ROLE OF DIETARY ZINC IN SHAPING THE GASTROINTESTINAL MICROBIOTA IN NURSERY PIGS

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IN NURSERY PIGS

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Abstract: Gut microbiota play an important role in extraction, synthesis and absorption of nutrients. Commensal bacteria prevent pathogenic bacteria colonization and maintain intestinal epithelium integrity. The most common families of commensal bacteria in nursery pigs are *Prevotellaceae, Clostridiaceae, Erysipelotrichaceae, Lachnospiraceae, Lactobacillaceae, Ruminicoccaceae* and *Streptoccocaceae*. Understanding the microbial abundance shifts that causes health disruption leading to diarrhea and stunted growth performance can be of great benefit for developing mitigation strategies. Next generation sequencing (NGS) technology facilitates metagenomic approaches, developing sequencing profiles representing any and all organisms within a sample. Electronic-probe Diagnostic Nucleic acid Analysis (EDNA) is a bioinformatic tool originally developed to detect species-specific plant pathogen targets in metagenomic databases. EDNA has been shown to reduce time to detect microbial signatures in large metagenomic sequence data. However, it has not previously been used as a metagenomic tool for assessing microbiome composition at the family level. Therefore, E-probes for detection of gut microbiota of the seven most common commensal families were developed. The hits were able to detect the relative abundance variations of the 4-time periods. The current study confirms the importance of bacterial taxa influencing growth and diarrhea, although data are conflicting in some cases. Nonetheless, as demonstrated by the E-probes, specific bacterial taxa are consistently associated with growth performance in the nursery phase. In conclusion, the growth performance and diarrhea-associated bacterial taxa identified in this research could potentially be used to identify microbiota changes promptly that could lead to health impairment and economic impact on pig production. Overall, the approach outlined here can reduce the time to detect microbiota changes essential for determining a pig's health status, decreasing the side effects of unhealthy animals in the swine production.

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CHAPTER I

1.1 INTRODUCTION

In the commercial swine industry, the weaning period is a vulnerable stage associated with several stress factors causing intestinal and immune system dysfunctions resulting in reduced feed intake, reduced growth, increased pathogen infections and intestinal diseases (Boudry et al., 2004; Campbell et al., 2013; Lallès et al., 2004) lasting days or weeks, depending on how susceptible the weaned pig becomes to the stress factors (Lallès et al., 2007). At weaning, the pig immune system is not fully competent yet, therefore, several nutritional strategies have been studied in order to minimize the effects of weaning by improving health status and growth performance (Heo et al., 2013).

In-feed antibiotics have long been used as growth promoters; however, since 2017 in the United States its use has been banned due to the increasing concern of antibiotic resistance genes transfer to humans from livestock products (Centner, 2016). The new regulations prompted the livestock industry, including swine, to propose alternative substances to control diseases and improve growth performance (Lallès et al., 2004).

Trace mineral supplementation has shown positive effects to improve the health and performance of pigs. The functions of minerals range from structural to regulatory, including efficient protein and energy usage (NRC, 2012).

Among trace minerals, zinc has been reported to have antimicrobial, immune modulation, gut integrity (Li et al., 2001), and feed-intake regulation properties (Yin et al., 2009). Furthermore, zinc is a cofactor of several metalloenzymes involved in protein, carbohydrate, lipid metabolism, and also oxidative enzymes (Underwood & Suttle, 1999), making it important to maintain normal metabolism and growth (Suttle, 2010). However, meeting the physiological mineral requirements of pigs depend on the bioavailability of the mineral source in the diet (Cohen, 2014; NRC, 2012).

Results from our lab (Schaaf, 2017) suggest that pigs fed Zn hydroxychloride (IBZ; Intellibond Z, Micronutrients, Indianapolis, IN) had improved growth performance compared to the those fed sulfate sources, even when sulfates are the source most widely used in swine diets (Villagómez-Estrada et al., 2020). However, the effects of zinc sources are still not well understood (Cromwell et al., 1998) in the indigenous gastrointestinal bacteria development and its relationship with growth performance and diarrhea appearance in nursery pigs (Soler et al., 2018).

Gut microbiota plays an important role in the extraction, synthesis, and absorption of nutrients. In the case of pigs and other farm animals, the gastrointestinal microbiota affects the animal health status, immune response, meat quality, and body weight (Knecht et al., 2020). Commensal bacteria prevent pathogenic bacteria colonization and maintain intestinal epithelium integrity, having a great role in preventing diseases. The relative

abundance of the commensal bacteria in the intestinal tract of pigs varies throughout their life (Isaacson & Kim, 2012; Kim et al., 2011; Li et al., 2017; Rinninella et al., 2019; Soler et al., 2018). The decrease in the number of commensal bacteria, including *Lactobacillus sobrius*, *L. acidophilusand, L. reuteri,* and an increase in pathogenic *E. coli* count accelerate the division of cells in the epithelium of the small intestine preventing them to reach full maturity, affecting carbohydrate digestion and overall digestive processes (Knecht et al., 2020). A better understanding of the microbial abundance shifts that cause health disruption leading to diarrhea and stunted growth performance will allow the swine industry to prevent negative impacts in the production system (Tran et al., 2018).

Next-generation sequencing (NGS) technology facilitates metagenomics approaches, developing sequencing profiles representing any and all organisms within a sample. Electronic-probe Diagnostic Nucleic acid Analysis (EDNA) is a bioinformatics tool originally developed to detect species-specific plant pathogen targets (bacteria, virus, fungi and oomycete) in metagenomics databases (Stobbe et al., 2013). EDNA has been demonstrated to decrease time of assembly and analysis of large amounts of sequence data (Espindola et al., 2015; Espindola & Cardwell, 2021). However, it has not previously been used as a metagenomics tool for assessing gastrointestinal microbiota composition.

Therefore, the objective of this study was to evaluate the role of two dietary zinc sources on growth performance, diarrhea presence, and in shaping the gastrointestinal microbiota in nursery pigs using E-probes to track changes in the most common gastrointestinal commensal families: *Prevotellaceae, Clostridiaceae,*

Erysipelotrichaceae, *Lachnospiraceae*, *Lactobacillaceae*, *Ruminicoccaceae and Streptoccocaceae*.

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CHAPTER II

2.1 NURSERY PIGS NUTRITION AND GROWTH

2.1.1 Biological stress of weaning

In intensive swine production systems, pigs are weaned at an early age leading to stress and higher susceptibility to infections (Lallès et al., 2007). The weaning period is one of the most challenging and stressful times for the pigs. Weaned pigs will be exposed to social and physical stressors, such as maternal and littermate separation, environmental changes, new diets (Moeser et al., 2007), and exposure to pathogenic microorganisms (Schokker et al., 2015) which can lead to detrimental effects on gastrointestinal health, immunity, and performance, resulting in reduced productivity and large economic losses (Campbell et al., 2013; Lallès et al., 2007).

2.1.1.1 Weaning and the adaptation to dry food

Sow milk contains secretory immunoglobulins and enzymes that help the piglets be less vulnerable to opportunistic pathogens. However, at weaning, pigs switch from

sow's milk to a more complex and less digestible solid feed (Heo et al., 2013; Lallès et al., 2007) resulting in effects on intestinal development, feed and water intake reduction, and leading to a post-weaning lag phase, diarrhea and high mortality (Campbell et al., 2013; Lallès et al., 2004).

2.1.1.2 Post-weaning diarrhea in nursery pigs

The physical presence of food in the gastrointestinal tract (GIT) is necessary for structural and functional maintenance of intestinal mucosa. Acute and long-lasting changes in the intestinal physiology and morphology, such as villous atrophy and crypt hyperplasia are observed after post-weaning fasting, followed by intestinal maturation upon feed intake restarts (Lallès et al., 2007). Short villous and deeper crypts have fewer absorptive and more secretory cells resulting in a decreased absorption and increased secretion capacity. A lower digestion and absorption capacity is associated with the development of an osmotic diarrhea (Boudry et al., 2004).

Additionally, enterotoxigenic *Escherichia coli* (*E. coli*) in the small intestine is associated with post-weaning diarrhea. Infected pigs have an affected absorption of nutrients, therefore, stunted growth is often observed in the first couple weeks after weaning (Hu et al., 2013).

2.1.1.3 Weaning and the gastrointestinal bacterial population

Early weaning is characterized by substantial changes in the gastrointestinal microbial population (Campbell et al., 2013). Following weaning, Lactobacilli were detected at significantly lower levels in the gastrointestinal tract when compared to unweaned piglets where Lactobacilli were abundant colonizers. Additionally, after the early post-weaning period, an increase of Clostridia spp and *E.coli* were observed

(Konstantinov et al., 2006). The presence of Lactobacilli, a beneficial microbe, is important during the weaning period when the immature immune system of the piglet depends on the sow's milk to prevent growth of opportunistic bacteria (Lallès et al., 2007).

Nutritional strategies to alleviate the weaning transition phase have been used (Campbell et al., 2013; Heo et al., 2013; Lallès et al., 2007) to enhance the functioning and optimum maintenance of the immune system (Chandra, 1997; Stafford et al., 2013; Wintergerst et al., 2007).

2.1.2 Zinc

In 1869, the importance of zinc was discovered due to its essential role for bread mold development, *Aspergillus niger* (Raulin, 1905). Zinc, the 24th trace element in abundance in the earth's crust, was found to have a growth promoting effect in rats followed by the recognition and confirmation of zinc deficiency in humans in later years. In 1973, the World Health Organization first established a dietary zinc requirement (WHO, 1996) in humans. Nowadays, in the European Union, the maximum recommended inclusion rate is 150 mg zinc/kg in pig diets (Pieper et al., 2020).

Zinc, in the cell, is found in the cytosol (50%), nucleus (30-40%), organelles and specialized vesicles, and the remainder is found in cell membranes (Bonaventura et al., 2014). Furthermore, zinc has catalytic, cocatalytic and structural function and works in cooperation with metalloproteins and metalloenzymes (Vallee & Falchuk, 1993). Additionally, zinc plays an important role in regulating gene expression, nutrient metabolism, immune function, and health (Rink & Kirchner, 2000).

2.1.2.1 Zinc and the gastrointestinal tract

The gastrointestinal tract is the major site for absorption of exogenous zinc and excretion of endogenous zinc (Krebs, 2000). The small intestine, especially duodenum and jejunum, are the primary absorption sites for zinc (Kambe et al., 2015). Absorption takes place by unsaturable mechanisms through passive transport, and saturable mechanisms by carrier mediated processes (Wang & Zhou, 2010). The saturable mechanism is controlled by the binding proteins of the lipid bilayer, ZIP and ZNT. ZIP acts as an importer by transporting zinc from extracellular space or cellular compartments into cytosol whereas ZNT acts as an exporter by exporting zinc out of the cytosol (Lichten & Cousins, 2009).

Zinc plays an important role in improving intestinal morphology (Carlson et al., 1999). Li et al. (2001) reported intestinal morphology modification; increased villus height and decreased crypt depth, in the jejunum when feeding pharmacological concentrations of zinc oxide (3000 ppm).

2.1.2.1.1 Zinc and the gastrointestinal microbiota

Zinc is essential for bacteria in the gastrointestinal tract. In rats, about twenty-percent of the dietary zinc is used by the gut microbiota (Sauer & Grabrucker, 2019).

2.1.2.2 Zinc and the immune system

Zinc availability plays an important role in the regulation of the immune system (Haase & Rink, 2014; Wellinghausen et al., 1997). Zinc is required in immune function since the immune cells contain a wide number of zinc containing enzymes, and zinc finger proteins (Haase & Rink, 2014). Additionally, zinc is required as enzymatic cofactor and immune cell signaling (Rink & Kirchner, 2000). The main functions of zinc are as a

neurotransmitter, being the first messenger in cell to cell communication, or as an intracellular signaling molecule (Bonaventura et al., 2014).

The innate immune system (polymorphonuclear cells, macrophages, and natural killer cells) is impaired by decreased zinc concentration (Jarosz et al., 2017; Rink & Haase, 2006). Polymorphonuclear cells directed movement is induced by a gradient of chemical substance; chemotaxis. Reduced concentration of polymorphonuclear cells is observed in zinc deficiency due to zinc's participation in the chemo attractant process between the immune cells (Rink & Kirchner, 2000).

Stafford et al. (2013) stated that a type of leukocyte, the monocyte, amounts and functions are affected by zinc availability. Macrophages enhance their microbicide activity (Stafford et al., 2013) and phagocytic capacity (van Heugten et al., 2003). Moreover, zinc induces monocytes to produce cytokines (Haase & Rink, 2014; Rink & Kirchner, 2000; Wellinghausen et al., 1997), moderating the overproduction of proinflamatory cytokines during immune response (Bin et al., 2003).

Zinc, an essential micronutrient (McDowell, 2003), is necessary for the proper development of immune cells, specially T-lymphocyte development and proliferation (Kubena & McMurray, 1996). T cell production and maturation in the thymus is influenced by zinc availability as a cofactor for thymulin enzyme (Jarosz et al., 2017; Wellinghausen et al., 1997). Moreover, supplementation of different sources of zinc significantly improved red blood cells antibody titer in goats, and the proliferation of cell-mediated and humoral components of the immune system in weaned pigs (Li et al., 2016) possibly affecting the negative effects of an immunological stress (Guo-jun et al., 2009).

Extreme zinc deficiency increases the susceptibility to bacteria, viral, and fungal infections due to a profound suppressive effect on thymic function (Kubena & McMurray, 1996; Sullivan et al., 1980). In the case of zinc deficiency in rats, increased lipid peroxidation in liver is observed (Sullivan et al., 1980).

2.1.2.3 Zinc and performance

The nutritional zinc requirement for weaned pigs with body weights ranging between 5 and 11 kg is 100 mg of zinc per kg of diet. At the end of the nursery phase, at around 25 kg of body weight, zinc requirements are reduced to 80 mg of zinc per kg of diet (NRC, 2012).

Zinc is a cofactor of more than 300 metalloenzymes (Suttle, 2010), particularly those involved in protein, carbohydrate and lipid metabolism such as carbonic anhydrase, alkaline phosphatase and zinc-binding proteins, including metallothionein (McDowell, 2003). DNA and RNA synthetases and transferases have zinc as a cofactor (NRC, 2012). Additionally, collagen and keratin, structural proteins, require zinc for their synthesis. Keratin is the major structural protein of the hoof and skin, while collagen is the major structural protein of the extracellular matrix and connective tissues, including cartilage and bone (Underwood & Suttle, 1999).

Zinc is needed for epithelial cell differentiation and wound healing promotion (Chandra, 1997; Jensen-Waern et al., 1998). The gastrointestinal tract and immune system have significant high turnover rates with high DNA and protein synthesis requirements; therefore, zinc has an important effect on them. Furthermore, zinc contributes to normal intestinal barrier function, and also to the regeneration of damaged gut epithelium.

Moreover, dietary zinc has been shown to reduce intestinal permeability after weaning (Zhang & Guo, 2009).

Zinc also plays a role in the production, storage, and secretion of individual hormones as well as in the effectiveness of receptor sites (McDowell, 2003). Insulin, adrenal corticosteroids, and testosterone production and secretion are primarily affected during zinc deficiency (NRC, 2012). Furthermore, zinc shortage results in impairment of immune function (Rink & Kirchner, 2000), parakeratosis, reduced feed intake, growth retardation and diarrhea in young pigs (McDowell, 2003; NRC, 2012). Mechanisms include alteration of the intestinal microbiota composition and activity (Pieper et al., 2020). Moreover, growth may be impaired by amino acid utilization and protein synthesis when in zinc deficiency (McDowell, 2003).

Zinc bioavailability is influenced by source and dietary levels, subsequently affecting plasma concentration. Cereal grains and plant protein have low bioavailability related to impaired zinc digestion due to their phytic acid content (McDowell, 2003); however, it may be enhanced by microbial phytase supplementation (NRC, 2012). Zinc sulfate, inorganic nutritional source of zinc (Hill et al., 2014), is highly hygroscopic, resulting in the breakdown of vitamins, fatty acid, and other nutrients in the diet, while tetrabasic zinc chloride (TBZC) is insoluble in water, therefore, more chemically stable (Zhang & Guo, 2007).

Pharmacological concentrations of zinc (1500 – 3000 mg Zn/kg) in nursery pig diets, tetrabasic zinc chloride or zinc oxide, increase weight gain and feed efficiency; however, tetrabasic zinc chloride has a higher feed efficiency (Mavromichalis et al., 2001) and may enhance weight gain at lower dosages (Zhang & Guo, 2007). Nevertheless,

pharmacological concentrations of zinc have been limited in nursery diets due to environmental concerns.

2.2 OBJECTIVE AND HYPOTHESIS

Recent results from our lab (Schaaf, 2017) suggest that pigs fed Zn hydroxychloride (IBZ; Intellibond Z, Micronutrients, Indianapolis, IN) had improved growth performance compared to those fed sulfate sources, even when sulfates are the source most widely used in swine diets (Villagómez-Estrada et al., 2020). However, the effects of zinc sources with growth performance and diarrhea appearance in nursery pigs is still not well understood (Cromwell et al., 1998; Soler et al., 2018). Therefore, the aim of our study was to compare the growth performance in nursery pigs supplemented with Zn Sulfate and Zn IBZ. We hypothesized that pigs fed Zn IBZ have a lower diarrhea incidence leading to a better performance assessed by end weight, average daily gain (ADG), average daily feed intake (ADFI) and feed conversion (F:G); in accordance to previous growth performance studies in our laboratory.

2.3 MATERIALS AND METHODS

Animals, housing and treatments

The experimental protocol was approved, and the pigs were handled and cared for according to the guidelines established by the Oklahoma State University Institutional Animal Care and Use Committee (IACUC).

A total of sixty crossbred weaned pigs (3 weeks of age; PIC®; 30 barrows and 30 gilts) were subject to a research trial performed for a period of 6 wk. Pigs with an average initial body weight (BW) of 5.4 kg were randomly allotted to one of two dietary zinc

treatments. Three replicate pens per treatment consisting of ten pigs per pen (5 barrows and 5 gilts) were blocked to minimize variations in gender, initial BW, and location of the pen in a randomized complete block design. Pigs were housed in an environmentally controlled nursery facility with slatted plastic flooring and a mechanical ventilation system. The environmental temperature decreased by 1˚C per week, starting at 30˚C in the first week. During the entire experiment, pigs were allowed to consume feed and water *ad libitum*. Each pen had a nipple waterer and a stainless steel feeder.

Form day 0 until day 42, the treatment diets were allotted to pens: Sulfate (100 ppm of added Zn Sulfate) or Zn IBZ (100 ppm of added Zinc hydroxychloride; Intellibond Z, Micronutrients, Indianapolis, IN). All remaining nutrients in the diet were added at or above the requirements listed in the NRC (2012). Both diets were formulated as basal diets, and each treatment mineral premix was added subsequently during the mixing process.

Diets

A four-phase nursery feeding program was employed in the experiment, with diets formulated to meet or exceed NRC (2012) nutrient requirements as presented in Table 1 and 2. Pigs were fed a common diet with added zinc from Zn Sulfate or Zn IBZ: phase 1 (100 mg Zn/kg), phase 2 (100 mg Zn/kg), phase 3 (100 mg Zn/kg), and phase 4 (100 mg Zn/kg). The experiment lasted for 42 days and all pigs received Phase 1 diet during d 0-7, Phase 2 during d 7- 14, Phase 3 during d 14-23 and Phase 4 during d 23-42.

Table 1. Nutrient composition of the diets

	Phase 1 d 0-7	Phase 2 d 7-14	Phase 3 d 14-21	Phase 4 d 21-42
Ingredient, %				
Corn, yellow dent	32.57	38.56	54.12	59.27
Soybean meal, 47.5% CP	15	20	26.32	34.3
Whey, dried	25	25	10	0
Lactose	7	0	0	0
Plasma spray-dried	6	2.5	0	0
Blood cell spray-dried	0	1.25	1.25	0
Fish meal, menhaden	6	4	2	0
Soy protein concentrate	2.21	2.12	0	0
Soybean oil	4	4	3	3
L-lysine HCl	0.17	0.21	0.27	0.25
DL-methionine	0.18	0.21	0.17	0.11
L-threonine	0.07	0.1	0.12	0.09
Dicalcium phosphate 18.5%	0.67	0.93	1.39	1.58
Limestone	0.45	0.44	0.72	0.74
Salt	0.5	0.5	0.5	0.5
Vitamin Premix	0.05	0.05	0.05	0.05
Mineral Premix[a]	0.1	0.1	0.1	0.1
Choline Cl	0.03	0.03	0	0
Total	100	100	100	100

[a]Mineral Premix:
Mineral premix containing 100 mg/kg Zn from zinc sulfate or IBZ (Intellibond Z, Micronutrients, Indianapolis, IN).

Table 2. Chemical composition of the basal diets

Calculated Analysis	N1	N2	N3	N4
ME, kcal/kg	1585	1577	1551	1557
CP, %	22.9	23	20.9	21.5
SID Lysine, %	1.56	1.51	1.31	1.25
Ca, %	0.9	0.85	0.85	0.75
Available P, %	0.6	0.55	0.45	0.37
Added Zn, ppm	100	100	100	100

Growth performance

Pigs BW and feeders were weighed weekly, starting on d 0 (initial weight), followed by d 7, 14, 21, 28, 35 and 42. Feed was weighed before feeding at every feeding time according to the treatment. The feed intake was calculated weekly by subtracting the feeder with the weekly remaining feed to the initial feeder weight. Average daily gain (ADG), average daily feed intake (ADFI) and feed efficiency (F:G) were determined.

Fecal consistency by pen

Fecal consistency scoring was visually assessed for pigs in each pen and scoring was done weekly from d 0 to d 42. Fecal scoring was according to the following scale: 1 = solid; 2 = semi-solid; and 3 = liquid. Diarrhea was considered when feces at level 2 or 3 were present for 2 continuous days (Liu et al., 2010).

Fecal consistency by pig size categories

Fecal consistency scoring was visually assessed to pigs in pen in the three different size categories (large, medium, and small) blinded to treatments on d 0, 14, 28, and 42. Pigs were categorized before trial and ear notched for identification throughout the experiment. Fecal scoring was according to the following scale: 1 = solid; 2 = semi-solid; and 3 = liquid. Diarrhea was considered when feces at level 2 or 3 were present for 2 continuous days (Liu et al., 2010).

2.4 STATISTICAL ANALYSIS

Performance data were analyzed as a randomized complete block design with initial body weight as the blocking effect. Growth performance was analyzed using a GLM procedure (SAS Institute, version 9.2). The data were analyzed as a randomized complete block design with the model including the effects of treatment (zinc source) in growth performance (weight at d 42, average daily gain (ADG), average daily feed intake (ADFI) and feed efficiency (F:G). The pen was considered the experimental unit. The variability of the data was expressed as the standard error (SE), the treatment means are presented as least square means, and the level of significance was set at $P \leq 0.05$, while ≤ 0.10 P-value > 0.05 was considered a tendency.

Fecal consistency data were analyzed as a randomized complete block design with initial body weight as the blocking effect. The normality and homoscedasticity of data were examined using the Shapiro-Wilk test and assessing the normal plot before statistical analysis. Fecal consistency scoring was rank-transformed and analyzed using the MANOVA repeated measures of the GLM procedure (SAS Institute, version 9.2). The pen was considered the experimental unit for the fecal consistency analysis and the pig was considered the experimental unit for the individual fecal consistency data. The data were analyzed as a randomized complete block design with the model including the effects of treatment by pen; however, treatment, size, and interactions were analyzed by pig. The variability of the data was expressed as the standard error (SE); the response means are presented as least-square means of the transformed data. Significantly different least square means were separated using Tukey adjust. The level of significance was set at P-value ≤ 0.05, while ≤ 0.10 P-value > 0.05 was considered a tendency.

2.5 RESULTS

Growth performance by zinc treatment

The initial body weight (BW) was significantly different ($P < 0.0001$), with a mean of 5.26 kg for pigs starting on Zn Sulfate diet and 5.58 kg for pigs starting on Zn IBZ diet on d 0. On d 42, BW, ADG, ADFI, and F:G ratio were different ($P < 0.01$); Table 3. For pigs fed Zn Sulfate, the body weight mean was lower when compared to the pigs fed Zn IBZ (BW = 21.02 kg and 21.87 kg; ADG = 0.375 kg and 0.388 kg; ADFI = 0.54 kg and 0.533 kg). There was a difference ($P = 0.0106$) for pigs fed the Zn IBZ source to have a better F:G ratio.

Table 3. Effect of zinc source on growth performance of nursery pigs[a]

	Zinc Sulfate	Zinc IBZ[b]	SE[c]	P-value
Initial BW, kg	5.26	5.58	0.032	< 0.0001
Final BW, kg	21.02	21.87	0.118	< 0.0001
ADG, kg	0.375	0.388	0.002	< 0.0001
ADFI, kg	0.54	0.533	0.006	0.0036
F:G	0.653	0.647	0.004	0.0106

[a]Least square means for 3 pens/ treatment.
[b]IBZ; Intellibond Z, Micronutrients, Indianapolis, IN.
[c]SE: Standard Error.

Fecal consistency scoring

Fecal consistency by pen

There are evidence of a time (day) effect ($P < 0.0001$) in the model. Additionally, the treatment effect ($P < 0.0001$) was dependent on time (day), Table 4.

Table 4. Time[a] and effect of treatment[b] on fecal consistency of nursery pigs by pen

	P-value
Time	< 0.0001
Time*Trt	< 0.0001

[a]Time (Day 0, Day 7, Day 14, Day 21, Day 28, Day 35, Day 42).
[b]Treatment (Zinc Sulfate or Zinc IBZ).

On d 0 and 42, there was no zinc source difference (P = 1.00) with a mean score of 2. On d 7, 14, 28, and 35 pigs fed Zn Sulfate presented a lower (P < 0.0001) fecal scoring when compared to those fed Zn IBZ. However, on d 21 pigs fed Zn IBZ presented a lower (P < 0.0001) fecal scoring (1.3 vs 2.3); Table 5.

Table 5. Effect of zinc source on fecal consistency[a] of nursery pigs by pen

	Zinc Sulfate	Zinc IBZ[b]	SE[c]	P-value
Day 0	2.0	2.0	0.00	1.00
Day 7	1.7	2.0	0.06	< 0.0001
Day 14	1.3	2.3	0.08	< 0.0001
Day 21	2.3	1.3	0.13	< 0.0001
Day 28	2.0	2.7	0.06	< 0.0001
Day 35	1.7	2.0	0.06	< 0.0001
Day 42	2.0	2.0	0.00	1.00

[a]Fecal consistency (1 = solid; 2 = semi-solid; and 3 = liquid).
[b]IBZ; Intellibond Z, Micronutrients, Indianapolis, IN.
[c]SE: Standard Error.

Fecal consistency by pig size categories

The scores decreased over time although inconsistent. There is evidence that there is a time (day) effect in the model. Additionally, the treatment by size effect was dependable on time (day); Table 6.

Table 6. Time[a] effect of treatment[b] and size[c] on fecal consistency of nursery pigs by categories

	Wilk's Λ	P-value
Time	0.1082	< 0.0001
Time*Trt*Size	0.6784	< 0.0001
Time*Trt	0.9362	0.0927
Time*Size	0.4057	< 0.0001

[a]Time (Day 0, Day 14, Day 28, Day 42).
[b]Treatment (Zinc Sulfate or Zinc IBZ).
[c]Size (Large, medium/average and small).

On d 0 and 42, there was a treatment by size difference ($P < 0.01$). On d 0, medium size pigs fed Zn IBZ presented a lower ($P < 0.0001$) fecal scoring when compared to those fed Zn Sulfate (2.0 vs 2.3), large and small pigs presented no difference ($P = 1.0$). On d 42, large size pigs fed Zn Sulfate presented a lower ($P < 0.05$) fecal scoring when compared to those fed Zn IBZ (1.0 vs 1.5). On the contrary, small size pigs fed Zn IBZ presented a lower ($P < 0.05$) fecal scoring when compared to those fed Zn Sulfate (1.0 vs 1.3), and medium pigs presented no difference ($P = 1.0$); Table 7.

On d 14, there was no difference ($P > 0.1$). On the contrary, on d 28, small size pigs fed Zn IBZ presented a lower ($P < 0.05$) fecal scoring when compared to those fed Zn Sulfate (1.3 vs 1.7), medium and large pigs presented no difference ($P = 1.0$); Table 7.

Table 7. Effect[a] of treatment[b] by size[c] on fecal consistency of nursery pigs by categories[c]

	Large			Medium			Small		
	$ZnSO_4$[d]	IBZ[e]	P-value	$ZnSO_4$[d]	IBZ[e]	P-value	$ZnSO_4$[d]	IBZ[e]	P-value
Day 0	2.0	2.0	1.000	2.3	2.0	< 0.0001	2.0	2.0	1.000
Day 14	3.0	2.5	0.700	1.3	1.7	0.700	2.0	2.0	1.000
Day 28	1.0	1.0	1.000	1.0	1.0	1.000	1.7	1.3	0.008
Day 42	1.0	1.5	0.004	1.0	1.0	1.000	1.3	1.0	0.004

[a]Equation: Day (0, 14, 28, and 42) = Treatment Size Treatment*Size
[b]Treatment (Zinc Sulfate or Zinc IBZ).
[c]Categories/Size (Large, medium/average and small).
[d]Zinc Sulfate
[e]IBZ; Intellibond Z, Micronutrients, Indianapolis, IN.

2.6 DISCUSSION

The weaning period is one of the most stressful phases in swine production. During this period, the piglets are more vulnerable and must rapidly adapt to a multitude of stressors often leading to impairment in intestinal epithelial barrier function causing digestive problems, post-weaning diarrhea, growth retardation, and increased mortality rate (Hu et al., 2012; Knecht et al., 2020).

For decades, zinc has been used in swine diets for growth performance improvement, post-weaning diarrhea reduction, and enhancement in immunity. We investigated the effect of Zn Sulfate and Zn IBZ (Intellibond Z, Micronutrients, Indianapolis, IN) on growth performance and diarrhea appearance. According to the Nutrient Requirements of Swine (NRC, 2012), the recommended concentrations of zinc in nursery diets suitable for normal growth and development is 100 ppm (5-11 kg BW) and 80 ppm (11-25 kg BW).

During the entire experiment, the dietary treatment diets met properly the nutritional requirements of zinc regardless of their source. In this experiment, we did find differences in growth performances among treatments. Moreover, there were significant differences in ADG, ADFI, and F:G. The Zn IBZ treatment was the one with the most favorable results.

Villagómez-Estrada et al. (2021) observed higher growth performance in pigs fed 80 ppm sulfate mineral at the end of the growing period (d 21 to d 84). However, the opposite was true in the finisher period. This is in accordance with Cemin et al. (2019), who observed in the finisher period an improved ADG at 100 mg/kg added in pigs fed Zn

IBZ in comparison with those fed Zn Sulfate. In agreement, Van Kuijk et al. (2019), observed a tendency for improved F:G and ADG in finisher pigs fed 80 ppm Zn IBZ in comparison to zinc counterparts. It is likely that the improvements in the last period of pig growth might be related to improvements in carcass characteristics, as a greater carcass yield (P = 0.017), greater (P = 0.058) hot carcass weight (Cemin et al., 2019), greater (P = 0.001) lean meat percentage (Van Kuijk et al., 2019) were observed.

Overall, the greater growth performance in Zn IBZ can be explained by a higher apparent digestibility compared to the pigs fed Zn Sulfate as suggested by Villagómez-Estrada et al. (2021).

To study the role of zinc source in shaping the presence of diarrhea in nursery pigs, fecal scoring throughout the experiment was performed. Zinc improves intestinal mucosal integrity, water and electrolyte absorption (Hedegaard et al., 2017; Hu et al., 2013). In the present research, lower fecal scores in pigs fed Zn Sulfate were observed most of the weeks, except on d 21 where pigs fed Zn IBZ presented a lower fecal scoring (Figure 1). Little research has been published in regards to feeding physiological levels of different sources of zinc and their effects on the control of diarrhea of weaned pigs. Indeed, there are some data available demonstrating the effects of zinc supplementation in controlling diarrhea but in pharmacological levels. A possible explanation for the diarrhea scoring changes on d 14 (Figure 2) and d 21 (Figure 1) is the enzyme development of starch and protein digestion in the gastrointestinal tract, increasing the protease, amylase, maltase, and sucrose enzymes by 6 to 8 weeks of age (Koepke et al., 2017). In phase 3, d 14 to 21, there is a notable increase in corn composition of the diet that could explain the higher fecal scoring observed in Figure 2. Additionally, in phase 4,

there is a change in fecal scoring among diets (Figure 1). Due to the lack of environmental changes or possible external influences affecting the gastrointestinal tract, we hypothesize that both changes could be related to the natural enzyme development of the pig and its ability to digest the new ingredients of the diet. In our current research, the pigs that suffered less change in their fecal score throughout the experiment were the small size pigs (Figure 2), however, the medium size pigs were the ones with the best-scored feces regardless of the zinc source supplemented in their diet.

Figure 1. Effect of zinc sources on fecal score in nursery pigs

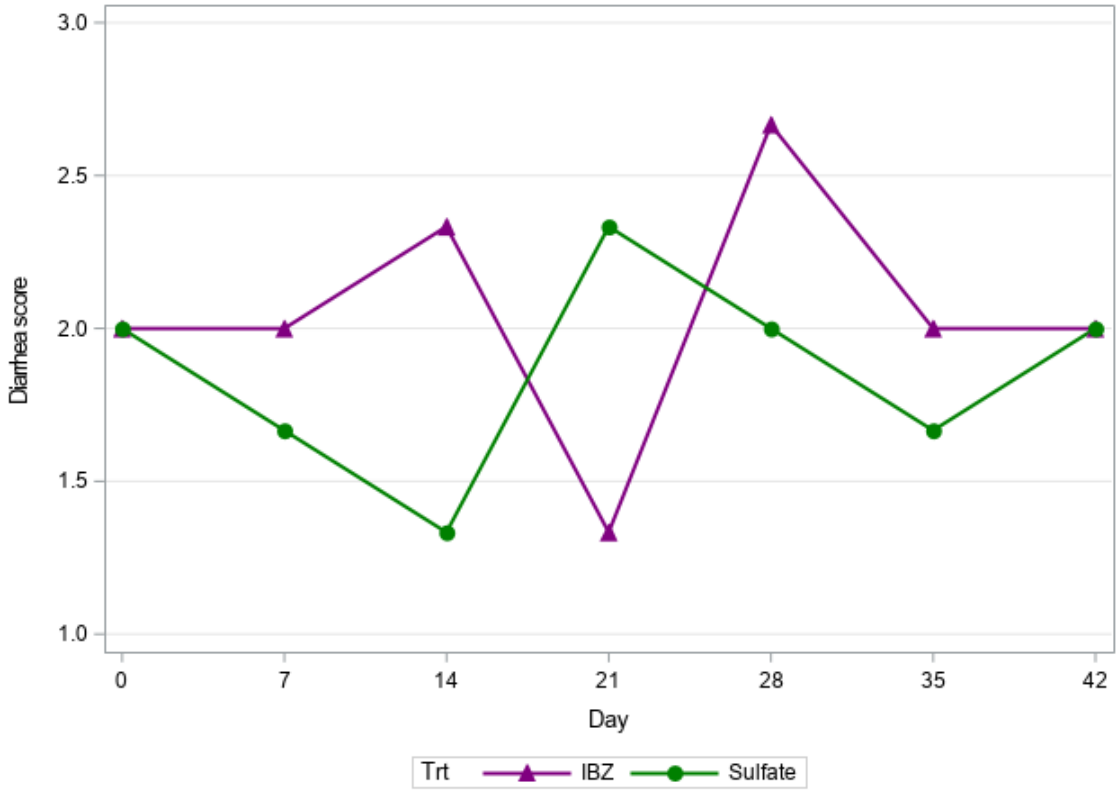
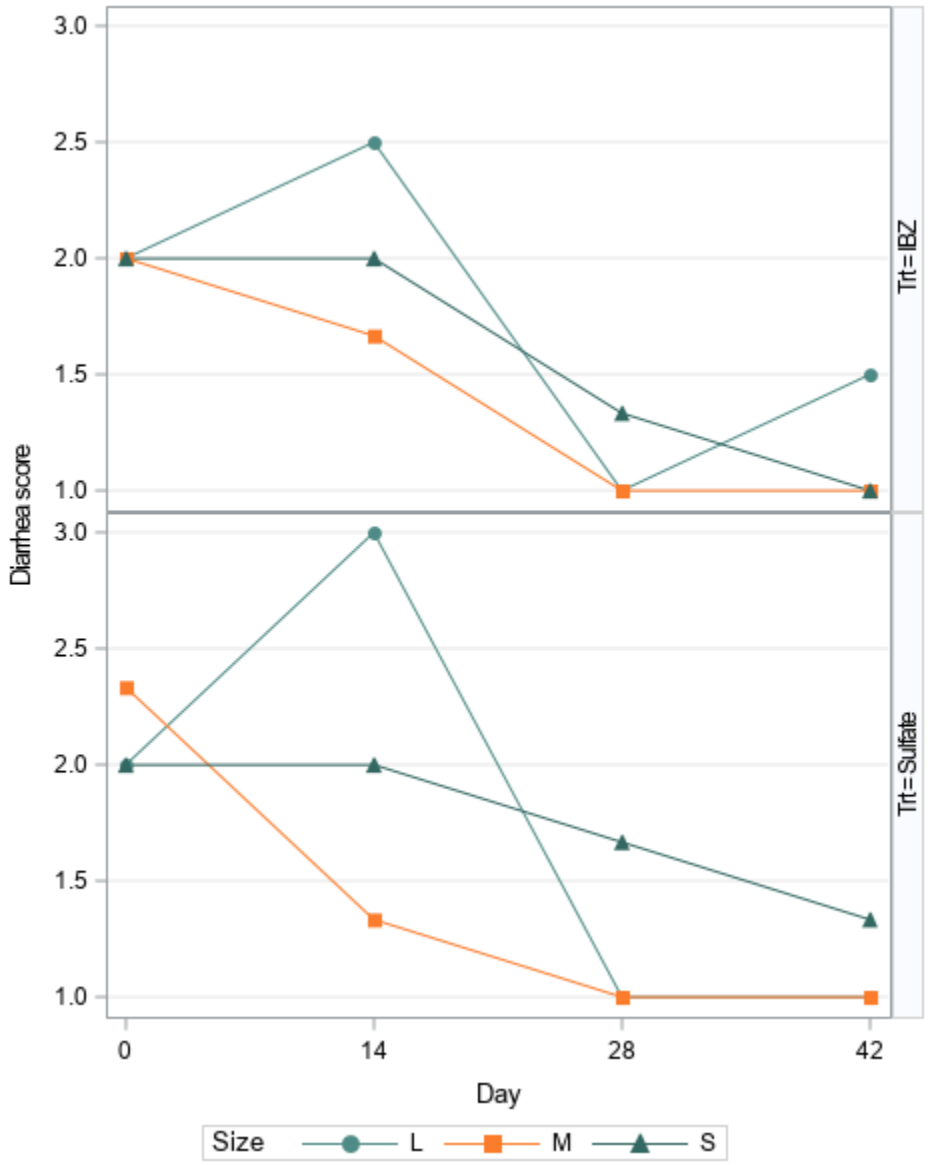


Figure 2. Effect of zinc source in fecal score on different sizes of nursery pigs



Although a level of zinc at 80 ppm is sufficient for optimum growth, numerous studies have found that pharmacological supplementation of 2,000-3,000 ppm of zinc oxide to the diet for two weeks after weaning effectively suppresses the incidence and severity of non-specific post-weaning diarrhea by up to 50% after inducing bacterial death (Case & Carlson, 2002; Hill et al., 2001; Hu et al., 2013; Hu et al., 2012; Stensland

et al., 2015; van Heugten et al., 2003; Wei et al., 2020). This in accordance with Hu et al. (2012) that observed decreased fecal scores on d 4, 8 and 14 post-weaning for pigs fed 2,000 mg/kg Zn Oxide compared to Zn Sulfate fed at 100 mg/kg. However, feeding high levels of zinc oxide for greater than 2 weeks reduces performance and increases bacterial shedding and may cause long term toxic effects on growth performance (Kim et al., 2015).

2.7 CONCLUSION

Zinc hydroxychloride (IBZ; Intellibond Z, Micronutrients, Indianapolis, IN) is able to positively affect the growth performance in nursery pigs. Besides that, pigs fed Zn IBZ showed more promising results in individual fecal scoring, indicating that the nutrient absorption needed for growth may be greater. However, the pen fecal mean scores do not contribute to this hypothesis. Therefore, further investigations on the effect of Zinc hydroxychloride on growth performance and diarrhea appearance in phase feeding in nursery phase piglets should be done to better explain our findings.

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CHAPTER III

3.1 GASTROINTESTINAL MICROBIOTA

3.1.1 Gastrointestinal microbiota

The gastrointestinal tract of swine is composed of a dense and complex community of bacteria, archaea, fungi, protozoa, and viruses at least ten times greater than the host cell number. The stomach and proximal small intestine contain 10^3-10^5 bacteria/g or ml of content, whereas the distal small intestine harbors 10^8 bacteria/g or ml of content. *Lactobacillus* spp. and *Streptococcus* spp. being the most dominant in the proximal small intestine (Kiarie et al., 2013).

There are four micro-habitats where the microbes are distributed within each specific segment of the gastrointestinal tract; the intestinal lumen, the mucus layer that covers the mucosal epithelium, the deep mucus layer in the crypts, and the surface of the intestinal epithelial cells. These micro-habitats have diverse bacterial populations influenced by the presence or absence of oxygen, pH, dietary subtracts, digesta flow rate, short-chain fatty acids (SCFA), toxins as bacteriocins, and competitive advantage. Consequently, any alteration in the micro-habitats can disrupt the microflora balance,

where a pathogenic bacterium may find the proper means to proliferate (Kiarie et al., 2013).

3.1.1.1 Bacterial families compose the gastrointestinal tract.

Relative abundance describes key elements of biodiversity. It is defined as the percentage of an organism of a particular kind relative to the total number of organisms in a given location, as shown in Table 8. It describes how common or rare a microorganism is relative to other species in the location (Lin & Peddada, 2020; Milanese et al., 2019). In pigs, high relative abundances of *Bacteroidaceae* in the colon at 10 d of age have been observed. Additionally, at d 21 (weaning age) in the colon, more than a 2-fold increase than d 10 was observed in the relative abundance of *Christensenellaceae, Paraprevotellaceae,* and *Ruminococcaceae*; nonetheless, significant decreases in relative abundance for family *Lachnospiraceae* (>2-fold change) were observed. In contrast, *Bacteroidaceae* abundance in the distal gastrointestinal (GI) tract decreased post-weaning (De Rodas et al., 2018).

At d 21 of age, a relative increase in *Clostridiaceae* or *Ruminococcaceae* and significant decreases in *Lactobacillaceae* were observed. *Lactobacillaceae* levels increased again at d 33 when the pigs were adapted to solid feed consumption (De Rodas et al., 2018).

The relative abundance of the main bacterial taxonomic groups in fecal samples at day 42 of age (21 days after weaning) were Firmicutes (~81 %) and Bacteroidetes (~16 %). The relative abundance from the Firmicutes at d 42*; Lactobacillaceae* (~45%), *Lachnospiraceae* (~13%), *Erysipelotrichaceae* (~2%), *Ruminococcaceae* (~6%), *Clostridiaceae* (~6%), *Peptostreptococcaceae* (~1%), and *Streptococcaceae* (~2%). The

relative abundance from the Bacteroidetes at d 42; *Prevotellaceae* (~14%), *Porphyromonadaceae* (~1%). *Lactobacillaceae* levels decreased with age (De Rodas et al., 2018; Poudel et al., 2020).

Table 8. Bacterial families composing the gastrointestinal tract

	Pig's age (days old)				
Taxonomy	21	28	35	42	49
Phylum					
Firmicutes	~40[1] - 59[2] %	~79[2] %	~82[2] %	~81[3] - 90[2] %	~93[2] %
Bacteroidetes	~27[2] %	~5[2] %	~5[2] %	~5[2]-16[3] %	~1[2] %
Family					
Bacteroidaceae	6.6[1] %	n/a	n/a	~0[1] %	n/a
Paraprevotellaceae	13[1] %	n/a	n/a	4.9[1]%	n/a
Ruminococcaceae	~4[2] %	~7[2] %	~6[2] %	~4[2] %	~4[2]-6[3] %
Lachnospiraceae	~10[2] %	~33[2] %	~18[2] %	~9[2] %	~9[2]-13[3] %
Clostridiaceae	~3[2] %	~1[2] %	~5[2] %	~4[2] %	~4[2]-6[3] %
Lactobacillaceae	~1[2] %	~20[2] %	~39[2] %	~17[2] %	~4[2]-45[3] %
Erysipelotrichaceae	~17[2] %	~1[2] %	~0.5[2] %	~0.2[2] %	~0.2[2] – 2[3] %
Streptococcaceae	~0.5[2] %	~1[2] %	~0.2[2] %	~2[3] - 41[2] %	~52[2] %
Prevotellaceae	~2[2] - 6[1] %	~0.9[2] %	~2[2] %	~1[2] - 33[1,3] %	~1[2] %
Genus					
Proteobacteria	13.8[1] %	n/a	n/a	n/a	n/a
Streptococcus	~0.5[2] %	~1[2] %	~0.5[2] %	~41[2] %	~52[2] %
Ruminococcus	n/a	n/a	~2[3] %	n/a	n/a

[1]Soler et al. (2018), [2]Tran et al. (2018), [3]De Rodas et al. (2018)

At day 63 of age (the end of the weaning period), the main taxonomic groups were Firmicutes (~82 %) and Bacteroidetes (~18 %). The relative abundance from the Firmicutes at d 63; *Lactobacillaceae* (~13%), *Lachnospiraceae* (~13%),

Erysipelotrichaceae (~4%), *Ruminococcaceae* (~6%), *Clostridiaceae* (~17%), *Peptostreptococcaceae* (~4%), and *Streptococcaceae* (~9%). The relative abundance from the Bacteroidetes at d 63; *Prevotellaceae* (~13%), and *Porphyromonadaceae* (~5%) (Poudel et al., 2020).

In the ileum of 120 d old pigs, the most abundant genera were *Streptococcus* (17.73%) and the unspecified genera of the *Clostridiaceae* family (17.10%). In the duodenum, the most abundant bacteria were *Cyanobacteria*, *Lactobacillales*, and the *Moraxellaceae* family. The jejunum had more Firmicutes and *Proteobacteria*. Significant differences between the proximal and distal colon were observed; however, the distal colon had more OTUs (Operational Taxonomic Unit) belonging to the *Ruminococcaceae* family (Crespo-Piazuelo et al., 2018).

In the ileum of 166-day-old pigs, Firmicutes and Actinobacteria were predominant (Li et al., 2017). However, Firmicutes and Bacteroidetes were the most abundant phyla in feces and colon. Moreover, Crespo-Piazuelo et al. (2018) observed a higher Shannon diversity measure in the large intestine compared to the small intestine section. However, *Proteobacteria* were relatively more abundant in the cecum and ileum than in the feces. Additionally, *Spirochsmaaetes* increased relative abundance in the feces in the cecum as the pigs aged (Mccormack et al., 2017).

3.1.1.2 Bacterial composition in good vs. poor gut health

At weaning age, the gut microbiota of healthy pigs is composed of a greater abundance of *Prevotellaceae*, *Lachnospiraceae*, *Ruminocacaceae,* and *Lactobacillaceae* when compared to diarrheic pigs (Guevarra et al., 2019; Pluske et al., 2018). Overall, higher Bacteroidetes abundance was observed in the healthy pigs (Pluske et al., 2018).

On the contrary, gastrointestinal inflammation after weaning has been characterized by the overgrowth of *Enterobacteriaceae* (Guevarra et al., 2019; Pluske et al., 2018). Guevarra et al. (2019) observed pigs with diarrhea to increase the relative abundance of *Prevotella*, *Sutterella*, *Campylobacter*, and *Fusobacteriaceae*.

Increases in Firmicutes and decreases in Bacteroidetes have been observed in humans and animal models with obesity. Obesity is associated with a decreased fecal Firmicutes: Bacteroidetes ratio relative to lean subjects (Conlon & Bird, 2014).

In adult humans, ~90% of the bacteria in the gastrointestinal tract are Bacterioidetes and Firmicutes, almost at the same proportions. However, in feces, the genera *Bacteroides*, *Bifidobacterium*, and *Eubacterium* are the 60% of numerically highest bacteria. *Clostridium*, *Enterobacteriaceae*, and *Streptococcus* are significant but less numerous (Conlon & Bird, 2014).

Diet affects the gut microbial population. Humans on Western-style diets have more Bacteroides than Eastern ones, and plant-based diets are more associated with a *Prevotella* prevalence (Conlon & Bird, 2014). Lower Bacteroidetes: Firmicutes ratio has been observed in children from Western countries. Additionally, differences in gut microbial composition depending on the diet were noticed. Diets rich in proteins and saturated fats like North America and Italy are dominated by *Bacteroides* and *Enterobacteriaceae* (respectively). In contrast, in Africa and South America, the stool is composed of Bacteroidetes, particularly *Prevotella*, due to the higher fiber levels in plant-based diets. Lower taxonomic diversity in fecal microbiota is prevalent in Western diets and associated with obesity, type II diabetes, and inflammatory diseases (Conlon & Bird, 2014).

3.1.1.3 Main functions of the microbiota in the gastrointestinal tract

In the Tan et al. (2017) study of caecal and colonic microbiota, annotated differentially expressed genes (DEGs) in pigs with high and low feed conversion ratio (FCR). Additionally, up-regulated DEGs are potentially directly or indirectly involved in pigs' FE (feed efficiency) regulation. Down-regulated DEGs were mainly related to immunity and disease in caecal mucosa or colonic mucosa tissues. GUCA2A, GUCA2B, HSP70.2, NOS2, PCK1 (Phosphoenolpyruvate carboxykinase-1), SLCs, and CYPs (cytochrome) from caecal and colonic mucosa are possible candidate genes for feed efficiency (FE) in pigs. These genes were functionally related to energy and lipid metabolism, short-chain fatty acids, gastrointestinal peristalsis, and biotransformation (Tan et al., 2017).

3.1.1.3.1 Gastrointestinal microbiota function in nutrient metabolism

Proteins

Protein metabolism efficiency is enriched by producing microbial proteinases and peptidases by the gut microbiota. Several transporters on the bacterial cell wall facilitate amino acid entry from the intestinal lumen into the gram-positive and gram-negative bacteria to convert the amino acids into small signaling molecules and antimicrobial peptides (bacteriocins) (Jandhyala, 2015).

Carbohydrates

Bacteroides are the predominant organisms that participate in carbohydrate metabolism (Jandhyala, 2015). Regarding the functional annotation of microbiomes, the abundance of carbohydrate-active enzymes (CAZy) families suggests that microbiota in the colon and cecum share carbohydrate enzyme profiles. Moreover, bacteria species present in the ileum and hindgut are related to polysaccharide metabolism, as expected

due to the physiological characteristics of the large intestine (Poudel et al., 2020). The ileum specializes in genetic information processing and nucleotide metabolism.

Lipids

Gut microflora aids lipid metabolism by suppressing the inhibition of lipoprotein lipase activity in adipocytes. Bacteroides improve lipid digestion by increasing lipid hydrolysis by up-regulating the expression of a colipase, required by pancreatic lipase (Jandhyala, 2015).

Short-chain fatty acids (SCFA) are synthesized by Bacteroides, *Roseburia*, *Bifidobacterium, Fecalibacterium* in the colon by the fermentation of carbohydrates that escaped proximal digestion and indigestible oligosaccharides. Acetate, butyrate, and propionate are rich energy sources for the host. This host energy balance is hypothesized to occur by the interaction of the SCFAs with a G protein-coupled receptor Gpr41 and the hormone Peptide Tyrosine Tyrosine/Pancreatic Peptide YY3-36, (PYY).

Vitamins

Synthesis of vitamin K and several components of vitamin B are another primary metabolic function of the gut microbiota (Jandhyala, 2015; Ramakrishna, 2013). These vitamins are directly absorbed from the bowel, contributing to nutrition in humans. Vitamin B12 produced by the gut microbiota requires binding to the R factor in the stomach and transfer to the intrinsic factor in the small intestine to be absorbed as a complex in the terminal ileum; therefore, it is not directly available to the host (Ramakrishna, 2013).

3.1.1.3.2 Gastrointestinal microbiota function in immunomodulation

The gut microbiota contributes to normal gut-associated lymphoid tissues by properly developing Peyer's patches. Additionally, they promote interleukins (important

immune cell messenger molecules), dendritic cell stimulation (antigen-presenting cells), development of T-reg cells (suppress immune response for homeostasis) by SCFA butyrate (Jandhyala, 2015). In the mouse colon, *Clostridia* strains can increase the number of T-reg cells (Honda & Littman, 2016).

Mucosal Immunoglobulin A (IgA) acts as a physical barrier and can control the expression of genes by microbes in the intestine. For instance, people with IgA deficiency have more bacteria taxa with potentially inflammatory properties. *Bacteroides thetaiotaomicron,* in the human gut, express pro-inflammatory signals in the host in the absence of IgA. In mice, reduced levels of IgA have resulted in aberrant expression of flagella-related genes in commensal bacteria. Changes in the gut microbiota composition dynamically adjust the IgA, and the more complex the gut microbiota is, the more diverse the IgA pool becomes (Honda & Littman, 2016).

3.1.1.3.3 Gastrointestinal microbiota function and the integrity of the gastrointestinal tract

Non-digestible dietary carbohydrates enter the colon, where colonic bacteria ferment resistant starch (RS) and fermentable non-starch polysaccharides (NSPs) to SCFA. SCFA promotes tight junction integrity, increases epithelial cell proliferation and repair rate, and facilitates epithelial cell differentiation (Ramakrishna, 2013). Moreover, gut microbiota aids in the maintenance of the epithelial villus, tight junction maintenance, and structural development of the intestinal microvasculature (Jandhyala, 2015).

3.1.1.3.4 Gastrointestinal microbiota function in growth

Like humans, adiposity in pigs has been related to the gut microbiota composition. When two breeds with different adiposity propensity, rapid growth - high

lean carcass in contrast to slow growth - high intramuscular fat were compared, the microbial composition significantly differed. Firmicutes (>75%) were the most abundant phylum in both breeds. Bacteroidetes were the second most abundant in the slow growth pigs, followed by Spirochaetes, and Proteobacteria accounted for less than 2%. *Clostridium*, *Treponema*, *Turicibacter*, and *Lactobacillus* were the top four genera (Yang et al., 2018), Table 9.

On the other hand, in pigs with rapid growth, Bacteroidetes and Spirochaetes were dramatically diminished. Proteobacteria accounted for nearly 18% of all fecal bacteria, and *Lactobacillus*, *Escherichia*, *Clostridium*, and *Turicibacter* were the top four genera (Yang et al., 2018), Table 9.

Short-chain fatty acid (SCFA) differences were also observed. In pigs with rapid growth, elevated levels of SCFAs were observed. It is known that enhanced SCFA synthesis is associated with improved energy extraction from the diet and prevents and counteracts obesity (Yang et al., 2018).

Table 9. Families of the top four genera in the slow in rapid growth pigs

Phylum	**Family**	**Genus**
Firmicutes	*Lactobacillaceae*	*Lactobacillus*
	Turicibacteraceae	*Turicibacter*
	Clostridiaceae	*Clostridium*
Spirochaetes	*Treponemataceae*	*Rectinema*
		Treponema
Proteobacteria	*Enterobacteriaceae*	*Escherichia*

Yang et al. (2018) and NCBI taxonomy

3.1.2 High Throughput Sequencing (HTS) gold standards

The gold standard for bacterial identification in fecal samples remains molecular identification. Molecular tools and sequence technologies, including 16S rRNA markers,

have increased the ability to detect and identify culturable and unculturable bacteria (Lagier et al., 2015). However, using markers relies on the specificity and sensitivity of the PCR-based assays (Khan et al., 2017).

The advert sequencing DNA molecules has had a tremendous impact on researchers and their understanding of microorganisms. Early sequencing technology started with the Maxam-Gilbert (Maxam & Gilbert, 1977) and Sanger sequencing (Sanger et al., 1977) methods. For decades, the gold standard technology was Sanger sequencing (Raza & Ahmad, 2016). Sanger sequencing, a low-throughput sequencing technology, was followed by developing a new type of high-throughput sequencing (HTS), the second-generation sequencing or Next Generation Sequencing (NGS), and a more recent third-generation sequencing.

High-throughput sequencing (HTS) technologies sequence multiple DNA molecules in parallel simultaneously (Churko et al., 2013) and are also known as deep sequencing technology or next-generation sequencing (NGS). In 2005, the Roche® 454 pyrosequencer was the pioneer of NGS. The second-generation sequencing technology is represented by 454 from Roche®, Ilumina® (formerly known as Solexa), and SOLiD Company. Single-molecule long reads and copious data characterize the third-generation sequencing, bringing about challenges in design, pre-processing, normalization, and analysis of the HTS data (Cao et al., 2017).

3.1.2.1 Commercially available HTS

3.1.2.1.1 Ilumina®

Currently, HTS is dominated by Ilumina®. Ilumina® processes involve clonal amplification by PCR. Several models are available (MiSeq, NextSeq 500, and HiSeq

series), but MiSeq and HiSeq are currently the best-established platforms. MiSeq is ideal for small genomes and target sequencing. On the other hand, HiSeq yields 1 Tb in 6 days, a powerful tool for de novo sequencing of species without a reference genome (Reuter et al., 2015).

3.1.2.1.2 Ion Torrent®

Ion Torrent® uses a similar concept as the 454 platform, which utilizes beads, and clonal amplification of DNA takes place by emulsion-PCR. The pH is measured by the release of hydrogen ions during DNA extraction and converted into a voltage signal. By avoiding optical scanning of nucleotides during sequencing cycles, the sequencing speed improves, and the cost is reduced. The maximum read lengths are 200 bp in the most current machine, which yields 10Gb in 2-8 hours of sequencing (Reuter et al., 2015).

3.1.2.1.3 Pacific Biosciences (PacBio)

PacBio was the pioneer of single-molecule read; real-time sequencing and amplification are not needed, allowing direct sequencing of DNA. The fluorescent signals allow for the reading in real-time of the DNA sequence. A typical output with the latest technology is ~50k reads, an average length of > 14kb, and up to 1Gb of data in 4 hours. However, it has a high error rate (~11%) randomly distributed. It is less sensitive to GC sequence content as compared to previous technologies. These characteristics make this valuable technology for de novo assembly of small bacterial and viral genomes and extensive genome finishing. It can be used for studying DNA and ribosomes (Reuter et al., 2015).

3.1.2.1.4 Oxford Nanopore Technologies

Nanopore sequencing is possible by transitioning nucleotides through a small channel (nanopores). It is characterized by minimal library preparation and can be done with or without PCR amplification. However, it has a high error rate and run failure rate. Both strands of DNA are sequenced, increasing accuracy and resulting in more than 90 Mbp of data, ~ 16,000 total reads, read lengths of ~6 kb, and >60 kb produced in 18 hours. Best used for determining the position and structure of bacterial resistance and resolving assembly gaps (Reuter et al., 2015) and now for diagnostics (Espindola & Cardwell, 2021).

3.1.3 Metagenomics

Metagenomics techniques with nucleic acid sequencing allow diversity analysis of culturable and unculturable microbes. It provides a complete profile of host, endogenous microorganisms, and pathogens in a sample. Multiple steps are needed for a metagenomics-based diagnosis, such as nucleic acid extraction, sequencing, sequence assembly, and BLAST analysis of the assembled contigs. Earth science, life science, biomedical sciences, energy, environmental remediation, biotechnology, agriculture, biodefense, and microbial forensics are some of the different application areas addressed with metagenomics (Stobbe et al., 2013).

3.1.3.1 Amplicon sequencing (16S rRNA)

Amplicon Sequencing is one of the earliest and most widely used techniques for microbial assessment (Gupta et al., 2019; NAP, 2018). This method effectively broad taxonomic characterization and comparison of microbial communities such as bacteria and fungi (Hodkinson et al., 2015) and does not rely on whether the sample is culturable

or not (Gupta et al., 2019). Bacteria are the organisms most often researched microbiota (Hodkinson & Grice, 2015). Amplicon sequencing targets the subunit 16S rRNA gene, which is almost universal in all bacteria (Gupta et al., 2019; Hodkinson & Grice, 2015; NAP, 2018; Poretsky et al., 2014; Wen et al., 2017), and it contains hypervariable regions that are widely divergent between taxa (Hodkinson & Grice, 2015). For eukaryote profiling, amplicon sequencing targets the subunit 18S rRNA gene and internal transcribed spacer (ITS) (NAP, 2018).

Amplicon sequencing can assess the relative abundance of all the organisms within a sample, allowing for simultaneous parallel sequencing of multiple samples. Due to the availability of PCR machines and increased DNA sequencing facilities, amplicon sequencing is used in research and clinical laboratories (Gupta et al., 2019). PCR amplification of the selected region (i.e., 16S rRNA) of a given DNA sample is needed, and bacterial-community-specific primers universally anneal to the target bacterial region, reflecting the bacterial taxonomic composition of the community (Hodkinson & Grice, 2015). High-quality, comprehensive, and curated 16S databases are currently available (Poretsky et al., 2014), and more are being added regularly.

Amplicon sequencing is limited by short read lengths, sequencing errors, and difficulties assessing operational taxonomic units (OTUs), providing less sense of community structure. It has the best results when analyzing samples with low microbial variation because as the database expands, the ability to accurately assign taxonomy to reads is compromised (Poretsky et al., 2014).

Despite the limitations of amplicon sequencing, it is less compromised when compared to other technologies (Poretsky et al., 2014). Moreover, there are great options

for analyzing amplicons that can be used for studies requiring high resolution with high sensitivity and excellent specificity, such as DADA2 and USEARCH-UNOISE3, to name some bioinformatics pipelines for amplicon sequence data (Prodan et al., 2020).

Amplicon sequencing requires target-specific PCR amplification. Some of the limitations of amplicon sequencing are the target amplification of a single genomic locus, providing only a small region (NAP, 2018). The specific primers used for amplification could bind to regions, not 100% conserved across all taxa in the multiple amplification cycles (Gupta et al., 2019; Hodkinson & Grice, 2015; Poretsky et al., 2014; Prodan et al., 2020). Therefore, closely related species are challenging to differentiate by amplicon sequencing due to the high similarity in the 16S rRNA gene (Gupta et al., 2019).

Assembly is necessary when the paired-end approach has been used; however, problems related to assembly, i.e., by bioinformatics pipelines such as QIIME or MOTHUR, are often reported and require some ability to do computer coding. These programs are often used for determining diversity metrics and taxonomic determination (Hodkinson & Grice, 2015).

Sensitivity and specificity among bioinformatics pipelines broadly vary (Prodan et al., 2020). Therefore, over-or under-estimation of the relative microbial abundance of specific taxa can be observed (Prodan et al., 2020; Wen et al., 2017). In order to account for this over-or underestimation of relative microbial abundance and validate the results, sample duplicates or triplicates are recommended with low deviation among them. (Poretsky et al., 2014; Wen et al., 2017).

The 16S rRNA gene is largely conserved throughout prokaryotes (NAP, 2018; Poretsky et al., 2014). In addition to that, the 16S rRNA gene has several regions with differential (V1-V9) targets of amplification (Gupta et al., 2019; Poretsky et al., 2014), providing different resolutions in each region depending on the bacteria of interest. More resolution is achieved when all the regions are targeted (Gupta et al., 2019). Amplicon sequencing does not rely on whether or not the bacteria in a sample is culturable. Additionally, amplicon sequencing can accurately (in most cases) generate taxonomic profiles at the genus level, whereas it is more challenging to achieve species or strain levels (Gupta et al., 2019; NAP, 2018).

Technical problems that compromise the sensitivity of the amplicon sequencing are nucleotide extraction (microorganism variation in reagent sensitivity), amplification conditions, primer selection/composition, polymerase enzyme, barcode pooling, library preparation, and sequencing, hindering the estimation of population abundance in microbial communities (NAP, 2018; Poretsky et al., 2014; Wen et al., 2017). Extra caution needs to be considered during nucleotide extraction when extracting RNA instead of DNA since RNA is very susceptible to degradation. In addition to the chemical instability of RNA and due to the ubiquitous RNases, working with RNA is more demanding, and it can compromise the results if not handled in an RNase-free environment (NAP, 2018).

3.1.3.2 Shotgun Metagenomics

Shotgun metagenomics is a non-targeted sequencing and an alternative to PCR uncultured microbiota approach (Hodkinson & Grice, 2015; NAP, 2018; Poretsky et al., 2014; Sharpton, 2014). It allows the identification of novel genes with robust estimation

of the whole microbial community composition (including viruses, archaea, and microeukaryotes) and diversity (Hodkinson & Grice, 2015; Poretsky et al., 2014; Roumpeka et al., 2017).

The DNA is shredded into fragments throughout the genome without amplification (Poretsky et al., 2014). These fragments are sequenced independently (Hodkinson & Grice, 2015; Sharpton, 2014). Once high-quality data are obtained, studying an organism with a reference genome makes it possible to map the reads to the reference genome or transcriptomes, allowing species and strain-level classification. If enough reference genomes exist, even organisms of modest abundance are detected (NAP, 2018; Poretsky et al., 2014).

Despite all the benefits of being a PCR-free method, shotgun metagenomics has its challenges. It requires more complex, computationally demanding, and expensive analytic approaches; therefore, this method is limited to technology and cloud-sharing availability (NAP, 2018; Sharpton, 2014).

In shotgun metagenomics, the DNA preparation methods, proper DNA quantity collection, sample complexity, and accurate sequence classification of taxa are crucial steps for an accurate result (Hodkinson & Grice, 2015; Poretsky et al., 2014; Sharpton, 2014). Additionally, classification of the taxa can be unreliable due to the limited currently available whole-genome reference sequences. Moreover, most communities are so diverse that most genomes are not entirely represented (Hodkinson & Grice, 2015).

Metagenomics approach by shotgun can be overwhelming because the sample contains the microbial community of interest and the host DNA and environmental

contamination. This can mislead the analyses; therefore, software tools for filtering are needed (Sharpton, 2014).

Shotgun sequencing is less tolerant of low biomass or contaminated samples (NAP, 2018). Phylogenetic classification methods have discrepancies in their level of resolution (Poretsky et al., 2014), and specialized assembly software is required to minimize assembly errors (Hodkinson & Grice, 2015). Therefore, the specificity of the results highly depends on the quality of the sample and the protocol used for sample analysis.

Shotgun sequencing allows for de novo assembly, characterization of diversity, and function of communities of microorganisms (new or uncultured ones). In addition to that, shotgun sequencing allows for identifying taxa and metabolic pathways to assess the community's function, community structure, and evolutionary relationships (Hodkinson & Grice, 2015; Poretsky et al., 2014; Roumpeka et al., 2017; Sharpton, 2014). The sensitivity of shotgun sequencing is not biased by amplification. However, the sensitivity is highly dependent on the sequencing (Hodkinson & Grice, 2015).

3.1.3.2.1 Community profiling bioinformatics methods

The National Center for Biotechnology Information (NCBI) has a series of databases significant to biotechnology and biomedicine and is a vital resource for bioinformatics tools and services. The most widely used bioinformatics research tool for analyzing metagenomics data is the NCBI's Basic Local Alignment Search Tool (BLAST). The BLAST tool can be used locally (on a personal computer); however, the large database size makes the computational analysis cumbersome for large datasets (Santamaria et al., 2012).

Diamond is a sequence aligner for protein and translated DNA searches, designed for high-performance analysis of big sequences. Diamond allows for sequence alignments of DNA protein-coding sequences and protein 20,000 times faster than traditional BLAST (Buchfink et al., 2015). Programs like the Diamond pipeline are used to improve the speed of BLAST.

Another program, Kraken2, assigns taxonomic labels to DNA sequences using k-mer based binning. A k-mer is a sequence of k nucleotides in a DNA sequence. Kraken2 can estimate read abundance using the Bracken program (Wood & Salzberg, 2014). Moreover, alignment programs like Bowtie2 are used for community profiling by comparing nucleotide or protein sequences to sequence databases (Johnson et al., 2008). The software contrasts nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches.

The functions and utilities of organisms and the ecosystem is assessed by querying sequence reads against databases. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database and Clusters of Orthologous Groups of proteins (COG) database are amongst the most widely used for potential functional assessment (Hodkinson & Grice, 2015).

3.1.3.2.2 Target profiling methods

Electronic probes

Timely and accurate detection and correct diagnoses are essential to prevent the spread of disease, especially after introducing an exotic pathogen and before the symptoms of the disease appear. Driven by agricultural biosecurity, plant pathologists at

Oklahoma State University acknowledged the need for a more timely response to disease diagnostics (Espindola & Cardwell, 2021; Stobbe et al., 2013).

Stobbe et al. (2013) developed E-probe Detection of Nucleic acid Analysis (EDNA), a bioinformatics pipeline that takes only minutes to run and focuses on target microorganisms. Target genomic sequences are queried in raw unassembled, non-quality-checked metagenomics sequence reads. This pipeline can be used on a standard personal computer. A BLAST against the metagenomics with target-specific E-probes (diagnostic signature sequence) is much faster than by BLAST of all sequences against nr/nt databases, such as GenBank, EMBL, or DDBJ for further taxonomic assignment of the read using software, such as MEGAN, Metaphlan, Kraken and QIIME (Espindola et al., 2015; Espindola & Cardwell, 2021; Melcher et al., 2014; Stobbe et al., 2013).

The E-probes are designed by comparing the target organism's sequence with that of near relatives (designated near neighbors); a set of oligonucleotide sequences of a specified length are then generated and tested for specificity against a public database, eliminating all but sequences unique to the target, called E-probes (Melcher et al., 2014). E-probes are validated for sensitivity, specificity, and limit of detection (Espindola & Cardwell, 2021; Espindola et al., 2018).

This modified bioinformatic approach rapidly detects microorganism-associated sequences without further computationally intensive metagenome analysis, allowing users to limit and control the size of the searchable database and the size of the searching query set (Espindola et al., 2015; Stobbe et al., 2013).

EDNA has been able to detect bacteria, viruses (RNA and DNA), fungi, and oomycetes in plants (Espindola et al., 2015; Espindola et al., 2018; Melcher et al., 2014;

Stobbe et al., 2013) and complex food matrices (Blagden et al., 2016) at a low, medium and high pathogen abundance levels (from 0.5% pathogen reads to 25% pathogen reads) in a mock sample database (Stobbe et al., 2013) and *in vivo* (Espindola et al., 2015). E-probe lengths rank from 40 to 140 nucleotides based on pathogen genomic size (Blagden et al., 2016).

EDNA can be used for the detection of human, animal, and plant pathogens as well as endosymbionts and commensals as long as the researcher knows the organisms to be tested and have a large (if not all) part of the metagenome of those organisms for E-probe design (Melcher et al., 2014; Stobbe et al., 2013). While EDNA does not provide a taxon profile of relative species abundance, it has the potential to detect a pathogen in a metagenomics dataset rapidly. Furthermore, EDNAtran is a theoretical approach to metatranscriptomics to detect metabolic functions associated with pathogenicity in other host-pathogen systems (Espindola et al., 2018).

When comparing EDNA with traditional bioinformatics tools used for diagnostics, such as minimap2 and BLAST, EDNA detected grapevine pathogens in metagenomes in 10 min compared to 13.14 min 5.3 h, respectively. Additionally, when using data from the first Critical Assessment of Metagenome Interpretation (CAMI) metagenomes, EDNA detected all 23 pathogens of the grapevine for each metagenome. On the contrary, Metaphlan3 failed to detect the pathogens, and Kraken2 failed to detect six of them.

Metaphlan3 and Kraken2, BLAST, and minimap2 are tools that provide a broader perspective of the metagenome composition and can be used for experimental purposes as long as an experienced bioinformatician handles them. EDNA can be used

for detection and diagnostics without specialized bioinformatics for data analysis (Espindola & Cardwell, 2021).

3.2 OBJECTIVE AND HYPOTHESIS

The objective of this study was to evaluate the role of two dietary zinc sources (Zinc Sulfate or Zinc Hydroxychloride - IBZ) in shaping the gastrointestinal microbiota in nursery pigs using E-probes to track changes in the most common gastrointestinal commensal families: *Prevotellaceae, Clostridiaceae, Erysipelotrichaceae, Lachnospiraceae, Lactobacillaceae, Ruminicoccaceae and Streptoccocaceae*. We hypothesized that family-specific E-probes are a valuable tool for detecting the microbial composition of the gastrointestinal tract of nursery pigs subject to both dietary treatments.

3.3 MATERIALS AND METHODS

3.3.1 Animals, housing, and treatments

The experimental protocol was approved by, and the pigs were handled and cared for according to the guidelines established by the Oklahoma State University Institutional Animal Care and Use Committee (IACUC).

A total of sixty crossbred weaned pigs (3 weeks of age; Pig Improvement Company (PIC®); 30 barrows and 30 gilts) were subject to research for 6 wk. Pigs with average initial body weight (BW) of 5.4 kg were randomly allotted to one of two dietary zinc treatments. Three replicate pens per treatment consisting of ten pigs per pen (5 barrows and 5 gilts) were blocked to minimize variations in gender, initial BW, and location of the pen in a randomized complete block design. Each pen had a nipple

waterer and a stainless-steel feeder. Pigs were housed in an environmentally controlled nursery facility with slatted plastic flooring and a mechanical ventilation system. The environmental temperature decreased by 1˚C per week, starting at 30˚C in the first week. During the entire experiment, pigs were allowed to consume feed and water *ad libitum*.

From day 0 until day 42, the dietary Zn treatments were allotted to pens: Zn Sulfate (100 ppm of added Zinc Sulfate) or Zn IBZ (100 ppm of added Zinc Hydroxychloride; Intellibond Z, Micronutrients, Indianapolis, IN). All remaining nutrients in the diet were added at or above the requirements listed in the NRC (2012). Both diets were formulated as basal diets, and each dietary Zn treatments mineral premix was added subsequently during the mixing process.

3.3.2 Diets

A four-phase nursery feeding program was employed in the experiment, with diets formulated to meet or exceed NRC (2012) nutrient requirements as presented in Chapter II, Tables 1 and 2. Pigs were fed a standard diet with either Zn Sulfate or Zn IBZ: phases 1-4 (100 mg Zn/kg). The experiment lasted for 42 days, and all pigs received Phase 1 diet during d 0-7, Phase 2 during d 7- 14, Phase 3 during d 14-23, and Phase 4 during d 23-42.

3.3.3 Fecal microbiota composition

A large, medium/average and a small pig per pen were visually selected on d 0. After selecting the three pigs per pen, the large, medium, and small pigs were ear-tagged for easier identification on subsequent evaluation on d 14, 28, and 42. The same pigs were sampled throughout the study.

On d 0, fecal samples were obtained by rectal stimulation with a dry transport system sterile flocked swab (Puritan Medical Products, Co., Guilford, ME). On d 14, 28, and 42, the sampling was done with tubes with a screw cap and a spoon (Growing Labs, Co., Suwanee, GA). After collection, the fecal samples were placed on ice, placed into an appropriately labeled sterile Eppendorf tube (Thermo Fisher Scientific, Waltham, MA), and stored at -80˚C until further analysis.

3.3.4 DNA extraction

DNA extraction from fecal samples was performed following the manufacturer's protocol using Quick-DNA™ Fecal/Soil Microbe Miniprep Kit (Zymo Research, Irvine, CA). Nucleic acid purity assessment was detected by NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE), and the DNA concentration was detected by Quantus™ Fluorometer (Promega, Madison, WI). The samples with DNA < 100 ng of molecular weight before library preparation were purified with DNeasy PowerClean Pro Cleanup Kit (Qiagen, Hilden, Germany).

3.3.5 Library preparation and sequencing

DNA multiplexing of 12 samples at a time was performed using the Rapid Barcoding Sequencing SQK-RBK004 (Oxford Nanopore Technologies, Oxford, UK). After the DNA barcoding step, sequencing was made by a MinION™ device (Oxford Nanopore Technologies, Oxford, UK).

Platform Quality Check (QC) was performed to determine the number of pores available for sequencing in the flow cell, and only flow cells with >1000 pores were used. The flow cells are manufactured with 2000 pores; however, receiving flow cells

with no more than 1500 active pores upon purchase is reasonably expected. The sequencing run was performed using the MinIONTM flow cell (FLO-MIN106D (R9.4.1); Oxford Nanopore Technologies, Oxford, UK). After reaching the quality for sequencing, the flow cell was primed following the Rapid Barcoding Sequencing SQK-RBK004 (Oxford Nanopore Technologies, Oxford, UK) protocol, and 75µl of the pooled library was carefully mixed through pipetting before loading it onto the SpotON port. The library was loaded dropwise, preventing loading air bubbles into the flow cell and closing the prime and SpotON cover back to its original position. Subsequently, the lit of the MinION^{TM} device was moved back to the closed position.

Once the sample was loaded into the flow cell, the MinKNOW software (Oxford Nanopore Technologies, Oxford, UK) was used. An experiment and sample ID name were given. Additionally, the used kit was selected - SQK-RBK004 (Oxford Nanopore Technologies, Oxford, UK). The sequencing run lasted for 72 hours or until no more pores were available for sequencing. Basecalling was disabled, and barcoding was enabled before starting the sequencing. The output format selected was Fast5 and Fastq.

The large datasets required a specific workstation computer (Oklahoma State University High Performance Computing Center) for processing the generated data. Guppy, a data processing tool, requires Fast5 files for basecalling. Basecalling is the computational procedure of translating the raw electrical signal of the sequencer into nitrogenous bases. A configuration file was used to set the basecalling parameters depending on the flow cell and library preparation kit utilized during sequencing (Wick et al., 2019). Several Fastq files with the basecalled reads were generated after basecalling; such files underwent demultiplexing. Demultiplexing is the process of

classifying the barcodes per read and assigning them to read groups (metagenomes of each sampled pig). The Fastq files per barcode were concatenated and compressed with GZIP (Fastq.gz) for later analysis.

3.3.6 E-Probe design

E-probe design at the family level required two sets of genomes, the target and near neighbor genomes. The target genome was used as a template to generate E-probes. The near neighbors helped eliminate redundant and duplicative genome regions in the target genome, leaving sequences unique to the target family available to generate the E-probes. The genomes from *Prevotellaceaee, Clostridiaceae, Erysipelotrichaceae, Lachnospiraceae, Lactobacillaceae, Ruminicoccaceae,* and *Streptoccocaceae* families served as target genomes. The genomes of such families were obtained from the National Center of Biotechnology Information (NCBI) GenBank; details of the genomes per family can be accessed in this dissertation (Chapter III, Appendix 2, Table 1 and 2). Genomes in a family were used as the target sequence. The rest of the family genomes were used in a compiled Fasta file of near neighbors. E-probes were generated using the E-probe pipeline for EDNA MiFi, where E-probe length determination occurs (Stobbe et al., 2013).

During E-probe development, redundant genome regions were eliminated by the modified Tools for Oligonucleotide Fingerprint identification (TOFI) pipeline (Satya et al., 2008) used by the EDNA MiFi pipeline (Stobbe et al., 2013). After TOFI comparison, designed E-probes underwent a specific Basic Local Alignment Search Tool Nucleotide (BLAST) to eliminate potentially cross-reacting homo-oligomers. E-probe specificity was verified by pairwise alignment of each E-probe with the intended

target family genome using stringency of 100% identity and query coverage through the partially non-redundant nucleotide collection (nr/nt) database (nt) by BLAST with the NCBI. Target-specific E-probes sets of different lengths (20nt, 30nt, 40nt, 60nt, 80nt, 100nt, 120nt) were generated depending on genome size with a minimum match for 15 nt probe sequence.

Four hundred twenty-nine thousand one hundred fifteen target-specific E-probes sequences of different lengths were generated. Each set corresponds to the target families: *Prevotellaceae, Clostridiaceae, Erysipelotrichaceae, Lachnospiraceae, Lactobacillaceae, Ruminicoccaceae, and Streptoccocaceae*. The original count of generated E-probes was as follows: *Prevotellaceae* 89,565, *Clostridiaceae* 58,554, *Erysipelotrichaceae* 195, *Lachnospiraceae* 87, *Lactobacillaceae* 211,507, *Ruminicoccaceae* 14,575, *and Streptoccocaceae* 54,632. The E-probe length for *Clostridiaceae* was 60nt and 80nt for the remaining families.

Each family-specific E-probe database was curated before being uploaded to EDNA MiFi to ensure E-probe specificity. A list of the genera in each family, referred to as a "friend" list, was created from the NCBI taxonomy database (Chapter III, Appendix 2, Table 3). Any E-probe matching to genus not in the "friend" list was eliminated from the E-probe database. Additionally, E-probe sequence datasets were mapped to the NCBI using BLASTn, and only uniquely taxa-specific sequences were retrieved after being curated at seven different E-values (10^{100}, 10^{30}, 10^{5}, 10^{-1}, 10^{-3}, 10^{-10}, 10^{-20}) and examined as separate E-probe sets. The result was curated target-specific E-probes further uploaded to EDNA MiFi to query raw unassembled sequence reads.

After comparing the resulting sets, E-probes curated at the e-value score $1x10^{-3}$ were selected. Any E-probe aligned with an e-value score lower than $1x10^{-3}$ was eliminated from the E-probe database. Lower e-values were not utilized to avoid eliminating an excessive number of family-specific E-probes. After curation, the original count was reduced to 413,601 E-probes divided as follows: *Prevotellaceae* 85,924, *Clostridiaceae* 57,115, *Erysipelotrichaceae* 188, *Lachnospiraceae* 85, *Lactobacillaceae* 206,301, *Ruminicoccaceae* 14,101, *and Streptoccocaceae* 49,887, respectively.

The ability of the developed E-probes to identify the families they were engineered for was verified *in silico*. For such, a sequencing simulator for metagenomics, MetaSim V0.9.5, was used to generate synthetic reads that resemble metagenome data sets (Ritcher et al., 2008). The simulated metagenomes were then queried against the generated E-probes. The E-probes detected the different concentrations of the seven families of interest in the simulated metagenomes; they were then uploaded to MiFi™ under the name EDNA-Gut Microbiota.

3.3.7 Rapid gastrointestinal microbiota detection and differentiation using EDNA-Gut Microbiota database

The EDNA-Gut Microbiota MiFi™ was used to identify the seven families of bacteria in the gastrointestinal tract. A 90% identity (%ID) and query coverage (QC) was selected for quality parameters in EDNA-Gut Microbiota MiFi™ to assess the sequenced metagenomes. A hit was defined as any instance where a raw read had a counterpart E-probe, and sensitivity of e-value 10^{-9} was used. The raw sequence data were queried against the EDNA-Gut Microbiota MiFi™, and the number of hits per

family generated per metagenome was obtained. The total number of reads per metagenome was calculated from the Fastq.gz files obtained after demultiplexing.

The total reads of each metagenome were used to calculate the relative abundance; Appendix 1, Table 1. Relative abundance was calculated by dividing the number of hits per family generated with the E-probes by the total number of reads per metagenome. The result was then multiplied by 100. For instance, when calculating the relative abundance for *Lactobacillaceae* in a metagenome with 1,182,511 total reads and having 201,537 hits related to the family, the relative abundance was calculated as 201,537 divided by 1,182,511 and multiplied by 100. When calculating the relative abundance for *Prevotellaceae* in a metagenome with 1,044,030 total reads and having 65,148 hits related to the family, the relative abundance was calculated as 65,148 divided by 1,044,030 and multiplied by 100.

3.4 STATISTICAL ANALYSIS

Fecal composition data were analyzed as a relative percentage of taxonomic abundance in a randomized complete block design with initial body weight as the blocking effect. Fecal microbiota composition was log10 transformed and analyzed using the MANOVA repeated measures of the GLM procedure (SAS Institute, version 9.2). The pig was considered the experimental unit. The comparative taxonomic abundance data were analyzed as a randomized complete block design with the model, including the effects of treatment, size, family, and interactions. The data variability was expressed as the standard error (SE); the response means are presented as the least-square means of the transformed data. Significantly different least-square means were

separated using Tukey adjust. The significance level was set at P-value ≤ 0.05, while ≤ 0.10 P-value > 0.05 was considered a tendency.

3.5 RESULTS

In this research, we hypothesized that family-specific E-probes are a valuable tool for detecting the microbial composition of the gastrointestinal tract of nursery pigs subject to two ZN dietary treatments. Consistent with our hypothesis, family-specific E-probes detected microbial abundance changes in the gastrointestinal microbe composition throughout the nursery phase.

Fecal microbiota composition

Analysis of the effect of ZN treatment, pig's size (large, medium, small), and time (day) on the relative abundance in the fecal microbiota of nursery pigs is in Table 10. There is no significant (P > 0.10) three-way interaction in the relative abundance of families in fecal microbiota in nursery pigs. However, there were significant two-way interactions in relative abundance of *Lactobacillaceae* by pig size category (P = 0.02) by the two dietary ZN treatments (P = 0.05) across days. There were significant interactions of dietary treatment by pig size category for *Clostrideaceae* and *Lactobacillaceae* (p = 0.03 and 0.08 respectively).

There was clearly significant difference in prevalence of all family members, except Erysipelotrichaceae across time (days). Fecal abundance by the size category of the pig was significantly different for *Clostridiaceae* (P = 0.002*); Lactobacillaceae* (P = 0.04*), and Ruminicoccaceae* (P = 0.001). However, the overall relative abundance of

the families of interest were not significantly different across the Zn Sulfate and Zn IBZ diet treatments at days 0, 14, 28, and 42. (Table 10).

Table 10. Summary of the effect of treatment[a], size[b], and day[c] on relative abundance[d] in the fecal microbiota of nursery pigs

	DF	*Clostridiaceae* p-value	*Erysipelotrichaceae* p-value	*Lachnospiraceae* p-value	*Lactobacillaceae* p-value	*Prevotellaceae* p-value	*Ruminicoccaceae* p-value	*Streptococcaceae* p-value
Trt*Size*Day	6	0.88	0.42	0.85	0.15	0.82	0.25	0.86
Size*Day	6	0.14	0.20	0.56	0.02	0.48	0.18	0.30
Trt*Day	3	0.67	0.80	0.63	0.05	0.28	0.20	0.38
Trt*Size	2	0.03	0.70	0.67	0.08	0.59	0.71	0.36
Day	3	< 0.0001	0.56	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Size	2	0.002	0.82	0.32	0.04	0.48	0.001	0.60
Trt	1	0.34	0.24	0.61	0.19	0.34	0.90	0.88

Equation: Relative abundance = Trt Size Day Trt*Size Trt*Day Size*Day Trt*Size*Day + ε

[a]Treatment (Zinc Sulfate or Zinc IBZ).

[b]Size (Large, medium/average, and small).

[c]Day (Day 0, Day 14, Day 28, Day 42).

[d]Relative abundance (Log10).

The relative abundance across families and the effect of treatment by day in nursery pigs are shown in Table 11. On d 0, 14, and 28, there was no significant difference (P > 0.1) observed between dietary treatments. However, on d 42, there was a significant difference (P = 0.03) observed, and the pigs fed Zn Sulfate presented a lower relative abundance across the families of interest when compared to those fed IBZ (Intellibond Z, Micronutrients, Indianapolis, IN), 0.130 % and 0.178 % respectively; Table 11.

Table 11. Effect of zinc source on relative abundance[a] of fecal microbiota composition across family of nursery pigs by day

	Zinc Sulfate	Zinc IBZ[b]	SE[c]	P-value
Day 0	0.027	0.020	0.36	0.13
Day 14	0.131	0.120	0.37	0.51
Day 28	0.236	0.166	0.37	0.12
Day 42	0.130	0.178	0.36	0.03

Equation: Relative abundance = Trt Day Trt*Day + ε
[a]Families (Clostridiaceae, Erysipelotrichaceae, Lachnospiraceae, Lactobacillaceae, Prevotellaceae, Ruminicoccaceae, and Streptoccocaceae).
[b]IBZ; Intellibond Z, Micronutrients, Indianapolis, IN.
[c]SE: Standard Error.

The relative abundance across families and the effect of pig size by day is shown in Table 12. There was a significant difference in the relative abundance of the families of interest on the different pig sizes on d 28 (P = 0.01) and 42 (P = 0.06); however, this was not observed on d 0 (P = 0.18) and 14 (P = 0.20). On d 28, the large pigs presented a significantly lower (P = 0.01) relative abundance when compared to the small ones. But, by d 42, the trend reversed and the small pigs presented a lower (P = 0.04) relative abundance when compared to the large ones; Table 12.

Table 12. Effect of size[d] on relative abundance[e] of the fecal microbiota of nursery pigs by day

	Large	Medium	Small	P-value
Day 0	0.019[a]	0.031[a]	0.021[a]	0.18
Day 14	0.106[a]	0.136[a]	0.135[a]	0.20
Day 28	0.129[bc]	0.204[ab]	0.294[a]	0.01
Day 42	0.175[a]	0.150[ac]	0.124[bc]	0.06

Equation: Relative abundance = Size Day Size*Day + ε

[abc]The values with different superscript letters in a row are significantly different ($P < 0.05$).

[d]Size (Large, Medium, and Small).

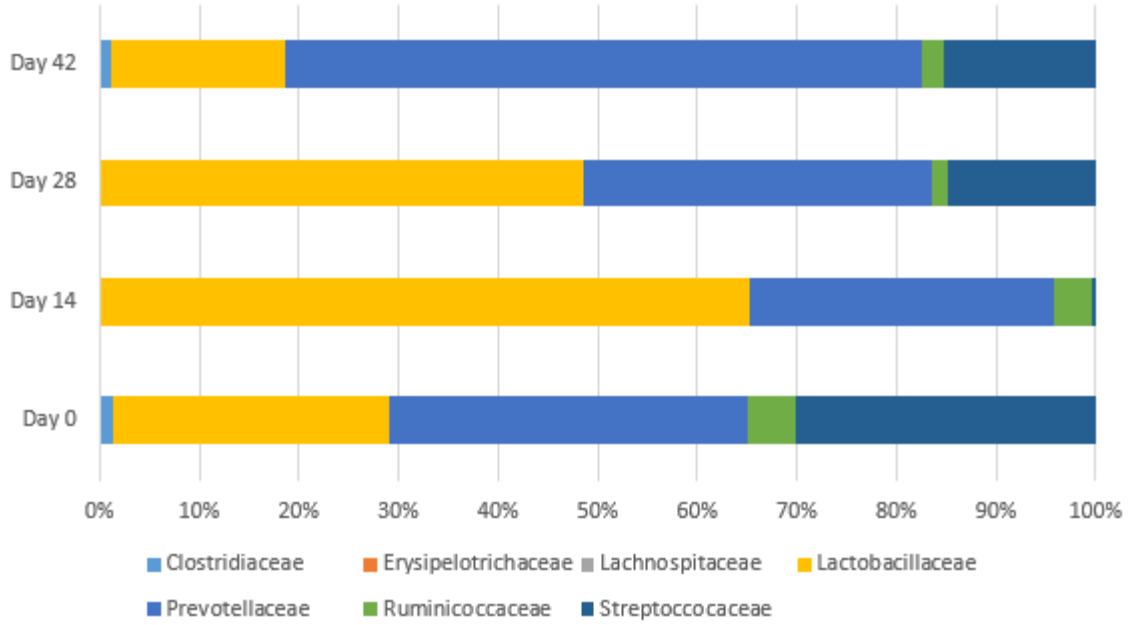
[e]Families (Clostridiaceae, Erysipelotrichaceae, Lachnospiraceae, Lactobacillaceae, Prevotellaceae, Ruminicoccaceae, and Streptoccocaceae).

On d 0, 14, 28, and 42, there was a significant difference ($P < 0.0001$) in *Clostridiacee, Lachnospiraceae, Lactobacillaceae, Prevotellaceae, Ruminicoccaceae, Streptoccocaceae* relative abundance in fecal microbiota assessed with E-probes of nursery pigs by days. However, this was not observed in *Erysipelotrichaceae* ($P > 0.1$), Figure 3; Appendix 3, Table 1.

On d 0, the families with the highest relative abundance in feces were *Prevotellaceae, Streptoccocaceae, and Lactobacillaceae,* 1.085 %, 0.906 %, and 0.834 %, respectively, and the lowest were *Erysipelotrichaceae, Lachnospiraceae, and Clostridiaceae,* 0.00002 %, 0.00003 %, and 0.042 % respectively. On d 14, the families with the highest relative abundance in feces were *Lactobacillaceae, Prevotellaceae, and Ruminicoccaceae,* 30.761 %, 14.415 %, and 1.732 %, respectively, and the lowest were *Erysipelotrichaceae, Lachnospiraceae and Clostridiaceae,* 0.00001 %, 0.005 %, and 0.047 % respectively. On d 28, the families with the highest relative abundance in feces were *Lactobacillaceae, Prevotellaceae, Streptoccocaceae,* 20.735 %, 14.962 %, and 6.380 %, respectively, and the lowest were *Erysipelotrichaceae, Lachnospiraceae and*

Clostridiaceae, 0.00003 %, 0.004 %, and 0.084 % respectively. On d 42, the families with the highest relative abundance in feces were *Prevotellaceae, Lactobacillaceae, and Streptoccocaceae,* 15.290 %, 4.206 %, and 3.641 %, respectively. On the other hand, the ones with the lowest were *Erysipelotrichaceae, Lachnospiraceae, and Clostridiaceae,* 0.00002 %, 0.004%, and 0.260 %, respectively.

Figure 3. Effect of relative abundance in fecal microbiota composition of nursery pigs



Equation: Relative abundance = Day + ε

3.6 DISCUSSION

The pig's gastrointestinal tract, particularly during early weaning and the weaning period, is a complex environment with a rapidly changing size and bacterial population (Campbell et al., 2013). Understanding changes in the gastrointestinal microbiome composition under different conditions is essential for studying diseases (Knecht et al.,

2020). In this study, the role of Zn Sulfate and Zn Hydroxychloride in shaping the gastrointestinal microbiota in nursery pigs was evaluated using E-probes to track changes in the most common gastrointestinal commensal families: *Prevotellaceae, Clostridiaceae, Erysipelotrichaceae, Lachnospiraceae, Lactobacillaceae, Ruminicoccaceae* and *Streptoccocaceae*. The data demonstrate that the family-specific E-probes could detect microbial abundance changes in the gastrointestinal microbe composition throughout the nursery phase. However, several significant differences between the current study and previous ones were observed. The inconsistencies may be related to differences, such as methodologies, weaning days, sampling time, fecal collection (from intestinal segments or rectal swabs), and nursing facility environments.

Nursery pigs usually require 80 to 100 ppm of Zn to meet their requirement for growth. In previous reports, more than 90% of the core bacterial taxa in fecal samples of pigs were supplemented with 300 mg/kg of zinc oxide from the 11 phyla identified in the study (Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Tenericutes, Synergistetes, Chlamydiae, and unclassified bacteria) were composed of Firmicutes and Bacterioidetes. As the pigs aged, the Firmicutes increased, and Bacterioidetes decreased (Tran et al., 2018). Our data did not generally support these proportions. In the current study, Firmicutes are represented by *Streptoccocaceae, Lactobacillaceae, Clostridiaceae, Lachnospiraceae, Ruminicoccaceae, and Erysipelotrichaceae;* and Bacterioidetes by *Prevotellaceae.* The differences observed may undoubtedly be related to the methodology used in both experiments. Tran et al. (2018) performed amplicon sequencing of the V3 region. The obtained sequences were filtered to remove low-quality reads and trimmed. Only sequences containing 80 to 176 base pairs (length of

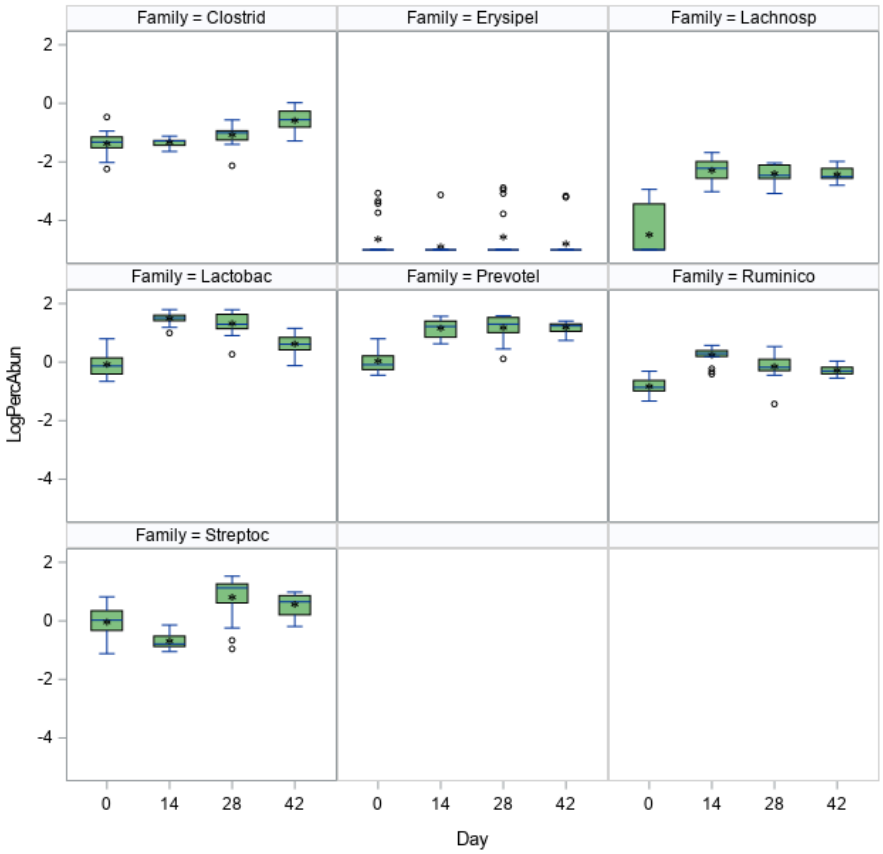
the amplified region) were selected for subsequent analyses. After being trimmed, the unique sequences were aligned, and the cleaned sequences were clustered into OTU. The sequence data were normalized by dividing the sequence reads by each sample's total number of reads. It is to note that Tran et al. (2018) supplementation was 300 mg/kg from zinc oxide compared to 100 mg/kg from Zn Sulfate or Zn IBZ as in our experiment. This may help explain the differences among results.

Bacterial changes will impact others and affect the entire gut ecosystem. Following weaning, we observed *Prevotellaceae, Streptoccocaceae,* and *Lactobacillaceae* as the families of highest relative abundance in the feces of nursery pigs (Figure 4), contrary to Tran et al. (2018) that Erysipelotrichaceae, Bacteroidaceae, and Lachnospiraceae were the families of highest relative abundance. During the weaning process, Konstantinov et al. (2006) detected in ileal samples significantly lower levels of *Lactobacillus* in the gastrointestinal tract compared to unweaned piglets where *Lactobacillus* were abundant colonizers. *Lactobacillus* is especially important during weaning. *Lactobacillus sobrius, L. reuteri*, and *L. acidophilus* may play a crucial role in establishing and maintaining the gastrointestinal tract bacterial homeostasis after birth (Konstantinov et al., 2006).

The piglet's immune system is underdeveloped during weaning and depends on the sow's milk. The glycans in the sow's milk control the microbiome of nursing animals and prevent opportunistic and pathogenic bacteria (Knecht et al., 2020; Lallès et al., 2007). Competitive exclusion and excretion of bacteriocins capable of bacterial lysis are used to prevent colonization and growth of pathogenic bacteria (Stensland et al., 2015).

Escherichia coli, Clostridium perfringens, Salmonella choleraesuis, and *Salmonella typhimurium* are the most common pathogenic bacteria causing impairments of the intestinal villi, leading to intestinal disorders in pigs (Knecht et al., 2020). In the current study, the effect of diarrhea presence was only assessed at the family level and not down to the specie level; therefore, no accurate comparison against literature can be made. In addition, the pathogenic bacteria causing impairments in the intestinal tract, according to Knecht et al. (2020), are from *Enterobacteriaceae* and *Clostridiaceae* families. The E-probes specific to the *Enterobacteriaceae* family were not developed in this study; therefore, the relative abundance of *Escherichia coli, Salmonella choleraesuis,* and *Salmonella typhimurium* were not assessed. On the contrary, E-probes specific to *the Clostridiaceae* family were indeed designed; however, our findings indicate complexity within the family, such that there may be beneficial effects of some family members.

Figure 4. Effect of relative abundance[a] in families[b] of interest of nursery pig feces



Equation: Relative abundance = Day + ε.
[a]Log10
[b]Families (Clostridiaceae, Erysipelotrichaceae, Lachnospitaceae, Lactobacillaceae, Prevotellaceae, Ruminicoccaceae, and Streptoccocaceae).

Knecht et al. (2020) stated that *Lactobacillus* is the prevalent bacterial community in the gastrointestinal tract of pigs and is always present in it throughout an animal's life. We demonstrated that *Lactobacillaceae* are present through all the nursery phases and are highly predominant. Our findings suggest that the relative abundance of *Lactobacillaceae* changes from being the 3rd most abundant after weaning (d 0) to being the most abundant on d 14 and 28, to being the second most abundant on d 42 (Figure 4). Presenting the highest relative abundance on day 14, in agreement with Tran et al. (2018), we observed

a 30.76% compared to 39.05% observed by them. However, Soler et al. (2018) observed a greater relative abundance of *Lactobacillaceae* on day 30.

In commercial farming, the pigs' stunted growth often is observed in the first couple of weeks after weaning. The emergence of *Clostridia spp* and *E. coli* in intestinal samples of piglets may result in diarrhea and decreased weight gain due to affected nutrient absorption caused by shorter villi and deeper crypts (Hu et al., 2013; Konstantinov et al., 2006). In our experiment, the designed E-probes were not designed to differentiate down to the genius level (i.e., *Clostridia spp*); therefore, a proper comparison with the literature cannot be accurately made. However, at a family level, in *Clostridiaceae,* we observed no significant difference in the first couple of weeks after weaning 0.042% on d 0 and 0.047% on d 14. Additionally, a set of E-probes targeting the *Enterobacteriaceae* family, where the *E. coli* belongs, was not designed. Therefore, due to the absence of data, comparison with the literature is not feasible.

Our data suggest that the *Ruminicoccaceae* family's highest relative abundance throughout the experiment was 1.73% on d 14 compared to the highest relative abundance (7.63%) on d 7 that Tran et al. (2018) observed. The decrease in the relative abundance of *Rumminicoccaceae* following d 14 may be related to the decrease in Oscillospira (genus of the *Rumminicoccaceae* family) usually observed in weaned pigs. Oscillospira is a butyrate producer that acts as an energy source for colonocytes and prevents intestinal inflammation and systemic infection (Wei et al., 2021).

Our data suggest that the *Clostridiaceae, Erysipelotrichaceae,* and *Lachnospiraceae*'s highest relative abundance throughout the experiment was 0.26% on d

42, 0.00003% on d 28, and 0.005% on d 14, relatively (Figure 4). However, Tran et al. (2018) reported the highest relative abundance of 10.74% on d 28, 17.54% on d 0, and 33.74% on d 7 of the same families. Even though the relative abundance calculated with the E-probes was not numerically similar to those reported in previous studies, we did observe an increase in the relative abundance of *Lachnospiraceae* which may correspond with the increase in *Blautia* (genus of the *Lachnospiraceae* family) which is involved in carbohydrate digestion known to increase post-weaning (Figure 4) (Wei et al., 2021).

We observed a significant enrichment of almost 13.8% in *Prevotellaceae* from d 0, starting at 1.08% to an almost steady average of 14.89% from d 14 onwards (Figure 4). This increase agrees with Soler et al. (2018) and Wei et al. (2021), who observed a dramatic increase in *Prevotellaceae* the first 7 days post-weaning. However, they reported an increase from 6.5% to more than 33% from day 15 onwards. The increment in *Prevotellaceae* may be explained since *Prevotella*'s relative abundance is rapidly increased after weaning, corresponding to the consumption of plant polysaccharides as *Prevotella* abundance is driven by them (Wei et al., 2021).

In the current study, the *Streptoccocaceae* family increased in the relative abundance of the family, with its highest at d 28 (6.38%), decreasing to 3.64% at d 42 (Figure 4). Our results might be explained by previous findings by Kim et al. (2011) and Tran et al. (2018), observing a fluctuation of the proportion of *Streptococcus* during the experiment, increasing the highest by d 28 and then decreasing.

Previous data have demonstrated that the gastrointestinal microbiota affects the animal's health status, immune response, meat quality, and body weight since the gut

microbiota plays a vital role in the extraction, synthesis, and absorption of nutrients (Knecht et al., 2020). Even though it has been observed that the commensal bacteria in the intestinal tract of pigs change their relative abundance throughout their life, conflicting data is observed (Isaacson & Kim, 2012; Kim et al., 2011; Li et al., 2017; Rinninella et al., 2019; Soler et al., 2018). In this research, contradictory data was observed in the relative abundance of the families of interest. The highest/lowest relative abundance between previous data differed numerically and in the age of the pigs where the bacterial abundances were observed; however, families like *Lactobacillaceae* and *Prevotellaceae* in the current research behaved similarly to previous ones. Whether the observed differences between our results and the literature are a function of the methodology or factual microbiota compositional differences between tested subjects is unclear. However, this research illustrates the possible usage of the EDNA MiFi pipeline as a metagenomics tool for assessing gastrointestinal microbiota composition.

3.7 CONCLUSION

The pig's gastrointestinal tract, particularly in the nursery phase, is a complex environment rapidly changing in the bacterial population. Overall the results from this study indicate that the designed E-probes were able to assess the microbial changes in the feces of pigs throughout the nursery phase and helped us understand the age-related bacterial diversity in commercial pig feces. However, the literature's gastrointestinal tract bacteria composition data is diverse and inconsistent. Therefore, further investigations on variables affecting the relative abundance of critical commensal bacteria should be done along with method comparison to explain our findings better.

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CHAPTER IV

4.1 RELATIONSHIP BETWEEN NURSERY PIGS NUTRITION AND GROWTH & GASTROINTESTINAL MICROBIOTA

4.1.1 The general importance of zinc

Micronutrients are substances necessary by the body in small amounts. These low concentration substances play a significant role in metabolism and normal tissue functions. As the second most abundant essential trace mineral, zinc is required for multiple metabolic processes in the body (Usama et al., 2018). Cell functions such as enzymatic reactions, DNA synthesis, and gene expression are mediated by zinc (Gielda & Dirita, 2012). Additionally, acquired and innate immunity require zinc, which acts as a coenzyme in many essential immune responses (Foligné et al., 2020). Zinc atoms are used in over 300 enzymes and thousands of transcription factors

Pharmacological levels of zinc oxide have been shown to reduce diarrhea and improve growth performance in weaning pigs. In experimental diets for pigs, zinc must be supplemented in small amounts to meet the nutritional requirements. One proposed mechanism is modifying the intestinal microbiota composition and activity (Pieper et al., 2020).

4.1.2 Intestinal zinc status

Intracellular zinc reduces the inflammation of intestinal mucosa and regulates intestinal permeability by occludin proteolysis, protecting the intestines from pathogens. Studies suggest that intracellular zinc stimulates the release of ghrelin, the hormone required for regulating appetite, in the stomach of pigs. Under zinc deficit, the intestinal tight junction and membrane function is impaired, leading to mucosal inflammation increasing permeability and potential for invading microorganisms (Usama et al., 2018).

The regulation of zinc is essential, as too little zinc does not support cellular growth, while too much zinc is toxic. Mammalian cells maintain zinc homeostasis through transport and export proteins (such as human ZIP proteins or ZnT-1) (Gielda & Dirita, 2012). Expression levels of zinc (Zn) transporters and metallothioneins (MTs) are greatly affected by zinc availability. The MTs expression in the gastrointestinal tract is enhanced by infection and inflammation. The MTs isoforms most abundant are MT-1 and MT-2 (Foligné et al., 2020). A high zinc oxide diet (2500 ppm) reveals higher exporter ZnT1, lower zinc importer ZIP4, and an increased expression of metallothioneins MT1A and MT2B. The aforementioned is a good reference point for homeostatic regulation. However, no difference is observed in 40 or 110 ppm diets (Pieper et al., 2020).

4.1.3 Impact of zinc on the gastrointestinal microbiota

Zinc has been shown to have profound effects on gut microbiota composition. It is suggested that there is an interaction between zinc and the gut epithelium, being a vital booster of normal intestinal barrier functions and the regeneration of epithelium (Usama et al., 2018). Zinc is essential for controlling the growth of most microorganisms. Many

species of bacteria require zinc uptake systems for growth and fitness (Foligné et al., 2020). However, elevated zinc levels are toxic to microbiota; therefore, zinc homeostasis must be tightly regulated. Under low-zinc conditions, zinc is brought into bacteria through the ZnuABC transporter. The ZnuABC is a transport system necessary for virulence and host colonization in several bacterial species (Gielda & Dirita, 2012).

Ileum biopsies of mice supplemented with Zn Sulfate presented downregulation of interferon-stimulated response element (ISRE) and interferon-regulatory factor (IRF) genes (Souffriau et al., 2020). The ISRE element serves as the binding site for interferon-regulatory factors (IRF) (Meraro et al., 2002). Interferon regulatory factors (IRFs) regulate different aspects of innate and adaptive immune responses, such as triggering anti-viral and pro-inflammatory responses and regulating immune cell differentiation by interacting in gene transcription (Jefferies, 2019). The gut commensal microbiota induces ISRE and IRF genes in the ileum, some of which are involved in necroptosis. Zinc lowers ISRE and IRF gene expression by actively and directly modulating the composition of the gut microbes. However, due to a lack of gut microbes (laboratory germ-free mice), mice with shallow ISRE/IRF gene expression are not influenced by the beneficial effects of zinc. The mechanism by which zinc reduces ISRE/IRF gene expression appears to relate to the direct antibacterial effects of zinc against *Staphylococcus sciuri* and *Staphylococcus nepalensis* (Souffriau et al., 2020). *Staphylococcus sciuri* has been isolated in an acute outbreak of exudative epidermitis in piglets (Lu et al., 2017). *Staphylococcus nepalensis,* on the other hand, was originally isolated from goats with pneumonia; however, in pigs, it has only been isolated in the skin (NovãKovã et al., 2006).

Additionally, zinc is essential for healthy intestinal homeostasis and Paneth cell function and survival (Souffriau et al., 2020). Paneth cells reside in the small intestine crypts and are highly specialized secretory epithelial cells implicated in balancing the ecology of the intestinal lumen through the release of cytokines and tumor necrosis factors. Defects in the Paneth cell's function are associated with microbial dysbiosis (Podany et al., 2016).

4.1.4 Impact of zinc on the fecal microbiota

Zinc release from host tissue has been proposed to be an important innate defense mechanism. It is suggested that zinc modulates the microbiome because zinc is irrelevant in the absence of gut flora (Souffriau et al., 2020). Excess zinc can be toxic to bacteria; therefore, intracellular levels must be tightly controlled. Zinc concentration in host fluids can rise in response to bacterial infection and inflammation by being released from damaged cells (Velasco et al., 2018).

The positive effects of zinc supplementation on intestinal bacterial populations make it a standard method for treating diarrhea (Gielda & Dirita, 2012). Microbes in the gastrointestinal tract lacking a high-affinity zinc uptake system are impaired for replication and persistent colonization (Gielda & Dirita, 2012). Zinc chloride modifies the composition of the gut microbial communities by the direct cytotoxic effect on certain bacterial species, such as *Staphylococci* (Firmicutes) and *Eschericchia coli* (Proteobacteria), while *Enterococcus faecalis* (Firmicutes) is not affected (Souffriau et al., 2020; Usama et al., 2018). Velasco et al. (2018) propose that zinc excess inhibits pathogen growth by competition for manganese (Mn) uptake.

Feces of zinc-treated mice presented a higher abundance of Actinobacteria (3.8% vs. 2%) and Bacteroidetes (66% vs. 46%). Substantial decreases in Firmicutes (24% vs. 42.2%) and Proteobacteria (2.1% vs. 8.1%) we apparent effects of zinc when compared to the control group (Souffriau et al., 2020). However, Foligné et al. (2020) did not observe changes in the microbiota composition in Firmicutes, Bacteroidetes, Tenericutes, and Proteobacteria after zinc supplementation in mice. Moreover, they observed very subtle decreases at the family level for the *Lactobacillaceae* and *Enterobacteriaceae* (Foligné et al., 2020).

Conclusions about the effects of zinc at the genus level might be misleading. Members of the same genus (i.e., *Bacteroides, Bifidobacterium, Clostridium*) have a higher tolerance, whereas others were more susceptible to zinc in vitro (Pieper et al., 2020). Lactobacillus and Streptococcus in the gastrointestinal tract have increased with zinc supplementation (Usama et al., 2018). Some bacteria are sensitive to zinc, whereas others are resistant. Greater microbial diversity is associated with high intestinal zinc concentrations that might impact other bacterial strains. For example, certain species would enable others to multiply more in the presence of excess zinc. Alternatively, displacing other bacterial groups would reduce competition in the intestine, acting as a potent modifier for certain bacterial groups (Foligné et al., 2020).

4.2 OBJECTIVE AND HYPOTHESIS

This study evaluates the role of two dietary zinc sources (Zn Sulfate or Zn Hydroxychloride - IBZ) in shaping the gastrointestinal microbiota and the growth performance and diarrhea prevalence in nursery pigs. The hypothesis is that pigs fed Zn

IBZ have a lower diarrhea incidence than pigs fed Zn Sulfate, leading to better performance metrics: end weight, average daily gain (ADG), average daily feed intake (ADFI), and feed efficiency (F:G), These metrics are correlated with gastrointestinal microbiota variations. The gastrointestinal commensal families assessed in this objective were: *Prevotellaceae, Clostridiaceae, Erysipelotrichaceae, Lachnospiraceae, Lactobacillaceae, Ruminicoccaceae,* and *Streptoccocaceae*.

4.3 MATERIALS AND METHODS

4.3.1 Animals, housing, and treatments

The experimental protocol was approved by, and the pigs were handled and cared for according to the guidelines established by the Oklahoma State University Institutional Animal Care and Use Committee (IACUC).

A total of sixty crossbred weaned pigs (3 weeks of age; Pig Improvement Company (PIC®); 30 barrows and 30 gilts) were subject to research for 6 wk. Pigs with average initial body weight (BW) of 5.4 kg were randomly allotted to one of two dietary zinc treatments. Three replicate pens per treatment consisting of ten pigs per pen (5 barrows and 5 gilts) were blocked to minimize variations in gender, initial BW, and location of the pen in a randomized complete block design. Each pen had a nipple waterer and a stainless steel feeder. Pigs were housed in an environmentally controlled nursery facility with slatted plastic flooring and a mechanical ventilation system. The environmental temperature decreased by 1˚C per week, starting at 30˚C in the first week. During the entire experiment, pigs were allowed to consume feed and water *ad libitum*.

From day 0 until day 42, the dietary Zn treatments were allotted to pens: Sulfate (100 ppm of added Zinc Sulfate) or Zn IBZ (100 ppm of added Zinc Hydroxychloride; Intellibond Z, Micronutrients, Indianapolis, IN). All remaining nutrients in the diet were added at or above the requirements listed in the NRC (2012). Both diets were formulated as basal diets, and each dietary Zn treatments mineral premix was added subsequently during the mixing process.

4.3.2 Diets

A four-phase nursery feeding program was employed in the experiment, with diets formulated to meet or exceed NRC (2012) nutrient requirements as presented in Chapter II, Tables 1 and 2. Pigs were fed a standard diet with either Zn Sulfate or Zn IBZ: phases 1-4 (100 mg Zn/kg). The experiment lasted for 42 days, and all pigs received Phase 1 diet during d 0-7, Phase 2 during d 7- 14, Phase 3 during d 14-23, and Phase 4 during d 23-42.

4.3.3 Growth performance

Pigs BW and feeders were weighed weekly, starting on d 0 (initial weight), followed by d 7, 14, 21, 28, 35, and 42. Average daily gain (ADG), average daily feed intake (ADFI), and feed efficiency (F:G) were determined. According to the dietary Zn treatment, the feed was weighed before every feeding time. The feed intake was calculated weekly by subtracting the feeder weight with the remaining weekly feed from the initial feeder weight. The starting and end weights were used to calculate the average daily gain when subtracting the starting weights from the end weights over a specified period. Feed efficiency is the ratio of feed consumed to body weight gain (Patience et al., 2015).

4.3.4 Fecal consistency by pen

Fecal consistency scoring was visually assessed on pigs in each pen, and scoring was done weekly from d 0 to d 42. Fecal scoring was according to the following scale: 1 = solid; 2 = semi-solid; and 3 = liquid. Diarrhea was considered when feces at level 2 or 3 were present for two successive days (Liu et al., 2010).

4.3.5 Fecal consistency by pig size categories

A large, medium/average and a small pig per pen were visually selected on d 0. After selecting the three pigs per pen, the large, medium, and small pigs were ear-tagged for easier identification on subsequent evaluation on d 14, 28, and 42. The same pigs were sampled throughout the study.

Fecal consistency scoring was visually assessed for the large, medium, and small pigs in each pen on d 0, 14, 28, and 42. Fecal scoring was as reported above (by pig size categories).

4.3.6 Fecal microbiota composition

The three pigs per pen (large, medium, and small) selected and ear-tagged on d 0 were subject to fecal microbiota composition assessment. On d 0, fecal samples were obtained by rectal stimulation with a dry transport system sterile flocked swab (Puritan Medical Products, Co., Guilford, ME). On d 14, 28, and 42, the sampling was done with tubes with a screw cap and a spoon (Growing Labs, Co., Suwanee, GA). After collection, the fecal samples were placed on ice, placed into an appropriately labeled sterile Eppendorf tube (Thermo Fisher Scientific, Waltham, MA), and stored at -80˚C until further analysis.

4.3.7 DNA extraction

DNA extraction from fecal samples was performed following the manufacturer's protocol using Quick-DNA™ Fecal/Soil Microbe Miniprep Kit (Zymo Research, Irvine, CA). Nucleic acid purity assessment was detected by NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE), and the DNA concentration was detected by Quantus™ Fluorometer (Promega, Madison, WI). The samples with DNA < 100 ng of molecular weight before library preparation were purified with DNeasy PowerClean Pro Cleanup Kit (Qiagen, Hilden, Germany).

4.3.8 Library preparation, sequencing, and post-processing

DNA multiplexing of 12 samples at a time was performed using the Rapid Barcoding Sequencing SQK-RBK004 (Oxford Nanopore Technologies, Oxford, UK). After the DNA barcoding step, sequencing was made by a MinION™ device (Oxford Nanopore Technologies, Oxford, UK).

Platform Quality Check (QC) was performed to determine the number of pores available for sequencing in the flow cell, and only flow cells with >1000 pores were used. The flow cells are manufactured with 2000 pores; however, receiving flow cells with no more than 1500 active pores upon purchase is reasonably expected. The sequencing run was performed using the MinIONTM flow cell (FLO-MIN106D (R9.4.1); Oxford Nanopore Technologies, Oxford, UK). After reaching the quality for sequencing, the flow cell was primed following the Rapid Barcoding Sequencing SQK-RBK004 (Oxford Nanopore Technologies, Oxford, UK) protocol, and 75µl of the pooled library was carefully mixed through pipetting before loading it onto the SpotON port. The library was loaded dropwise, preventing loading air bubbles into the flow cell and closing the prime

and SpotON cover back to its original position. Subsequently, the lit of the MinION™ device was moved back to the closed position.

Once the sample was loaded into the flow cell, the MinKNOW software (Oxford Nanopore Technologies, Oxford, UK) was used. An experiment and sample ID name were given. Additionally, the used kit was selected - SQK-RBK004 (Oxford Nanopore Technologies, Oxford, UK). The sequencing run lasted for 72 hours or until no more pores were available for sequencing. Basecalling was disabled, and barcoding was enabled before starting the sequencing. The output format selected was Fast5 and Fastq.

The large datasets required a specific workstation computer (Oklahoma State University High Performance Computing Center) for processing the generated data. Guppy, a data processing tool, requires Fast5 files for basecalling. Basecalling is the computational procedure of translating the raw electrical signal of the sequencer into nitrogenous bases. A configuration file was used to set the basecalling parameters depending on the flow cell and library preparation kit utilized during sequencing Wick et al., 2019). Several Fastq files with the basecalled reads were generated after basecalling; such files underwent demultiplexing. Demultiplexing is the process of classifying the barcodes per read and assigning them to read groups (metagenomes of each sampled pig). The Fastq files per barcode were concatenated and compressed with GZIP (Fastq.gz) for later analysis.

4.3.9 E-Probe design

E-probe design at the family level required two sets of genomes, the target and near neighbor genomes. The target genome was used as a template to generate E-probes.

The near neighbors helped eliminate redundant and duplicative genome regions in the target genome, leaving sequences unique to the target family available to generate the E-probes. The genomes from *Prevotellaceaee, Clostridiaceae, Erysipelotrichaceae, Lachnospiraceae, Lactobacillaceae, Ruminicoccaceae,* and *Streptoccocaceae* families served as target genomes. The genomes of such families were obtained from the National Center of Biotechnology Information (NCBI) GenBank; details of the genomes per family can be accessed in this dissertation (Chapter III, Appendix 2, Table 1 and 2). Genomes in a family were used as the target sequence. The rest of the family genomes were used in a compiled Fasta file of near neighbors. E-probes were generated using the E-probe pipeline for EDNA MiFi, where E-probe length determination occurs (Stobbe et al., 2013).

During E-probe development, redundant genome regions were eliminated by the modified Tools for Oligonucleotide Fingerprint identification (TOFI) pipeline (Satya et al., 2008) used by the EDNA MiFi pipeline (Stobbe et al., 2013). After TOFI comparison, designed E-probes underwent a specific Basic Local Alignment Search Tool Nucleotide (BLAST) to eliminate potentially cross-reacting homo-oligomers. E-probe specificity was verified by pairwise alignment of each E-probe with the intended target family genome using stringency of 100% identity and query coverage through the partially non-redundant nucleotide collection (nr/nt) database (nt) by BLAST with the NCBI. Target-specific E-probes sets of different lengths (20nt, 30nt, 40nt, 60nt, 80nt, 100nt, 120nt) were generated depending on genome size with a minimum match for 15 nt probe sequence.

Four hundred twenty-nine thousand one hundred fifteen target-specific E-probes sequences of different lengths were generated. Each set corresponds to the target families: *Prevotellaceae, Clostridiaceae, Erysipelotrichaceae, Lachnospiraceae, Lactobacillaceae, Ruminicoccaceae and Streptoccocaceae*. The original count of generated E-probes was as follows: *Prevotellaceae* 89,565, *Clostridiaceae* 58,554, *Erysipelotrichaceae* 195, *Lachnospiraceae* 87, *Lactobacillaceae* 211,507, *Ruminicoccaceae* 14,575, *and Streptoccocaceae* 54,632. The E-probe length for *Clostridiaceae* was 60nt and 80nt for the remaining families.

Each family-specific E-probe database was curated before being uploaded to EDNA MiFi to ensure E-probe specificity. A list of the genera in each family, referred to as a "friend" list, was created from the NCBI taxonomy database (Chapter III, Appendix 2, Table 3). Any E-probe matching to genus not in the "friend" list was eliminated from the E-probe database. Additionally, E-probe sequence datasets were mapped to the NCBI using BLASTn, and only uniquely taxa-specific sequences were retrieved after being curated at seven different E-values (10^{100}, 10^{30}, 10^{5}, 10^{-1}, 10^{-3}, 10^{-10}, 10^{-20}) and examined as separate E-probe sets. The result was curated target-specific E-probes further uploaded to EDNA MiFi to query raw unassembled sequence reads.

After comparing the resulting sets, E-probes curated at the e-value score $1x10^{-3}$ were selected. Any E-probe aligned with an e-value score lower than $1x10^{-3}$ was eliminated from the E-probe database. Lower e-values were not utilized to avoid eliminating an excessive number of family-specific E-probes. After curation, the original count was reduced to 413,601 E-probes divided as follows: *Prevotellaceae* 85,924,

Clostridiaceae 57,115, *Erysipelotrichaceae* 188, *Lachnospiraceae* 85, *Lactobacillaceae* 206,301, *Ruminicoccaceae* 14,101, *and Streptoccocaceae* 49,887, respectively.

The ability of the developed E-probes to identify the families they were engineered for was verified *in silico*. For such, a sequencing simulator for metagenomics, MetaSim V0.9.5, was used to generate synthetic reads that resemble metagenome data sets (Ritcher et al., 2008). The simulated metagenomes were then queried against the generated E-probes. The E-probes detected the different concentrations of the seven families of interest in the simulated metagenomes; they were then uploaded to MiFi^{TM} under the name EDNA-Gut Microbiota.

4.3.10 Rapid gastrointestinal microbiota detection and differentiation using EDNA-Gut Microbiota database

The EDNA-Gut Microbiota MiFi^{TM} was used to identify the seven families of bacteria in the gastrointestinal tract. A 90% identity (%ID) and query coverage (QC) was selected for quality parameters in EDNA-Gut Microbiota MiFi^{TM} to assess the sequenced metagenomes. A hit was defined as any instance where a raw read had a counterpart E-probe, and sensitivity of e-value 10^{-9} was used. The raw sequence data were queried against the EDNA-Gut Microbiota MiFi™, and the number of hits per family generated per metagenome was obtained. The total number of reads per metagenome was calculated from the Fastq.gz files obtained after demultiplexing.

The total reads of each metagenome were used to calculate the relative abundance; Appendix 1, Table 1. Relative abundance was calculated by dividing the number of hits per family generated with the E-probes by the total number of reads per

metagenome. The result was then multiplied by 100. For instance, when calculating the relative abundance for *Lactobacillaceae* in a metagenome with 1,182,511 total reads and having 201,537 hits related to the family, the relative abundance was calculated as 201,537 divided by 1,182,511 and multiplied by 100. When calculating the relative abundance for *Prevotellaceae* in a metagenome with 1,044,030 total reads and having 65,148 hits related to the family, the relative abundance was calculated as 65,148 divided by 1,044,030 and multiplied by 100.

4.4 STATISTICAL ANALYSIS

The statistical association, the magnitude of the association, and the direction of the relationship between variables for both dietary Zn treatments were calculated by the Spearman's correlation coefficient (ρ) (RStudio, version 1.4.1717). Correlation between two variables is expressed as either positive (+x) or negative (-x). Correlation coefficients magnitude indicates: very highly correlated (±0.9 to ±1.0), considered highly correlated (±0.7 to ±0.9), moderately correlated (±0.5 to ±0.7), and low correlation (±0.3 to ±0.5). The complete absence of correlation is represented by 0.

4.5 RESULTS

The pig intestinal microbiota is undoubted of critical importance to its host, considering that it contributes to nutrient digestion (Gardiner et al., 2020). During the weaning period, the pig's gastrointestinal tract is a complex environment with a rapidly changing size and bacterial population (Campbell et al., 2013). Zinc has been used in swine diets for growth performance improvement, post-weaning diarrhea reduction, and enhancement in immunity. However, the mode of action is still not well understood.

In this research, we hypothesized that pigs fed Zn IBZ would have a lower diarrhea incidence leading to better performance metrics than those fed Zn Sulfate, and that end weight, average daily gain (ADG), average daily feed intake (ADFI), and feed efficiency (F:G) were correlated with gastrointestinal microbiota variations of the families of interest.

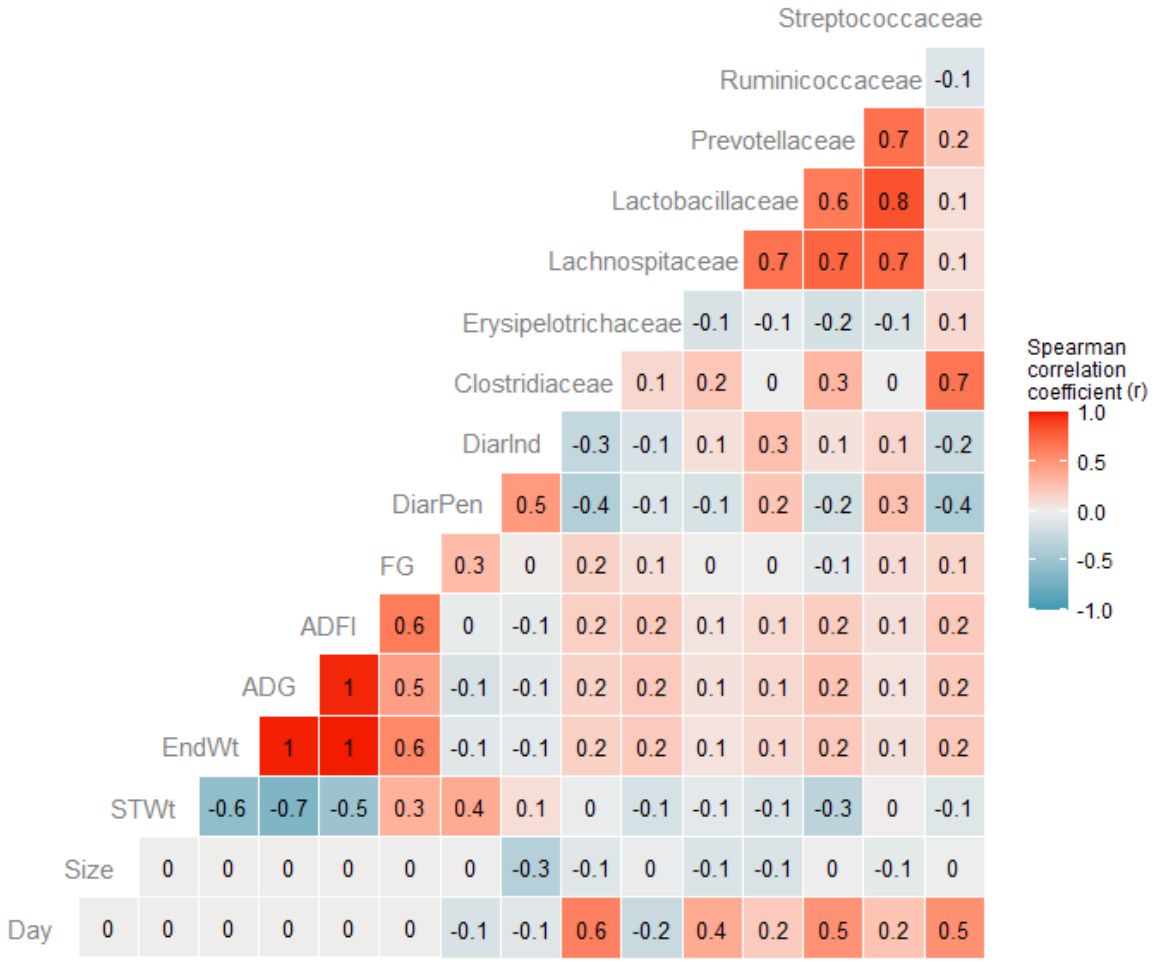
Throughout the research, treatment diets met the nutritional requirements of zinc regardless of source. Indeed, we did find differences in growth performances and gastrointestinal microbiota variations of the families of interest among dietary Zn treatments.

The pigs fed the diets supplemented with Zn Sulfate showed a positive correlation ($r > 0.5$) between growth factors (end weight, ADG, ADFI, and FG) as expected. As expected, diarrhea per pen and individual diarrhea were also correlated ($r = 0.5$). The diarrhea presence was negatively correlated ($r = -0.1$) with the sampling day; therefore, as the pigs aged, the presence of diarrhea decreased. Additionally, the smallest pigs presented more diarrhea when compared to the other pig sizes ($r = -0.3$) (Figure 5).

Regarding the gastrointestinal microbial composition in pigs supplemented with Zn Sulfate, we observed that *Lachnospiraceae*, *Lactobacillaceae*, *Prevotellaceae*, and *Ruminicoccaceae* are highly correlated ($r > 0.6$) with each other. *Streptococcaceae* was highly correlated ($r = 0.7$) with *Clostridiaceae* but only presented modest impact ($r = 0.2$) on growth factors. *Clostridiaceae* had negative influence on diarrhea ($r = -0.3$), positive on growth factors ($r = 0.2$), and its relative abundance increased in the stool throughout time ($r = 0.6$). *Erysipelotrichaceae* had a mild ($r = 0.1$) correlation with diarrhea, a

positive effect (r = 0.2) on growth factors, and was the only family that decreased over time (r = -0.2). *Lachnospiraceae* presented no strong effects (r = 0.1) in growth factors but did increase (r = 0.4) with age. *Lactobacillaceae* (r = 0.3) and *Ruminicoccaceae* (r = 0.1) had a positive correlation with diarrhea. In general, most relative abundance of the families of interest increased with age. Moreover, all had a modest positive effect on growth factors (Figure 5).

Figure 5. Correlation between all variables by Zinc Sulfate

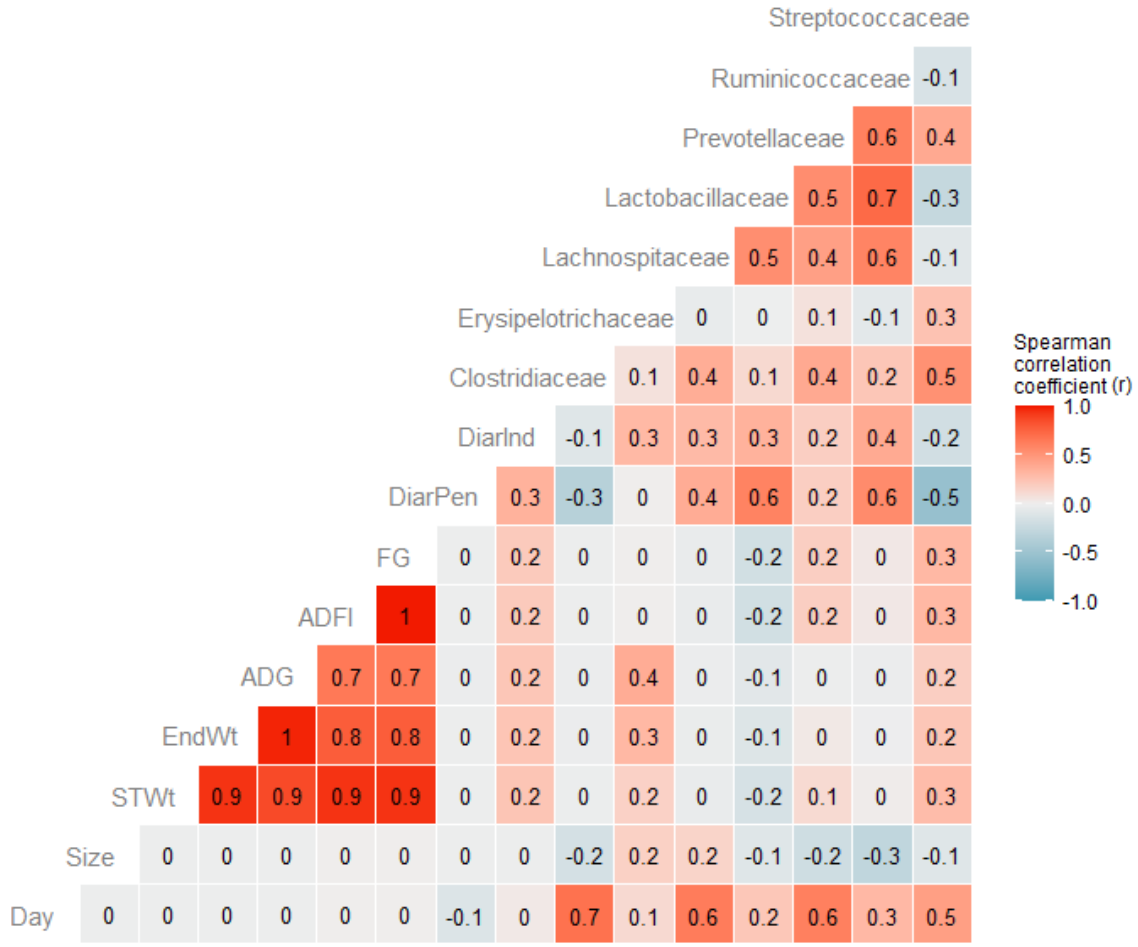


Variables: Day, Size, STWt = Starting weight (kg), EndWt = End weight (kg), ADG = Average Daily Gain (kg), ADFI = Average Daily Feed Intake (kg), FG = Feed efficiency (kg), DiarPen = Diarrhea Pen (score), DiarInd = Diarrhea Individual (score), *Clostridiaceae* (relative abundance, %), *Erysipelotrichaceae* (relative abundance, %), *Lachnospiraceae* (relative abundance, %), *Lactobacillaceae* (relative abundance, %), *Prevotellaceae* (relative abundance, %), *Ruminicoccaceae* (relative abundance, %), *Streptococcaceae* (relative abundance, %).

The pigs fed the diets supplemented with Zn IBZ showed a positive correlation (r > 0.7) between growth factors (end weight, ADG, ADFI, and FG) as expected. As expected, diarrhea per pen and individual diarrhea are also correlated (r = 0.3). Diarrhea had no impact at all on growth parameters. The diarrhea presence was negatively correlated (r = -0.1) with the sampling day; therefore, as the pigs aged, the presence of diarrhea decreased. Additionally, the pig sizes had no impact on diarrhea presence (Figure 6).

Regarding the gastrointestinal microbial composition in pigs supplemented with Zn IBZ, we observed that diarrhea was strongly influenced (r = 0.6) by *Lactobacillaceae* and *Ruminicoccaceae*. In contrast, diarrhea presence was less with higher *Streptococcaceae* (r = -0.5) and *Clostridiaceae* (r = -0.3). In general, most relative abundance of the families of interest increased with age (Figure 6).

Figure 6. Correlation between all variables by Zinc IBZ



Variables: Day, Size, STWt = Starting weight (kg), EndWt = End weight (kg), ADG = Average Daily Gain (kg), ADFI = Average Daily Feed Intake (kg), FG = Feed efficiency (kg), DiarPen = Diarrhea Pen (score), DiarInd = Diarrhea Individual (score), *Clostridiaceae* (relative abundance, %), *Erysipelotrichaceae* (relative abundance, %), *Lachnospiraceae* (relative abundance, %), *Lactobacillaceae* (relative abundance, %), *Prevotellaceae* (relative abundance, %), *Ruminicoccaceae* (relative abundance, %), *Streptococcaceae* (relative abundance, %).

When comparing the results of Zn Sulfate and Zn IBZ supplementation, we noticed that the pigs in the Zn IBZ diet had a bigger starting weight, which was highly correlated with overall growth performance. On the contrary, larger pigs in the Zn Sulfate diet had lower ADG and ADFI. The pigs supplemented with Zn IBZ had a slightly higher correlation with diarrhea, contrary to our hypothesis that pigs fed Zn IBZ would have lower diarrhea incidence. However, we observed that their growth was not compromised

despite diarrhea, consistent with our hypothesis. Pigs in the Zn IBZ diet had stronger microbial growth over time. The gastrointestinal microbiota was correlated with growth factors consistent with our hypothesis. Most families of interest in pigs supplemented with Zn Sulfate presented a modest impact on growth factors compared to their counterparts.

4.6 DISCUSSION

Micronutrients are substances necessary by the body in small amounts. Zinc modifies the composition of the gut microbial communities on certain bacterial species. It has been shown to profoundly affect gut microbiota composition as it is essential for the growth of microorganisms (Foligné et al., 2020; Usama et al., 2018). In this study, the role of Zn Sulfate and Zn Hydroxychloride in shaping the gastrointestinal microbiota and the growth performance and diarrhea prevalence in nursery pigs was evaluated. The data demonstrate a correlation between different zinc sources supplementation in the diet and performance metrics, diarrhea, and gastrointestinal commensal microbiota as revealed by the pigs supplemented with Zn Sulfate presenting less diarrhea than those supplemented with Zn IBZ. However, the growth performance of the pigs fed Zn IBZ was not compromised, which may indicate the role of Zn in modifying gut microbiota composition. The correlation towards better growth performance in the heavier pigs at starting weight in the Zn IBZ group reflects the importance of weaning weight in pigs.

In contrast, in the Zn Sulfate group, the heavier pigs had lower ADG and ADFI. A likely explanation is that the smaller pigs commonly strive to consume enough feed to meet their energy needs for maintenance and growth. The data suggest that only minor

differences existed between the growth performance parameters, supporting previous findings where the improvement of feed efficiency has been observed to be complex and multivariable in the pig industry, and the feed composition, genetics, unnecessary social stress, environmental constraints, and the diseases need to be addressed to observed sustainable improvement (Patience et al., 2015). However, the study was not designed to evaluate all those variables.

In pigs, stress is a factor that significantly disturbs the intestinal microbial ecosystem. Stress - such as weaning, transport, and feed reduction - challenges gastrointestinal microorganism stability allowing pathogens to thrive, leading in most cases to the presence of diarrhea. Diarrhea - a multifactorial disease - has been the leading cause of morbidity and mortality in the swine industry, with increasing evidence that suggests that pathogens and the commensal gut microbiota contribute to the development of diarrhea in weaned piglets (Guevarra et al., 2019). Diarrhea impairs nutrient absorption and weight gain due to the damage of the intestinal villi in the small intestine and the underdeveloped microbiota in the large intestine (Knecht et al., 2020). The data suggest that the pigs in the Zn Sulfate group presented a negative correlation between ADG and diarrhea, which complies with previous findings, contrariwise to the observed with pigs supplemented Zn IBZ.

Contrary to our hypothesis, the pigs supplemented with Zn IBZ had a slightly higher correlation with diarrhea. However, it is to note that despite the stool consistency and frequency observed in the current research, no characteristics indicative of disease (blood, mucous or yellow coloration) were observed in any pen or individual pig's feces. Therefore, it is a good indicator that the increased bowel movements were unrelated to a

specific pathogen. However, a proper diagnosis is suggested to assess if the diarrhea frequency and other symptoms observed in the animals are related to a pathogen.

In order to analyze the role of Zn Sulfate and Zn Hydroxychloride in shaping the gastrointestinal microbiota and diarrhea prevalence in nursery pigs, we used a novel approach allowing us to identify bacterial families - *Prevotellaceaee, Clostridiaceae, Erysipelotrichaceae, Lachnospiraceae, Lactobacillaceae, Ruminicoccaceae,* and *Streptoccocaceae* - by a bioinformatics pipeline that focuses on target genomic sequences that are queried in raw unassembled, non-quality-checked metagenomics sequence reads. Spearman's correlation coefficient was used to identify the magnitude of the association and direction of the relationship between variables.

The digestive system's microbiota is likely to impact growth and feed efficiency in pigs; actually, increased health and production indices are observed when appropriate microbiota resides in the gastrointestinal tract (Knecht et al., 2020). For example, the dietary fiber distinctly impacts the microbiota composition, increasing xylanolytic (i.e., *Bacteroides ruminicola*) and cellulolytic (i.e., *Bacteroides succinogenes*) bacteria count (Varel et al., 1987). The genus *Prevotella,* for instance, has an essential impact on ADFI (Gardiner et al., 2020), but this increment in feed intake is suggested to be a product rather than a driver of feed intake (Amat et al., 2020). Our findings are similar to those from Gardiner et al. (2020); the ADFI presented a positive correlation with *Prevotellaceae* despite the dietary Zn treatment, the ADG presented a positive correlation in pigs supplemented with Zn Sulfate, and F:G presented a positive correlation in pigs supplemented with Zn IBZ. A previous study suggests *Streptococcus* ability to produce lactic acid and antimicrobials bestowing a beneficial role in the gut. This could explain

the observed higher relative abundance of *Streptococcus* in more feed-efficient pigs (Gardiner et al., 2020). Consistent with Gardiner et al. (2020), *Streptococcaceae* in the present research has the strongest positive correlation with ADFI and feed efficiency in pigs supplemented with Zn IBZ in their diet. Regarding ADG, one previous observation was an increased back fat in pigs associated with *Clostridium, Prevotella, Lactobacillus, Ruminococcus, Faecalibacterium, and Streptococcus* (Gardiner et al., 2020). In our data, *Erysipelotrichaceae* had the strongest positive correlation in ADG for pigs supplemented with Zn IBZ; this could be since *Faecalibacterium* belongs to the *Erysipelotrichaceae* family, a bacterial family associated with host lipid metabolism and linked to inflammation.

A previous study has postulated that a decrease in *Lactobacillaceae* counts and proliferation in *Prevotella* is a characteristic in piglets with diarrhea. This could be due to *Lactobacillus* association with beneficial gastrointestinal tract effects and better feed efficiency (Amat et al. 2020; Knecht et al. 2020). However, these studies predicted microbial presence based on 16S rRNA gene sequencing. Here, we could not find a decrease in *Lactobacillaceae*; on the contrary, there was an increase in *Prevotella* in pigs with diarrhea. A previous study has postulated a higher abundance of *Prevotellaceae, Lachnospiraceae, Ruminococcaceae*, *Streptococcus*, and *Lactobacillaceae* and a decrease of *Sarcina* (*Clostridiaceae* family) in healthy piglets when compared to diarrheic ones (Gardiner et al., 2020; Guevarra et al., 2019; Knecht et al., 2020; Wang et al., 2019). However, it is to note that in such studies, the pigs were supplemented with Zn oxide, the DNA was sequenced with Illumina, and traditional methods performed the microbiome data analysis. On the other hand, we observed that most of the relative abundance of the

families of interest increased with age. Comparable to our findings, a higher amount of *Clostridiaceae, Prevotellaceae*, and *Erysipelotrichaceae* in older pigs was observed (Amat et al., 2020; Guevarra et al., 2019; Wang et al., 2021). This could be due to *Prevotella spp* association with a plant food-based diet and fiber digestion, characteristic of later production stages (Wang et al., 2021). In addition, *Prevotella*, for example, is associated with metabolizing plant-derived polysaccharides, making otherwise indigestible substrates available to the host, resulting in short-chain fatty acids (SCFAs). Moreover, *Prevotella* breakdowns complex polysaccharides in the plant cell wall by enzymes, such as xylanases, mannases, and β-glucanases (Amat et al., 2020; Gardiner et al., 2020; Guevarra et al., 2019).

In the current research, shotgun metagenomics was used, where the DNA preparation methods, proper DNA quantity collection, sample complexity, and accurate sequence classification of taxa are crucial steps for an accurate result (Hodkinson & Grice, 2015; Poretsky et al., 2014; Sharpton, 2014). The specificity of the results highly depends on the quality of the sample and the protocol used for sequencing and sample analysis (Hodkinson & Grice, 2015). In this research, substantial variability in the amount of DNA extracted within several samples was noted. This variation could be due to the diet or the amount of actual microbial biomass loaded with each sample, as fecal samples contained substantial amounts of sizeable cracked grain particles. Although measurement of sample mass was done with caution, these particles may have affected the quality and quantity of extracted fecal DNA. More importantly, the DNA extraction method influences the performance of the following processes, potentially affecting the relative abundance of the families of interest.

The use of EDNA MiFi pipeline in this research was highly influenced by its speed since it works with raw unassembled databases, and its accuracy in disease diagnosis in plant pathology. However, this research is the first of its kind, and we encounter several challenges with the selected methodology. Comparison of the relative abundance of the families of interest with the existing literature became challenging since the vast majority of gastrointestinal microbiota research is based on 16S rRNA sequencing for bacterial identification. Some of the benefits of amplicon sequencing are that it is a well-established method that only amplifies the target; therefore, very little to no contamination is observed (Yap et al., 2020). In order to validate the EDNA MiFi pipeline microbiota methodology, it is advised to perform comparison studies between methodologies with standard tools known by their precision and speed, i.e., Kraken2 or MetaPhlAn3. However, it is worth noting that the increment of relative abundance as the pigs aged assessed with E-probes are in agreement with other published reports for *Clostridiaceae* and *Prevotellaceae.* A positive correlation was observed between ADG and *Prevotellaceae* for pigs supplemented with Zn IBZ. Additionally, the pigs with a higher presence of diarrhea had a higher relative abundance of *Ruminococcaceae and Streptococcaceae.*

Whether our results differences with the literature are a function of extraction method, detection method, or due to a difference in the populations tested is unclear. This research illustrates the importance of careful consideration of DNA extraction, sequencing and data analysis methods when designing experiments and interpreting metagenomics data.

4.7 CONCLUSION

The potential of the composition and abundance of gut microbiota to impact pig growth, diarrhea appearance, and risk of disease cannot be ignored. The increased interest in investigating the link between the gut microbiome and growth demonstrates the vast impact of feed on bacterial taxa associated with growth performance. The microbiota is known for interacting between taxa and zinc for being required for multiple metabolic processes in the body, often making the cause-and-effect relationships not distinguishable. Despite advances in technologies, the link between diet, microbiome composition, and pig growth is still difficult to anticipate.

The current study confirms the importance of bacterial taxa influencing growth and diarrhea, although data are conflicting in some cases. Nonetheless, as demonstrated by the E-probes, specific bacterial taxa are consistently associated with growth performance in the nursery phase. In conclusion, the growth performance and diarrhea-associated bacterial taxa identified in this research could potentially be used to identify microbiota changes promptly that could lead to health impairment and economic impact on pig production. However, given the multiple variables affecting the gastrointestinal microbiota, it may not be a simple process. It may be more appropriate to develop genera and species-specific E-probes known to impact growth performance and diarrhea appearance or the genera and species-specific of healthy pigs to assess the impact of specific supplements in their diet. Alternatively, considering that the E-probes are a novel approach for microbiota assessment and were developed for specific families rather than to genera or species level, this research allows us to have a big picture of the gastrointestinal microbiota and its effect on growth performance and diarrhea appearance.

Overall, if successful, the approach outlined here can reduce the time to detect microbiota changes essential for determining a pig's health status, decreasing the side effects of unhealthy animals in the swine production.

4.8 PITFALLS AND CORRECTIONS

This study has one main limitation, the normalization method selected in the research removes technical bias related to sequencing and yields a relative proportion of counts to that gene. However, further normalization should be done to avoid introducing bias across-sample variations. Applying normalization to the lowest feature might address the research problem more effectively by preventing systematic bias that could increase false discovery rate of the families of interest.

4.9 REFERENCES

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CHAPTER V

“MOTHERHOOD – Philosophy and Science”

One of the activities that every person performs is to think, question, and try to obtain the truth about everything that surrounds them. In fact, it is one of the differential characteristics of humans to other beings that exist on this planet: to observe something and think about it beyond what is concretely evident. That is philosophy. Philosophizing is something that humankind has always done, how so much knowledge has been accumulated throughout history. Although another name might be assigned in a different time and place.

In Greece, philosophy begins with the pre-Socratic period, led by Thales of Miletus in the 6th century BC. C, who would later be followed by Socrates, is considered the father of philosophy, his disciple Plato and Aristotle. In the hopes to better understand the word, the Greeks looked away from mythology and religion and more in touch with rational thought. Said Plato, "Ignorance is the root of all evil" and "They are true philosophers, who enjoy contemplating the thruth.”

The word philosophy derives from the Latin "Philosophia," apparently coined by Pythagoras in Ancient Greece; it means "love of wisdom" or "friend of wisdom." Philosophy is characterized by covering various areas of study, emphasizing critical thinking, seeking logical answers to multiple questions, and not accepting absolute truths. In Plato's disciple Aristotle, "Doubt is the beginning of wisdom."

In the XVII century, the new science had its most significant impulse, where the triumph of experimentation over philosophy was marked. The new language of science gave the new scientists tools to handle evidence. Galileo Galilei, recognized as the father of modern science, is the one who formulated the method of empirical verification of the facts through experimentation, direct observation, and logical reasoning. Like Leonardo da Vinci, Pascal, and Diderot, Galileo affirmed that the experience marked the difference between the new and the old sciences.

Science is called all the knowledge or knowing constituted through observation and the systematic and reasoned study of nature, society, and thought. Its objective is to discover the laws that govern the phenomena of reality, understand them and explain them. From there, it follows that the function of science is to describe, explain and predict such phenomena to improve human life. To be able to assign the name of scientific knowledge, it is a requirement that it has been obtained through the scientific method, that is, systematic observation and analysis. Consequently, scientific knowledge offers reasoned and valid conclusions that can be tested. For what is called science, all knowledge or knowledge is constituted through observation and systematic and reasoned study of nature, society, and thought.

In this sense, science comprises all the fields of knowledge and study that lead to developing particular theories and methods for each area. It is cumulative and systematic. Science values the accumulated knowledge of previous investigations, the background.

Scientific knowledge must comply with the so-called "scientific method," which is based on: observation, proposition, formulation of hypotheses, experimentation, demonstration, and conclusions. Thus, the scientific knowledge is systematic and values the accumulated knowledge of previous investigations. For example, Copernicus' heliocentric theory superseded Ptolemy's geocentric theory, while Kepler's laws of elliptical orbits perfected the Copernican theory. Showing the importance of previous investigations, Newton expressed, "To me, there has never been a higher source of earthly honor or distinction than that connected with advances in science."

The term Philosophy of Science is then coined, which is dedicated to studying scientific knowledge and methodology. Science is also closely related to technology, especially since the second half of the 19th century. Hence the importance of scientific studies aimed at creating or perfecting the technology.

Since we are talking about science, a crucial element is experimentation. Experimentation refers to creating tests that verify the established hypotheses about the causes of a specific topic under study. The experimenter chooses certain factors and alters them in a controlled way to see the resulting consequences. In general, an experiment is performed for one of the following purposes; a) Determine the reasons for variation of a response, b) Compare the responses at different levels of observation of the variables, and

c) Obtain a relationship that allows predictions of future responses. Implicit in every experiment is the need for measurement.

The English scientist William Thomson Kelvin (1824-1907) summed up the importance of measurement as an essential part of the development of science in the following comment: "When you can measure what you are speaking about, and express it in numbers, you know something about it. When you cannot express it in numbers, your knowledge is of meager and unsatisfactory kind. It may be the beginning of knowledge, but you have scarcely, in your thoughts advanced to the stage of science." Galileo Galilei had previously said, "Measure what is measurable, and make measurable what is not so."

The measurement consists of comparing the magnitude of a variable with a unit of measure. The greater the precision in an experiment, the greater the need to improve the measurements. Therefore, the importance to be careful when making measurements, as they support the reasoning, hypotheses, and explanations of an experiment.

The measurements and the size of the sample are required for the acquisition of scientific knowledge. It is also necessary to replicate or repeat the experiment to calculate a more precise response of the effect to study. The greater the number of repetitions for each experiment, the better the result. Of course, it is not recommended for time and economy to repeat an experiment indefinitely.

Since the present study is of a biological nature, mainly related to an abiotic limitation in the health, pigs let us address the importance of nutrition in living organisms. Living organisms inhabit suitable places for living, have adapted, and evolved

to live in a specific environment. However, changes occur that will prevent a particular process from happening. These changes have been classified into biotic and abiotic.

Biotic limiting factors include interactions of organisms, such as competition or predation. Abiotic factors are physical factors found in the ecosystem that affect living organisms' ability to survive and reproduce; it includes the entire inert environment. Nutrients as a chemical factor are a necessary form of sustenance for living organisms. They are required by all living organisms to grow and thrive. I.e., the lack of vitamin C produces scurvy, and the chronic lack of iron produces anemia. Just as in the eighteenth century, James Lind demonstrated that oranges and lemons could cure scurvy, preventing the death of hundreds of men on expeditions in the Pacific Ocean. Christiaan Eijkman, in 1886, noticed the symptoms of beriberi in some chickens. During the months when the birds developed beriberi, the birds were fed polished rice. When the birds' diet was changed back to unpolished rice, the birds recovered within a few days. Eijkman conjectured that polished rice lacked a dietary component found in unpolished rice causing beriberi. He called this component “the anti-beriberi factor," now known as Thiamin.

The balance of nutrients is of great importance in animal science, where it always seeks to improve the sustainability of animal production to increase production at the lowest possible costs. We want to demonstrate the effect a nutrient such as zinc has on the body and health of pigs. Also, the impact in the microbiota in the health and in productive parameters favoring or harming them.

With the current research, we can go back to the well-known phrase of the "father of medicine, Hippocrates," "Let food be thy medicine." He formulated the humoral theory of disease, referring to four humors in the body that needed to be mixed in exact proportions; otherwise, they would produce diseases. This theory can be observed in our research, the importance of nutrition and supplementation of essential nutrients to prevent diseases and improve the health of animals, which in turn will enhance production. On the contrary, an imbalance of the same causes an opposite effect.

All the main biomes on the Earth are characterized by that dependence on the medium, the substrate, and its environmental elements that indicate the type of life that can exist according to the present circumstances. Charles Darwin, the father of the theory of evolution, emphasizes, "It is not the strongest of the species that survives, nor the most intelligent that survives. It is the one that is most adaptable to change."

Anton van Leeuwenhoek's curiosity, in the XVII century, taught him to "watch." He made the microscope that increased the size of particles up to 270 times. He was the first human able to observe tiny "animals," which generated many questions and skepticism that significantly contributed to the knowledge of microbiology.

The microbiota is the set of microorganisms that reside in the human/animal body, which can be differentiated according to their behavior in commensals, mutualists, and pathogens. Nowadays, it is currently accepted that to achieve a state of comprehensive health, it is necessary that the microbiota, mainly that associated with the gastrointestinal tract, also be healthy. The primary health indicators of the microbiota are its richness (number of microorganisms) and its biodiversity (number of species). The

knowledge of the microbiota has been considerably expanded after the use of molecular techniques of massive sequencing.

We overlooked for decades what has recently been proven, not all bacteria are enemies, not all are pathogenic, and not all cause disease. Especially in the gastrointestinal tract, thousands of species inhabit and are mostly harmless.

The gastrointestinal microbiota is so diverse and rapidly evolving that it makes them as crucial for the body's functioning as if it were any other organ since they have necessary virtues for life. Currently, some experts can assure us that bacteria help us much more than they harm us. Virtues as assisting in the digestion of food, production of vitamins and minerals lacking in the diet, break down toxins and protect from more dangerous microorganisms among many other things to discover.

Looking back in time to date, all that has been discovered just makes you think how exciting it must have been at the time. And even more, it makes us think about how exciting the knowledge generated and the contributions that this research has to animal science are currently for us. Without a doubt, this research answers questions. Still, at the same time, it opens the door to many more unanswered questions we hope to resolve over time. It is known that, in medicine and health, there are many variables to consider which can affect research; however, for years, zinc's role in improving pigs' productive parameters has been known without explicitly knowing the "how" this improvement is achieved. Nevertheless, its participation in numerous enzymatic processes and intracellular communication is recognized. Perhaps this research brings us closer to the answer sought for years or led us to it!

Albert Einstein: "The important thing is not to stop questioning."

APPENDICES

APPENDIX 1

Table 1. Reads in metagenomes by sampling day and pig sizes

Day	Sample #	Sample size	Reads in metagenome	Day	Sample #	Sample size	Reads in metagenome	Day	Sample #	Sample size	Reads in metagenome	Day	Sample #	Sample size	Reads in metagenome
1	1	L	511726	2	1	L	193608	3	1	L	161554	4	1	L	177406
1	1	M	675810	2	1	M	31962	3	1	M	99728	4	1	M	145548
1	1	S	591625	2	1	S	210514	3	1	S	142066	4	1	S	129348
1	2	L	540399	2	2	L	63086	3	2	L	282806	4	2	L	251980
1	2	M	215794	2	2	M	154196	3	2	M	295641	4	2	M	245104
1	2	S	1044030	2	2	S	193290	3	2	S	282112	4	2	S	154667
1	3	L	503377	2	3	L	172777	3	3	M	120448	4	3	L	324205
1	3	M	139147	2	3	M	101517	3	3	S	96099	4	3	M	243443
1	3	S	186033	2	3	S	115907	3	3	L	1182511	4	3	S	144653
1	4	L	230968	2	4	L	253165	3	4	L	26781	4	4	L	288652
1	4	M	264828	2	4	M	133272	3	4	M	238533	4	4	M	231320
1	4	S	789867	2	4	S	77495	3	4	S	356785	4	4	S	279092
1	5	L	101707	2	5	L	207845	3	5	M	188531	4	5	L	228222
1	5	M	153963	2	5	M	178567	3	5	L	215937	4	5	M	450089
1	5	S	295703	2	5	S	182122	3	5	S	151582	4	5	S	425392
1	6	M	172949	2	6	S	99326	3	6	L	191543	4	6	L	160076
1	6	S	409590	2	6	L	275414	3	6	M	323891	4	6	M	231802
1	6	L	86908	2	6	M	214366	3	6	S	67143	4	6	S	414503

APPENDIX 2

Appendix 2 - Table 1. Target genome information used for the e-probe design

Organism	Near neighbor	E-probe length	RefSeq category	Assembly level	Anomalous exclusion
Prevotellaceae	*Clostridiaceae, Erysipelotrichaceae, Lachnospiraceae, Lactobacillaceae, Ruminococcaceae, Streptococcaceae*	80	Reference and representative	Complete genome, chromosome, scaffold, contig	Yes
Clostridiaceae	*Prevotellaceae, Erysipelotrichaceae, Lachnospiraceae, Lactobacillaceae, Ruminococcaceae, Streptococcaceae*	60	Reference and representative	Complete genome, chromosome, scaffold, contig	Yes
Erysipelotrichaceae	*Prevotellaceae, Clostridiaceae, Lachnospiraceae, Lactobacillaceae, Ruminococcaceae, Streptococcaceae*	80	Reference and representative	Complete genome, chromosome, scaffold, contig	Yes
Lachnospiraceae	*Prevotellaceae, Clostridiaceae, Erysipelotrichaceae, Lactobacillaceae, Ruminococcaceae, Streptococcaceae*	80	Reference and representative	Complete genome, chromosome, scaffold, contig	Yes
Lactobacillaceae	*Prevotellaceae, Clostridiaceae, Erysipelotrichaceae, Lachnospiraceae, Ruminococcaceae, Streptococcaceae*	80	Reference and representative	Complete genome, chromosome, scaffold, contig	Yes
Ruminococcaceae	*Prevotellaceae, Clostridiaceae, Erysipelotrichaceae, Lachnospiraceae, Lactobacillaceae, Streptococcaceae*	80	Reference and representative	Complete genome, chromosome, scaffold, contig	Yes
Streptococcaceae	*Prevotellaceae, Clostridiaceae, Erysipelotrichaceae, Lachnospiraceae, Lactobacillaceae, Ruminococcaceae,*	80	Reference and representative	Complete genome, chromosome, scaffold, contig	Yes

Appendix 2 - Table 2. Genomes from *Prevotellaceaee, Clostridiaceae, Erysipelotrichaceae, Lachnospiraceae, Lactobacillaceae, Ruminicoccaceae* and *Streptoccocaceae* families

Organism	Reference number or GenBank ID
Lactobacillaceae	
Lactobacillus parabuchneri strain FAM21731	NZ_CP018796.1 - NZ_CP018798.1
Lactobacillus jensenii strain SNUV360	NZ_CP018809.1
Lactobacillus amylophilus DSM 20533	NZ_CP018888.1
Lactobacillus crustorum strain MN047	NZ_CP017996.1 - NZ_CP017998.1
Lactobacillus amylolyticus strain L6	NZ_CP020457.1 - NZ_CP020458.1
Lactobacillus mixtipabuli strain IWT30	NZ_BCMF01000001.1 - NZ_BCMF01000087.1
Lactobacillus silagei JCM 19001 strain IWT126	NZ_BCMG01000001.1 - NZ_BCMG01000087.1
Lactobacillus curvatus strain MRS6	NZ_CP022474.1
Lactobacillus sakei strain FAM18311	NZ_CP020459.1 - NZ_CP020461.1
Lactobacillus bombicola strain R-53102	NZ_FOMN01000001.1 - NZ_FOMN01000020.1
Lactobacillus acidipiscis strain ACA-DC 1533	NZ_LT630287.1
Lactobacillus zymae strain ACA-DC 3411	NZ_LT854705.1
Lactobacillus salivarius UCC118	NC_007929.1 - NC_006530.1
Lactobacillus fermentum IFO 3956	NC_010610.1
Lactobacillus acidophilus NCFM	NC_006814.3
Lactobacillus gasseri ATCC 33323	NC_008530.1
Lactobacillus brevis ATCC 367	NC_008497.1 - NC_008499.1
Lactobacillus paracasei ATCC 334	NC_008502.1, NC_008526.1
Lactobacillus reuteri DSM 20016	NC_009513.1
Lactobacillus rhamnosus GG	NC_013198.1

Appendix 2 - Table 2. Genomes from *Prevotellaceaee, Clostridiaceae, Erysipelotrichaceae, Lachnospiraceae, Lactobacillaceae, Ruminicoccaceae* and *Streptoccocaceae* families

Organism	Reference number or GenBank ID
Lactobacillus delbrueckii subsp. bulgaricus ATCC 11842	NC_008054.1
Lactobacillus crispatus ST1	NC_014106.1
Lactobacillus hilgardii DSM 20176	NZ_GG669992.1 - NZ_GG670104.1
Lactobacillus vaginalis DSM 5837	NZ_GG693412.1 - NZ_GG693523.1
Lactobacillus antri DSM 16041	NZ_GG700732.1 - NZ_GG700751.1
Lactobacillus iners DSM 13335	NZ_GG700801.1 - NZ_GG700812.1
Lactobacillus coleohominis 101-4-CHN	NZ_GG698802.1 - NZ_GG698813.1
Lactobacillus farciminis KCTC 3681	NZ_GL575016.1 - NZ_GL575020.1
Lactobacillus amylovorus strain 30SC	NC_015213.1, NC_015214.1, NC_015218.1
Lactobacillus plantarum WCFS1	NC_004567.2, NC_006375.1 - NC_006377.1
Lactobacillus kefiranofaciens ZW3	NC_015598.1, NC_015602.1, NC_015603.1
Lactobacillus ruminis ATCC 27782	NC_015975.1
Lactobacillus sanfranciscensis	NC_015978.1 - NC_015980.1
Lactobacillus mucosae LM1	NZ_CP011013.1 - NZ_CP011014.1
Lactobacillus vini DSM 20605	NZ_AHYZ01000001.1 - NZ_AHYZ01000220.1
Lactobacillus hominis DSM 23910	NZ_CAKE01000001.1 - NZ_CAKE01000036.1
Lactobacillus pasteurii DSM 23907	NZ_CAKD01000001.1 - NZ_CAKD01000030.1
Lactobacillus buchneri CD034	NC_016034.1 - NC_01603.1, NC_018610.1 - NC_018611.1
Lactobacillus ingluviei str. Autruche 4	NZ_HE997173.1 - NZ_HE997180.1
Lactobacillus pobuzihii E100301	NZ_APCP01000004.1, NZ_APCP01000031.1, NZ_APCP01000040.1 - NZ_APCP01000044.1, NZ_APCP01000049.1 - NZ_APCP01000051.1, NZ_APCP01000057.1, NZ_APCP01000063.1 - NZ_APCP01000066.1, NZ_APCP01000070.1 - NZ_APCP01000071.1, NZ_APCP01000074.1 -

Appendix 2 - Table 2. Genomes from *Prevotellaceaee, Clostridiaceae, Erysipelotrichaceae, Lachnospiraceae, Lactobacillaceae, Ruminicoccaceae* and *Streptoccocaceae* families	
Organism	**Reference number or GenBank ID**
	NZ_APCP01000075.1, NZ_APCP01000081.1 - NZ_APCP01000082.1, NZ_APCP01000087.1 - NZ_APCP01000088.1, NZ_APCP01000097.1 - NZ_APCP01000113.1, NZ_KB714685.1 - NZ_KB714706.1
Lactobacillus parabrevis ATCC 53295	NZ_KB911365.1 - NZ_KB911458.1
Lactobacillus ceti DSM 22408	NZ_AUHP01000001.1 - NZ_AUHP01000019.1, NZ_KE383993.1
Lactobacillus saerimneri DSM 16049	NZ_AUHQ01000001.1 - NZ_AUHQ01000050.1, NZ_KE383994.1 - NZ_KE383996.1, NZ_AUHQ01000027.1 - NZ_AUHQ01000050.1, NZ_KE383994.1 - NZ_KE383996.1
Lactobacillus harbinensis DSM 16991	NZ_KE384471.1 - NZ_KE384472.1, NZ_AUEH01000001.1 - NZ_AUEH01000103.1
Lactobacillus psittaci DSM 15354	NZ_AUEI01000001.1 - NZ_AUEI01000026.1, NZ_KE384473.1 - NZ_KE384474.1
Lactobacillus rossiae DSM 15814	NZ_AUAW01000001.1, NZ_AUAW01000004.1 - NZ_AUAW01000040.1, NZ_KE386820.1
Lactobacillus shenzhenensis LY-73 LY73	NZ_KI271582.1 - NZ_KI271643.1
Lactobacillus fabifermentans DSM 21115	NZ_AYGX02000001.1 - NZ_AYGX02000181.1
Lactobacillus farraginis DSM 18382	NZ_BAKI01000001.1 - NZ_BAKI01000129.1
Lactobacillus curieae strain CCTCC	NZ_CP018906.1
Lactobacillus paracasei subsp. paracasei JCM 8130	NZ_AP012541.1 - NZ_AP012543.1
Lactobacillus hokkaidonensis	NZ_AP014680.1 - NZ_AP014682.1
Lactobacillus heilongjiangensis strain DSM 28069	NZ_CP012559.1
Lactobacillus kunkeei strain MP2	NZ_CP012920.1
Lactobacillus paracollinoides strain TMW 1.1994	NZ_CP014915.1 - NZ_CP014923.1
Lactobacillus lindneri strain TMW 1.481	NZ_CP014873.1 - NZ_CP014880.1, NZ_CP014907.1 - NZ_CP014911.1
Lactobacillus senmaizukei DSM 21775	NZ_BCWD01000001.1 - NZ_BCWD01000074.1
Lactobacillus fructivorans strain ATCC 27394	NZ_JQAS01000001.1 - NZ_JQAS01000012.1

Appendix 2 - Table 2. Genomes from *Prevotellaceaee, Clostridiaceae, Erysipelotrichaceae, Lachnospiraceae, Lactobacillaceae, Ruminicoccaceae* and *Streptoccocaceae* families

Organism	Reference number or GenBank ID
Lactobacillus secaliphilus strain DSM 17896	NZ_JQBW01000001.1 - NZ_JQBW01000011.1
Lactobacillus senioris DSM 24302	NZ_AYZR01000001.1 - NZ_AYZR01000009.1
Lactobacillus brantae DSM 23927	NZ_AYZQ01000001.1 - NZ_AYZQ01000012.1
Lactobacillus diolivorans DSM 14421	NZ_AZEY01000001.1 - NZ_AZEY01000109.1
Lactobacillus composti DSM 18527	NZ_BAMK01000001.1 - NZ_BAMK01000094.1
Lactobacillus sucicola DSM 21376	NZ_BALC01000001.1 - NZ_BALC01000015.1
Lactobacillus hamsteri DSM 5661	NZ_BALY01000001.1 - NZ_BALY01000081.1
Lactobacillus equi DSM 15833	NZ_BAMI01000001.1 - NZ_BAMI01000158.1
Lactobacillus fuchuensis DSM 14340	NZ_BAMJ01000001.1 - NZ_BAMJ01000071.1
Lactobacillus hayakitensis DSM 18933	NZ_BAML01000001.1 - NZ_BAML01000082.1
Lactobacillus nodensis DSM 19682	NZ_BAMN01000001.1 - NZ_BAMN01000052.1
Lactobacillus oryzae JCM 18671	NZ_BBJM01000001.1 - NZ_BBJM01000093.1
Lactobacillus wasatchensis strain WDC04	NZ_AWTT01000001.1 - NZ_AWTT01000105.1
Lactobacillus kullabergensis strain Biut2	NZ_KQ033870.1 - NZ_KQ033876.1
Lactobacillus mellis strain Hon2	NZ_KQ033877.1 - NZ_KQ033883.1
Lactobacillus apis strain Hma11	NZ_KQ033999.1 - NZ_KQ034005.1
Lactobacillus herbarum strain TCF032-E4	NZ_LFEE01000001.1 - NZ_LFEE01000055.1
Lactobacillus mellifer strain Bin4	NZ_KQ034028.1 - NZ_KQ034038.1
Lactobacillus acetotolerans	NZ_AP014808.1
Lactobacillus koreensis strain 26-25	NZ_CP012033.1
Lactobacillus ginsenosidimutans strain EMML	NZ_CP012034.1

Appendix 2 - Table 2. Genomes from *Prevotellaceaee, Clostridiaceae, Erysipelotrichaceae, Lachnospiraceae, Lactobacillaceae, Ruminicoccaceae* and *Streptoccocaceae* families

Organism	Reference number or GenBank ID
Lactobacillus apinorum strain Fhon13	NZ_KQ440395.1 - NZ_KQ440403.1
Lactobacillus helveticus strain CAUH18	NZ_CP012381.1
Lactobacillus similis DSM 23365	NZ_BBAD01000001.1 - NZ_BBAD01000176.1
Lactobacillus pantheris DSM 15945	NZ_BBAI01000001.1 - NZ_BBAI01000081.1
Lactobacillus camelliae DSM 22697	NZ_BBAJ01000001.1 - NZ_BBAJ01000161.1
Lactobacillus parafarraginis DSM 18390	NZ_BBAR01000001.1 - NZ_BBAR01000236.1
Lactobacillus xiangfangensis strain LMG 26013	NZ_JQCL01000001.1 - NZ_JQCL01000103.1
Lactobacillus kimchiensis strain DSM 24716	NZ_JQCF01000001.1 - NZ_JQCF01000071.1
Lactobacillus siliginis strain DSM 22696	NZ_JQCB01000001.1 - NZ_JQCB01000052.1
Lactobacillus selangorensis strain DSM 13344	NZ_JQAZ01000001.1 - NZ_JQAZ01000032.1
Lactobacillus paucivorans strain DSM 22467	NZ_JQCA01000001.1 - NZ_JQCA01000167.1
Lactobacillus aquaticus DSM 21051	NZ_AYZD01000001.1 - NZ_AYZD01000036.1
Lactobacillus cacaonum DSM 21116	NZ_AYZE01000001.1 - NZ_AYZE01000017.1
Lactobacillus florum DSM 22689	NZ_AYZI01000001.1 - NZ_AYZI01000050.1
Lactobacillus floricola DSM 23037	NZ_AYZI01000022.1 - NZ_AYZI01000050.1
Lactobacillus equigenerosi DSM 18793	NZ_BBAS01000001.1 - NZ_BBAS01000038.1
Lactobacillus saniviri JCM 17471	NZ_BBBX01000001.1 - NZ_BBBX01000067.1
Lactobacillus thailandensis DSM 22698	NZ_BBER01000001.1 - NZ_BBER01000021.1
Lactobacillus gallinarum strain HFD4	NZ_CP012890.1 - NZ_CP012896.1
Lactobacillus coryniformis subsp. coryniformis KCTC 3167	NZ_AZCN01000001.1 - NZ_AZCN01000198.1

Appendix 2 - Table 2. Genomes from *Prevotellaceaee, Clostridiaceae, Erysipelotrichaceae, Lachnospiraceae, Lactobacillaceae, Ruminicoccaceae* and *Streptoccocaceae* families

Organism	Reference number or GenBank ID
Lactobacillus kimchicus JCM 15530	NZ_AZCX01000001.1 - NZ_AZCX01000045.1
Lactobacillus nagelii DSM 13675	NZ_AZEV01000001.1 - NZ_AZEV01000044.1
Lactobacillus mindensis DSM 14500	NZ_AZEZ01000001.1 - NZ_AZEZ01000103.1
Lactobacillus versmoldensis DSM 14857	NZ_AZFA01000001.1 - NZ_AZFA01000062.1
Lactobacillus oligofermentans DSM 15707	NZ_AZFE01000001.1 - NZ_AZFE01000032.1
Lactobacillus kalixensis DSM 16043	NZ_AZFM01000001.1 - NZ_AZFM01000108.1
Lactobacillus gastricus DSM 16045	NZ_AZFN01000001.1 - NZ_AZFN01000066.1
Lactobacillus suebicus DSM 5007	NZ_AZGF01000001.1 - NZ_AZGF01000081.1
Lactobacillus animalis KCTC 3501	NZ_AYYW01000001.1 - NZ_AYYW01000058.1
Lactobacillus bifermentans DSM 20003	NZ_AZDA01000001.1 - NZ_AZDA01000147.1
Lactobacillus tucceti DSM 20183	NZ_AZDG01000001.1 - NZ_AZDG01000051.1
Lactobacillus algidus DSM 15638	NZ_AZDI01000001.1 - NZ_AZDI01000028.1
Lactobacillus nasuensis JCM 17158	NZ_AZDJ01000001.1 - NZ_AZDJ01000035.1
Lactobacillus alimentarius DSM 20249	NZ_AZDQ01000001.1 - NZ_AZDQ01000046.1
Lactobacillus equicursoris DSM 19284	NZ_AZDU01000001.1 - NZ_AZDU01000182.1
Lactobacillus hordei DSM 19519	NZ_AZDX01000001.1 - NZ_AZDX01000137.1
Lactobacillus odoratitofui DSM 19909	NZ_AZEE01000001.1 - NZ_AZEE01000031.1
Lactobacillus capillatus DSM 19910	NZ_AZEF01000001.1 - NZ_AZEF01000061.1
Lactobacillus uvarum DSM 19971	NZ_AZEG01000001.1 - NZ_AZEG01000164.1
Lactobacillus manihotivorans DSM 13343	NZ_AZEU01000001.1 - NZ_AZEU01000335.1
Lactobacillus spicheri DSM 15429	NZ_AZFC01000001.1 - NZ_AZFC01000037.1

Appendix 2 - Table 2. Genomes from *Prevotellaceaee, Clostridiaceae, Erysipelotrichaceae, Lachnospiraceae, Lactobacillaceae, Ruminicoccaceae* and *Streptoccocaceae* families

Organism	Reference number or GenBank ID
Lactobacillus satsumensis DSM 16230	NZ_AZFQ01000001.1 - NZ_AZFQ01000055.1
Lactobacillus ghanensis DSM 18630	NZ_AZGB01000001.1 - NZ_AZGB01000033.1
Lactobacillus intestinalis DSM 6629	NZ_AZGN01000001.1 - NZ_AZGN01000055.1
Lactobacillus pontis DSM 8475	NZ_AZGO01000001.1 - NZ_AZGO01000068.1
Lactobacillus gigeriorum DSM 23908	NZ_AYZO01000001.1 - NZ_AYZO01000116.1
Lactobacillus rennini DSM 20253	NZ_AYYI01000001.1 - NZ_AYYI01000106.1
Lactobacillus graminis DSM 20719	NZ_AYZB01000001.1 - NZ_AYZB01000072.1
Lactobacillus malefermentans DSM 5705	NZ_AZGJ01000001.1 - NZ_AZGJ01000152.1
Lactobacillus aviarius subsp. aviarius DSM 20655	NZ_AYZA01000001.1 - NZ_AYZA01000024.1
Lactobacillus vaccinostercus DSM 20634	NZ_AYYY01000001.1 - NZ_AYYY01000072.1
Lactobacillus oeni DSM 19972	NZ_AZEH01000001.1 - NZ_AZEH01000043.1
Lactobacillus rapi DSM 19907	NZ_AZEI01000001.1 - NZ_AZEI01000087.1
Lactobacillus sharpeae JCM 1186	NZ_AYYO01000001.1 - NZ_AYYO01000057.1
Lactobacillus dextrinicus DSM 20335	NZ_AYYK01000001.1 - NZ_AYYK01000026.1
Lactobacillus frumenti DSM 13145	NZ_AZER01000001.1 - NZ_AZER01000026.1
Lactobacillus ozensis DSM 23829	NZ_AYYQ01000001.1 - NZ_AYYQ01000036.1
Lactobacillus collinoides DSM 20515	NZ_AYYR01000001.1 - NZ_AYYR01000130.1
Lactobacillus panis DSM 6035	NZ_AZGM01000001.1 - NZ_AZGM01000150.1
Lactobacillus concavus DSM 17758	NZ_AZFX01000001.1 - NZ_AZFX01000096.1
Lactobacillus perolens DSM 12744	NZ_AZEC01000001.1 - NZ_AZEC01000101.1
Lactobacillus paralimentarius DSM 13961	NZ_AZDH01000001.1 - NZ_AZDH01000028.1

Appendix 2 - Table 2. Genomes from *Prevotellaceaee, Clostridiaceae, Erysipelotrichaceae, Lachnospiraceae, Lactobacillaceae, Ruminicoccaceae* and *Streptoccocaceae* families

Organism	Reference number or GenBank ID
Clostridiaceae	
Clostridium chauvoei JF4335	NZ_LT799839.1
Clostridium acidisoli DSM 12555	NZ_FWXH01000001.1 - NZ_FWXH01000062.1
Clostridium merdae strain Marseille-P2953	NZ_FXLN01000001.1 - NZ_FXLN01000004.1
Khelaifiella massiliensis strain Mt13	NZ_LT854616.1 - NZ_LT854640.1
Mordavella sp.	NZ_LT990039.1
Senegalia massiliensis strain SIT17	NZ_LR130785.1 - NZ_LR130799.1
Clostridium tetani E88	NC_004557.1, NC_004565.1
Clostridium acetobutylicum ATCC 824	NC_003030.1, NC_001988.2
Clostridium perfringens ATCC 13124	NC_008261.1
Clostridium novyi	NC_008593.1
Clostridium kluyveri DSM 555	NC_009466.1, NC_009706.1
Alkaliphilus metalliredigens	NC_009633.1
Clostridium botulinum A	NC_009698.1
Alkaliphilus oremlandii	NC_009922.1
Clostridium botulinum B	NC_010674.1, NC_010680.1
Clostridium botulinum A	NC_009495.1 - NC_009496.1
Clostridium cellulovorans 743B	NC_014393.1
Hungatella hathewayi DSM 13479	NZ_GG667607.1 - NZ_GG668320.1
Clostridium botulinum BKT015925	NC_015417.1 - NC_015418.1, NC_015425.1 - NC_015427.1
Hungatella hathewayi WAL-18680	NZ_JH379027.1 - NZ_JH379044.1
Clostridium arbusti SL206	NZ_BAEV01000001.1 - NZ_BAEV01000243.1
Clostridium senegalense JC122	NZ_HE611050.1 - NZ_HE611063.1, NZ_CAEV01000123.1 -NZ_CAEV01000191.1
Caloramator australicus RC3	NZ_CAKP01000001.1 - NZ_CAKP01000161.1
Clostridium celatum DSM 1785	NZ_KB291596.1 - NZ_KB291717.1
Clostridium saccharoperbutylacetonicum N1-4(HMT)	NC_020291.1 - NC_020292.1

Appendix 2 - Table 2. Genomes from *Prevotellaceaee, Clostridiaceae, Erysipelotrichaceae, Lachnospiraceae, Lactobacillaceae, Ruminicoccaceae* and *Streptoccocaceae* families

Organism	Reference number or GenBank ID
Caldisalinibacter kiritimatiensis strain L21-TH-D2	NZ_ARZA01000001.1 - NZ_ARZA01000289.1
Clostridium pasteurianum BC1	NC_021182.1 - NC_021183.1
Butyricicoccus pullicaecorum 1.2	NZ_KB976103.1 - NZ_KB976106.1
Clostridium sartagoforme AAU1	NZ_ASRV01000001.1 - NZ_ASRV01000323.1
Clostridium paraputrificum AGR2156	NZ_AUJC01000001.1 - NZ_AUJC01000032.1, NZ_KE384122.1 - NZ_KE384123.1
Clostridium cadaveris AGR2141	NZ_AUJL01000001.1 - NZ_AUJL01000045.1, NZ_KE384148.1 - NZ_KE384150.1
Thermobrachium celere DSM 8682	NZ_HF951986.1 - NZ_HF952041.1
Clostridium intestinale URNW	NZ_KI273146.1, NZ_KI273148.1
Clostridium saccharobutylicum DSM 13864	NC_022571.1
Clostridium autoethanogenum DSM 10061	NC_022592.1
Youngiibacter fragilis 232.1	NZ_AXUN02000001.1 - NZ_AXUN02000240.1
[Clostridium] ultunense DSM 10521	NZ_AZSU01000001.1 - NZ_AZSU01000012.1
Clostridium bornimense	NZ_HG917868.1 - NZ_HG917869.1
Fervidicella metallireducens AeB	NZ_AZQP01000001.1 - NZ_AZQP01000164.1
Clostridium ihumii AP5	NZ_CCAT010000004.1
Clostridium lundense DSM 17049	NZ_JHVC01000001.1 - NZ_JHVC01000054.1, NZ_KK211043.1 - NZ_KK211044.1
Caloramator quimbayensis strain USBA 833	NZ_FUYH01000001.1 - NZ_FUYH01000067.1
Maledivibacter halophilus strain M1	NZ_FUZT01000001.1 - NZ_FUZT01000039.1
Paramaledivibacter caminithermalis DSM 15212	NZ_FRAG01000001.1 - NZ_FRAG01000155.1
Hathewaya proteolytica DSM 3090	NZ_FRAD01000001.1 - NZ_FRAD01000045.1
Lutispora thermophila DSM 19022	NZ_FQZS01000001.1 - NZ_FQZS01000063.1

Appendix 2 - Table 2. Genomes from *Prevotellaceaee, Clostridiaceae, Erysipelotrichaceae, Lachnospiraceae, Lactobacillaceae, Ruminicoccaceae* and *Streptoccocaceae* families

Organism	Reference number or GenBank ID
Clostridium amylolyticum strain DSM 21864	NZ_FQZO01000001.1 - NZ_FQZO01000020.1
Clostridium cavendishii DSM 21758	NZ_FQZB01000001.1 - NZ_FQZB01000056.1
Clostridium collagenovorans DSM 3089	NZ_FQXP01000001.1 - NZ_FQXP01000030.1
Clostridium grantii DSM 8605	NZ_FQXM01000001.1 - NZ_FQXM01000087.1
Clostridium fallax strain DSM 2631	NZ_FQVM01000001.1 -NZ_FQVM01000064.1
Caloramator proteoclasticus DSM 10124	NZ_FQVG01000001.1 - NZ_FQVG01000122.1
Lactonifactor longoviformis DSM 17459	NZ_FQVI01000001.1 - NZ_FQVI01000076.1
Clostridium uliginosum strain DSM 12992	NZ_FOMG01000001.1 - NZ_FOMG01000078.1
Clostridium frigidicarnis strain DSM 12271	NZ_FOKI01000001.1 - NZ_FOKI01000112.1
Clostridiisalibacter paucivorans DSM 22131	NZ_JHVL01000001.1 - NZ_JHVL01000091.1, NZ_KK211075.1 - NZ_KK211080.1
Alkaliphilus transvaalensis ATCC 700919	NZ_JHYF01000001.1 - NZ_JHYF01000047.1, NZ_KK211286.1 - NZ_KK211288.1
Clostridium tetanomorphum DSM 665	NZ_APJS01000001.1 - NZ_APJS01000224.1
Clostridium hydrogeniformans DSM 21757	NZ_JMLJ01000003.1, NZ_JMLJ01000006.1 - NZ_JMLJ01000008.1, NZ_JMLJ01000011.1 - NZ_JMLJ01000034.1, NZ_KK366004.1 - NZ_KK366006.1
Clostridium akagii DSM 12554	NZ_JMLK01000006.1 - NZ_JMLK01000008.1, NZ_JMLK01000011.1 - NZ_JMLK01000055.1, NZ_KK366007.1 - NZ_KK366010.1
Proteiniclasticum ruminis DSM 24773	NZ_JNKC01000001.1 - NZ_JNKC01000025.1, NZ_KL370832.1 - NZ_KL370834.1
Clostridium algidicarnis strain B3	NZ_JNLN01000001.1
Clostridium sulfidigenes strain 113A	NZ_JPMD01000001.1 - NZ_JPMD01000096.1
Clostridium baratii str.	NZ_CP006905.1 - NZ_CP006906.1
Clostridium pasteurianum DSM 525	NZ_CP009268.1
Clostridium polynesiense strain MS1	NZ_CCXI01000001.1 - NZ_CCXI01000161.1

Appendix 2 - Table 2. Genomes from *Prevotellaceaee, Clostridiaceae, Erysipelotrichaceae, Lachnospiraceae, Lactobacillaceae, Ruminicoccaceae* and *Streptoccocaceae* families

Organism	Reference number or GenBank ID
Beduini massiliensis strain GM1	NZ_CDPP01000001.1 - NZ_CDPP01000006.1
Clostridium beijerinckii strain NCIMB 14988	NZ_CP010086.2
Clostridium carboxidivorans P7	NZ_CP011803.1 - NZ_CP011804.1
Clostridium aceticum strain DSM 1496	NZ_CP009687.1 - NZ_CP009688.1
Clostridium cylindrosporum DSM 605	NZ_LFVU01000001.1 - NZ_LFVU01000028.1
Clostridium phoceensis strain GD3	NZ_LN866265.1 - NZ_LN866274.1
Natronincola peptidivorans strain DSM 18979	NZ_FOHU01000001.1 - NZ_FOHU01000056.1
Tindallia californiensis strain APO	NZ_FNPV01000001.1 - NZ_FNPV01000027.1
Clostridium gasigenes strain DSM 12272	NZ_FNJM01000001.1 - NZ_FNJM01000042.1
Alkaliphilus peptidifermentans DSM 18978	NZ_FMUS01000001.1 - NZ_FMUS01000073.1
Natronincola ferrireducens strain DSM 18346	NZ_FNFP01000001.1 - NZ_FNFP01000024.1
Massilioclostridium coli strain Marseille-P2976	NZ_FMIZ01000001.1 - NZ_FMIZ01000007.1
Fonticella tunisiensis strain DSM 24455	NZ_SOAZ01000001.1 - NZ_SOAZ01000064.1
Serpentinicella alkaliphila strain DSM 100013	NZ_SLYC01000001.1 - NZ_SLYC01000105.1
Marinisporobacter balticus strain DSM 102940	NZ_SLWV01000001.1 - NZ_SLWV01000074.1
Keratinibaculum paraultunense strain DSM 26752	NZ_SMAE01000001.1 - NZ_SMAE01000035.1
Thermohalobacter berrensis strain CTT3	NZ_MCIB01000001.1 - NZ_MCIB01000040.1
Anaeromicrobium sediminis strain DY2726D	NZ_NIBG01000001.1 - NZ_NIBG01000055.1
Clostridium botulinum strain 89G	NZ_CP014175.1 - NZ_CP014176.1
Clostridium thermobutyricum DSM 4928	NZ_LTAY01000001.1 - NZ_LTAY01000111.1

Appendix 2 - Table 2. Genomes from *Prevotellaceaee, Clostridiaceae, Erysipelotrichaceae, Lachnospiraceae, Lactobacillaceae, Ruminicoccaceae* and *Streptoccocaceae* families

Organism	Reference number or GenBank ID
Clostridium chromiireducens strain DSM 23318	NZ_MZGT01000001.1 - NZ_MZGT01000188.1
Clostridium oryzae strain DSM 28571	NZ_MZGV01000001.1 - NZ_MZGV01000176.1
Clostridium tepidum strain IEH 97212	NZ_MRAE01000001.1 - NZ_MRAE01000138.1
Clostridium felsineum DSM 794	NZ_LZYT01000001.1 - NZ_LZYT01000100.1
Clostridium puniceum strain DSM 2619	NZ_LZZM01000001.1 - NZ_LZZM01000245.1
Clostridium estertheticum subsp. estertheticum strain DSM 8809	NZ_CP015756.1 - NZ_CP015757.1
Clostridium formicaceticum strain ATCC 27076	NZ_CP017603.1
Clostridium acetireducens DSM 10703	NZ_LZFO01000001.1 - NZ_LZFO01000085.1
Geosporobacter ferrireducens strain IRF9	NZ_CP017269.1 - NZ_CP017270.1
Clostridium taeniosporum strain 1/k	NZ_CP017253.2, NZ_CP017254.1, NZ_CP017255.2 - NZ_CP017256.2
Clostridium homopropionicum DSM 5847	NZ_LHUR01000001.1 - NZ_LHUR01000048.1
Inediibacterium massiliense strain Mt12	NZ_LN876574.1 - NZ_LN876587.1
Oxobacter pfennigii strain DSM 3222	NZ_LKET01000001.1 - NZ_LKET01000069.1
Desnuesiella massiliensis strain mt10	NZ_LN879444.1 - NZ_LN879457.1
Clostridium ventriculi strain 2789STDY5834858	NZ_CYZR01000001.1 - NZ_CYZR01000031.1
Clostridium disporicum strain 2789STDY5834856	NZ_CYZX01000001.1 - NZ_CYZX01000057.1
Caloramator mitchellensis strain VF08	NZ_LKHP01000001.1 - NZ_LKHP01000053.1
Clostridium butyricum strain KNU-L09	NZ_CP013252.1, NZ_CP013489.1
Clostridium neonatale strain LCDC no.99-A-005	NZ_LN890312.1 - NZ_LN890328.1
Thermotalea metallivorans strain B2-1	NZ_LOEE01000001.1 - NZ_LOEE01000110.1

Appendix 2 - Table 2. Genomes from *Prevotellaceaee, Clostridiaceae, Erysipelotrichaceae, Lachnospiraceae, Lactobacillaceae, Ruminicoccaceae* and *Streptoccocaceae* families

Organism	Reference number or GenBank ID
Clostridium colicanis DSM 13634	NZ_LTBB01000001.1 - NZ_LTBB01000083.1
Clostridium tepidiprofundi DSM 19306	NZ_LTBA01000001.1 - NZ_LTBA01000175.1
Clostridium magnum DSM 2767	NZ_LWAE01000001.1 - NZ_LWAE01000025.1
Clostridium tyrobutyricum strain KCTC 5387	NZ_CP014170.1 - NZ_CP014171.1
Clostridium pasteurianum strain GL11	NZ_MCGV01000001.1 - NZ_MCGV01000002.1
Caloranaerobacter ferrireducens strain DY22619	NZ_MDJR01000001.1 - NZ_MDJR01000028.1
Erysipellaceae	
Absiella dolichum DSM 3991	NZ_DS483460.1 - NZ_DS483478.1
Erysipelatoclostridium ramosum DSM 1402	NZ_DS499649.1 - NZ_DS499660.1
[Clostridium] spiroforme DSM 1552	NZ_DS562843.1 - NZ_DS562854.1
Holdemanella biformis DSM 3989	NZ_DS996836.1 - NZ_DS996879.1
Catenibacterium mitsuokai DSM 15897	NZ_ACCK01000001.1 - NZ_ACCK01000475.1
Turicibacter sanguinis PC909 contig00098	NZ_ADMN01000001.1 - NZ_ADMN01000125.1
Eubacterium cylindroides T2-87	NC_021019.1
Erysipelothrix rhusiopathiae str. Fujisawa	NC_015601.1
Dielma fastidiosa strain type strain: JC13	NZ_HE578923.1 - NZ_HE578944.1
Dielma fastidiosa strain JC13	NZ_CAEN01000073.1 - NZ_CAEN01000145.1
Holdemania massiliensis AP2	NZ_HE998567.1 - NZ_HE998583.1
Eggerthia catenaformis OT 569	NZ_KB446646.1 - NZ_KB446653.1
[Clostridium] innocuum 2959	NZ_KB850943.1 - NZ_KB850949.1
Faecalicoccus pleomorphus DSM 20574	NZ_ATUT01000001.1 - NZ_ATUT01000047.1
Solobacterium moorei DSM 22971	NZ_AUKY01000001.1 - NZ_AUKY01000031.1, NZ_KE384293.1, NZ_AUKY01000034.1 - NZ_AUKY01000170.1
[Clostridium] saccharogumia DSM 17460	NZ_JMLH01000001.1 - NZ_JMLH01000008.1, NZ_KK365998.1 - NZ_KK365999.1, NZ_JMLH01000011.1

Appendix 2 - Table 2. Genomes from *Prevotellaceaee, Clostridiaceae, Erysipelotrichaceae, Lachnospiraceae, Lactobacillaceae, Ruminicoccaceae* and *Streptoccocaceae* families	
Organism	**Reference number or GenBank ID**
	- NZ_JMLH01000064.1, NZ_JMLH01000067.1 - NZ_JMLH01000135.1
Kandleria vitulina DSM 20405	NZ_JNKN01000001.1 - NZ_JNKN01000079.1, NZ_KL370857.1 - NZ_KL370860.1
Turicibacter sanguinis strain 2789STDY5834851	NZ_CYZQ01000001.1 - NZ_CYZQ01000059.1
Erysipelothrix larvae strain LV19	NZ_CP013213.1 - NZ_CP013214.1
Faecalibaculum rodentium strain Alo17	NZ_CP011391.1
Ileibacterium valens strain NYU-BL-A3	NZ_MPJW01000001.1 - NZ_MPJW01000291.1
Massiliomicrobiota timonensis strain An13	NZ_NFLJ01000001.1 - NZ_NFLJ01000099.1
[Clostridium] cocleatum strain DSM 1551	NZ_FOIN01000001.1 - NZ_FOIN01000088.1
Alterileibacterium massiliense strain Marseille-P3115	NZ_FNWE01000001.1- NZ_FNWE01000002.1
Massiliomicrobiota timonensis strain SN16	NZ_FQLU01000001.1 - NZ_FQLU01000002.1
Lachnospiraceae	
Lachnoclostridium phytofermentans ISDg	NC_010001.1
[Clostridium] saccharolyticum WM1	NC_014376.1
Butyrivibrio proteoclasticus B316	NC_014387.1 - NC_014390.1
Blautia obeum ATCC 29174	NZ_DS264289.1 - NZ_DS264342.1
[Ruminococcus] torques ATCC 27756	NZ_DS264343.1 - NZ_DS264383.1
Dorea longicatena DSM 13814	NZ_DS264384.1 - NZ_DS264419.1
Anaerostipes caccae DSM 14662	NZ_DS499719.1 - NZ_DS499744.1
Coprococcus eutactus ATCC 27759	NZ_DS483520.1 - NZ_DS483542.1
[Clostridium] scindens ATCC 35704	NZ_DS499678.1 - NZ_DS499718.1
Coprococcus comes ATCC 27758	NZ_GG662005.1 - NZ_GG662017.1

Appendix 2 - Table 2. Genomes from *Prevotellaceaee, Clostridiaceae, Erysipelotrichaceae, Lachnospiraceae, Lactobacillaceae, Ruminicoccaceae* and *Streptoccocaceae* families

Organism	Reference number or GenBank ID
Butyrivibrio crossotus DSM 2876	NZ_GG663534.1 - NZ_GG663504.1
[Clostridium] hylemonae DSM 15053	NZ_GG657759.1 - NZ_GG657881.1
Blautia hansenii DSM 20583	NZ_GG698588.1 - NZ_GG698595.1
Blautia hydrogenotrophica DSM 10507	NZ_GG657678.1 - NZ_GG657710.1
[Clostridium] asparagiforme DSM 15981	NZ_GG657586.1 - NZ_GG657677.1
Catonella morbi ATCC 51271	NZ_KI535366.1 - NZ_KI535373.1
Oribacterium sinus F0268	NZ_GG668533.1 - NZ_GG668575.1
Dorea formicigenerans ATCC 27755	NZ_AAXA02000001.1 - NZ_AAXA02000016.1
Anaerobutyricum hallii DSM 3353	NZ_ACEP01000001.1 -NZ_ACEP01000175.1
Clostridium lentocellum DSM 5427	NC_015275.1
Lachnoanaerobaculum saburreum DSM 3986	NZ_GL622296.1 - NZ_GL622332.1
[Clostridium] symbiosum WAL-14163	NZ_GL834305.1 - NZ_GL834356.1
[Clostridium] citroniae WAL-17108	NZ_JH376420.1 - NZ_JH376460.1
Oribacterium parvum ACB1	NZ_KE148312.1 - NZ_KE148313.1
[Clostridium] bolteae 90A9	NZ_KB851182.1
Blautia producta ATCC 27340	NZ_KB892637.1 - NZ_KB892704.1
Lachnospira multipara ATCC 19207	NZ_AUJG01000001.1 - NZ_AUJG01000020.1, NZ_KE384131.1, NZ_AUJG01000023.1 - NZ_AUJG01000032.1
Butyrivibrio hungatei NK4A153	NZ_AUJY01000001.1 - NZ_AUJY01000002.1, NZ_KE384194.1 - NZ_KE384199.1, NZ_AUJY01000007.1 - NZ_AUJY01000008.1, NZ_AUJY01000011.1 - NZ_AUJY01000012.1, NZ_AUJY01000020.1 - NZ_AUJY01000038.1
[Ruminococcus] gnavus AGR2154	NZ_JAGQ01000001.1 - NZ_JAGQ01000005.1
Butyrivibrio proteoclasticus P6B7	NZ_JHWL01000001.1 - NZ_JHWL01000006.1, NZ_KK211395.1 - NZ_KK211398.1, NZ_JHWL01000009.1 - NZ_JHWL01000014.1, NZ_JHWL01000018.1 - NZ_JHWL01000022.1, NZ_JHWL01000025.1 -

Appendix 2 - Table 2. Genomes from *Prevotellaceaee, Clostridiaceae, Erysipelotrichaceae, Lachnospiraceae, Lactobacillaceae, Ruminicoccaceae* and *Streptoccocaceae* families

Organism	Reference number or GenBank ID
	NZ_JHWL01000028.1, NZ_JHWL01000031.1 - NZ_JHWL01000058.1
[Clostridium] aerotolerans DSM 5434	NZ_JHWJ01000001.1 - NZ_JHWJ01000050.1
Blautia schinkii DSM 10518	NZ_KL370850.1 - NZ_KL370852.1, NZ_JNKJ01000003.1, NZ_JNKJ01000006.1 - NZ_JNKJ01000011.1, NZ_JNKJ01000014.1 - NZ_JNKJ01000068.1
[Clostridium] aminophilum DSM 10710	NZ_JONJ01000001.1 - NZ_JONJ01000004.1, NZ_KL543982.1, NZ_JONJ01000007.1 - NZ_JONJ01000037.1
Faecalicatena fissicatena strain KCTC 15010	NZ_LDAQ01000001.1 - NZ_LDAQ01000184.1
[Clostridium] glycyrrhizinilyticum JCM 13369	NZ_BBAB01000001.1 - NZ_BBAB01000065.1
Cellulosilyticum ruminicola JCM 14822	NZ_BBCG01000001.1 - NZ_BBCG01000194.1
Faecalicatena contorta strain 2789STDY5834876	NZ_CYZU01000001.1 - NZ_CYZU01000139.1
Blautia obeum strain 2789STDY5608838	NZ_CYZA01000001.1 - NZ_CYZA01000058.1
Fusicatenibacter saccharivorans strain 2789STDY5608849	NZ_CYYV01000001.1 - NZ_CYYV01000040.1
Lachnoanaerobaculum saburreum strain DNF00896	NZ_KQ959772.1 - NZ_KQ959853.1
[Clostridium] propionicum DSM 1682	NZ_CP014223.1
Anaerotignum neopropionicum strain DSM-3847	NZ_LRVM01000001.1 - NZ_LRVM01000029.1
Eisenbergiella tayi strain NML 110608	NZ_MCGH01000001.1 - NZ_MCGH01000005.1
Butyrivibrio hungatei strain MB2003	NZ_CP017830.1 - NZ_CP017833.1
Anaerostipes hadrus strain BPB5	NZ_CP012098.1
[Desulfotomaculum] guttoideum strain DSM 4024	NZ_FOIP01000001.1 - NZ_FOIP01000002.1
Lachnobacterium bovis DSM 14045	NZ_FNPG01000001.1 - NZ_FNPG01000057.1

Appendix 2 - Table 2. Genomes from *Prevotellaceaee, Clostridiaceae, Erysipelotrichaceae, Lachnospiraceae, Lactobacillaceae, Ruminicoccaceae* and *Streptoccocaceae* families	
Organism	**Reference number or GenBank ID**
[Clostridium] fimetarium strain DSM 9179	NZ_FOJI01000001.1 - NZ_FOJI01000048.1
[Clostridium] polysaccharolyticum strain DSM 1801	NZ_FOHN01000001.1 - NZ_FOHN01000054.1
[Clostridium] populeti strain 743A	NZ_FOYZ01000001.1 - NZ_FOYZ01000044.1
Anaerocolumna aminovalerica strain DSM 1283	NZ_FOWD01000001.1 - NZ_FOWD01000083.1
Butyrivibrio proteoclasticus strain P18	NZ_FOXO01000001.1 - NZ_FOXO01000075.1
Eubacterium sp. Marseille-P3177	NZ_LT635479.1
Butyrivibrio fibrisolvens DSM 3071	NZ_FQXK01000001.1 - NZ_FQXK01000083.1
Anaerocolumna jejuensis DSM 15929	NZ_FRAC01000001.1 - NZ_FRAC01000071.1
Anaerotignum lactatifermentans DSM 14214	NZ_FRAH01000001.1 - NZ_FRAH01000163.1
Anaerosporobacter mobilis DSM 15930	NZ_FRCP01000001.1 - NZ_FRCP01000045.1
Anaerocolumna xylanovorans DSM 12503	NZ_FRFD01000001.1 - NZ_FRFD01000023.1
Prevotellaceae	
Prevotella ruminicola 23	NC_014033.1
Prevotella marshii DSM 16973	NZ_GL397214.1 - NZ_GL397224.1
Prevotella copri DSM 18205	NZ_GG703852.1 - NZ_GG703878.1
Alloprevotella tannerae ATCC 51259	NZ_GG700642.1 - NZ_GG700647.1
Prevotella bergensis DSM 17361	NZ_GG704780.1 - NZ_GG704809.1
Prevotella buccalis ATCC 35310	NZ_ADEG01000001.1 - NZ_ADEG01000118.1
Prevotella bryantii B14	NZ_ADWO01000001.1 -NZ_ADWO01000098.1
Prevotella buccae ATCC 33574	NZ_GL586311.1 - NZ_GL586331.1
Prevotella oralis ATCC 33269	NZ_GL833116.1 - NZ_GL833119.1
Prevotella salivae DSM 15606	NZ_GL629647.1 - NZ_GL629686.1

Appendix 2 - Table 2. Genomes from *Prevotellaceaee, Clostridiaceae, Erysipelotrichaceae, Lachnospiraceae, Lactobacillaceae, Ruminicoccaceae* and *Streptoccocaceae* families

Organism	Reference number or GenBank ID
Prevotella multiformis DSM 16608	NZ_GL872282.1 - NZ_GL872306.1
Prevotella denticola F0289	NC_015311.1
Paraprevotella xylaniphila YIT 11841	NZ_GL883805.1 - NZ_GL883891.1
Prevotella multisaccharivorax DSM 17128	NZ_GL945015.1 - NZ_GL945016.1
Prevotella dentalis DSM 3688	NZ_GL982488.1 - NZ_GL982512.1
Prevotella nigrescens ATCC 33563	NZ_GL982464.1 - NZ_GL982487.1
Prevotella pallens ATCC 700821	NZ_GL982513.1 - NZ_GL982552.1
Alloprevotella rava F0323	NZ_JH376827.1 - NZ_JH376856.1
Prevotella stercorea DSM 18206	NZ_JH379330.1 - NZ_JH379477.1
Prevotella bivia DSM 20514	NZ_JH660658.1 - NZ_JH660660.1
Prevotella paludivivens DSM 17968	NZ_KB890626.1 - NZ_KB890686.1
Prevotella veroralis DSM 19559	NZ_KB898325.1 - NZ_KB898352.1
Prevotella loescheii DSM 19665	NZ_KB899210.1 - NZ_KB899240.1
Prevotella amnii DSM 23384	NZ_KB905265.1 - NZ_KB905341.1
Prevotella nanceiensis DSM 19126	NZ_KB904327.1 - NZ_KB904338.1
Prevotella maculosa DSM 19339	NZ_KB908319.1 - NZ_KB908378.1
Prevotella albensis DSM 11370	NZ_AUFP01000001.1 - NZ_AUFP01000002.1, NZ_KE384540.1 - NZ_KE384543.1, NZ_AUFP01000005.1 - NZ_AUFP01000014.1, NZ_AUFP01000017.1 - NZ_AUFP01000018.1, NZ_AUFP01000021.1 - NZ_AUFP01000027.1, NZ_AUFP01000030.1 - NZ_AUFP01000048.1,
Prevotella baroniae DSM 16972	NZ_AUFQ01000001.1 - NZ_AUFQ01000009.1, NZ_KE384544.1, NZ_AUFQ01000012.1 - NZ_AUFQ01000048.1
Prevotella corporis DSM 18810	NZ_AUME01000001.1 - NZ_AUME01000002.1, NZ_KE387156.1 - NZ_KE387162.1, NZ_AUME01000007.1 - NZ_AUME01000014.1, NZ_AUME01000019.1 - NZ_AUME01000021.1, NZ_AUME01000025.1 - NZ_AUME01000070.1, NZ_AUME01000075.1 - NZ_AUME01000092.1,

Appendix 2 - Table 2. Genomes from *Prevotellaceaee, Clostridiaceae, Erysipelotrichaceae, Lachnospiraceae, Lactobacillaceae, Ruminicoccaceae* and *Streptoccocaceae* families

Organism	Reference number or GenBank ID
Prevotella timonensis 4401737	NZ_HG417090.1 - NZ_HG417114.1
Prevotella disiens JCM 6334	NZ_BAIS01000001.1 -NZ_BAIS01000187.1
Prevotella shahii DSM 15611	NZ_BAIZ01000001.1 -NZ_BAIZ01000142.1
Prevotella oris DSM 18711	NZ_BAJC01000001.1 - NZ_BAJC01000094.1
Prevotella oulorum JCM 14966	NZ_BAJQ01000001.1 - NZ_BAJQ01000138.1
Prevotella histicola JCM 15637	NZ_BAJX01000001.1 - NZ_BAJX01000055.1
Prevotella falsenii DSM 22864	NZ_BAJY01000001.1 - NZ_BAJY01000092.1
Prevotella aurantiaca JCM 15754	NZ_BAKF01000001.1 - NZ_BAKF01000148.1
Prevotella dentasini JCM 15908	NZ_BAKG01000001.1 - NZ_BAKG01000041.1
Prevotella micans DSM 21469	NZ_BAKH01000001.1 - NZ_BAKH01000108.1
Prevotella saccharolytica JCM 17484	NZ_BAKN01000001.1 - NZ_BAKN01000082.1
Prevotella brevis ATCC 19188	NZ_JHXM01000001.1 - NZ_JHXM01000004.1, NZ_KK211333.1 - NZ_KK211334.1, NZ_JHXM01000007.1 - NZ_JHXM01000014.1, NZ_JHXM01000017.1 - NZ_JHXM01000034.1,
Prevotella fusca JCM 17724	NZ_CP012074.1 - NZ_CP012075.1
Prevotella enoeca strain F0113	NZ_CP013195.1
Prevotella scopos JCM 17725	NZ_CP016204.1 - NZ_CP016206.1
Prevotella intermedia ATCC 25611	NZ_CP019300.1 - NZ_CP019301.1
Prevotella aff. ruminicola Tc2-24	NZ_FOIQ01000001.1 - NZ_FOIQ01000006.1
Prevotella ihumii strain Marseille-P3385	NZ_LT706987.1 - NZ_LT707005.1
Ruminicoccaceae	
[Clostridium] leptum DSM 753	NZ_DS480331.1 - NZ_DS480351.1
Anaerotruncus colihominis DSM 17241	NZ_DS544167.1 - NZ_DS544194.1
Ruminococcus lactaris ATCC 29176	NZ_DS990163.1 - NZ_DS990209.1
Subdoligranulum variabile DSM 15176	NZ_GG704769.1 - NZ_GG704779.1

Appendix 2 - Table 2. Genomes from *Prevotellaceaee, Clostridiaceae, Erysipelotrichaceae, Lachnospiraceae, Lactobacillaceae, Ruminicoccaceae* and *Streptoccocaceae* families

Organism	Reference number or GenBank ID
[Clostridium] methylpentosum DSM 5476	NZ_EQ973338.1 - NZ_EQ973354.1
Faecalibacterium prausnitzii A2-165	NZ_GG697149.2 - NZ_GG697168.2
Pseudoflavonifractor capillosus ATCC 29799	NZ_AAXG02000001.1 - NZ_AAXG02000066.1
Ruminococcus flavefaciens FD-1	NZ_ACOK01000001.1 - NZ_ACOK01000119.1
Ethanoligenens harbinense	NC_014828.1
Ruminococcus albus 7	NC_014827.1 - NC_014833.1
[Eubacterium] siraeum DSM 15702	NZ_KB907512.1 - NZ_KB907545.1
[Clostridium] sporosphaeroides DSM 1294 strain VPI 4527	NZ_KB911066.1 - NZ_KB911086.1
Ruminococcus gauvreauii DSM 19829	NZ_AUDP01000001.1 - NZ_AUDP01000071.1, NZ_KE384395.1
Ruminococcus callidus ATCC 27760	NZ_KI260285.1 - NZ_KI260510.1
Ruminococcus flavefaciens ATCC 19208	NZ_KI912489.1 - NZ_KI912494.1, NZ_JAEF01000006.1 - NZ_JAEF01000007.1, NZ_JAEF01000015.1 - NZ_JAEF01000017.1, NZ_JAEF01000023.1 - NZ_JAEF01000036.1,
[Clostridium] viride DSM 6836	NZ_KK211198.1, NZ_JHZO01000004.1 - NZ_JHZO01000009.1
Agathobaculum desmolans ATCC 43058	NZ_JNJN01000001.1 - NZ_JNJN01000012.1, NZ_KL370811.1 - NZ_KL370812.1, NZ_JNJN01000015.1 - NZ_JNJN01000042.1, NZ_JNJN01000045.1 - NZ_JNJN01000097.1,
Ruminococcus flavefaciens MC2020	NZ_JNKE01000001.1 - NZ_JNKE01000007.1, NZ_KL370840.1 - NZ_KL370842.1, NZ_JNKE01000016.1 - NZ_JNKE01000018.1, NZ_JNKE01000021.1 - NZ_JNKE01000023.1
Ruminococcus bicirculans	NZ_HF545616.1 - NZ_HF545617.1
Ruthenibacterium lactatiformans strain 585-1	NZ_JXXK01000001.1 - NZ_JXXK01000108.1
[Clostridium] cellulosi	NZ_LM995447.1
Anaeromassilibacillus senegalensis strain mt9	NZ_LN868527.1 - NZ_LN868538.1
Ruminococcus faecis JCM 15917	NZ_BBDW01000001.1 - NZ_BBDW01000045.1

Appendix 2 - Table 2. Genomes from *Prevotellaceaee, Clostridiaceae, Erysipelotrichaceae, Lachnospiraceae, Lactobacillaceae, Ruminicoccaceae* and *Streptoccocaceae* families

Organism	Reference number or GenBank ID
Fournierella massiliensis strain AM2	NZ_LN908946.1 - NZ_LN908964.1
Acutalibacter muris strain KB18	NZ_CP021422.1
Neglecta timonensis strain SN17	NZ_LT160627.1 - NZ_LT160640.1
Anaerotruncus rubiinfantis strain MT15	NZ_FKLA01000001.1 - NZ_FKLA01000009.1
Ruminococcus bromii strain YE282	NZ_FMUV01000001.1 - NZ_FMUV01000081.1
Hydrogenoanaerobacterium saccharovorans strain CGMCC 1.5070	NZ_FOCG01000001.1 - NZ_FOCG01000012.1
Ruminococcus flavefaciens strain XPD3002	NZ_FPJT01000001.1 - NZ_FPJT01000039.1
Negativibacillus massiliensis strain Marseille-P3213	NZ_FTRU01000001.1 - NZ_FTRU01000008.1
Massilimaliae massiliensis strain Marseille-P2963	NZ_FUHT01000001.1 - NZ_FUHT01000004.1
Gemmiger formicilis strain ATCC 27749	NZ_FUYF01000001.1 - NZ_FUYF01000092.1
Ruminococcaceae bacterium strain KHP2	NZ_FWXP01000001.1 - NZ_FWXP01000020.1
Streptococcaceae	
Streptococcus pyogenes M1 GAS	NC_002737.2
Lactococcus lactis subsp. lactis Il1403	NC_002662.1
Streptococcus pneumoniae	NC_003098.1
Streptococcus agalactiae 2603V/R	NC_004116.1
Streptococcus mutans UA159	NC_004350.2
Streptococcus uberis 0140J	NC_012004.1
Streptococcus sanguinis SK36	NC_009009.1
Streptococcus gordonii str. Challis	NC_009785.1
Streptococcus equi	NC_012470.1
Streptococcus suis BM407	NC_012923.1, NC_012926.1
Streptococcus mitis B6	NC_013853.1
Streptococcus parasanguinis ATCC 15912	NC_015678.1
Streptococcus criceti HS-6	NZ_AEUV02000001.1 - NZ_AEUV02000002.1

Appendix 2 - Table 2. Genomes from *Prevotellaceaee, Clostridiaceae, Erysipelotrichaceae, Lachnospiraceae, Lactobacillaceae, Ruminicoccaceae* and *Streptoccocaceae* families

Organism	Reference number or GenBank ID
Streptococcus macacae NCTC 11558	NZ_AEUW02000001.1
Streptococcus ictaluri	NZ_AEUX02000001.1 - NZ_AEUX02000008.1
Streptococcus pseudoporcinus LQ 940-04	NZ_AEUY02000001.1 - NZ_AEUY02000005.1
Streptococcus urinalis 2285-97	NZ_AEUZ02000001.1
Streptococcus parauberis KCTC 11537	NC_015558.1
Streptococcus thermophilus JIM 8232	NC_017581.1
Lactococcus garvieae ATCC 49156	NC_015930.1
Streptococcus ratti FA-1	NZ_AJTZ01000001.1 - NZ_AJTZ01000006.1
Streptococcus dysgalactiae subsp. equisimilis AC-2713	NC_019042.1
Streptococcus ferus DSM 20646	NZ_AQVD01000001.1 - NZ_AQVD01000012.1
Streptococcus henryi DSM 19005	NZ_AQYA01000001.1 - NZ_AQYA01000037.1
Streptococcus minor DSM 17118	NZ_AQYB01000001.1 - NZ_AQYB01000033.1
Streptococcus caballi DSM 19004	NZ_KB904062.1 - NZ_KB904123.1
Streptococcus didelphis DSM 15616	NZ_KB904188.1 - NZ_KB904202.1
Streptococcus entericus DSM 14446	NZ_KB904155.1 - NZ_KB904187.1
Streptococcus marimammalium DSM 18627	NZ_KB904339.1 - NZ_KB904363.1
Streptococcus massiliensis DSM 18628	NZ_KB904364.1 - NZ_KB904447.1
Streptococcus merionis DSM 19192	NZ_KB904538.1 - NZ_KB904561.1
Streptococcus orisratti DSM 15617	NZ_KB904448.1 - NZ_KB904537.1
Streptococcus ovis DSM 16829	NZ_KB904562.1 - NZ_KB904585.1
Streptococcus cristatus AS 1.3089	NC_021175.1
Streptococcus plurextorum DSM 22810	NZ_AUIO01000001.1 - NZ_AUIO01000016.1, NZ_KE384086.1 - NZ_KE384090.1, NZ_AUIO01000021.1 -

Appendix 2 - Table 2. Genomes from *Prevotellaceaee, Clostridiaceae, Erysipelotrichaceae, Lachnospiraceae, Lactobacillaceae, Ruminicoccaceae* and *Streptoccocaceae* families

Organism	Reference number or GenBank ID
	NZ_AUIO01000025.1, NZ_AUIO01000028.1 - NZ_AUIO01000036.1,
Streptococcus porci DSM 23759	NZ_AUIP01000001.1, NZ_KE384089.1 - NZ_KE384090.1, NZ_AUIP01000006.1 - NZ_AUIP01000037.1
Streptococcus intermedius B196	NC_022246.1
Streptococcus anginosus C238	NC_022239.1
Streptococcus sobrinus DSM 20742	NZ_JMLC01000001.1 - NZ_JMLC01000006.1, NZ_KK365991.1, NZ_JMLC01000009.1 - NZ_JMLC01000055.1
Streptococcus phocae subsp. salmonis strain C-4	NZ_JSAP01000001.1 - NZ_JSAP01000105.1
Streptococcus salivarius strain NCTC 8618	NZ_CP009913.1
Streptococcus iniae strain YSFST01-82	NZ_CP010783.1
Streptococcus equinus strain AG46	NZ_JNLO01000001.1
Lactococcus piscium MKFS47	NZ_LN774769.1 - NZ_LN774771.1
Streptococcus varani strain FF10	NZ_CTEN01000001.1 - NZ_CTEN01000016.1
Streptococcus halotolerans strain HTS9	NZ_CP014835.1
Streptococcus marmotae strain HTS5	NZ_CP015196.1 - NZ_CP015196.1
Streptococcus pantholopis strain TA 26	NZ_CP014699.1
Streptococcus himalayensis strain HTS2	NZ_CP016953.1
Streptococcus gallolyticus subsp. gallolyticus DSM 16831	NZ_CP018822.1

Appendix 2 - Table 3. Genus in family used as "friend" list for e-probe curation	
Family	**Genus**
Streptococcaceae	*Lactococcus*
	Streptococcus
Ruminococcaceae	*Acetanaerobacterium*
	Acetivibrio
	Acutalibacter
	Agathobaculum
	Anaerofilum
	Anaerolactibacter
	Anaeromassilibacillus
	Anaerotruncus
	Angelakisella
	Bittarella
	Candidatus soleaferrea
	Ethanoligenens
	Faecalibacterium
	Flavonifractor
	Fournierella
	Gemmiger
	Hydrogenoanaerobacterium
	Marasmitruncus
	Massilimaliae
	Negativibacillus
	Neglecta
	Neobitarella
	Papillibacter
	Phocea
	Provencibacterium
	Pseudoflavonifractor
	Pygmaiobacter
	Ruminococcus
	Ruthenibacterium
	Sporobacter
	Subdoligranulum
Prevotellaceae	*Alloprevotella*
	Metaprevotella
	Paraprevotella
	Prevotella
	Prevotellamassilia
Lactobacillaceae	*Lactobacillus*
	Pediococcus
	Sharpea
Lachnospiraceae	*Acetatifactor*
	Acetitomaculum
	Agathobacter

Appendix 2 - Table 3. Genus in family used as "friend" list for e-probe curation

	Anaerobium
	Anaerobutyricum
	Anaerocolumna
	Anaerosporobacter
	Anaerostipes
	Anaerotignum
	Bariatricus
	Blautia
	Butyrivibrio
	Catonella
	Cellulosilyticum
	Coprococcus
	Cuneatibacter
	Dorea
	Eisenbergiella
	Faecalicatena
	Frisingicoccus
	Fusicatenibacter
	Hespellia
	Johnsonella
	Lachnoanaerobaculum
	Lachnobacterium
	Lachnoclostridium
	Lachnospira
	Merdimonas
	Mobilisporobacter
	Muricomes
	Niameybacter
	Oribacterium
	Parasporobacterium
	Pseudobutyrivibrio
	Robinsoniella
	Roseburia
	Sellimonas
	Shuttleworthia
	Stomatobaculum
	Tyzzerella
	Unclassified lachnospiraceae
	Environmental samples
Erysipelotrichaceae	*Absiella*
	Allobaculum
	Breznakia
	Bulleidia
	Catenibacterium
	Dielma

Appendix 2 - Table 3. Genus in family used as "friend" list for e-probe curation	
	Dubosiella
	Eggerthia
	Erysipelatoclostridium
	Erysipelothrix
	Faecalibaculum
	Faecalicoccus
	Faecalitalea
	Galactobacillus
	Holdemanella
	Holdemania
	Ileibacterium
	Kandleria
	Lactimicrobium
	Longibaculum
	Longicatena
	Massiliomicrobiota
	Merdibacter
	Solobacterium
	Traorella
	Turicibacter
	Unclassified erysipelotrichaceae
	Environmental samples
Clostridiaceae	*Alkaliphilus*
	Anaeromicrobium
	Beduini
	Butyricicoccus
	Caldisalinibacter
	Caloramator
	Caloranaerobacter
	Clostridiisalibacter
	Clostridium
	Desnuesiella
	Fervidicella
	Fonticella
	Geosporobacter
	Hathewaya
	Hungatella
	Inediibacterium
	Keratinibaculum
	Khelaifiella
	Lactonifactor
	Lutispora
	Maledivibacter
	Marinisporobacter
	Massilioclostridium

Appendix 2 - Table 3. Genus in family used as "friend" list for e-probe curation
Mordavella
Natronincola
Oxobacter
Paramaledivibacter
Proteiniclasticum
Senegalia
Serpentinicella
Thermobrachium
Thermohalobacter
Thermotalea
Tindallia
Youngiibacter

APPENDIX 3

Appendix 3 - Table 1. Relative abundance of families[h] in fecal microbiota composition[i] of nursery pigs

	Clostridiaceae	*Erysipelotrichaceae*	*Lachnospiraceae*	*Lactobacillaceae*	*Prevotellaceae*	*Ruminicoccaceae*	*Streptoccocaceae*	P-value
Day 0	0.04222[b]	0.00002[c]	0.00003[d]	0.83445[a]	1.08543[a]	0.14819[e]	0.90573[a]	< 0.0001
Day 14	0.04677[b]	0.00001[c]	0.00512[d]	30.76097[a]	14.41451[e]	1.73181[f]	0.20460[g]	< 0.0001
Day 28	0.08381[b]	0.00003[c]	0.00399[d]	20.73481[a]	14.96236[a]	0.70616[e]	6.37970[a]	< 0.0001
Day 42	0.25888[b]	0.00002[c]	0.00373[d]	4.20630[e]	15.28974[a]	0.51983[b]	3.64083[e]	< 0.0001

Equation: Relative abundance = Trt Size Family Trt*Size Trt*Family Size*Family Trt*Size*Family

[abcdefg]The values with different superscript letters in a row are significantly different (P < 0.05).

[h]Family (Clostridiaceae, Erysipelotrichaceae, Lachnospiraceae, Lactobacillaceae, Prevotellaceae, Ruminicoccaceae, and Streptoccocaceae).

[i]Relative abundance (%).

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