
His	0.45	0.42	0.35

* SID= Standard Ileal Digestible

Table 2.2 Amino acid inclusion levels (ratio to SID* Lys) for growing pigs according to body weight (kg) as recommended by AMINOPig® 1.0 (2011)

Amino acid	Pre-Starter	Starter	Grower 1	
	5-10 kg	10-20 kg	20-40 kg	
			Boars	Gilts
Met: Lys	33	33	34	34
Met + Cys: Lys	60	60	62	62
Thr: Lys	63	63	65	65
Trp: Lys	22	22	20	20
Ile: Lys	55	55	55	55
Val: Lys	68	68	68	68
Leu: Lys	100	100	100	100
Arg: Lys	42	42	42	42
Phe: Lys	60	60	60	60
Phe + Tyr: Lys	95	95	95	95
His: Lys	32	32	32	32

* SID= Standard Ileal Digestible

2.2.1 The role of the first five essential amino acids on the growth, immunity and gut health of pigs

Lysine

Lysine (Lys) is the first limiting amino acid in typical pig diets and therefore the requirements of other amino acids are always given according to Lys (Kahindi *et al.*, 2014). A deficiency in Lys can result in severe impairment of growth performance (Hamilton & Veum, 1986). Lysine requirements of the modern pig have changed dramatically over time due to the intensive breeding selection for leaner animals, and

therefore this increased ability for lean muscle growth requires higher levels of amino acids to support such growth (Htoo, 2013). Lysine requirements of the modern pig may be as much as 30% higher than what was recommended in the NRC (1998) guidelines (Gaines *et al*., 2003). Although Lys in the animal body is primarily used for protein synthesis (Ball *et al*., 2007), there are several important physiological functions of this amino acid as well (Htoo, 2013). Lysine is an important constituent of carnitine synthesis and the supplementation of Lys is necessary in plant-based diets (Vaz & Wanders, 2002). Carnitine has an important function in energy production via the citric acid cycle through the transportation of long-chain fatty acids from the cytoplasm into the mitochondria of cells for β-oxidation (Htoo, 2013; Liao *et al*., 2015). Other important roles of carnitine include the protection against oxidative stress, maintenance of normal cholesterol levels and the control of oxidation of substrates in adipose tissue (Liao *et al*., 2015).

Antibiotic growth promoters have been used in the majority of pig trials determining the optimal SID Lys levels in growing pigs; however, with increased pressure to reduce the usage of AGPs in swine diets, it is necessary to determine the SID Lys requirements without AGP supplementation (Kahindi *et al*., 2014). Antibiotics are routinely used in weaner diets to prevent diarrhea and promote increased growth performance, and pigs fed antibiotics have significantly higher growth performance than their counterparts on antibiotic- free diets (Dibner & Richards, 2005; Bikker *et al*., 2006). Although Lys does not typically have an immunological function, feeding AGP-free diets under unsanitary conditions can result in increased AA requirements, including Lys (Bikker *et al*., 2006). In a trial done by Kahindi *et al*. (2014), piglets were either housed in clean or unclean environmental conditions. Piglets raised in unclean houses had a significant growth reduction as a result of increased diarrhea and decreased feed intake. However, the increased supplementation of Lys resulted in increased ADG and G: F ratio, and the optimal SID Lys requirement for these pigs was possibly even higher than the highest level used in the study (1.43%).

Methionine and cysteine

The sulphur-containing amino acids, methionine (Met) and cysteine (Cys), have important functions in the health status and growth of the pig. Methionine is an essential amino acid that cannot be produced within the animal's body from precursors, and therefore needs to be supplied in the diet (Wang *et al*., 2009). Cysteine is classified as a semi-essential amino acid for neonatal pigs as these young animals have a low inherent capability to convert Met to Cys (Finkelstein, 2000). The metabolic end products of Met and Cys such as glutathione (GSH), homocysteine (Hcy) and taurine (Tau) play crucial roles in the immune response within the intestine (Grimble, 2006). Glutathione is an important antioxidant and when present in the gut lumen and within the intestinal cells, it protects the epithelium from damage from reactive oxygen species. A deficiency of GSH due to inadequate methionine supplementation in the diet can lead to

extensive damage to the mucosa and epithelium, due to loss of crucial antioxidant protection (Wang *et al*., 2009). As a result, the intestinal villi may be damaged and experience atrophy, ultimately negatively impacting digestion and absorption. Taurine is imperative in detoxification, osmoregulation, membrane stabilisation and antioxidation (Huxtable, 1992). Research shows that Tau prevents cellular lipid peroxidation in the intestinal cells of humans by counteracting the negative effects of hydrogen peroxide (Roig-Pérez *et al*., 2004). In addition, Tau plays an important role in immune and inflammatory responses since it comprises more than 50% of the free amino acids in lymphocytes (Wang *et al*., 2009).

Threonine

Threonine is particularly essential for the synthesis of glycoproteins, such as mucins, on the surface of the small intestine and in turn, maintains the integrity of the gut barrier by preventing injury. Threonine comprises about 30% of the amino acid composition of mucin (Faure *et al*., 2007). It has been demonstrated in a trial done by Stoll *et al*. (1998) that the gastrointestinal tract metabolises 61% of dietary Thr, and 90% of this metabolised Thr is either secreted in the form of mucosal protein or is catabolised. In another study done by Bertolo *et al*. (1998) in which piglets were parentally infused with Thr via the left jugular vein, the gut experienced atrophy, indicating that the route of nutrient delivery (in particular Thr) has a significant impact on the morphology and function of the gut. Goblet cells are found along the small and large intestines and are responsible for the secretion of mucin (Specian & Oliver, 1991).

Threonine has shown to be in high demand by the gastrointestinal tract due to the high turnover rate of intestinal mucosa, and therefore a significant proportion of the total Thr requirement for pigs is accounted for by the GIT (Bertolo *et al*., 1998). Lack of adequate enteral Thr nutrition leads to a reduction in intestinal epithelium and goblet cells. This inevitably results in reduced secretion of mucin (Bengmark & Jeppson, 1995). During times of inflammation, Thr requirement increases, because the synthesis of mucins is amplified and the acute-phase proteins that are stimulated in response to inflammation also contain high levels of Thr (Faure *et al*., 2007). In an experiment done by Faure *et al*. (2007), rats were infected with *E. coli* and as a result, plasma protein synthesis was increased by up to 6-fold compared with rats that were uninfected. When these rats were parentally given labelled Thr, the utilisation of Thr for plasma protein synthesis was 360% higher and the utilisation for mucin production was more than 70% higher in infected rats. On the second day following infection, the additional Thr demand was 2.6 times higher than the dietary Thr intake, implying that Thr was mobilised from other sources within the body, such as muscle protein. Therefore, dietary Thr was not used for anabolic processes such as growth and muscle development but was used to assist in fighting an infection. In situations such as infection or sepsis,

the increased Thr demand can be satisfied by increasing the dietary supply of Thr, in turn reducing the negative effects of muscle atrophy (Faure *et al*., 2007).

Tryptophan

Tryptophan is a crucial component of certain biological processes in the body such as the metabolic pathway involved in the production of serotonin. Serotonin is an important neuromediator associated with the production of melatonin, the regulation of stress responses and sleep (Esteban *et al*., 2004; Le Floc'h & Seve, 2007). Supplementing Trp into the diet of pigs can modulate their response to stress as demonstrated in a trial done by Koopmans *et al*. (2006). In this experiment, weaner pigs that had been supplemented with 5 g/kg Trp (to raise the level of ileal digestible Trp to 7 g/kg) had lower salivary cortisol levels compared to their counterparts receiving the control diet (normal dietary supply of Trp of 2 g/kg ileal digestible Trp) upon mixing with unfamiliar littermates at weaning. This process typically results in significant amounts of stress. The intestinal morphology was also affected by the increased dietary Trp level, with a significant increase in the villi height to crypt depth ratio (VH: CD) in both the distal and proximal sections of the small intestine. Supplementing Trp into the diet of weaner pigs for more than 10 days resulted in more relaxed behaviour in the weaner house, as seen through a greater amount of time pigs spent lying down as opposed to standing up (Koopmans *et al*., 2006).

A deficiency of Trp in the diet is linked to depressed feed intake in pigs (Eder *et al*., 2001). Le Floc'h & Seve (2007) discussed that depressed feed intake is a physiological response initiated by the brain due to the detection of an imbalance of the AA concentrations in the brain versus the free AA pool in the blood serum. The exact mechanisms between Trp, serotonin levels and appetite are not fully understood, but it is thought that serotonin is a mediator of satiety (Le Floc'h & Seve, 2007).

It was observed in a study done by Henry *et al*. (1996) that supplementing Trp at 0.16% into a low CP diet of 12.9% increased the ADFI, ADG as well as feed efficiency when compared to pigs receiving lower Trp supplementation at 0.12%. When Trp was supplemented at 0.12% in a high CP diet (16.7%), growth performance and feed intake was significantly negatively affected, but this was counteracted when dietary Trp supplementation was increased to 0.16%. The depressive effect on feed intake and growth performance is as a result of an imbalance in other AA, namely the large neutral amino acids (LNAA) which includes the branched chain amino acids (BCAA) and the aromatic amino acids (Phe, Tyr). With the increased dietary CP level, Leu supply was increased by 50% and Tyr by 33%, which resulted in imbalances between these AA and Trp (Henry *et al*., 1996). This imbalance resulted in increased competition between Trp and the LNAA for access into the brain via the blood-brain barrier. Therefore, an increased supply of

LNAA in the diet of pigs compared to Trp will negatively affect the production of serotonin and thus decrease voluntary feed intake and reduce growth performance (Henry *et al*., 1996).

Tryptophan is also involved in the regulation of the immune response and is associated with the indoleamine 2,3 dioxygenase (IDO, EC 1.13.11.42) pathway (Moffett & Namboodiri, 2003). During an inflammatory response, the IDO pathway is activated by pro-inflammatory cytokines which results in the catabolism of Trp into its metabolites and therefore lower free Trp in the circulation of the affected animal (Christen *et al*., 1990). Le Floc'h *et al*. (2004) found that pigs suffering from lung inflammation but fed a diet well balanced for Trp had lower levels of haptogloblin (acute phase protein indicative of inflammation in pigs) concentration in blood plasma compared to their counterparts who were given Trp-deficient diets. Other noteworthy observations were lower IDO activity in the lungs and lymph nodes as well as decreased lung lesions in pigs consuming diets with sufficient Trp supplementation. Therefore, increasing the dietary Trp supplementation can assist in maintaining the free Trp pool in times of inflammatory stress (Le Floc'h & Seve, 2007).

Branched-chain amino acids

The branched chain amino acids (BCAAs) include Val, Leu and Ile. Not only are these three amino acids important substrates for protein deposition, but they have also proven to have very important functions in the development and maintenance of intestinal integrity (Mou *et al*., 2019). The BCAAs are responsible for the production of glutamine which has several important functions in the body. These include protein synthesis, antioxidation and the regulation of genes involved in protecting the intestinal cells against oxidative stress (Wu, 2009).

Leucine was found to reduce the negative impacts of intestinal disease in weaned pigs challenged with rotavirus in the study done by Mao *et al*. (2015). In addition, Leu can reduce the activation of the immune system during the stressful weaning period preventing the negative impact on growth but does not impact protein deposition pathways during this time (Rudar *et al*., 2017). In a trial done by Hu *et al*. (2017) it was discovered that Leu is able to reduce the amount of reactive oxygen species (ROS) in the enterocytes of weaner pigs. Reactive oxygen species can cause significant damage to intestinal integrity (Circu & Aw, 2012), and therefore the effect of supplemental Leu on villi height and crypt depth was measured. The weaner pigs that received additional Leu supplementation had shallower crypts and increased VH: CD, indicative of improved intestinal integrity (Hu *et al*., 2017).

2.3 The early development and maturation of the digestive system in piglets

Commercial pig farming has dictated a far earlier weaning age of piglets at only 3-4 weeks-of-age, as opposed to natural weaning that occurs only around 12 weeks-of-age or older, coinciding with the period when the consumption of milk has ceased (Weary & Fraser, 1997). Due to this drastic reduction in weaning age, the weaned piglet's full digestive capabilities are not yet reached, such as the limited secretion of hydrochloric acid (HCl), leading to a myriad of digestive irregularities, including the condition known as dysbiosis (also referred to dysbacteriosis), which is the imbalance of the microbial population in the gastrointestinal system (Ravindran & Kornegay, 1993; Gresse *et al*., 2017). The gastrointestinal tract (GIT) refers to the stomach, the small intestine and the large intestine.

During the first three weeks of life, there is remarkably faster growth and development of the GIT in comparison to total body weight gain, which is referred to as positive allometric growth (Pluske, 2016). During the first day following birth, the stomach of the piglet increases in weight by 26-28% compared to total body weight gain of only 7-8%. This is combined with the fast maturation of the functional aspects of the stomach, such as the capacity for gastric acid secretions tripling within the first three days following birth (Widdowson *et al*., 1976; Xu *et al*., 1992).

The small intestine undergoes a variety of morphological changes within the first few days postpartum, including a 20% increase in length, 15% increase in diameter and a 70% increase in tissue weight, mainly in the mucosa (Pluske, 2016). During the neonatal period, colostrum induces gut closure as well as increases the absorptive area of the intestine and the activity of the enzymes on the brush border (Everaert *et al*., 2017). The surface area of suckling pigs increases by 50% on the first day, rising to 100% during the remaining 10 days after parturition (Smith & Jarvis, 1978; Xu *et al*., 1992). Other adaptations that occur during the first four weeks of life include changes in the enterocyte brush border disaccharidases, causing a shift from lactase enzymatic activity to higher activities of sucrase and maltase, in order to cope with the abrupt change in diet from a highly digestible milk-based diet to the solid weaning diet (Le Huërou-Luron, 2002).

The pancreas also plays a major role in assisting the entire GIT to function optimally in the days preceding weaning (Pluske, 2016). Although the pancreas' development begins in the prenatal phase, it is only after birth that the pancreatic weight rises as a result of the increase in number and size of the pancreatic cells (Corring *et al*., 1978). There is also a linear increase in the activity of the digestive enzymes secreted by the pancreas from birth until six weeks of age; namely lipase, amylase, chymotrypsin and trypsin; but this rate of increase declines slightly in the days before weaning. After weaning, however, there is a sudden

and remarkable increase in their activity (except for lipase) in response to the weaning ration, which consists primarily of plant-based feed ingredients (Lindemann *et al*., 1986).

Neonatal pigs are known to be in an immunosuppressive state directly after birth (Butler *et al*., 2009). This is for the gastrointestinal tract to fully develop and for the piglet to receive passive immunity from the colostrum during suckling. Sows' milk contains immunoglobulins, leukocytes as well as milk glycans that are able to neutralise and control the microbial population in the gut of the piglet (Newburg & Walker, 2007). Several other changes occur in the immune system during the early stages of the piglet's life to achieve gut homeostasis, and therefore, this immunosuppressive state is crucial for the complete maturation and lifelong function of the immune system. If there is any form of improper immune stimulation or activation; this will affect the optimum development of the pig and have far reaching consequences for later in life (Moeser e*t al.*, 2017).

The GIT is sterile at the time of birth and relies heavily on the inoculation with microbial populations from the sow as well as from the piglet's immediate environment during the first few crucial days of life. This is especially true for the large intestine, as its main role in digestion is to absorb electrolytes, water and short chain fatty acids from the digestive action of the microbial population (Pluske, 2016). The intimate relationship that exists between the pig and the microbiome develops according to the host's early environment it is exposed to, diet, as well as phylogenetic background (Ley *et al*., 2006). The adult pig's gut houses over 1000 diverse bacterial species, most of which are part of the *Bacteroidetes*, *Actinbacteria*, *Spirochaetes*, *Proteobacteria* and *Firmicutes* genetic phyla (Kim *et al*., 2011; Ramayo-Caldas *et al*., 2016). Studies done by Bian *et al*. (2016) and Kubasova *et al*. (2017) show that the early colonising microbiota of piglets are not directly related to the intestinal microbial community of the sow but interestingly, the sow's microbiome only influences the piglet's intestinal microbiota later in life through the environment the piglet is exposed to postpartum (Thompson *et al*., 2008). The subsequent artificial removal of piglets from the sow and placement into weaning houses results in a completely different intestinal microbial community developing within the piglet (Schmidt *et al*., 2011).

2.4 The influence of weaning stressors on the gastrointestinal tract

Weaning introduces several psychological and external stressors to the piglet; including separation from the sow and movement into the new weaner house, vaccination and the mixing with unfamiliar pigs which may encourage fighting in order to establish the new social structure in weaner groups (Moeser *et al*., 2017). At weaning, passive immunity from the sow slowly declines putting the piglet under additional pressure and risk of infection (Moeser *et al*., 2017). In addition, the piglet's own body produces

inflammatory cells in response to the stressors of weaning, indicative of the activation of its own immune system, specifically in the GIT (McCracken *et al*., 1999).

The initial, and arguably the most profound, effect of weaning is a sudden drop in feed intake leading to malnutrition and a dip in growth performance. The metabolisable energy (ME) intake at seven days post-weaning is only 60-70% of the ME content of sow's milk before weaning. The pre-weaning energy intake is only achieved again after approximately 14 days post-weaning (Le Dividich & Sève, 2000). The lack of feed in the GIT for extended periods of time results in a disruption of the intestinal barrier; and when feed is consumed again, an immune response is elicited in the lamina propria due to the foreign bodies in the feed permeating the membrane too readily (McCracken *et al*., 1999).

The proliferation of the intestinal cells is stimulated by the consumption and flow of feed along the length of the GIT (Diamond & Karasov, 1983). Therefore, during times of anorexia as noticed during the first few days following weaning, the rate of cell differentiation will be drastically reduced, negatively impacting the height of the villi as well as the proliferation of the crypt cells, affecting crypt depth (Altmann, 1972; Pluske *et al*., 1997). Villus height and crypt depth are crucial for adequate digestion and absorption in the small intestine, and atrophy of the villi combined with increased crypt depth can cause post-weaning diarrhoea (PWD) (Nabuurs *et al*., 1993). This could potentially result in long-term alterations in intestinal morphology as demonstrated by Boudry *et al*. (2004). Villus atrophy is caused by one of two processes: either there is an increased rate of cell loss such as in the case of inflammation during a microbial challenge or in the presence of antigens; or due to a decreased rate of cell proliferation that occurs during periods of anorexia. In the first instance, there will also be an increased crypt cell proliferation that results in increased crypt depths which reduce the VH:CD (Pluske *et al*., 1997).

After weaning, the diet changes from a highly digestible, nutritious, palatable liquid diet to a less digestible (usually plant-based) solid diet (Le Dividich & Sève, 2000; Pluske, 2016). Kim *et al*. (2001) found that piglets that consumed a liquid milk replacer diet after weaning had an ADG of 44% higher at 14 days post-weaning compared to their counterparts who received dry pellets with identical nutritional content to the milk replacer. In an experiment done by Montagne *et al*. (2004), highly digestible animal proteins were replaced by less digestible plant proteins such as full fat soya, rapeseed meal and lupins in a weaner diet. In one of the treatments, animal protein was fed along with carboxymethylcellulose which caused a disturbance in the gut homeostasis by increasing the viscosity of the digesta. Faeces of the piglets fed the different diets were compared nine days after weaning. The piglets which received diets with animal proteins but no carboxymethylcellulose had the hardest faecal consistency compared to their counterparts that received the diets with the carboxymethylcellulose, with 50% showing signs of wet faeces, and the

remaining 50% having diarrhoea. The piglets that were fed the plant-based weaner ration instead of the animal protein diet also showed signs of gut disturbance, with 50% of these piglets having wet faeces. A fourth diet was also tested that replaced the animal proteins with wheat, creating a wheat-plant protein diet. On the eighth day after weaning, there were more haemolytic *E. coli* found in the faeces from the piglets fed the wheat-plant protein diet compared to the pigs on the plant protein-based diet. 28% of these piglets had wet faeces, and it was therefore deduced that haemolytic *E. coli* was positively correlated with wetter faeces. The viscosity of the ileal digesta was higher in the wheat-plant protein diet fed pigs compared to the pigs fed either the animal or the more digestible plant protein-based diet. Therefore, a diet based on highly digestible animal proteins can protect the gut from pathogenic bacterial colonisation by limiting the amount of available fermentable substrate in the proximal small intestine (Montagne *et al*., 2004).

In the same trial by Montagne *et al*. (2004), inclusion of the plant-based protein sources increased beneficial carbohydrate fermentation in the hindgut which produced volatile fatty acids (VFA), lowering the pH of the digesta. This fermentative activity also increased the weights of the caeca and colons of the pigs fed the plant protein diets as well as the wheat-based diet. The inclusion of fermentable carbohydrates from plant protein sources are beneficial for newly weaned pigs as it encourages the gut microflora to develop and improves gut integrity. The VFA, including acetate, propionate and butyrate, have several beneficial effects in the GIT of piglets. These acids have a growth-stimulatory effect on the intestinal barrier, thereby protecting the pig against pathogenic bacteria invasion (Williams *et al*., 2001). The low pH environment created by the VFA have an inhibitory effect on *E. coli* and other harmful bacteria in the gut. Lastly, a low pH stimulates the absorption of sodium and water from the large intestine which can reduce the threat of post-weaning diarrhoea (Argenzio & Whipp, 1979). Trying to balance the benefits and risks of including less digestible plant proteins in the diets of weaners, especially without the inclusion of antibiotic growth promoters to assist in stabilising the gut environment, is a complex task.

The effect of weaning stress on the permeability of the gastrointestinal barrier

The intestinal barrier in pigs is made up of a single layer of columnar epithelial cells and acts as the animal's first line of defense against any pathogens or antigens present within the gut lumen and is arguably the most important component in the piglet's immune system (Moeser *et al*., 2017). The intestinal barrier is regulated by tight junctions, which are cellular membrane proteins that control the pore size and movement of ions, and ultimately control the level of permeability from the gut into the bloodstream (Edelblum & Turner, 2009). In most animal species, the intestinal barrier development and functionality occurs at 14 to 21 days postpartum (Catassi *et al*., 1995), and is clearly outlined in Figure 2.1. If this barrier is compromised in any way by an increase in permeability; antigens, toxins or bacteria present in the lumen

can 'leak' or cross over the barrier and into the tissues and result in inflammation, diarrhoea and disease (Smith *et al*., 2010). This mode of action for causing diarrhoea and disease is renowned for bacteria such as *E. coli* as well as *Clostridium difficile* (Moeser *et al*., 2007).

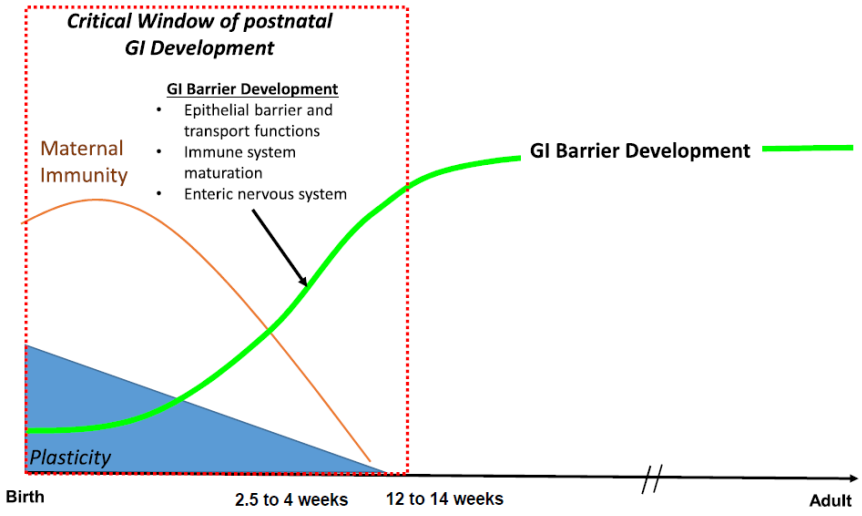


Figure 2.1 The postpartum development of the gastrointestinal barrier in pigs (Moeser *et al*., 2017)

Any stress caused by the weaning process can cause a cascade of mechanisms that increase the permeability of the gut as seen in Figure 2.2. Moeser *et al*. (2007) investigated the role of stress mediators such as cortisol, corticotropin releasing factor (CRF) and CRF receptor signaling pathways in the permeability of the intestinal barrier. Pigs were either kept with the sow to provide unweaned controls or weaned at 19 days-of-age. Permeability was tested *in vitro* by mounting the various intestinal tissues (jejunum, ileum and colon) inside Ussing chambers after excision from the weaned and unweaned pigs. This was done by clamping the spontaneous electrical potential that drives the passive ion transfer to zero using an external current that is passed through the epithelium. This current is known as the short circuit current (I_{sc}) and is the measure of the net active ion transport. The amount of active ion transport is then calculated by measuring the isotopic tracers (I_{sc}) in the Ussing chamber after the external current has eliminated the passive ion transport as a result of diffusion (Clarke, 2009).Transepithelial resistance is a measure of the 'tightness' of the membrane and is calculated using the I_{sc} and the potential difference in the Ussing Chamber (Moeser *et al*., 2007).

In the experiment by Moeser *et al*. (2007), weaning caused a reduction in the transepithelial resistance in the jejuna and colons of weaned piglets. This resulted in an increase in permeability, observed

through an increase of [^{3}H] mannitol flow through the jejunum and colon. Weaning also resulted in increased levels of CRF and cortisol measured 24 hours after weaning, signifying the activation of the central stress pathway in the newly weaned pig (Moeser *et al*., 2007).

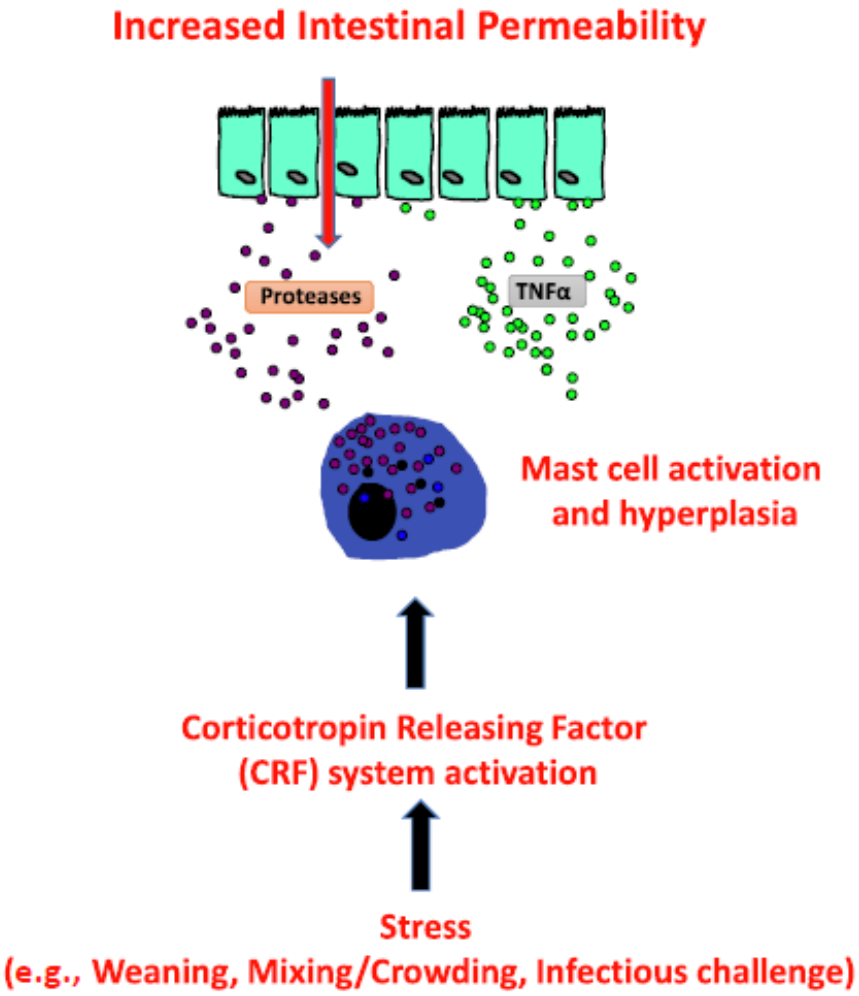


Figure 2.2 The impact of external stressors during early weaning on the gastrointestinal barrier function and therefore intestinal permeability (Moeser *et al*., 2017)

Corticotropin releasing factor receptor expression was found to be higher in the jejunum of pigs that were weaned at 19 days-of-age compared to the control unweaned pigs (Moeser *et al*., 2007). The upregulation of the CRF system has also occurred under other stressors including stress associated with mixing with other pigs as well as disease challenges (Boyer *et al*., 2015; Li *et al*., 2017).

The CRF system facilitates its effects through the activation of mast cells, which when activated, release several mediators that cause inflammation, including prostaglandins, cytokines, histamine and proteases. These mediators increase the secretory activity and motility of the intestine in an attempt to 'flush out' the detected pathogens and increase the recruitment of other inflammatory cells into the affected area to manage the infection (Smith *et al*., 2010).

Smith *et al*. (2010) demonstrated that as weaning age increased from 15 to 23 days-of-age, jejunal barrier function was improved as seen through higher transepithelial resistance and lower [^{14}C] inulin and [^{3}H] mannitol permeability in Ussing chambers. These authors also demonstrated that any stress occurring during the early neonatal period has long-lasting effects on the function of the intestinal barrier in pigs. Piglets that were weaned at 15 days-of-age had lower levels of transepithelial resistance and increased permeability at nine weeks-of-age compared to their counterparts that were weaned at 28 days; signifying disturbances persisting into adult life. Mast cell numbers were also still elevated 20 days post-weaning in the jejunal tissues of pigs that were weaned earlier (Smith *et al*., 2010).

Pohl *et al*. (2017) demonstrated that piglets which are weaned earlier (15 days-of-age) compared to their later-weaned counterparts (28 days-of-age) exhibit higher frequencies of diarrhoea that persist into adulthood, and 40% of piglets that had early weaning stress had chronic relapsing diarrhoea until 20 weeks-of-age. In addition to this, piglets exposed to early weaning stress had a higher number of intestinal mast cells in their colon (at seven weeks-of-age) as well as the ileum (at both seven and 20 weeks-of-age). Disturbances in the gastrointestinal barrier structure and function early in the pig's life can have consequences on disease susceptibility and immune and nervous system function later in life, negatively affecting growth rate and feed efficiency (Smith *et al*., 2010; Pohl *et al*., 2017).

2.5 The effect of excess crude protein on the gut health of weaned piglets

The general term 'gut health' refers to the multifaceted interactions between the gut mucosa, nutrition and the microbiota present within the GIT and how these influence one another. 'Good' gut health can be achieved when all these components are in balance and there is homeostasis within the GIT. Any disturbance of the delicate balance may result in sub-clinical disease (Pluske *et al*., 2007).

High CP diets are routinely fed to weaned piglets due to their limited capacity for feed intake during this critical growth period as well as their high inherent capability for protein accretion (Nyachoti & Jayaraman, 2016). The development of the piglet's digestive system is incomplete at earlier commercial weaning and full maturation of the gut and intestinal enzymes only occurs at 12-14 weeks-of-age (Moeser

et al., 2017). Incomplete digestion of dietary CP and the surplus of protein in the distal portion of the small intestine, results in excessive microbial proliferation of pathogenic bacteria such as enterotoxigenic *Escherichia coli* (ETEC) (Prohaska & Baron, 1980; Wellock *et al*., 2008). Enterotoxigenic *Escherichia coli* is a group of bacteria that produce enterotoxins (either heat-labile (LT) or heat-stable (ST)) that act on enterocytes (Nagy & Fekete, 1999). In addition, ETEC have a very specific pathogenesis; the *E. coli* organisms colonise the surface mucosa of the small intestine and secrete either LT or ST toxins, which result in a net secretory state, as seen in Figure 2.3. Once the ETEC are bound to the intestinal cell membranes, the enterotoxin is endocytosed by the cell and disrupts the metabolism and processes within the cell. Due to increased presence of ions in the lumen, water is drawn out of the cells and into the gut, resulting in osmotic diarrhoea (Nataro & Kaper, 1998).

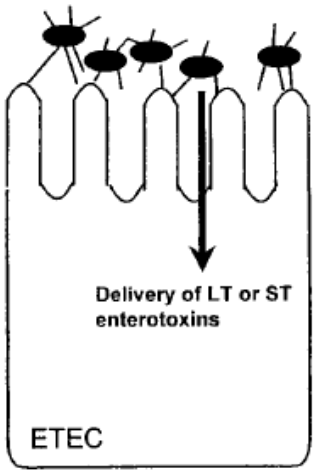


Figure 2.3 The pathogenesis of enterotoxigenic *Escherichia coli* (ETEC) (Nataro & Kaper, 1998)

Colibacillosis is the disease most frequently associated with ETEC and is debilitating for the pig industry worldwide due to its negative effects on the growth and performance of weaners, as well as the costs involved in treatment of the disease (Opapeju *et al*., 2010). Dysbiosis in the GIT of pigs after weaning is commonly referred to as post-weaning diarhoea (PWD) (Kim *et al*., 2011) or post-weaning enteric disorders (PWED) (Wellock *et al*., 2006). Post-weaning diarrhoea manifests itself in the pig by causing either mild or severe diarrhoea, commonly referred to as scours. By analysing the faecal score of young

pigs, one is able to rapidly determine the health status of the herd, upon which the causative effects of a detected problem can be investigated (Kim *et al*., 2011; Gresse *et al*., 2017).

Diets high in CP provide an ideal substrate for fermentation by pathogenic bacteria and this fermentation produces several toxic metabolites. The metabolites are harmful to intestinal cells, causing damage and irregular changes in the intestinal morphology such as increased crypt depths which in turn negatively affect digestion and absorption (Nyachoti & Jayaraman, 2016). The reduction of the VH:CD causes increased water secretion from the crypt into the gut lumen, causing diarrhoea. In addition, the decreased absorption in the small intestine results in a significant amount of unabsorbed nutrients entering the hindgut of the pig (Montagne *et al*., 2004). In a trial conducted by Htoo *et al*. (2007), the concentration of microbial metabolites produced from the degradation of excess CP in the diet was higher in the caecum than in the ileum, indicating that the caecum is the main site of microbial fermentation of excess undigested protein due to the longer digestal retention time.

In an experiment by Opapeju *et al*. (2008), pigs fed diets high in CP (21%) had higher ammonia nitrogen concentrations in their caeca compared to pigs fed lower CP diets. These pigs also had deeper crypts and reduced VH:CD in the ileum, duodenum and jejunum. Pigs that had the highest level of ammonia in their caeca also had the worst faecal scores, indicative of diarrhoea. Thus, high dietary CP increased ammonia levels in the gut, which resulted in increased intestinal damage and diarrhoea. A high dietary CP level has the ability to increase intestinal pH due to its buffering capabilities (Partanen & Mroz, 1999), and to compensate, the stomach secretes more HCl in an attempt to lower the pH to favourable levels in order to ensure the efficient digestion of protein. Early-weaned pigs are incapable of producing sufficient levels of HCl in the stomach to counteract this increase in pH, which is one of the causative factors of gut disturbances in young pigs after weaning (Htoo *et al*., 2007).

Tumor necrosis factor-α, an important pro-inflammatory cytokine, is present in the serum of piglets fed high CP diets even in the absence of an ETEC infection, due to its sensitivity to specific and non-specific stimuli. This indicates that high levels of protein in the diet can initiate an acute phase immune response in the animal (Opapeju *et al*., 2010).

Lactobacilli have proven to promote good intestinal health and maintain the delicate balance of microbial populations due to their ability to produce lactic acid. Lactic acid decreases the intestinal pH and therefore provides unfavourable growth conditions for enterobacteria such as *E. coli* (Canibe & Jensen, 2003; Wellock *et al*., 2006). The ratio between the *lactobacilli* and coliforms (L:C) is considered an indicator of gastrointestinal health (Muralidhara, 1977). Suckling piglets have high concentrations of *lactobacilli* in their upper GIT due to the fermentation of lactose present in sow milk, which is their primary

nutrient source during this period. During the first seven days after weaning, *lactobacilli* numbers drastically drop, which results in a decrease in the L:C (Wellock *et al*., 2006). Under normal intestinal conditions (in the absence of a disease challenge), pigs adapt quickly to diets comprising solely of solid feed, the gut microflora stabilises and the L:C increases to satisfactory levels once again. However, diets too high in protein cause a drop in the favourable *lactobacilli* population, and the excess protein available for fermentation increases intestinal pH, causing the proliferation of ETEC and other pathogens, increasing the pig's risk for PWD dramatically (Wellock *et al*., 2006).

2.6 The effects of feeding low crude protein diets supplemented with crystalline amino acids

Post-weaning diarrhoea

Kim *et al*. (2011) conducted an experiment to ascertain whether feeding a reduced CP diet to pigs after experimental infection with *E. coli* would reduce the amount of PWD cases during the first two weeks after weaning. Fifty percent of the pigs fed the high protein experimental diet (23%) had PWD during the experimental period, whereas only 16-18% of pigs on the lower CP diet (18.5%) had diarrhoea. In a trial done by Wellock *et al*. (2008), the *E. coli* challenge had a much larger detrimental effect on the weaned pigs that consumed high protein diets (23%) compared to the pigs who were offered the low CP diets (13%). Pigs that were infected and received the high CP diet experienced a 42% decrease in ADG compared to their non-infected counterparts on day 3-6 of the trial. The pigs on the low CP diet, however, only had a 25% decrease in ADG compared to the uninfected pigs. This can be attributed to excess protein availability in the distal portion of the small intestine in pigs fed the high CP diet providing a favourable substrate for the increased proliferation of ETEC. Therefore, pigs that consume high CP diets will experience greater penalties on growth and performance during post-weaning colibacilliosis than pigs on a lower CP diet. The potential benefit of feeding low CP diets is greater in sub-optimal environments that pose a higher infection risk such as commercial pig facilities and especially in early weaning systems where weaning occurs at 28 days or earlier (Wellock *et al*., 2008).

As previously discussed, the ability of ETEC to cause disease relies on the necessity to colonise the gut through initial adherence to the enterocytes. In an experiment done by Opapeju *et al*. (2015), ETEC K88 organisms which had successfully adhered to intestinal mucosa were identified in 80% of pigs receiving a high CP (22.2%) diet, compared to only 20% of pigs who consumed the low CP (17.3%) diet. This indicates that the colonisation of ETEC in the small intestine was drastically reduced by decreasing

the dietary CP content and thereby proving that protein content in the diet is a crucial factor governing the adhesion of ETEC through surface fimbriae on the enterocytes.

Performance implications for weaned pigs

Dietary CP can be reduced by four percentage points without affecting the growth performance and carcass quality in pigs provided that the diets are adequately balanced for essential amino acids (Yue & Qiao, 2008). In swine, Lys is the first limiting amino acid, followed by Thr, Met, Trp, Val and Ile (Htoo, 2017). These amino acids form part of the essential amino acids (EAA), meaning that the animal's body is incapable of producing these endogenously and therefore, need to be supplied in the correct proportions and ratios to one another in the diet (Mavromichalis *et al*., 1998; Theil *et al*., 2004). Since Lys is the first limiting amino acid in pigs, diets are formulated on a standardised ileal digestible (SID) basis for amino acids in relative proportion to Lys (NRC, 1998). It is important to note that when dietary CP is reduced beyond 4% points, pig performance may be markedly reduced due to one or more of the NEAA or other EAA (beyond the first five limiting) becoming limiting. Nitrogen may become deficient for the synthesis of NEAA, and other important physiological functions are also negatively impacted, such as the formation of digestive enzymes and mucosal proteins (Htoo, 2017).

An experiment was conducted by Yue & Qiao (2008) to determine the effect of low CP diets on the serum levels of Arg, proline (Pro), Gln, Glu and the total concentration of NEAA. When reducing dietary CP and balancing for EAA in starter pig diets, it is important not to exceed a SID Lys: CP of 6.9% (or 7.4% on a total amino acid basis) as this ensures that the NEAA do not become limiting (Htoo, 2017). The performance of weaner pigs (7-10 kg) was not affected when dietary CP was reduced by 4% when sufficiently balanced for EAA on a SID basis. However, when the reduction in dietary CP was in excess of 5%, the growth performance of pigs was markedly reduced and there were negative alterations in intestinal morphology and functionality. These included a reduction in jejunal and duodenal villi height and diminished activity of lactase and sucrase in the jejunum, which are essential for the digestion of already-limited nutrients. These enzymes, together with maltase, are important indicators of the level of gut maturity in piglets, and therefore is indicative of the negative impact a too drastic reduction of CP can have on the development of the GIT in young piglets after weaning (Yue & Qiao, 2008).

Arginine, although not typically classified as an EAA, is one of the conditionally essential amino acids, meaning that it is essential in certain circumstances, one of them being in immature animals. Arginine plays a crucial role in the synthesis of protein, creatine and nitric oxide to name a few (Wu *et al*., 2004);

and piglets are capable of synthesising some of their requirement for Arg from Pro in the small intestine (Kristine *et al*., 2006). However, because weaning results in atrophy of some important morphological structures in the intestine, the rate at which Arg is produced from Pro is decreased. Supplementing Arg into the diet of weaned pigs may be necessary to prevent a sudden drop in growth and performance after weaning (Yue & Qiao, 2008).

In a study done by Peng *et al.* (2016), growth performance of starter pigs was significantly reduced when dietary CP was reduced from 20% to 13.9%, but performance was not negatively impacted when reducing the CP to 15.3%. This was due to insufficient total N presence in the 13.9% CP diet. In fact, the total N was 30.5% lower than the diet containing 20% dietary CP, which influenced the ratio between N from EAA and NEAA. This ratio should ideally be in a constant proportion of 50:50 for growing pigs (Lennis *et al*., 1999) but was subsequently a lot higher at 62: 38 in their study.

By reducing the dietary CP level too drastically, the weights of organs such as the liver and pancreas may be negatively impacted, which could result in the reduced secretion of pancreatic protease crucial for the digestion of already-limited protein in the diet (Peng *et al*., 2016). However, Kerr *et al*. (2003) found a positive impact of reduced CP on kidney weights, in that a reduction of dietary CP results in less workload for the kidneys to excrete nitrogenous waste, and therefore the weight of the kidneys was lower. In the trial done by Peng *et al*. (2016), feeding a low CP diet had negative repercussions on the innate immunity of pigs even though all the EAA were balanced. This was seen through a lower percentage of $CD3^{+}$T cells as the CP level in the diets declined.

Intestinal health

Htoo *et al*. (2007) conducted a trial in which newly weaned pigs were given either a diet comprising of 24% CP or a reduced-protein diet of 20% CP supplemented with Lys, Met, Thr, Ile, Trp and Val. The growth performance of the two different groups of pigs did not differ significantly over the experimental period in terms of ADG, G: F, ADFI as well as final body weight (BW). Additionally, reducing CP did not have a negative impact on the standard ileal digestibility of amino acids when diets were balanced to achieve a similar SID amino acid supply. Reducing the CP content of the diet did however result in a 32% reduction of ammonia N in the caeca as well as lowering the concentration of putrescine by 39%. Ammonia has a negative impact on the growth and differentiation of the epithelial cells in the small intestine and as a result, is one of the causative factors of PWD (Dong *et al*., 1996; Gaskins *et al*., 2001). In addition, putrescine along with cadavarine are toxic amines that cause a reduction in growth performance in pigs and are produced as a result of microbial fermentation of excess dietary CP. Dietary protein is broken down via

proteolysis into individual amino acids which can either be further degraded through deamination, which produces ammonia; or via decarboxylation which produces amines (Urlings *et al.*, 1993; Htoo *et al*., 2007).

Nyachoti *et al*. (2006) observed a steady decline of ammonia (measured as mg/L) in the duodenum, jejunum and ileum as dietary CP was reduced by 2% points each time from 23% to 17%. The largest reduction in ammonia occurred in the ileum, from 71.96 mg/L to 37.83 mg/L when CP was decreased from 23% to 17%. The detoxification of ammonia by the liver places a high metabolic demand on the animal's body; therefore, by reducing the ammonia concentration in intestinal contents, the efficiency by which the animal utilises other nutrients such as energy and amino acids is improved (Jensen, 1998).

Environmental pollution

Feeding diets high in CP not only results in disturbances in the digestive system of the newly weaned piglet, but also results in excess harmful nitrogenous compounds to be excreted into the environment. Every 1% reduction in dietary CP results in a 9% reduction in N excretion from pigs in a commercial environment (Htoo, 2017). Nitrogen emissions from pig houses in the form of ammonia pose a substantial environmental risk in terms of atmospheric quality (Apsimon & Kruse-Plass, 1990). In an experiment done by Canh *et al*. (1998) with growing pigs, reducing the dietary CP content from 16.5% to 12.5%, but maintaining similar levels of EAA, did not result in a reduction in N content of the faeces, but did cause a substantial reduction in urinary N content by 45%. Additionally, decreasing the CP to 12.5% resulted in a reduction of the total N concentration of the slurry by 36% and reduction of slurry pH by 1 unit. The pH of slurry is affected by the level of ammonium in the slurry content, and a higher level of ammonium results in a higher pH (Sommer & Husted, 1995). Therefore, the trial conducted by Canh *et al*. (1998) showed that the reduction of slurry pH was due to a reduction in the slurry ammonium content. There is a balance between ammonia and ammonium concentrations in the slurry, and these are usually in equilibrium (NH_3: NH_4). At higher pH values, this balance is disrupted and more NH_3 is produced and therefore emitted into the atmosphere (Canh *et al*., 1998). Therefore, reducing the CP content of the diet caused a decline the NH_4 content, which resulted in reduced NH_3 emissions into the environment. Ammonia emissions from pig houses represent 64% of the total NH_3 emissions of a pig farm (Pain *et al*., 1997), and therefore, reducing these emissions can limit the negative impact intensive pig production has on the environment.

Reducing the dietary CP level from 19.6% to 16.8% had no effect on the growth performance or the amount of N retained in 10-20 kg pigs in a trial done by Gloageun *et al*. (2014), but reduced N excretion by 29%. The pigs that were fed the 16.8% CP diet retained 71% of the N consumed and 83.8% of the N absorbed, compared to their counterparts on the 19.6% CP diet who only retained 63.9% of the consumed

N and 74.4% of the absorbed N. The N digestibility was not negatively impacted by reducing the dietary CP, which confirmed that the excess N was primarily excreted in the urine. The reduction of N excretion by 29% when CP was decreased by 3% points is in agreement with Htoo (2017) which states that a reduction in dietary CP by 1% reduces the N excretion into the environment by 9%. Therefore, reducing the CP by 4% points in young piglet diets has the massive potential to reduce N excretion by at least 40%. As expected, Gloaguen *et al*. (2014) saw a reduction in growth performance when the CP reduction exceeded 4% points. Gloaguen *et al*. (2014) therefore concluded from their trial that it is possible to reduce the dietary CP in 10-20 kg pigs by up to 4% points, so long as in addition to the usual supplemental amino acids that are routinely added (Lys, Met, Thr and Trp); Val, Leu, Ile, His and Phe are also considered.

2.7 Antibiotic growth promoters and their impact on gut health

Antibiotics have been used for around 50 years in animal agriculture particularly in swine and poultry production, for their direct and indirect effects on improving animal production by modulating the microbial population within the GIT. Antibiotic growth promoters have consistently demonstrated beneficial effects on pig weight gain and feed efficiency, which although more commonly used in younger piglets, still infers beneficial effects in grower and finisher pigs (Dibner & Richards, 2005).

The rationale behind using AGPs in agriculture is based on the assumption that any intestinal bacteria, either beneficial or pathogenic, has a negative impact on growth and performance due to their metabolic activities. There are several suggested modes of action of AGPs for modulating their effects within the animal body, such as reducing the microbial metabolites that are thought to depress growth performance, decreasing the use of nutrients by microbial populations, preventing sub-clinical infections, and lastly causing thinning of the intestinal wall which enhances nutrient uptake and therefore better utilisation of dietary nutrients (Gaskins *et al*., 2002). Microbial activity in the large intestine (caecum and colon) is beneficial to the host as it contributes 5-20% of the pig's energy supply through the fermentation of previously undigested feed ingredients (high fibrous material) (Friend *et al*., 1963).

The small intestine however, is the major site of nutrient absorption and therefore high microbial activity in this section of the gut could influence the uptake of nutrients compared to the alternate use by microbes (Anderson *et al*., 1999; Gaskins *et* al., 2001). The proximal portion of the small intestine (duodenum and jejunum) is characterised as having a faster rate of digesta movement compared to the ileum, and therefore, the amount of bacterial colonisation is lower in this region (the rate of digestal flow and bacterial washout is faster than the growth rates of most bacterial species). The ileum has much higher

counts of bacteria (108 per mL of intestinal contents) and produces a substantial amount of hydrogen gas, indicative of high microbial activity (Jensen & Jorgensen, 1994; Gaskins *et al*., 2002). Bacteria in the small intestine contend with the pig for dietary AA and energy, and as much as 6% of the ingested net energy in a typical swine diet may be utilised by the intestinal bacteria instead of by the host itself (Vervaek *et al*., 1979; Hedde *et al*., 1986). Beneficial bacteria in the gut convert glucose into lactic acid which increases the peristalsis of the intestine, further limiting the time which digesta spend in the intestine and therefore negatively affecting absorption even more (Saunders & Sillery, 1982). Certain bacteria stimulate the breakdown of mucus in the small intestine, which negatively affects the gut barrier and causes the repartition of nutrients away from growth into the production of more mucus to protect the barrier from further damage (Deplancke & Gaskins, 2001). Lastly, intestinal bacteria degrade the dietary AA into toxic metabolites such as ammonia, amines and phenols which further negatively affect the gut due to their inflammatory effects (Macfarlane & Macfarlane, 1995).

Most AGPs target gram-positive bacteria in the intestine of pigs as these bacteria are facultative anaerobes (meaning they can survive with or without oxygen), and due to their abundance in the small intestine, have been linked to the depression of growth performance in pigs (Stewart, 1997; Gaskins *et al*., 2002). One of the gram-positive bacteria of interest is *Lactobacillus*, which is responsible for bile acid biotransformation in the small intestine. The degradation of bile acid by this group of bacteria results in impaired lipid breakdown and absorption as well as the production of toxic products, which negatively affects the growth of the animal (Gaskins *et al*., 2002). Interestingly, many gram-positive bacteria are routinely used as probiotics (*Lactobacillus* and *Enterococcus*) for improving the health status of production animals and thereby improving growth and performance (Jonsson & Conway, 1992).

As of the 1st of January 2006, the European Union (EU) banned the inclusion of all antibiotics used as AGPs in animal diets (Heo *et al*., 2013). Some countries such as Sweden had already refrained from AGP use since 1986 and Denmark in 1998. Other countries such as Finland banned certain antibiotics such as carbadox and olaquindox in September 1999 (Laine *et al*., 2004). North America began following the EU by initiating bans on certain, if not all, AGPs used in animal agriculture. This was due to increased public pressure and concerns surrounding antibiotic resistance and residues in animal products, as well as what effect their continued use will have on the international trade of meat products, especially into countries where AGPs are forbidden (Liao & Nyachoti, 2017).

The result of the sudden removal of AGPs in Sweden and Denmark without proper strategic adjustments to diets or the use of other potential additives, caused a drastic increase in PWD and mortalities after weaning (Heo *et al*., 2013). The age at which pigs reached 25 kg body weight increased by 5-6 days

in Sweden and the use of therapeutic antibiotics was elevated by a substantial 25% in order to treat sick animals and prevent mortalities (Wierup, 2001b). As alternatives to AGPs in animal diets, some farmers include ZnO and $CuSO_4$ in order to control PWD, but due to the more stringent regulations on their accumulation in the environment, these too have been excluded from swine diets in some countries (Verstegen & Williams, 2002; Heo *et al*., 2013).

The complete removal of AGPs from swine diets, especially in weaners, who are notoriously sensitive to diarrhoea after weaning, is met with apprehension. Farms that do attempt to go AGP-free have in the past had outbreaks of diarrhoea, which resulted in stronger doses of therapeutic antibiotics to try and curb a potential disaster on their farms (Wierup, 2001a). Laine *et al*. (2004) conducted a study in Finland on 73 pig farms after the use of two commonly used AGPs, olaquindox and carbadox, was banned in 1999. The production data from these farms was evaluated over a one-year period (divided into three four-month periods) in order to ascertain the possible negative effects of AGP removal. The parameters that were measured in this study for each of the 73 farms were the number of sows on each farm, the number of piglets weaned per sow per year, the number of piglets weaned in each four-month period, the age at weaning and the percentage mortalities. On 29 farms, the four months' production data before the AGP withdrawal was also included and interestingly; after the discontinuation of AGPs, there was no significant increase in PWD. Only four farms participating in the 16-month follow up period (May 1999 - August 2000) had a higher use of antibiotics during May - August 2000 compared to May - August 1999 for the treatment of PWD. Of the group of 40 farms participating in the 12-month follow up period after the ban of AGPs (September 1999 - August 2000), only four farms experienced higher use of therapeutic antibiotics during May - August 2000 compared to September - December 2000. There were no other pertinent problems that occurred after the exclusion of AGPs from the diet, such as an increase in the incidences of diarrhoea or an increase in the age of weaning. It is important to note that before the removal of AGPs from the diets, farmers were informed of correct weaning procedures to improve the health status of the piglets and give the weaners a better chance of survival. These procedures included weaning the piglets slightly later (around 30 days-of-age) at a minimum weaning weight of 7.9 kg to optimise their performance. Weaning at an earlier age places the piglets under a significant amount of stress which results in higher incidences of PWD (Smith *et al*., 2010), and the adverse effects of PWD may be even further exacerbated in an environment where AGPs are prohibited. This study demonstrated that AGPs can be successfully removed from the diets of weaner pigs, but the diets, health status, and farm management practices must be optimised in order to avoid the increase in PWD and therefore the unavoidable use of therapeutic antibiotics (Laine *et al*., 2004).

2.8 Alternative dietary additives to antibiotics

There are several possible dietary strategies and additives that can be used as alternatives when AGPs are banned from pig diets that can assist in improving gut health, reduce mortalities and increase performance of weaners, and these will be discussed briefly below.

Zinc oxide (ZnO)

Zinc is an essential trace mineral for swine and if it is deficient in pig diets, will negatively affect growth and deplete the enzymatic activity of tissues. It is important in DNA and RNA synthesis and is a component in enzymes such as transferases and certain digestive enzymes. Lastly, it has critical functions in protein, carbohydrate and lipid metabolism as well as insulin activity (Prasad *et al*., 1969; Li *et al*., 2006). According to the National Research Council (NRC), zinc is only required in small doses in weaner pigs and its recommended level in weaner diets is only 100 mg/kg. However, much higher doses (up to 3000 mg/kg) are occasionally used in order to have a growth promoting function and to drastically reduce the incidences of PWD (Hill *et al*., 2000; Højberg *et al*., 2005). Some of the proposed modes of action for these extremely high levels of zinc include modulating and stabilising the microbiota in the intestine, increasing gene expression of insulin-like growth factor (IGF) I and II in the mucosa, increasing the expression of antimicrobial peptides and lastly, possible bactericidal effects (Heo *et al*., 2013). Interestingly, in a trial done by Højberg *et al*. (2005), pigs fed ZnO had lower numbers of *Lactobacilli* but higher numbers of *Enterococci* and coliforms, which was not the desired outcome, as the beneficial bacteria were suppressed but the pathogenic bacteria were not. This coincides however with the effects of AGPs, which suppress gram-positive bacteria but not routinely the group of gram-negative bacteria. Due to the potential environmental pollution of zinc, the use of ZnO as an alternative to AGPs has also been banned in some parts of the world (Carlson *et al*., 2004).

Organic acids

The digestive system of pigs whether they are weaned at 21 or 28 days is still immature and the secretion of digestive enzymes and HCl is limited. The gastric pH of suckling and weaner pigs is 2.6-5 compared to adult pigs that have a gastric pH of 2-3, which is essential for the effective digestion of nutrients (Ravindran & Kornegay, 1993). Suckling pigs maintain a lower pH than that of weaned pigs due to their consumption of lactose in sow milk, which ferments to lactic acid in the gut, thereby lowering the pH (Cranwell *et al*., 1976). To lower the gastric pH of weaner pigs, organic acids such as citric, butyric, lactic, fumaric and formic acids are routinely added in diets to improve gut health and performance (Tsiloyiannis

et al., 2001). There are different modes of action for each of the organic acids that are used in pig diets, which will affect the efficacy of each. The age of the animal in which each is used and the buffering capacity of the diets in which they are included may counteract the organic acids. It is therefore important to use each organic acid in the appropriate manner (Ravindran & Kornegay, 1993).

Li *et al*. (2008) conducted an experiment whereby certain organic acids were able to reduce the *E. coli* numbers in the stomach of piglets that had been infected with ETEC but did not have any effect on the pH of the gut. This suggests that certain organic acids may modulate their effects against pathogenic bacteria by having direct antibacterial activities unrelated to the effect on pH. One of the proposed modes of action is the direct effect organic acids have on the bacterial cell itself, by diffusing across the cell membrane and causing a disruption in the pH of the cytoplasm. Organic acids are able to do this by dissociating to produce protons (H^+) and anions ($RCOO^-$) which will alter the ionic balance within the cell. This increase in cellular pH causes the denaturation of enzymes, elevates the turgor pressure within the cell and negatively affects the purine base integrity. All these processes are necessary for the growth and viability of bacterial cells and therefore organic acids provide a potential alternative for AGPs in swine diets (Heo *et al*., 2013).

Prebiotics

Prebiotics are a group of non-digestible feed ingredients that have been selectively fermented and cause specific changes in the microbiota of the digestive system to improve gut health (Gibson & Roberfroid, 1995). For an ingredient to be classified as a prebiotic, it has several criteria which it needs to fulfill: it needs to withstand the acidity of the stomach, not be hydrolysed by the animal's digestive enzymes and not be absorbed into the bloodstream via the gut wall (Roberfroid, 2007). In addition, it needs to remain in the GIT and exert its effects on the intestinal bacteria by providing a fermentable substrate to the beneficial bacteria associated with good gut health such as *Lactobacilli*, *Bifidobacteria* as well as *Eubacteria*. Based on these criteria, only fructooligosaccharides (FOS) and transgalactooligosaccharides (TOS) qualify as true prebiotics (Heo *et al*., 2013). Houdijk *et al*. (2002) found that FOS and TOS can reduce the number of aerobic bacteria and reduce the pH in the ileum of weaned pigs but not in the faeces; therefore, their effects may be modulated before the caecum. In a trial done by Mikkelson *et al*. (2004), FOS increased the population of yeast in the GIT of piglets. There are also other experiments in which oligosaccharides had other beneficial effects on the GIT of weaner pigs, such as improving the morphology of the gut (Liu *et al*., 2008) and increasing butyric acid concentration (Shim *et al*., 2005), but there are several discrepancies among different trials. Therefore, data on prebiotics should be used with caution, as

the effects of these products differ according to the type and dosage of the oligosaccharides used and age of the animals (Heo *et al*., 2013).

Dietary fibre and resistant starch may have potential prebiotic abilities. Kiarie *et al*. (2008) found that when piglet diets were supplemented with products from non-starch polysaccharide enzyme hydrolysis, a reduction in the severity of enteritis in piglets who had been infected with ETEC was observed (Kiarie *et al*., 2008). There may also be some synergistic effects that exist between prebiotics and probiotics exerting their benefits in conjunction with one another (Heo *et al*., 2013).

Probiotics

Probiotics are a group of live microorganisms that are routinely used as feed additives and if supplemented in sufficient quantities, can positively influence the gut health status of the host animal by modifying the microbiota in the gut (Angelakis, 2016). Probiotics need to withstand the acidic environment of the stomach and bile acids so that they can pass through into the intestine where they can exert their beneficial effects (Hossain *et al*., 2017; Liao & Nyachoti, 2017). The three types of organisms that are frequently included under the probiotic group include yeast, certain *Bacillus* strains and lactic acid-producing bacteria, such as *Bifidobacterium, Enterococcus* and *Lactobacillus* strains (Stein & Kil, 2006). The *Bacillus* species are soil bacteria that are carefully selected for their use as probiotics for the antibiotic substances which they produce (Cho *et al*., 2011).

There are numerous proposed modes of action for probiotics that enable them to promote gut health and performance as depicted in Figure 2.4. Certain probiotics can modulate the immune system without initiating activation, which would expend a lot of energy in the pig. Probiotics enhance macrophage activity and recruit anti-inflammatory cytokines to prepare the immune system for a possible infection (Cho *et al*., 2011). Probiotics can also produce bacteriocins and organic acids which are microbicidal against pathogens in the gut and can increase the production of short chain fatty acids which reduce digesta pH and therefore assist in depressing the proliferation of pathogenic bacteria (Heo *et al*., 2013). The reduction in gut pH through the fermentation of carbohydrates such as lactose to lactic and acetic acid by lactic acid bacteria, may potentially compensate for the low HCl secretion in young weaner pigs (Liao & Nyachoti, 2017).

Competitive exclusion is arguably the most important mode of action of probiotics whereby beneficial bacteria either compete with the pathogenic bacteria for nutrients in the gut or inhibit the adhesion of pathogenic bacteria such as ETEC on the intestinal mucosa (Cho *et al*., 2011; Heo *et al*., 2013). Not all probiotics are identical, and as a result, there are many varying scientific opinions whether treatment with probiotics will result in any improvement in gut health and performance. It is therefore crucial that the

correct strain of probiotic is used, at the correct concentration and lastly at the correct time in the animal's life (at weaning or before weaning) (Cho *et al*., 2011).

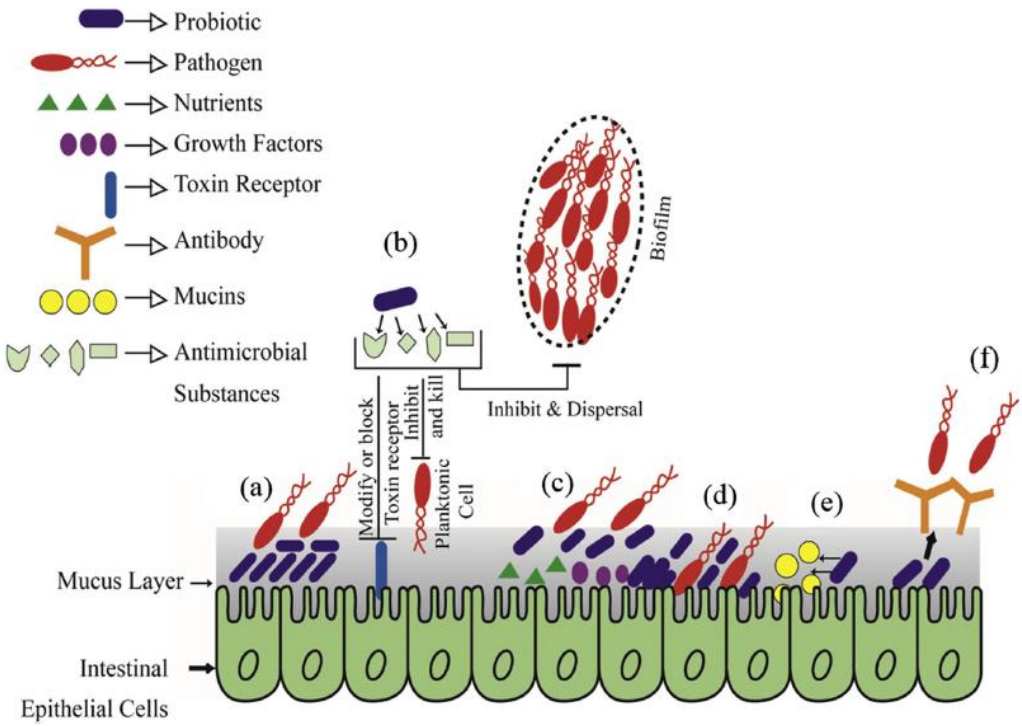


Figure 2.4 The various modes of action of probiotics used in animal agriculture: (a) competitive exclusion, (b) the production of antimicrobial substances, (c) competition against pathogenic bacteria for nutrients, (d) adhesion to mucosa, (e) enhancing the functionality of the barrier & (f) stimulating the immune system by increasing IgA secretion (Hossain *et al*., 2017)

2.9 Probiotics as a suitable alternative to antibiotic growth promoters

The correct balance of microflora in the GIT of animals is essential for optimum health, welfare and performance, and play an important role in the innate immune system (Cho *et al*., 2011). The GIT of the pig is the animal's first line of defense against any invading pathogens, particularly from the gut lumen and therefore it is especially crucial that this part of the immune system is not compromised in any way and that the delicate microbiota of the gut is in balance (Liao & Nyachoti, 2017).

Selecting the correct probiotic depends on the intended use in the animal diet. There are several characteristics of probiotics that need to be considered in order to use the selected probiotic as efficiently as possible. As described by Liao & Nyachoti (2017), probiotics can either be sold as single strain or multi-species micro-organisms and can either be found naturally within the digestive system of the animal of interest (referred to as autochthonous) or be a 'foreign' organism (referred to as allochthonous). Probiotics can either be of bacterial or non-bacterial origin such in the case of yeast or fungal probiotics and can either be spore-forming or not. Bacteria which can sporulate are able to protect themselves from otherwise harsh and unfavourable conditions, and therefore can resist high temperatures, dessication as well as ultraviolet (UV) exposure from direct sun. It is due to these characteristics that spore-forming bacteria such as *Bacillus* species are used in animal feeds as a large majority of pig and poultry feeds are pelleted at high temperatures (Liao & Nyachoti, 2017).

Probiotics have shown to significantly improve the ADG, ADFI as well as FCR in pigs (Liao & Nyachoti, 2017). In the study done by Liu *et al*. (2015), the supplementation of *Lactobacillus brevis* into the diets of weaned pigs significantly improved ADG, ADFI and G: F. In addition, pigs had improved faecal scores with less diarrhoea incidences as well as reduced faecal coliform load. Le Bon *et al*. (2010) had similar findings after the supplementation with a probiotic combination consisting of *Saccharomyces cerevisiae spp. boulardii* and *Pediococcus acidilactici* organisms. These authors found that the supplementation of the probiotic combination not only improved the FCR of weaned pigs, but also reduced the faecal *E. coli* levels.

Nutrient digestion can be explained simply by splitting it into two processes: enzymatic degradation of nutrients and the fermentation of less digestible feed ingredients by the intestinal microbes particularly in the hindgut of the pig (Williams *et al*., 2001; Liao & Nyachoti, 2017). Several studies on probiotics have shown that they are able to improve the digestibility of CP, energy and crude fibre from the diet (Giang *et al*., 2010; Meng *et al*., 2010). The improvements in digestibility are possibly due to the heightened levels and activity of digestive enzymes as a result of the increased fermentation and digestion associated with probiotic supplementation (Cho *et al*., 2011; Upadhaya *et al*., 2015). An example is *Lactobacillus*, which produces proteolytic enzymes and lactic acid (Yu *et al*., 2008).

As probiotics are live, viable microorganisms, their safety needs to be established before they can be successfully used in animal feeds and therefore, there are a few crucial points to consider in order to verify whether a probiotic is stable and safe to use. The microorganism should not cause any skin, eye or mucosal membrane irritation to the workers who handle the product, cause gastrointestinal or systemic infection in the pigs or workers, release any enterotoxins and lastly, the strain that is used should not be

associated with any human or animal disease. The probiotic should also not release any infectious or toxic substances into the environment and lastly, should not confer any antibiotic resistant genes to other microorganisms (Doron & Snydman, 2015; Bajagai *et al*. 2016).

The use of *Bacillus* species of bacteria as probiotics in animal diets are steadily increasing due to their robustness under multiple conditions. These bacteria can produce spores which are able to withstand high temperatures encountered during pelleting and feed production and can survive in extended periods of storage. Spore-forming bacteria such as *Bacillus amyloliquefaciens* also have the ability to produce extracellular enzymes that could potentially improve nutrient digestion, such as cellulase, α-amylase, protease and metalloproteases (Lee *et al*., 2008; Bajagai *et al*., 2016).

Bacillus amyloliquefaciens is a single strain probiotic which is registered with the European Food Safety Authority as 2 x 10^9 CFU/g *Bacillus amyloliquefaciens*. The product complies with the European Union feed additive standards with regards to microbial quality, heavy metals, toxins as well as any other undesirable substances (EFSA, 2008).

A trial was conducted by de Oliviera *et al*. (2019) whereby the efficacy of *Bacillus amyloliquefaciens* was compared to an AGP, bacitracin methylene disalicylate (BMD) under an enteric pathogen challenge in broilers. *Bacillus amyloliquefaciens* has demonstrated bacteriocidal effects on pathogenic bacteria such as *E. coli* and *Clostridium perfringens* in previous trials conducted by Diaz (2007) and Lei *et al*. (2015). In the study by de Oliviera *et al*. (2019), the authors observed a significant improvement in FCR in broilers infected with *Eimeria maxima* and *Clostridium perfringens* after supplementation with *Bacillus amyloliquefaciens* compared with the positive control broilers. The significant improvement was in line with the birds receiving the AGP treatment. These findings indicate that *Bacillus amyloliquefaciens* can successfully replace AGPs in broiler diets with no adverse effect on performance.

In addition, Lei *et al*. (2015) found that villi height, crypt depth and VH:CD was significantly improved in broilers supplemented with *Bacillus amyloliquefaciens*. In addition, caecal *E. coli* count was reduced in broilers either supplemented with virginiamycin or *Bacillus amyloliquefaciens*, compared to the control group. In a study conducted by Kinh *et al*., (2019), weaner piglets receiving *Bacillus amyloliquefaciens* supplementation had significantly improved growth performance as seen through increased ADG and feed efficiency, compared to their counterparts on the AGP-supplemented diet.

The interaction between protein level and the addition of probiotics

Several studies have been discussed thus far exploring either the effect of the reduction of dietary CP in weaner pig rations (Htoo *et al*., 2007; Peng *et al*., 2016), or the addition of probiotics as a suitable replacement for AGPs (Lei *et al.*, 2015; de Oliviera *et al*., 2019) but the interaction between low CP and probiotics is a very interesting concept and has not been well explored thus far. Bhandari *et al*. (2010) conducted a trial whereby the interaction between the addition of a non-pathogenic *E. coli*-based probiotic and protein levels in a weaner diet were explored. Pigs were either fed a diet consisting of 21% CP or 17% CP and either had no additional additives, an antibiotic additive or a probiotic additive to their feed. The findings of the trial were that the pigs on the low CP diets had the best performance compared to the high CP pigs, with or without probiotic supplementation. In addition, they found that there was a significant interactive effect between protein level and probiotic addition, with piglets consuming low CP diets plus a probiotic having improved ADG before and after inoculation with *E. coli*. The positive effects of the low CP and probiotic interaction was also inferred into gut health with pigs receiving low CP diets having significantly improved faecal scores after infection than high CP pigs. *Escherichia coli* K88 count in the ileum was also measured and it was observed the adhesion of these organisms was reduced in the low CP diets as well as in diets supplemented with either the probiotic or the AGP (Bhandari *et al.*, 2010). Garcia *et al.* (2014) also observed a positive interaction between low CP diets and the addition of a probiotic on faecal score improvement. These studies clearly demonstrate positive synergistic effects between low CP and the addition of a probiotic to weaner pigs rations.

CHAPTER 3: MATERIAL AND METHODS

This trial was approved by the Animal Ethics Committee of the University of Pretoria and the ethical clearance number of this trial is EC027-18.

3.1 Facilities

The trial was carried out in a pig grower house that contained 58 pens, but only 54 of the 58 pens were used. The pens were 3.5 m^2 and three pigs per pen were placed at a stocking density of 1.17 m^2/pig. There were nine replicate pens per treatment. Each pen was fitted with a water nipple, a single feeder trough (CAWI feeder) and partially slatted concrete floors. The solid floors of the pens were insulated with rubber mats ensuring that the newly weaned pigs were provided with a comfortable, warm environment at placement. The entire pig house and each pen was thoroughly washed and disinfected before the arrival of the new pigs. The environment of the pig house was closely controlled, and pigs were adequately protected from the elements. The temperature of the house was monitored throughout the day to ensure that the house temperature was correct for the growing pigs. Infrared heating lamps were fitted in each pen to provide a heat source to the small piglets and were removed after 21 days when the pigs were a bit older and the temperature in the house had reached a point where the pigs were generating sufficient heat without the need for additional heat sources. The temperature of the house started at 28°C for the first week and declined by 2°C each week as per the PIC Early Pig Care Manual (2017). Adequate ventilation of the pig house was maintained through the usage of three fans; two smaller fans that remained operational throughout the day and one large fan that automatically turned on when the temperature exceeded the appropriate temperature set for the week. Windows were also kept open during the day and closed to an appropriate level during the night to ensure that sufficient heat was maintained in the house, whilst ensuring adequate ventilation for the animals.

A high level of biosecurity was maintained throughout the trial to ensure a high level of health among the pigs during the trial period. All farm workers and students had to take a shower and change into overalls and gumboots, used only within the confines of the house. Farm workers and students were also forbidden to have been around any other domesticated or wild pigs within three days prior entering the pig house.

It is well accepted that pigs are highly intelligent animals and need adequate mental stimulation in order to ensure optimum welfare and well-being. For this reason, metal chains were hung between each pen so that pigs were able to interact with neighbouring pigs and play with the chains to alleviate boredom and to prevent any negative behaviour occurring; such as tail-biting and aggression.

3.2 Animals

One hundred and sixty-two Camborough-line genetic pigs were sourced from PIC Concord Piggery in Bapsfontein, South Africa. This line is obtained by crossing Landrace and Large White pig breeds. Pigs were weaned at 28 days-of-age and transported to the experimental farm on the same day. Only intact boars were used in the trial in order to exclude the additional variable of sex in the measurement of growth performance parameters. A consulting veterinarian was available during the trial to provide advice and to ensure the humane treatment of the pigs. Feed and water were provided *ad libitum* and pigs were checked twice daily. Pens were cleaned twice weekly to remove solid waste and the waste drainage system was flushed once a week with clean water.

3.3 Experimental design and dietary treatments

The experiment consisted of six dietary treatments. Crude protein level was reduced by 3% (21% to 18% in the pre-starter diets and 18% to 15% in the starter diets), two amino acid levels (recommended amino acid level or elevated by 10%) and with or without the supplementation of a probiotic additive (*Bacillus amyloliquefaciens*). The individual treatments are represented in Table 3.1 below.

Table 3.1 Description of the individual dietary treatments used in the experiment

Treatment	CP level*	Amino acid level$	Probiotic Inclusion#
1: HP	High	Recommended	-
2: HP + Pro	High	Recommended	+
3: LP	Low	Recommended	-
4: LP + Pro	Low	Recommended	+
5: LPAA	Low	110% of Recommended	-
6: LPAA + Pro	Low	110% of Recommended	+

* High CP diets are 21% and 18% for pre-starter and starter respectively. Low CP diets are 18% and 15% for pre-starter and starter diets respectively

$ Recommended amino acid level refers to levels currently utilised in South Africa

Probiotic contains *Bacillus amyloliquefaciens* bacteria and was supplemented at 500g/ton feed

A two-phase diet programme was followed from 28 days-of-age until 70 days-of-age. A pre-starter diet was fed for 21 days (from 28 to 49 days-of-age) after which a starter diet was fed for the remaining 21 days (from 50 to 70 days-of-age). The trial was run for a total of 42 days.

The high CP level of the pre-starter diet for Treatment 1 and 2 was 21%, as this was representative of a 'high CP diet' in South Africa. The level of CP was then reduced by three percentage points to 18% in Treatments 3 to 6. The high CP diet in the starter phase was 18% in Treatments 1 and 2 and was reduced to 15% for Treatments 3 to 6. The first five limiting amino acids were increased by 10% of typical South African pre-starter and starter levels in order to reach or even exceed Evonik recommended levels. Because Evonik recommendations are grouped according to body weights (from 5-10 kg, 10-20 kg and 20-40 kg BW), and in this trial the pre-starter diets were fed from 7-15 kg BW and the starter diets from 15- 30 kg, there was a compromise with the 'recommended' SID lysine level. The recommended SID lysine for Treatments 1 to 4 in the pre-starter diet was 1.23% and was increased to 1.37% in Treatments 5 and 6. In the starter diets, the recommended SID lysine level was 1.08% in Treatments 1 to 4, increasing to 1.20% in Treatments 5 and 6.

The diets contained no organic acids, zinc oxide or antibiotics. Highly digestible raw materials were used because the usual gut health-promoting additives (antibiotics and zinc) were absent from the diets. This included the use of lactose products as well as a highly digestible cooked cereal mix comprising of a mixture between cooked maize and barley. *Bacillus amyloliquefaciens* was added on top of the formulated diets for Treatments 2, 4 and 6 in both the pre-starter and starter phases. The complete ingredient list for the pre-starter and starter diets can be found in Table 3.2 and Table 3.3 below. The expected nutrient specifications for the pre-starter and the starter diets can be found in Tables 3.4 and 3.5, respectively.

Table 3.2 Raw material composition of the pre-starter diets

Ingredient (%)	Treatment 1	Treatment 2	Treatment 3	Treatment 4	Treatment 5	Treatment 6
Cereal Mix[1]	25.00	25.00	25.00	25.00	25.00	25.00
Maize	33.00	33.00	43.00	43.00	44.00	44.00
Nosilac HL[2]	5.00	5.00	5.00	5.00	5.00	5.00
Full Fat Soya	14.00	14.00	8.00	8.00	6.00	6.00
Fishmeal	3.50	3.50	3.50	3.50	3.50	3.50
Soya Oilcake	8.70	8.70	4.30	4.30	4.60	4.60
Wheat Bran	5.50	5.50	5.00	5.00	5.00	5.00
Lactofeed 70[3]	2.00	2.00	2.00	2.00	2.00	2.00
Monocalcium Phosphate	0.90	0.90	0.97	0.97	1.00	1.00
Salt	0.38	0.38	0.40	0.40	0.40	0.40
Limestone	0.95	0.95	0.98	0.98	0.99	0.99
Biolys® 70[4]	0.45	0.45	0.83	0.83	1.14	1.14
DL-Methionine	0.14	0.14	0.22	0.22	0.31	0.31
L-Tryptophan	0.05	0.05	0.10	0.10	0.14	0.14
Threonine	0.12	0.12	0.25	0.25	0.35	0.35
Valine	0.00	0.00	0.15	0.15	0.26	0.26
Premix	0.20	0.20	0.20	0.20	0.20	0.20
Mycofix® Plus[5]	0.10	0.10	0.10	0.10	0.10	0.10
Axtra®Phy[6] (10 000 FTU)	0.01	0.01	0.01	0.01	0.01	0.01
Probiotic[7]	0	0.05	0	0.05	0	0.05

1 Cereal mix: Cooked cereal mix consisting of cooked maize and barley

2 Nosilac HL: High lactose product

3 Lactofeed 70: Lactose source consisting of whey permeate and Hi-Pro soybean meal

4 Biolys® 70: Lysine sulphate containing 54.6% L-lysine

5 Mycofix® Plus: Mycotoxin binder

6 Axtra®Phy: Phytase enzyme

7 Probiotic: *Bacillus amyloliquefaciens*

Table 3.3 Raw material composition of the starter diets

Ingredient (%)	Treatment 1	Treatment 2	Treatment 3	Treatment 4	Treatment 5	Treatment 6
Cereal Mix[1]	15.00	15.00	15.00	15.00	27.00	27.00
Maize	54.30	54.30	61.40	61.40	51.60	51.60
Nosilac HL[2]	0.00	0.00	0.00	0.00	0.00	0.00
Full Fat Soya	2.00	2.00	0.00	0.00	0.00	0.00
Fishmeal	2.00	2.00	2.00	2.00	2.00	2.00
Soya Oilcake	16.20	16.20	8.20	8.20	4.50	4.50
Wheat Bran	7.00	7.00	9.00	9.00	10.00	10.00
Monocalcium Phosphate	0.73	0.73	0.80	0.80	0.77	0.77
Salt	0.50	0.50	0.50	0.50	0.50	0.50
Limestone	1.00	1.00	1.01	1.01	1.00	1.00
Biolys® 70[3]	0.58	0.58	0.97	0.97	1.23	1.23
DL-Methionine	0.15	0.15	0.25	0.25	0.31	0.31
L-Tryptophan	0.05	0.05	0.09	0.09	0.12	0.12
Threonine	0.16	0.16	0.29	0.29	0.38	0.38
Valine	0.02	0.02	0.18	0.18	0.28	0.28
Premix	0.20	0.20	0.20	0.20	0.20	0.20
Mycofix® Plus[4]	0.10	0.10	0.10	0.10	0.10	0.10
Axtra®Phy[5] (10 000 FTU)	0.01	0.01	0.01	0.01	0.01	0.01
Probiotic[6]	0	0.05	0	0.05	0	0.05

1 Cereal mix: Cooked cereal mix consisting of cooked maize and barley

2 Nosilac HL: High lactose product

3 Biolys® 70: Lysine sulphate containing 54.6% L-lysine

4 Mycofix® Plus: Mycotoxin binder

5 Axtra®Phy: Phytase enzyme

6 Probiotic: *Bacillus amyloliquefaciens*

Table 3.4 The formulated nutrient levels (g/kg on 'as is' basis) of the pre-starter diets

Nutrient (g/kg)	Treatment 1	Treatment 2	Treatment 3	Treatment 4	Treatment 5	Treatment 6
Net Energy (MJ/kg)	10.7	10.7	10.7	10.7	10.7	10.7
SID* Lysine	12.3	12.3	12.3	12.3	13.7	13.7
SID Lysine / Net Energy	1.15	1.15	1.15	1.15	1.28	1.28
Crude Protein	210	210	180	180	180	180
SID Lys: Crude Protein	0.56	0.56	0.68	0.68	0.76	0.76
Crude Fat	61.0	61.0	52.0	52.0	49.0	49.0
Crude Fibre	29.0	29.0	25.0	25.0	26.0	26.0
Calcium	8.0	8.0	8.0	8.0	8.0	8.0
Total Phosphorus	6.10	6.10	6.10	6.10	6.10	6.10
Sodium	2.20	2.20	2.20	2.20	2.20	2.20
Digestible Phosphorus	4.00	4.00	4.00	4.00	4.00	4.00
SID Met	4.69	4.69	5.07	5.07	5.97	5.97
SID Met + Cys	7.38	7.38	7.38	7.38	8.22	8.22
SID Thr	7.74	7.74	7.74	7.74	8.63	8.63
SID Trp	2.70	2.70	2.70	2.70	3.01	3.01
SID Val	8.36	8.36	8.36	8.36	9.31	9.31
SID Ile	7.37	7.37	5.94	5.94	5.76	5.76
SID His	4.48	4.48	3.71	3.71	3.60	3.60
SID Arg	11.26	11.26	8.68	8.68	8.35	8.35
SID Phe	7.07	7.07	5.53	5.53	5.33	5.33
SID Leu	13.56	13.56	11.70	11.70	11.44	11.44
SID Tyr	0.77	0.77	0.77	0.77	0.77	0.77
SID Ala	0.89	0.89	0.89	0.89	0.89	0.89
SID Asp	2.40	2.40	2.40	2.40	2.40	2.40
SID Glu	7.42	7.42	7.42	7.42	7.42	7.42
SID Gly	0.88	0.88	0.88	0.88	0.88	0.88
SID Pro	1.11	1.11	1.11	1.11	1.11	1.11
SID Ser	1.09	1.09	1.09	1.09	1.09	1.09
SID Met/SID Lys	0.40	0.40	0.40	0.40	0.40	0.40
SID Met + Cys/SID Lys	0.60	0.60	0.60	0.60	0.60	0.60
SID Thr/SID Lys	0.63	0.63	0.63	0.63	0.63	0.63
SID Trp/SID Lys	0.22	0.22	0.22	0.22	0.22	0.22
SID Val/SID Lys	0.68	0.68	0.68	0.68	0.68	0.68
SID Ile/SID Lys	0.60	0.60	0.48	0.48	0.42	0.42
SID Leu/SID Lys	1.10	1.10	0.95	0.95	0.84	0.84
SID Arg/SID Lys	0.92	0.92	0.71	0.71	0.61	0.61
SID Phe/SID Lys	0.57	0.57	0.45	0.45	0.39	0.39
SID His/SID Lys	0.36	0.36	0.30	0.30	0.26	0.26

* SID= Standard Ileal Digestible

Table 3.5 The formulated nutrient levels (g/kg on 'as is' basis) of the starter diets

Nutrient (g/kg)	Treatment 1	Treatment 2	Treatment 3	Treatment 4	Treatment 5	Treatment 6
Net Energy (MJ/kg)	10.6	10.6	10.6	10.6	10.6	10.6
SID Lysine	10.8	10.8	10.8	10.8	12	12
SID Lysine / Net Energy	1.02	1.02	1.02	1.02	1.13	1.13
Crude Protein	180	180	150	150	150	150
SID Lys:CP	0.6	0.6	0.72	0.72	0.80	0.80
Crude Fat	45.00	45.00	42.00	42.00	42.00	42.00
Crude Fibre	27.00	27.00	25.00	25.00	25.00	25.00
Calcium	7.00	7.00	7.00	7.00	7.00	7.00
Total Phosphorus	5.30	5.30	5.30	5.30	5.30	5.30
Sodium	2.30	2.30	2.30	2.30	2.30	2.30
Digestible Phosphorus	3.30	3.30	3.30	3.30	3.30	3.30
SID Met	4.30	4.30	4.67	4.67	5.44	5.44
SID Met + Cys	6.69	6.69	6.69	6.69	7.44	7.44
SID Thr	7.02	7.02	7.02	7.02	7.80	7.80
SID Trp	2.16	2.16	2.16	2.16	2.40	2.40
SID Val	7.34	7.34	7.34	7.34	8.16	8.16
SID Ile	6.20	6.20	4.65	4.65	4.46	4.46
SID His	4.19	4.19	3.38	3.38	3.29	3.29
SID Arg	10.20	10.20	7.56	7.56	7.27	7.27
SID Phe	6.81	6.81	5.13	5.13	4.57	4.57
SID Leu	13.41	13.41	11.23	11.23	10.75	10.75
SID Tyr	0.46	0.46	0.46	0.46	0.85	0.85
SID Ala	0.54	0.54	0.54	0.54	0.98	0.98
SID Asp	1.44	1.44	1.44	1.44	2.62	2.62
SID Glu	4.52	4.52	4.52	4.52	8.14	8.14
SID Gly	0.53	0.53	0.53	0.53	0.96	0.96
SID Pro	0.67	0.67	0.67	0.67	1.21	1.21
SID Ser	0.67	0.67	0.41	0.41	1.19	1.19
SID Met/SID Lys	0.39	0.39	0.43	0.43	0.45	0.45
SID Met + Cys/SID Lys	0.62	0.62	0.62	0.62	0.62	0.62
SID Thr/SID Lys	0.65	0.65	0.65	0.65	0.65	0.65
SID Trp/SID Lys	0.20	0.20	0.20	0.20	0.20	0.20
SID Val/SID Lys	0.68	0.68	0.68	0.68	0.68	0.68
SID Ile/SID Lys	0.57	0.57	0.43	0.43	0.37	0.37
SID Leu/SID Lys	1.24	1.24	1.04	1.04	0.90	0.90
SID Arg/SID Lys	0.94	0.94	0.7	0.7	0.61	0.61
SID Phe/SID Lys	0.63	0.63	0.48	0.48	0.38	0.38
SID His/SID Lys	0.39	0.39	0.31	0.31	0.27	0.27

* SID= Standard Ileal Digestible

3.4 Feed analysis

The major raw materials used in the feed formulation (maize, cereal mix, Nosilac HL, full fat soya, fishmeal, soya oilcake, wheat bran and Lactofeed 70) were analysed beforehand either via near infrared spectroscopy (NIRS) at the Evonik Africa laboratory in Midrand, South Africa, or via wet chemical methods (high-performance liquid chromatography (HPLC) or ion chromatography) at the laboratories of Evonik Nutrition and Care GmbH in Hanau, Germany. The nutrients measured in the raw materials were the complete amino acid profile (essential and non-essential), SID levels of amino acids as well as the individual proximate and energy values.

The feed was manufactured at Simple Grow, South Africa, and after feed production, a 1 kg sample of each of the treatments for both the pre-starter and starter diets were sent to Evonik GmbH for analysis of amino acid levels via HPLC, and NIRS analysis for the proximate and energy levels in the feed. The analysed nutrient content of the feeds is provided in Tables 3.6 and 3.7.

3.4.1 The determination total amino acid content (oxidation and hydrolysis)

The amino acids which are present in feed and raw materials are bound to protein and need to be released via hydrolysis before they can be measured by ion chromatography using an Amino Acid Analyser. The methods used for amino acid determination in feed and raw materials comply with the requirements of the AOAC Official Method 994.12 (Llames & Fontaine, 1994) as well as the Commission Regulation (EC) No. 152/2009 of January 2009 (Official Journal of the European Union, 1994); and are described below. It is important to note that an additional oxidation step is needed in order to hydrolyse methionine and cysteine; but this process destroys tyrosine. In addition, tryptophan is destroyed during hydrochloric acid hydrolysis and therefore a separate analysis was needed for the determination of this amino acid.

The feed or raw material samples were ground to a homogenous particle size (0.5 mm) using a Retsch ZM 200 grinder. This sample was then weighed out to ensure 10 mg nitrogen was present in the sample (according to the prior Kjeldahl or Dumas analysis of the sample) into a 50 mL laboratory bottle (this should be between 100 and 800 mg of sample). A metal container was packed with 20 L of ice to allow for cooling after which the bottle with the sample was placed on the magnetic stirrer (along with the magnetic rod) inside the metal container. 5 mL of oxidation reagent (performic acid-phenol) was added to the sample bottle and stirred for a total of 15 minutes. The bottle was then covered and left for 16 hours at 0°C to allow for adequate oxidation to occur (in particular for methionine and cysteine).

The sample bottle was then removed from the metal container, placed in an ice bath under a fume hood and 0.84 g of sodium disulphite was added to destroy the excess performic acid. The sample was

stirred using the stirrer for another 30 minutes with the cover off the bottle in order to allow for sulphur dioxide to escape. 25 mL of hydrochloric acid-phenol reagent was added to the sample and the bottle placed in a heating oven (thermostatically controlled heating oven UT 6060 AR, Thermo Electron LED) at 110°C. The screw top was loosely applied, and the sample left for 1 hour in the oven, after which the lid was tightened completely and left for a further 23 hours to allow for proper hydrolysation. In order to determine the content of tyrosine in the sample, the process as described above was repeated, however, the oxidation step with performic acid-phenol was removed and only the hydrolysis step with hydrochloric acid- phenol was performed.

The sample bottle was removed from the oven, opened and allowed to cool. 15 mL of norleucine standard solution was pipetted into the bottle, mixed and transferred to a 250 mL plastic beaker. 125 mL of citrate buffer and 19 mL of sodium hydroxide solution were added, and the beaker placed into the sample changer (Metrohm 789 Robotic Sample Processor with autodoser Titrino 719 S) for pH adjustment with a sodium hydroxide solution to achieve a pH of 2.20. A portion of the solution was then added into a 50 mL polyethylene bottle and analysed using chromatography. In this process, the sample was passed over a membrane filter and applied to the column of the Amino Acid Analyser. The individual amino acids were mixed with nonhydrin, causing a reaction in a heated reaction loop. This caused a violet dye (post column derivatisation) to form, which was detected via photometric analysis at wavelengths of 570 nm.

3.4.2 The determination of tryptophan content (alkaline hydrolysis)

Since tryptophan is destroyed during hydrochloric acid hydrolysis, as previously mentioned, alkaline hydrolysis using an autoclave was needed instead. The sample was analysed using HPLC (high-performance liquid chromatography). This method was developed using the methods by Bech-Anderson (1991), Slump *et al*. (1991) and Landry & Delhaye (1992) and described in detail below. The feed or raw material samples were ground to a homogenous particle size (0.5 mm) using a Retsch ZM 200 grinder. The sample was then weighed out into a 125 mL polypropylene bottle (this should be between 100 and 1000 mg of sample) ensuring that 10 mg nitrogen was present in the sample (according to the prior Kjeldahl or Dumas analysis of the sample. 8.4 g of barium hydroxide (barium hydroxide-octahydrate) and 12 mL of water were added to the bottle and stirred with the magnetic stirrer until sufficiently wet and no sample remained on the side of the bottle (a small amount of water was used to wash down any residual sample off the sides of the bottle). The sample bottle's lid was applied loosely and was then placed into a wire basket at a slightly tilted angle. This basket was placed in the autoclave when the appropriate temperature was reached.

Three litres of water were poured into the autoclave (SANOclav LA M-3-20-MSC-J), the metal lid loosely fitted, and the temperature set to 110°C. As soon as the water reached boiling point, the wire basket containing the sample bottle was lowered into the autoclave and the lid was tightly shut, but the waste steam valve remained open to allow the steam to escape for 60 minutes. After the 60 minutes had elapsed, the valve was closed, and the samples were allowed to hydrolyse in the autoclave for a further 20 hours at the temperature of 110°C and pressure of 0.4 bar.

After the necessary hydrolysation time, the autoclave was cooled to 90°C before opening. 30 mL of water and 2 mL of internal standard were added to the sample bottle and then cooled in an ice-water bath whilst being stirred. 5 mL of phosphoric acid and 5 mL of hydrochloric acid (6 mol/L) were added successively; after which the pH was adjusted using hydrochloric acid (1 mol/L) to pH 3 ± 0.2. A portion of the solution was poured into a 100 mL glass beaker through a folded piece of filter paper, after which 1 mL of filtrate was mixed with 4 mL aqueous methanol in a 25 mL glass beaker. The resultant solution is a 1:5 diluted stabilised hydrolysate solution and was passed through a 0.45 um filter to obtain the final HPLC measuring solution. Tryptophan and the internal standard were then separated from one another via reverse-phase chromatography RP-18 using a HPLC column. A fluorescence detector was used for selective detection to prevent any interference from other feed components or amino acids.

3.4.3 The determination of proximate parameters (Weende analysis)

The proximate analysis of the feed and raw materials was done via NIRS (Near-Infrared Spectroscopy) analysis using Evonik Nutrition & Care GmBH proximate NIR calibrations. These NIR calibrations are based on various ISO (International Organization for Standardization), AOAC (The Association of Official Analytical Chemists) and VDLUFA (Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten (Association of German Agricultural Analytic and Research Institutes)) - approved methods and is described below.

The sample was dried for 4 hours at 103°C and the weight of water lost from the sample was used to calculate the crude water fraction. The sample was then heated for a further 4 hours at 550°C in order to remove all the organic components in the feed, i.e. the carbon. Using this weight loss of the sample after the heating period, the organic matter fraction of the sample was calculated. The remaining fraction of the feed sample was considered to be the crude ash. In order to determine the CP content of the feed sample, the nitrogen content was determined through the Dumas method (method nr. 990.03) (AOAC, 1990). The sample was combusted by exposing it to a temperature of 1000°C for 1 minute in the presence of oxygen. This combustion process produced N_2 and NO_x as gaseous by-products. NO_x was reduced to N_2 in the

presence of tungsten or copper and the subsequent N_2 levels were measured using a Thermal Conductivity Detector (TCD).

The fat and lipid contents were extracted from the sample in the following manner. Hydrochloric acid was used to hydrolyse the fat from the cell matrix as per the Weibull- Stoldt method (Ulberth & Henniger, 1992); after which petrol ether was used for fat extraction as per the Soxhlet method (Ulberth & Henniger, 1992). The solvent evaporated from the sample and the remaining filtrate was considered to be the ether extract fraction. Crude fibre was considered to be the component of carbohydrates not soluble in either acid or alkali detergents and comprised of cellulose, hemicellulose and lignin. In order to determine the total cell wall contents of the sample, the sample was boiled in a neutral detergent and the remaining fraction was considered the neutral detergent fibre. The acid detergent fibre fraction of the sample was determined by digesting the sample in sulphuric acid and this fraction was comprised of cellulose and lignin and was considered to be the most indigestible part of the carbohydrate fractions in the sample. The nitrogen free extract (NFE) of the sample consists of the sugars, starch, hemicellulose and pectin within the sample and its content was determined by means of a calculation rather than through chemical analysis. The formula used for this determination was as follows: NFE= Organic matter- CP- EE- CF.

The Luff-Schoorl method (Blanco *et al*., 2000) was used to determine the reducing sugars component of the sample. An ethanolic solution was used to extract the sugars after which Carrez reagent I and II were added to the sample. The ethanol was removed and an iodometric titration was performed using the Luff-Schoorl reagent. The total content of the reducing sugars in the sample was specified as the glucose content of the sample. The starch content of the sample was determined by means of polarimetric measurement in the method described by Ewers (Mitchell, 1990). The sample was treated with boiling, diluted hydrochloric acid and the first optical rotation was performed. The sample was then treated with 40% ethanol and hydrochloric acid was added once again to the sample, after which a second rotation was performed.

Lastly, the phosphorus content of the sample was determined by weighing 2 g of homogenised sample into a crucible and ashing it at 550°C for a total of 5 hours. Hydrochloric acid was added to dissolve the sample and the phosphorus content was then measured using an inductively coupled plasma optical emission spectrometer (ICP-MS). The phytic phosphorus content of the sample was determined by multiplying the total phosphorus content by the relevant phytic phosphorus factor from the INRA tables for the raw material in question (Sauvant & Perez, 2004).

3.4.4 The determination of probiotic content in the feed samples

The total content of probiotic spores (*Bacillus amyloliquefaciens*) as well as the background aerobic microbes in the feed samples were determined according to the methods described by VDLUFA 28.2.2 (Kaewtapee *et al*., 2017) and VDLUFA 28.1.2 (Nesic *et al*., 2019) respectively, and is provided in Table 3.8.

Ten grams of each feed sample was weighed and 90 g of NaOH solution (0.05 mol/L) was added in order to prepare the initial suspension for analysis. The sample was then placed in a stomacher for 1 minute to ensure adequate homogenisation. A pH of ≥ 8.5 was ensured in the solution to prevent the adsorption of the *Bacilli* spores onto the agar and the vegetative cells being destroyed when sodium hydroxide was used. The sample was plated onto an agar plate (PO5013A, Oxoid) using an automatic spiral plater (0.1 mL) and then incubated at 30°C for 18-24 hours, or a further 24 hours if no colonies formed on the agar in the first 24-hour period. The colonies present were counted, and their morphological characteristics considered in order to identify the specific bacteria which has grown on the plate. This also aided in identifying any cross contamination of the probiotic in the feed. The colony forming units (CFUs) were determined using a logarithmic transformation and reported as CFU per gram of feed (CFU/g feed).

In any typical feed sample, the presence of background aerobic microbes is inevitable, and this was determined using the same method as previously described, however the preparation of the sample differs slightly. Ten grams of the feed sample was mixed with 90 g casein peptone solution (1 g/L) and NaCl (8.5 g/L) and homogenised using a stomacher for 1 minute. The colonies that formed on the agar plate were again counted and their CFUs recorded. In feed samples that contain a probiotic, the total background aerobic bacteria should be equal to the probiotic spore count, and in feed samples with no probiotic supplementation, the background spore count is usually in the region of 10^4 or 10^5 CFU/g feed.

Table 3.6 The analysed proximate nutrient and amino acid concentrations (on 'as is' basis) of the pre-starter diets

Nutrients (%) #	**Treatment 1**	**Treatment 2**	**Treatment 3**	**Treatment 4**	**Treatment 5**	**Treatment 6**
Crude Protein	20.44	20.44	17.57	17.68	17.63	17.77
Acid Detergent Fibre	4.24	4.16	3.89	3.61	3.39	3.47
Neutral Detergent Fibre	10.49	11.11	10.39	9.58	8.97	9.17
Ash	5.33	5.35	4.88	4.83	4.70	4.63
Ether Extract (HCl)	5.64	5.48	4.89	4.68	4.45	4.42
Fibre	2.82	2.94	2.62	2.39	2.25	2.31
Total Phosphorus	0.59	0.59	0.52	0.54	0.50	0.50
Starch	38.12	37.52	40.73	42.62	43.47	43.44
Sugar	5.13	5.27	4.86	4.74	4.79	4.77
Gross Energy (MJ/kg)	17.18	17.16	17.03	16.97	16.91	16.93
Digestible Energy (MJ/kg)	14.58	14.44	14.31	14.43	14.58	14.58
Metabolisable Energy (MJ/kg)	13.98	13.83	13.81	13.92	14.07	14.07
Net Energy (MJ/kg)	10.42	10.30	10.36	10.47	10.59	10.58
Analysed amino acids (%) *						
Met	0.45	0.47	0.49	0.49	0.55	0.55
Met + Cys	0.77	0.78	0.76	0.76	0.81	0.81
Lys	1.37	1.35	1.35	1.33	1.45	1.51
Thr	0.88	0.89	0.86	0.87	0.90	0.90
Trp	0.28	0.28	0.28	0.27	0.31	0.32
Val	0.98	0.97	0.94	0.93	0.98	0.99
Ile	0.86	0.85	0.70	0.68	0.66	0.65
Arg	1.34	1.33	1.07	1.05	1.00	0.99
Leu	1.65	1.62	1.44	1.41	1.37	1.37
His	0.52	0.52	0.45	0.44	0.42	0.42
Phe	1.00	0.99	0.84	0.82	0.79	0.78
Gly	0.88	0.88	0.73	0.72	0.69	0.68
Ser	0.96	0.96	0.80	0.79	0.75	0.75
Pro	1.17	1.17	1.03	1.05	0.99	1.01
Ala	1.02	1.02	0.91	0.89	0.87	0.86
Asp	2.02	2.00	1.61	1.58	1.50	1.49
Glu	3.51	3.46	2.94	2.89	2.77	2.77

Values determined by near-infrared spectroscopy (NIRS)

* Values determined by High performance liquid chromatography (HPLC)

Table 3.7 The analysed proximate nutrient and amino acid concentrations (on 'as is' basis) of the starter diets

Nutrients (%) #	**Treatment 1**	**Treatment 2**	**Treatment 3**	**Treatment 4**	**Treatment 5**	**Treatment 6**
Crude Protein	17.83	17.96	15.14	14.81	14.74	14.96
Acid Detergent Fibre	4.02	4.22	3.53	3.32	3.31	3.59
Neutral Detergent Fibre	10.36	10.86	9.59	9.05	9.38	10.21
Ash	4.71	4.45	3.70	4.00	4.15	4.16
Ether Extract (HCl)	3.89	3.66	3.51	3.72	3.89	4.15
Fibre	2.76	2.86	2.38	2.16	2.13	2.43
Total Phosphorus	0.51	0.48	0.41	0.41	0.41	0.40
Starch	44.90	45.86	50.43	49.56	48.62	47.13
Sugar	3.33	3.15	2.91	2.96	3.15	3.20
Gross Energy (MJ/kg)	16.84	16.81	16.69	16.66	16.75	16.78
Digestible Energy (MJ/kg)	14.20	14.20	14.38	14.35	14.31	14.27
Metabolisable Energy (MJ/kg)	13.69	13.68	13.96	13.95	13.91	13.86
Net Energy (MJ/kg)	10.28	10.27	10.61	10.61	10.57	10.52
Analysed amino acids (%)*						
Met	0.42	0.42	0.46	0.50	0.51	0.51
Met + Cys	0.69	0.69	0.70	0.74	0.75	0.74
Lys	1.19	1.20	1.15	1.22	1.27	1.29
Thr	0.79	0.79	0.76	0.80	0.82	0.80
Trp	0.25	0.25	0.22	0.24	0.26	0.24
Val	0.84	0.84	0.80	0.84	0.88	0.87
Ile	0.70	0.70	0.53	0.54	0.52	0.52
Arg	1.12	1.10	0.82	0.83	0.79	0.80
Leu	1.46	1.46	1.22	1.22	1.17	1.18
His	0.45	0.45	0.36	0.37	0.35	0.35
Phe	0.87	0.85	0.67	0.67	0.65	0.65
Gly	0.73	0.73	0.58	0.59	0.57	0.57
Ser	0.82	0.81	0.65	0.64	0.61	0.62
Pro	1.06	1.06	0.93	0.94	0.92	0.91
Ala	0.90	0.90	0.77	0.77	0.74	0.74
Asp	1.63	1.62	1.20	1.20	1.14	1.14
Glu	3.01	2.99	2.43	2.44	2.38	2.39

Values determined by near-infrared spectroscopy (NIRS)

* Values determined by High performance liquid chromatography (HPLC)

Table 3.8 The analysed microbial levels of the pre-starter and starter diets

Feed	*Bacillus amyloliquefaciens* (CFU/g feed)	Total aerobic microorganisms (CFU/g feed)
Pre-Starter Treatment 1	<100	3.4×10^3
Pre-Starter Treatment 2	8.6×10^5	8.6×10^5
Pre-Starter Treatment 3	<100	5.2×10^3
Pre-Starter Treatment 4	8×10^5	8×10^5
Pre-Starter Treatment 5	<100	3.2×10^3
Pre-Starter Treatment 6	8.1×10^5	8.1×10^5
Starter Treatment 1	<100	4.4×10^3
Starter Treatment 2	1.8×10^6	1.8×10^6
Starter Treatment 3	<100	4.1×10^3
Starter Treatment 4	8.3×10^5	8.3×10^5
Starter Treatment 5	<100	1.5×10^4
Starter Treatment 6	1.4×10^6	1.4×10^6

3.5 Animal husbandry and measurements

Prior to the arrival of the pigs, the pig house was adequately prepared as described in section 3.2. The temperature of the house was set at 28°C and all infrared lights were switched on and warmed up to ensure a smooth transition from Concord Piggery to the farm facilities. The feed was weighed out into individual buckets with the respective treatment number labelled on the bucket. After the pens had been allocated to a specific treatment, the weighed-out feed was placed into the CAWI feeder. Upon arrival at the pig house, pigs were weighed individually using an animal scale. The scale had been accurately calibrated prior to the arrival of the pigs and proven to be in good order. Each pig was tagged using an ear tagger and allocated a number which was then the animal's number for the duration of the trial. Pigs were handled in a calm manner to reduce the already stressful situation of weaning and placement into a new environment. Pigs were randomly allocated into the six dietary treatments and pens, and there were three pigs per pen. Pigs were then left alone to acquaint themselves with their new surroundings and were not handled any further in order to minimise any further stress.

Pigs were individually weighed on a weekly basis starting one week after the arrival of the pigs. It was decided to take the first seven days of the trial as an adaptation period and to only consider the performance data from day seven onwards. The motivation behind this decision was due to the studies done by Le Dividich & Sève (2000) and Dong & Pluske (2007) who discussed the absence of adequate feed intake and higher maintenance requirements during the first week post-weaning, which inevitably results in a reduction in growth during this period. In order to avoid any inaccuracies in the growth performance data, a period of seven days was allocated to the pigs to acquaint themselves with their environment, reduce their stress load, and increase feed intake. Average daily gain was calculated by dividing the body weight gain over the 7-day period by seven. In addition, the remaining feed in the feeders were also weighed on the same day the pigs were weighed in order to determine the weekly feed intake. Using these figures, the G: F could be calculated. Each pen was considered the experimental unit of the trial, therefore the growth performance parameters of the three pigs in the pen were averaged and these values used to determine the results for each treatment.

The average faecal score of each pen was determined daily for the first 21 days of the trial (including the adaptation period), after which, every second day for the remaining 21 days of the trial. Two trained persons gave each pen a score from 0 to 4. A score of 0 was indicative of no faecal presence in the pen, 1 being faeces of a hard and firm nature, 2 having a soft putty-like appearance, 3 being slightly loose but not completely diarrhoeal and 4 being very loose and watery. These scores were then averaged for both people to give one score per pen. The faecal scores were used to indicate gut health during the trial.

3.6 The collection and analysis of intestinal tissue and digesta samples

The collection of intestinal tissue and digesta samples

After 21 days of the trial (including the 7-day adaptation period), 54 pigs were humanely euthanised and tissue from the duodenum and ileum, as well as digesta from the caecum and ileum, was removed from these animals. Caecal digesta was analysed for NH_3 concentration, the presence of *Salmonella spp.* and the enumeration of *E. coli* and used to measure the pH. The digesta collected from the ileum was analysed only for *Salmonella spp.* presence and *E. coli* count. One pig of average body weight was chosen per pen and removed after 21 days of the trial (49 days-of-age). Each pig was injected with sodium pentobarbital into the jugular vein by a veterinarian. This ensured the minimal suffering and stress endured by each pig and a quick demise. The pigs were then brought to the abattoir section of the farm where they were dissected, the necessary parameters measured and relevant tissues and digesta removed.

5 mL of digesta was collected from the colon, caecum and ileum immediately after euthanasia and exposure of the peritoneal cavity. Due to the fact that the pigs had been weighed in the morning prior to the euthanasia which inevitably caused a fair amount of stress, the pigs had not eaten sufficiently to allow for adequate ileal digesta to be collected from all the pigs. Digesta samples were placed in sample bottles and immediately placed into a freezer at -15 ℃ to prevent any further microbial action and fermentation in the sample that could influence the results of analysis. Samples were defrosted at a later stage for analysis.

The determination of liver and kidney weights

During dissection, the liver and both kidneys from each pig were carefully excised. These organs were then weighed, and their weights recorded on a data sheet. This data was used to determine the organ weights relative to total body weight.

The preparation of intestinal tissue for histomorphological assessment

In order to perform the histomorphology and histopathology on the intestinal tissue, a 2 cm segment of both the ileum and duodenum was removed and immediately placed into jars containing 10% neutral buffered formalin to ensure instant preservation of the tissue for later analysis. The section from the pylorus to the ligament of Treitz was considered the duodenum and 10 cm proximal to the ileocaecal junction considered the ileum. Samples were embedded into paraffin wax and sectioned into 5 µm slices after which they were stained with haematoxylin and eosin for histological analysis. The measurements for villus height and crypt depth were performed using a light microscope. Cross sections of intestinal segments were photographed using a light microscope under the 5x magnification and 15 villi and their respective crypts per sample were measured using ImageJ software. Measurements were compared among treatments to ascertain whether the different diets influenced the villi height and/or crypt depths.

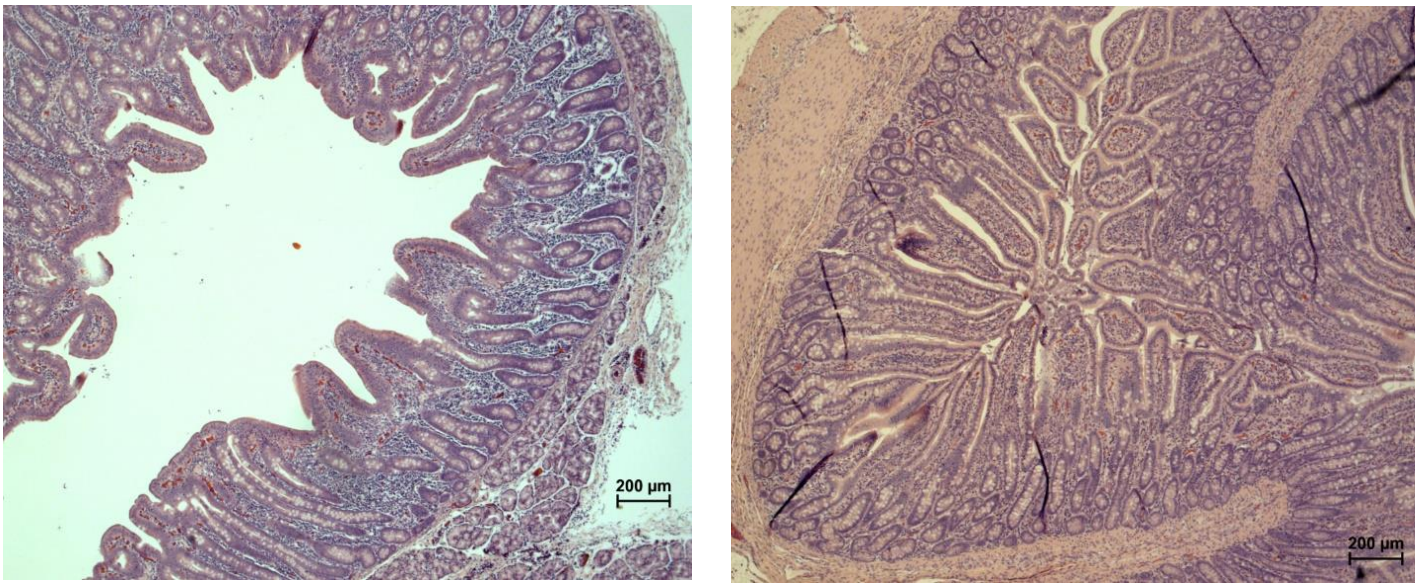


Figure 3.1 Photographs depicting duodenal (left) and ileal (right) tissue samples (5 x magnification using a light microscope)

The preparation and analysis of caecal digesta for ammonia nitrogen (NH_3-N) content

The caecal digesta samples were thawed overnight in preparation for the analysis. Once defrosted, the pH of the caecal digesta was measured using a pH meter and recorded. The method used to analyse the ammonia nitrogen content of the caecal digesta was taken from Broderick & Kang (1980) and is described below. 50 µL of the test standard (ammonium sulphate and 0.1 N HCl), the control sample and the sample to be tested were pipetted into test tubes in duplicate along with 50 µL of 0.1 N HCl (which was the blank for the analysis). 2.5 mL of phenol reagent (sodium nitroferricyanide, phenol, 90% w/v and deionised water) was added to each of the test tubes and thoroughly mixed; after which 2 mL of hypochlorite reagent (sodium hydroxide, disodium phosphate, 5.25% sodium hypochlorite and deionised water) was added to the tubes and mixed further.

Test tubes were placed in a water bath at a temperature 95°C for a total of 5 minutes. The tubes were removed from the hot water bath and placed into an ice bath for cooling for 5-7 minutes, or until the temperature of the samples reached 25-30°C. In order to determine the absorbance readings of the samples to calculate the sample's ammonia-N content, the tubes were analysed using a spectrophotometer at 630

nm. The ammonia concentrations (in mM NH_3/L) of the samples were determined using a standard curve computing the various absorbance readings and their corresponding mM NH_3/L concentration.

The preparation and analysis of caecal digesta for Escherichia coli and Salmonella spp. count

Ileal and caecal samples intended for microbial analysis were not frozen but instead placed in a fridge at 5°C for 24 hours to prevent any further microbial growth in the samples. The samples were then taken to Deltamune laboratories where the analyses took place. The method used by Deltamune for the *Salmonella spp.* presence in the samples was ISO 6579-1 (Mooijman *et al*., 2019). The samples were enriched in Buffered Petone Water as well as selectively enriched in Rappaport Vassiliadis soya peptone broth. The samples were then plated on XLD & Hektoen Enteric (HE) agar and their presence or absence in each sample noted.

The method used for the enumeration of *E. coli* in each sample as described by Baylis (2007) and is given below. Crystal violet and bile salts were used as selective agents in the analysis and lactose was used as the fermentative agent in order to diagnose *E. coli*. Brilliance *E. coli*/ coliform agar was used to plate the samples for the identification of *E. coli*. In order to differentiate between *E. coli* and other coliforms present in the samples, the agar contains two enzyme substrates. The first enzyme, glucuronidase, cleaves the chromogenic substrate that is specific for *E. coli* and is produced by approximately 97% of *E. coli* strains. The second enzyme is galactosidase and is produced by most coliforms. This enzyme cleaves the other chromogenic substrate. This creates on the agar either purple or pink colonies. The purple colonies are *E. coli* colonies, as they are able to cleave both the chromogenic substrates; and the pink colonies are the other coliform colonies, as they are only able to cleave the galactosidase chromogen. This enables the laboratory technician to distinguish between the different colonies and only count the *E. coli* colonies to accurately determine the colony forming units (CFUs) in the sample.

3.7 Statistical analyses

All statistically analyses were performed using SAS and SPSS software (Arbuckle, 1997; SAS Institute, 2017). Data for all parameters (except for *Salmonella spp.* content and faecal score) was subjected to analysis of variance (ANOVA). Gain to feed ratio (G: F) was given “as is” and was corrected for mortality. Probability of error $p<0.05$ was considered significant. Body weight at day 7 was used as a covariate in the analysis. The following model was used for ANOVA:

$$X_{ijk} = \mu + \alpha_i + \beta_j + \gamma_{ij} + \varepsilon_{ijk},$$

Where:

μ is grand mean,

α = the effect of type of diet (the CP and amino acid combination)

β = the effect of the probiotic

γ = the interaction between the diet and the probiotic

ε = the error of variability

The weekly faecal score data was analysed using chi-square. The chi-square test is used when data is of a nominal/categorical nature (McHugh, 2013). Each faecal score was taken as a category and the mean values of each of these scores were calculated per pen. Multi-comparison analysis between each treatment was performed in order to determine significance between treatments. The same test was applied to the *Salmonella spp.* presence in the caecal and ileal digesta because these parameters were already of a categorical nature; either *Salmonella spp.* was present in the digesta, or not. The percentages of each treatment which represented either the presence or absence of *Salmonella spp.* was then subjected to chi-square analysis with the following equation:

$$\sum X^2_{i\text{-}j} = \frac{(O - E)^2}{E}$$

Where:

X^2 = the cell chi-square value

i-j = represents all the cells. From the first cell (i) to the last cell (j)

O = the observed value

E = the expected value

The Kruskal- Wallis (KW) H test was used to determine statistical significance for the total faecal score comparisons among treatments over the whole experimental period. This test is a rank-based

nonparametric test which is used to determine statistical significance when there are two or more groups of independent variables of ordinal data (over time) (Chan & Walmsley, 1997). The KW test is represented with the following formula:

$$H = \left[\frac{12}{n(n+1)} \sum_{j=1}^{c} \frac{T_j^2}{n_j} \right] - 3(n+1)$$

Where:

n = sum of sample sizes for all samples

c = number of samples

T_j = sum of ranks in the j^{th} sample

n_j = size of the j^{th} sample

CHAPTER 4: RESULTS

4.1 Growth performance parameters

Table 4.1 gives the body weights and Table 4.2 outlines the ADG of each treatment for the pre-starter and starter phases. Dietary CP level had a significant effect on the BW of the pigs at day 7, 14, 21, 28 and 35, with pigs consuming the high CP diets having higher ($p < 0.05$) BW at these time periods. For this reason, BW at day 7 was used as a covariate in the statistical analyses of the results as this was our starting period for performance measurement after the adaptation phase (day 0-7). The 10% increase in amino acids supplemented in the low CP diets did not result in any differences in BW compared to the low CP diets without additional amino acids (LP), but interestingly, the low CP + AA (LPAA) diets also resulted in lower ($p < 0.05$) BW than the high CP (HP) diets. It can be deduced from Table 4.2 that the level of CP in the diet had a significant influence on ADG in pigs between day 7-14 and day 7-21. In the period of day 7-14 of the trial, pigs receiving the HP diets had significantly higher ($p < 0.0001$) ADG than the pigs in the lower CP treatment groups, even when diets were supplemented with additional amino acids. Table 4.2 also demonstrates that the HP diets resulted in significantly higher ($p < 0.05$) ADG for the pre-starter period (day 7-21) compared to the LPAA diets, but no significant difference compared to the LP diets. The addition of the probiotic did not result in any significant improvements in ADG or BW throughout the trial period.

Table 4.3 presents the ADFI of the pigs in both the pre-starter and starter periods. From the table it can deduced that there were no significant differences in feed intake among treatment groups. However, there was a trend ($p = 0.10$) for increased feed intake in the HP diet. In addition, the supplementation of the probiotic did not have any significant effect on feed intake both in the pre-starter and starter phases.

There was only one mortality during the trial but there were an additional two pigs that were treated with antibiotics for a respiratory infection and thus removed from the trial. This was due to the trial being based on antibiotic-free conditions and thus leaving these pigs in the trial after treatment with antibiotics may have influenced the results. All growth performance data (ADG, ADFI and G: F) accounted for these removals.

Table 4.1 Body weight (kg) of pigs receiving either high or low crude protein diets, supplemented with additional amino acids and/or *Bacillus amyloliquefaciens* (Probiotic)

Treatments*	Days of trial						
	0	**7**	**14**	**21**	**28**	**35**	**42**
1: HP	7.41	8.36	11.23	15.42	18.70	22.93	28.03
2: HP + Pro	7.33	8.43	11.03	14.86	17.50	21.38	26.65
3: LP	7.33	8.08	10.75	14.62	17.33	21.38	26.62
4: LP + Pro	7.37	8.25	10.67	14.58	17.21	20.48	25.73
5: LPAA	7.33	8.02	10.42	14.23	16.73	20.72	26.01
6: LPAA + Pro	7.33	8.08	10.55	14.27	16.81	20.98	25.68
SEM[#]	0.44	0.39	0.14	0.22	0.39	0.54	0.72
CP Main Effect							
High CP	7.37	8.40[a]	11.13[a]	15.14[a]	18.10[a]	22.16[a]	27.34
Low CP	7.35	8.17[ab]	10.71[b]	14.60[ab]	17.27[ab]	20.93[b]	26.17
Low CP + AA	7.33	8.05[b]	10.49[b]	14.25[b]	16.77[b]	20.85[b]	25.84
SEM[#]	0.43	0.38	0.10	0.16	0.28	0.39	0.51
Probiotic Main Effect							
Without Probiotic	7.36	8.16	10.80	14.76	17.59	21.68	26.89
With Probiotic	7.35	8.25	10.75	14.57	17.18	20.94	26.02
SEM[#]	0.43	0.37	0.90	0.13	0.23	0.31	0.42
Probability (p-value)							
CP	0.75	0.04	<.0001	0.001	<0.01	0.04	0.11
Probiotic	0.78	0.37	0.64	0.31	0.20	0.11	0.15
CP* Probiotic	0.52	0.90	0.41	0.36	0.22	0.25	0.77

[a, b]: values with differing superscripts within a column are statistically significant ($p < 0.05$)

* HP: High CP; HP +Pro: High CP + Probiotic; LP: Low CP; LP + Pro: Low CP + Probiotic; LPAA: Low CP + AA; LPAA + Pro: Low CP + AA + Probiotic

[#] SEM: Standard error of the mean

Table 4.2 Average daily gain (kg) of pigs receiving either high or low crude protein diets, supplemented with additional amino acids and/or *Bacillus amyloliquefaciens* (Probiotic)

Treatments*	Days of the trial				
	7-14	14-21	7-21	21-42	7-42
1: HP	0.43	0.60	0.52	0.60	0.57
2: HP + Pro	0.40	0.55	0.48	0.56	0.53
3: LP	0.36	0.55	0.46	0.57	0.53
4: LP + Pro	0.35	0.56	0.46	0.53	0.50
5: LPAA	0.32	0.54	0.43	0.56	0.51
6: LPAA + Pro	0.34	0.53	0.43	0.55	0.50
SEM#	0.02	0.02	0.02	0.03	0.02
CP Main Effect					
High CP	0.42[a]	0.57	0.50[a]	0.58	0.55
Low CP	0.36[b]	0.56	0.46[ab]	0.55	0.51
Low CP + AA	0.33[b]	0.54	0.43[b]	0.55	0.50
SEM#	0.02	0.01	0.01	0.02	0.01
Probiotic Main Effect					
Without Probiotic	0.37	0.57	0.47	0.58	0.53
With Probiotic	0.36	0.55	0.45	0.55	0.51
SEM#	0.01	0.01	0.01	0.02	0.01
Probability (p-value)					
CP	<.0001	0.23	0.002	0.53	0.09
Probiotic	0.64	0.23	0.31	0.17	0.17
CP* Probiotic	0.42	0.36	0.36	0.88	0.70

[a, b]: values with differing superscripts within a column are statistically significant ($p < 0.05$)

* HP: High CP; HP +Pro: High CP + Probiotic; LP: Low CP; LP + Pro: Low CP + Probiotic; LPAA: Low CP + AA; LPAA + Pro: Low CP + AA + Probiotic

SEM: Standard error of the mean

The G: F for the pre-starter and starter diets are given in Table 4.4 and represents the feed conversion of the consumed feed to average daily weight gain. From Table 4.4 it can be seen that there were significant CP effects exerted during the time periods day 7-14, 7-21, 21-42 and 7-42. The pigs consuming the HP diets had higher ($p < 0.01$) G: F compared to the pigs receiving either the LP or LPAA diets for day 7-14. During the pre-starter period (day 7-21), the pigs receiving the HP diet had higher ($p < 0.01$) G: F than the LP and LPAA pigs. The HP pigs also had increased ($p < 0.05$) G: F between day 21-42 compared to the LP pigs and lastly, for the overall trial period (day 7-42), HP diets resulted in higher ($p < 0.001$) G: F compared to their LP counterparts.

There was a trend ($p = 0.09$) observed in the periods between day 14-21 and 7-21 for improved G: F with the addition of the probiotic.

Table 4.3 Average daily feed intake (kg) of pigs receiving either high or low crude protein diets, supplemented with additional amino acids and/or *Bacillus amyloliquefaciens* (Probiotic)

Treatments*	Days of the trial				
	7-14	14-21	7-21	21-42	7-42
1: HP	0.43	0.71	0.57	1.00	0.83
2: HP + Pro	0.42	0.67	0.55	0.95	0.79
3: LP	0.43	0.68	0.55	1.05	0.85
4: LP + Pro	0.41	0.70	0.56	0.97	0.81
5: LPAA	0.35	0.67	0.51	0.97	0.79
6: LPAA + Pro	0.39	0.67	0.53	0.96	0.79
SEM#	0.03	0.03	0.02	0.04	0.03
CP Main Effect					
High CP	0.43	0.69	0.56	0.98	0.81
Low CP	0.42	0.69	0.55	1.01	0.83
Low CP + AA	0.37	0.67	0.52	0.96	0.79
SEM#	0.02	0.02	0.01	0.03	0.02
Probiotic Main Effect					
Without Probiotic	0.40	0.69	0.54	1.01	0.82
With Probiotic	0.41	0.68	0.55	0.96	0.80
SEM#	0.01	0.01	0.01	0.02	0.02
Probability (p-value)					
CP	0.10	0.68	0.15	0.44	0.34
Probiotic	0.75	0.77	0.97	0.15	0.25
CP* Probiotic	0.53	0.45	0.59	0.67	0.64

* HP: High CP; HP +Pro: High CP + Probiotic; LP: Low CP; LP + Pro: Low CP + Probiotic; LPAA: Low CP + AA; LPAA + Pro: Low CP + AA + Probiotic

SEM: Standard error of the mean

Table 4.4 Gain to feed ratio (G: F) of pigs receiving either high or low crude protein diets, supplemented with additional amino acids and/or *Bacillus amyloliquefaciens* (Probiotic)

Treatments	Days of the trial				
	7-14	**14-21**	**7-21**	**21-42**	**7-42**
1: HP	0.99	0.84	0.91	0.60	0.68
2: HP + Pro	0.95	0.82	0.87	0.59	0.67
3: LP	0.88	0.83	0.83	0.54	0.62
4: LP + Pro	0.83	0.80	0.82	0.54	0.62
5: LPAA	0.82	0.82	0.86	0.58	0.65
6: LPAA + Pro	0.83	0.80	0.81	0.57	0.63
SEM[#]	0.04	0.02	0.02	0.02	0.01
CP Main Effect					
High CP	0.97[a]	0.83	0.89[a]	0.59[a]	0.68[a]
Low CP	0.85[b]	0.81	0.83[b]	0.54[b]	0.62[b]
Low CP + AA	0.82[b]	0.81	0.84[b]	0.58[ab]	0.64[ab]
SEM[#]	0.03	0.02	0.02	0.01	0.01
Probiotic Main Effect					
Without Probiotic	0.90	0.83	0.87	0.58	0.65
With Probiotic	0.87	0.81	0.84	0.57	0.64
SEM[#]	0.03	0.02	0.01	0.01	0.01
Probability (p-value)					
CP	0.002	0.35	<0.01	0.03	<0.001
Probiotic	0.37	0.09	0.09	0.59	0.35
CP* Probiotic	0.70	0.99	0.72	0.91	0.67

[a, b]: values with differing superscripts within a column are statistically significant ($p < 0.05$)

* HP: High CP; HP +Pro: High CP + Probiotic; LP: Low CP; LP + Pro: Low CP + Probiotic; LPAA: Low CP + AA; LPAA + Pro: Low CP + AA + Probiotic

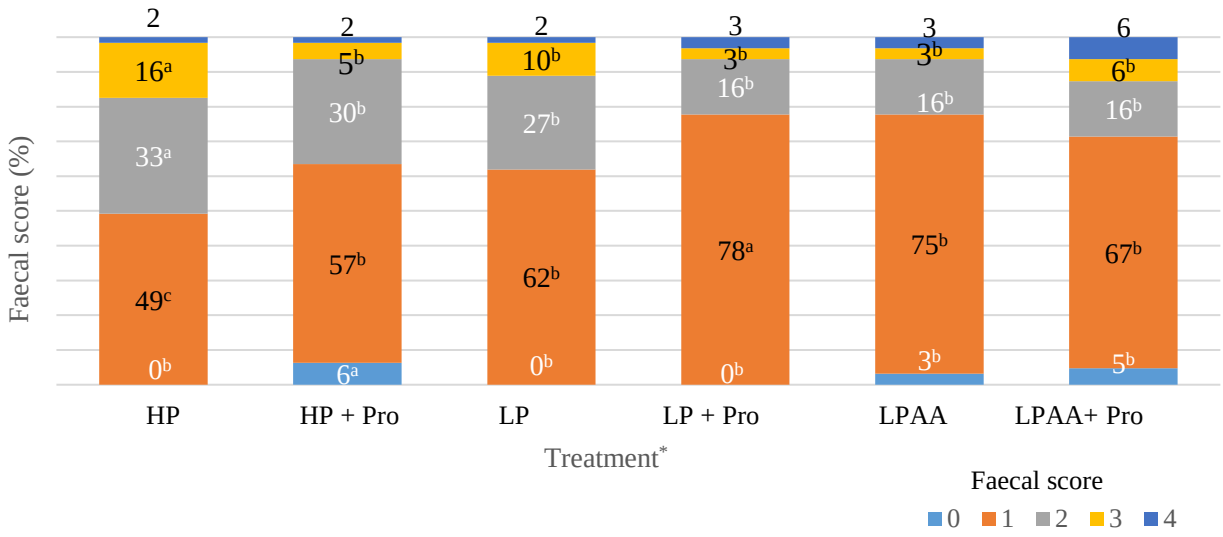
[#] SEM: Standard error of the mean

4.2 Gut health parameters

Faecal score

Faecal score was used as a measure to determine the gut health of the piglets every day for the first 21 days of the trial starting the day after placement (day 1-21) and every second day for the remainder of the trial (day 21- 41). These faecal scores are represented from day 1-7, day 7-14, day 14-21 and day 21-41 in Figures 4.1, 4.2, 4.3 and 4.4, respectively. The faecal scores for the entire experimental period (day 1-41) are given in Figure 4.5 to demonstrate the trend over time. Significant differences in faecal scores among treatments were noted during day 1-7 and day 7-14 only. When considering the figures, take note that each faecal score was compared individually across treatment groups, considering the percentage of

each faecal score recorded for each treatment. Figure 4.1 depicts the faecal score data for day 1-7. It can be observed from the graph that there were significant differences among treatments for faecal scores from 0-3 but not for faecal scores of 4. Pigs on Treatment 1 (HP) had the highest number of faecal scores of 3 between the groups, indicative of some diarrhoea during the first seven days. The reduction of CP improved faecal scores by significantly increasing the number of faecal scores of 1 and reducing the number of faecal scores of 3. In addition, the pigs consuming the LPAA diets also had significantly higher numbers of faecal scores of 1 and lower scores of 3, compared to the HP pigs. The addition of the probiotic into the HP diets resulted in an increase in the number of faecal scores of 0 and 1, and a reduction in faecal scores of 3, indicative of an improvement in gut health. In the LP diets, the addition of the probiotic resulted in an increase in faecal scores of 1 and a reduction in faecal scores of 2 and 3. Treatment 4 (LP + Probiotic) resulted in significantly the highest amount of faecal scores of 1 than the other treatments, this indicates a positive interaction effect between low CP and the probiotic addition in the diet.

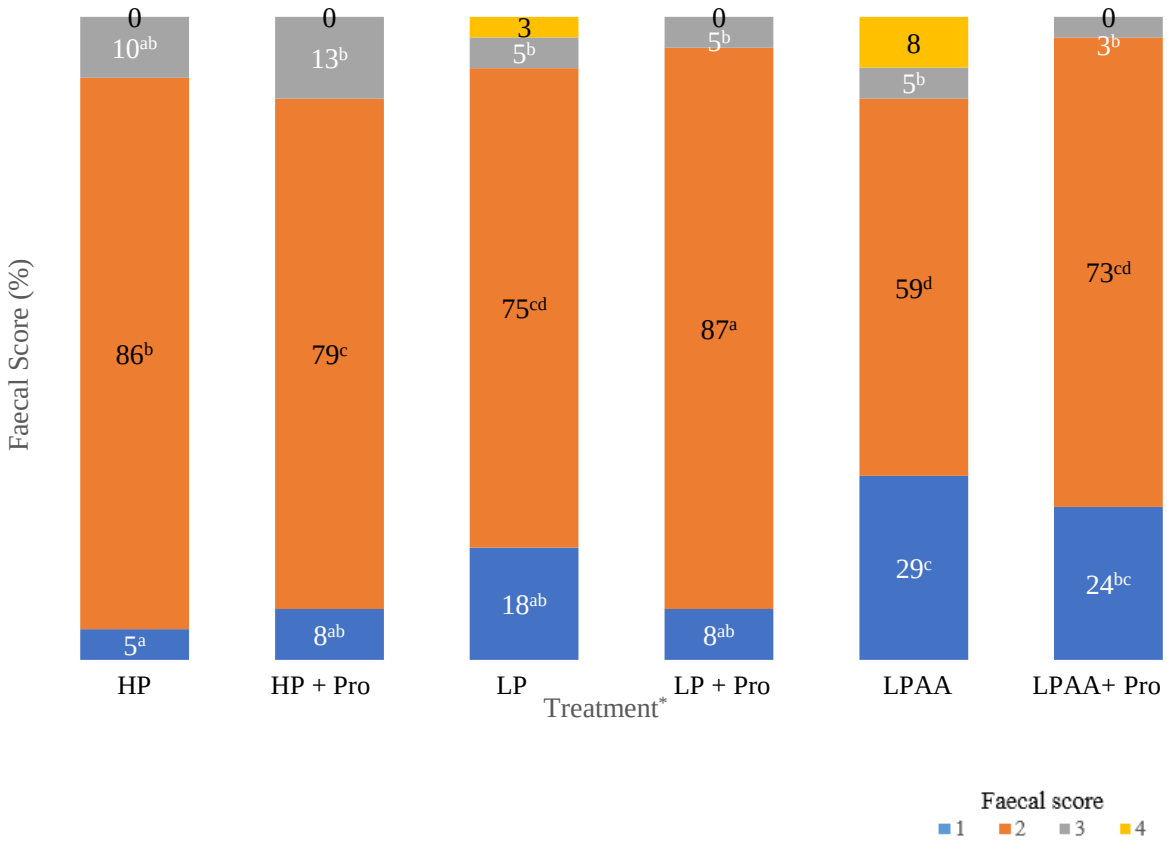


[a, b]: values with differing superscripts between adjacent columns of the same faecal score are statistically significant ($p < 0.05$)

*HP: High CP; HP + Pro: High CP + Probiotic; LP: Low CP; LP + Pro: Low CP + Probiotic; LPAA: Low CP + AA; LPAA + Pro: Low CP + AA + Probiotic

Figure 4.1 Faecal score data (%) for day 1-7 of pigs receiving either high or low crude protein diets, supplemented with additional amino acids and/or *Bacillus amyloliquefaciens* (Probiotic)

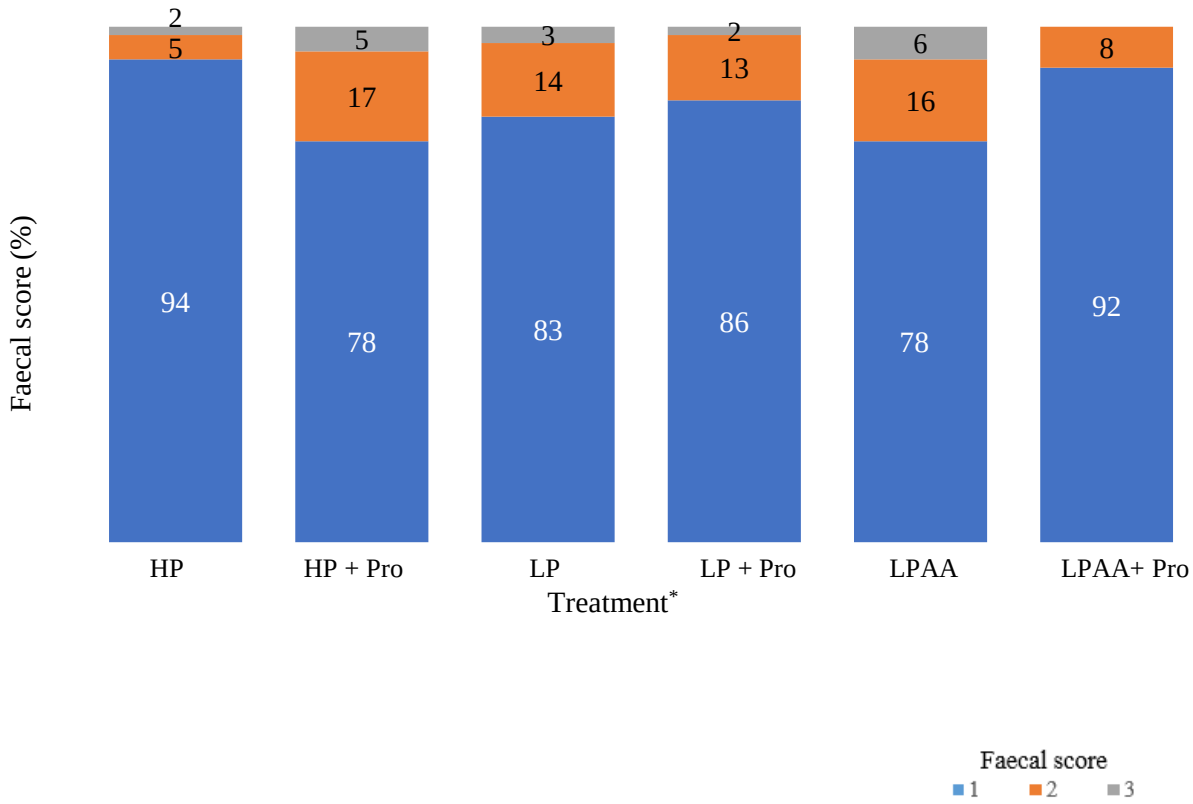
The data in Figure 4.2 demonstrates a similar tendency for the period between day 7-14 for Treatment 4 (LP + Probiotic), with these pigs showing the highest number of faecal scores of 2, (which again is considered the most 'normal' faeces of weaned pigs). This once again demonstrates a positive interaction between reduced CP and the addition of the probiotic. Pigs receiving Treatment 5 (LPAA) also had significantly the highest number of hard stools. Interestingly, the addition of the probiotic resulted in significantly higher faecal scores of 2 in the low CP diets. The time periods day 14-21 and day 21-41 did not have any significant differences among treatments when considering individual faecal scores as seen in both Figure 4.3 and Figure 4.4.



[a, b]: values with differing superscripts between adjacent columns of the same faecal score are statistically significant ($p < 0.05$)

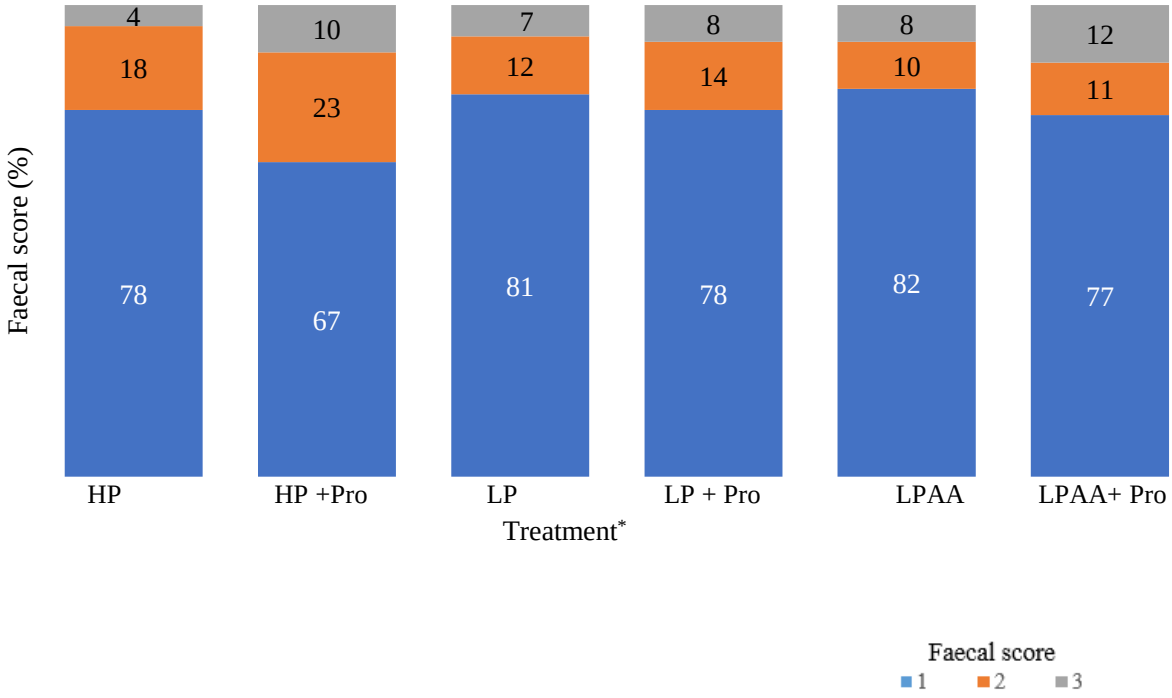
*HP: High CP; HP + Pro: High CP + Probiotic; LP: Low CP; LP + Pro: Low CP + Probiotic; LPAA: Low CP + AA; LPAA + Pro: Low CP + AA + Probiotic

Figure 4.2 Faecal score data (%) for day 7-14 of pigs receiving either high or low crude protein diets, supplemented with additional amino acids and/or *Bacillus amyloliquefaciens* (Probiotic)



*HP: High CP; HP + Pro: High CP + Probiotic; LP: Low CP; LP + Pro: Low CP + Probiotic; LPAA: Low CP + AA; LPAA + Pro: Low CP + AA + Probiotic

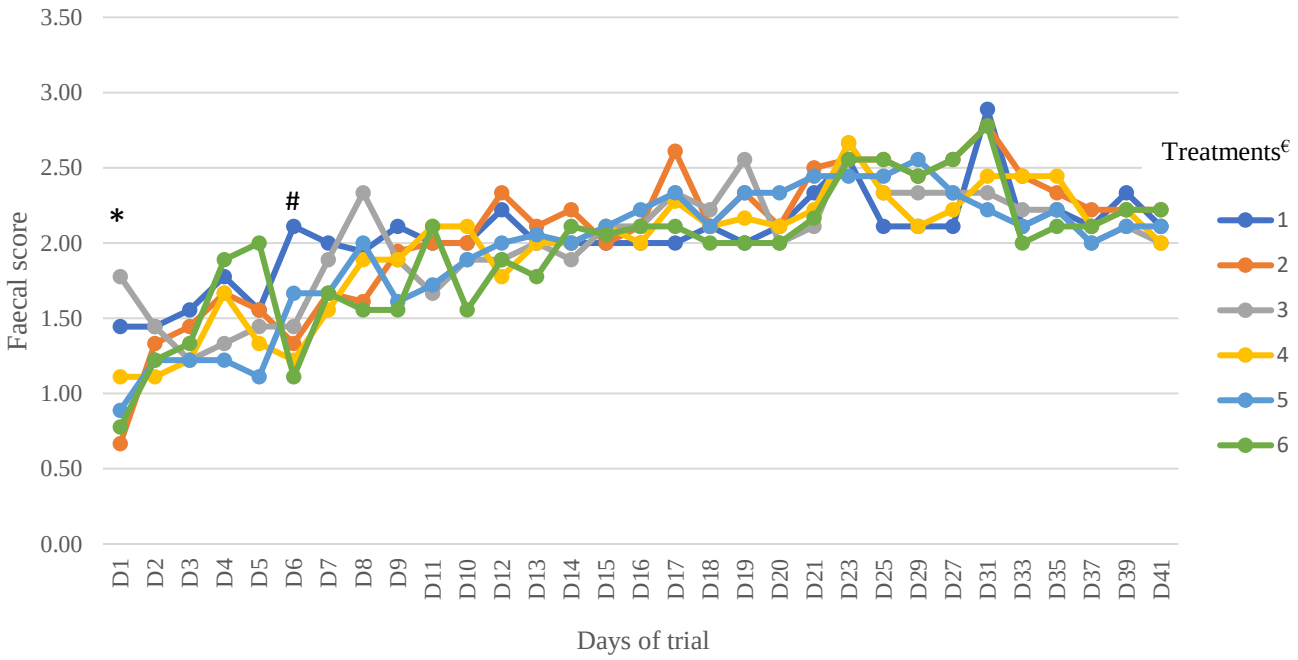
Figure 4.3 Faecal score data (%) for day 14-21 of pigs receiving either high or low crude protein diets, supplemented with additional amino acids and/or *Bacillus amyloliquefaciens* (Probiotic)



*HP: High CP; HP + Pro: High CP + Probiotic; LP: Low CP; LP + Pro: Low CP + Probiotic; LPAA: Low CP + AA; LPAA + Pro: Low CP + AA + Probiotic

Figure 4.4 Faecal score data (%) for day 21-41 of pigs receiving either high or low crude protein diets, supplemented with additional amino acids and/or *Bacillus amyloliquefaciens* (Probiotic)

Figure 4.5 depicts the daily faecal score over the entire experimental period (day 1-41). It can be seen from the graph that there were significant effects exerted by the dietary treatments on faecal score for day 1 and day 6 only. On day 1, pigs on Treatment 3 (LP) had significantly higher faecal scores than pigs from Treatments 2 (HP + Probiotic) and 6 (LPAA + Probiotic). On day 6, only Treatments 1 (HP) and Treatment 6 (LPAA + Probiotic) were significantly different to one another in terms of faecal score, where pigs receiving Treatment 1 had significantly higher faecal scores than Treatment 6 pigs.



* Pigs in Treatment 3 had significantly higher ($p < 0.05$) faecal score than pigs in Treatments 2 and 6

Pigs in Treatment 1 had significantly higher ($p < 0.05$) faecal scores than pigs in Treatment 6

€ Treatment 1: High CP; Treatment 2: High CP + Probiotic; Treatment 3: Low CP; Treatment 4: Low CP + Probiotic; Treatment 5: Low CP + AA; Treatment 6: Low CP + AA + Probiotic

Figure 4.5 Faecal score data for day 1-41 of pigs receiving either high or low crude protein diets, supplemented with additional amino acids and/or *Bacillus amyloliquefaciens* (Probiotic)

Ammonia concentration and pH of caecal digesta

Table 4.5 depicts the ammonia (NH_3) concentration and pH of the caecal digesta collected from each of the pigs. None of the individual Treatments exerted significant responses for either the caecal ammonia concentration or pH. Interestingly, the higher CP diets did not appear to increase hindgut fermentation of the excess proteinous substrate, which may have been observed as increased NH_3 production.

Table 4.5 Caecal pH values and ammonia concentrations extracted from pigs receiving either high or low crude protein diets, supplemented with additional amino acids and/or *Bacillus amyloliquefaciens* (Probiotic)

Treatments*	pH	Ammonia (mg NH_3- N/100 mL)
1: HP	5.52	17.72
2: HP + Pro	5.44	20.15
3: LP	5.43	18.15
4: LP + Pro	5.46	19.88
5: LPAA	5.45	23.14
6: LPAA + Pro	5.38	19.42
SEM#	0.04	2.04
CP Main Effect		
High CP	5.48	18.94
Low CP	5.44	19.02
Low CP + AA	5.42	21.28
SEM#	0.03	1.45
Probiotic Main Effect		
Without Probiotic	5.47	19.82
With Probiotic	5.43	19.67
SEM#	0.02	1.18
Probability (p-value)		
CP	0.32	0.44
Probiotic	0.21	0.93
CP* Probiotic	0.26	0.27

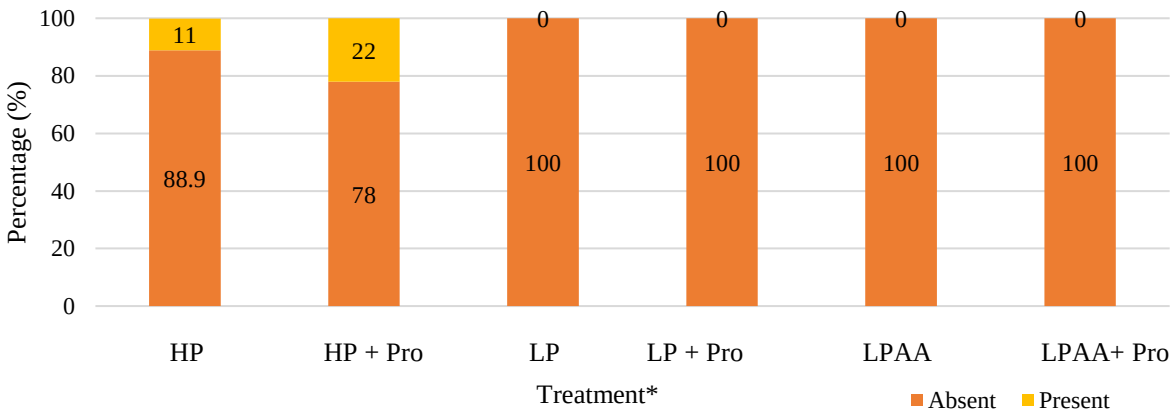
* HP: High CP; HP +Pro: High CP + Probiotic; LP: Low CP; LP + Pro: Low CP + Probiotic; LPAA: Low CP + AA; LPAA + Pro: Low CP + AA + Probiotic

SEM: Standard error of the mean

Presence of Salmonella spp. and Escherichia coli in ileal and caecal digesta

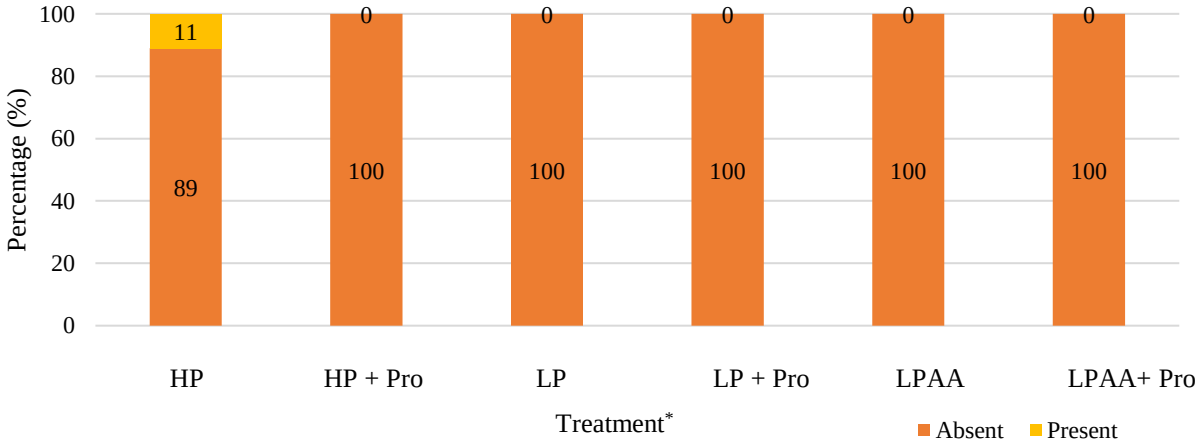
Figure 4.6 and 4.7 represent the presence or absence of *Salmonella spp.* in the ileal and caecal digesta. From the data, it can be seen that *Salmonella spp.* was present in 11% of caecal digesta from pigs in Treatment 1 (HP); and in the ileal digesta of 11% of pigs in Treatment 1 and 22% of pigs in Treatment 2 (HP + Probiotic). However, none of these differences among treatments were significant.

The concentration of *E. coli* in the caecal and ileal digesta was determined and are shown in Table 4.6. There were no significant differences noted in the enumeration of *E. coli* bacteria among any of the treatments.



*HP: High CP; HP + Pro: High CP + Probiotic; LP: Low CP; LP + Pro: Low CP + Probiotic; LPAA: Low CP + AA; LPAA + Pro: Low CP + AA + Probiotic

Figure 4.6 The presence or absence of *Salmonella spp.* in ileal digesta of pigs receiving either high or low crude protein diets, supplemented with additional amino acids and/or *Bacillus amyloliquefaciens* (Probiotic)



*HP: High CP; HP + Pro: High CP + Probiotic; LP: Low CP; LP + Pro: Low CP + Probiotic; LPAA: Low CP + AA; LPAA + Pro: Low CP + AA + Probiotic

Figure 4.7 The presence or absence of *Salmonella spp.* in caecal digesta of pigs receiving either high or low crude protein diets, supplemented with additional amino acids and/or *Bacillus amyloliquefaciens* (Probiotic)

Table 4.6 The enumeration of *E. coli* in the ileal and caecal digesta (converted to log values) of pigs receiving either high or low crude protein diets, supplemented with additional amino acids and/or *Bacillus amyloliquefaciens* (Probiotic)

Treatments*	Ileum E coli (CFUs/g)	Caecum E coli (CFUs/g)
1: HP	5.51	5.95
2: HP + Pro	5.85	5.21
3: LP	4.79	6.02
4: LP + Pro	5.56	5.74
5: LPAA	5.59	4.77
6: LPAA + Pro	5.84	6.15
SEM#	0.15	0.23
CP Main Effect		
High CP	5.68	5.58
Low CP	5.17	5.88
Low CP + AA	5.71	5.46
SEM#	0.16	0.24
Probiotic Main Effect		
With Probiotic	5.75	5.70
Without Probiotic	5.30	5.58
SEM#	0.16	0.24
Probability (p-value)		
CP	0.30	0.75
Probiotic	0.14	0.80
CP* Probiotic	0.76	0.16

* HP: High CP; HP +Pro: High CP + Probiotic; LP: Low CP; LP + Pro: Low CP + Probiotic; LPAA: Low CP + AA; LPAA + Pro: Low CP + AA + Probiotic

SEM: Standard error of the mean

Intestinal histomorphology

The intestinal tissue extracted from the ileum and duodenum was prepared and measured as previously described. The measurements for villi height, crypt depth and villi height to crypt depth ratio (VH:CD) of the different dietary treatments are given in Table 4.8. There was a significant CP*Probiotic interaction effect noted with ileal crypt depth. Treatment 1 (HP) had deeper crypts ($p = 0.01$) compared to Treatment 5 (LPAA). Crypt depth for pigs in Treatments 2 (HP + Probiotic), 3 (LP), 4 (LP + Probiotic) and 6 (LPAA + Probiotic) were not statistically different to one another. Crude protein level had a significant effect on ileal villi height. Pigs receiving HP diets had increased ($p = 0.01$) villi height compared to the pigs on the LPAA diets.

Table 4.7 The histomorphology at 49 days of age of pigs receiving either high or low crude protein diets, supplemented with additional amino acids and/or *Bacillus amyloliquefaciens* (Probiotic)

Treatments*	Duodenum VH (µm)	Duodenum CD (µm)	Duodenum VH:CD	Ileum VH (µm)	Ileum CD (µm)	Ileum VH:CD
1: HP	699.73	347.06	2.17	470.56	219.38[a]	2.23
2: HP + Pro	624.35	355.43	1.96	483.55	169.73[ab]	3.01
3: LP	665.13	319.97	2.63	446.48	171.67[ab]	2.76
4: LP + Pro	663.27	341.40	2.17	455.12	192.06[ab]	2.51
5: LPAA	661.88	300.26	2.41	408.21	155.96[b]	2.76
6: LPAA + Pro	663.42	327.15	2.16	428.33	195.89[ab]	2.37
SEM#	35.75	34.06	0.36	19.26	14.35	0.27
CP Main Effect						
High CP	662.04	351.25	2.06	477.06[a]	194.55	2.62
Low CP	664.20	330.68	2.40	450.80[ab]	181.87	2.63
Low CP + AA	662.65	313.70	2.29	418.27[b]	175.93	2.57
SEM#	25.28	24.08	0.25	13.57	10.00	0.19
Probiotic Main effect						
Without Probiotic	675.58	322.43	2.40	441.75	182.34	2.58
With Probiotic	650.35	341.33	2.10	455.67	185.89	2.63
SEM#	20.64	19.66	0.21	11.08	8.17	0.16
Probability (p value)						
CP	0.10	0.55	0.63	0.01	0.41	0.97
Probiotic	0.39	0.50	0.30	0.38	0.76	0.84
CP* Probiotic	0.48	0.96	0.93	0.96	0.01	0.07

a, b: values with differing superscripts within a column are statistically significant ($p < 0.05$)

* HP: High CP; HP +Pro: High CP + Probiotic; LP: Low CP; LP + Pro: Low CP + Probiotic; LPAA: Low CP + AA; LPAA + Pro: Low CP + AA + Probiotic

SEM: Standard error of the mean

Liver and kidney weights

The relative weights of the liver and each kidney are given in Table 4.8. The absolute weights were the actual weights measured; whereas the relative weights are the organ weights relative to total body weight at slaughter. The relative organ weights are more representative and give a better understanding of the treatment effects on organ weight. This is because it removes the variability of body weights between pigs. Interestingly, it can be seen from the table that there was a significant effect exerted by probiotic inclusion which resulted in increased liver weight. There were no significant differences among treatments for kidney weights.

Table 4.8 Relative organ weights (expressed as % of body weight) at 49 days-of-age of pigs receiving either high or low crude protein diets, supplemented with additional amino acids and/or *Bacillus amyloliquefaciens* (Probiotic)

Treatments*	Relative weight-Liver (%)	Relative weight-Kidney 1 (%)	Relative weight-Kidney 2 (%)
1: HP	3.71	0.28	0.27
2: HP + Pro	3.72	0.28	0.27
3: LP	3.66	0.27	0.28
4: LP + Pro	3.79	0.28	0.27
5: LPAA	3.66	0.27	0.27
6: LPAA + Pro	4.02	0.30	0.30
SEM#	0.13	0.02	0.01
CP Main Effect			
High CP	3.71	0.28	0.27
Low CP	3.72	0.27	0.27
Low CP + AA	3.84	0.29	0.28
SEM#	0.11	0.01	0.01
Probiotic Effect			
With Probiotic	3.84[a]	0.29	0.28
Without Probiotic	3.68[b]	0.27	0.27
SEM#	0.10	0.01	0.01
Probability (*p value)			
CP	0.38	0.57	0.55
Probiotic	0.05	0.21	0.47
CP * Probiotic	0.24	0.47	0.35

a, b: values with differing superscripts within a column are statistically significant ($p < 0.05$)

* HP: High CP; HP +Pro: High CP + Probiotic; LP: Low CP; LP + Pro: Low CP + Probiotic; LPAA: Low CP + AA; LPAA + Pro: Low CP + AA + Probiotic

SEM: Standard error of the mean

CHAPTER 5: DISCUSSION

5.1 Growth performance parameters

Performance parameters

The significant effect exerted by the higher CP diets on body weight and ADG is contrary to what was expected. The pigs consuming the HP diets had higher ($p < 0.05$) body weights at day 7, 14, 21, 28 and 35, and greater ADG ($p < 0.01$) for the periods between days 7-14 and 7-21. In addition, high CP diets resulted in increased ($p < 0.05$) G: F in the periods 7-14, 7-21, 21-42 and 7-42. The reduction of dietary CP from 21% to 18% in the pre-starter period was done in accordance with the study done by Yue & Qiao (2008) which specified that dietary CP can be reduced by up to 4% without having any significant effect on growth performance; provided that the essential amino acids were all in balance. Opapeju *et al*. (2008) however, observed reduced growth performance in pigs when dietary CP was reduced from 21% to 19%, even when diets were supplemented with additional amino acids. It was concluded in their study, and in ours, that there must have been a deficiency in some of the other nutrients or amino acids which caused this poorer performance.

Upon revision of our pre-starter and starter diets it became evident that there were several deficiencies for some of the treatments in both phases. In the pre-starter diets, there was a deficiency in Ile, Leu and His in Treatments 3-6, as well as a deficiency in phenylalanine (Phe) in all the dietary treatments, which ultimately resulted in unbalanced amino acid ratios. For the pre-starter diets, the achieved SID Ile levels for Treatments 3 and 4 were 5.94 g/kg with these levels dropping even further in Treatments 5 and 6 with achieved levels of 5.76 g/kg. The target level as per Evonik recommendations is 7.2- 7.8 g/kg for pre-starter pigs. The achieved SID Leu levels were 11.70 g/kg for Treatments 3 and 4 and 11.44 g/kg for Treatments 5 and 6. The target level for Leu is 13.2- 14.2 g/kg. The SID His levels were also far below target at 3.71 g/kg for Treatments 3 and 4 and 3.60 g/kg for Treatments 5 and 6. The target level for His in pre-starter pigs is 4.2- 4.5 g/kg. And lastly, the SID Phe levels were greatly deficient across all the diets at levels of 7.07 g/kg for Treatments 1 and 2, 5.53 g/kg for Treatments 3 and 4, and 5.33 g/kg for Treatments 5 and 6. The target SID Phe level for pre-starter is 8.0- 8.5 g/kg. These deficiencies almost certainly resulted in the poorer growth performance in these treatment groups.

In the starter diets, Phe and His were deficient in Treatments 3-6, Leu in Treatments 5 and 6 and Ile in all diets. The formulated Phe levels were 5.13 g/kg for Treatments 3 and 4 and 4.57 g/kg for Treatments 5 and 6. The target value for Phe for starter pigs as per Evonik recommendations is 6.5- 8.0

g/kg. The achieved His levels were 3.38 g/kg for Treatments 3 and 4 and 3.29 g/kg for Treatments 5 and 6. The target His level is 3.5- 4.2 g/kg. The achieved Leu level in Treatments 5 and 6 were 10.75 g/kg, where the target level for this amino acid is 10.8- 13.2 g/kg. And lastly, the formulated Ile levels were deficient across all dietary treatments in the starter phase with levels of 6.20 g/kg in Treatments 1 and 2, 4.65 g/kg in Treatments 3 and 4 and 4.46 g/kg in Treatments 5 and 6. Target Ile levels for starter pigs is 6.0- 7.2 g/kg.

All these deficiencies were unfortunately not detected during the formulation of the diets as emphasis was placed on the first 5 limiting amino acids, ensuring that none of these became limiting during the reduction of dietary CP. This unfortunately created an oversight whereby the other essential amino acids were not considered and reviewed. It was only upon revision of the results and the evaluation of poor performance in treatment groups with lower CP, that these deficiencies were detected. In addition, in hindsight, perhaps using SID AA levels currently employed by some nutritionists in South Africa as a starting point was the incorrect approach, as PIC intact boars may have a higher SID Lys requirement for lean growth and therefore, we may have not allowed these animals to reach their full potential by starting at such a low starting point. In the PIC (2016) nutrient guidelines manual, the SID Lys recommendation for pre-starter pigs is 1.42% and starter pigs 1.33%, which is significantly higher than our starting point of 1.23% for pre-starter phase and 1.08% for starter pigs.

Another possible causative effect of the significantly different performance among treatments could be as a result of the variations in ingredient inclusion. When formulating the diets, it was challenging to keep the NE constant, while at the same time reducing CP and increasing AA levels. This resulted in fluctuating inclusions of each raw material and the possible negative effects of varying starch and lipid contents in the diets on pig performance was not considered. In the studies by Zijlstra *et al*. (2012), Moss *et al*. (2018) and Selle *et al*. (2018), increased starch levels in reduced CP diets negatively impacted protein and AA digestibility. Moss *et al*. (2018) further elaborates that glucose and AA compete against one another for uptake within the intestine. The reduced CP diets had higher maize and cereal mix inclusions, and subsequently higher starch content.

There were no significant effects exerted by any of the dietary treatments on the ADFI among groups and therefore, a reduction in ADG in the treatments with lower CP was mirrored in the G: F. However, significant reductions were only seen in the period between day 7-14, 7-21, 21-42 and 7-42. From the results of the current study, and confirmed by Nyachoti and Jayaraman (2016), it appears that in order to take full advantage of the high protein accretion of weaner pigs and to avoid negative consequences on growth performance, dietary CP levels should remain elevated during this early growth phase. However,

perhaps if diets were correctly balanced with no deficiencies in any essential or NEAA, we would not have seen such a drastic effect on BW, ADG and G: F with CP reduction.

The lower growth performance observed in pigs consuming low CP diets compared to high CP diets can be further explained in the study by Htoo (2017). In this paper, Htoo pointed out that while it is important to bear the essential amino acids in mind when reducing dietary CP, one must also remember not to exceed the SID Lys to CP ratio of 6.9% (or 7.4% on a total amino acid basis). This rule was unfortunately overlooked when formulating the diets of the current study. The SID Lys: CP for Treatment 5 and 6 for the pre-starter diets was 7.6% and this ratio deviated even further in the starter diets when the CP was reduced to 15%. The SID Lys: CP ratio was 7.2% in Treatments 3 and 4 and as high as 8.0% in Treatments 5 and 6 in the starter diets. The reduction of CP resulted in an unbalanced ratio between SID Lys and total CP needed for the synthesis of the NEAA. Furthermore, when the SID Lys level was increased by 10% in Treatments 5 and 6, the deficiencies were intensified, resulting in the observed poorer performance of pigs in these treatment groups.

The lack of significant response on growth performance after supplementation with a probiotic (even though there was a trend observed for G: F between day 14-21 and 7-21), is contrary to the findings by Le Bon *et al*. (2010), Liu *et al*. (2015) and Kinh *et al*. (2019). In the study by Liu *et al*. (2015), a significant improvement in ADG, ADFI and G: F was observed after supplementation with *Lactobacillus brevis* into the diets of weaner pigs. The FCR of weaned pigs was also improved in the trial done by Le Bon *et al*. (2010) after the supplementation of a combination probiotic consisting of *Saccharomyces cerevisiae spp. boulardii* and *Pediococcus acidilactici*. In a trial by Lei *et al*. (2015), the supplementation of *Bacillus amyloliquefaciens* resulted in significant improvements in body weight gain and feed efficiency of broiler chickens. Kinh *et al* (2019) observed significant improvements in growth performance after the supplementation with *Bacillus amyloliquefaciens* to weaner pigs. The lack of any significant improvements in growth performance in the current study is not known, however this could be attributed to the generally high herd health exhibited by the pigs during the course of the trial.

Bhandari *et al*. (2010) demonstrated a positive synergistic effect on growth performance between low CP and a probiotic in weaner pig diets, who observed an increased ADG before and after an enteric pathogen challenge of *E. coli* when pigs consumed lower CP diets compared to high CP diets. In addition, the pigs that were on the low CP plus probiotic diets had the best ADG before and after inoculation. In the current study however, we did not observe the same positive interactive effect on performance parameters between low CP and the addition of a probiotic into the diets of weaner pigs, which may have been attributable to the lack of challenge.

Liver and kidney weights

The effect of a 3% reduction in CP level on organ weights was also explored in our study due to the correlation previous researchers have seen. Nyachoti *et al.* (2006) observed a significant reduction in liver weight when CP level in the diet was reduced from 23% to 17%. Kerr *et al*. (2003) saw a significant reduction in kidney weights when CP was reduced from 16% to 12% but observed no significant effect on liver weights. In this study, kidney weights were not significantly different among dietary treatments which is in contradiction with previous researchers. We also saw no significant difference in liver weights among treatments when considering protein level, which is in accordance with the studies done by Opapeju *et al.* (2008) and Kerr *et al*. (2003) for liver weights. The reasoning behind the lack of response is unknown, perhaps the reduction in dietary CP again was not severe enough to see these significant changes. Interestingly, the addition of the probiotic resulted in a significant increase in liver weights. This was a very fascinating finding and is contrary to the findings by Tortuero *et al*. (1995) who found that the supplementation of lactic acid bacteria (*Lactobacillus casei, Lactobacillus bulgaricus spp.* and *Streptococcus thermophilus spp*.) resulted in reduced liver weights compared to the control (although these findings were not significant).

5.2 Gut health parameters

Faecal score

The period between day 7-14 revealed that Treatment 4 (LP + Probiotic) resulted in the highest number of pigs with faecal scores of 2, (which again is considered the most 'normal' faeces of weaned pigs). This indicates a positive interaction between reduced CP and the addition of the probiotic. This is in alignment with the trials done by Bhandari *et al*. (2010) and Garcia *et al*. (2014) who both also demonstrated a positive synergistic effect between low CP diets and the addition of a probiotic.

In the literature study, the numerous negative impacts of high CP in weaner pigs were extensively discussed in terms of poor faecal scores, increased caecal NH_3 concentration, reduced VH: CD, deeper intestinal crypts and higher counts of *E. coli* and *Salmonella spp.* in ileal and caecal digesta. In the current study, many of these expected outcomes did not materialise and conflicting trends were noted. Opapeju *et al*. (2008) observed increasing faecal scores as dietary CP increased from 17% to 21%. In our study, we considered a faecal score of 2 to be the most 'normal' and indicative of good gut health. There were significant effects of CP on faecal scores during day 1-7 where pigs receiving Treatment 1 (HP) had the highest number of diarrhoeal scores in the first seven days and the reduction of CP significantly improved faecal scores by resulting in an increase in the number of faecal scores of 1 and reducing the number of

faecal scores of 3. This finding is in alignment with Opapeju *et al*. (2008) when considering the negative impact of high CP on faecal score.

For day 1-7, the addition of the probiotic into the HP diets resulted in an increase in the number of faecal scores of 0 and 1, and a reduction in faecal scores of 3, indicative of decreased diarrhea incidence. Bhandari *et al*. (2010) observed the same trend whereby probiotic addition into the diets improved the faecal scores of pigs on high CP diets. In the low CP diets in the present study, the addition of the probiotic resulted in an increase in faecal scores of 1 and a reduction in faecal scores of 2 and 3. The addition of the probiotic in the diets from day 7-14 also resulted in significantly higher faecal scores of 2 in the low CP diets. This may demonstrate an improvement in gut health, but only in Treatments 4 and 6 compared to Treatments 3 and 5, respectively. These findings are in alignment with the work done by both Le Bon *et al*. (2010) and Liu *et al*. (2015), who both saw an improvement in faecal score with the inclusion of a probiotic into the diets of weaned pigs.

The overall findings for the entire experimental period (d1-41) was that the faecal scores worsened; showing an upward movement towards the higher scores of 3, indicative of slight diarrhoea. The causative effect for this is unknown, but our conclusion could be in alignment with Mou *et al*. (2019), that the longer the pigs were on the unbalanced diets, the worse their faecal scores and therefore gut health became. Mou *et al*. (2019) discussed that the BCAAs are important for adequate gut health integrity. The deficiency in Leu and Ile in the diets in the low CP diets could explain the worsening faecal scores, possibly caused by an underdeveloped intestinal barrier due to the importance role Leu has in intestinal development (Sun *et al*., 2015).

Significant effects were exerted by the dietary treatments on faecal score for day 1 and day 6 only. On day 1, pigs on Treatment 3 (LP) had significantly higher faecal scores than pigs from Treatment 2 (HP + Probiotic) and Treatment 6 (LPAA + Probiotic). This indicates that the addition of Probiotic may have resulted in improved faecal scores in certain treatments. On day 6, pigs receiving Treatment 1 (HP) had significantly higher faecal scores than those in Treatment 6 (LPAA + Probiotic). This is in line with what we would expect, pigs on higher CP diets have worse faecal scores than lower CP diets as demonstrated by Opapeju *et al*. (2008). The supplementation of the probiotic in this manner appears to result in improved faecal scoring.

The overall findings for low levels of diarrhoea during the study was not what we expected due to the absence of AGPs, organic acids and zinc in any of the treatment diets. This finding is therefore contrary to the study by Wierup (2001a), which stated that the complete removal of AGPs has in the past resulted in an increased use of therapeutic antibiotics due to outbreaks of diarrhoea. The lack of any prominent

diarrhoea in the trial could also be attributed to high biosecurity measures that were implemented during the trial; such as restricted movement of visitors into the pig house and the implementation of a shower-in policy. In addition, the trial was run under experimental conditions and therefore the probability of an *E. coli* outbreak was small. The pig house was also left open for a few months after the previous trial. Therefore, the likelihood of any remaining pathogens in the house that could have negatively impacted the pigs were minimal, and this also explains the lack of significant responses seen with the supplementation of the probiotic on the majority of faecal score data as well as the pathogenic bacterial loads.

Caecal ammonia and pH

There were no significant differences in caecal pH measurements among dietary treatments. Higher pH levels were expected in the higher CP diets in accordance with the studies done by Partanen & Mroz (1999) and Wellock *et al*. (2008). The lack of any effect of CP on pH correlates with the study done by Htoo *et al*. (2007), who also saw no significant differences in pH when reducing the CP level in the diet. The level of ammonia (NH_3) also did not differ among treatments, which is in contradiction to the findings by Htoo *et al*. (2007) and Opapeju *et al*. (2008). Htoo *et al*. (2007) found that there was a higher number of toxic nitrogenous metabolites (including NH_3) in the caecum resulting from increased fermentation of excess protein by pathogenic bacteria when the level of CP in the diet was elevated. In our study, it is uncertain as to why there was no increased NH_3 production from the higher CP diet. One possible explanation could be that the initial level of CP was not high enough to see any marked effects. In the study by Htoo *et al.* (2007), the CP level started at 24% and was reduced to 20%, whereas in this study, CP levels were reduced from 21% to 18% for the pre-starter phase, wherein the measurement of NH_3 occurred.

Salmonella and Escherichia coli in ileal and caecal digesta

Wellock *et al*. (2008) observed increased proliferation of *E. coli* in higher CP diets, and although these findings were not significant, there was a distinct tendency for increased shedding of these pathogenic bacteria. Prohaszka and Baron (1980) also observed a correlation between high CP diets and levels of haemolytic *E. coli* in the small intestine of weaner pigs. In our study, we observed higher ileal *E. coli* levels in the high CP diets compared to the low CP treatments, but these observations were however not significant. Although also not significant, the increased prevalence of *Salmonella spp.* in the caecal digesta of 11% of pigs in Treatment 1 (HP) and in the ileal digesta from pigs from both Treatment 1 (11%) and Treatment 2 (22%) (HP + Probiotic), may indicate increased proliferation of pathogenic bacteria in the higher CP diets due to the excess proteinous substrate. The lack of significant effect on *E. coli* levels after the addition of the probiotic is in contradiction to the studies by Le Bon *et al*. (2010) and Liu *et al*. (2015),

who both observed a reduction in *E. coli* and coliform bacteria in the intestinal tract of weaned pigs after the addition of a probiotic.

Intestinal histomorphology

There was a significant CP*Probiotic interaction effect observed for ileal crypt depth. Pigs in Treatment 1 (HP) had deeper ($p< 0.05$) crypts than pigs in Treatment 5 (LPAA), but interestingly, were both treatments that did not contain any probiotic supplementation. The other four treatments were not significantly different to one another or to Treatment 1 and Treatment 5. Increased crypt depth in high CP diets is in accordance with the paper by Nyachoti & Jayaraman (2016). These authors discussed the damage to intestinal villi caused by high CP diets is due to the production of toxic metabolites, and as a result, in an attempt to repair the damage, cell proliferation in the crypts is increased, resulting in deeper crypts. However, due to the lack of significantly higher NH_3 levels in the caeca of pigs on the high CP diets and the absence of recurring diarrhoea in these treatment groups in the current study, it is unlikely that the deeper crypts were as a result of intestinal damage by toxic metabolites.

High CP diets resulted in significantly longer villi compared to the LPAA treatment groups, which is completely contrary to what was expected. The increased amino acid content in the LPAA group was expected to result in increased mucosal thickness due to the positive effect threonine in particular has on gut integrity as explored by Bertolo *et al*. (1998), Stoll *et al*. (1998) and Faure *et al*. (2007). However, the significantly shorter villi in the LPAA diets could be explained by the imbalance of NEAA resulting from a SID Lys: CP of above 6.9%, which Yue & Qiao (2008) also observed in their study. Mou *et al*. (2019) discussed the importance of the branched chain amino acids (Ile, Val and Leu) on intestinal development in piglets through the proliferation of enterocytes. The lack of sufficient Ile in the majority of diets could have therefore also been a causative factor in the reduced villi height observed in the LPAA groups. The lack of significant improvement in intestinal morphology with the supplementation of a probiotic is in contradiction to the study by Lei *et al.* (2015). These authors observed significant improvements in villi height, crypt depth and VH:CD with the supplementation of *Bacillus amyloliquefaciens*.

CHAPTER 6: CONCLUSION AND CRITICAL EVALUATION

High CP diets are still widely used in weaner pigs in order to exploit the young piglet's high inherent ability for protein deposition during this time. This is especially true in countries which are still able to make use of AGPs on a regular basis, in order to counteract any possible negative effects high CP diets could have, particularly on gut health. Since the use of AGPs is becoming more widely monitored and controlled, there is a significant drive to seek alternative measures to improve gut health. One of the topics being explored more frequently is the reduction of dietary CP in weaner diets and accurately balancing for individual amino acids. This is done in an attempt to reduce protein wastage and avoid the subsequent passage of undigested protein into the hindgut of the pig, resulting in unwanted fermentation. Fermentation of protein in the hindgut results in higher pH and provides a perfect substrate and environment for pathogenic bacteria proliferation, which disrupts the intestinal barrier and causes diarrhoea. With the addition of crystalline amino acids, one can reduce the CP level of the diet without resulting in any negative impact on growth performance. With the movement towards more prudent use of AGPs in swine diets, alternatives are being explored which exhibit similar effects to antibiotics, in order to combat intestinal disease. Probiotics have been recognised as one of the best suited alternatives to AGPs compared to other gut health additives, due to their clearly defined modes of action, particularly against pathogenic bacteria presence in the gut.

In our study we explored the effect of reducing dietary CP by 3% from 21% to 18% in the pre-starter diets and from 18% to 15% in the starter diets. In addition, due to modern pig genetics dictating a higher lysine supplementation in order to accommodate increased lean muscle growth, the SID lysine level and the first five limiting amino acids (Met, Lys, Thr, Trp and Val) were elevated by 10%. Lastly, the diets were supplemented with a probiotic product, *Bacillus amyloliquefaciens*, to ascertain what effect beneficial bacteria would have on gut health and performance of weaned pigs. It was hypothesised that a better-balanced gut would result in increased utilisation of nutrients and therefore improved performance.

The reduction of dietary CP resulted in significant negative impacts on BW, ADG and G: F in the pre-starter period and on the BW and G: F in the starter period. This was completely contrary to what was expected, as it was assumed that all nutrients were properly balanced. Upon further examination of the diets, it was observed that there were significant deficiencies in Ile, Leu, Phe and His in the pre-starter and starter diets. In addition, the upper limit of SID Lys: CP of 6.9% was not maintained and was exceeded in both the pre-starter and starter diets, which almost certainly resulted in deficiencies in the non-essential amino acids (NEAA), resulting in poorer growth performance.

It is imperative to closely review and consider all the essential and non-essential amino acids when reducing the CP in weaner diets, and not just the first five limiting amino acids. Too much emphasis was placed on the first five limiting amino acids in our study, neglecting the other important amino acids. It was clearly observed that this resulted in a significant negative impact on performance.

There were no significant effects of dietary CP level on ammonia excretion, pH in caecal digesta, organ weights or pathogenic bacteria in ileal and caecal digesta, as expected. Perhaps in order to see more of a pronounced effect of excess protein, there needs to be a higher starting CP, in alignment with other weaner pig studies on CP. It is concluded that a starting CP of 21%, although representative of weaner diets in South Africa, was not high enough to see any significant effects on these parameters.

There was a significant effect of CP level on faecal score, indicative of gut health, especially in the first few weeks of the trial. This is in alignment with what was expected and is an indication of a possible oversupply of protein to the hindgut, subsequent unwanted fermentation and disruption of the gut balance. However, this was not severe at any point during the trial. In addition, the faecal score of all treatments worsened as the trial progressed. This could be as a result of deficiencies in essential nutrients in the diet for optimal gut integrity, such as the BCAAs. Crypt depth was also increased on the high CP diets, indicative of possible increased damage due to toxic metabolites from excess protein fermentation. This is a further indication of improved gut health (in this case measured as intestinal integrity) with reduced CP. Villus height was the highest in the high CP diets, perhaps attributed to increased nutrient availability in the higher CP diets. The shorter villi in the low CP + AA diets could once again be attributed to the deficiency in important nutrients (NEAA and BCAA) in these diets.

The health status of the pigs was at a very high level during the trial, and this could be the reasoning behind a lack of significant response on growth performance with the supplementation of the probiotic. Probiotics exert their largest effect if there is a disruption in the gut microbiota which they can act on and resolve. With no challenge exposed to the piglets, or lack of severe diarrhoea caused by excessive protein in the gut, the gut health of all pigs was generally of a high level. Perhaps for a more indicative trial of probiotic performance in the future, the pigs should be challenged with an enteric pathogen such as *E. coli* in order to truly ascertain the effects of a probiotic under a severe challenge.

A positive synergistic effect between the probiotic and low CP was observed between day 7-14 for faecal score. This is in alignment with what was expected as per other researchers' findings, that low CP diets and the addition of a probiotic can maintain good gut health in weaner pigs in the absence of AGPs. The addition of the probiotic into the high CP diets also appeared to mitigate some of the negative effects of increased protein fermentation, by improving faecal score in these pigs.

Due to the negative effect lowering the dietary CP by 3% had on pig performance, we reject the first alternate hypothesis (H_{A1}). Lowering the CP by 3% and increasing the amino acid levels by 10% also resulted in reduction of pig performance and therefore we also reject the fourth alternate hypothesis (H_{A4}). However, these hypotheses state that there should not be any deficiencies in the standard ileal digestible amino acids, which we know was not the case when CP was reduced, as some essential and non-essential amino acids became deficient. Due to the positive impact reducing CP had on some aspects of gut health (by improving faecal score and resulting in reduced ileal crypt depth), we reject the second null hypothesis (H_{02}).

The supplementation of the probiotic in the low CP diets resulted in a perceived improvement in gut health as seen through improved faecal score, therefore we reject the third null hypothesis (H_{03}). Lastly, due to the lack of significant effect being observed in the LPAA diets after the supplementation of the probiotic on both gut health and performance, we reject the fifth alternate hypothesis (H_{A5}).

The following final recommendations can be drawn from the current study. Reducing dietary CP is an effective strategy to prevent digestive imbalances in weaner pigs, especially when AGPs are not permitted in feed. It is however imperative that when CP is reduced, the essential and non-essential amino acids do not become limiting. This is in order to avoid any potential negative impacts these deficiencies may have on growth performance and gut integrity. The supplementation of a probiotic into low CP diets may be another potentially viable strategy to employ in the face of antibiotic bans, in order to take full advantage of the positive synergistic effects that exist between low CP diets and probiotics. The maintenance of good biosecurity should also not be overlooked during an AGP ban, as reduced exposure to external pathogens maintains a high herd health and ensures subsequent good growth performance among weaned pigs.

REFERENCES

Altmann, G.G., 1972. Influence of starvation and refeeding on mucosal size and epithelial renewal in the rat small intestine. Am. J. Anat. 133, 391-400

AMINOPig® 1.0. 2011. Evonik Degussa GmbH, Hanau-Wolfgang, Germany

Angelakis, E., 2016. Weight gain by gut microbiota manipulation in productive animals. Microb. Pathogenesis 106, 162-170

Anderson, D.B., McCracken, V.J., Aminov, R.I., Simpson, J.M., Mackie, R.I., Verstegen, M.W.A. & Gaskins, H.R., 1999. Gut microbiology and growth-promoting antibiotics in swine. Nutrition Abstracts and Reviews. Series B: Livestock Feeds and Feeding 70, 101-188

AOAC Authors, 1990. Official methods of analysis of the association of official analytical chemists. 15th edition

Apsimon, H.M. & Kruse-Plass, M., 1990. The role of ammonia as an atmospheric pollutant. In: Nielsen, V.C., Voorburg, J.H., L'Hermite, P. (Eds.), Odour and Ammonia Emissions from Livestock Farming. Elsevier Applied Science, London and New York. 17-19

Arbuckle, J.L., 1997. IBM® SPSS® Amos™ 23 User's Guide. Amos Version 3.6 (SmallWaters Corporation)

Argenzio, R.A. & Whipp, S.C., 1979. Inter-relationship of sodium, chloride, bicarbonate and acetate transport by the colon of the pig. J. Physiol. 295, 365-381

Bajagai, Y.S., Klieve, A.V., Dart, P.J., & Bryden, W.L., 2016. Probiotics in animal nutrition – Production, impact and regulation. FAO Animal Production and Health Paper No. 179. Rome

Ball, R.O., Urschel, K.L. & Pencharz, P.B., 2007. Nutritional consequences of interspecies differences in arginine and lysine metabolism. J. Nutr. 137, 1626-1641

Baylis, C.L., 2007. Manual of microbiological methods for the food and drinks industry, 5th edition. Campden BRI

Bech-Anderson, S., 1991. Determination of Tryptophan with HPLC after alkaline hydrolysis in autoclave using α-Methyl-tryptophan as internal standard. Acta Agric. Scand. 41, 305-309

Bengmark, S. & Jeppsson, B., 1995. Gastrointestinal surface protection and mucosa reconditioning. J. Parent. Enteral Nutr. 19, 410-415

Bertolo, R.F.P., Chen, C.Z.L., Law, G., Pencharz, P.B. & Ball, R.O., 1998. Threonine requirement of neonatal pigs receiving total parental nutrition is considerably lower than that of piglets receiving an identical diet intragastrically. J. Nutr. 128, 1752-1759

Bhandari, S.K., Opapeju, F.O., Krause, D.O. & Nyachoti, C.M., 2010. Dietary protein level and probiotic supplementation effects on piglet response to Escherichia coli K88 challenge: Performance and gut microbial population. Livest. Sci. 133, 185-188

Bian, G., Ma, S., Zhu, Z., Su, Y., Zoetendal, E.G., Mackie, R., Liu, J., Mu, C., Huang, R., Smidt, H. & Zhu, W., 2016. Age, introduction of solid feed and weaning are more important determinants of gut bacterial succession in piglets than breed and nursing mother as revealed by a reciprocal cross-fostering model. Environ. Microbiol. 18, 1566-1577

Bikker, P., Fledderus, J., Le Bellego, L. & Rovers, M. 2006. AGP ban effects on amino acid requirements. Feed Mix 14, 22-24.

Blanco, M., Coello, J., Iturriaga, H., Maspoch, S. & González Bano, R., 2000. On-line monitoring of starch enzymatic hydrolysis by near-infrared spectroscopy. Analyst. 125, 749-752

Boudry G., Peron V., Le Huerou-Luron I., Lalles J.P. & Sève B., 2004. Weaning induces both transient and long-lasting modifications of absorptive, secretory, and barrier properties of piglet intestine. J. Nutr. 134, 2256-2262

Boyer, P.E., D'Costa, S., Edwards, L. L., Milloway, M., Susick, E., Borst, L.B., Thakur, S., Campbell, J.M., Crenshaw, J.D., Polo, J. & Moeser, A.J., 2015. Early-life dietary spray-dried plasma influences immunological and intestinal injury responses to later-life *Salmonella typhimurium* challenge. Brit. J. Nutr. 113, 783-793

Broderick, G.A. & Kang, J.H., 1980. Automated simultaneous determination of ammonia and total amino acids in ruminal fluid and in vitro media. J. Dairy Sci. 63, 64-75

Butler J.E., Lager K.M., Splichal I., Francis D., Kacskovics I., Sinkora M., Wertz N., Sun. J., Zhao, Y., Brown, W.R., DeWald, R., Dierks, S., Muyldermans,S., Lunney, J.K., McCray, P.B., Rogers, C.S., Welsh, M.J., Navarro, P., Klobasa, F., Habe, F., & Ramsoondar, J., 2009. The piglet as a model for B cell and immune system development. Vet. Immunol Immunop. 128 (1-3), 147-170

Canh, T.T., Aarnink, A.J.A., Schutte, J.B., Sutton, A., Langhout, D.J. & Verstegen, M.W.A., 1998. Dietary protein affects nitrogen excretion and ammonia emission from slurry of growing-finishing pigs. Livest. Prod. Sci. 56, 181-191

Canibe, N. & Jensen, B.B., 2003. Fermented and nonfermented liquid feed to growing pigs: Effect on aspects of gastrointestinal ecology and growth performance. J. Anim. Sci. 81, 2009-2031

Carlson, D., Poulsen, H. D., & Vestergaard, M., 2004. Additional dietary zinc for weaning piglets is associated with elevated concentrations of serum IGF-I. J. Anim. Physiol. Anim. Nutr. 88, 332-339

Catassi, C., Bonucci, A., Coppa, G.V., Carlucci, A. & Giorgi, P.L., 1995. Intestinal permeability changes during the first month: effect of natural versus artificial feeding. J. Pediatr. Gastr. Nutr. 21, 383-386

Chan, Y. & Walmsley, R.P., 1997. Learning and understanding the Kruskal-Wallis one-way analysis-of-variance-by-ranks test for differences among three or more independent groups. Phys. Ther. 77, 1755-1762

Cho, J.H., Zhao, P.Y. & Kim, I.H., 2011. Probiotics as a dietary additive for pigs: A review. J. Anim. Vet. Adv. 10 (16), 2127-2134

Christen, S., Peterhans, E. & Stocker, R., 1990. Antioxidant activities of some tryptophan metabolites: possible implication for inflammatory diseases. Proc. Natl. Acad. Sci. U. S. A. 87, 2506-2510

Circu, M.L. & Aw, T.Y., 2012. Intestinal redox biology and oxidative stress. Semin. Cell Dev. Biol. 23, 729-737

Clarke, L.L., 2009. A guide to Ussing chamber studies of mouse intestine. Am. J. Physiol.-Gastr. L. 296, 1151-1166

Corring, T., Aumaitre, A. & Durand. G., 1978. Development of digestive enzymes in the piglet from birth to 8 weeks. Nutr. Metab. 22, 231-243

Cranwell, P. D., Noakes, D. E. & Hill, K. J., 1976. Gastric secretion and fermentation in suckling pig. Brit. J. Nutr. 36, 71-86

De Oliveira, M.J.K., Sakomura, N.K., de Paula Dorigam, J.C., Doranalli, K., Soares, L. & da Silva Viana, G., 2019. Bacillus amyloliquefaciens CECT 5940 alone or in combination with antibiotic growth

promoters improves performance in broilers under enteric pathogen challenge. Poultry Sci. 98, 4391-4400

Deplancke, B. & Gaskins, H.R., 2001. Microbial modulation of innate defense: Goblet cells and the intestinal mucus layer. Am. J. Clin. Nutr. 73 (suppl), 1131-1141

Diamond, J.M., Karasov, W.H., 1983. Trophic control of the intestinal mucosa. Nature (London) 304, 18

Diaz, D. 2007. Effect of *Bacillus amyloliquefaciens* CECT-5940 spores on broiler performance and digestibility. Accessed Mar. 2020. https://en.engormix.com/poultry-industry/articles/ bacillus-amyloliquefaciens-cect-5940-spores-on-broiler-t33979.htm

Dibner, J.J. & Richards, J.D., 2005. Antibiotic growth promoters in agriculture: history and mode of action. Poultry Sci. 84, 634-643

Dong, G., Zhou, A., Yang, F., Chen, K., Wang, K. & Dao, D., 1996. Effect of dietary protein levels on the bacterial breakdown of protein in the large intestine, and diarrhoea in early weaned piglets. Acta Vet. Zootec. Sin. 27, 293-302

Dong, G.Z. & Pluske, J.R., 2007. The low feed intake in newly-weaned pigs: problems and possible solutions. Asian Australas. J. Anim.Sci. 20 (3), 440-452

Doron, S. & Snydman, D.R., 2015. Risk and safety of probiotics. Clin. Infect. Dis. 60 (Suppl. 2), 129-134

Edelblum, K.L. & Turner, J.R., 2009. The tight junction in inflammatory disease: communication

breakdown. Curr. Opin. Pharmacol. 9, 715-720

Eder, K., Peganova, S. & Kluge, H., 2001. Studies on the tryptophan requirement of piglets. Arch. Tierernahr. 55, 281-297

EFSA., 2008. Scientific opinion of the panel on additives and products or substances used in animal feed (FEEDAP) on a request from the European Commission on the safety and efficacy of Probiotic (*Bacillus amyloliquefaciens*) as feed additive for chickens for fattening. The EFSA Journal 773, 1-13

Esteban, S., Nicolaus, C., Garmundi, A., Rial, R.V., Rodriguez, A.B., Ortega, E. & Ibars, C.B., 2004. Effect of orally administered L-tryptophan on serotonin, melatonin, and the innate immune response in the rat. Mol. Cell. Biochem. 267, 39-46

Everaert, N., Van Cruchten, S., Weström, B., Bailey, M., Van Ginneken, C., Thymann, T. & Pieper, R., 2017. A review on early gut maturation and colonization in pigs, including biological and dietary factors affecting gut homeostasis. Anim. Feed Sci. Tech. 233, 89-103

Faure, M., Choné, F., Mettraux, C., Godin, J., Béchereau, F., Vuichoud, J., Papet, I., Breuillé, D. & Obled, C., 2007. Threonine utilization for synthesis of acute phase proteins, intestinal proteins, and mucins in increased during sepsis in rats. J. Nutr. 137, 1802-1807

Finkelstein, J.D., 2000. Pathways and regulation of homocysteine metabolism in mammals. Semin. Thromb. Hemost. 26, 219-225

Friend, D.W., Cunningham, H.M., Nicholson, J.W.G., 1963. The production of organic acids in the pig. Can. J. Anim. Sci. 43, 156-168

Gaines, A.M., Yi, G.F., Ratliff, B.W., Srichana, P., Allee, G.L., Knight, C.D. & Usry, J.L., 2003. Estimation of the true ileal digestible (TID) lysine and sulfur amino acid requirements for nursery pigs weighing 12 to 24 kilograms. J. Anim. Sci. 82 (2), 66

Garcia, K.E., de Souza, T.C.R., Landin, G.M., Barreyro, A.A., María Guadalupe Bernal Santos, M.G.B. & Soto, J.G.G., 2014. Microbial fermentation patterns, diarrhea incidence, and performance in weaned piglets fed a low protein diet supplemented with probiotics. Food Nutr. Sci 5, 1776-1786

Gaskins, H.R., 2001. Intestinal bacteria and their influence on swine growth. Swine Nutrition, 585-608

Gaskins, H.R., Collier, C.T. & Anderson, D.B., 2002. Antibiotics as growth promotants: mode of action. Anim. Biotechnol. 13 (1), 29-42

Giang, H.H., Viet, T.Q., Ogle, B. & Lindberg, J.E., 2010. Growth performance, digestibility, gut environment and health status in weaned piglets fed a diet supplemented with potentially probiotic complexes of lactic acid bacteria. Livest. Sci. 129, 95-103.

Gibson, G. R. & Roberfroid, M. B., 1995. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. J. Nutr. 125, 1401-1412.

Gloaguen, M., Le Floc'h, N., Corrent, E., Primot, Y. & van Milgen, J., 2014. The use of free amino acids allows formulating very low crude protein diets for piglets. J. Anim. Sci. 92 (2), 637-644

Gresse, R., Chaucheyras-Durand, F., Fleury, M.A., Van de Wiele, T., Forano, E. & Blanquet-Diot, S., 2017. Gut microbiota dysbiosis in postweaning piglets: understanding the keys to health. Trends Microbiol. 25 (10), 851-873

Grimble, R.F., 2006. The effects of sulfur amino acids intake on immune function in humans. J. Nutr. 136, 1660-1665

Hamilton, C.R. & Veum, T.L., 1986. Effect of biotin and (or) lysine additions to corn-soybean meal diets on the performance and nutrient balance of growing pigs. J. Anim. Sci. 62, 155-162

Hedde, R.D. & Lindsey, T.O., 1986. Virginiamycin: A Nutritional Tool for Swine Production. Agri-Practice 7, 3-4.

Henry, Y., Sève, B., Mounier, A. & Ganier, P., 1996. Growth performance and brain neurotransmitters in pigs as affected by Tryptophan, protein and sex. J. Anim. Sci. 74, 2700-2710

Heo, J.M., Opapeju, F.O., Pluske, J.R., Kim, J.C., Hampson, D.J & Nyachoti, C.M., 2013. Gastrointestinal health and function in weaned pigs: a review of feeding strategies to control post-weaning diarrhoea without using in-feed antimicrobial compounds. J. Anim. Physiol Anim. Nutr. 97, 207-237

Hill, G. M., Cromwell, G.L., Crenshaw, T.D., Dove, C.R., Ewan, R.C., Knabe, D.A., Lewis, A.J., Libal, G.W., Mahan, D.C., Shurson, G.C., Southern, L.L. & Veum, T.L., 2000. Growth promotion effects and plasma changes from feeding high dietary concentrations of zinc and copper to weanling pigs (regional study). J. Anim. Sci. 78, 1010-1016

Højberg, O., Canibe, N., Poulsen, H. D., Hedemann, M. S. & Jensen, B. B., 2005. Influence of dietary zinc oxide and copper sulfate on the gastrointestinal ecosystem in newly weaned piglets. Appl. Environ. Microb. 71, 2267-2277

Hossain, M.I., Sadekuzzaman, M. & Ha, S., 2017. Probiotics as potential alternative biocontrol agents in the agriculture and food industries: a review. Food Res. Int. 100, 63-73

Houdijk, J. G. M., Hartemink, R., Verstegen, M. W. A. & Bosch, M., 2002. Effects of dietary non-digestible oligosaccharides on microbial characteristics of ileal chyme and faeces in weaner pigs. Arch. Anim. Nutr. 56, 297-307

Htoo, J.K., Araiza, B.A., Sauer, W.C., Rademacher, M., Zhang, Y., Cervantes, M. & Zijlstra, R.T., 2007. Effect of dietary protein content on ileal amino acid digestibility, growth performance, and

formation of microbial metabolites in ileal and cecal digesta of early- weaned pigs. J. Anim. Sci. 85, 3303-3312

Htoo, J.K., 2013. Requirements for lysine for modern high lean pigs- an update. AMINONews® 17 (2)

Htoo, J.K., 2017. The potential for feeding low crude protein-amino acid supplemented diets to starter and growing-finishing pigs. AMINONews® 21 (1)

Hu, J., Nie, Y., Chen, S., Xie, C., Fan, Q., Wang, Z., Long, B., Yan, G., Zhong, Q. & Yan, X., 2017. Leucine reduces reactive oxygen species levels via an energy metabolism switch by activation of the mTOR-HIF-1alpha pathway in porcine intestinal epithelial cells. Int. J. Biochem. Cell B. 89, 42-56

Huxtable, R.J., 1992. Physiological actions of taurine. Physiol. Rev. 72, 101-163

Jensen, B.B. & Jorgensen, H., 1994. Effect of dietary fiber on microbial activity and microbial gas production in various regions of the gastrointestinal tract of pigs. Appl. Environ. Microbiol. 60, 1897-1904

Jensen, B. B. 1998. The impact of feed additives on the microbial ecology of the gut in young pigs. J. Anim. Feed Sci. 7, 45-64

Jonsson, E. & Conway, P. 1992. Probiotics for pigs. In Probiotics: The Scientific Basis; Fuller, R., Ed.; Chapman and Hall: London, 260-316

Kaewtapee, C., Burbach, K., Tomforde, G., Hartinger, T., Camarinha-Silva, A., Heinritz, S., Seifert, J., Wiltafsky, M., Mosenthin, R. & Rosenfelder-Kuon, P., 2017. Effect of Bacillus subtilis and Bacillus licheniformis supplementation in diets with low- and high-protein content on ileal crude protein and amino acid digestibility and intestinal microbiota composition of growing pigs. J. Anim. Sci. Biotechno. 8, 37

Kahindi, R.K., Htoo, J.K. & Nyachoti, C.M., 2014. Effect of dietary lysine content and sanitation conditions on performance of weaned pigs fed antibiotic-free diets. Can. J. Anim. Sci. 94, 115-118

Kendall, D.C., Allee, G.L., Usry, J.L., Ward, M.M. & Cook, D.R., 2008. Evaluation of the lysine requirement for 11 to 20 kg pigs. J. Anim. Sci. 80 (1), 132

Kerr, B.J., Yen, J.T, Nienaber, J.A. & Easter, R.A., 2003. Influences of dietary protein level, amino acid supplementation and environmental temperature on performance, body composition, organ weights and total heat production of growing pigs. J. Anim. Sci. 81, 1998-2007

Kiarie, E., Slominski, B.A., Krause, D.O. & Nyachoti, C.M., 2008. Acute phase response of piglets fed diets containing non-starch polysaccharide hydrolysis products and egg yolk antibodies following an oral challenge with *Escherichia coli* (K88). Can. J. Anim. Sci. 89(3), 353-360

Kim, J.H., Heo, K.N., Odle, J., Han, I.K. & Harrell, R.J., 2001. Liquid diets accelerate the growth of early-weaned pigs and the effects are maintained to market weight. J. Anim. Sci. 279, 427-434

Kim, H.B., Borewicz, K., White, B.A., Singer, R.S., Sreevatsan, S., Tu, Z.J. & Isaacson, R.E., 2011. Longitudinal investigation of the age-related bacterial diversity in the feces of commercial pigs. Vet. Microbiol. 153, 124-133

Kim, J.C., Heo, J.M., Mullan, B.P. & Pluske, J.R., 2011. Efficacy of a reduced protein diet on clinical expression of post-weaning diarrhea and life-time performance after experimental challenge with an enterotoxigenic strain of *Escherichia coli*. Anim. Feed Sci. Tech. 170, 222-230

Kinh, L.V., Jayaraman, B., Ninh, P.H., Vinh, D., Huyen, L.T.T., Piriyabenjawat, Doranalli, K., Channarayapatna, G. & Thammathipborworn, T. Responses of *Bacillus amyloliquefaciens* CECT 5940 supplementation in weaned pig diets [abstract]. In: 3rd International Symposium on Alternatives to Antibiotics. Challenges and Solutions in Animal Health and Production. 2019 December 16-18. Bangkok, Thailand

Koopmans, S.J., Guzik, A.C., van der Meulen, J., Dekker, R., Kogut, J., Kerr, B.J. & Southern, L.L., 2006. Effects of supplemental L- Tryptophan on serotonin, cortisol, intestinal integrity, and behavior in weanling piglets. J. Anim. Sci. 84, 963-971

Kristine, L.U., Pencharz, P.B. & Ball, R.O., 2006. Intestinal atrophy decreases arginine synthesis in neonatal piglets. Adv. Pork Product. 17, 16

Kubasova, T., Davidova-Gerzova, L., Merlot, E., Medvecky, M., Polansky, O., Gardan-Salmon, D., Quesnel, H. & Rychlik, I., 2017. Housing systems influence gut microbiota composition of sows but not of their piglets. PLoS One 12, e0170051

Laine, T., Yliaho, M., Myllys, V., Pohjanvirta, T., Fossi, M. & Anttila, M., 2004. The effect of antimicrobial growth promotor withdrawal on the health of weaned pigs in Finland. Prev. Vet. Med. 66, 163-174

Landry, J. & Delhaye, S., 1992. Simplified procedure for the determination of tryptophan of food and feedstuffs from barytic hydrolysis. J. Agric. Food Chem. 40, 776-779

Le Bon, M., Davies, H.E., Glynn, C., Thompson, C. & Madden, M., Wiseman, J., Dodd, C.E.R., Hurdidge, L., Payne, G., Le Treut, Y., Craigon, J., Tötemeyer, S. & Mellits, K.H., 2010. Influence of probiotics on gut health in the weaned pig. Livest. Sci. 133, 179-181

Le Dividich, J. & Sève, B., 2000. Effects on underfeeding during the weaning period on growth, metabolism, and hormonal adjustments in the piglet. Domest. Anim. Endocrin. 19, 63-74

Le Floc'h, N., Melchior, D. & Sève, B., 2004. The importance of dietary tryptophan for preserving growth and controlling inflammatory response of weaned pigs submitted to immune stress. In: Madec, F., Clement, G. (Eds.), Animal Production in Europe: The Way Forward in a Changing World, Proceedings of International Society for Animal Hygiene, 239-240

Le Floc'h, N. & Seve, B., 2007. Biological roles of tryptophan and its metabolism: Potential implications for pig feeding. Livest. Sci. 112, 23-32

Le Huërou-Luron, I., 2002. Production and gene expression of brush border dissaccharidases and peptidases during development in pigs and calves. In: Zabielski, R., Gregory, P.C., Weström, B. (Eds.), Biology of the intestine in growing animals. Elsevier Science, Amsterdam, 491-513

Lee, Y.J., Kim, B.K., Lee, B.H., Jo, K.I., Lee, N.K., Chung C.H., Lee, Y.C. & Lee, J.W., 2008. Purification and characterization of cellulase produced by Bacillus amyoliquefaciens DL-3 utilizing rice hull. Bioresource Technol. 99, 378-386

Lei, X., X. Piao, Y. Ru, H. Zhang, A. P´eron, and H. Zhang. 2015. Effect of *bacillus amyloliquefaciens*-based direct-fed microbial on performance, nutrient utilization, intestinal morphology and cecal microflora in broiler chickens. Asian Australas. J. Anim. Sci. 28, 239-246

Lenis, N.P., van Diepen, H., Bikker, P., Jongbloed, A.W. & van der Meulen, J., 1999. Effect of the ratio between essential and nonessential amino acids in the diet on utilization of nitrogen and amino acids by growing pigs. J. Anim. Sci. 77, 1777-1787

Ley, R.E., Peterson, D.A. & Gordon, J.I., 2006. Ecological and evolutionary forces shaping microbial diversity in the human intestine. Cell 124, 837-848

Li, X. L., Yin, J. D., Li, D. F., Chen, X. J., Zang, J. J. & Zhou, X., 2006. Dietary supplementation with zinc oxide increases IGF-I and IGF-I receptor gene expression in the small intestine of weanling piglets. J. Nutr. 136, 1786-1791

Li, Z. J., Yi, G. F., Yin, J. D., Sun, P., Li, D. & Knight, C., 2008. Effects of organic acids on growth performance, gastrointestinal pH, intestinal microbial populations and immune responses of weaned pigs. Asian Australas. J. Anim. Sci. 21, 252-261.

Li, Y., Song, Z., Kerr, K.A. & Moeser, A.J., 2017. Chronic social stress in pigs impairs intestinal barrier and nutrient transporter function, and alters neuro-immune mediator and receptor expression. PLoS ONE 12(2), e0171617

Liao, S.F., Wang. T. & and Regmi, N., 2015. Lysine nutrition in swine and the related monogastric animals: muscle protein biosynthesis and beyond. SpringerPlus 4, 147

Liao, S.F. & Nyachoti, M., 2017. Using probiotics to improve swine gut health and nutrient utilization. Anim. Nutr. 1-13

Lindemann, M. D., Cornelius, S.G., El Kandelgy, S.M., Moser, R.L. & Pettigrew, J.E., 1986. Effect of age, weaning and diet on digestive enzyme levels in the piglet. J. Anim. Sci. 62, 1298-1307

Liu, P., Piao, X.S., Kim, S.W., Wang, L., Shen, Y.B., Lee, H.S. & Li, S.Y., 2008: Effects of chito-oligosaccharide supplementation on the growth performance, nutrient digestibility, intestinal morphology, and fecal shedding of Escherichia coli and lactobacillus in weaning pigs. J. Anim. Sci. 86, 2609-2618

Liu, H., Ji, H.F., Zhang, D.Y., Wang, S.X., Wang, J., Shan, D.C. & Wang, Y.M., 2015. Effects of *Lactobacillus brevis* preparation on growth performance, fecal microflora and serum profile in weaned pigs. Livest. Sci. 178, 251-254

Llames, C.R. & Fontaine, J., 1994. Determination of amino acids in feeds: Collaborative study. J. AOAC. Int. 77, 1362-1402

Macfarlane, S. & Macfarlane, G.T., 1995. Proteolysis and amino acid fermentation. In Human Colonic Bacteria: Role in Nutrition, Physiology, and Pathology; Gibson, G.R., Macfarlane, G.T., Eds.; CRC Press: Boca Raton, 75-100

Main, R.G., Dritz, S.S., Tokach, M.D., Goodband, R.D. & Nelssen, J.L., 2008. Determining an optimum lysine: calorie ratio for barrows and gilts in a commercial finishing facility. J. Anim. Sci. 86, 2190-2207

Mao, X., Liu, M., Tang, J., Chen, H., Chen, D., Yu, B., He, J., Yu, J. & Zheng, P., 2015. Dietary leucine supplementation improves the mucin production in the jejunal mucosa of the weaned pigs challenged by porcine rotavirus. PLoS ONE 10, e0137380

Mavromichalis, I., Webel, D.M., Emmert, J.L., Moser, R.L. & Baker, D.H., 1998. Limiting order of amino acids in a low-protein corn soybean meal-whey-based diet for nursery pigs. J. Anim. Sci. 76, 2833-2837

McCracken, B.A., Spurlock, M.E., Roos, M.A., Zuckermann, F.A. & Gaskins, H.R., 1999. Weaning anorexia may contribute to local inflammation in the piglet small intestine. J. Nutr. 129, 613-619

McHugh, M.L., 2013. The Chi-square test of independence. Biochemia Medica 23(2), 143-149

Meng, Q.W., Yan, L., Ao, X., Zhou, T.X., Wang, J.P., Lee J.H. & Kim, I.H., 2010. Influence of probiotics in different energy and nutrient density diets on growth performance, nutrient digestibility, meat quality, and blood characteristics in growing-finishing pigs. J. Anim. Sci. 88, 3320-3326

Mikkelson, L.L. & Jensen, B.B., 2004. Effect of fructooligosaccharides and transgalacto-oligosaccharides on microbial populations and microbial activity in the gastrointestinal tract of piglets post-weaning. Anim. Feed Sci. Tech. 117, 107-119

Mitchell, G.A., 1990. Methods of starch analysis. Starch. 42 (4), 131-134

Moeser, A.J., van der Klok, C., Ryan, K.A., Wooten, J.G., Little, D., Cook, V.L. & Blikslager, A.T., 2007. Stress signaling pathways activated by weaning mediate intestinal dysfunction in the pig. Am. J. Physiol. - Gastr. L. 292, 173-181

Moeser, A.J., Pohl, C.S. & Rajput, M., 2017. Weaning stress and gastrointestinal barrier development: Implications for lifelong gut health in pigs. Anim. Nutr. 3(4), 313-321

Moffett, J.R. & Namboodiri, A., 2003. Tryptophan and the immune response. Immunol. Cell. Biol. 81, 247–265

Montagne, L., Cavaney, F.S., Hampson, D.J., Lallès, J.P. & Pluske, J.R., 2004. Effect of diet composition on post-weaning colibacillosis in piglets. J. Anim. Sci. 82, 2364-2374

Mooijman, K.A., Pielaat, A. & Kuijpers, A.F.A., 2019. Validation of EN ISO 6579-1 - Microbiology of the food chain - Horizontal method for the detection, enumeration and serotyping of Salmonella - Part 1 detection of Salmonella spp. Int. J.Food Microbiol. 288, 3-12

Moss, A.F., Sydenham, C.J., Khoddami, A., Naranjo, V.D., Liu, S.Y. & Selle, P.H., 2018. Dietary starch influences growth performance, nutrient utilization and digestive dynamics of protein and amino acids in broiler chickens offered low-protein diets. Anim. Feed Sci. Tech. 237, 55-67.

Mou, Q., Yang, H.S., Yin, Y.L. & Huang, P.F., 2019. Amino acids influencing intestinal development and health of the piglets. Animal 9, 302

Muralidhara, K.S., Sheggeby, G.G., Elliker, P.R., England, D.C & Sandine, W.E., 1977. Effect of feeding lactobacilli on the coliform and lactobacillus flora of intestinal tissue and feces from piglets. J. Food Protect. 40 (5), 288-295

Nabuurs, M.J., Hoogendoom, A., Molen, E.J. van der, Osta, A.L., 1993. Villus height and crypt depth in weaned and unweaned pigs, reared under various circumstances in the Netherlands. Res. Vet. Sci. 55, 78-84

Nagy, B. & Fekete, P.Z., 1999. Enterotoxigenic Escherichia coli (ETEC) in farm animals. Vet. Rese. 30 (2-3), 259-284

Nataro, J.P. & Kaper, J.B., 1998. Diarrheagenic *Escherichia coli.* Clin. Microbiol. Rev. 11, 142-201

National Research Council (1998): Nutrient requirements of swine. 10th revised edition. National Academy Press, Washington, DC

Nesic, K., Pavlovic, M. & Ivanovic, S., 2019. Assessment of the microbiological quality of feed using the Verbands Deutscher Landwirdschaftlicher Untersuchungs Und Forschungsanstalten (VDLUFA) method. Veterinarski Glasnik, 00: 1-9

Newburg, D.S. & Walker, W.A., 2007. Protection of the neonate by the innate immune system of developing gut and of human milk. Pediatr. Res. 61, 2-8

Nyachoti, C.M., Omogbenigun, F.O., Rademacher, M. & Blank, G., 2006. Performance responses and indicators of gastrointestinal health in early-weaned pigs fed low-protein amino acid-supplemented diets. J. Anim. Sci. 84, 125-134

Nyachoti, M. & Jayaraman, B., 2016. Low crude protein diet and its effect on diarrhea. 16th Annual Midwest Swine Nutrition Conference Proceedings

Official Journal of the European Union, 1994

Opapeju, F.O., Rademacher, M., Blank, G & Nyachoti, C.M., 2008. Effect of low-protein amino acid-supplemented diets on the growth performance, gut morphology, organ weights and digesta characteristics of weaned pigs. Animal 2 (10), 1457-1464

Opapeju, F.O., Rademacher, M., Payne, R.L., Krause, D.O. & Nyachoti, C.M., 2010. Inflammation-associated responses in piglets induced with post-weaning colibacillosis are influenced by dietary protein level. Livest. Sci. 131, 58-64

Opapeju, F.O., Rodriguez-Lecompte, J.C., Rademacher, M., Krause, D.O. & Nyachoti, C.M., 2015. Low crude protein diets modulate intestinal responses in weaned pigs challenged with *Escherichia coli* K88. Can. J. Anim. Sci. 95, 71-78

Pain, B.F., van der Weerden, T.J., Chambers, B.J., Phillips, V.R. & Jarvis, S.C., 1997. A new inventory for ammonia emissions from UK agriculture. Atmos. Environ. 32(3), 309-313

Partanen, K. H., & Mroz, Z., 1999. Organic acids for performance enhancement in pig diets. Nutr. Res. Rev. 12, 117–145

Peng, X., Hu, L., Liu, Y., Yan, C., Fang, Z.F., Lin, Y., Xu, S.Y., Li, J., Wu, C.M., Chen, D.W., Sun, H., Wu, D. & Che, L.Q., 2016. Effects of low-protein diets supplemented with indispensable amino acids on growth performance, intestinal morphology and immunological parameters in 13 to 35kg pigs. Animal 10 (11), 1812-1820

PIC Nutrient Specifications Manual, 2016. https://www.picrsa.co.za/manuals/

PIC Early Pig Care Manual, 2017. https://www.picrsa.co.za/manuals/

Pohl, C.S., Medland, J.E., Mackey, E., Edwards, L.L., Bagley, K.D., DeWilde, M.P., Williams, K.J. & Moeser, A.J., 2017. Early weaning stress induces chronic functional diarrhea, intestinal barrier defects, and increased mast cell activity in a porcine model of early life adversity. Neurogastroent. Motil. e13118

Pluske, J.R., Hampson, D.J. & Williams, I.H., 1997. Factors influencing the structure and function of the small intestine in the weaned pig: a review. Livest. Prod. Sci. 51, 215-236

Pluske, J. R., Hansen, C. F., Payne, H. G., Mullan, B. P., Kim, J. C., Hampson, D. J., Paterson, J. E. & Barker, J. A. (Ed), 2007. Gut health in the pig. In: Manipulating pig production XI.: Proceedings of the Eleventh Biennial Conference of the Australasian Pig Science Association (APSA), held in Brisbane, Australia, 25th to 28th November, 147-158

Pluske, J.R., 2016. Invited review: Aspects of gastrointestinal tract growth and maturation in the pre- and postweaning period of pigs. J. Anim. Sci. 94, 399-411

Prasad, A. S., Oberleas, D., Wolf, P., Horwitz, J. P., Miller, E. R. & Luecke, R. W., 1969. Changes in trace elements and enzyme activities in tissues of zinc-deficient pigs. Am. J. Clin. Nutr. 22, 628-637

Prohaszka, L. & Baron, F., 1980. The predisposing role of high dietary protein supplies in enteropathogenic E. coli infections of weaned pigs. Zbl. Veterinarmedizin. 27, 222-232

Ramayo-Caldas, Y., Mach, N., Lepage, P., Levenez, F., Denis, C., Lemonnier, G., Leplat, J.J., Billon, Y., Berri, M., Doré, J., Rogel-Gaillard, C. & Estellé, J., 2016 Phylogenetic network analysis applied to pig gut microbiota identifies an ecosystem structure linked with growth traits. ISME J. 10 (12), 2973-2977

Ravindran, V. & Kornegay, E. T., 1993. Acidification of weaner pig diets: a review. J. Sci. Food Agric. 62, 313-322

Roberfroid, M., 2007. Prebiotics: the concept revisited. J. Nutr. 137, 830-837

Roig-Pérez, S., Guardiola, F., Moretó, M., & Ferrer, R., 2004. Lipid peroxidation induced by DHA enrichment modifies paracellular permeability in Caco-2 cells: protective role of taurine. J. Lipid. Res. 45, 1418-1428

Rudar, M., Zhu, C.L. & de Lange, C.F., 2017. Dietary leucine supplementation decreases whole-body protein turnover before, but not during, immune system stimulation in pigs. J. Nutr. 147, 45-51

SAS Institute Inc. 2017. SAS/STAT® 14.3 User's Guide: High-Performance Procedures. Cary, NC: SAS Institute Inc.

Saunders, D.R. & Sillery, J. Effect of Lactate on Structure and Function of the Rat Intestine. Digest Dis. 27, 33-41

Sauvant, J.M. & Perez, G., 2004. Tables of composition and nutritional value of feed materials. INRA

Schmidt, B., Mulder, I.E., Musk, C.C., Aminov, R.I., Lewis, M., Stokes, C.R., Bailey, M., Prosser, J.I., Gill, B.P., Pluske, J.R., & Kelly, D., 2011. Establishment of normal gut microbiota is compromised under excessive hygiene conditions. PLoS One 6, e28284

Selle, P.H., Sydenham, C.J., Moss, A.F., Khoddami, A., Naranjo, V.D., & Liu, S.Y., 2018. Dietary starch influences performance of broiler chickens offered low-protein diets. In: PRC (ed.) 29th Annual Australian Poultry Science Symposium. 12-15. Poultry research Foundation and World's Poultry Science Association, Sydney, NSW, Australia

Shim, S.B., Williams, B.A. & Verstegen, M.W.A., 2005. Effects of dietary fructose-oligosaccharide on villous height and disaccharidase activity of the small intestine, pH, VFA and ammonia concentrations in the large intestine of weaned pigs. Acta Agriculturae Scandinavica, Section A – Animal Science 55, 91-97

Slump, P., Flissebaalje, T.D. & Haaksman, I.K., 1991. Tryptophan in food proteins: A comparison of two hydrolytic procedures. J. Sci. Food Agric. 55, 493-496

Smith, M. W. & L. G. Jarvis. 1978. Growth and cell replacement in the new-born pig intestine. P. Roy. Soc. B-Biol. Sci. 203, 69-89

Smith, F., Clark, J.E., Overman, B.L., Tozel, C.C., Huang, J.H., Rivier, J.E.F., Blisklager, A.T. & Moeser, A.J., 2010. Early weaning stress impairs development of mucosal barrier function in the porcine intestine. Am. J. Physiol. - Gastr. L. 298, 352-363

Sommer, S.G. & Husted, S., 1995. The chemical buffer system in raw and digested animal slurry. J. Agric. Sci. 124, 45-53

Specian, R. D. & Oliver, M. G., 1991. Functional biology of intestinal goblet cells. Am. J. Physiol. 260, 183-193

Stein, H.H. & Kil, D.Y., 2006. Reduced use of antibiotic growth promoters in diets fed to weanling pigs: dietary tools, part2. Anim. Biotechnol. 17(2), 217-31

Stewart, C.S., 1997. Microorganisms in hindgut fermentors. In Gastrointestinal Microbiology; Mackie, R.I., White, B.A., Isaacson, R.E., Eds.; Chapman and Hall: New York, 2, 142-175

Stoll, B., Henry, J., Reeds, P.J., Yu, H., Jahoor, F. & Burrin, D.G., 1998. Catabolism dominates the first-pass intestinal metabolism of dietary essential amino acids in milk protein-fed piglets. J. Nutr. 128, 606-614

Sun, Y., Wu, Z., Wei Li, W., Zhang, C., Sun, K., Ji, Y., Wang, B., Jiao, N., He, B., Wang, W., Dai, Z. & Wu, G., 2015. Dietary l-leucine supplementation enhances intestinal development in suckling piglets. Amino acids, 47, 1517-1525

Theil, P.K., Fernandez, J.A. & Danielsen, V., 2004. Valine requirement for maximal growth rate in weaned pigs. Livest. Prod. Sci. 88, 99-106

Thompson, C.L., Wang, B. & Holmes, A.J., 2008. The immediate environment during postnatal development has long-term impact on gut community structure in pigs. ISME J. 2, 739-748

Tortuero, F., Rioperez, J., Fernandez, E. & Rodriguez, M.L., 1995. Response of Piglets to Oral Administration of Lactic Acid Bacteria. J. Food Protect. 58 (12), 1369-1374

Tsiloyiannis, V. K., Kyriakis, S. C., Vlemmas, J. & Sarris, K., 2001. The effect of organic acids on the control of porcine post-weaning diarrhoea. Res. Vet. Sci. 70, 287-293

Ulberth, F. & Henniger, M., 1992. One-step extraction/ methylation method for determining the fatty acid composition of processed foods. J. Am. Oil. Chem. Soc. 69,2

Upadhaya, S.D., Kim, S.C., Valientes, R.A. & Kim, I.H., 2015. The effect of Bacillus-based feed additive on growth performance, nutrient digestibility, fecal gas emission, and pen cleanup characteristics of growing-finishing pigs. Asian Australas. J. Anim. Sci. 28, 999-1005

Urlings, H.A.P., Fransen, N.G., Bijker, P.G.H. & van Logtestijn, J.G., 1993. Proteolysis and amino acid breakdown of heated and irradiated poultry byproducts and muscle tissue. J. Anim. Sci. 71, 2432-2438

Vaz, F.M. & Wanders, R.J., 2002. Carnitine biosynthesis in mammals. Biochem. J. 361, 417-429

Verstegen, M. W. A. & Williams, B. A., 2002. Alternatives to the use of antibiotics as growth promoters for monogastric animals. Anim. Biotechnol. 13, 113-127.

Vervaeke, I.J., Decuypere, J.A., Dierick, N.A. & Henderickx, H.K., 1979. Quantitative in vitro Evaluation of the Energy Metabolism Influenced by Virginiamycin and Spiramycin Used as Growth Promoters in Pig Nutrition. J. Anim. Sci. 49, 846-856

Wang, W.W., Qiao, S.Y. & Li, D.F., 2009. Amino acids and gut function. Amino acids 37, 105-110

Weary, D.M. & Fraser, D., 1997. Vocal response of piglets to weaning: effect of piglet age. Appl. Anim. Behav. Sci. 54, 153-160

Wellock, I.J., Fortomaris, P.D., Houdijk, J.G.M. & Kyriazakis, I., 2006. The effect of dietary protein supply on the performance and risk of post-weaning enteric disorders in newly weaned pigs. Anim. Sci. 82, 327-335

Wellock, I.J., Fortomaris, P.D., Houdijk, J.G.M. & Kyriazakis, I., 2008. Effects of dietary protein supply, weaning age and experimental enterotoxigenic *Escherichia coli* infection on newly weaned pigs: performance. Animal (2008), 2 (6), 825-833

Widdowson, E. M., Colombo, V.E. & and Artavanis, C.A., 1976. Changes in the organs of pigs in response to feeding for the first 24 h after birth. II. The digestive tract. Biol. Neonate 28, 272-281

Wierup, M., 2001a. The experience of reducing antibiotics used in animal production in the Nordic countries. Int. J. Antimicrob. Ag. 18, 287-29

Wierup, M., 2001b. The Swedish experience of the 1986 year ban of antimicrobial growth promoters, with special reference to animal health, disease prevention, productivity, and usage of antimicrobials. Microb. Drug Resist. 7, 183-190

Williams, B.A., Verstengen, M.W.A. & Tamminga, S., 2001. Fermentation in the large intestine of single-stomached animals and its relationship to animal health. Nutr. Res. Rev. 14, 207-227

Wu, G., Knabe, D.A. & Kim, S.W., 2004. Arginine Nutrition in Neonatal Pigs. J. Nutr. 134, 2783-2790

Wu, G., 2009. Amino acids: metabolism, functions, and nutrition. Amino Acids 37, 1-17

Xu, R. J., Tungthanathanich, P., Birtles, M.J., Mellor, D.J., Reynolds, G.W. & Simpson, H.V., 1992. Growth and morphological changes in the stomach of piglets during the first three days after birth. J. Dev. Physiol. 17, 7-14.

Yu, H.F., Wang, A.N., Li, X.J. & Qiao, S.Y., 2008. Effect of viable Lactobacillus fermentum on the growth performance, nutrient digestibility and immunity of weaned pigs. J. Anim. Feed Sci. 17, 61-69

Yue, L.Y. & Qiao, S.Y., 2008. Effects of low-protein diets supplemented with crystalline amino acids on performance and intestinal development in piglets over the first 2 weeks after weaning. Livest. Sci. 115, 144 -152

Zijlstra, R.T., Jha, R., Woodward, A.D., Fouhse, J. & van Kempen, T.A.T.G., 2012. Starch and fiber properties affect their kinetics of digestion and thereby digestive physiology in pigs. J. Anim. Sci. 90, 49-58