Impact of *Bacillus* probiotics on production performance and microbial gut diversity in weaner pigs

By

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Declaration

I, Judith Maryna Rosenstrauch hereby declare that this thesis, submitted for the MSc (Agric) Animal Science: Animal Nutrition degree at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at any other University.

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“Victory belongs to the most persevering” – Napoleon Bonaparte

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Abstract

In this project the impact of *Bacillus* probiotics on the production performance and microbial gut diversity in weaner pigs were investigated. Two production units were used, namely individual housing units and group housing units each with a probiotic treatment and a control. A total of 167 weaner pigs, 21-days of age, were purchased from a commercial farm with 40 pigs allocated to individual pens and the remainder of animals housed in group pens with ±15 pigs per pen and a stocking density of 0.95 m^2 per pig. Group housed pigs were kept in the trial until 57 days of age and pigs in individual pens were kept in the trial until 63 days of age. Three products from Chrisal Africa (Pty) Ltd were used, which included a PIP Animal Housing cleaner containing probiotic enzymes (3 % solution applied at 1 L/100 m^2 for cleaning and 5 % solution for flushing the waterlines), PIP Environmental Enhancer containing *Bacillus* spores (applied as a mist at 1 L/200 m^2) and PIP Water Plus containing *Bacillus* spores (0.02 % solution once a day). Growth performance was measured as well as microbial gut diversity using 16S rRNA sequencing analysis on faecal samples of the individually housed pigs. Group housed pigs on the probiotic treatment gained less weight than the control at 21-28 days (-230 g) and 49-57 days (-1420 g), with no significant difference in feed intake and higher feed conversion ratios at 21-28 days (+0.19 g/g) and 35-42 days (+0.11 g/g). Individually housed pigs on the probiotic treatment gained less weight than the control at 35-42 days (-0.73 g), had higher feed intake at 49-60 days (+1530 g) and higher feed conversion ratios at 35-42 days (+0.19 g/g) as well as 49-60 days (+0.3 g/g). Changes that were noted between treatments in the microbial populations included significantly more *Ruminococcaceae* (+4.58 %) and *Streptococcaceae* (+2.49 %) in the probiotic treatment at 35 days of age as well as significantly more *Erysipelotrichaceae* (+0.7 %), *Lactobacillaceae* (+3.1 %) and *Streptococcaceae* (+2.67 %) in the probiotic treatment at 49 days of age. No differences were detected at 63 days of age. Results indicated negative effects on growth and performance for probiotic treated pigs, although microbial gut populations of probiotic treated pigs showed increased amounts of beneficial microbes during the first four weeks after weaning.

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List of abbreviations

ADG: Average daily gain

AMR: Antimicrobial resistance

ATTD: Apparent total tract digestibility

BWG: Body weight gain

CE: Competitive exclusion

CP: Crude Protein

DNA: Deoxyribonucleic acid

EDTA: Ethylenediaminetetraacetic acid

EU: European Union

FCR: Feed conversion ratio

FI: Feed intake

gDNA: Genomic deoxyribonucleic acid

GIT: Gastrointestinal tract

IFN-ɣ: Interferon gama

IgA: Immunoglobulin A

LAB: Lactic acid bacteria

NaCl: Sodium Chloride

OTU: Operational taxonomic unit

PCR: Polymerase chain reaction

PWD: Post weaning diarrhoea

RNA: Ribonucleic acid

rRNA: Ribosomal ribonucleic acid

SA: South Africa

SCFA: Short chain fatty acids

SDS: Sodium dodecyl sulfate

SI: Small Intestine

Tris-HCl: Tris Hydrochloride

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Chapter 1: Introduction

1.1 Introduction

Domestication of the pig (*Sus scrofa*) was most likely a complex process that took place over many millennia. Evidence suggests domestication occurred at the upper Tigris and Euphrates regions (Wu *et al.*, 2007). The domestication process for pigs was different compared to other farm animals. Unlike herbivores they were not able to convert inedible plant materials into valuable protein sources, they could not be herded over long distances, they could not be used as working animals such as horses or oxen and most importantly they competed directly with humans for food sources (White, 2011). Pig farming historically was associated with peasant subsistence rather than a valuable farming commodity. In the late 17th and 18th centuries, however, pig production developed into a more intensive system where they were fed more nutritious feed sources such as potatoes, peas and beans (Arbuckle, 2013; Cucchi *et al.*, 2016).

Modern pig production is much more sophisticated and holds many advantages, such as less land needed per unit animal when compared to large livestock. The short generation intervals and large litters make it an extremely efficient animal with much faster improvement in production due to genetic selection (Neeteson-van Nieuwenhoven *et al.*, 2013) Pig production also has an economic impact through the employment sector, as work opportunities are created not only on farm level but also in processing and trading. Modern day pig production units are intensive with high throughputs. Sows are bred intensively, making use of artificial insemination with superior genetic material to yield large fast growing litters. Short dry periods and short lactation periods of between 21-28 days ensures fast turnover.

Modern pig farms focus on high growth rate and throughput and therefore require a high level of management with regards to feeding and health. Farms aim to reduce the risk of pathogenic infections by using all-in-all-out systems, showering and disinfecting before entering facilities, constant cleaning, disinfecting and keeping closed herds with little opportunity for contamination from outside threats (Jayaraman & Nyachoti, 2017).

In a pig production system weaning is considered as one of the most stressful events in a young pig's life (Smith *et al.*, 2010). A number of factors play a role including the change of diet, handling, new litter mates and hierarchy that needs to be established together with many other factors. These changes can exert harmful effects on the gut morphology, especially at early weaning, through shortening of the villi, shape of the villi, crypt depth and greater epithelial cell mitosis (Dong & Pluske, 2007). Further negative effects are observed on the digestive capacity through reduced enzyme activity and absorption which in turn have its own negative consequences, such as diarrhoea and reduced performance (Pluske *et al.*, 1997; Pluske *et al.*, 2018). Reduced feed intake (FI) during the first 48h after weaning is one of the main reasons for these destructive morphological changes, since it exposes the gut to antigens. Proper feed intake after weaning not only prevents poor gut health, but also improves weight gain (Jayaraman & Nyachoti, 2017). Low feed intake also results in an energy deficiency for maintenance. Several factors may lead to reduced FI that can be categorised into factors associated with environment (lighting, allocated space), diet (nutritional balance, feed palatability, physical form of feed, water availability and quality), management (housing, age at weaning, grouping of piglets into eight classes, feeder space, temperature and ventilation) and disease or immune responses (Jayaraman & Nyachoti, 2017).

For many years, intensive pig farming has relied on the use of in-feed antimicrobials to control diseases and maintain healthy immune systems in herds (Jahanbakhsh *et al.*, 2015; Cameron-Veas *et al.*, 2016). In 2006 the EU banned the use of all in-feed antibiotics as growth promoters, due to fear of microbial resistance. From the late 1980's pharmaceutical companies have not invested enough resources into research and development of new antibiotics and new development in this field is desperately needed (Silver, 2011; Aminov,

2017), providing a supporting argument in favour of the ban. In SA, no such bans have been applied, but such measures might follow in the future. Since the cost of feed is the main expense in SA pig production, the use of antimicrobials is important to improve feed efficiency and therefore make pig production more financially viable. It is thus imperative to seek alternative methods to improve health, prevent disease and maintain good feed efficiency in the livestock sector.

Probiotics is one such alternative with numerous possibilities, as there exists a wide range of different probiotics with varying efficacies and modes of action (Klose *et al.*, 2010; Liu *et al.*, 2015; Liao & Nyachoti, 2017). Probiotics can be described as living microbes added to feed with the purpose of enhancing the host animal's microbiome and thereby exerting favourable growth and performance effects (Fuller, 1989). One such bacterial probiotic genus of interest to the feed and animal production industry is *Bacillus*. These bacteria are aerobic bacteria with rod shaped, spore-forming capabilities that is widely distributed in soil (Lawrence, 2008). Endospores are highly differentiated cells produced by some bacteria and are exceptionally resilient against harsh environmental conditions such as heat, chemicals and radiation and should be viewed as a dormant stage in the bacterial life cycle (Nicholson *et al.*, 2000). The endospore thus functions as a survival mechanism for the bacteria in times of depleted nutrition or harsh conditions. For this reason, *Bacillus* species should be excellent candidates as probiotics in animal feeds, as they will be able to survive harsh processing conditions of feed manufacturing or chemicals in the water and surroundings as well as survival in the animal gut (Khochamit *et al.*, 2015; Manhar *et al.*, 2016) The vegetative bacterial cell will only sporulate when cell growth ceases. The resulting endospores can stay dormant for many years until conditions become more favourable and enables them to germinate (Madigan *et al.*, 2009).

The benefits of *Bacillus* probiotics include improvement in gut health, growth and performance, bowel movements (Ng *et al.*, 2009; Giang *et al.*, 2012), total tract digestibility (Liu *et al.*, 2015) as well as reductions in pathogenic bacteria in the gut (Choi *et al*., 2011a), shedding of pathogens and allergic reactions (Schrezenmeir & Vrese, 2001; Lee *et al*., 2015). This can become an important field of interest for improving weaner production by altering the gut microbiome in a beneficial manner.

1.2 Aim

In this study a mixture of *Bacillus* species were evaluated in weaner pigs with regards to production performance and gut microbial diversity. Both the housing and water in the facility was pre-treated with a probiotic product range from Chrisal Africa (Pty) Ltd. Gut microbial diversity was measured through analysis of the 16S ribosomal RNA from rectal faecal samples. Faecal sampling is the least invasive procedure that could be used to obtain a good representation of the microbial diversity present in the gut.

The purpose of this research was to test the efficacy of a range of probiotic products containing a mixture of *Bacillus* spores at 5×10^7 CFU/mL concentrated product. The first product, PIP Animal Housing cleaner (Chrisal Africa (Pty) Ltd), was used before arrival of the animals and has replaced general cleaners and disinfectants, as prescribed by the company recommendations. The second product, PIP Environmental Enhancer (Chrisal Africa (Pty) Ltd) was sprayed in the direct environment of the pigs to reduce the number of pathogens in the environment. The last product, PIP Water Plus (Chrisal Africa (Pty) Ltd), was added to the water supply and was intended to improve the animals' intestinal microbiome.

The overall aim of the research was therefore to evaluate the impact of *Bacillus* probiotics on production performance and microbial gut diversity in weaner pigs and the following objectives were set:

- Comparison of growth and production between treated and untreated animals by recording body weight and feed intake.

• Comparing the faecal microbial diversity between treated and untreated animals based on 16S rRNA analysis.

1.3 Hypothesis

0 Hypotheses:

Bacillus probiotics will have no positive effect on growth and performance of weaner pigs.

Bacillus probiotics will have no effect on the microbial diversity in the gut of weaner pigs.

Alternative Hypotheses:

Bacillus probiotics will improve growth and performance of weaner pigs

Bacillus probiotics will have an impact on the gut microbial diversity of weaner pigs.

Chapter 2: Literature review

2.1 Introduction

Commercial pig production consists of various phases of which weaning is one of the most challenging with regard to extreme stressors. The young weaner piglets are moved into new, larger groups with non-litter mates and are abruptly deprived from maternal milk and moved on to a solid diet that they are often not adapted to. Gastro-intestinal balance is disrupted and they may become more susceptible to infection by pathogens (Smith *et al.*, 2010; Moeser *et al.*, 2017).

Several factors need to be considered when referring to post-weaning performance such as management factors (age of weaning, diet quality, temperature, ventilation stocking density and sanitation) (Buddington & Sangild, 2011), herd health (Fablet *et al.*, 2018), nutrition (feed intake before weaning, accessibility to feed, feed quality and palatability, water intake and nutritional content of feed) (Lallès *et al.*, 2007) as well as genetic factors (breed, selection within the herd) (Neeteson-van Nieuwenhoven *et al.*, 2013). Any number of stressors can cause poor growth and post weaning diarrhoea (PWD) which can eventually lead to great economic losses. The process of weaning requires close management of the feed and environment to limit these potential stressors and to equip the piglet to reach its genetic potential. In modern pig rearing this implies high capital inputs to create a favourable environment as well as high quality feed ingredients that can be utilised by the piglet's immature digestive tract.

In this section literature was reviewed on the factors influencing the health and production performance of weaning pigs, with special reference to the use of probiotics in weaner diets.

2.2 Pork production in South Africa

For pigs to have become domesticated and utilised as a valuable farming commodity required finding a way around the complications these animals posed, such as destruction of human plantations and interference of early human settlements (White, 2011). The benefits of keeping pigs was their ability to utilise excess produce for human consumption and convert it into a food source that could be kept for longer periods of time. At first they utilised human food wastes and refuse as they foraged for food, but later became actual farm animals, evident from Chinese history around eight thousand years ago (Larson *et al.*, 2007; Ottoni *et al.*, 2012). Eventually they were kept and fed in paddocks, where they became separated from their wild predecessors, enabling humans to select for desirable traits (Giuffra *et al.*, 2000).

Historical evidence for pig domestication in Europe indicates that wild boars were kept in large groups that were herded in forests where they would feed on beechnuts and acorns. This practice was known as pannage which never truly made use of the pig's full potential as they had to forage over large areas leading to low feed efficiency while still interbreeding with the wild pig population (Parsons, 1962).

Limited research is available on the history of pig breeds in South African (SA), but the most prominent commercial pig breeds in SA had their origins from all over the world (Visser, 2014). A few breeds were imported to SA from England in the 1800's that included the Large White, Berkshire, Tamsworth and Large Black breeds. Another very popular breed was the Landrace that was developed in Denmark and was imported to SA from Denmark in 1953 for the purpose of research at the University of Pretoria (Pig breeders society of South Africa ©, 2013 http://www.studbook.co.za/Society/pigsa/?p=12). The Landrace was the universal dam line of SA pigs and resulted in F1 females of excellent reproductive capability when crossed with the Large White breed. A few breeds were also imported from the United States of America which included the Chester White, Duroc and Hampshire. The Duroc is a red coloured pig with good meat quality and is an important terminal sire in many breeding programmes

across the world today. The Piètrain pig breed is another imported breed that was developed in Belgium and imported to SA in 1997 from Germany. They were used as a sire breed that can produce extremely lean meat thereby improving meat yield. They used to carry the undesirable malignant hyperthermia gene, also called the stress gene, leading to pale soft and exudative meat (MacLennan *et al.*, 1990). A stress negative genotype was bred to eliminate the gene and still keep most of the desirable traits for which the breed was known (Blakely & Bade, 1976).

South Africa's only indigenous pig breed is the Kolbroek. The origin of this breed is not well documented, but it is speculated that the Kolbroek possibly came with a British East India Company ship that ran shipwreck at the coast of Cape Hangklip in 1778. As a recognised local breed in SA, it is a hardy breed with excellent foraging capabilities and will eat almost anything. They are therefore suited for extensive and small scale farming where they perform well in cross breeding with Duroc and Large White breeds (Visser, 2014).

Modern pig breeding is characterised by good female traits such as mothering ability and litter size with male traits that include growth rate, carcass quality and efficiency (Zak *et al.*, 2017). Crossbreeding utilises the heterosis effect to generate offspring with exceptional performance called the F1 progeny (Visser & Hofmeyr, 2014; Yadav *et al.*, 2018). Breeds that had a significant impact on the SA pig market include the Large White, Landrace, Chester White, Duroc, Hampshire, Large Black, indigenous Kolbroek and the Piètrain (Visser, 2014).

In a global context, SA is not a major pork producing country with the SA domestic sow herd estimated at 103 000 sows and 7000 boars owned by an estimated 240 pork producers. The majority of pork is produced in North West, Kwazulu-Natal and Western Cape. In contrast to the SA market, the top six pork producing countries include China (53.5 million tonnes), the European Union (EU) (23.4 million tonnes), the USA (11.72 million tonnes), Brazil (3.73 million tonnes), Russia (2.96 million tonnes) and Vietnam (2.75 million tonnes). Together these six countries produce around 88% of the global pork production. Capper (2013) reported that the annual production of slaughter pigs in the United States increased by 25 million pigs from 1959 to 2009 while the breeding herd decreased by 39% simultaneously. According to the world markets and trade report of the Foreign Agriculture service, USDA (2017), global pork production was estimated to increase by 2% in 2018 resulting in 113.1 million tonnes of pork production. This can be ascribed to better genetics and management practices.

In SA and other African countries pork production are challenging compared to the global pork production due to environmental concerns, compliance to welfare regulations, less competitive feed prices and efficiency of production (Davids & Van der Westhuizen, 2014). According to the SA Department of Agriculture, Forestry and Fisheries (Department of agriculture, forestry and fisheries, 2013) the pig industry contributes approximately 2.15% to the agriculture sector with a gross value of R19.8 billion. An upward trend in the value of pork production has been observed from 2001 and is still increasing. In 2011 an estimated 2 million tons of pork meat were produced with less than 250 tons of this consumed locally. Despite of these figures, SA remains a net importer of pork, due to the higher demand for processed products. The majority of pork is exported to SADC countries with Mozambique being the main importer of SA pork. The importance of maintaining a healthy gut environment can not be underestimated when producing economically viable pork meat.

2.3 Factors influencing pig gut development and function at weaning

Newborn piglets possess foetal enterocytes that are endocytotically active and replaced by adult enterocytes with reduced activity within the first three weeks of life (Smith & Peacock, 1980; Ferenc *et al.*, 2017). At the same time changes in the digestive enzymes occur, for example a reduction in lactase and increase in sucrase and maltase as piglet matures, which are most pronounced at weaning. During weaning gut digestion and absorption rapidly adjust to accommodate the new weaning diet that replaces the easy digestible sow's milk by increasing stomach acid secretion, enzyme production and pancreatic function (Everaert *et al.*, 2017).

Intense stomach development occurs in the third month of gestation and again at ten and twenty days after birth. Mucous secretion is already initiated at 45 to 50 days of gestation in very small amounts (Patt, 1977). Gastric acid secretion is low at birth, but increases as parietal cells mature, reaching optimum levels at 5-6 weeks. Gastric protease secretion is linked to nutrition, with high levels of cymosin for milk clotting at birth. Cymosin decreases up to 3-4 weeks and becomes virtually undetectable after 2 months (Sangild *et al.*, 2000). Pepsinogen A has an opposite response with very low traces at birth, but becomes one of the main proteases from 4-5 weeks of age. Creep feeding before weaning increases gastric acid and protease secretion in the stomach (Efird *et al.*, 1982; Cranwell, 1985). At birth, the brush border enzyme peptidase is secreted at high levels, but decreases with age. Carbohydrase activity on the other hand is substrate independent and genetically programmed (Sangild *et al.*, 1995; Everaert *et al.*, 2017). For example, Lactase decrease within 2-5 weeks, whereas maltase and sucrase increase from week one (Patt, 1977). Around day 40 of gestation the jejunum in the small intestine starts to form villi and in the third month crypts and muscularis mucosae starts to develop. During this time, luminal epithelium cells start to differentiate into enterocytes, goblet cells and enteroendocrine cells. There is a 70-80% increase in weight of the small intestine at the end of gestation.

Mucosal growth and gut closure are stimulated by colostrum and milk intake after birth (Salcedo *et al.*, 2016). In the foetus and neonatal suckling piglet, macromolecules are taken up through endocytosis. This is essential for uptake of immunoglobulins when acquiring passive immunity through consumption of colostrum (Rooke & Bland, 2002). Macromolecules such as hormones and growth factors are also taken up in a similar manner through the milk. This feature is only active for 18-36h after birth until onset of gut closure. As this ability reduces, adult enterocytes increase and are better capable of enzyme secretion and absorption of nutrients (Patt, 1977; Palmer, 2011).

In adult pigs, apoptosis of villi cells occurs at the tip of the villi, whereas in neonatal pigs the villi tend to house groups of apoptotic cells throughout the length of the villi (Skrzypek *et al.*, 2010). Apoptosis also occurs significantly less in neonatal pigs and mitosis significantly more in the first few days after birth, resulting in mucosal growth and villi length increase (Zabielski *et al.*, 2008). Three days after birth the villi length starts to decrease and villi diameter increase. Up to 3 days after birth crypt depth will increase, but will then gradually decrease with age. Intake of colostrum results in increased intestinal weight, absorptive area and brush border enzyme activities, with continual milk intake stimulating gut maturation (Skrzypek *et al.*, 2010). The first day after birth, the large intestinal weight will already have increased by 30%, and will have doubled by day 3. At birth, the large intestine has immature colonic epithelium cells, with the architecture of the proximal large intestine resembling that of the small intestine, with an undefined function and villi resembling that of the small intestine (Patt, 1977). This enables a newborn piglet to take up amino acids through the large intestine up to 2 weeks after birth. Peyer's patches are poorly developed at birth and antigen-specific immune responses are not fully developed until six weeks of age (Everaert *et al.*, 2017). Antigen presenting T-cells, CD4, develop in the intestinal mucosa in the first two weeks and CD8 T-cells in the epithelium from 6 weeks of age. The absence of microbiota can limit antigen- presenting cells from developing (Willing & van Kessel, 2007).

Early weaning can lead to changes in the development of the microbiome that can in turn have a detrimental impact on the immune system (Buddington & Sangild, 2011). In the same way, the environment in which the piglet is raised can impact the microbiome. Management practices therefore can influence the health of piglets and their potential to grow optimally (Smith, 1988; Bianchi *et al.*, 1992; van Ginneken & Weyns, 2004). Early weaning can cause significant growth stunts and diarrhoea that can be ascribed to more than one aspect. Anorexia and malnutrition after weaning can be major causes of disease and fasting during weaning can lead to alterations in gut architecture. As the weaned piglet starts to eat again, gut development will also be stimulated (Lallès *et al.*, 2007).

Major factors affecting gut structure and function after weaning include reduced feed intake (Verdonk *et al.*, 2007), dietary changes (Jha & Berrocoso, 2016), infectious bacteria in the small intestine (Derrien *et al.*, 2010), withdrawal of sow's milk (Albrecht *et al.*, 2014) and cytocines (Pluske *et al.*, 1997; Luissint *et al.*, 2016). The most pronounced effect of stress at weaning is reduced feed intake that leads to reduced protein and energy availability that causes reduced cell development and increased loss of mature enterocytes (Verdonk *et al.*, 2007). Reduced cell development has a greater impact on nutrient availability compared to apoptosis of mature enterocytes, although both have a significant effect (Pluske *et al.*, 1997).

Boudry *et al.* (2004) found that weaning had significant short-term and long-term effects on intestinal physiology. Jejuna villous height decreased by 40% between 2 to 8 days, with no effect on the distal ileum, while crypt length in the distal ileum increased with time from day 2 by 35% up to day 15. Similar effects occurred in the proximal jejunum. These negative effects of weaning were attributed to psychological stress and food deprivation. It was theorised that these stressors could lead to reduced absorption in the ileum at weaning resulting in increased incidences of osmotic diarrhoea while low transmucosal resistance in the ileum 2 days after weaning could lead to electrolyte and inflammatory cell secretion into the lumen, resulting in secretory diarrhoea (Field, 2003). Therefore, increasing feed consumption and reducing stressors could potentially alleviate these problems by maintaining intestinal integrity. Similar results were observed by Verdonk *et al.* (2007), who found that intake levels after weaning influenced villous height and crypt depth, but not intestinal permeability. Low intakes at weaning had a detrimental impact on gut structure, whereas high intakes reduced the impact of weaning on intestinal structure (Spreeuwenberg *et al.*, 2001; Dong & Pluske, 2007; Pluske *et al.*, 2018).

Dietary change is a second factor that can influence gut structure and function in weaner pigs. A change in the physical form of the feed can influence FI, for example, slurry increases intake compared to pelleted feed, and is also less abrasive to the intestines (Jha & Berrocoso, 2016). Physical presence of feed in the intestines is important not only for cell differentiation, but also for maintenance of structure and function of the mucosa (Jayaraman & Nyachoti, 2017). High FI before weaning could potentially favour immune tolerance and reduce hypersensitivity to antigenic compounds. Many antinutritional factors could also be present in feed that needs to be taken into consideration. Dietary change could also lead to increased amounts of material flowing to the large intestine, causing increased microbial fermentation, which could have beneficial or detrimental effects. It has been proven that diets higher in fibre increases the amount of short chain fatty acids produced in the hindgut, in turn stimulating proliferation of crypt-cells, whereas excess protein flowing to the hindgut could lead to production of detrimental compounds such as ammonia, hydrogen sulphide, amines as well as phenols and indoles (Davila *et al.*, 2013; Zhang *et al.*, 2018)

A third factor is infectious bacteria in the small intestine that reside in the gut of sick animals as well as healthy animals. However, pigs with PWD tend to have shorter villi and deeper crypts, leading to reduced absorption of nutrients and increased secretion of substances into the gut lumen, which in turn could lead to osmotic diarrhoea (Derrien *et al.*, 2010). Unabsorbed feed could furthermore serve as an energy substrate for pathogenic bacteria, worsening the problem. The diet could potentially impact the structure of the mucosa and mucin glycoconjugates that has an impact on the adhesion of microflora in the gut.

Intake of colostrum and milk also has an impact on bacterial growth in the gastrointestinal tract that can be altered at weaning, impacting not only immunity, but also gut morphology and function (Albrecht *et al.*, 2014). Sows' milk contain many substances beneficial to gut health and development, such as epidermal growth factor, polyamines, insulin like growth factor and glutamine (Bauer *et al.*, 2006). In Table 2.1 the potential effects of porcine milk components on intestinal microbes were summarised.

Table 2.1. Potential effects of porcine milk components on intestinal microbes (Adapted from Bauer *et al.*, 2006)

Component in milk	Potential Effect on intestinal microbes
Lysozyme	Bactericidal
Transferrin	Antimicrobial
Lactoferrin	Antimicrobial & Growth promotion for beneficial microbes
Milk lipids	Antimicrobial
Oligosaccharides	Growth promotion for beneficial microbes
'Bifidus factor'	Growth promotion for beneficial microbes
Casein	Growth promotion for beneficial microbes
Peptide from casein digestion (casomorphin)	Immunomodulatory effect

The mucin layer has two important functions to the host animal by serving as a protective layer or barrier to the gut epithelium and to act as a prebiotic that can feed beneficial microbes. Mucin production in the GIT has three major advantages for gut microbes, namely 1) serving as a binding structure for gut microbes, 2) serving as a layer of protection for some microbes and 3) aiding as a nutrient source for other microbes (Derrien *et al.*, 2010). Mammalian milk can often also serve a similar purpose as the mucin layer by providing a substrate for microbes to bind to, thereby reducing the risk of microbial invasion of the gut epithelium (Allen, 2005; Cone, 2009). Bacteria with the ability to degrade mucin include *Akkermansia muciniphila*, *Bacteroides fragilis*, *Bacteroides thetaiotaomicron*, *Bacteroides vulgatus*, *Bifidobacterium* spp., *Bifidobacterium bifidum* , *Clostridium cocleatum*, *Clostridium septicum*, *Helicobacter pylori*, *Prevotella sp. RS2*, *Ruminococcus torques*, *Streptomyces* spp. and *Vibrio cholera* (Wright *et al.*, 2000; Rho *et al.*, 2005).

Cytocines originate from lymphocytes and macrophages and are also produced by other cells such as epithelium, endothelium and fibroblasts and they assist in regulating inflammatory responses. (Eckmann *et al.*, 1995). Intestinal epithelial cells produce cytokines and chemokines in response to the presence of antigens and then summon and activate immune cells such as neutrophils, macrophages, T-cells, B-cells and dendritic cells. Inflammation of the gut is a common occurrence in newly weaned animals and in pigs this can happen as soon as 2 days after weaning (McCracken *et al.*, 1999; Hedemann *et al.*, 2003) Inflammation brought about by these cytokines can cause significant damage to the intestinal structure by impacting villi height, crypt depth, epithelial tight junctions and enzyme activity (Pié *et al.*, 2004; Luissint *et al.*, 2016).

2.4 Microbiome diversity in the pig

Marchesi and Ravel (2015) defined the microbiome as a complete habitat containing all microorganisms as well as their genomes in the environment that surrounds them. In other words, a microbiome includes all microorganisms and their surroundings, and thus both biotic and abiotic factors. The gut microbiome is not a new field of study and has been in existence for at least 50 years, however, new developments in high throughput sequencing that is also less expensive, have caused a revival in the field, with formerly unculturable microbes that can now be identified (Prescott, 2017).

Culture based techniques had a phenomenal influence on research in the field of microbiology and science, however, it was insufficient in many ways, as it was not always reliable, due to variations between techniques and difficulty in growing certain cultures (Isaacson & Kim, 2012). As DNA sequencing techniques developed it was possible to study the complete microbiome that included new and unfamiliar microbial species. It was soon realised that the gut microbiome was far greater and more diverse than previously thought (Chen *et al.*, 2018). The first description of the pig gastrointestinal microbiome based on 16S rRNA gene sequencing was by Pryde *et al.* (1999). Although this approach was one of the

first of its kind, valuable information was gained and it was reported that about 59% of sequences were of unknown bacterial origin.

Woese & Fox (1977) were of the first to categorise prokaryotic cells into Bacteria and Archaea based on the 16S rRNA genes. 16S rRNA that encodes the small ribosomal subunit is ideal for identifying bacterial species, as it is present in all prokaryotes and is approximately 1550 base pairs long, consisting of several conserved regions that flank nine hypervariable regions (Bhattacharya & Medlin, 1995). These hypervariable regions can be used to identify unique species, whereas the conserved regions can be used for the creation of universal PCR primers that will be suitable for bacterial species that are closely related and have similar hypervariable regions. Variations in these genes indicate evolutionary distance and relatedness of organisms, but does not indicate the rate of change in the gene sequences. Hypervariable regions V2 and V4 were found to have the lowest error rate of the nine regions (Wang *et al.*, 2007). Advantages of using 16S rRNA sequencing include identifying and describing rare strains, routine identification of bacteria, discovery of new pathogens and identifying bacteria that could previously not be cultured (van de Peer *et al.*, 1996; Clarridge, 2004). Next-generation sequencing platforms are used when sequencing the 16S rRNA. Various platforms exist such as Roche 454, Illumina, SOLiD, and IonTorrent (Quail *et al.*, 2012).

The microbial community in the gut of most mammals, including pigs, is a dynamic system influenced by many factors. The microbial population tend to reach a stable climax community during gut maturation as the animal matures (Spor *et al.*, 2011). Even after disturbances in the gut community, the climax community will usually return to its previous state. Some microbes are opportunistic in nature and passes through the system without becoming part of the more permanent climax community. Microbes will also differ through the length of the intestinal tract, as conditions in these areas favour some microbes more than others (Isaacson & Kim, 2012). Evidence even suggests that the host genotype can have a significant effect on the gut microbiome, but external factors can also influence microbial composition, such as the use of antibiotics, a specific diet, stress and disease as well as the microbial community of the sow with which the piglet comes in contact (Konstantinov *et al.*, 2004). Thompson *et al.* (2008) proposed that there exists a very short period during which a piglet acquires its own climax microbial community, that will then reside in the gut of the adult pig. This window presumably occurs from ages 2-3weeks and reaches stability at about 5 weeks of age

Konstantinov *et al.* (2006) used 16S rRNA and denaturing gradient gel electrophoresis in a study to describe the microbial diversity of piglets at two different ages. At 2 days of age *Lactobacillus sobrius* and *L*. *reuteri* dominated the ileum, whereas the number of these microbes decreased after weaning. It was also found at 2 days of age that 45% of all microbes were pathogenic, such as *E*. *coli* and *Shigella flexneri* belonging to the phylum Bacteroides. Besides these two pathogenic species three *Lactobacillus* species were also identified namely *L*. *sobrius*, *L*. *reuteri* and *L*. *acidophilus* which are thought to competitively exclude pathogens. In weaned pigs the *Lactobacillus* levels were significantly lower, thus implying that the gut microbiome significantly changes as a result of weaning stress (Konstantinov *et al.*, 2004; Castillo *et al.*, 2007).

Literature shows the impact of weaning stress on the gut had the greatest influence on the ileum of the piglet, with the microbiome in the gut that changed significantly 4 days after weaning and favourable *Lactobacillus* populations that decreased sharply 5 days after weaning, especially in the ileum (Tao *et al.*, 2015). Weaning decreased the intestinal permeability allowing pathogens and feed antigens to cross the intestinal barrier. Microbial diversity decreased at weaning, but despite this, the number of pathogenic microbes increased in relation to total microbes, because of a reduction in probiotic microbes (Janczyk *et al.*, 2007).

Microbial populations can also vary greatly between individuals with colonisation depending on various interacting factors such as natural selection, genetics, physiology and microbial interactions. Contact with the environment and birth canal during and directly after birth initialises the colonisation process (Chen *et al.*, 2018). Consumption of milk conveys passive immunity to the suckling piglet through various substances in milk that could potentially have antimicrobial effects as well as growth promoting effects for beneficial organisms (Bauer *et al.*, 2006). The digestive tract of the suckling piglet is adapted to digest substances in milk such as lactose, hexoses and oligosaccharides. During the process of weaning the gut must adapt to digest plant carbohydrates, which results in a shift of microbial colonies. It was reported by Pajarillo *et al.* (2014) that the order of most abundant microbes changed from 4 to 6 weeks of age with the most pronounced effect between *Bacteriodetes* and *Firmicutes*, It was concluded that an age effect was involved in the establishment of the microbiota.

Intestinal epithelium, for example Paneth cells, can secrete antimicrobial substances such as Angiogenin-4 into the gut lumen and these substances tend to increase during weaning (Bauer *et al.*, 2006). Early interactions between bacteria and Paneth cells can assist in forming the antimicrobial repertoire during intestinal development. Introduction to solid feeds during suckling could potentially aid in the stimulation and establishment of microbial species needed at weaning (Hooper, 2004). Similar reports have been made by Stappenbeck *et al.* (2002) who reported that microbes in the gut influence Paneth cell differentiation. In conjunction with this, Paneth cells secretes antimicrobial proteins affecting microbial populations. These cells also directly affect microvasculature and indirectly affects expression of regulators in epithelium or mesenchymal cells. Microbes not only stimulate expression of genes responsible for absorption of digesta by breaking down indigestible substances, they also increase intestinal absorptive capacity through amplified angiogenesis, creating an extraordinary symbiotic relationship with the host (Sommer & Bäckhed, 2013). The presence of microbes in the gut influences blood flow in the gut, as shown in a study on gnotobiotic adult mice. Bacterial colonisation of the gut assisted in resuming angiogenesis rapidly in the small intestine (Reinhardt *et al.*, 2012).

In a study analysing microbial genes present in pig faeces, Xiao *et al.* (2016) was able to taxonomically classify half of the non-redundant genes. Of the classifiable genes, 98% could be allocated to the *Bacteria* super kingdom, with the rest belonging to *Archaea* and Eukaryotes. Further classification into the phylum revealed that the majority of genes belonged to *Firmicutes* followed by *Bacteroidetes*. Only a small fraction of genes could be classified into a bacterial genera and species and of these the majority were *Prevotella*, *Bacteroides*, *Clostridium*, *Ruminococcus* and *Eubacterium*, with age, sex and breed also having an impact on the composition. In a study by Pajarillo *et al.* (2014) 16S rRNA pyrosequencing was used to analyse pig faeces and it was observed that microbial diversity increased from 4 to 6 weeks of age with the dominant species belonging to the phyla *Firmicutes*, *Bacteriodetes*, *Proteobacteria*, *Spirochaetes* and *Tenericutes*.

Microbiome studies have been linked to traits such as back fat in pigs as indicated in a comparison study between Landrace and Meishan pigs. A correlation between backfat depth and level of *Bacteroidetes* in the caeca was reported, with Meishan pigs having 48% greater backfat depth than Landrace pigs and a corresponding decrease in *Bacteroidetes* levels (Guo *et al.*, 2008a). *Firmicutes* did not increase with obesity, indicating that there might be other factors influencing *Firmicute* levels. Reports by Guo *et al.* (2008b) found significantly lower levels of *Bacteroidetes* in obese pigs with no corresponding increase in the amount of *Firmecutes*. Ban-Tokuda *et al.* (2017) also found decreasing levels of *Bacteroidetes* with fattening, but in that study levels of *Firmicutes* also increased with fattening although feed efficiency was not correlated with these two bacteria.

The role of diet has been linked to microbial diversity, with the balance between *Bacteroidetes* and *Firmicutes* influenced by high fat content as has been reported by Ley *et*

al. (2005) in a study on obesity in mice. Obese mice had 50% less *Bacteroidetes* in the caeca with a direct increase in the level of *Firmicutes*. Research by de Vadder *et al.* (2016) reported that *Prevotella copri* was able to produce succinate which in turn stimulated intestinal gluconeogenesis in humans. *Prevotella* species might therefore exert a beneficial effect on the host. This might, however, be host specific and might vary between animals with different microbiomes. In human subjects the ratio between *Prevotella* and *Bacteriodes* was higher in humans who consumed kernel-based bread for three days (Kovatcheva-Datchary *et al.*, 2015). Higher levels of *Prevotella copri* increased the potential to ferment complex polysaccharides which could potentially be beneficial. When germ-free mice were given the same *Prevotella copri* from the human subjects, they exhibited improved glucose metabolism and liver glycogen.

2.5 Preventing post-weaning diarrhoea

At weaning the microbiome of the piglets is placed under stress as they tend to stop eating in anticipation of getting sow's milk. Piglets that were exposed to creep feed will be used to the smell and texture of feed and will start eating sooner (Sulabo *et al.*, 2010). As the stomach and intestines starts to empty its digestive content, the piglet will consume feed for the first time that will then pass into the large intestine rather quickly. The abnormally fast passage rate will result in higher than normal pH levels that will in turn result in poor digestion with a more basic environment, failing to prevent pathogenic bacterial growth, which will eventually pass to the lower intestine (Drasar *et al.*, 1969; Suiryanrayna & Ramana, 2015). Table 2.2 refers to the specific disease causing bacteria in the pig's digestive tract. Pathogenic bacteria in the lower intestine will utilise the undigested proteins, multiply, secrete toxins and damage the structure of the intestines (Heo *et al.*, 2012). Water will be secreted into the large intestine to try and rid the body of the undigested feed which in turn could result in scours. The limited amount of water that will be left in the piglet's body will be excreted in the faeces and urine, which in turn could lead to dehydration. Feeding strategy becomes an important factor to ensure optimal adaptation to the new diet while still supplying the correct nutrients. Creep diets tend to contain larger portions of milk and animal derived products for easier digestion together with feed substrates with an appealing smell. Therefore, plant based substrates should be limited and slowly introduced in the weaner diet until the bulk of the diet consists of plant based substrates in the grower and finisher feed (Pretorius, 2014).

Table 2.2. Specific disease-causing bacteria in pigs (Adapted from Klose *et al.*, 2010)

Indicator serogroup/pathotype	Disease and associated swine period
Clostrdium perfringens type A	Neonatal diarrhoea
Salmonella enterica serovar choleraesuis	Salmonellosis (all ages), zoonosis
Salmonella enterica serovar Enteritidis	Salmonellosis (all ages), zoonosis
Salmonella enterica serovar Typhimurium	Salmonellosis (all ages), zoonosis
Escherichia coli O8 K88 F4	Neonatal and post-weaning diarrhoea
*Escherichia coli*O138 K81	Neonatal and post-weaning diarrhoea
Escherichia coli O139 K82	Neonatal and post-weaning diarrhoea
Escherichia coli O147 H19	Neonatal and post-weaning diarrhoea
Escherichia coli O157 H7 933	Hemorrhagic colitis, edema disease, zoonosis
Brachyspira pilosicoli	Grower scour at finishing stage, zoonosis

Nutritional strategies have been recommended to stimulate gut development and health, such as the inclusion of small amounts of insoluble fibre like oat hulls (Pluske, 2013). Soluble fibre tend to have the opposite effect, by slowing down the passage rate and having a viscous effect on the digesta (Kim *et al.*, 2012). Southgate (1977) defined dietary fibre as all

indigestible polysaccharides and lignin that will reach the large intestine, where it will either be digested by microbes in the large intestine or excreted in the faeces.

The benefits of including specific fibre sources such as pea and wheat bran fibre in weaning diets have resulted in improved villus height and crypt depth (Chen *et al.*, 2014; Wu *et al.*, 2018). Pea and wheat bran fibre stimulated enzyme secretion, particularly of sucrase and maltase with pea fibre and only sucrase with wheat bran fibre. Fibre also had the ability to influence SCFA production such as soybean fibre that tended to stimulate acetate production, whereas wheat bran fibre stimulated butyrate production (Chen *et al.*, 2014). The production of SCFA correlated with microbial populations in the gut where soybean fibre diets resulted in lower *Lactobacillus* population and higher *E. coli* populations and pea and wheat fibre increased *Lactobacillus* and *Bifidobacterium* populations, respectively (Chen *et al.*, 2013). Glucose transporter gene expression was also up-regulated by wheat bran fibre and pea fibre. In addition, soybean fibre negatively affected FI whereas FI with addition of pea fibre to the diet was unaffected. Pea fibre however, improved feed conversion, without improvement in average daily feed intake and average daily gain (ADG) indicating that some fibre types have the ability to alter the gut digestive physiology (Montagne *et al.*, 2003).

Studies on the effect of particle size of wheat bran on the gut microflora in pigs infected with an *E. coli* strain (K88+), reported that inclusion of wheat bran fibre at 4% of the weaner diet reduced *E. coli* populations in the ileum, respective if finely ground or coarse (Molist *et al.*, 2010). Coarse wheat bran reduced the adhesion of *E. coli* to the gut and reduced the faecal score as well as the enterobacteria populations (Mikkelsen *et al.*, 2004). Opposed to coarse wheat bran (1088 µm), fine wheat bran (445 µm) has increased microbial diversity, but reduced short chain fatty acid levels in the ileum. Coarse wheat bran reduced the ability of *E. coli* to attach to the gut, thereby decreasing the severity of diarrhoea. (Hamaker & Tuncil, 2014). High fibre diets tended to increase the weight of the large intestine of growing pigs, since it promoted greater fermentation in the colon (Hermes *et al.*, 2010). High fibre also reduced the amount of ammonia produced in the large intestine, since less protein fermentation occured in the large intestine compared to increased carbohydrate fermentation, thereby increasing the amount of favourable lactic acid producing bacteria (LAB) (Awati *et al.*, 2006).

High protein levels in feed, as well as the source of protein, can have a significant effect on gut health and the incidence of diarrhoea, as indicated by Wu *et al.* (2015). Pigs fed on lower protein diets had lower incidences of diarrhoea opposed to pigs fed protein rich diets. It was also shown that increasing the protein level had no beneficial effect on growth performance. Efficiency of protein utilisation was also compromised by increased protein levels, evident from high plasma urea nitrogen levels. Similar results were obtained in a study by Bhandari *et al.* (2010) in which low protein diets reduced the level of pathogenic *E. coli*. Lower protein levels as well as animal protein sources such as fish meal also improved gut morphology in terms of villus height, crypt depth and lower colonic Cl^- content when compared to high protein levels or plant protein sources (Yu *et al.*, 2010). Amino acid composition and protein balance seems to be of greater concern, as indicated by Nyachoti *et al.* (2006). By lowering the crude protein (CP) levels and supplementing the diet with essential amino acids, they were able to significantly improve performance and gut health. In a similar study by Wu *et al.* (2015) pigs on a low soya protein diet had higher levels of IFN-ɣ in the jejunum and colon than those on the low fish meal diet and consequently also had higher incidences of diarrhoea. Increased expression of these pro-inflammatory cytokines can cause down-regulation of tight junction proteins (Al-sadi *et al.*, 2013), resulting in increased incidences of diarrhoea (Kim *et al.*, 2011; Heo *et al.*, 2012; Jha & Berrocoso, 2016).

2.6 In-feed antibiotics

For many years, in-feed antibiotics were used as both growth promoters and prophylactics. Antibiotics in feed not only offers a health benefit to animals but also has a growth promoting effect by improving weight gain and feed efficiency (Gaskins *et al.*, 2002). The effect seems to be more significant in younger animals. Antibiotics enhance growth

through four main mechanisms namely reduced sub-clinical infections, reduced growth depressing microbial metabolites, reduced competition for nutrients, and a thinner intestinal wall that allows greater nutrient uptake (Anderson *et al.*, 2000). It therefore has a direct effect through reduced competition for nutrients as well as an indirect effect through reduced growth depressing metabolites produced by the microbes. Microbes in the SI usually compete for nutrients and results in negative growth effects. These microbes are usually gram positive aerobes, whereas the microbes in the large intestine have a beneficial effect as it digests the indigestible substrates that passes from the SI and are not target microbes for antibiotics (Dibner & Richards, 2005). Despite many beneficial effects of feed antibiotics, there is growing concern about development of resistance to antibiotics across humans and animals. Bans on these substances will soon become a reality as it already has in the EU since 2006. Table 2.3 summarises some feed antibiotics and their modes of action.

Jahanbakhsh *et al.* (2015) showed that most *E. coli* resistance to antimicrobials were towards tetracycline (92.5%), ampicillin (83.7%), sulfisoxazole (80%), trimethoprim–sulfamethoxazole (77.5%), streptomycin (77.5%), amoxicillin/clavulanic acid (65%), chloramphenicol (40%), kanamycin (22.5%), ceftriaxone (20%), ceftiofur (20%), cefoxitin (20%), and gentamicin (18.75%). Of the antimicrobial resistant (AMR) strains, 87.5% were multi-drug resistant. These high resistance rates could be attributed to in-feed supplementation of chlortetracycline and penicillin. An added danger lies in the possibility of co-selection with other resistant genes. Co-resistance of streptomycin, trimethoprim–sulfamethoxazole, sulfisoxazole, amoxicillin/clavulanic acid, ampicillin, chloramphenicol, and tetracycline was shown by Jahanbakhsh *et al.* (2015). From a medical point of view this is alarming evidence.

Table 2.3. Feed antibiotics and their mode of action (Gaskins *et al.*, 2002)

Class	Trade name	Generic name	Spectrum	Mode of action
Diterpene	Tiamulin	tiamulin	Gram+	Protein synthesis inhibition
Glycopeptide	Avotan	avoparcin	Gram+	Cell wall synthesis inhibition
Lincosaminides	Lincomix	lincomycin	Gram+	Protein synthesis inhibition
Macrolide	Tylan	tylosin	Gram+	Protein synthesis inhibition
	Spira 200	spiramycin		
Oligosaccharide	Maxus	avilamycin	Gram+	Protein synthesis inhibition
β-lactam	Penicillin	penicillin	Gram+	Cell wall synthesis inhibition
	Bacitracin	bacitracin	Gram+	Cell wall synthesis inhibition
	Zn Bacitra	bactitractin		
Streptogramin	Stafac	virginiamycin	Gram+	Protein synthesis inhibition
Phosphoglycolipid	Flavomycin	bambermycin	Gram+	Cell wall synthesis inhibition
Polyether	Salocin	salinomycin	Gram+	Membrane alterations
	Monteban	nerasin		
Quinoxalines	Mecadox	carbadox	Broad	DNA synthesis inhibition
	Bayonox	olaquindox		
Sulfonamides	Sulfamethazine	sulfamethazine	Broad	Metabolic inhibition
	Sulfa thizole	sulfathiazole		
Tetracycline	Aureomycin	chlortetracycline	Broad	Protein synthesis inhibition
	Terramycin	oxytetracyline		

Significant differences were found in the occurrence of resistant *E. coli* between different farms and was not necessarily connected to specific antibiotics given. It was revealed that the degree of *E. coli* resistance was not only age dependent, but also more likely to occur in

progeny from sows carrying resistant *E. coli*. These piglets were also more likely to retain the resistant *E. coli* until slaughter (Cameron-Veas *et al.*, 2016). Preventing *E. coli* resistance in sows could reduce the introduction of new-born piglets with resistant *E. coli*, as results from this trial indicated that the prevalence of resistant *E. coli* was reduced after weaning in piglets that did not acquire resistant *E.* coli from maternal sows and developed resistant *E. coli* only after introduction to antimicrobials at 7 days of age. This could possibly be due to development of intestinal microbial populations in the gut with age (Agga *et al.*, 2014).

Keelara & Thakur (2014) describes how *Salmonella* serotypes can spread from pig to human, with the farm environment acting as a reservoir for resistant bacteria, leading to rapid spread into the human food chain. A combination between resistance in animals due to antibiotic use in the animal husbandry and resistance in humans due to medical treatment of infectious diseases could exacerbate the problem with multidrug resistance in various pathogenic bacteria, as these two sectors frequently come in contact. The spread of diseases between different countries can easily occur with effortless travel possibilities for people and global trading of fresh products and live animals all around the world (Holmes *et al.*, 2016). Therefore, occurrence of resistant bacteria can no longer be confined to certain locations, necessitating multi-industry and cross continental awareness and action (Rotimi *et al.*, 2008; Cameron-Veas *et al.*, 2016; Lopes *et al.*, 2016).

More than 98.9% of all *Camphylobacter* isolates found in Chinese pig herds belonged to *C. coli* (Qin *et al.*, 2011). From these isolates, 76.8% were multi-drug resistant, numbers much higher than recorded by Shin and Lee (2007) in pig herds in Korea. In a comparison of data from other countries, they found that resistance varied among countries, depending on specific antimicrobials used. They conclude that multi-drug resistance might become a problem with regards to food-borne diseases transmittable to humans, especially in countries such as Korea and China with high per capita consumption of pork. A significantly higher level of antimicrobial resistant genes were found in Chinese pigs compared to Danish and French pig genes, indicating that elimination of antibiotics in feed can significantly reduce antimicrobial resistance genes (Xiao *et al.*, 2016)

2.7 Non-antibiotic feed additives

Feed additives exert beneficial effects to animals by either improving the gut physiology and morphology or modulating the immune function and intestinal microbiome. The main feed additives that beneficially affect health and performance of production animals include gut acidifiers, minerals, prebiotics, probiotics and yeast, nucleotides, plant extracts and enzymes (Liu *et al.*, 2017). Combinations of these additives could potentially reduce or even eliminate the use of antibiotics in feed. Not only will farmers and feed producers be forced to use alternatives to antibiotics, but it will become even more important to maintain a healthy pig herd and prevent infection through improved management practices as reliance on antimicrobials lessens. It is already becoming more evident in the feed manufacturing industry that combinations of different products can be used together fairly effectively (de Lange *et al.*, 2010).

Additives such as gut acidifiers have been shown to have positive effects through different modes of action, for example decreasing and stabilising the gut pH, increasing pepsin activity, reducing pathogenic microbes and promoting populations of beneficial microbes (Guggenbuhl *et al.*, 2007). Other modes of action may include increased FI, improved nutrient digestibility and improved growth and performance. Gut acidifiers include organic acids, inorganic acids, salts of acids and blends of acids. Gut acidifiers seem to be inconsistent and benefits restricted to feeding it for short periods of time (Partanen & Mroz, 1999; Papatsiros & Billinis, 2012; Lei *et al.*, 2017; Luise *et al.*, 2017).

Minerals are also used as feed additives with zinc and copper as the two main minerals that have major effects on gut health (Heo *et al.*, 2010). Zinc is an activator of metalloenzymes, affects hormone secretion, affects skin and wound healing and is vital for the functioning of the immune system. It has also been proven to help reduce post-weaning diarrhoea, improve

growth and performance and increase FI (Heo *et al.*, 2010, Hu *et al.*, 2012; Upadhaya *et al.*, 2018). These positive effects can be attributed to increased villus height and villus height to crypt depth ratio, reduced intestinal permeability, a stabilised gut microbiome and molecular signalling of immune cells. Zinc can however have a negative effect on Ca and P digestibility (Walk *et al.*, 2013). Copper is also a component of metalloenzymes such as cytochrome oxidase and lysyl oxidase and is important for oxidation reduction reactions, transport of oxygen and electrons and protection against oxidative stress. Copper is involved in various metabolic reactions and has growth promoting effects due to its bactericidal properties (Højberg *et al.*, 2004; Pérez *et al.*, 2011; Ma *et al.*, 2015).

Nucleotides are molecules with a nitrogenous purine or pyrimidine base linked to a pentose sugar and a phosphate group and have been used as feed additives. Nucleotides can be synthesised *de novo* by using glutamine, which is an energy expensive process (Domeneghini *et al.*, 2004). Nucleotides are not only building blocks for DNA and RNA, but also have physiological roles as energy sources, cofactors in redox reactions, physiological regulators, carriers for intermediate substrates and acyl groups, development of the immune system and intestinal tract (Hess & Greenberg, 2012). Beneficial effects of nucleotides include absorption of iron, metabolism of lipoproteins and long chain polyunsaturated fatty acids and enhanced mucosal and liver development and maintenance (Carver & Walker, 1995; Martinez-Puig *et al.*, 2007).

Plant extracts has been used as feed additives successfully and include essential oils which consists of water-insoluble plant extracts (Baydar *et al.*, 2004). Beneficial properties include antiviral, antimicrobial, antioxidant, anti-inflammatory and anti-oxidative effects (Sökmen *et al.*, 2004; Liu *et al.*, 2013b). Plant extracts include extracts from herbs and spices such as oregano, thyme, clove, pepper, lavender, garlic, basil and others (Zhai *et al.*, 2018). They tend to have a positive effect on production by increasing ADG, feed intake and feed efficiency.

Feed enzymes are known to enhance the nutritive value of the feed by reducing anti-nutritive factors thereby improving the availability of feed substrates and are used in most monogastric feeds (Torres-Pitarch *et al.*, 2017). They break indigestible bonds in feeds that would otherwise not be broken down by digestive enzymes and results in beneficial polysaccharide hydrolysis products for gut microbes. It can also reduce PWD and the negative effects of feed substrates with high viscosity (de Lange *et al.*, 2010; Chen *et al.*, 2016).

Prebiotics are indigestible oligosaccharides that can be added to feed to increase the amount of beneficial microbes in the gut and thereby provide a benefit to the host organism (Gibson & Roberfroid, 1995). Most prebiotics consists of indigestible carbohydrates with beneficial properties for gut microbes. Prebiotics includes inulin, fructo-oligosaccharides, transgalacto-oligosaccharides and lactulose. Other carbohydrates which might also have a prebiotic effect include arabino-xylans, xyloglucans and resistant starches (Liu *et al.*, 2017; Suthongsa *et al.*, 2017). Prebiotics promote fermentation in the gut that results in the production of SCFA which in turn reduces the pH in the gut, thereby decreasing the amount of pathogenic microbes while increasing beneficial microbes. SCFA such as butyrate has been shown to beneficially effect epithelial health in both the small and large intestines (Awati *et al.*, 2006; Wu *et al.*, 2017).

Probiotics consist of three main groups namely *Bacillus* bacteria, LAB and yeast (Liu *et al.*, 2017). Yeast products include live yeast cells, heat treated yeast cells, ground yeast, purified yeast cell cultures and yeast extracts (Shurson, 2018). They tend to increase FI, growth and performance, mucosal immunity, intestinal development, protect against mycotoxins, reduce diarrhoea and affects the gut microbes. Mannans present in yeast cell walls are able to bind to mannan receptors of bacteria such as *E. coli* and *Salmonella*, preventing adhesion to the gut lumen (Bontempo *et al.*, 2006; Jiang *et al.*, 2015). Yeast probiotics will not be discussed in detail in this paper and the remainder of the paper will focus on bacterial probiotics.

2.8 Bacterial probiotics

The use of probiotics was common practice even before the discovery of bacteria. It dates back as far as 10 000BC with the first reports of fermented milk (Gasbarrini *et al.*, 2016). Although ancient people did not know exactly what caused fermentation, it was well known that these products had beneficial health effects. It is believed that the first fermented milk originated in ancient Egypt and Eastern cultures where shepherds used to keep milk in bags made of animal stomachs, which resulted in yogurt that they believed had beneficial health effects and called it the elixir of life (McFarland, 2015). Fermented products developed in nearly all cultures since the earliest of times, and mention of such products such as cheese, wine and sour milk can be found in many historical books. More modern references to probiotics refer to faecal transplantation as a remedy for food poisoning and transfaunation in the case of rumen dysfunction in cattle (Kelly, 2013). It has even been documented that German soldiers stationed in Africa in the Second World War mimicked the local inhabitants by eating fresh camel dung to treat gastric dysentery. Today it is known that the cure was due to *Bacillus subtillis* in the faeces. In the 1900s Elie Metchnikoff opened the way for scientific evidence on the effects of fermentation microbes on human health (Mackowiak, 2013).

Fuller (1989) made one of the first comprehensive descriptions of probiotics when he proposed that a probiotic is a live microbial feed supplement that can improve the microbial balance in the host's gut. Six modes of action can be identified (Table 2.4) when dealing with most probiotics (Liao & Nyachoti, 2017).

Table 2.4 Probiotic modes of action in humans and animals (Adapted from Liao & Nyachoti, 2017)

Mode of action	Description
Change in microbial populations together with competitive exclusion	Competition for space & adhesion sites on the gastro-intestinal epithelium
	Competition for nutrients
Bacteriostatic effect	Secretion of bactericidal & bacteriostatic substrates
	Lower pH through probiotic fermentation end products
	Bacteriostatic effects against gram-negative bacteria through hydrogen peroxide production
	Impacts metabolism and toxin production of pathogens
Impact host immunity	Improves intestinal barrier function and health, thus impacting innate immunity
	Impacts innate immunity through increased mucus and chloride secretion
	Selective stimulation & suppression of acquired immune responses.
	Impacting the immune system through secretion products such as metabolites, cell wall components & DNA
Reduce diarrhoea & antitoxin effects	Inhibition of pathogenic toxin production
	Reduced effects of pathogenic enterotoxins
Improved nutrient digestibility	Increased fermentation capacity
	Increased digestive enzyme production & activity
	Improved absorption and secretion activities in the gut
	Production of selective vitamins
Other modes of action	Antioxidative effects & stress relief
	Changes in bacterial & host gene expression

Most probiotics change the microbiome allowing more beneficial bacteria to populate the gastrointestinal tract and eliminating pathogenic bacteria through competitive exclusion, as they compete for adhesion sites and organic substrates (Bhandari *et al.*, 2010; Liu *et al.*, 2015). They can also directly suppress the growth of pathogens through secretion of

substrates such as bacteriocins and organic acids. Many probiotics have the ability to produce bacteriocins, which are peptides synthesised ribosomally and has antimicrobial effects (Sawa *et al.*, 2013). The extent of the antimicrobial effect and its target bacteria depends on the specific probiotic and the specific strain of bacteria used as a probiotic (Klose *et al.*, 2010; Kumar *et al.*, 2016). These pepsins have the ability to stimulate the adaptive immune system and prevent secretion of pro-inflammatory cytokines such as interleukin-17 (Mazmanian *et al.*, 2008). Bacteriocins have various other non-nutritive applications as described by Ahmed *et al.* (2010) and Abriouel *et al.* (2011) that will not be discussed in this review. Secretion of organic acids such as lactic acid produced by *Lactobacillus* through fermentation can reduce the pH and therefore reduce the growth of pathogens that grow at higher pH ranges (Lema *et al.*, 2001).

Probiotics have the ability to regulate the host immune responses by either improving the innate immunity or by modulating the acquired immunity (Lee *et al.*, 2015; Liao & Nyachoti, 2017). Probiotics furthermore have the ability to regulate phosphorylation of cytoskeletal and tight junction proteins in the intestinal barrier, which is the first line of defence against pathogens (García-Lafuente *et al.*, 2001; Bontempo *et al.*, 2006; Yang *et al.*, 2015). Microbes in the gut can communicate with the host immune system through microbe-associated molecular patterns that interact with pattern recognition receptors in the intestinal mucosa (Lebeer *et al.*, 2010). Probiotics potentially stimulate immune responses in the GIT through upregulation of antibody production and phagocytic activity resulting in development of the acquired immune system (Schierack *et al.*, 2007). They impact the immune system through various mechanisms such as stimulation of epithelial lymphocytes (Yu *et al.*, 2008), stimulation of pro-inflammatory as well as anti-inflammatory cytokines (O'Hara *et al.*, 2006), upregulation of interleikin-6 resulting in B-cell differentiation and production of Immunoglobulin A and M, increased populations of CD4+ and CD8+ T-cells (Vinderola, 2005) and increased mucin production (Takahashi, 1997). Probiotic products such as metabolites, cell wall components and DNA also impact the immune system through the above mechanisms (Oelschlaeger, 2010), for this reason even dead probiotic cells can still influence the immune system.

Probiotics have an antidiarrheal effect in most cases (Kyriakis *et al.*, 1999; Alexopoulos *et al.*, 2004; Zeyner & Boldt, 2006; Trckova *et al.*, 2014). There are two general thoughts as to the mode of action through which probiotics can reduce diarrhoea, the first being modulations of gut microbes and competitive exclusion and the second through modulation of the immune system (Guarino *et al.*, 2008).

Various studies have shown improvements in nutrient digestibility through the use of different probiotics. These include improvement in CP digestibility (Yu *et al.*, 2008), total phosphorus digestibility (Huang *et al.*, 2004), energy (Zhao & Kim, 2015), fibre and organic matter (Giang *et al.*, 2010). Improved nutrient digestibility could partially be due to improved enzyme secretion, as shown by Collington *et al.* (1990) when they reported increased levels of sucrose, lactase and tripeptidase. An *in vitro* study with a specific *Lactobacilli* sp G3_4_1TO2 showed significant amylase production (Tallapragada *et al.*, 2018). Kim *et al.* (2007) was able to isolate a *Lactobacilli* sp. PSC101 that could produce amylase, lipase, phytase and protease. Huang *et al.* (2004) used a multi-strain *Lactobacilli* probiotic and found improved nitrogen and phosphorus digestibility as well as increased feed intake and a more favourable microbiome. Zhao & Kim (2015) also found significant improvements in apparent total track digestibility of N as well as gross energy when using *Lactobacillus reuteri* and *Lactobacillus plantarum* probiotics on weaner pigs. Lower *E. coli* and increased *Lactobacilli* levels in the gut with lower diarrhoea scores and noxious gas emissions were observed. Yu *et al*. (2008) reported improved ADG, apparent digestibility of CP and increased levels of IgG when using *Lactobacillus fermentum*. Cai *et al.* (2015) reported increased feed efficiency, ADG and lower blood urea nitrogen and ammonia emissions, they in part ascribed to longer duodenal and jejunal villi height.

The two most popular groups of probiotics used commercially include LAB, such as *Lactobacillus*, *Bifidobacterium* and *Enterococcus*; as well as different bacilli strains such as *Bacillus subtilis*, *Bacillus lenchiniformis*, *Bacillus pumilus* and *Bacillus megaterium* (de Lange *et al.*, 2010; Liu *et al.*, 2017*)*. Timmerman *et al.* (2004) described how probiotics can be used

as single strain, multi strain or multi species preparations where single strain preparations consists of a specific strain from a specific specie in isolation. Multi-stain refers to multiple stains from the same species for example *Bacillus subtilis*, *Bacillus lenchiniformis*, *Bacillus pumilus* and *Bacillus megaterium*, whereas multi-species probiotics can contain stains from different species such as *Bacillus*, *Bifidobacterium* and *Lactobacillus* all mixed into one preparation. Mixtures of different strains and species can be more effective opposed to a single strain preparation, as these have a greater change of populating the gastrointestinal tract and might have synergistic effects (Jin *et al.*, 2000; Lema *et al.*, 2001). Table 2.5 gives an indication of commercial probiotics used throughout the animal husbandry industry.

Table 2.5 Probiotics used throughout the animal husbandry industry (Adapted from Gaggìa *et al.*, 2010)

Genus	Species
Bifidobacterium	*B animalis subsp. animalis (B. animalis)a*
	B. lactis subsp. lactis (B. lactis)
	B. longum subsp. longum(B. longum)
	B. pseudolongum subsp. pseudolongum (B. pseudolongum)
	B. thermophilum
Enterococcus	*E. faecalis (Streptococcus faecalis)*
	E. faecium (Streptococcus faecium)
Lactobacillus	*L. acidophilus*
	L. amylovorus
	L. brevis
	L. casei subsp. casei (L. casei)
	L. crispatus
	L. farmicinis
	L. fermentum
	L. murinus
	L. plantarum subsp. plantarum (L. plantarum)
	L. reuteri
	L. rhamnosus
	L. salivarius
	L. amylovorus (L. sobrius)
Lactococcus	*L. lactis subsp. cremoris (Streptococcus cremoris)*
	L. lactis subsp. lactis
Leuconostoc	*L. citreum*
	L. lactis
	L. mesenteroides
Pediococcus	*P. acidilactici*
	P. pentosaceus subsp. pentosaceous
Propionibacterium	*P. freudenreichii*
Streptococcus	*S. infantarius*
	S. salivarius subsp. salivarius
	S. thermophilus (S. salivarius subsp. thermophilus)
Bacillus	*B. cereus (B. cereus var. toyoi)*
	B. licheniformis
	B. subtilis
Saccharomyces	*S. cerevisiae (S. boulardii)*
	S. pastorianus (S. carlsbergensis)
Kluyveromyces	*K. fragilis*
	K. marxianus
Aspergillus	*A. orizae*
	A. niger

Multi-species probiotics have advantages over single strain and multi-strain probiotics. Multi-species probiotics can overcome colonisation resistance from commensal bacteria more effectively and they are able to survive the hostile conditions in the gastro-intestinal tract to a greater extent since a variety of strains can survive in different areas of the gastrointestinal tract (Bezkorovainy, 2001). *Bifidobacterium* for instance tends to live in the small intestine, whereas *Lactobacilli* lives in the colon. *Bifidobacterium* will therefore be able to withstand the high pH in the small intestine better than *Lactobacilli*. Probiotic species can influence each other synergistically through different mechanisms. For example, lactate produced by *Lactobacilli* can be used by propionibacteria as a substrate for propionic acid production (Fröhlich-Wyder *et al.*, 2002). Giang *et al.* (2012) reported that a combination between different *Lactobacilli*, *Bacillus subtilis* and *Saccharomyces boulardii* had greater effects than the multi-strain *Lactobacilli* probiotic on its own. The multi-species probiotic improved digestibility of feed, reduced diarrhoea and reduced faecal scores.

2.8.1 Impact of probiotics on gut health and microbiome

Common measurements of gut health include villus height and crypt depth, brush-border enzyme activity and the presence of pathogens (Lallès *et al.*, 2007). It is clear that longer villi increase the absorption area of the intestines, thereby making absorption of nutrients more effective (Nabuurs, 1998). Villi height to crypt depth ratio can also be negatively impacted by weaning as the villi length tends to degenerate forcing immature enterocytes, with lower digestive capacity, to move into the villus column, thereby increasing the crypt depth (Pluske *et al.*, 1996). With regards to the immune system a common method used to determine immune function is enumeration of different types of white blood cells in the peripheral blood, for instance monocytes or lymphocytes and the cells produced by them (Herbert & Cohen, 1993). Lymphocytes commonly reported include natural killer cells, T and B cells and the cells derived from them such as CD4+ and CD8+ cells. A second type of enumerative essay is to count the antibodies produced by B cells in the circulating blood, such as Immunoglobulins A, G and M (Murphy, 2012).

It was reported that *L. jensenii* was able to exert a beneficial health effect and effectively modulate the mucosal immune system in pigs by upregulating pro-inflammatory mediators in porcine intestinal epithelium of cells challenged with enterotoxigenic *E. coli* (Suda *et al.*, 2014). *L. jensenii* offered simultaneous protection against inflammatory damage as well as improved immunity. In a study with a multi-specie probiotic consisting of *Bifidobacterium animalis* and *Lactobacillus* (*L. acidophilus, L. casei, L. pentosus, L. plantarum*) the small intestine of formula-fed, new born piglets were heavier when ingesting the probiotic opposed to those in the control (Siggers *et al.*, 2008). Other effects included reduced degeneration of small intestinal mucosa and villi length (Cao *et al.*, 2016) and increased aminopeptidase A and N enzyme activity at the brush border (Siggers *et al.*, 2008) Table 2.6 shows the efficacy of various pobiotic strains agains some patogens.

Probiotics such as *Lactobacillus brevis* have the ability to reduce inflammation and infection as shown by Liu *et al.* (2015) who indicated that supplementation of feed for weaned pigs with *Lactobacillus brevis* probiotics caused an increase in the serum pro-inflammatory cytokine IFN-ɣ secreted by immune cells to activate innate immune responses (Dalton *et al.*, 1993). In addition hepatoglobin decreased, indicating reduced inflammation and infection (Huzzey *et al.*, 2009). Intestinal morphology was also improved with longer villi, deeper crypt depth and reduced villi to crypt depth ratios (Dowarah *et al.*, 2017). Rodrigues *et al.* (2007) reported increased amounts of immunoglobulin A (IgA) expressing cells in the mesenteric lymph nodes, small intestine and blood of pigs on a multi-specie probiotic containing *Lactobacillus acidophilus, Enterococcus faecium e Bifidobacterium bifidum*. In addition they reported longer villi and deeper crypts. Toth *et al.* (2015) reported increased goblet cell numbers in the jejunum and decreased intraepithelial lymphocytes on the day of weaning when using a *lactobacillus* probiotic. Prieto *et al.* (2014) reported similar results on *Bacillus pumilus* probiotics. In addition, Toth *et al.* (2015) reported increased numbers of CD 163-expressing macrophages, confirming the positive effects of *lactobacilli*, as CD 163 can be associated with binding of both gram positive and negative bacteria as well as activating pro-

inflammatory production pathways. Some studies found higher IgG, IgM and IgA antibody levels in probiotically fed pigs, proving that probiotics had a positive effect on the humoral immune system (Naqid *et al.*, 2015). Lower numbers of eosinophils have also been reported, indicating lower pathogenic infections (Ross *et al.*, 2010) In addition to this, probiotics have been shown to be effective in treating and reducing the effects of many liver diseases (Nardone *et al.*, 2010; Bajaj *et al.*, 2014; Chávez-Tapia *et al.*, 2015)

Table 2.6 *In vitro* activities of various probiotic strains against pathogenic strains measured as radius of inhibition zone in mm (Adapted from Klose *et al.*, 2010)

		Pathogenic microbes									
Probiotic species	**Strain**	*Clostrdium perfringens* type A	*Salmonella enterica* serovar choleraesuis	*Salmonella enterica* serovar Enteritidis	*Salmonella enterica* serovar Typhimurium	*Escherichia coli* O8 K88 F4	*Escherichia coli* O138 K81	*Escherichia coli* O139 K82	*Escherichia coli* O147 H19	*Escherichia coli* O157 H7 933	*Brachyspira pilosicoli*
Bacillus subtilis	AE1	3.5	0	0	1	0	0	0	0	0	5
Enterococcus faecium	FASI13b	5.5	2	1.5	2.5	10	9.5	9	8	10	4
Bifidobacterium thermophilum	AN2	3	0	0	0	0	0	0	0	0	5
Bifidobacterium thermophilum	BTS	4.5	6	1	1	2	1	1.5	3	2.5	5
Lactobacillus salivarius	FACA12	11	13	6	5	14	12	14	9	17	7
Lactobacillus salivarius	LLS	11	11.5	8	5	11	14	15	9	11	6
Lactobacillus amylovorus	FACA8a	9.5	1	1	0	9	10	10	1	5.5	5
Lactobacillus amylovorus	FACO13	0.5	3	2	1.5	9	9.5	7.5	1	2	4
Lactobacillus reuteri	FACA2	1	6	3.5	4	5	6.5	6.5	0	6	0
Lactobacillus reuteri	LRS	13	14	6	3	11	9	10	7	7.5	0
Lactobacillus mucosae	FACO11a	13	3.5	0.5	3	5	4	4.5	3	4	0

Positive results with two different probiotic strains were reported by Dowarah *et al.* (2017) who used *Lactobacillus acidophilus* NCDC-15 and *Pediococcus acidilactici* FT28. Beneficial LAB bacteria as well as bifidobacteria were increased whereas *E. coli* and clostridia numbers decreased. Similar results were reported by Chiang *et al.* (2015) using *Lactobacillus johnsonii* x-1d-2 and *Lactobacillus mucosae* x-4w-1in weaned pigs with increased lactobacilli

numbers and lower *E. coli* numbers. Liu *et al.* (2015) reported lower faecal coliform population than normal when fed *Lactobacillus brevis*. No differences were observed between different levels of probiotic, indicating that a base level might be sufficient in altering microbial gut populations. There seems to be definite differences between probiotic species as seen by the study of Suda *et al.* (2014) where *L. jensenii* was able to suppress 3 different strains of enterotoxigenic *E. coli*, whereas *L. plantarum* only suppressed one of these strains. Giang *et al.* (2011) reported an increase in LAB and a decrease in *E. coli* in the faeces of grower pigs when fed a combination of *Bacillus subtilis* H4, *Saccharomyces boulardi* Sb, *Enterococcus faecium* 6H2, *Lactobacillus acidophilus* C3, *Pediococcus pentosaceus* D7, and *Lactobacillus fermentum* NC1 and similar results were reported by Giang *et al.* (2010). Many other studies also reported increased beneficial populations in the gut and reduced pathogenic population (Rodrigues *et al.*, 2007; Choi *et al.*, 2011b; Prieto *et al.*, 2014; Naqid *et al.*, 2015; Pajarillo *et al.*, 2015; Zhao & Kim, 2015).

The effect of a certain probiotic on a specific bacterial species is strain dependant. One strain might prove to be effective against a certain bacterium, whereas another might have little to no effect. In an *in vitro* study with two different strains of *L. reuteri*, Klose *et al.* (2010) showed that one strain had a significant effect on *C. perfringens*, while the other one had virtually no effect as demonstrated in Table 2.6. From this *in vitro* data, it is clear that Lactobacilli species (*L. salivarius*, *L. reuteri* and *L. mucosae*) had strong inhibitory effects against *C. perfringens* type A, whereas other probiotics had minor effects. *L. salivarius* and *L. reuteri* were also antagonistic towards most *Salmonella* and *E. coli* strains, with *E. faecium*, in addition, having moderate effects against *E. coli*. From this it seems that *B. subtilis* had a preference for *C. perfringens* inhibition similar to results by Teo & Tan (2005), but in comparison to most *Lactobacilli* species, had minor effects against any of these pathogens. Most of the strong inhibiting probiotic species were able to secrete organic acids and hydrogen peroxide as the inhibitory substrates, but since *B. subtilis* did not secrete these acids, it was postulated that another mode of action was involved, for instance bacteriocin secretion (Parisot *et al.*, 2008). LAB with the ability to secrete bacteriocins showed promising results against infection with *Campylobacter* as well as other food-borne pathogens, making it ideal for use as probiotics in animal feeds (Messaoudi *et al.*, 2012). However, despite positive *in vitro* results, Dobson *et al.* (2012) questioned the significance of bacteriocin production and its efficiency *in vivo*, since probiotics in the gut might not produce significant quantities of bacteriocins, and therefore exert their effect through other modes of action.

2.8.2 Impact of probiotics on growth and performance

It is likely that different strains of a specific probiotic will exert different effects on growth and performance to varying degrees (Modesto *et al.*, 2009; Klose *et al.*, 2010). Most probiotics tend to improve growth and performance as reported in numerous trials (Alexopoulos *et al.*, 2004; Bontempo *et al.*, 2006; Choi *et al.*, 2011b). A meta-analysis on the application of various probiotics indicated that probiotics had the potential to increase ADG and feed intake in pigs. These probiotics include *Lactobacillus* spp., *Enterococcus* spp., *Saccharomyces* spp., *Bacillus* spp. and *Pediococcus* spp. (Zimmermann *et al.*, 2016). Weaned pigs fed a combination of LAB together with *Bacillus subtilis* resulted in increased average daily feed intake and weight gain as well as lower feed conversion ratios (FCR) (Giang *et al.*, 2012). These groups also had lower incidences of diarrhoea and lower faecal scores (Giang *et al.*, 2012). Similar results were found by other authors (Le Bon *et al.*, 2010; Veizaj-Delia *et al.*, 2010; Lee *et al.*, 2015; Liu *et al.*, 2015; Dowarah *et al.*, 2017). Improved FCR and ADG were reported by Giang *et al.* (2011) on a multi-species probiotic containing *Bacillus subtilis* H4, *Saccharomyces boulardi* Sb, *Enterococcus faecium* 6H2, *Lactobacillus acidophilus* C3, *Pediococcus pentosaceus* D7, and *Lactobacillus fermentum* NC1. No improvements in average daily feed intake, ADG or FCR were observed when *Bacillus subtilis* H4 was fed alone or in combination with *Saccharomyces boulardi* Sb. Chiang *et al.* (2015) reported increased BWG, feed intake and lower FCR. Various production parameters were improved on both *Lactobacillus acidophilus* NCDC-15 and *Pediococcus acidilactici* FT28.

Giang *et al.* (2011) conducted a trial with different combinations of probiotics and found that CP and organic matter digestibility was improved on the complete combination probiotic containing *Bacillus subtilis* H4, *Saccharomyces boulardi* and LAB. Crude fibre digestibility was also improved on the complete combination probiotic and on the probiotic containing a combination of *Bacillus subtilis* H4 and *Saccharomyces boulardi*. Nitrogen retention was not improved by any probiotic and *Bacillus subtilis* H4 alone had no effect on any nutrient digestibility, with similar results reported in other studies (Giang *et al.*, 2010; Giang *et al.*, 2012).

Other research reported improvements in nitrogen and total phosphorus digestibility on multi-strain *lactobacilli* (Huang *et al.*, 2004) as well as improved energy digestibility on *Bacillus subtilis* probiotics, but concluded that the nutrient density of the diet might have an impact on the degree of digestibility exerted by the probiotic (Meng *et al.*, 2010). Kohn *et al.* (2005) also reported higher total protein in the serum and lower blood urea nitrogen, indicating better protein metabolism. Liu *et al.* (2013a) reported similar results with improved protein utilisation and deposition and in addition found improved metabolism of cholesterol.

Significant improvements in growth parameters of newly weaned pigs were reported when fed probiotics containing *Lactobacilli* spp. (Zhao & Kim, 2015). Improvements in apparent total tract digestibility (ATTD) of nitrogen and gross energy, as well as reduced noxious gas emissions on higher dosages of *Lactobacillus* were also reported. Contrary to this, Ross *et al.* (2010) found no difference in weight gain of weaned pigs, but did report significant improvements in feed efficiency. Similar results were reported by Shon *et al.* (2005) who observed slight to no improvement in growth parameters on *Lactobacilli* probiotics, but did find improvements in dry matter and nitrogen digestibility.

Improved pork meat through reduced drip loss, cooking loss and increased tenderness were reported by Liu *et al.* (2013a) when testing an unknown commercial probiotic. Suda *et al.* (2014) reported similar results with lower backfat thickness and improved carcass grading through improving meat colour, intramuscular fat, softness and succulence when fed *L. jensenii*. It is interesting to note that in the same study *L. plantarum* reduced carcass quality by reducing the bright colour of the meat and reducing intramuscular fat.

Various *Lactobacillus* spp. have shown to be effective against noxious gasses by improving protein metabolism and lowering blood urea nitrogen levels. This can have a significant impact on control of air pollution in agriculture, and treatment of manure (Liu *et al.*, 2013a; Liu *et al.*, 2015; Naqid *et al.*, 2015; Lan *et al.*, 2016)

2.9 *Bacillus* species as probiotics

Bacillus species as well as spores of *Bacillus* species as probiotics could have an added advantage since they are relatively resistant to environmental pressure (Nicholson *et al.*, 2000). Manufacturing of animal feeds requires a product that is resistant to mechanical processing and in some cases high temperatures and humidity (Amerah *et al.*, 2013). The optimum temperature for *B. subtilis* strain KKU213 was found to be between 30-37ºC, where these bacteria showed the highest antibacterial activity and bacteriocin production (Khochamit *et al.*, 2015). Above 42ºC bacteriocin production ceased, although these protein molecules remained stable at higher temperatures (Khochamit *et al.*, 2015). However, it is possible that bacteriocin activity can be lost above 87ºC (Bizani & Brandelli, 2002). *Bacillus* spores are able to survive extremely high and low temperatures, UV and gamma radiation, vacuums, oxidising agents and hash chemicals and detect when conditions become favourable for germinations (Nicholson *et al.*, 2000). These spores can survive for thousands of years before sporulation with the oldest recorded viable spores dating back to between 25 to 40 million years ago (Cano & Borucki, 1995) Barbosa *et al.*(2005) reported that some *Bacillus* spp obtained from the guts of broilers can germinate under anaerobic conditions of the gut for example, *B. licheniformis* and *B. cereus*. *Bacillus subtilis,* however, showed limited growth anaerobically, although some strains had better growth capability than others. Live *Bacillus* cells were not able to survive the harsh gastrointestinal conditions such as acidic pH and bile secretions in vitro, but their spores however, did survive (Barbosa *et al.*, 2005).

Jørgensen *et al.* (2016) found no significant effect of *Bacillus* probiotics (*B. subtilis* and *B. pumilus*) on growth and performance in the pre-starter phase of weaned pigs (28-42d), but did report increased growth and FCR during the starter and grower phase, with the grower exhibiting the greatest improvement. During the finisher period, there were improvements in growth, but to a lesser extent than the starter and grower phase and FCR was negatively impacted. Balasubramanian *et al.* (2016) also found significant improvements in ADG and FCR, but not feed intake in growing and finishing pigs. It seems that *Bacillus* probiotics have the ability to improve the digestibility of various nutrients including fat, phosphorus, CP, calcium (Jørgensen *et al.*, 2016), dry matter, nitrogen, fibre and energy (Meng *et al.*, 2010; Giang *et al.*, 2011; Balasubramanian *et al.*, 2016) depending on age, nutrient density of the diet and concentration of the probiotic administered (Jørgensen *et al.*, 2016; Kaewtapee *et al.*, 2017). In addition to positive growth effects, Kyriakis *et al.* (1999) also reported reduced incidences of PWD on various *Bacillus* probiotic species indicating health benefits of using *Bacillus* probiotics. Alexopoulos *et al.* (2004) also reported improved growth and FCR with lower incidences of diarrhoea on a *Bacillus licheniformis* and *B. subtilis* probiotic.

It seems that probiotics from *Bacillus* spp. have broad spectrum inhibitory activity dependent on the specific strain in question (Cladera-Olivera *et al.*, 2004). Reports by Xie *et al.*, 2009 found that bacteriocins from certain *Bacillus* spp. had a wider range of action, targeting organisms such as *Escherichia coli*, *Salmonella enteritidis*, *Salmonella typhimurium* and *Clostridium perfringens*. *Bacillus* bacteriocins further had the advantage of not inhibiting beneficial gut microbes such as *Lactobacillus plantarum* and *Bifidobacterium pullorum* and remained stable after exposure to various gastrointestinal substances and environments. In another study with two different strains of *Bacillus*, each strain had a different mode of action against pathogens, with one impacting cell-wall biosynthesis and the other inhibiting RNA synthesis (Nithya & Halami, 2012). It was found that antimicrobial compounds secreted by these *Bacillus* spp., are quite resistant to proteolytic enzymes and bile and can therefore survive harsh conditions in the gut (Bizani & Brandelli, 2002). They were also considerably resistant to organic solvents (Cladera-Olivera *et al.*, 2004) and had some cellulolytic activity (Manhar *et al.*, 2016). Similar results were reported by Ariffin *et al*. (2006) with a *Bacillus pumilus* strain that produced cellulase, an enzyme responsible for the breakdown of cellulose. This could be beneficial for monogastric feed production, since monogastric animals are unable to digest cellulose. Another study by Wang *et al.* (2017), found that a strain of *Bacillus pumilus* (ES-21) occurring in soil, had high esterase activity and was able to degrade mycotoxins secreted by Fusarium, called zearalenone. This mycotoxin is a health risk in both humans and animals and can cause reproductive toxicity and cytogenetic toxicity.

Bacillus could also be applied as biosurfactants due to their enzyme producing capabilities. *Bacillus subtilis* and *Bacillus licheniformis* have been shown to produce amylase and cellulase, rendering them effective biosurfactants (Rivardo *et al.*, 2009). Treatment of surfaces with these probiotics before inoculation with pathogens were effective in reducing *E. coli* by 97% and *S. aureus* by 90%. To date very limited research exists on the application of probiotics as cleaning agents in the animal husbandry industry, but has been used successfully in hospital settings (Vandini *et al.*, 2014a; Vandini *et al.*, 2014b). Microbial cleaning products consisting of *Bacillus subtilis, Bacillus pumilus* and *Bacillus megaterium* were used in hospital trials to evaluate the effectiveness and sustainability of these products opposed to chemical cleaning products (Vandini *et al.*, 2014a). *Bacillus* strains tended to have a significant impact on hard surfaces due to its sporulation capacity. Regular cleaning with microbial cleaners significantly reduced the pathogenic load in the environment and the longer it was used, the greater the effect, for example when microbial cleaners were used on a regular basis, the reduced pathogenic load was maintained over time and no bacterial resistance was apparent (Vandini *et al.*, 2014a). Similar results were found by Vandini *et al.* (2014b). Contradictory results were reported by Luyckx *et al.* (2016) when *Bacillus* spores were used in a competitive exclusion (CE) trial in a pig production unit as cleaning products to assess its efficacy in eliminating pathogens opposed to normal disinfectants. Six pig nurseries participated in the study for the duration of 3 production cycles. Probiotics were supplied by

Chrisal Africa (Pty) Ltd and products were used in accordance with company recommendations. Although spore enumeration increased after use of CE probiotics, pathogenic bacteria numbers were higher for the CE treatments than for controls. Disinfection proved to be more effective in controlling pathogens than CE products. There was also no significant difference in feed efficiency or faecal consistency between treatments (Luyckx *et al.*, 2016).

In a chicken trial it was reported that *Bacillus subtilis* reduced the amount of *Salmonella enteritidis* shed in the environment long after withdrawal of the probiotic (La Ragione & Woodward *et al.*, 2003). It was hypothesised that the probiotics might have stimulated macrophage infiltration, causing the immune system to adapt. La Ragione *et al.* (2001) found similar results with *B. subtilis* and *E. coli* infections. In a similar trial *B. subtilis* was fed to chickens on a regular basis resulting in significant improvements in the innate immunity against both *Elmeria* spp. and *Clostridium* spp. (Lee *et al.*, 2015). Rivardo *et al.* (2011) found synergistic effects when a combination of *Bacillus licheniformis* and various antibiotics were used *in vitro*. They hypothesised that the probiotic disrupts the cell integrity of biofilm, forming pores through which the antibiotic can then penetrate and kill all pathogens. Living cell numbers were reduced to below detection levels *in vitro*.

Another possible effect of probiotics could be on carcass parameters and quality (Alexopoulos *et al.*, 2004). Cui *et al*. (2013) postulated that gut populations could have an impact on fat deposition in pigs. They examined the effect of *B. subtilis* on *Bacteriode* and *Fermicute* populations as well as fat metabolism and found significant improvements in ADG, FI and FCR. Probiotics had no effect on dressing percentage of leaf lard weight, but increased the area of the longissimus muscle and back fat. Probiotics tended to increase serum fatty acid synthase and decrease serum triglycerides and glucose, with no effect on total cholesterol or hormone sensitive lipases. This indicates that *B. subtilis* has the ability to regulate the site of fat deposition. Kim *et al.* (2017) found that supplementation of broiler diets with *B. subtilis* reduced the decline in quality of frozen chicken meat by reducing lipid peroxidation and improving water holding capacity. Ice crystal damage in frozen meat causes oxidants to be released from cells, leading to lipid peroxidation and meat quality deterioration. Probiotic supplementation also reduced the amount of non-heme iron in the breast meat.

2.10 Conclusion

Early weaning can have a detrimental impact on the development of the gut through reduced feed intake together with dietary changes and withdrawal of sow's milk, bacterial infections and activation of cytokines. These changes in turn affect the gut through reduced nutrient availability at a cell level that leads to reduced cell development and increased cell apoptosis that negatively effects the gut barrier function. The establishment of a gastrointestinal microbiome is another important factor in maintaining gut health, with establishment of the microbiome occurring around 2 to 5 weeks of age in the piglet. Weaning during this time could have a detrimental effect on the microbiome as a shift in microbial populations could favour opportunistic pathogens and negatively impact beneficial microbes. As a result of the weakened gut barrier, these pathogens could easily cross the gut barrier and cause intestinal infections, and disease.

Feeding strategies and additives could potentially alleviate the negative impact of weaning stress by providing support to the gastrointestinal tract. Inclusion of insoluble fibre and reduction of total protein in diets contribute to a healthier gut environment by reducing feed sources to pathogenic microbes and supporting the growth of beneficial microbes. Other strategies include addition of in feed antibiotics, gut acidifiers, minerals, nucleotides, plant extracts, feed enzymes, prebiotics and probiotics.

From literature it is clear that probiotics have the ability to significantly influence growth, performance and health of pigs in production systems. In most cases probiotics will have some effect on weight gain, feed intake, FCR, nutrient digestibility, gut health and the immune system. No negative effects have been reported to date with a few studies showing no significant effects. Probiotics also have no negative effects on the development of bacterial resistance, making it a possible candidate for sustainably replacing antibiotics in animal feeds,

although to date the same efficacy has not been achieved. When considering probiotics as cleaning products, it is important to consider the bacterial load in the environment as well as specific microbes that it will need to target. Limiting factors need to be considered when dealing with these live microbial organisms.

Chapter 3: Materials and methods

3.1 Introduction

The aim of the study was to evaluate the efficacy from a combination of three *Bacillus* probiotic products from Chrisal Africa (Pty) Ltd on growth and performance of weaner pigs and on the gut microbiome. The efficacy of the probiotics was tested through measuring weight gain and feed intake of the pigs as well as analysing microbial populations in the faeces using 16s RNA sequencing. The trial consisted of two different types of housing systems, namely individual housing and group housing to evaluate the effect under different housing systems with different environmental conditions and stressors. Each of these units had two treatments per unit, namely control and probiotic treatment. Ethical approval was granted by the Animal Ethics Committee of the University of Pretoria (EC051-15).

3.2 Materials

3.2.1 Animal housing units

A total of 167 weaner pigs, 21-days of age, were purchased from a commercial farm. Pigs were housed at the pig unit on the experimental farm of the University of Pretoria. Forty pigs were allocated to individual pens and the remainder were housed in group pens. The entire housing unit were empty for more than a year before the commencement of the trial. Individually housed pigs were each placed in a 3.5 m^2 pen with partially slatted concrete floors and metal bars separating pens. Each pen was fitted with a water nipple, infrared heat lamp and a single feeder trough. All pigs in a treatment received water from a single water tank, making it possible to dose the water source of treated pigs. The unit was not environmentally controlled. Windows and extraction fans along the side of the unit were the only means of ventilation. Temperature loggers recorded temperature data in the unit throughout the duration of the trial. Individually housed pigs were weighed and equally distributed according to weight to a treatment and pen. In Figure 3.1 the pen arrangement and allocation of pigs in the individual pens according to treatment and control are illustrated. The weight of pigs when allocated to the pens were summarised in Appendix A Table A1, giving an indication of tag numbers, body weights and pen numbers according to control and treatment.

Control

C1	C11
C2	C12
C3	C13
C4	C14
C5	C15
C6	C16
C7	C17
C8	C18
C9	C19
C10	C20

Probiotic

P1	P11
P2	P12
P3	P13
P4	P14
P5	P15
P6	P16
P7	P17
P8	P18
P9	P19
P10	P20

Figure 3.1 Pen arrangements and blocking allocation of pigs in individual housing

*C1-C20 Indicates the treatment number of control pigs and P1-P20 indicates the treatment number of probiotic treatment pigs

Group housed pigs were weighed and allocated to a pen according to weight to avoid any dominance between pigs of different weights. Pig weights were distributed into four different weight classes, with each treatment receiving pigs from all four weight classes. Within weight classes pigs were randomly distributed between treatments

Pigs were kept at a constant stocking density of 0.95 m^2 per pig, therefore the quantity of pigs per pen varied with pen size, as illustrated in Figure 3.2. Two separate rooms were used for the group housed pigs with room one used for the probiotic group and room two used for the control. The basic features of the group housed pens were the same as the individual with windows and extraction fans along the side of the house and temperature loggers to keep record of house temperatures. Both rooms also had partially slatted floors with metal bars separating pens. Each pen had two nipple drinkers, three feed troughs, four infrared heat lamps and all four pens in a treatment received water from a single water tank, making it possible to dose the water with probiotics similar to the individual pens. Major differences between individual housing and group housing were the two separate rooms in the group housing, size of the room, pen sizes and pen arrangements.

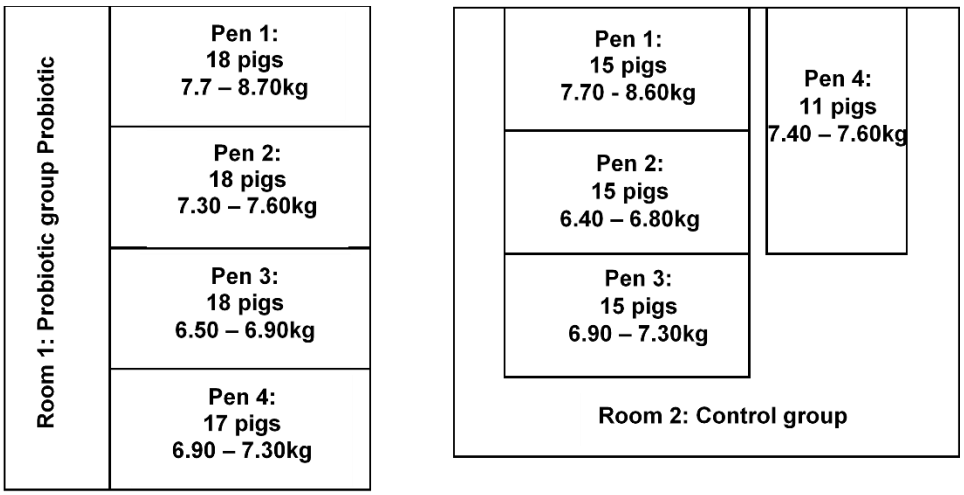

Figure 3.2 Pen arrangements of group housed pigs with the number of pigs per pen and weight class of each pen

3.2.2 Probiotic products used

The following products were manufactured by the company Chrisal Africa (Pty) Ltd and was used as the probiotic treatment for the treatment groups of both individually housed and group housed pigs, namely

a) PIP Animal Housing cleaner for the physical cleaning of the house. This product contained the following ingredients: Sodiumlaurylethersulphate, 2-(2-butoxyethoxy) ethanol, Isopropanol, Lauryldiethanolamide, Subtilisin and Amylase.

b) PIP Environmental Enhancer with *Bacillus* spores - Information of contents remains the intellectual property of Chrisal Africa (Pty) Ltd.

c) PIP Water Plus with *Bacillus* spores - Information of contents remains the intellectual property of Chrisal Africa (Pty) Ltd.

3.3 Methods

3.3.1 Probiotic application

Three different probiotic products were used in a two stage process. Stage one was the initial cleaning of the house before the arrival of the pigs and stage two was the continual probiotic application after arrival of the pigs. The application of the three products in stages are summarised in Table 3.1.

Table 3.1 Two-stage application of probiotic products

	Action	Product
Stage 1: Initial Cleaning	Washing of house	PIP AHC
	Flush waterlines	PIP AHC
Stage 2: Continuous probiotic application	Dosing of drinking water	PIP Plus Water
	Environmental spraying of probiotic	PIP EE

Treatments for both individually housed and group housed probiotic were the same. Before arrival of the pigs, the animal housing units of treated groups were thoroughly washed with a high-pressure washer at 3% PIP AHC concentration, with 1L covering 100 m^2. After application of the solution it was left for 20 minutes before rinsing the area with clean water. Waterlines of probiotic treatment groups were flushed with the same PIP AHC product at a 5% solution. The solution was kept in the waterlines for 3 days, where after it was rinsed again with clean water.

Shortly before arrival the water of probiotic treatment groups were dosed with a 0.02% solution of PIP Plus Water. Thereafter water was dosed with this concentration once a day. Pigs had to finish all the dosed water, where after they were given clean water for the rest of the day.

Shortly before arrival of animals, pens of probiotic treatment groups were sprayed with a mist sprayer containing PIP EE, with 1 L covering 200 m^2. Thereafter pens of treated pigs were sprayed once a day for the first 7 days and 3 times a week thereafter.

3.3.2 Performance recording

All pigs were identified with an ear tag at the start of the trial. Pigs were weighed at arrival and thereafter once a week to determine weight gain. In the individual housing each individual pig was viewed as an experimental unit whereas in the group housing the pen was considered as the experimental unit. A commercial diet, without any in-feed antibiotics was used for all pigs in the trial. Pigs received feed *ad libitum* and all feed was weighed before allocated to the pens. Once a week the remaining feed was weighed back to determine feed intake (FI) for individual and group housed pigs per week. Measurements for individual pigs were taken at the following days of age: days 21, 28, 35, 42, 60 and 63 and measurements for group housed pigs were taken at days 21, 28, 35, 42, 49 and 57. Due to a disease outbreak in the group pigs' unit on day 55, which was adjacent to the individual pigs, it was decided not to weigh individual pigs on day 56, as a precautionary measure to prevent unnecessary disease spread. Individual pigs were only weighed on day 60 and on day 63 they also started to fall ill, whereupon it was decided to terminate the trial.

Data were analyzed statistically as a randomized block design with the GLM model (Statistical Analysis System, 2017) for the average effects. Means and standard error were calculated and significance of difference ($P<0.05$) between means was determined by Fischer's test (Samuels, 1989).

The linear model used is described by the following equation:

$$Yij = \mu + Ti + Ek + TSik+ + eijk$$

where Yijk = variable studied during the period
μ = overall mean of the population
Ti = effect of the ith treatment
Ek = effect of the kth environment, pen location within the house
TSik = effect of the ikth interaction between treatment and environment
eijk = error associated with each Y

3.3.3 Collection of faecal samples

Faecal samples were only taken for individually housed pigs. Faecal samples were collected once a week for three weeks, starting on the second week after arrival, including sampling at 35, 49 and 63 days of age. Samples were taken directly from the anus by means of rectal stimulation. Each pen was sampled in duplicate so that a total of 80 samples were obtained per sample day and 240 for the three sample days. Samples were kept on ice directly after sample taking and frozen at -40 ºC further processing, whereupon 15 samples from each treatment per sample day were selected based on visual quantity and quality of the sample for deoxyribonucleic acid (DNA) extractions. In total 90 samples from the three sample days were used for DNA extractions.

3.3.4 DNA extraction

A Phenol-chloroform extraction method was used with the DNA extraction protocol adapted from Pinto *et al.* (2012) with modifications from Urakawa *et al*. (2010). In contrast to the methods previously described, no filter membranes were used.

A total of 3.0 mg of faecal matter was placed in a 2 mL Lysing Matrix Tube E with sterilised tweezers. 300 µL of 2 x Tens buffer (100 mM Tris-HCl [pH 8.0], 40 mM EDTA, 200 mM NaCl, 2 % SDS) was added to the tubes and vortexed vigorously. 900 µL of phenol:Chloroform:isoamyl alcohol (25:24:1, pH 8) was then added to the tube and vortexed vigorously. Tubes were placed in a Tissue-Lyser II (Qiagen, South Africa) and bead beat for 40 seconds at setting 6 and then centrifuged at 14 000 x g for 10 min to pellet the beads. The aqueous phase was consequently transferred into a 2 mL Matrix tube E. A second round of 200 µL 2 x Tens buffer was added to the 2 mL Matrix tube E and bead beat at the same settings as before. Another centrifugation step followed at 12 500 x g for 10 min. The aqueous phase was transferred again and the same steps repeated.

After transferring the aqueous phase for the last time, 700 µL of phenol: chloroform: isoamyl alcohol (25:24:1) was added to the tube containing the aqueous phase and mixed by means of repeated inversions. The solution was centrifuged at 14 000 x g for 5 min after which the aqueous phase was transferred into a new 2 mL Matrix tube E. 350 µL 7.5 M NH_4Ac was added to the aqueous phase and mixed with repeated inversions followed by the addition of 600 µL of chloroform, that was also mixed by repeated inversions and centrifuged at 14 000 x g for 5 min.

The aqueous phase was transferred into a new 1.5 mL Matrix tube E and nucleic acid precipitated from the final aqueous phase by adding 600 µL of isopropanol and 5 µL of 15 mg/ mL cold GlycoblueTM (Life Technologies, South Africa) that was mixed gently and kept at -80 ºC for 10 min. Samples were then centrifuged at 12 000 x g for 30 min.

The supernatum was discarded and the pellet washed with 1 mL of 80 % cold ethanol followed by centrifugation at 12 000 x g for 30 min. The supernatum was discarded again and the pellet left to air dry for 5-10 min. The pellet was re-suspended in 50 µL of DNAse free water (Qiagen, South Africa).

DNA concentration was measured using a Nanodrop NA-1000TM Spectrophotometer (Thermo Scientific™, South Africa). Electrophoresis was performed using 5 µL of gDNA together with 1 µL of loading dye in a 1 % agarose gel stained with ethidium bromide in order to visualise the DNA concentrations. Samples were run at 90 V and 400 A for 40 min.

3.3.5 16S rRNA amplification

A polymerase chain reaction (PCR) was performed to test whether 16S rRNA amplification would be successful. Universal primers flanking the 16S rRNA sequence were used as adapted by Edward *et al.* (1989). Primer sequences were as follows: 27 F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492 R (5'-GGT TAC CTT GTT ACG ACT-3'). A BIO-RAD T100™ Thermal Cycler was used for PCR reactions. A 25 µL master mix consisting of 2.5 µL reaction buffer, 2 µL dNTP, 2.0 µL $MgCl_2$, 0.5 µL forward primer as well as 0.5 µL

reverse primer, 16.85 µL nuclease free water, 0.15 µL Taq DNA polymerase (Qiagen, South Africa) and 0.5 µL DNA.

Initial denaturation occurred at 94 ºC for 10 min, followed by 30 cycles of denaturation at 94 ºC for 1 min, annealing at 58 ºC for 1 min, extension at 75 ºC for 1 min and final extension at 75 ºC for 5 min. After the 30 min cycle samples were kept at 4 ºC. Agarose gel electrophoresis was performed on the 16S rRNA samples to determine whether the 16S rRNA amplification was successful. Cycles were ran for 30min at 80V and 400mA on 1% agarose gel.

After confirmation that 16S RNA amplification was successful, the original genomic DNA was sent to the University of Michigan Medical School (USA) for analysis of the VP 4 region of the 16S rRNA gene to determine the microbial diversity using the method described by Kozich *et al.* (2013).

3.3.6 Sequencing and statistical analysis

Raw 16S RNA sequencing data were processed using MOTHUR v.1.35.1. software (Schloss *et al.*, 2009). Analysis included relative abundance, Braycurtis and Jaccard indexes, Shannon index, unweighted phylip and taxonomy.

The first step in analysis of the data was linking forward and reverse sequences through generating contigs by means of the Illumina Miseq platform. Sequences were filtered to remove undefined bases as well as those longer than 275 base pairs. Identical sequences were then clustered (unique.seqs) and the remaining sequences aligned (align.seqs) to the SILVA database to eliminate unfit sequences. A second filtering step was implemented (filter.seq) gaps and overhangs followed by realigning (unique.seqs) (Schloss, 2009, Schloss, 2010, Schloss *et al.*, 2011, Schloss, 2013)

Chimera sequences were then screened with Uchime (chimera.uchime) (Edgar *et al.*, 2011) and removed (remove.seq). Sequences were classified by means of the Greengene database (classify.seqs) (http://greengenes.lbl.gov/Download/) and undesirable sequences belonging to chloroplasts, mitochondria or Eukaryota, discarded (remove.lineage). Clustering of sequences into operational taxonomic units (OTUs) followed (dist.seqs) with a maximum distance of 0.20. OTUs with a similarity of 97% or greater were clustered (make.shared) and classified (classify.otu).

Alpha diversity was obtained through the summary.single command and the following calculations: Good's coverage (coverage); number of sequences (nseqs); observed species (Sobs); Shannon diversity (Shannon) and Plielou's evenness (Shannoneven). A subsample set of 2349 sequences and 1000 iterations were used.

To acquire beta-diversity the dist.shared command was used and the following calculations for dissimilarity pairwise distance metrics were made with the Mothur software: Bray Curtis (bray, community structure-based) and Jaccard (jest, community membership-based).

Operational taxonomic units with relative abundance of less than 1% of all OTUs observed were discarded from the statistical analysis. Dominant OTUs occurring at levels more than 1% were statistically analysed using IBM® SPSS® statistics software version 24. To determine the effect of treatment on the microbial population a sample independent Nonparametric Mann-Whitney U test was performed with a 95% significance level (Mann & Whitney, 1947). To determine the effect of time on the microbial population a nonparametric related-samples Friedman's Two-way analysis of variance by rank was performed with a 95% significance level (Friedman, 1936).

Chapter 4: Results

4.1 Production parameters

4.1.1 Weight gain

For the individual pigs weight gain was only significantly different between treatments at 35-42 days of age, when the ADG of the treatment group was 10 g less (P<0.05) compared to the control. Between 21-28 days and 28-35 days there was a tendency for the probiotic group to be slightly heavier than the control (P>0.05). From 42 days of age there was no significant difference between treatments. Results of weight gain is summarised in Table 4.1 and Table A2 in Appendix A summarises the weights of the individually housed pigs per weighing day.

Table 4.1 Comparison of weight gain per treatment for individually housed pigs

Days of age	Average weekly gain control (kg)	Standard Error	Average weekly gain probiotic treatment (kg)	Standard Error	P<0,05
21-28	1.49	± 0.11	1.67	± 0.11	0.26
28-35	3.09	± 0.22	3.55	± 0.21	0.14
35-42	4.98	± 0.23	4.25	± 0.22	**0.03**
42-49	4.55	± 0.25	4.55	± 0.23	1.00
49-60	9.64	± 0.44	9.03	± 0.42	0.33
60-63	2.95	±0.30	3.23	± 0.28	0.51

For the group housed pigs significant differences were seen between 21-28 days of age with pigs in the control having 3 g higher ADG than pigs in the probiotic treatment (P< 0.05). From 49-57 days of age the control had 180 g higher ADG than probiotic pigs (P< 0.05). Between 28-49 days of age there was a slight tendency for control pigs to be heavier than probiotic pigs. The comparison of weight gain for group housed pigs per treatment is indicated in Table 4.2. Table A3 in Appendix A summarises the average pig weight per pen for the control and treatment per weighing day.

Table 4.2 Comparison of weight gain per treatment for group housed pigs

Days of age	Average weekly gain control (kg)	Standard Error	Weekly Gain Probiotic Treatment (kg)	Standard Error	P<0,05
21-28	1.60	± 0.05	1.37	± 0.05	**0.02**
28-35	2.58	± 0.14	2.85	± 0.14	0.23
35-42	4.16	± 0.18	3.69	± 0.18	0.11
42-49	4.51	± 0.28	4.39	± 0.28	0.78
49-57	4.53	± 0.34	3.11	± 0.34	**0.03**

4.1.2 Feed intake

There was a slight tendency for individually housed pigs in the probiotic group to have greater feed intakes than the control group, with the only significant difference at 49-60 days of age (P<0.05) when probiotic treated pigs ate 0.14 g per day more as indicated in Table 4.3. Feed intake per week for each pen is summarised in Table A4 of Appendix A.

Table 4.3 Comparison of average feed intake per pig between treatments for individually housed pigs

Days	Average weekly FI control (kg)	Standard Error	Average weekly FI probiotic treatment (kg)	Standard Error	P<0,05
FI 21-28	1.70	± 0.09	1.81	± 0.08	0.36
FI 28-35	4.89	± 0.22	5.30	± 0.21	0.19
FI 35-42	5.16	± 0.28	5.21	± 0.27	0.92
FI 42-49	10.46	± 0. 76	10.77	± 0.74	0.77
FI 49-60	16.97	± 0.54	18.50	± 0.53	**0.05**
FI 60-63	4.81	± 0.31	5.18	± 0.31	0.40

There was no significant difference between FI of treatments for the group housed pigs at any day as shown in Table 4.4. Average FI per pig per pen for the group housed pigs over the entire trial period is summarised in Table A5 in Appendix A.

Table 4.4 Comparison of average feed intake per pig between treatments for group housed pigs

Days	Average weekly FI/Pig control	Standard Error	Average weekly FI/Pig probiotic treatment	Standard Error	P<0,05
21-28	1.78	± 0.06	1.80	± 0.06	0.86
28-35	3.06	± 0.12	3.13	± 0.12	0.70
35-42	4.44	± 0.18	4.38	± 0.18	0.82
42-49	6.32	± 0.46	5.15	± 0.46	0.12
49-57	5.40	± 0.33	5.28	± 0.33	0.80

4.1.3 Feed conversion

Feed efficiency was significantly different for individually housed pigs between treatments at 35-42 days and 49-60 days (P<0.05). Pigs in the control at 35-42 days of age had a FCR of 0.19 lower than the treatment group. At 49-60 days pigs in the control had a FCR of 0.3 lower than the treatment group as shown in Table 4.5.

Table 4.5 Average weekly FCR (kg/kg) of individually housed pigs

Days	Average weekly FCR control	Standard Error	Average weekly FCR probiotic treatment	Standard Error	P<0,05
FCR 21-28	1.17	± 0.05	1.12	± 0.05	0.51
FCR 28-35	1.65	± 0.14	1.63	± 0.13	0.92
FCR 35-42	1.05	± 0.05	1.24	± 0.05	**0.02**
FCR 42-49	2.35	± 0.12	2.39	± 0.12	0.82
FCR 49-60	1.81	± 0.09	2.11	± 0.09	**0.02**
FCR 60-63	1.82	± 0.34	2.09	± 0.33	0.58

From 21-28 days of age there was a significant difference for the group housed pigs between treatments, with the control having a 0.19 lower FCR (P<0.05). Between 35-42 days the FCR for the control was once again 0.11 lower (P<0.05). Between 28-35 days and again between 42-49 days the control tended to have higher FCRs (P>0.05) as can be seen from Table 4.6.

Table 4.6 Average weekly FCR (kg/kg) of group housed pigs

Days	Average weekly FCR control	Standard Error	Average weekly FCR probiotic treatment	Standard Error	P<0,05
FCR 21-28	1.12	± 0.04	1.31	± 0.04	**0.02**
FCR 28-35	1.19	± 0.04	1.10	± 0.04	0.19
FCR 35-42	1.07	± 0.01	1.18	± 0.01	**<0.015**
FCR 42-49	1.40	± 0.09	1.18	± 0.09	0.12
FCR 49-57	1.19	± 0.24	1.82	± 0.24	0.12

4.2 Gut microbiome

4.2.1. Overall results

After DNA extraction of 40 samples per sample day (20 per treatment), a total of 10 samples (5 per treatment) were rejected based on the lowest DNA concentration and electrophoresis data. In total 90 samples were sequenced of which there were 1229551 sequence reads that varied between 242 and 258 base pairs with an average length of 253 base pairs. Undesirable reads amounted to 0.01%, therefore resulting in 1229412 final sequence reads that were used in the data set. In total for all three sample days 3275 Operational taxonomic units (OTUs) were detected, with only *Archaea* and *Bacteria* detected at a Kingdom level as demonstrated in Table 4.7.

Table 4.7 Percentage (%) microbial composition at a kingdom level

Kingdom	Day 35 %	Day 49%	Day 63%
Archaea	0.04	0.19	0.19
Bacteria	99.96	99.81	99.81

In Table 4.8 the bacterial phyla percentages of all 90 samples analysed were summarised over the three sample periods. *Firmicutes* was the most abundant phylum followed by *Bacteroidetes*, *Proteobacteria* and *Tenericutes.* The total microbial OTUs detected as a percentage is summarised in Table B1 of Appendix B.

Table 4.8 Composition of bacterial phyla in percentage (%)

Phyla	% Bacteria at day 35	% Bacteria at day 49	% Bacteria at day 63
Firmicutes	60.66	55.00	68.43
Bacteroidetes	34.03	36.68	24.75
Proteobacteria	0.94	3.71	2.00
Tenericutes	0.10	0.47	1.23

Table 4.9 shows the major phyla divided into the different families that will be presented throughout the next section.

Table 4.9 Dominant bacterial phyla and the corresponding bacterial families

Phylum	Family
Firmicutes	*Clostridiaceae*
	Erysipelotrichaceae
	Lachnospiraceae
	Lactobacillaceae
	Ruminococcaceae
	Streptococcaceae
	Veillonellaceae
Bacteroidetes	*Paraprevotellaceae*
	Prevotellaceae
	S24-7
Proteobacteria	*Succinivibrionaceae*

4.2.2. Diversity indices

Calculations for Good's coverage exceeded 0.95 for all bacterial libraries over all 3 sample days and there were no significant differences between treatments (P>0.05). The Shannon diversity index was similar between samples with an average Shannon evenness of 0.72 (P>0.05).

4.2.3. Treatment specific results

The composition of microbes as a percentage of total detected OTUs per treatment over the three sampling periods is summarised in Table B1 of Appendix B.

4.2.4. Control

The main microbial populations that occurred at a level greater than 1% of all microbes at any sampling day between 35-63 days, varied with age within the control as indicated in Table 4.9 for the entire trial from 35-63 days of age. Microbial populations that varied significantly (P<0.05) between the three different sampling days included *Clostridiaceae*, *Erysipelotrichaceae*, *Lachnospiraceae*, *Ruminococcaceae*, *Streptococcaceae*, *Succinivibrionaceae* and *Veillonellaceae* as seen in Table 4.10.

Table 4.10 Significant differences from 35-63 days of microbial populations in the control

Dominant Microbes of Control	Friedman's Test	Significance
Paraprevotellaceae	1.20	0.55
Clostridiaceae	12.64	**<0.001[a]**
Erysipelotrichaceae	17.23	**<0.001[a]**
Lachnospiraceae	10.13	**0.01[a]**
Lactobacillaceae	2.53	0.28
Prevotellaceae	1.73	0.42
Ruminococcaceae	15.60	**<0.001[a]**
S24-7	0.93	0.63
Streptococcaceae	17.73	**<0.001[a]**
Succinivibrionaceae	16.71	**<0.001[a]**
Veillonellaceae	7.60	**0.02[a]**

Significant difference (P<0.05) within treatments between sampling days indicated with [a], where significant difference means there were significant differences between sampling days.

Microbial populations that varied significantly (P<0.05) between the three different sampling days included *Clostridiaceae* between 35-49 days, *Erysipelotrichaceae* between 35-49 days, *Lachnospiraceae* between 35-49 days as well as 35-63 days, *Ruminococcaceae* between 35-49 days as well as 49-63 days, *Streptococcaceae* between 49-63 days, *Succinivibrionaceae* between 35-49 days as well as 35-63 days and *Veillonellaceae* between 35-63 days as seen in Table 4.11.

Table 4.11 Pairwise comparison for the control of the dominant microbes from 35-63 days

	Day	Test Statistic	± Standard Error	Standard. Test Statistic	P<0.05	Adjusted. P<0.05
	35 - 49	**1.27**	**0.37**	**3.47**	**0.001**	**0.002[a]**
Clostridiaceae	35 - 63	0.43	0.37	1.19	0.235	0.706
	49 - 63	-0.83	0.37	-2.28	0.022	0.067
	35 - 49	**1.53**	**0.37**	**4.20**	**<0.001**	**<0.001[a]**
Erysipelotrichaceae	35 - 63	0.67	0.37	1.83	0.068	0.204
	49 - 63	-0.87	0.37	-2.37	0.018	0.053
	35 - 49	**1.07**	**0.37**	**2.92**	**0.003**	**0.010[a]**
Lachnospiraceae	**35 - 63**	**0.93**	**0.37**	**2.56**	**0.011**	**0.032[a]**
	49 - 63	-0.13	0.37	-0.37	0.715	1.000
	35 - 49	**1.00**	**0.37**	**2.74**	**0.006**	**0.019[a]**
Ruminococcaceae	35 - 63	-0.40	0.37	-1.10	0.273	0.820
	49 - 63	**-1.40**	**0.37**	**-3.83**	**<0.001**	**<0.001[a]**
	35 - 49	0.87	0.37	2.37	0.018	0.053
Streptococcaceae	35 - 63	-0.67	0.37	-1.83	0.068	0.204
	49 - 63	**-1.53**	**0.37**	**-4.20**	**<0.001**	**<0.001[a]**
	35 - 49	**-1.40**	**0.37**	**-3.83**	**<0.001**	**<0.001[a]**
Succinivibrionaceae	**35 - 63**	**-1.00**	**0.37**	**-2.74**	**0.006**	**0.019[a]**
	49 - 63	0.40	0.37	1.10	0.273	0.820
	35 - 49	-0.40	0.37	-1.10	0.273	0.820
Veillonellaceae	**35 - 63**	**-1.00**	**0.37**	**-2.74**	**0.006**	**0.019[a]**
	49 - 63	-0.60	0.37	-1.64	0.100	0.301
	35 - 49	0.20	0.37	0.55	0.584	1.000
unclassified	35 - 63	-0.80	0.37	-2.19	0.028	0.085
	49 - 63	**-1.00**	**0.37**	**-2.74**	**0.006**	**0.019[a]**

Significance values have been adjusted by the Bonferroni correction for multiple tests.
Significant differences (P<0.05) indicated by [a]

Table 4.12 indicates the level of microbes as a percentage of all detected bacterial OTUs, and shows which microbes were the most dominant, such as *Prevotellaceae*, *Lachnospiraceae* and *Veillonellaceae*.

Table 4.12 Change in microbial populations of the control from 35-63 days in percentage (%)

	35 Days (% of total)	49 Days (% of total)	63 Days (% of total)
Clostridiaceae	4.32	0.92	1.76
Erysipelotrichaceae	2.92	0.59	1.01
Lachnospiraceae	**20.78**	**9.54**	**10.73**
Lactobacillaceae	11.18	6.95	9.36
Paraprevotellaceae	2.80	5.26	4.55
Prevotellaceae	**28.68**	**35.02**	**18.79**
Ruminococcaceae	12.33	7.78	**12.70**
S24-7	1.28	1.83	1.65
Streptococcaceae	0.98	0.16	4.91
Succinivibrionaceae	0.27	4.68	1.28
unclassified	3.04	3.55	4.66
Veillonellaceae	**5.94**	**20.06**	**22.66**

Figure 4.1 gives a visual representation of the change in microbial composition over time. An increase in some microbes such as *Veillonellaceae* can be observed, with a decrease in others such as *Clostridiaceae*. Other microbes such as *Lactobacillaceae* decreases at 49 days, but then increases again towards 63 days, indicating a variation in microbial composition with age depending on the specific bacterial family.

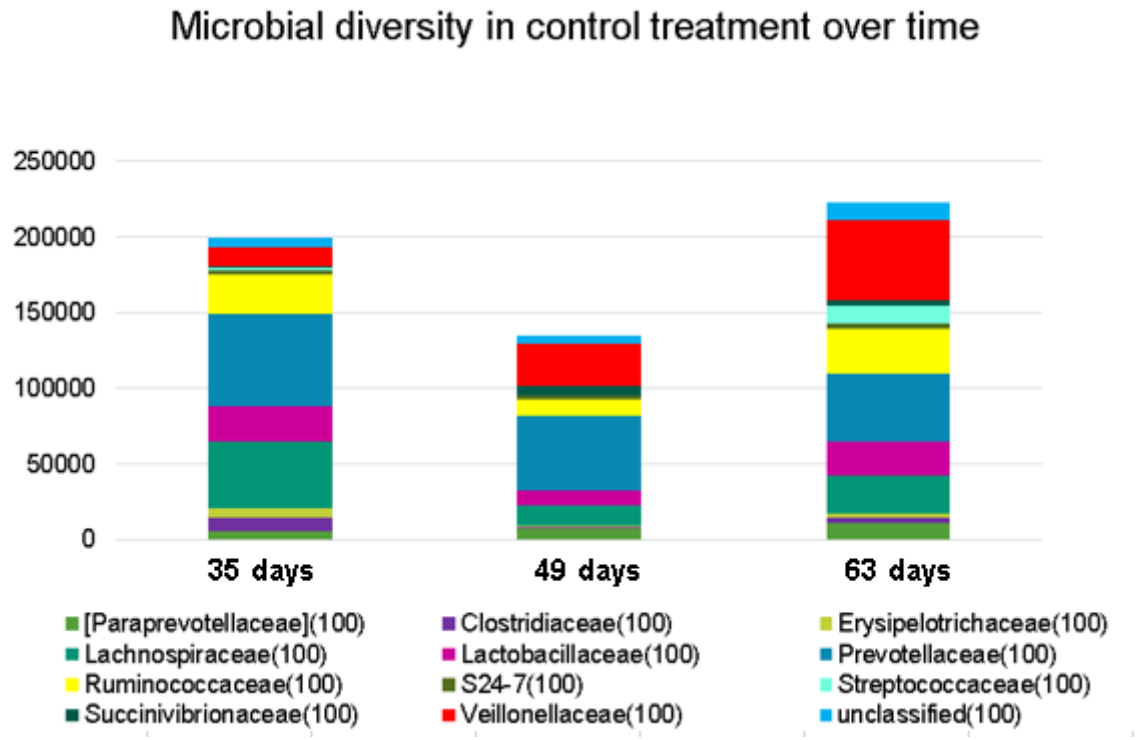

Figure 4.1 Microbial population diversity between 35-63 days for the control

Figure 4.2 illustrates the change in microbial quantities between 35-63 days for the individual microbes that changed significantly between the different sampling days. Each microbial family is unique regarding the size of the population and the way in which it behaves over time. It also shows the impact that the probiotic treatment had at different ages when compared to the control.

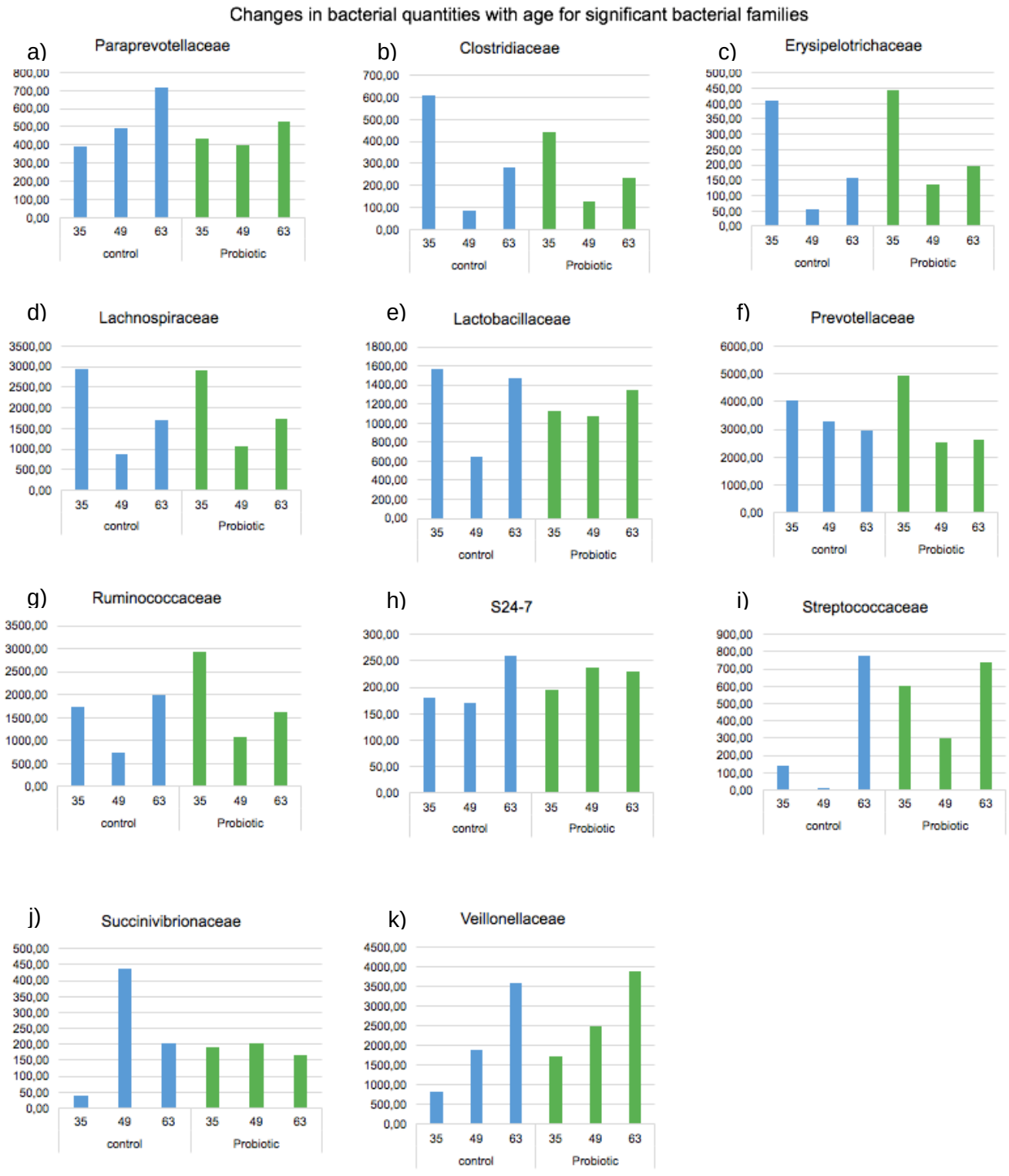

Figure 4.2 Microbial change with age for bacterial families that had significant variation between different ages for both the control and probiotic treatment

4.2.5. Probiotic treatment

Significant differences were seen in the populations of *Erysipelotrichaceae*, *Lachnospiraceae*, *Prevotellaceae*, *Ruminococcaceae*, *Streptococcaceae* and *Veillonellaceae* for the probiotic treatment from 35-63 days of age as indicated in Table 4.13.

Table 4.13 Significant differences from 35-63 days of microbial populations in the probiotic treatment

Microbial Populations of Probiotic Treatment	Friedman's Test	Significance
Paraprevotellaceae	1.73	0.420
Clostridiaceae	1.05	0.590
Erysipelotrichaceae	**16.53**	**<0.001[a]**
Lachnospiraceae	**13.33**	**0.001[a]**
Lactobacillaceae	1.20	0.550
Prevotellaceae	**6.93**	**0.031[a]**
Ruminococcaceae	**17.73**	**<0.001[a]**
S24-7	0.93	0.630
Streptococcaceae	**11.90**	**0.003[a]**
Succinivibrionaceae	5.41	0.070
Veillonellaceae	**8.93**	**0.011[a]**

Significant difference (P<0.05) within treatments from 35-63 days indicated with [a], where significant difference means there were significant differences between the sampling days

Populations of *Erysipelotrichaceae* differed significantly (P<0.05) between 35-49 days and between 35-63 days as well as *Lachnospiraceae* between 35-49 days, *Prevotellaceae* between 35-49 days, *Ruminococcaceae* between 35-49 days, *Streptococcaceae* between 35-49 days and between 49-63 days and *Veillonellaceae* between 35-63 days as indicated in Table 4.14.

Table 4.14 Pairwise comparison for the probiotic treatment between 35-63 days of age

	Day	Test Statistic	± SE	Std. Test Statistic	P<0.05	Adj. P<0.05
	35 - 49	**1.47**	**0.37**	**4.02**	**<0.001**	**<0.001[a]**
Erysipelotrichaceae	**35 - 63**	**0.93**	**0.37**	**2.56**	**0.011**	**0.032[a]**
	49 - 63	-0.53	0.37	-1.46	0.144	0.432
	35 - 49	**1.33**	**0.37**	**3.65**	**<0.001**	**0.001[a]**
Lachnospiraceae	35 - 63	0.67	0.37	1.83	0.068	0.204
	49 - 63	-0.67	0.37	-1.83	0.068	0.204
	35 - 49	**0.93**	**0.37**	**2.56**	**0.011**	**0.032[a]**
Prevotellaceae	35 - 63	0.67	0.37	1.83	0.068	0.204
	49 - 63	-0.27	0.37	-0.73	0.465	1.000
	35 - 49	**1.53**	**0.37**	**4.20**	**<0.001**	**<0.001[a]**
Ruminococcaceae	35 - 63	0.67	0.37	1.83	0.068	0.204
	49 - 63	-0.87	0.37	-2.37	0.018	0.053
	35 - 49	**0.90**	**0.37**	**2.47**	**0.014**	**0.041[a]**
Streptococcaceae	35 - 63	-0.30	0.37	-0.82	0.411	1.000
	49 - 63	**-1.20**	**0.37**	**-3.29**	**0.001**	**0.003[a]**
	35 - 49	-0.73	0.37	-2.01	0.045	0.134
Veillonellaceae	**35 - 63**	**-1.07**	**0.37**	**-2.92**	**0.003**	**0.01[a]**
	49 - 63	-0.33	0.37	-0.91	0.361	1.000
	35 - 49	0.00	0.37	0.00	1.000	1.000
unclassified	35 - 63	-0.80	0.37	-2.19	0.028	0.085
	49 - 63	-0.80	0.37	-2.19	0.028	0.085

Significance values have been adjusted by the Bonferroni correction for multiple tests. Significant differences (P<0.05) indicated by [a]

Table 4.15 shows the level of microbes as a percentage of all detected bacterial OTUs, and shows which microbes were the most dominant between 35-63 days of age, such as *Prevotellaceae*, *Lachnospiraceae, Ruminococcaceae* and *Veillonellaceae*.

Table 4.15 Microbial population change of the probiotic treatment between 35-63 days of age in percentage (%)

	35 Days (% of total)	49 Days (% of total)	63 Days (% of total)
Clostridiaceae	2.55	1.17	1.59
Erysipelotrichaceae	2.57	1.29	1.34
Lachnospiraceae	**16.75**	**10.00**	**11.72**
Lactobacillaceae	6.52	10.05	9.11
Paraprevotellaceae	2.53	3.73	3.56
Prevotellaceae	**28.60**	**23.59**	**17.91**
Ruminococcaceae	**16.91**	**10.04**	**11.02**
S24-7	1.13	2.21	1.55
Streptococcaceae	3.47	2.83	4.98
Succinivibrionaceae	1.11	1.89	1.12
unclassified	2.91	4.69	4.10
Veillonellaceae	**9.87**	**23.42**	**26.19**

Figure 4.3 shows that there was an increase in some microbial families such as *Veillonellaceae* between 35-63 days and a decrease in others populations such as *Lachnospiraceae*, *Prevotellaceae* and *Ruminococcaceae* for the probiotic treatment. The concentration of microbes differs between ages, with a decrease in at 49 days of age and a slight increase at 63 days of age.

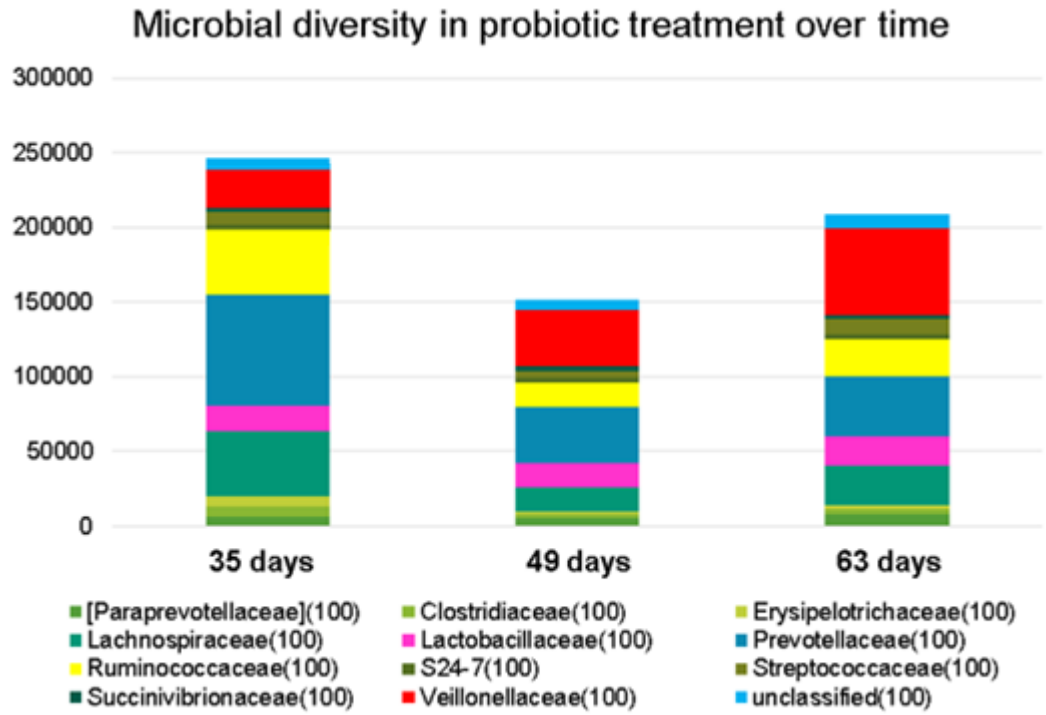

Figure 4.3 Microbial population diversity between 35-63 days for the probiotic treatment

4.2.6. Microbial population variations between treatments

It is clear from Figure 4.4 that *Firmicutes* were the most abundant bacteria at a phylum level with *Bacteroidetes* as the second most abundant phylum for both control and treatment at 35 days of age. During this time *Proteobacteria* was slightly more abundant in the probiotic treatment at a phylum level.

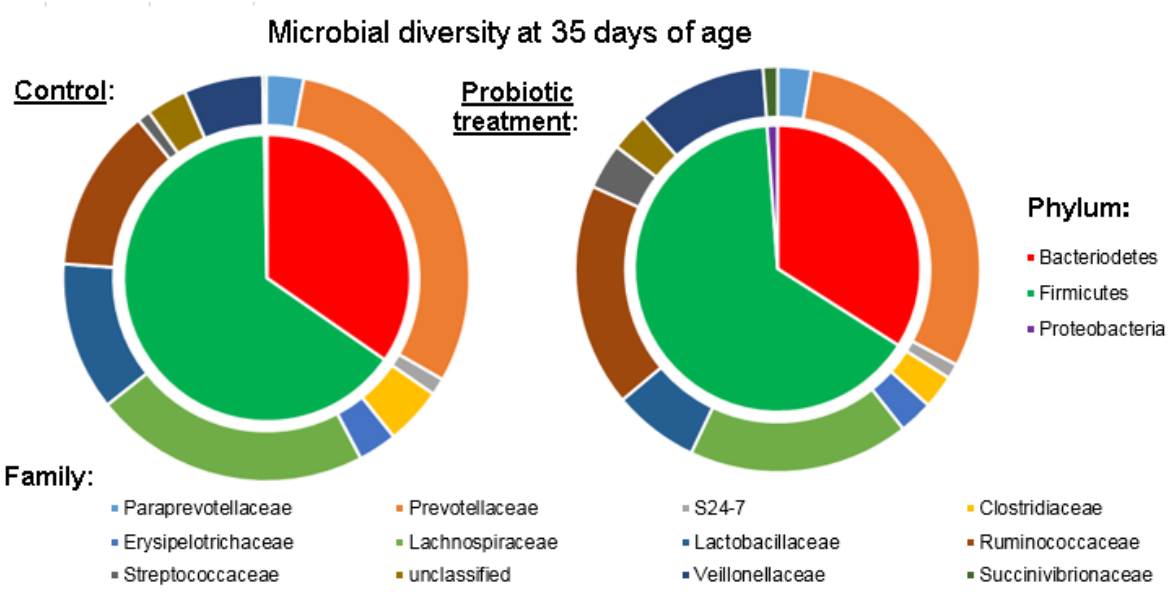

Figure 4.4 Microbial diversity of the control (left) and the probiotic treatment (right) at 35 days of age Inner circle indicates microbes at a phylum level and outer circle indicates microbes at a family level. Phyla and Families occurring less than 1% of all microbes were disregarded

At a family level differences were observed in the probiotic treatment with significant differences observed in the faecal *Ruminococcaceae* and *Streptococcaceae* population between the treatment and control, with 4.58% more *Ruminococcaceae* ($P<0.05$) and 2.49% more *Streptococcaceae* ($P<0.05$) in the probiotic treatment at 35 days of age as summarised in Table 4.16.

Table 4.16 Percentage (%) relative abundance of microbes between treatments at 35 days of age

	% Contribution Control	% Contribution Probiotic	Mann-Whitney U	Significance
Paraprevotellaceae	2.80	2.53	109.00	0.900
Clostridiaceae	4.32	2.55	107.50	0.840
Erysipelotrichaceae	2.92	2.57	118.00	0.840
Lachnospiraceae	20.78	16.75	110.00	0.940
Lactobacillaceae	11.18	6.52	82.00	0.220
Prevotellaceae	28.68	28.60	140.00	0.270
Ruminococcaceae	**12.33**	**16.91**	**170.00**	**0.020[a]**
S24-7	1.28	1.13	126.50	0.570
Streptococcaceae	**0.98**	**3.47**	**171.50**	**0.010[a]**
Succinivibrionaceae	0.27	1.11	131.50	0.440
Veillonellaceae	5.94	9.87	159.00	0.060

Significant difference ($P<0.05$) between treatments indicated with [a]

At 49 days of age the most abundant phylum for both control and treatment remained *Firmicutes*, followed by *Bacteroidetes*, although the treatment group had greater amounts of *Firmibutes* and less *Bacteroidetes* and *Proteobacteria* than the control as illustrated in Figure 4.5 and summarised in Table 4.17.

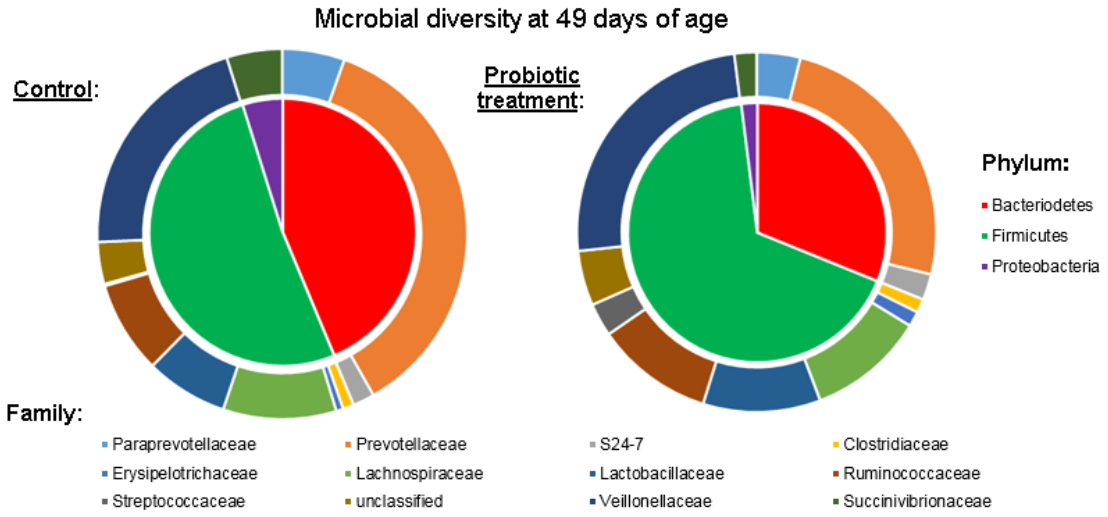

Figure 4.5 Microbial diversity of the control (left) and the probiotic treatment (right) at 49 days of age Inner circle indicates microbes at a phylum level and outer circle indicates microbes at a family level. Phyla and Families occurring less than 1% of all microbes were disregarded

Table 4.17 Percentage (%) relative abundance of microbes between treatments at 49 days of age

	% Contribution Control	% Contribution Probiotic	Mann-Whitney U	Significance
Paraprevotellaceae	5.26	3.73	95.00	0.490
Clostridiaceae	0.92	1.17	160.00	0.050
Erysipelotrichaceae	**0.59**	**1.29**	**178.00**	**0.010[a]**
Lachnospiraceae	9.54	10.00	137.00	0.330
Lactobacillaceae	**6.95**	**10.05**	**162.00**	**0.040[a]**
Prevotellaceae	35.02	23.59	84.00	0.250
Ruminococcaceae	7.78	10.04	150.00	0.130
S24-7	1.83	2.21	133.50	0.390
Streptococcaceae	**0.16**	**2.83**	**187.00**	**<0.001[a]**
Succinivibrionaceae	4.68	1.89	71.00	0.090
Veillonellaceae	20.06	23.42	135.00	0.370

Significant difference (P<0.05) between treatments indicated with [a]

Figure 4.6 shows the most abundant phylum at 63 days remains *Firmicutes*, followed by *Bacteroidetes* and *Proteobacteria*, with no compositional differences between treatment and control.

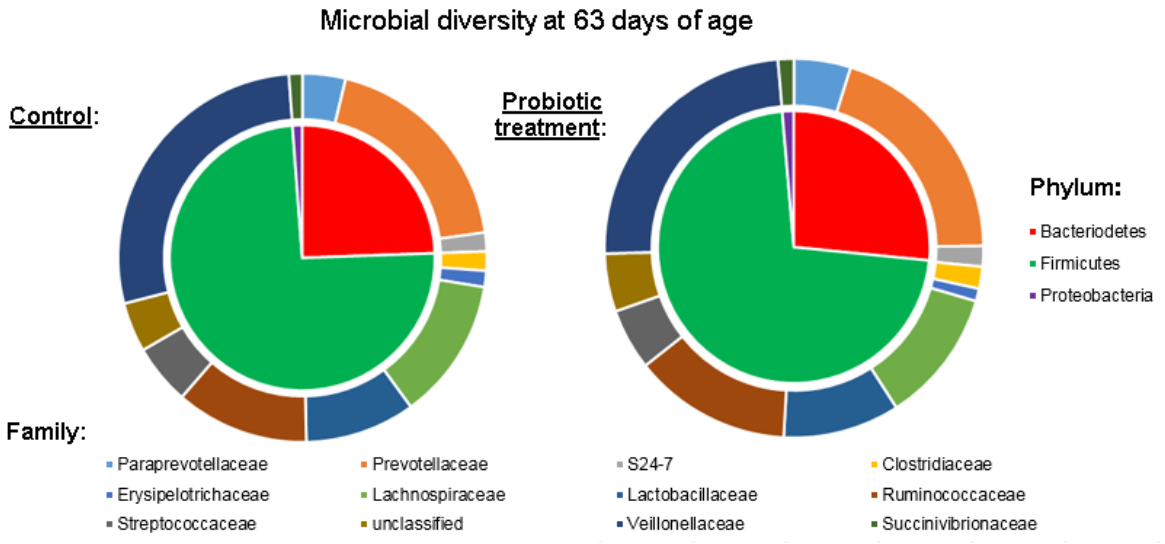

Figure 4.6 Microbial diversity of the control (left) and the probiotic treatment (right) at 63 days of age Inner circle indicates microbes at a phylum level and outer circle indicates microbes at a family level. Phyla and Families occurring less than 1% of all microbes were disregarded

At day 63 no significant differences were observed between treatments, summarised in Table 4.18.

Table 4.18 Percentage (%) relative abundance of microbes between treatments at 63 days of age

	% Contribution Control	% Contribution Probiotic	Mann-Whitney U	Significance
Paraprevotellaceae	4.55	3.56	82.00	0.220
Clostridiaceae	1.76	1.59	107.00	0.840
Erysipelotrichaceae	1.01	1.34	126.00	0.600
Lachnospiraceae	10.73	11.72	118.00	0.840
Lactobacillaceae	9.36	9.11	90.00	0.370
Prevotellaceae	18.79	17.91	114.00	0.970
Ruminococcaceae	12.70	11.02	83.00	0.230
S24-7	1.65	1.55	90.00	0.370
Streptococcaceae	4.91	4.98	104.00	0.740
Succinivibrionaceae	1.28	1.12	117.50	0.840
Veillonellaceae	22.66	26.19	129.00	0.510

Significant difference ($P<0.05$) between treatments indicated with [a]

From Figure 4.7 it is clear that the microbiome of pigs follow a general trend that changes with age. At 35 days of age *Veillonellaceae* populations are relatively small, but then increases with age, while Prevotellaceae populations tend to decrease with age. It can also be seen that each pig has a unique microbiome that differs to various extents from each other, as for example in the case with *Lactobacillaceae* where some pigs regardless of treatment had high levels of *Lactobacillaceae*, while others had lower levels. It is also noticeable that the treatment had an effect on some microbial populations such as *Streptococcaceae* at 35 and 49 days of

age, *Erysipelotrichaceae* at 49 days and *Lactobacillaceae* at 49 days. It is clear from Figure 4.7 that the pig microbiome is a dynamic system that can be influenced by many factors.

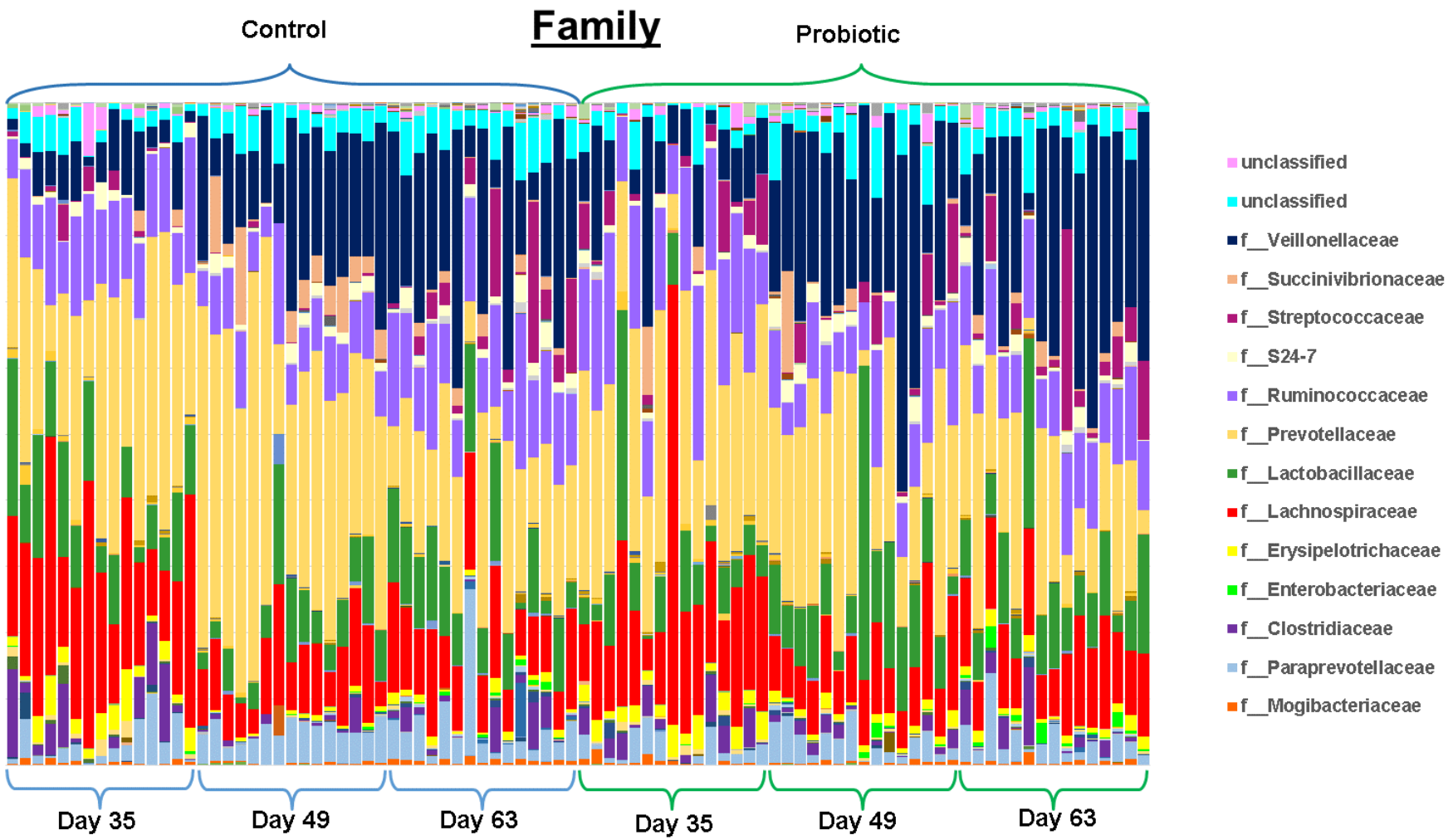

Figure 4.7 Heat map of microbial population diversity between treatment and control at a family level between 35-63 days of age

Chapter 5: Discussion

5.1 Introduction

In this project the impact of *Bacillus* probiotics on the production performance and microbial gut diversity in weaner pigs were investigated. Three products from Chrisal Africa (Pty) Ltd were used, which included a PIP Animal Housing cleaner, PIP Environmental Enhancer with probiotics and PIP Water Plus with probiotics. The purpose of using three products in combination was firstly to clean the unit properly with a housing cleaner, thereby removing any debris and pathogens, thereafter the environmental enhancer was used for the purpose of populating the environment with probiotic bacteria and eliminating any pathogens by means of competitive exclusion. Lastly, the purpose of dosing the water was to ensure that the animals ingest the probiotic. Growth performance was measured as well as microbial gut diversity using 16S rRNA sequencing analysis.

One of the major health benefits of probiotics is modulation of the gut microbiome to include healthier gut microbes and exclude potential pathogens (Ng *et al*, 2009). It is also known to improve growth and performance in some cases together with numerous other health benefits. Probiotics that result in beneficial effects on growth performance hold potential for complete or partial replacement of antibiotic growth promoters in the future (Silva *et al.*, 2010; Dlamini *et al.*, 2017).

The role of the complete gut microbiome in the pig is a fairly recent field of study which is not yet fully understood. It has been shown that there is variation between animals with regard to their microbiome and this phenomenon becomes even greater between different sexes, herds and breeds (Konstantinov *et al.*, 2004; Xiao *et al.*, 2016; Chen *et al.*, 2018).

5.2 Probiotic effect on growth performance

In this study no significant effects on growth and performance were observed for the probiotic treatment. The animals exposed to the probiotic did not perform better compared to the control, therefore the null hypothesis was accepted that the probiotic treatment will have no beneficial significant effect on growth and performance. During the first week of the trial (21-28 days) the group that received the probiotic treatment gained less weight compared to the control in the group housed pigs. In the individually housed pigs no significant differences were observed in the animals receiving the probiotic treatment.

In contrast to studies by Giang *et al.* (2012), Liu *et al.* (2015) and Dowarah *et al.* (2017) who reported positive performance results when using *Bacillus* probiotics, in this study pigs in the probiotic group did not outperform pigs in the control. Higher FI was noted for individually housed pigs in the probiotic group compared to pigs in the control group at 49-60 days with no significant difference between treatments in the group housed pigs. Average daily gain for the individual pig in the control was greater at 35-42 days and for the group pigs at 21-28 days as well as 49-57 days. Individual pigs in the probiotics group had higher FCR compared to the control at 35-42 days as well as 49-60 days and in the group pigs FCR was higher for the probiotic group at 21-28 days and again at 35-42 days.

Studies that reported positive effects of *Bacillus* probiotics for both growth and feed efficiency, also provided evidence that *Bacillus* probiotics had the ability to improve the function of the immune system. (Schrezenmeir & de

Vrese, 2001; Choi et al., 2011a; Lee et al., 2015; Liu et al., 2015; Jørgensen *et al.*, 2016; Gadde *et al.*, 2017), however, some species and strains of *Bacillus*, for example *B. cereus*, have been known to cause undesired immune modulating properties (Altmeyer *et al.*, 2014)

Despite studies reporting beneficial effects, the study by Luyckx *et al.* (2016), found no significant effect of *Bacillus* probiotics on growth and performance and they also used a similar product to this study from Chrisal (Pty) Ltd. A number of studies reported no negative effects, but did not find any significant effect on growth parameters using various probiotics (Shon *et al.*, 2005; Ross *et al.*, 2010; Zhao & Kim, 2015).

There are a number of factors that may influence the efficacy of probiotics, such as the efficacy of the specific strain (Klose *et al.*, 2010), possible interactions among the specific strains and species (Gareau *et al.*, 2010) or the physical form in which it is presented to the animal (Torshizi *et al.*, 2010). By far the majority of research reports the benefit of using probiotic products, but it should be noted, that not all probiotics have equal efficacy, with different strains within the same species that have vastly different efficacies.

Furthermore, factors such as the age of the animal, environment and management may contribute to the outcome of probiotics on the growth and performance of the animals. The results from this study also shows that outcomes may vary between different environments. Although the overall results for the treatments were the same, the degree to which the pigs responded and the time frame in which they experienced the effects differed between individual housing and group housing. The type and level of stressors might therefore affect the outcome of the probiotic effect and results might not always be repeatable.

5.3 Gut microbiome diversity

The results for the gut microbial diversity in this study was based on 16S rRNA sequencing and a Good's coverage of 0.95 was calculated for all bacterial libraries as part of quality control. The majority of the phylogenetic groups present in the sequencing libraries were represented and the Shannon diversity index confirmed minimum variation in diversity across samples.

Data seems to indicate that microbes at a phylum level are relatively constant, however no study has the same microbes at a genus level both in order and in quantity. This confirms that the microbiome is a dynamic system that is probably influenced by many different interactions such as environment, feed and FI, breed, origin of the animal, genetics, maternal effect, sex and many other such as probiotics as can be seen from the present study (Konstantinov et al., 2004; Spor et al., 2011; Isaacson & Kim, 2012; Verschuren et al., 2018).

The most abundant phylum observed for faecal samples analysed at 35 day of age, were *Firmicutes* (60.66 %) followed by *Bacteroidetes* (34.03 %) and *Proteobacteria* (0.94 %), irrespective of treatment. In a study by Xiao *et al.* (2016) *Firmicutes* was also identified as the most abundant phylum followed by *Bacteroidetes*. Results by Pajarillo *et al.*, (2014) indicated a shift between *Fermicutes* and *Bacteriodetes* at 6 weeks of age, which was not observed in the present study. At 49 days of age *Firmicutes* remained the most abundant, but decreased with 5.67%. *Bacteroides* remained the second most abundant group, but unlike *Firmicutes*, it increased slightly by 2.65%, whereas *Proteobacteria* increased by 2.46%. In contrast to the study by Pajarillo *et al.*, (2014) where the levels of *Proteobacteria* decreased, in this study the levels of *Proteobacteria* increased at 49 days of age.

At 63 days of age *Firmicutes* increased (68.43%), whereas *Bacteroidetes* decreased (24.75%) and *Proteobacteria* (2.00%) also decreased, respectively. In a study by Yan *et al.* (2016) it was reported that obese pigs had a higher *Firmicutes* to *Bacteroidetes* ratio compared to lean pigs. The increase in the amount of *Firmicutes* in the present study at 63 days of age, might therefore be due to the increased amount of body fat as the animal ages, since fat accumulation increases and growth efficiency decreases with age (Dunshea & D'Souza, 2003). This could possibly explain the reason why *Firmicutes* slightly decreased at 49 days of age with *Bacteroiodes* that slightly increased, as pigs at this stage were already weaned for more than a month and were probably adapted to the diet, therefore growing optimally in lean muscle and accumulating less fat than older pigs at 63 days of age. Similar studies also reported the increased ratio of *Firmicutes* to *Bacteroidetes* in obese pigs and mice (Ley *et al.*, 2005; Guo *et al.*, 2008b).

Within treatments for the period between day 35 and 49 there was a decrease in microbial populations for the control treatment in the phylum *Firmicutes* for the following bacterial families: *Clostridiaceae, Erysipelotrichaceae*, *Lachnospiraceae* and *Ruminococcaceae* while *Succinivibrionaceae* populations from the phylum *Proteobacteria* increased significantly. Over the same period the probiotic treatment populations of

Erysipelotrichaceae, *Lachnospiraceae*, *Ruminococcaceae* and *Streptococcaceae* belonging to the *Firmicutes* phylum as well as *Prevotellaceae* belonging to the *Bcateroidetes* phylum also decreased significantly. It was reported by Quan *et al.* (2018) that *Lachnospiraceae*, *Ruminococcaceae* and *Prevotellaceae* were more abundant in pigs with superior FCR, since these three families are associated with fermentation of various polysaccharides and proteins as well as production of beneficial short chain fatty acids like butyric acid in the case of *Lachnospiraceae*.

The family *Succinivibrionaceae* includes anaerobic gram-negative bacteria and is commonly found in ruminants where they ferment carbohydrates to succinate and acetate (de Oliveira Santos & Thompson, 2014) which could be used for gluconeogenesis (Imoto & Namioka, 1978; De Vadder *et al.*, 2016). The microbes that decreased significantly from 35-49 days of age are associated with improved digestion and performance. The decrease in these microbes could potentially indicate that pigs between 35-49 days of age might have experienced some digestive challenges in both treatments which could possibly be explained by heat stress during this time, due to a heatwave with maximum temperatures reaching 33.6°C inside the pig unit the day before sampling (Table B7, Appendix B).

There were significant increases in *Ruminococcaceae*, *Streptococcaceae* belonging to the phylum *Firmicutes* and Unclassified microbes in the control group between 49-63 days. In the probiotic group there was a significant increase in *Streptococcaceae* population also belonging to the phylum *Fermicutes* at 49-63 days. Increased levels of *Ruminococcaceae* and *Streptococcaceae* could indicate better digestion of fibre and polysaccharides (Biddle et al., 2013; Wu *et al.*, 2017) during this time. Over the entire period between 35-63 days *Lachnospiraceae* belonging to the phylum *Fermicutes* decreased significantly, *Succinivibrionaceae* belonging to the phylum *Proteobacteria* increased significantly and *Veillonellaceae* belonging to the phylum *Fermicutes* also increased significantly in the control, whereas for the probiotic treatment populations of *Erysipelotrichaceae* and *Veillonellaceae* belonging to the phylum *Fermicutes* also decreased significantly.

There were a few significant differences between the microbiomes of the two treatments, therefore rejecting the null hypothesis that the probiotic treatment will have no significant effect on faecal microbial diversity. Changes that were noted between treatments in the microbial populations included significantly more *Ruminococcaceae* and *Streptococcaceae* belonging to the *Fermicutes* phyla in the probiotic treatment compared to the control at 35 days of age as well as significantly more *Erysipelotrichaceae*, *Lactobacillaceae* and *Streptococcaceae* all of which belongs to the phyla *Fermicutes* in the probiotic treatment at 49 days of age. No differences were detected at 63 days of age, which could explain a disease outbreak amongst both treatments around this time, hence the reason why the trial was ended at 63 days of age. This is in agreement with Thompson *et al.* (2008), who proposed that probiotics will only have a significant effect on the gut microbiome up to a certain point when the climax community will stabilise, however in that study this point was reached much sooner.

High levels of *Erysipelotrichaceae* in the present study could not entirely be explained, as it has increased numbers in cases of chronic diseases such as HIV, colorectal cancer, inflammatory bowel disease and Crohn's disease in humans, although results have been inconsistent. Since the disease outbreak occurred in both treatments, this would not explain the cause of the outbreak, since only the probiotic treatment had significantly higher levels. High levels of *Erysipelotrichaceae* has also been found with diets rich in fats (Kaakoush, 2015) and in dogs *Erysipelotrichaceae* seem to play an important role in gut health and function (Bermingham *et al.*, 2017). Negative correlations were found between *Erysipelotrichaceae* and protein content in diets of dogs and positive correlations were observed with higher carbohydrate digestion and fibre content of the diets. Microbial populations will vary between the different segments of the GIT and will be dependent on the type of feed the animal has consumed (Jensen & Jørgensen, 1994). Since all pigs received the same feed, high fat levels could not be the cause of increased levels in the probiotic treatment.

Increased levels of *Ruminococcus, Streptococcus* and *Lactobacillus* were reported by Verschuren *et al.* (2018) to correlate with increased feed efficiencies in wheat-barley based diets fed to pigs, but did not find any relationship between FCR and microbiome in a maize-soya based diet. Currently in SA most diets are maize-soya based as in the case of this trial, which could explain why the microbiome of the pigs in the probiotic group of the current study did not result in improved FCR although some of the same microbes were present in both studies. In agreement with the results of the current study, Guo *et al.* (2006) also found increased *Lactobacillus* upon application of *B. subtilis* in pigs. Further results were reduced *E. coli* levels and improved performance, both of which were not proven in the present study. In a study by Ober *et al.*, (2017), increased numbers of *Ruminococcaceae* and *Streptococcaceae* correlated to high growth rates in pigs since *Ruminococcaceae* is an important fibre digesting family containing high levels of fibre degrading enzymes and can degrade a wide variety of polysaccharides (Biddle et al., 2013). Increased levels of *Streptococcus* belonging to the *Lactobacillaceae* family were reported by Wu *et al.* (2017) when diets high in isomalto-oligosaccharides were fed. It was concluded that *Streptococcus* possibly contributed to utilisation of these non-digestible isomalto-oligosaccharides in the lower gut. Increased levels of *Streptococcus* in the present study could possibly indicate better digestion of isomalto-oligosaccharides in the probiotic treatment at both 35 and 49 days of age. It is unlikely that any of the positive effects of the previous studies regarding better digestion or improved immune function were achieved in the present study, since none of the production parameters were simultaneously improved.

Species from the *Lactobacillaceae* family have been used frequently as probiotics in pig diets with numerous reports of success (Liu *et al.*, 2013a; Liu *et al.*, 2015; Naqid *et al.*, 2015; Zhao & Kim, 2015; Lan *et al.*, 2016). Bacteria from the family *Lactobacillaceae* have the ability to adhere to the intestinal tract, enabling it to reside in the gut of the animal (Turpin *et al.*, 2012). Many species under the family *Lactobacillaceae* belongs to the group of bacteria known for producing lactic acid (LAB) (Ljungh & Wadström, 2006). These bacteria can exert multiple health benefits when ingested such as upregulation of genes involved in immune responses (Di Caro *et al.*, 2005), antimicrobial effects (Yang *et al.*, 2012), growth performance, digestibility and general intestinal health (Giang *et al.*, 2015). From the microbial data in the current study it seems as if the ingestion of *Bacillus* probiotics beneficially affected the *Lactobacillaceae* populations at 49 days of age, which could have had an additional beneficial effect on intestinal health, although this could not be confirmed.

The disease outbreak at 63 days could not be explained by the 16S rRNA data, probably because the pathogenic microbes responsible for the disease did not form part of the major microbes in the analysed data, since only microbes that were present at levels greater than 1% for one of the three sampling days were considered.

Some unanswered questions with regards to probiotic products still remain. For instance, it might be possible that the competitive exclusion that results from the application of some probiotics might exclude bacteria that is beneficial to the gut microbiome, as some aspects of the microbiome is still not clearly understood, the role of some minor microbial populations might become apparent as more research follows. Williams (2010) warns about possible bloating and flatulence that may occur due to the use of probiotics and cautions the use of it in human patients with compromised immune systems. Another possibility is that probiotics might protect the gut from harmful pathogens up to a certain point where the bacterial load might simply be too large for the probiotic to efficiently eliminate it and prevent disease especially when the concentration of probiotic organisms are not high enough. It might be necessary to develop a fast method of testing the microbial activity of probiotics in the future before feeding it to animals to ensure that the correct concentration of microbes will reach the animal gut. Currently, no data exist to determine at what level of pathogenicity probiotics will be effective in preventing disease, and this will vary with species and strain.

The *Bacillus* probiotics are not derived from animal origin, but rather common bacteria found in soil or other environments (Lawrence, 2008). A minor concern might be that such probiotics under certain conditions could exert negative effects on the animal by causing

allergic reactions in the gut, especially if incorrect strains are fed. Some strains might even be more virulent than previously thought and when the animal is exposed to these strains for longer periods of time or suffers from a compromised immune system, as can be the case during weaning, it might have a negative impact on growth and performance. Three cases of bacteraemia in preterm human infants were reported in a case study by Zbinden *et al.* (2015) when *Bifidobacterium longum* was administered to these infants. Translocation of bacteria in a severely immune compromised child was also reported, when the boy suffered bacteraemia due to resistant *L. rhamnosus*, without consuming any probiotics (Robin *et al.*, 2010). Although these cases are rare it is possible that the occurrence of such cases are more prevalent than reported, since not all cases might result in clinical signs. To date no such reports have been made for animal models and could probably be ascribed to the fact that intense diagnosis is rarely performed on farm animals. It might therefore be possible in some cases that immune compromised animals (during weaning) could suffer from infections due to translocated bacteraemia. It might also be possible that the symptoms could be subclinical and therefore never properly diagnosed.

The screening process for the organisms should therefore be strict with sufficient research completed over various time intervals to determine what the long term effect will be when using these products, not only on the animal, but also on the environment (Anadón *et al.*, 2006; Donohue, 2006). A study by Bafeta *et al.* (2018), showed how poorly the adverse effects and safety results of probiotics have been recorded or even considered in research articles of human medicine. It was shown that 90% of the 347 studies did not have a clear definition of adverse effects due to the use of probiotics and 97% of studies did not describe any method for collecting harms-related information. This shows that stricter and more transparent methods of assessing safety of these products are needed.

Another possibility is that the specific probiotic in question might carry resistance genes enabling it to withstand pathogens and thereby ensure its survivability, these resistant genes however, could be transferred to other bacteria and pathogens resulting in resistant disease outbreaks (Ishibashi & Shoji Yamazaki, 2001; Anadón *et al.*, 2006). *Bacillus* spores can survive in the environment for many years under harsh conditions, therefore high doses in the environment could potentially be hard to eliminate should there be some concern as in the case with resistant microbes (Nicholson *et al.*, 2000).

Chapter 6: Conclusion

The focus of the study was to determine the impact of *Bacillus* probiotics on the production performance and microbial gut diversity in weaner pigs by using three products from Chrisal Africa (Pty) Ltd. The first product used was PIP Animal Housing cleaner, for the purpose of cleaning the house of any dirt or debris, the second product was a PIP Environmental Enhancer with probiotics for the purpose of applying probiotics to the environment to competitively exclude pathogens and the third product was PIP Water Plus with probiotics for the purpose of putting probiotics in the water for the animals to ingest. These products were tested in combination and were compared to a control with no exposure to the probiotic treatment or in-feed antibiotics.

A total of 167 weaner pigs, 21-days of age were placed in two housing units, the first of which consisted of individual pens and the second of group pens. In total 40 pigs were allocated to the individual pens and the remainder were placed in the group pens with a stocking density of 0.95 m^2 per pig. After the initial cleaning op the entire pig unit with PIP Animal Housing cleaner, the house was sprayed with PIP Environmental Enhancer at a concentration of 1L/200 m^2 from the day that the pigs arrived. This was done on a daily basis for the first 7 days and thereafter 3 times per week. The drinking water was dosed with PIP Water Plus at a concentration of 0.02% once per day for the duration of the trial. Pigs were weighed once per week to determine body weights and average daily gain and feed intake was measured by weighing out the allotted feed at the beginning of the week and then weighing back the remaining feed at the end of the week.

Faecal samples were collected from individual pigs only, for the purpose of 16S rRNA sequencing. A total of 40 samples were taken per sampling day (120 samples in total) at day 35, 49 and 63 of age. Of these samples 30 samples per sampling day were selected (90 samples in total) for DNA extractions using a Phenol-chloroform extraction method. Genomic DNA was send to the University of Michigan Medical School (USA) for analysis of the VP 4 region of the 16S rRNA gene to determine the microbial diversity. The raw 16S rRNA data was processed using MOTHUR V.1.35.1 software.

There were few significant differences between the treatment and control, with only slight negative effects of the probiotic on growth and feed efficiency. Body weight gain was negatively affected between 35-42 days of age for the individually housed pigs and between 21-28 days as well as 49-57 days in the group housed pigs. Feed intake was not affected by treatment, but FCR was greater for the probiotic treatment at 35-42 days of age as well as 49-60 days in the individual housing and between 21-28 days as well as 35-42 days for the group housing.

For the 16S rRNA data, Good's coverage was calculated at 0.95 with an average Shannon diversity index of 0.72. The most abundant phylum observed for faecal samples analysed at 35 day of age, was *Firmicutes* followed by *Bacteroidetes* and *Proteobacteria*, irrespective of treatment. At 49 days of age *Firmicutes* decreased while remaining the most abundant phylum, whereas *Bacteroides* and *Proteobacteria* increased. At 63 days of age *Firmicutes* increased again, whereas *Bacteroidetes* and *Proteobacteria* decreased.

Between 35-49 days of age there was a decrease *Clostridiaceae, Erysipelotrichaceae*, *Lachnospiraceae* and *Ruminococcaceae* at a family level, all of which belong to the phylum *Firmicutes*, whereas *Succinivibrionaceae* populations from the phylum *Proteobacteria* increased significantly for the control. For the probiotic treatment populations of *Erysipelotrichaceae*, *Lachnospiraceae*, *Ruminococcaceae* and *Streptococcaceae* belonging to the *Firmicutes* phylum as well as *Prevotellaceae* belonging to the *Bcateroidetes* phylum also decreased significantly.

Between 49-63 days there were significant increases in *Ruminococcaceae*, *Streptococcaceae* belonging to the *Fermicutes* phylum and Unclassified microbes in the control group and in the probiotic group there was a significant increase in *Streptococcaceae*

population at 49-63 days. Over the entire period between 35-63 days *Lachnospiraceae* belonging to the *Fermicutes* phylum decreased significantly whereas *Succinivibrionaceae* belonging to the phylum *Proteobacteria* and *Veillonellaceae* belonging to the *Bacteroidetes* phylum increased significantly in the control. For the probiotic treatment populations of *Erysipelotrichaceae* and *Veillonellaceae* belonging to the *Fermicutes* phylum also decreased significantly.

Significant differences between the two treatments were seen at 35 days of age, with increased levels of *Ruminococcaceae* and *Streptococcaceae* belonging to the phylum *Fermicutes* in the probiotic treatment compared to the control and increased *Erysipelotrichaceae*, *Lactobacillaceae* and *Streptococcaceae* also belonging to the phylum *Fermicutes* in the probiotic treatment at 49 days of age. No differences were detected at 63 days of age.

No negative effects were prevalent from the data and overall the probiotic treatment seemed to have improved the quantity of beneficial microbes in the faeces, however no such improvements were observed in the production data, with results even showing negative effects of the probiotic treatment on the production performance.

6.1. Recommendations

Current data could be used to expand the research to include smaller microbial populations which occurred at a level lower than 1% and thereby finding a correlation between the performance of the animals and the presence of minor populations. This could possibly also reveal the cause of the disease outbreak towards the end of the trial.

It would also be recommended that the production recording and faecal sample taking should be done at the same time so that a proper correlation could be drawn between production data and microbial diversity. Since the production data and faecal microbial diversity data did not originally form part of the same trial and data was not taken at the same days, it had to be combined when the disease outbreak resulted in the trial being ended prematurely. This made it difficult to interpret the data as a whole. It would also be preferable to use data from a trial with a healthy pig herd since the effect of the disease on the data could not be quantified neither for the production data nor for the microbial diversity. It is possible that the pigs were infected for a longer period of time with subclinical symptoms and therefore only displayed disease symptoms at a later stage.

A possible improvement on the trial design would have been to analyse the probiotic products at the start of the trial and at each sampling day to determine the concentration of viable spores or live microbes in the product at different stages and to measure the amount of probiotic bacteria and spores in the environment and drinking water of the pigs as a quality control measure. It is unknown if the product used had enough viable spores or microbes in the product to create a probiotic effect.

It would also have been beneficial to investigate the microbial diversity at different segments of the digestive tract, since faecal diversity might be irrelevant as it is only a measurement of the microbial diversity in the colon. Since the colon acts as a fermentation reservoir instead of a digestion chamber, microbes from the upper digestive tract might be vastly different from faecal microbes.

If the trial facility had the capacity and financial funding were not a limiting factor it would have been interesting to include an antibiotic treatment and a combination treatment of an antibiotic and probiotic to the trial to investigate the effect of the combination and also how the probiotic compared to the antibiotic with regards to performance and microbial diversity. As a control measure the bacterial load of the facility should have been tested before the start of the trial and again after the end of the trial to establish a base line from which to work.

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Appendix A

Table A1 Pig weights at placement for individually housed pigs

Control Treatment			Probiotic Treatment		
Tag #	**Weight (kg)**	**Assigned Pen**	**Tag #**	**Weight (kg)**	**Assigned Pen**
146	9.7	C-1	72	9.7	P-1
143	6.2	C-10	157	6.2	P-10
113	6.3	C-11	84	6.3	P-11
63	9	C-12	17	9.1	P-12
65	9.6	C-13	138	9.4	P-13
96	6.2	C-14	109	6.2	P-14
34	9.1	C-15	37	9.1	P-15
77	6.3	C-16	73	6.3	P-16
6	9.3	C-17	116	9.1	P-17
88	6	C-18	93	6	P-18
27	6.4	C-19	166	6.3	P-19
33	6	C-2	41	6	P-2
62	8.9	C-20	106	8.9	P-20
149	6.4	C-3	42	6.4	P-3
78	8.7	C-4	55	8.8	P-4
8	6.1	C-5	123	6.1	P-5
104	9.4	C-6	90	9.3	P-6
144	6.3	C-7	122	6.3	P-7
14	9	C-8	19	9	P-8
105	9.1	C-9	54	9.1	P-9

Table A2 Pig weights of individually housed pigs from 21-63 days of age

		Weight/pig (kg)						
	Pen	**Day 21**	**Day 28**	**Day 35**	**Day 42**	**Day 49**	**Day 60**	**Day 63**
Control Treatment	1.00	9.70	10.75	13.60	19.80	24.00	31.20	35.00
	2.00	6.00	7.23	9.50	12.70	16.50	23.40	26.40
	3.00	6.40	8.30	11.70	16.10	20.60	30.10	32.70
	4.00	8.70	10.48	13.80	18.00	21.80	31.40	34.20
	5.00	6.10	8.35	10.80	15.20	20.80	30.50	33.40
	6.00	9.40	10.09	13.40	18.30	22.20	31.50	34.80
	7.00	6.30	7.48	9.90	13.70	16.50	22.60	26.20
	8.00	9.00	10.13	14.10	19.10	22.80	32.50	33.50
	9.00	9.10	10.55	14.30	19.40	23.70	35.40	38.00
	10.00	6.20	7.10	9.20	12.00	17.70	24.40	
	11.00	6.30	7.90	11.90	16.60	21.50	33.60	37.00
	12.00	9.00	10.80	13.50	18.10	22.60	32.40	34.70
	13.00	9.60	11.60	15.10	21.90	28.30	38.30	41.80
	14.00	6.20	7.90	11.80	17.30	21.60	31.30	30.40
	15.00	9.10	10.50	14.20	19.80	25.30	37.50	41.10
	16.00	6.30	7.70	9.90	14.20	17.30	27.00	28.80
	17.00	9.30	10.40	13.20	18.40	23.40	33.50	37.00
	18.00	6.00	7.40	9.50	14.50	20.00	28.00	31.40
	20.00	8.90	10.90	14.70	21.90	27.80	40.00	43.10
Probiotic Treatment	1.00	9.70	12.20	16.00	Eliminated from trial			
	2.00	6.00	8.20	11.30	15.20	19.90	30.70	34.90
	3.00	6.40	9.00	12.50	16.80	20.50	29.60	37.70
	4.00	8.80	10.40	14.20	19.90	24.00	32.10	34.60
	5.00	6.10	7.30	10.40	14.90	18.90	29.10	29.60
	6.00	9.30	10.40	13.80	18.30	23.00	33.00	35.50
	7.00	6.30	7.80	9.20	12.50	16.00	21.30	23.40
	8.00	9.00	10.60	13.40	16.50	21.40	31.50	33.50
	9.00	9.10	11.20	15.70	20.60	26.70	34.70	39.20
	10.00	6.20	7.50	10.50	14.40	18.70	25.50	28.20
	11.00	6.30	7.40	11.80	15.30	20.40	33.50	35.30
	12.00	9.10	11.50	15.40	20.60	25.10	37.00	39.30
	13.00	9.40	10.70	14.20	17.90	22.40	31.70	35.00
	14.00	6.20	7.30	9.10	11.90	16.20	23.50	26.90
	15.00	9.10	10.40	13.80	18.70	21.60	29.40	32.90
	16.00	6.30	7.80	10.90	14.00	17.60	26.40	29.20
	17.00	9.10	10.60	14.70	19.10	25.30	33.40	37.00
	18.00	6.00	7.60	10.90	15.50	20.90	28.70	32.80
	19.00	6.30	8.60	13.90	18.50	21.70	30.20	33.40
	20.00	8.90	11.30	17.30	23.10	29.90	40.40	44.60

***** The pig in pen 19 was removed from the trial.

Table A3 Average pig weight per pen of group housed pigs from 21-57 days of age

		Average weight/pen (kg)					
	Pen	**Day 21**	**Day 28**	**Day 35**	**Day 42**	**Day 49**	**Day 57**
Control Treatment	1	8.09	9.79	12.32	17.03	21.50	26.54
	2	6.65	8.06	10.33	13.97	18.12	22.40
	3	7.11	8.82	11.58	15.69	20.49	24.43
	4	7.52	9.08	11.85	16.04	20.65	25.50
Probiotic Treatment	1	8.14	9.47	12.76	16.58	21.98	23.94
	2	7.48	8.85	11.72	15.67	19.93	23.18
	3	6.67	8.06	10.59	14.17	17.82	21.16
	4	7.07	8.45	11.14	14.55	18.82	22.72

Table A4 Feed intake per pig per pen of individually housed pigs from 21-63 days of age

		Feed intake pig/week (kg)					
	Pen	**21-28**	**28-35**	**35-42**	**42-49**	**49-60**	**60-63**
Control	1	1.47	4.25	6.18	8.78	13.33	4.50
	2	1.27	5.48	5.04	7.44	10.84	4.40
	3	1.76	4.30	5.21	9.87	15.94	3.45
	4	1.98	4.24	3.04	10.63	14.83	5.35
	5	2.46	4.06	4.85	11.15	16.10	5.05
	6	1.11	6.36	5.10	10.28	14.62	5.40
	7	1.25	5.54	4.11	6.96	11.93	4.00
	8	1.45	4.85	5.77	10.01	15.29	4.80
	9	1.75	4.94	3.40	13.40	14.53	5.90
	10	1.08	5.33	6.59	7.09	13.88	
	11	1.50	4.37	5.63	10.90	10.30	6.20
	12	1.99	4.03	4.65	9.64	15.21	5.20
	13	2.14	5.13	6.57	13.01	17.22	6.10
	14	1.81	4.79	5.48	9.36	15.68	3.60
	15	1.53	4.48	6.76	12.01	13.49	3.30
	16	1.31	5.83	4.20	8.66	13.48	3.84
	17	1.91	4.27	5.00	10.06	16.23	6.00
	18	1.91	4.09	4.90	13.51	11.40	2.80
	20	1.97	6.99	7.07	12.56	11.08	6.70
Probiotic Treatment	1	2.35	5.89	2.81		Eliminated from trial	
	2	2.06	4.53	5.53	8.58	15.82	5.85
	3	2.50	5.01	4.85	8.02	13.14	5.05
	4	1.88	4.53	5.94	10.29	14.83	4.60
	5	1.02	6.09	6.09	9.17	11.90	4.40
	6	1.52	4.11	4.15	10.86	14.69	4.50
	7	1.74	4.76	2.84	9.37	9.51	3.35
	8	1.89	3.65	5.65	10.30	14.78	2.90
	9	2.26	6.69	5.92	10.31	15.29	4.60
	10	1.22	6.06	5.03	10.18	13.18	9.70
	11	1.64	4.64	4.62	11.13	17.34	5.70
	12	2.09	5.38	5.42	10.82	16.66	4.60
	13	1.50	6.50	4.45	9.38	13.64	5.21
	14	1.42	5.41	3.76	8.98	11.90	4.90
	15	2.03	4.69	8.32	11.36	13.25	7.02
	16	1.55	6.17	3.33	8.39	14.63	4.50
	17	1.96	4.99	6.29	11.90	7.33	4.80
	18	1.87	4.56	4.82	9.96	14.39	4.25
	19	2.04	5.33	5.75	8.55	13.90	5.50
	20	2.23	7.66	6.15	27.16	10.32	7.05

***** The pig in pen 19 was removed from the trial.

Table A5 Average weekly FI per pig per pen for group housed pigs from 21-57 days of age

		Average feed intake per pen (kg)				
	Pen	**21-28 days**	**28-35 days**	**35-42 days**	**42-49 days**	**49-57 days**
Control	1	1.88	3.36	4.95	5.21	6.46
	2	1.67	2.80	3.97	5.89	5.21
	3	1.76	3.04	4.41	6.32	4.76
	4	1.80	3.04	4.42	7.86	5.18
Probiotic Treatment	1	1.76	3.50	4.71	5.75	5.56
	2	1.92	3.11	4.56	5.38	5.16
	3	1.63	2.95	4.21	4.28	4.51
	4	1.86	2.96	4.02	5.21	5.88

Appendix B

Table B1 Composition of microbes as percentage (%) of total detected OTUs from 35-63 days of age

Classification	% Contribution day 35	% Contribution day 49	% Contribution day 63
k__Archaea	0.04	0.19	0.19
k__Bacteria	99.96	99.81	99.81
p__Bacteroidetes	34.03	36.68	24.75
c__Bacteroidia	33.73	36.03	24.42
o__Bacteroidales	33.73	36.03	24.42
f__Paraprevotellaceae	2.65	4.45	4.07
g__Prevotella	1.93	3.39	3.59
s__unclassified	1.93	3.39	3.59
g__CF231	0.59	1.03	0.41
s__unclassified	0.59	1.03	0.41
f__Prevotellaceae	28.63	28.92	18.36
g__Prevotella	28.63	28.92	18.36
s__copri	15.01	16.83	11.16
s__stercorea	0.70	1.69	0.75
s__unclassified	12.90	10.33	6.42
f__S24-7	1.20	2.03	1.60
g__unclassified	1.20	2.03	1.60
p__Firmicutes	60.66	55.00	68.43
c__Bacilli	11.05	10.21	14.24
o__Lactobacillales	10.97	10.19	14.20
f__Lactobacillaceae	8.61	8.60	9.24
g__Lactobacillus	8.60	8.60	9.24
s__delbrueckii	0.10	1.02	0.86
s__mucosae	0.38	0.23	0.20
s__reuteri	3.44	1.78	1.72
s__unclassified	4.66	5.56	6.45
f__Streptococcaceae	2.36	1.58	4.94
g__Streptococcus	2.35	1.58	4.94
s__unclassified	2.35	1.58	4.94
c__Clostridia	46.79	43.46	52.52
o__Clostridiales	46.78	43.45	52.50
f__Mogibacteriaceae	0.46	0.34	0.52
g__unclassified	0.34	0.26	0.38
f__Clostridiaceae	3.34	1.06	1.68
g__Clostridium	3.34	1.05	1.68
s__celatum	3.14	0.87	0.97
f__Lachnospiraceae	18.56	9.79	11.21
g__Ruminococcus	0.88	0.21	0.35
s__gnavus	0.88	0.21	0.35
g__Blautia	10.41	1.89	1.94
s__obeum	0.54	0.30	0.22
s__unclassified	9.87	1.59	1.72
g__Coprococcus	0.73	0.61	1.04
s__catus	0.22	0.18	0.44
s__unclassified	0.51	0.38	0.54
g__Coprococcus	0.01	0.01	0.57
s__unclassified	0.01	0.01	0.57
g__Dorea	1.39	0.54	0.62
s__unclassified	1.16	0.37	0.44
g__Oribacterium	0.01	0.33	0.16
s__unclassified	0.01	0.33	0.16
g__Roseburia	1.83	2.89	2.54
s__faecis	1.83	2.89	2.54
g__unclassified	2.04	1.81	2.27
f__Peptostreptococcaceae	0.61	0.20	0.16
g__unclassified	0.61	0.20	0.16
f__Ruminococcaceae	14.86	8.99	11.89

g__Butyricicoccus	1.07	0.42	0.56
s__pullicaecorum	1.07	0.42	0.56
g__Butyricicoccus	0.32	0.26	0.29
s__pullicaecorum	0.32	0.26	0.29
g__Faecalibacterium	5.54	2.05	2.75
s__prausnitzii	5.54	2.05	2.75
g__Oscillospira	1.74	1.84	2.38
s__unclassified	1.74	1.84	2.38
g__Ruminococcus	0.84	0.71	0.86
s__unclassified	0.65	0.32	0.40
g__Ruminococcus	0.04	0.37	0.42
s__unclassified	0.01	0.33	0.34
g__unclassified	4.29	2.46	3.76
g__unclassified	0.48	0.09	0.12
f__Ruminococcaceae	0.12	0.23	0.40
f__Veillonellaceae	8.11	21.86	24.37
g__Acidaminococcus	0.65	1.45	0.36
s__unclassified	0.65	1.45	0.36
g__Anaerovibrio	0.40	4.04	7.67
s__unclassified	0.40	4.04	7.67
g__Dialister	0.30	0.09	0.16
s__unclassified	0.30	0.09	0.16
g__Megasphaera	2.60	6.78	8.33
s__unclassified	2.60	6.78	8.33
g__Mitsuokella	0.08	1.19	1.53
s__multacida	0.08	1.19	1.53
g__Phascolarctobacterium	3.66	5.04	4.72
s__unclassified	3.66	5.04	4.72
g__unclassified	0.38	2.88	1.31
f__unclassified	0.07	0.39	0.84
c__Erysipelotrichi	2.73	0.97	1.17
o__Erysipelotrichales	2.73	0.97	1.17
f__Erysipelotrichaceae	2.73	0.97	1.17
g__Eubacterium	1.04	0.18	0.32
s__biforme	0.76	0.18	0.31
g__Bulleidia	0.78	0.28	0.40
s__p-1630-c5	0.28	0.15	0.29
s__unclassified	0.46	0.09	0.03
g__Catenibacterium	0.33	0.09	0.07
s__unclassified	0.33	0.09	0.07
g__Sharpea	0.31	0.09	0.03
s__azabuensis	0.31	0.08	0.03
c__unclassified	1.80	1.10	0.92
p__Proteobacteria	0.94	3.71	2.00
c__Gammaproteobacteria	0.84	3.42	1.81
o__Aeromonadales	0.73	3.19	1.20
f__Succinivibrionaceae	0.73	3.19	1.20
g__Succinivibrio	0.73	3.19	1.20
s__unclassified	0.73	3.19	1.20
o__Enterobacteriales	0.03	0.19	0.59
f__Enterobacteriaceae	0.03	0.19	0.59
g__Escherichia	0.03	0.19	0.59
s__coli	0.03	0.19	0.59
p__Tenericutes	0.10	0.47	1.23
c__Mollicutes	0.10	0.47	1.23
o__RF39	0.10	0.47	1.23
f__unclassified	0.10	0.47	1.23

*Only phyla with a contribution of >1% were included as well as all classes with a family level of >0.30%. *Prefixes before names can be interpreted as follows: k__ kingdom; p__ phylum; c__ class; o__ order; f__ family; g__ genus; s__ specie

Table B2 Composition of microbes as percentage (%) of total detected OTUs per treatment from 35-63 days of age

Classification	Control Day 35 %	Probiotic Day 35 %	Control Day 49 %	Probiotic Day 49 %	Control Day 63 %	Probiotic Day 63 %
k__Archaea	0.01	0.06	0.16	0.22	0.16	0.23
k__Bacteria	99.99	99.94	99.84	99.78	99.84	99.77
p__Bacteroidetes	34.11	33.97	43.34	30.85	25.71	23.73
c__Bacteroidia	33.90	33.59	42.59	30.30	25.36	23.42
o__Bacteroidales	33.90	33.59	42.59	30.30	25.36	23.42
f__Paraprevotellaceae	2.80	2.53	5.26	3.73	4.55	3.56
g__Prevotella	2.04	1.83	4.15	2.73	4.08	3.08
s__unclassified	2.04	1.83	4.15	2.73	4.08	3.08
g__CF231	0.49	0.68	1.10	0.98	0.43	0.38
s__unclassified	0.49	0.68	1.10	0.98	0.43	0.38
f__Bacteroidaceae	0.34	0.13	0.01	0.09	0.06	0.01
g__Bacteroides	0.31	0.13	0.01	0.08	0.06	0.01
s__unclassified	0.30	0.06	0.01	0.06	0.06	0.01
f__Prevotellaceae	28.68	28.60	35.02	23.59	18.79	17.91
g__Prevotella	28.68	28.60	35.02	23.59	18.79	17.91
s__copri	15.32	14.76	21.01	13.18	11.44	10.87
s__stercorea	0.51	0.86	2.36	1.11	0.58	0.94
s__unclassified	12.83	12.96	11.57	9.25	6.73	6.08
f__S24-7	1.28	1.13	1.83	2.21	1.65	1.55
g__unclassified	1.28	1.13	1.83	2.21	1.65	1.55
f__unclassified	0.24	0.46	0.26	0.42	0.10	0.18
c__unclassified	0.20	0.34	0.74	0.55	0.34	0.31
p__Firmicutes	60.62	60.70	47.80	61.29	67.97	68.93
c__Bacilli	12.27	10.06	7.13	12.91	14.32	14.15
o__Lactobacillales	12.16	10.00	7.11	12.88	14.28	14.11
f__Lactobacillaceae	11.18	6.52	6.95	10.05	9.36	9.11
g__Lactobacillus	11.17	6.52	6.95	10.04	9.36	9.11
s__delbrueckii	0.05	0.15	0.99	1.04	0.82	0.90
s__mucosae	0.22	0.52	0.16	0.29	0.25	0.15
s__reuteri	4.81	2.32	1.44	2.07	1.80	1.63
s__unclassified	6.06	3.52	4.35	6.61	6.48	6.42
f__Streptococcaceae	0.98	3.47	0.16	2.83	4.91	4.98
g__Streptococcus	0.98	3.47	0.16	2.83	4.91	4.98
s__unclassified	0.98	3.47	0.16	2.83	4.91	4.98
c__Clostridia	45.29	48.00	39.80	46.66	52.14	52.93
o__Clostridiales	45.29	48.00	39.79	46.65	52.12	52.91
f__Mogibacteriaceae	0.39	0.52	0.27	0.40	0.60	0.44
g__unclassified	0.25	0.41	0.24	0.28	0.43	0.33
f__Clostridiaceae	4.32	2.55	0.92	1.17	1.76	1.59
g__Clostridium	4.32	2.55	0.92	1.17	1.76	1.58
s__celatum	4.21	2.26	0.71	1.00	1.34	0.57
s__unclassified	0.03	0.24	0.18	0.13	0.30	0.82
f__Clostridiaceae	0.53	0.16	0.02	0.07	0.08	0.05
g__SMB53	0.53	0.16	0.02	0.07	0.08	0.05
s__unclassified	0.53	0.16	0.02	0.07	0.08	0.05
f__Lachnospiraceae	20.78	16.75	9.54	10.00	10.73	11.72
g__Ruminococcus	1.31	0.53	0.18	0.24	0.30	0.41
s__gnavus	1.31	0.53	0.18	0.24	0.30	0.41

g__Blautia	12.28	8.89	0.96	2.69	1.88	2.00
s__obeum	0.46	0.61	0.14	0.43	0.17	0.27
s__unclassified	11.82	8.28	0.82	2.26	1.71	1.73
g__Coprococcus	1.05	0.46	0.68	0.55	1.26	0.81
s__catus	0.39	0.07	0.23	0.14	0.53	0.34
s__unclassified	0.65	0.39	0.39	0.37	0.63	0.44
g__Coprococcus	0.02	0.00	0.02	0.01	0.01	1.17
s__unclassified	0.02	0.00	0.02	0.01	0.01	1.17
g__Dorea	0.97	1.74	0.29	0.77	0.71	0.52
s__unclassified	0.70	1.54	0.19	0.52	0.54	0.33
g__Lachnospira	0.00	0.01	0.34	0.18	0.22	0.29
s__unclassified	0.00	0.01	0.34	0.18	0.22	0.29
g__Oribacterium	0.00	0.03	0.11	0.53	0.13	0.20
s__unclassified	0.00	0.03	0.11	0.53	0.13	0.20
g__Roseburia	0.33	0.21	0.25	0.06	0.04	0.04
s__inulinivorans	0.33	0.21	0.25	0.05	0.04	0.04
g__Roseburia	1.79	1.87	3.88	2.03	2.39	2.71
s__faecis	1.79	1.87	3.88	2.03	2.39	2.71
g__unclassified	1.95	2.12	1.78	1.84	2.11	2.44
f__Peptostreptococcaceae	0.74	0.50	0.17	0.23	0.18	0.15
g__unclassified	0.74	0.50	0.17	0.23	0.18	0.15
f__Ruminococcaceae	12.33	16.91	7.78	10.04	12.70	11.02
g__Butyricicoccus	1.45	0.77	0.35	0.48	0.53	0.58
s__pullicaecorum	1.45	0.77	0.35	0.48	0.53	0.58
g__Butyricicoccus	0.22	0.41	0.21	0.30	0.27	0.31
s__pullicaecorum	0.22	0.41	0.21	0.30	0.27	0.31
g__Faecalibacterium	3.66	7.06	1.89	2.19	2.93	2.55
s__prausnitzii	3.66	7.06	1.89	2.19	2.93	2.55
g__Oscillospira	1.56	1.90	1.98	1.71	2.75	1.97
s__unclassified	1.56	1.90	1.98	1.71	2.75	1.97
g__Oscillospira	0.10	0.32	0.13	0.13	0.18	0.10
s__unclassified	0.10	0.32	0.13	0.13	0.18	0.10
g__Ruminococcus	0.77	0.89	0.53	0.86	0.86	0.85
s__unclassified	0.64	0.65	0.25	0.37	0.44	0.36
s__unclassified	0.01	0.10	0.13	0.33	0.21	0.35
g__Ruminococcus	0.01	0.05	0.39	0.35	0.39	0.46
s__unclassified	0.00	0.01	0.35	0.31	0.31	0.38
g__unclassified	3.96	4.55	1.67	3.16	3.93	3.57
f__Ruminococcaceae	0.03	0.18	0.23	0.23	0.40	0.39
f__Veillonellaceae	5.94	9.87	20.06	23.42	22.66	26.19
g__Acidaminococcus	0.38	0.87	1.23	1.64	0.31	0.41
s__unclassified	0.38	0.87	1.23	1.64	0.31	0.41
g__Anaerovibrio	0.58	0.26	5.73	2.56	8.11	7.21
s__unclassified	0.58	0.26	5.73	2.56	8.11	7.21
g__Dialister	0.59	0.07	0.05	0.13	0.04	0.28
s__unclassified	0.59	0.07	0.05	0.13	0.04	0.28
g__Megamonas	0.02	0.00	0.02	0.27	0.09	0.11
s__unclassified	0.02	0.00	0.02	0.27	0.09	0.11
g__Megasphaera	1.47	3.51	6.60	6.95	6.17	10.63
s__unclassified	1.47	3.51	6.60	6.95	6.17	10.63
g__Mitsuokella	0.01	0.14	0.63	1.67	1.28	1.81
s__multacida	0.01	0.14	0.63	1.67	1.28	1.81

g__Phascolarctobacterium	2.77	4.38	4.98	5.09	4.92	4.50
s__unclassified	2.77	4.38	4.98	5.09	4.92	4.50
g__unclassified	0.12	0.60	0.65	4.83	1.62	0.98
f__unclassified	0.03	0.11	0.29	0.48	1.15	0.51
c__Erysipelotrichi	2.92	2.57	0.59	1.29	1.01	1.34
o__Erysipelotrichales	2.92	2.57	0.59	1.29	1.01	1.34
f__Erysipelotrichaceae	2.92	2.57	0.59	1.29	1.01	1.34
g__Eubacterium	1.25	0.87	0.11	0.25	0.32	0.31
s__biforme	0.85	0.68	0.11	0.23	0.31	0.30
s__cylindroides	0.40	0.19	0.00	0.01	0.01	0.01
g__Bulleidia	0.72	0.83	0.21	0.35	0.42	0.38
s__p-1630-c5	0.17	0.37	0.11	0.19	0.33	0.24
s__unclassified	0.51	0.42	0.06	0.11	0.05	0.01
g__Catenibacterium	0.32	0.34	0.04	0.13	0.05	0.08
s__unclassified	0.32	0.34	0.04	0.13	0.05	0.08
g__p-75-a5	0.04	0.05	0.04	0.18	0.11	0.38
s__unclassified	0.04	0.05	0.04	0.18	0.11	0.38
g__Sharpea	0.41	0.24	0.05	0.12	0.01	0.05
s__azabuensis	0.41	0.23	0.05	0.11	0.01	0.05
c__unclassified	2.35	1.34	0.69	1.45	0.79	1.07
p__Proteobacteria	0.42	1.36	5.24	2.37	1.76	2.26
c__Gammaproteobacteria	0.35	1.24	4.85	2.16	1.60	2.02
o__Aeromonadales	0.27	1.11	4.68	1.89	1.28	1.12
f__Succinivibrionaceae	0.27	1.11	4.68	1.89	1.28	1.12
g__Succinivibrio	0.27	1.11	4.68	1.89	1.28	1.11
s__unclassified	0.27	1.11	4.68	1.89	1.28	1.11
o__Enterobacteriales	0.06	0.01	0.10	0.26	0.31	0.90
f__Enterobacteriaceae	0.06	0.01	0.10	0.26	0.31	0.90
g__Escherichia	0.05	0.01	0.10	0.26	0.30	0.89
s__coli	0.05	0.01	0.10	0.26	0.30	0.89
p__Tenericutes	0.04	0.15	0.38	0.55	1.35	1.10
c__Mollicutes	0.04	0.14	0.38	0.55	1.34	1.10
o__RF39	0.04	0.14	0.38	0.55	1.34	1.10
f__unclassified	0.04	0.14	0.38	0.55	1.34	1.10
p__unclassified	0.13	0.27	0.37	0.58	0.33	0.30
p__unclassified	1.08	0.61	0.52	0.82	0.56	0.76

*Only phyla with a contribution of >1% were included as well as all classes with a family level of >0.30%.
*Prefixes before names can be interpreted as follows: k__ kingdom; p__ phylum; c__ class; o__ order; f__ family; g__ genus; s__ specie

Table B3 Relative abundance of dominant OTUs at 35 days

Treatment	Sample	*Paraprevotellaceae*	*Clostridiaceae*	*Erysipelotrichaceae*	*Lachnospiraceae*	*Lactobacillaceae*	*Prevotellaceae*	*Ruminococcaceae*	*S24-7*	*Streptococcaceae*	*Succinivibrionaceae*	*Veillonellaceae*	unclassified
Control	**1C1**	125	2337	243	3218	4189	4518	1042	75	129	0	281	297
	1C2	581	150	47	1956	852	3029	1309	157	8	0	195	470
	1C3	230	273	748	4433	3468	4484	1804	208	9	11	1232	986
	1C4	23	473	609	3708	1167	711	1668	84	79	4	539	562
	1C5	412	1707	426	2414	3077	3804	1364	34	976	11	1207	984
	1C6	617	127	536	4916	2309	7634	3724	559	13	0	2252	1262
	1C7	22	6	122	3735	1390	1059	1493	35	340	2	132	14
	1C8	207	1	656	3538	670	6407	1866	681	18	0	994	308
	1C9	237	175	402	1330	1150	4264	1597	181	321	4	1011	98
	1C10	334	0	1094	3618	1051	3524	1710	57	5	0	1568	232
	1C11	769	702	156	1785	261	3822	1290	80	95	360	1361	241
	1C12	1402	1205	88	1301	868	5074	1621	67	49	12	408	308
	1C13	514	1891	452	1719	817	7281	2054	97	16	5	531	285
	1C14	402	59	66	993	780	1774	450	56	3	147	594	231
	1C15	45	17	516	5213	1545	3152	3043	324	7	10	243	144
Probiotic	**1P1**	586	631	415	1352	622	5230	2413	307	1070	40	1175	676
	1P2	82	16	494	2239	341	4293	2980	132	68	11	3082	324
	1P3	119	483	173	1656	1829	4783	3875	163	871	7	1288	719
	1P4	32	263	142	1642	2273	1090	629	13	15	0	7	90
	1P5	286	22	132	761	386	1793	989	41	58	0	194	389
	1P6	1002	877	269	891	100	3889	2159	171	285	1943	6024	339
	1P7	1200	366	458	2963	2225	10176	4158	530	18	30	2096	1044
	1P8	6	1	423	5777	678	443	644	4	10	0	503	23
	1P9	2	262	386	4153	2762	7971	4206	35	359	0	1623	171
	1P10	242	4	1126	3541	1283	3184	5641	386	97	768	2680	1840
	1P11	1464	1802	258	4662	597	9061	4695	250	643	1	538	200
	1P12	209	710	485	1879	1237	4680	3344	164	1872	26	1326	590
	1P13	235	3	537	3240	629	5053	2786	138	118	3	1237	406
	1P14	374	1124	378	3842	857	4060	3537	403	962	54	1805	321
	1P15	735	54	1008	4938	1118	8611	1895	209	2579	5	2061	435

Table B4 Relative abundance of dominant OTUs at 49 days

Treatment	Sample	*Paraprevotellaceae*	*Clostridiaceae*	*Erysipelotrichaceae*	*Lachnospiraceae*	*Lactobacillaceae*	*Prevotellaceae*	*Ruminococcaceae*	*S24-7*	*Streptococcaceae*	*Succinivibrionaceae*	*Veillonellaceae*	unclassified
Control	**2C1**	40	3	0	81	23	491	50	6	0	0	207	16
	2C2	425	59	18	761	177	3043	627	203	10	825	400	327
	2C3	21	13	0	92	83	633	121	22	0	23	221	44
	2C4	52	0	11	122	19	1007	271	17	0	344	259	170
	2C5	353	25	25	345	378	5919	573	49	87	21	990	512
	2C6	119	29	0	219	93	1064	87	8	0	3	269	8
	2C7	1	0	0	4	4	3	4	0	0	0	2	2
	2C8	649	1	54	720	1271	2556	609	301	8	470	2908	196
	2C9	857	28	93	1446	1088	4496	929	258	53	585	3067	485
	2C10	982	73	151	1896	845	6878	1390	351	1	700	3405	338
	2C11	844	114	93	744	542	5164	1370	217	2	648	2883	717
	2C12	692	50	58	1800	1061	4638	1109	241	5	1267	3281	485
	2C13	774	881	125	2441	1093	4536	1300	399	3	631	3064	631
	2C14	583	14	97	2142	1920	3880	1460	321	46	381	2540	663
	2C15	971	3	107	543	1132	4693	990	164	5	654	4579	368
Probiotic	**2P1**	817	132	229	1079	1387	2905	1539	558	5	45	2213	1153
	2P2	1058	92	79	1129	974	3783	727	500	10	2331	3365	248
	2P3	596	266	124	870	1164	3615	870	367	802	32	3812	416
	2P4	34	51	29	232	316	981	329	81	1	6	653	69
	2P5	479	314	278	1409	924	2525	1256	399	27	39	2980	544
	2P6	340	194	79	607	260	3892	554	249	7	79	2417	356
	2P7	699	42	40	960	530	3172	679	184	7	314	1585	932
	2P8	8	0	47	442	2119	101	326	1	133	4	1193	10
	2P9	288	66	153	1584	1231	1420	2023	92	847	43	1646	1209
	2P10	154	5	186	682	1922	3112	546	125	28	8	2701	146
	2P11	38	5	23	204	456	376	293	31	21	6	1826	247
	2P12	566	4	195	1489	2086	2373	1602	634	156	16	6716	494
	2P13	570	300	177	2590	1204	962	2169	191	1150	18	913	1112
	2P14	185	159	192	1175	1281	6600	1463	65	112	19	4459	349
	2P15	149	249	239	1563	233	1953	1701	68	1229	68	1034	227

Table B5 Relative abundance of dominant OTUs at 63 days

Treatment	Sample	*Paraprevotellaceae*	*Clostridiaceae*	*Erysipelotrichaceae*	*Lachnospiraceae*	*Lactobacillaceae*	*Prevotellaceae*	*Ruminococcaceae*	*S24-7*	*Streptococcaceae*	*Succinivibrionaceae*	*Veillonellaceae*	unclassified
Control	**3C1**	759	143	215	2176	1830	1169	2215	60	103	0	3373	380
	3C2	575	522	122	1761	1630	2643	1817	428	20	7	2367	1139
	3C3	823	118	143	1197	1301	2237	1152	171	341	248	3105	671
	3C4	131	72	152	1750	1545	1231	2067	30	421	58	2549	432
	3C5	27	3	1	25	34	86	29	5	11	6	59	20
	3C6	1001	45	84	2590	2051	5402	2239	187	297	716	10292	759
	3C7	3188	29	103	2269	2072	783	1994	221	500	20	598	306
	3C8	821	23	112	2439	1912	9269	2343	349	539	974	8050	774
	3C9	182	1358	437	3372	3517	968	3057	196	3227	12	812	1403
	3C10	695	27	346	1590	2147	7335	1931	213	487	8	9303	626
	3C11	444	197	114	1153	137	3101	3966	701	50	422	1902	1473
	3C12	168	752	81	941	1351	1046	1229	206	2579	23	424	884
	3C13	848	849	249	1507	361	4021	2516	641	800	343	3069	1174
	3C14	438	20	196	941	1837	3286	2301	244	687	171	5740	346
	3C15	678	15	33	1688	427	1886	1192	244	1560	22	1996	646
Probiotic	**3P1**	1106	1061	245	2648	1678	5105	2375	726	88	199	1531	1420
	3P2	344	24	301	1275	1242	5662	1269	613	144	491	4249	650
	3P3	2239	528	426	2360	1034	2931	2218	57	1677	53	1426	747
	3P4	180	380	75	1028	1112	1386	764	36	30	44	2575	310
	3P5	614	32	128	1008	806	3975	1160	329	520	209	3167	567
	3P6	17	544	106	753	1334	82	693	25	7	5	115	518
	3P7	386	1	71	949	1252	3969	1040	57	186	542	4536	359
	3P8	384	0	23	455	823	1404	632	33	68	32	2239	169
	3P9	134	27	109	1247	660	746	1553	198	3093	0	1407	398
	3P10	160	202	43	2282	827	1285	1560	406	322	43	4468	862
	3P11	676	33	149	2389	1609	1905	2588	74	140	158	9124	481
	3P12	94	515	233	3388	2524	2618	1766	147	403	277	6489	875
	3P13	786	49	243	2051	488	4235	2174	145	1188	404	5512	679
	3P14	476	117	444	1825	1429	3738	2700	559	955	19	4196	870
	3P15	293	3	376	2327	3373	667	1945	32	2222	2	7023	178

Table B6 Shannon diversity index

Group	Good's Coverage	Number of Sequences	Shannon Index	Shannon Evenness
1C1	0.98	2349	3.23	0.678
1C2	0.98	2349	3.99	0.79
1C3	0.98	2349	3.58	0.737
1C4	0.98	2349	3.56	0.726
1C5	0.99	2349	3.46	0.729
1C6	0.98	2349	3.58	0.724
1C7	0.99	2349	2.63	0.602
1C8	0.99	2349	3.53	0.78
1C9	0.99	2349	3.54	0.742
1C10	0.99	2349	3.27	0.713
1C11	0.97	2349	3.78	0.742
1C12	0.98	2349	3.5	0.704
1C13	0.98	2349	3.46	0.71
1C14	0.98	2349	3.83	0.756
1C15	0.98	2349	3.44	0.708
1P1	0.98	2349	3.93	0.761
1P2	0.97	2349	3.67	0.728
1P3	0.98	2349	3.54	0.716
1P4	0.99	2349	3.07	0.7
1P5	0.99	2349	3.58	0.761
1P6	0.98	2349	3.54	0.725
1P7	0.98	2349	3.87	0.763
1P8	0.99	2349	2.38	0.556
1P9	0.99	2349	3.15	0.719
1P10	0.98	2349	3.72	0.761
1P11	0.99	2349	3.33	0.726
1P12	0.98	2349	3.62	0.72
1P13	0.98	2349	3.32	0.692
1P14	0.98	2349	3.76	0.742
1P15	0.99	2349	3.19	0.68
2C2	0.97	2349	3.91	0.745
2C4	0.98	2349	3.33	0.661
2C5	0.98	2349	3.19	0.646
2C8	0.98	2349	3.47	0.689
2C9	0.97	2349	3.75	0.724
2C10	0.97	2349	3.49	0.687
2C11	0.96	2349	4.03	0.747
2C12	0.97	2349	3.79	0.725
2C13	0.96	2349	4.11	0.762
2C14	0.96	2349	4.09	0.75
2C15	0.97	2349	3.47	0.667
2P1	0.96	2349	4.48	0.805
2P2	0.96	2349	3.91	0.732
2P3	0.97	2349	3.71	0.707
2P4	0.98	2349	3.4	0.696
2P5	0.96	2349	4.13	0.765
2P6	0.97	2349	3.6	0.694
2P7	0.97	2349	4	0.755
2P8	0.99	2349	2.86	0.664
2P9	0.98	2349	3.89	0.786
2P10	0.98	2349	3.45	0.709
2P11	0.98	2349	3.28	0.654
2P12	0.98	2349	3.78	0.737
2P13	0.97	2349	4.25	0.801
2P14	0.98	2349	3.39	0.683
2P15	0.98	2349	3.97	0.768

3C1	0.97	2349	4.05	0.761
3C2	0.96	2349	4.34	0.788
3C3	0.96	2349	4.13	0.759
3C4	0.97	2349	3.86	0.739
3C6	0.96	2349	3.46	0.657
3C7	0.97	2349	3.67	0.701
3C8	0.97	2349	3.67	0.716
3C9	0.97	2349	3.87	0.742
3C10	0.97	2349	3.41	0.668
3C11	0.97	2349	4.14	0.771
3C12	0.97	2349	3.73	0.71
3C13	0.96	2349	4.44	0.803
3C14	0.98	2349	3.44	0.684
3C15	0.97	2349	3.9	0.738
3P1	0.96	2349	4.44	0.811
3P2	0.97	2349	3.82	0.733
3P3	0.97	2349	4	0.76
3P4	0.97	2349	3.62	0.698
3P5	0.95	2349	4.19	0.757
3P6	0.97	2349	3.88	0.728
3P7	0.98	2349	3.49	0.695
3P8	0.97	2349	3.48	0.664
3P9	0.98	2349	3.41	0.659
3P10	0.97	2349	3.98	0.757
3P11	0.97	2349	3.2	0.624
3P12	0.97	2349	3.55	0.677
3P13	0.97	2349	3.88	0.726
3P14	0.96	2349	4.12	0.769
3P15	0.99	2349	2.92	0.633

Table B7 Temperature recording (°C) inside the pig unit between 47-49 days of age

Age (Days)	Date	Time	Temperature (°C)
Day 47	2015/11/10	06:00:16	21.80
	2015/11/10	07:00:16	23.70
	2015/11/10	08:00:16	25.90
	2015/11/10	09:00:16	27.70
	2015/11/10	10:00:16	28.80
	2015/11/10	11:00:16	29.80
	2015/11/10	12:00:16	31.00
	2015/11/10	13:00:16	32.00
	2015/11/10	14:00:16	32.50
	2015/11/10	15:00:16	31.60
	2015/11/10	16:00:16	31.80
	2015/11/10	17:00:16	31.50
	2015/11/10	18:00:16	30.90
	2015/11/10	19:00:16	28.90
	2015/11/10	20:00:16	27.60
	2015/11/10	21:00:16	26.90
	2015/11/10	22:00:16	25.60
	2015/11/10	23:00:16	26.10

Day 48	2015/11/11	00:00:16	25.40
	2015/11/11	01:00:16	24.40
	2015/11/11	02:00:16	23.40
	2015/11/11	03:00:16	23.60
	2015/11/11	04:00:16	23.20
	2015/11/11	05:00:16	21.70
	2015/11/11	05:25:16	**21.50**
	2015/11/11	06:00:16	22.10
	2015/11/11	07:00:16	24.30
	2015/11/11	08:00:16	26.80
	2015/11/11	09:00:16	28.60
	2015/11/11	10:00:16	29.90
	2015/11/11	11:00:16	31.10
	2015/11/11	12:00:16	31.90
	2015/11/11	13:00:16	32.70
	2015/11/11	13:50:16	**33.60**
	2015/11/11	14:00:16	33.50
	2015/11/11	15:00:16	33.10
	2015/11/11	16:00:16	32.60
	2015/11/11	17:00:16	32.20
	2015/11/11	18:00:16	31.50
	2015/11/11	19:00:16	30.10
	2015/11/11	20:00:16	29.00
	2015/11/11	21:00:16	29.40
	2015/11/11	22:00:16	29.20
	2015/11/11	23:00:16	28.40
Day 49	2015/11/12	00:00:16	29.30
	2015/11/12	01:00:16	27.90
	2015/11/12	02:00:16	26.70
	2015/11/12	03:00:16	24.70
	2015/11/12	04:00:16	23.30
	2015/11/12	05:00:16	23.00
	2015/11/12	06:00:16	23.40