EFFECTS OF POLYSACCHARIDE IRON COMPLEXES ON GROWTH, GUT HEALTH AND GUT MICROBIOTA IN NURSERY PIGS RAISED UNDER DIFFERENT SANITARY CONDITIONS

By

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ABSTRACT

This study evaluated the effect of different iron sources, including iron polysaccharide complexes (SQM®·iron) and ferrous sulfate (FS) on growth performance, gut health, pathogenic bacteria load, colon microbiota, and fecal iron concentration in nursery pigs. A total of 320 weaned pigs were randomly assigned to four treatments arranged in a 2 × 2 factorial design, with the factors being sanitation conditions (clean vs. dirty) and iron sources (SQM® iron vs. FS, each at 100 mg iron/kg) to give 8 pens (10 piglets per pen) per treatment. Pigs were fed their respective diets in two phases over a 28-d period. Fecal scores, body weight (BW), and feed intake were recorded weekly. Iron content, gene expression of butyryl-CoA:acetate CoA-transferase and bisulfite reductase, and pathogenic bacteria quantification were determined in fresh fecal samples collected on d 0, d 14, and d 21. On d 28, one pig randomly selected from each pen was euthanized to sample colon digesta for 16S rRNA analysis and sample jejunal tissue and serum for gut integrity and permeability analysis. Overall, SQM® iron resulted in higher average daily gain (ADG) from d 0 to14 and average daily feed intake (ADFI) from d 0 to 28 ($P < 0.05$). SQM® iron resulted in higher fecal iron at d 21 and improved fecal scores at d 27 ($P < 0.05$). Dietary iron treatments did not significantly affect gastrointestinal integrity and permeability in jejunum and did not alter fecal microbiota composition on d 28. In conclusion, substituting 100 mg/kg added iron as ferrous sulfate with equal concentration of SQM® iron improved ADG in the phase 1 (d 0 to 14) and overall ADFI (d 0 to 28) in weaned pigs.

Key words: polysaccharide iron complexes, ferrous sulfate, nursery pigs, growth, sanitation conditions

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LIST OF ABBREVIATIONS

ABT	Acetate-Coa Butyrate-Coa, Transferase
ADFI	Average daily feed intake
ADG	Average daily gain
B^0AT-1	Neutral amino acid transporter
BW	Body weight
CAT-1	Cationic amino acid transporter
CDH-1	Cadherin 1
CLDN-1	Claudin 1
DM	Dry matter
DMT-1	Divalent metal transporter 1
EAAC-1	Excitatory amino acid carrier 1.
ETEC	Enterotoxigenic Escherichia coli
FCR	Feed conversion ratio
FD4	Fluorescein isothiocyanate dextran – 4kDa
FEO	Ferrous iron transport
GIT	Gastrointestinal tract
IBW	Initial body weight
KEGG	Kyoto encyclopedia of genes and genomes
KRB	Krebs ringer buffer
MG	Maltase-glucoamylase
OCLN	Occludin

FS	Ferrous sulfate
OTU	Operational taxonomic unit
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PIC	Polysaccharide iron complex
PepT-1	Peptide transporter 1
PWD	Post-weaning diarrhea
QIIME	Quantitative insights into microbial ecology
RDP	Ribosomal database project
SEM	Standard error of the mean
SGLT-1	Sodium-dependent glucose transporter 1
xCT	Cysteine/glutamate antiporter
ZO	Zonula occludens

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Appendix 2. Statistics, P values and FDR adjusted P values for DESeq2 analysis at the genus level for pig's colon digesta microbiota. Only significant genera are presented ($P \leq 0.05$). IC= inorganic_clean, ID = inorganic_dirty, OC = organic_clean, OD = organic_dirty. There was no diet effect ($P > 0.05$).

CHAPTER 1 GENERAL INTRODUCTION

Modern swine production practices subject weaned pigs to numerous stressors. Transitioning from easily digestible sow milk to solid food stresses the digestive system, especially when the gastrointestinal tract and microbial balance haven't fully matured. Separation from the sow and relocation to a new pen exposes piglets to social stressors as they establish new hierarchies. As the passive immunity from sow milk decreases before full immunological development, growth performance suffers, often manifesting as post-weaning diarrhea (PWD) fueled by pathogens like enterotoxigenic (ETEC) *Escherichia coli.*

While antibiotics administered at subtherapeutic levels can mitigate bacterial outbreaks and promote growth, their prolonged use raises concerns about the emergence of antibiotic-resistant zoonotic bacteria, posing significant threats to food safety. Consequently, research into antibiotic alternatives in animal production has become imperative.

Due to their rapid growth, weaned pigs are supplemented with iron in their feed to support their development. Iron, an essential mineral, plays pivotal roles in oxygen exchange, mitochondrial respiratory chain function, and the activity of enzymes like ribonucleotide reductase (Valerio et al., 2007). However, excess iron induces oxidative stress which lead to cell and tissues damaged; Also, since many pathogenic microorganisms also require iron for growth, excess dietary iron in gut fosters pathogenic bacterial proliferation (Kortman et al., 2012; Pilo, Federica, et al., 2022).

While inorganic sources of iron, such as ferrous sulfate, effectively boost hematological parameters in pigs, their increased inclusion in diets correlates linearly with increased incidence of diarrhea in nursery pigs (Lee et al., 2008). Polysaccharide iron complexes (PIC) present a promising alternative, for their high iron content and bioavailability, along with minimal

gastrointestinal irritation, as demonstrated in human studies (Jing et al., 2022). However, there's limited information regarding the impact of PIC in conjunction with environmental sanitation on growth performance and gut health in post-weaned pigs. Furthermore, the relationship between PIC supplementation and alterations in the gut microbiome profile of post-weaned pigs warrants investigation.

A stable rich and diverse microbiome, characterized by a prevalence of commensal and beneficial bacteria, correlates with reduced gut dysbiosis and improved growth performance and feed efficiency (Vasquez et al., 2022). Understanding how iron supplementation, particularly through PIC, influences the gut microbiome could provide valuable insights into optimizing pig health and productivity.

The primary aim of this study was, therefore, to assess the impact of PIC on weaned piglets in conjunction with the effects of different environmental conditions; Specifically, how different iron sources (organic vs. inorganic) and sanitation levels (clean vs. dirty) affected the growth performance of weaned pigs, relative organ weights, fecal iron excretion and indicators of gut integrity and permeability. In addition, the gene expression of jejunum nutrient transporters and gut tight junction proteins were examined to understand their response to different iron sources and environmental conditions.

Fecal bacteria were quantified as an indicator of response to iron source treatments through gene expression analysis as well as of analyzing 16S rRNA sequencing data to summarize composition of the swine gut microbiota, to identify factors influencing its community structure, and to elucidate the diversity and functionalities of the microbiota in pigs receiving organic (PIC) and inorganic iron (ferrous sulfate) supplementation.

CHAPTER 2 LITERATURE REVIEW

2.1 Introduction

Iron is a trace metal that plays critical roles in the development, growth, and metabolism, participating in various metabolic processes. Deficiency or excess of iron can impact animal health and productivity. Iron body homeostasis is a tightly regulated process that requires the crosstalk between the host, the metal itself, and the gut. The gut is home to trillions of microorganisms and microbial genes (the gut microbiome) that can regulate the metabolism and transport of iron and contribute to the bioavailability of iron through their assimilation from food sources or by competing with the host. On the other hand, deficiency or excess of iron can modulate the gut microbiota and affect nutrient availability, immunity, and host health status. This literature review explores functional aspects of iron utilization in mammals with an emphasis on pigs. Key pathways of uptake, metabolism, and utilization of iron by the host and gut bacteria are discussed. Furthermore, the role of the gut microbiota in the metabolism of iron to the host and the effects of inorganic versus organic iron sources on pig iron homeostasis and the gut microbiota is discussed.

2.2 Functions and metabolism of iron

2.2.1 Mammals

Iron is an essential micronutrient with diverse functions in mammalian physiology, including oxygen transport, energy production, enzyme activation, immune function, neurological development, and storage; The distribution in the body varies to specific functions (Seyoum et al., 2021). Mammalian erythrocytes hold the largest pool of iron in the hosts, which is constituted by proteins such as hemoglobin that facilitates oxygen transportation to cells (Wilson et al., 2008). Liver and spleen account for significant percentage of bodily iron for storage. Muscles contains

iron protein such as myoglobin to facilitate oxygen diffusion. Finally, splenic reticuloendothelial macrophages in spleen recycle iron from dead red blood cells for new red blood cell synthesis (Pantopoulos et al., 2012). Iron can also be found in non-heme proteins such as the lipoxygenases or the rate limiting enzyme in DNA synthesis, i.e., ribonucleotide reductase among others (Anderson and Vulpe, 2009).

Table 2. 1 Iron requirement in different production stages of swine

Production stages	Weight (kg)	Iron requirement (mg/kg)	Method of delivery	References
Suckling	N/A	200 mg from single injection	Iron dextran intramuscular injection	(Heidbüchel et al., 2019)
Nursery	7 - 25	100	Diet/supplement	(Williams et al., 2020)
Grower/finisher	25 - 125	50	Diet	(Deng et al., 2021)

To optimize growth, pigs are supplemented with iron to meet the requirement, which is summarized in Table 2.1. Different dietary iron sources are absorbed differently by the hosts. Heme iron, derived from animal sources, such as hemoglobin and myoglobin, has high bioavailability as it can enter duodenal enterocytes through heme carrier protein 1 (HCP1) as intact metalloporphyrin (Harvey, 2012). Ferric iron in the non-heme iron source is reduced by ferrireductase of the brush border membrane of the enterocyte (DcytB) to more soluble ferrous iron before being transported across membranes via the DMT-1 transport system (Figure 2.1). When required, ferrous iron in the enterocytes is exported into the blood circulation by ferroportin, then oxidized by hephaestin into ferric iron before being carried by transferrin in the plasma and distributed to various organs for uptake.

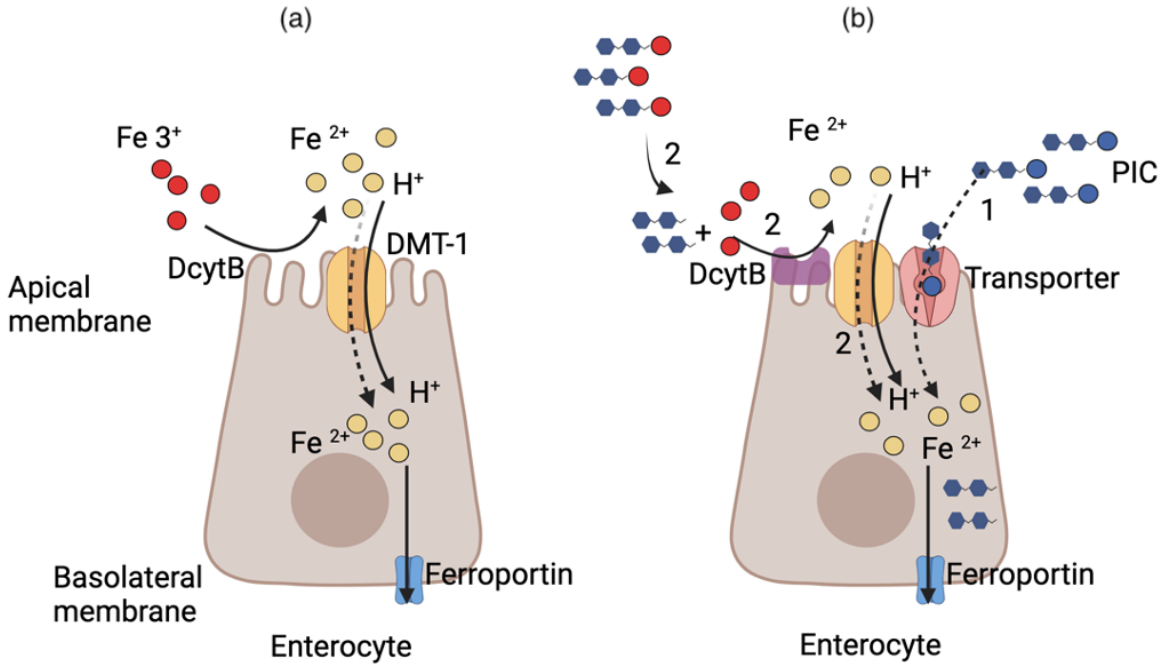


Figure 2. 1 Intestinal absorption of ferrous sulfate (a) and polysaccharide iron complex (b). Dietary iron is taken up across the brush border membrane of enterocytes through the divalent iron membrane transporter (DMT-1); Only ferrous sulfate can enter the cell through DMT-1. This iron reduction is done by an iron -regulated reductase of the brush border membrane (DcytB). The absorption of polysaccharide iron complex is not well understood but it is believed the organic iron enter the cell through either a specific transmembrane transporter and ferrous iron is released from the polysaccharide chain inside the cell or iron in polysaccharide iron complex is the ferric form and is reduced to ferrous iron and transported into the cell the same way as ferrous sulfate. Ferrous iron can then leave the cell through an iron regulated transporter, i.e., ferroportin.

Most of the iron found in the circulation, however, is not bound to transferrin but is found within hemoglobin of red cells. Under normal physiological conditions, this iron can only be utilized when senescent erythrocytes are broken down and heme is released. Oxygenases then release the iron from heme, and this is by far the most important route of iron entry into the plasma.

Iron that is taken up by cells and not required immediately for metabolic functions is stored within ferritin. Higher loads of iron can be further stored as hemosiderin (Koorts and Viljoen,

2007). Plasma iron is bound to diferric transferrin, which act as an innate defense against microorganisms resulting in extremely low free iron concentration of about 10^{-24} M, i.e., almost zero in the plasma (Raymond et al., 2003). In addition, once in the erythrocytes, the iron can only be utilized when senescent erythrocytes are broken down and heme is released. Bacteria can secrete siderophores to acquire iron from the host. As a defense mechanism, the host produces a siderophore-binding antimicrobial protein, i.e., lipocalin 2, that binds to siderophores preventing the bacterial uptake of iron and therefore, controlling this way systemic infections by pathogens (Guo et al., 2017). Both iron uptake and efflux are also tightly regulated to ensure iron homeostasis for the host. Under iron-deficient conditions, both plasma membrane protein transferrin receptor 1 and DMT-1 are increased on the cell surface and the expression of ferroportin is also up-regulated while the expression of the iron storage protein ferritin is decreased under these conditions (Anderson and Vulpe, 2009; Pantopoulos et al., 2012). Under iron-excess conditions or tissue injury, gene expression of liver-derived peptide hepcidin is up-regulated, which binds to ferroportin to prevent iron release from enterocyte to the plasma; thus, facilitating its internalization and degradation (Nemeth et al., 2004). Also, membrane protein transferrin receptor 1 and DMT-1 are reduced while ferritin production increases.

2.2.2 Gut microorganisms

Like higher organisms, iron is also required to perform various biochemical functions, namely respiration, gene regulation, and DNA biosynthesis in microorganisms (Skaar, 2010). Bacterial cells contain 10^5 to 10^6 iron ions per cell which are required for these functions (Wandersman and Stojiljkovic, 2000). Iron has different oxidation states. Ferrous ions are more soluble, but it is unstable as they undergo oxidation to form ferric ions under aerobic conditions

(Krewulak and Vogel, 2008). Ferric ions, on the other hand, are redox stable and exist in an equilibrium concentration of approximately 10^{-17} M which is well below the optimal value for microbial growth (10^{-8} to 10^{-6} M) (Andrews et al., 2003). Most bacteria develop several mechanisms to acquire iron from different sources. They can either obtain iron directly from host proteins or secrete ferric ion chelators, i.e., siderophores, to capture insoluble Fe (III) from the environment (Wandersman and Delepelaine, 2004). The main difference between siderophores and the acquisition of iron from host proteins is that siderophores are taken up by the bacteria as intact molecules whereas iron is first extracted from transferrin and lactoferrin before subsequent transport into the cell (Krewulak and Vogel, 2008). Iron acquisition by bacteria is receptor-mediated with various specific receptors located on the outer surface to recognize a wide range of substrates. In Gram-negative bacteria, iron uptake involves the translocation of subtract via the outer membrane receptors using energy-dependent mechanisms provided by a 3 protein TonB complex anchored to the cytoplasm membrane. Periplasmic binding protein (PBP) then conveys the subtract to an inner membrane ATP-binding cassette (ABC system) which channels it to the cytoplasm for cellular function. Gram-positive bacteria lacking the outer membrane take up iron involving a membrane-anchored binding protein that resembles the PBP (Krewulak and Vogel, 2008). Bacteria also utilise ferrous iron via Feo (Ferrous iron Transport) system (Cartron et al., 2006). Ferrous iron is the preferred iron form due to its high solubility which allows direct transport. Through evolution, bacteria developed mechanism to sequester iron from host iron sequestering proteins. Heme-containing proteins, for example, are abundant in the host and a valuable iron source for bacteria. Bacteria secrete exotoxins such as cytolysins and haemolysins which lyse cells and release the enclosed heme compounds. Subsequently bacteria have specific outer membrane receptors that can bind directly to the released heme or heme-containing proteins

as well as haemophores (siderophore-like molecules) which bind heme (Krewulak and Vogel, 2008). In these bacteria, iron is taken up by membrane-anchored binding proteins that direct the iron to a permease and ABC transporter system for further storage. Three types of iron storage proteins have been identified in bacteria including, ferritin, heme-containing bacterioferritin's and the smaller iron detoxification proteins that protect the chromosome from iron-induced damage by free radicals (Andrews et al., 2003). These proteins sequester iron, preventing its toxicity to the bacteria and serving as a reserve for times of iron scarcity. Bacteria tightly regulate iron homeostasis through complex regulatory networks. This involves sensing intracellular iron levels and adjusting gene expression accordingly. For example, in response to iron starvation, bacteria upregulate iron acquisition systems and downregulate iron-utilizing pathways (Andrews et al., 2003). In *E. coli* (and many other bacteria) the regulation of iron homeostasis is mediated by the ferric-uptake regulator protein (Fur) that controls the iron-dependent expression of more than 90 genes in *E coli*. The main physiological function of Fur is to repress the iron acquisition genes under iron sufficiency, thus ensuring that the iron transport systems are induced by iron restriction (Andrews et al., 2003).

2.3 Pig gut microbiome

The pig gastrointestinal tract (GIT) harbours a diverse and complex microbial community including bacteria, viruses, archaea and fungi. Among the microorganism, the number of bacteria outnumbers other microorganisms. The total number of bacteria in the pig colon has been estimates to be $1\times10^{10} – 1\times10^{11}$ per gram of gut content (Gaskins et al, 2002). The collective microbes found in the gut are termed the gut microbiota. Gut microbiota influences swine physiology and homeostasis, especially in energy harvest, nutrient digestion, and intestinal health, and is likely to affect pig's feed efficiency and growth (McCormack et al., 2017). Commensal relationship between the host and microbiota benefits the host in protective function, structural function and various metabolic functions (O'Hara and Shanahan, 2006). Each bacterium contains thousands of functionally relevant genes and pathways to support essential gut functions and prevent intestinal dysbiosis (Pickard et al., 2017). Commensal bacteria and its microbial genes (the gut microbiome) promote a healthy gut by maintaining the diversity of metabolic functions, preserving the integrity of the gut mucosa and enhancing host immunity (Pickard et al., 2017). *Lactobacillus*, for example, is known for its beneficial effects on gut health and digestion, including the production of short-chain fatty acids (SCFAs) and modulation of immune responses (Bergamasschi et al., 2020).

Advances in molecular techniques, make it now possible to determine and classify the microbial community present in the pig gut. The use of 16S rRNA together with various sequencing methods allows the detection of cultivable and uncultivatable species in the gut. Although there are many different species, they mainly belong to two major phyla that dominate the pig gut, namely Firmicutes and Bacteroidetes (e.g., Lamendella et al. 2011; Yang et al. 2017; Wang et al. 2021). Wang et al. (2019) also reported that Firmicutes, Bacteroidetes, and Actinobacteria were the most abundant phyla across each stage in the life of a pig, including

nursery pigs. Other major phyla in pig gut include Actinobacteria, Proteobacteria, Verrucomicrobia and Fusobacteria (Luo et al., 2022). At the genus level, *Prevotella*, *Runinococcaceae*, and *Lactobacillus* were reported as the core bacteria for fecal samples collected at 80, 120, and 240 days of age (Ke et al., 2019). Higher microbial diversity is often considered indicative of good gut health, as it reflects a more resilient and balanced microbial ecosystem capable of efficiently utilizing nutrients and resisting pathogen colonization (Lozupone, 2012).

2.3.1 Age effects

Microorganisms colonize the sterile gastrointestinal tract of the pig immediately after birth and take several weeks to fully develop (Mackie et al., 1999). Sow's milk is the primary nutrient source for both newborn piglets and their microbiota. During this stage, *Prevotella stecorea* and *Escherichia coli* are abundant (Wang et al., 2019). The dominant bacterial genera found during sucking period are reported to be Clostridium, Bacteroides and Bifidobacterium (Sword et al., 1993). At weaning, piglets are suddenly shifted to a solid diet rich in carbohydrates and the gut microbiota must develop and learn to adapt to the new nutrient sources. The weaning transition is characterized by a loss of microbial diversity, a decrease in the abundance of bacteria belonging to the Lactobacillus group and an increase in the abundance of facultative anaerobes, including bacteria belonging to the Enterobacteriaceae, *Proteobacteriaceae*, *Clostridiaceae* and *Prevotellaceae* families (Chen et al., 2017; Greese et al., 2017). Moreover, the phylogenetic composition of fecal microbial community is dominated by Bacteroidetes, Firmicutes, Proteobacteria, and Spirochaetes at the phylum level and, at the genus level, *Prevotella*, *Lactobacillus*, and *Treponema* were the three most abundant genera (Greese et al., 2017). Wei et al. (2021) found that the relative abundance of *Prevotella* increased dramatically and rapidly after

introducing solid plant-based feed: from 8.9% on the day of weaning to 17.6% on day 7 post-weaning. The relative abundance of *Prevotella* is closely related to the consumption of plant polysaccharides and is enriched by plant polysaccharides (Ivarsson et al., 2014). In a longitudinal investigation of the swine gut microbiome from birth to market by Wang et al. (2019, 2021), Firmicutes and Bacteroidetes were the most abundant phyla representing more than 80% of the total bacteria in the gut of swine. Firmicutes/ Bacteroidetes ratio increased from 2.58 to 3.17 from d90 to d128 of age. Sciellour et al. (2019) also reported an increase of the Firmicutes/Bacteroidetes ratio with age from 3.84 at d52 to 6.66 at d154. On the other hand, Ke et al. (2019) and Sciellour et al. (2019) reported a decreased of Bacteroidetes and Proteobacteria with age. Relative abundance of Spirochaetes increased as pigs aged from weaning age to 42d post-weaning and 138d post-weaning (McCormack et al., 2017). Sciellour et al., (2019) reported that pig fecal microbiota evolved strongly from 52 to 99 days of age with an increased abundance of *Streptococcaceae* and a decreased abundance of *Lactobacillaceae.* Increases in *Ruminoccocacea,* and *Clostrideacea* and *Paraprevotelleacea* agree with the higher cellulytic activity of adult pigs compared with young pigs as reviewed by Lindberg (2014). Ke et al. (2019) also reported that *Prevotella* and *Treponema* increased with age from 25 to 120 days, and they were the dominant bacteria for pigs at the age of 80, 120, and 240 days. Chao1 and Shannon index significantly increased with age. This increase agrees with the results from Wang et al. (2019). These authors studied the fecal microbiota composition from the pig's lactation stage to finishing stage, and it was found that alpha diversity increased over the entire period. Beta diversity analysis by McCormack et al. (2017) also showed that samples clustered according to age (weaning, day 42 post-weaning and day 138 post-weaning). Wang et al. (2019) observed an increasing trend in alpha diversity with pig age. Sciellour et al. (2019) demonstrated that pig fecal microbiota composition strongly depends on age. They

observed changes in the abundance of specific bacterial families and the presence of different enterotypes dominated by *Lactobacillus*, *Prevotella-Sarcina*, or *Ruminococcus* at different ages. These findings suggest that age-related shifts in gut microbiota composition may influence growth performance and feed efficiency in pigs.

2.3.2 Gut sections and microbiota

Bacterial composition varies along the gut (Adhikari et al., 2019; Quan et al., 2018; Yang et al., 2016.; Gresse et al., 2019). Also, the functional capacities of the localized microbiota varied according to gut locations and from mucosal to luminal samples (Mu et al., 2017). Firmicutes were the dominant phylum all along the GIT (e.g., Gresse et al., 2019; Adhikari et al., 2019; Gardiner et al., 2020). Despite that, in these studies, the relative abundances of major phyla differed among sites, with the cecum being like the colon and different from the jejunum and ileum. Greese et al. (2019), using Principal coordinate analysis (PcoA) analysis, also found distinct clusters of microbial numbers and diversity between the large and small intestines. At the phylum level, the cecum and colon were dominated by *Firmicutes* and *Bacteroidetes*, whereas the jejunum and ileum were dominated by Firmicutes ($P > 98\%$) and to a very small extent by *Proteobacteria* ($P < 1\%$). *Bacteroidetes* were almost nonexistent in the small intestine (Crespo-Piazuelo et al., 2019). Cecum and colon often show higher richness and diversity compared to the small intestine (e.g., Gardiner et al., 2020; Crespo-Piazuelo et al., 2019; Quan et al., 2018). This pattern is commonly reported and reflects the dynamic nature of microbial communities along the gastrointestinal tract, with distinct niches and environmental conditions influencing microbial diversity. This separation could arise from variations in physicochemical conditions, such as pH, oxygen levels, and nutrient availability, as well as differences in substrate utilization between the foregut and hindgut. The

fact that the physicochemical conditions and substrate availability vary greatly among different components of the GIT, and this can affect resident microbial communities (Adhikari et al., 2019, Gresse et al., 2019) makes the selection of a unique section of the gut to be of limited info when investigating the gut microbiota.

Nevertheless, fecal samples are often collected to investigate the gut microbiota. According to Zhao et al. (2015), the similarity of the microbiota in the feces is 0.75 and 0.38 compared with the luminal microbiota of the large intestine and small intestine, respectively. This high degree of similarity with the large intestine makes feces a good representative sample for the large intestine microbiota. Furthermore, the collection of feces is non-invasive, and it can be done repeatedly over time.

2.3.3 Other factors affecting gut microbiome

Host genetics, and diet, and other environment factors can greatly modulate the diversity of the gut microbiome (Wang et al., 2019; Gardiner et al., 2020). Various host genetics have various degrees of susceptibility to colonization by specific microbial species. Host genetics can also influence factors such as gut morphology, immune function, and susceptibility to diseases, all of which can indirectly affect the gut microbiota. Although Bacteroidetes and Firmicutes were the predominant phyla between Duroc, Landrace and Yorkshire pigs, their abundances differed. Furthermore, Landrace contained the most diverse microbiome composition and *Catenibacterium*, *Blautia*, *Dialister*, and *Sphaerochaeta* were found to vary among breeds (Pajarillo et al., 2014). Varying gut microbiome composition was also found different among three breeds of Duroc, Landrace, and Large White (Bergamaschi et al., 2020). The Landrace pigs had the highest alpha

diversity of fecal microbiota, and the genera *Catenibacterium*, *Clostridium*, and *Bacteroides* were the main drivers for the differences among the three breeds (Bergamaschi et al., 2020).

Diet also affects the composition of gut microbiota of both neonatal pigs and growing-finisher pigs by promoting a healthy gut or inducing intestinal dysbiosis in pigs (Verschuren et al., 2018, Deru et al., 2022, Gardiner et al., 2020). Fiber sources such as non-starch polysaccharides (NSP) are one of the major food sources for intestinal bacteria, and it can significantly alter the composition and structure of the gut microbiota (Pu et al., 2020; Wang et al., 2019; Zhang et al. 2016, 2018). An increase in the levels of dietary fibre could increase the bacteria with cellulolytic and hemicellulolytic activities in growing pigs (Metzler and Mosenthin., 2008). High fiber diet was linked to a greater relative abundance of *Prevotella* which is a group of bacteria with an essential role in the process of complex dietary polysaccharides (Ellekilde et al., 2014; Ramayo-Caldas et al., 2016).

Other environmental factors such as transportation, changes in housing conditions, hygiene practices, exposure to pathogens and social stress can disrupt the balance of the gut microbiota and compromise gut health and immune function leading to increased susceptibility to diseases (Gardiner et al., 2020).

2.4 Post weaning diarrhea

In Manitoba, common practices include removing piglets from the sow at 21d to a separate nursery facility (Dewey et al., 2006). This is earlier than conventional systems where pigs are weaned at 30 d of age and raised in the same barn until reaching market weight. Isolating the piglets into a separate nursery facility prevents exposure of piglets to pathogens endemic to the parent herd. Higher growth rates have been observed among the iso-weaned pigs as much as over

10 percent over the conventional systems (Dritz et al., 1994). Moreover, higher feed intake and improved feed conversion ratio and leaner carcasses have been reported in iso-weaned pigs (Patience et al., 2000; Whiting and Pasma, 2008). Despite of all these advantages, the isolated weaning practice is often associated with additional stressors to the piglets.

Weaning imposes stressors to piglets. Piglets are abruptly separated from the sow, introduced to new environments, and transitioned from a milk-based to a solid diet. Often, piglets suffer from decreased feed intake and lack digestive ability of disaccharidases, proteases, and lipases. These decreased enzyme activities negatively affect intestinal morphology and apoptosis, which results in growth retardation in weaned piglets and more susceptibility to pathogens (Tang et al., 2022). Further, weaning stresses can compromise the piglet's immune function and gut integrity, making them more susceptible to diarrhea-causing pathogens. Also, improper sanitation protocols, overcrowding, inadequate ventilation, and temperature fluctuations in the pig housing environment can further contribute to stress and increase the transmission of pathogens, exacerbating the risk of post-weaning diarrhea outbreaks which coincides with compromised intestinal physiological development, gut integrity, and disrupted gut microbiota (Cao et al., 2022; Tang et al., 2022).

Post-weaning diarrhea (PWD) is a common and significant issue in pig farming, especially, it can result in economic losses due to reduced growth rates, increased mortality, and the need for therapeutic interventions (Tang et al., 2024, Dewey et al., 2006). It is, therefore, of paramount importance to reduce pathogen exposure to its best by weaning pigs and increasingly important for early segregated piglets.

2.4.1 Gut dysbiosis

In general, the swine gut bacterial population is dominated by two phyla, Firmicutes and Bacteroidetes, followed by Proteobacteria, Actinobacteria and Spirochaetes (Wei et al., 2021). Although such general profile is relatively stable throughout most of the pig's growth stages, some variations occur when the gut microbiota experiences dysbiosis. Weaned piglets are at a higher risk of dysbiosis from stressors which manifests in gut dysbiosis and potential growth performance issues such as post-weaning diarrhea and enteric infections (Wei et al., 2019, 2021; Gardiner et al., 2020). Dysbiosis would decrease functional richness and microbial diversity as well as cause impairment in metabolic functions. During dysbiosis, pathogenic bacteria colonize epithelial cells, leading to local inflammation and the immature immune system of newly weaned pigs can aggravate their inflammatory response and cause sepsis. (Berkes et al., 2003). Disturbed gut microbiota of stressed weaned piglets was characterized by a decreased relative abundance of *Alloprevotella* and *Oscillospira* and increased *Campylobacterales* (Li et al., 2018). *Alloprevotella*, for example, is a succinic acid producer. Succinate was found to upregulate the expression of tight-junction proteins (claudin-1, ZO-1, and ZO-2) and positively modulate inflammatory cytokine (IL-6 and IL-18) expression in the growing pig jejunum. *Oscillospira*, is a butyrate producer which is the dominant energy source for colonocytes. The decrease in these two bacteria, rendered piglets prone to inflammatory disease (Gophna et al., 2017, Wei et al., 2021). Furthermore, an increase in the relative abundance of *Campylobacter*, an opportunistic pathogen, is known to cause acute bacterial diarrhea (Janssen et al., 2008) and this increase may be one of the factors triggering post-weaning diarrhea. The review by Greese et al. (2017) on microbiota changes during weaning showed that most of the studies reported a decrease in bacteria of the Lactobacillus group and a loss of microbial diversity, whereas *Clostridium* spp., *Prevotella* spp.

or facultative anaerobes such as *E. coli* were increased at weaning. Among the *E. coli* species, enterotoxigenic *E. coli* (ETEC) is known as one of the main infection's agents of postweaning diarrhea in piglets being responsible for 50% of piglet's death worldwide per year (Gyles, 1994).

2.4.2 Damage to gut lining from stressful weaning

Gut dysbiosis from weaning is characterized by shifts in microbial populations and reduced diversity. Dysbiosis impairs gut barrier function and increases susceptibility to colonization by pathogenic bacteria and contributes to gut damage and inflammation. Nutrient absorption and transport across the epithelium are tightly regulated by the intestinal epithelial barrier, which consists of the apical plasma membrane of the enterocytes and the intercellular tight junctions (TJs). TJs regulate epithelial permeability and paracellular diffusion (Wang et al., 2014). Certain transmembrane proteins, such as claudin, determine junctional permeability (Zhao et al., 2021). Peripheral membrane proteins, such as zonula occludens1 (ZO-1) are crucial for tight junction assembly and maintenance. TJs proteins limit intestinal epithelial cell permeability and protect mucosal cells from being exposed to bacteria and toxins (Zhao et al., 2021). Disrupted TJs would result in impaired intestinal function, leading to obstructed nutritional absorption and reduced growth performance of pigs. Weaning has been associated with the disruption of the intestinal barrier integrity, which lead to increased gut permeability (Boudry et al, 2004; Spreeuwenberg et al., 2001). One way to test the intestinal mucosal barrier function involves measuring the transepithelial electrical resistance (TEER) and paracellular flux of dextran (4kDa) in the Ussing chamber (Hu et al., 2013). Compared to preweaning stage, jejunal TEER and gene expressions of occludin and claudin-1 and ZO-1 were reduced in the postweaning stage and paracellular flux of dextran was increased. Similarly, using ^{3}H-mannitol and ^{14}C -inulin as marker probes, Moeser et

al. (2007) and Smith et al. (2010) found that the flux of mannitol and inulin over the jejunum was significantly increased, and TEER was significantly decreased when pigs were weaned at 15 to 21 d of age. Other adverse changes that occur in the GIT of weaning pigs post weaning include crypt hyperplasia and intestinal inflammation (Bomba et al., 2014, Hu et al., 2013, Boudry et al., 2004) as well as villus atrophy which is caused by an increased rate of cell loss (apoptosis) and a reduced rate of cell renewal (mitosis) (Modina et al., 2021; Tang et al., 2022).

2.5 Iron supplementation in nursery pig diets

Supplementing pig diets with iron prevents anemia, supports growth and development and optimizes production performance in intensive pig farming systems. Different dietary iron sources, including inorganic and organic iron sources, also influence gut microbiota in different ways, which may be utilized as a tool to reduce PWD and to improve gut health in weaned piglets.

2.5.1. Inorganic iron sources – ferrous sulfate

Ferrous sulfate, as iron source, is readily available and cost-effective. Other sources of inorganic iron supplemented in pig diets include ferrous carbonate, ferrous fumarate and iron phosphate among others. The absorption of ferrous sulfate supplement by the host often is limited due to lower bioavailability and antagonistic competition with other trace metals and some macromolecules.

Most of the supplemented iron is not absorbed and passes to the colon to be discharged consequently. In rats, it was estimated that on average 85% of dietary iron supplied as ferrous sulfate, remains unabsorbed, and colonic iron concentrations are around 25 nM of which only approximately 0.4 mM is in the form of readily absorbable Fe^{2+} (Abbas et al., 2022). The fecal iron

concentration of healthy pigs was on average 100 ppm in wet-weight fecal samples and the value could increase up to four times with increasing ferrous sulfate supplementation (Lee et al., 2008). According to these authors, fecal iron increased linearly and quadratically from 108 to 474 ppm when ferrous sulphate supplementation increased from 0, 50, 100 to 250 mg/kg in the diet.

In addition, the absorption and activity of ferrous sulphate are often compromised by the antagonism among trace mineral such as zinc and copper, and macromolecules (Angelova et al., 2014; Umbreit, 2005). Dietary components, such as tannins, polyphenols, calcium and phytates can inhibit iron absorption and bioavailability by forming insoluble iron-containing complexes (Piskin et al., 2022; Sun et al., 2024).

Most gut bacteria in the pig have essential requirements for iron and supplementation of ferrous sulfate in piglets affects microbiota profile in the gut. In addition, microbiota responses to ferrous sulfate are dose dependent.

Early-life iron deficiency alters the specific bacteria genera in the colon, promoting the growth of iron-independent bacterial communities such as *Lactobacillus*; however, iron deficiency decreases immunity functions, rendering susceptibility to infections (Abbaz et al., 2022). Increased iron availability in the gut from supplementation may improve immune functions, but it also favours iron dependent gut pathogenic bacteria, such as *Salmonella*, *Shigella* and pathogenic *E. coli* (Kortman et al., 2014). Increased ferrous sulfate supplementation also increased free radicals that damage biological components leading to higher risks of infection and inflammation (Abbaz et al., 2022; Nairz and Weiss, 2020).

Alpha diversity indexes, including Chao, ACE, Shannon and Simpson decreased with increasing iron supplementation of ferrous sulphate from 50 ppm to excess dietary iron levels of 500 and 800 ppm, indicating that both microbial community richness and diversity were reduced

by high dietary iron (Chen et al., 2020). Ding et al., (2020) found that dietary iron as ferrous sulphate at higher dose (3000 ppm) decreased Clostridiales, *Faecalibacterium* and *Prevotellaceae*, and favoured *Desulfovibrio* and *Anaerovibrio* in the cecum of piglets compared with control pigs fed a diet supplemented with a lower dose (100 ppm) of ferrous sulfate.

High dietary inorganic iron inclusion has also been associated with the impairment of the gut lining by increasing intestinal permeability, diarrheal incidence, and reducing villus height of duodenum in weaning pigs (Chen et al., 2020). The addition of 3000 ppm ferrous sulphate significantly decreased the villus height of weaned pigs compared to control pigs fed with 100 ppm of ferrous sulphate (Ding et al., 2022). These authors also reported that the higher iron level supplementation, significantly reduced the expression of tight junction proteins Claudin-1, Occludin and ZO-1 in the duodenal mucosa of weaned pigs compared to control pigs, indicative of a compromised gut barrier function.

2.5.2 Organic iron sources – polysaccharide iron complexes

Organic iron sources are compounds where iron is complexed with organic molecules like amino acids, carbohydrates, and proteins. Compared to ferrous sulfate, organic iron sources exhibit fewer diarrhea in human studies as well as increased tissue iron status and activities of iron containing enzymes in pigs (Hu et al., 2023; Huang et al., 2023). Different organic iron sources also affect gut microbiota differently. Piglets supplemented with yeast iron exhibited higher abundance of Firmicutes, Blautia, and Peptococcus compared to those supplemented with iron sulfate on cecum microflora (Zeng et al., 2023). Conversely, the abundance of *Acinetobacter*, *Streptococcus,* and *Prevotella* was lower in piglets fed yeast iron than in the control group. Moreover, no significant changes of commensal bacteria such as *Bifidobacterium*, *Roseburia,* and

Ruminococcus between piglets fed yeast iron and control pigs (Zeng et al., 2023) were reported. Another organic iron source: ferrous glycine chelates led to an increase in the abundance of short-chain fatty acid-producing bacteria such as T*ezzerella* in pigs and decreased the abundance in potential pathogenic bacteria including *Slackia*, *Olsenella*, and *Prevotella* (Ma et al., 2022).

Polysaccharide iron complexes, a type of organic iron, comes in many forms such as iron dextran, sucrose iron, sodium iron gluconate, carboxyl maltose iron, polysaccharide superparamagnetic iron oxide nanoparticles, isomaltose iron, chitosan iron, and plant polysaccharide iron (Jin et al., 2022). The structure of PIC can vary significantly and exhibit distinct physiochemical properties depending on such the monosaccharide composition, molecular weight, and stability, as well as differences in metabolism and functional effects (Jing et al., 2022). Previous studies on PIC showed promising application of PIC in animal productions. The chelation of metal ion by the hydroxyl and carboxyl group from the polysaccharides may hinder bacterial sequestration of iron for growth, which could potentially exert antibacterial activities by limiting the essential mineral to the bacteria (Liang et al., 2024). Gao et al. (2020) demonstrated that a soluble soybean polysaccharide-iron complex exhibited inhibitory effects on pathogenic bacteria like *E. coli* and S. *aureus*, while enhancing the growth of commensal bacteria such as *B. licheniformis* in an *in vitro* study. Structure of PIC also minimizes potential negative antagonistic interactions between trace minerals at absorption, which allows the iron to be better delivered and absorbed by pigs. Furthermore, PIC demonstrated the ability to either mitigate or prevent the overproduction of free radicals and reactive oxygen species, including superoxide anion, hydroxyl radical, and hydrogen peroxide, which are typically generated during normal cellular metabolism and growth (Tang et al., 2022).

2.5.3 Polysaccharide iron complex research gaps

SQM®(Sea-Questra-Min) iron is a commercially available PIC manufactured by QualiTech (Chaska, MN, USA). Previous *in-vitro* and *in-vivo* studies suggested promising results in improving growth performance and animal health.

A previous study on human colonic adenocarcinoma (CaCo-2) cell model to investigate intestinal iron uptake and utilization found that CaCo-2 cells exposed to SQM® iron exhibited higher ferritin formation without cytotoxic effects compared to cells treated with reduced iron (Glahn et al., 2009; Wortley et al., 2005). A previous study on broiler chicken showed improvement in growth performance and reduced pathogen levels in the intestine (Garrett and McNaughton, 2019). However, the effects of SQM® iron on pig growth performance and gut microbiota are not known. Investigating the effects of SQM® iron on the gut microbiota of pigs may offer insight, particularly among weaned piglets to explore the effects of SQM® iron on gut microbiota.

In summary, based on the iron-restricting nature of SQM® iron to bacteria and previous studies on broiler chicken, it is reasonable to hypothesize that SQM® iron restricts the growth of pathogenic bacteria with higher iron requirement in pig's colon over beneficial bacteria such as *Lactobacillus* spp, which require little iron to grow (Abbaz et al., 2022). In particular, the effects of SQM® iron (QualiTech) on postweaning pig's growth performance, gut health, and microbiota and pathogens load needs to be compared with responses to $FeSO_4$ iron source. Furthermore, it is important to investigate how different the above responses are when pigs are raised in different hygiene/ sanitation conditions and therefore different levels of pathogens exposure.

CHAPTER 3 HYPOTHESES AND OBJECTIVES

3.1 **Hypotheses**

Following hypotheses were tested in this study:

Feeding polysaccharide iron complex supplemented diet to weaned piglets would show positive and better effect on gut health and growth performance than feeding ferrous sulfate supplemented diet due to:

1. Improved nutrient absorption capacity.
2. Improved membrane barrier integrity in weaned pigs under different environmental factors (i.e., sanitary conditions)
3. Positive modulation of microbial structure in weaned pigs.

Growth performance and gut barrier function would be negatively affected by the poor sanitation condition.

3.2 **Objectives**

The specific objectives were:

1. To determine the effect of ferrous sulfate and iron polysaccharide complex on growth performance, relative organ index, of 7- to 15-kg weanling pigs (TN70 × TN Tempo; Topigs Norsvin) fed a corn-soybean meal-based diet under different sanitary conditions.

2. To evaluate the effects of ferrous sulfate and iron polysaccharide complex and sanitary conditions on gene expression of nutrient transporters and gene expression of tight junction proteins of weaned pigs under different sanitary conditions.

3. To characterize the composition and predicted functionality of gut microbiome in the weaned pigs because of ferrous sulfate and iron polysaccharide complex supplemented diets under different sanitary conditions.

CHAPTER 4

EFFECTS OF SQM® IRON ON GROWTH, GUT HEALTH AND GUT MICROBIOTA OF NURSERY PIGS RAISED UNDER CLEAN OR DIRTY CONDITONS

4.1 Abstract

SQM® iron reduced abundance of certain pathogenic bacteria in broiler; however, the effect of SQM® iron on nursery pigs have not been investigated. The experiment was conducted to evaluate SQM® iron (organic) as an alternative source of ferrous sulfate (inorganic) for nursery pigs in different sanitation conditions (clean vs. dirty). A total of 320 nursery pigs weaned at 21 ± 2 d of age were assigned to four treatment groups: 1) clean rearing conditions/ SQM® iron supplemented diet; 2) clean rearing conditions/iron sulfate supplemented diet; 3) dirty rearing conditions/ SQM® iron supplemented diet; and 4) dirty rearing conditions/iron sulfate supplemented diet. Treatments were mash feed that were supplemented with different sources of iron (organic vs. inorganic). Piglets had access to feed and water *ad libitum* and their body weight (BW) and feed intake were measured weekly for the experiment. Fresh fecal samples were collected on days 0, 14, and 21 to measure iron content levels, relative quantification of pathogenic bacteria and relative gene expression of acetate-CoA butyrate-CoA transferase and bisulfite reductase level. Fecal scores per pen were recorded weekly. Out of 32 pigs euthanized at the end of 28 days, jejunum samples were used for transepithelial electrical resistance (TEER) and permeability analysis; Heart, liver, and spleen were weighted to calculate relative organ weight and colon digesta were collected for microbiome analysis. Blood serum samples were collected to analyze d-lactate and diamine oxidase levels. The results showed that piglets fed with SQM® iron had significantly higher ADG in phase I (d 0- 14) ($P < 0.05$) and tended to have higher ADG than

the piglets fed with ferrous sulfate (d 0 – 28) (P = 0.0908). ADFI was significantly higher among piglets fed with SQM® iron from d 0 – 28 (P < 0.05). Higher fecal iron content (dry matter basis) was observed in the SQM® iron treatment group under clean conditions (P < 0.05). Fecal scores improved (P < 0.05) on d 27 for the organic iron treatment group than the inorganic iron treatment groups under the dirty condition. Higher transepithelial electrical resistance (TEER) was observed in piglets fed with SQM® iron under the clean condition than piglets fed with SQM® iron under the dirty condition (P < 0.05). The relative gene expression of PepT -1 tended to be higher in the piglets fed with the SQM® iron than in the piglets fed with the ferrous sulfate (P = 0.088). In conclusion, improved hygiene conditions positively impacted overall ADG and FCR in nursery pigs. Substituting 100 mg/kg of added iron as ferrous sulfate with an equivalent concentration of SQM® iron enhanced ADG during the phase 1 (d 0 to 14) and overall ADFI (d 0 to 28).

Key words: polysaccharide iron complexes, ferrous sulfate, nursery pigs, growth, sanitation conditions

4.2 Introduction

Post-weaning piglets undergo significant nutritional and environmental transitions. Abrupt separation from the mother, introduction to new litters in unfamiliar surroundings, and the shift from highly digestible liquid milk to less digestible solid feed marks this critical phase (Suiryanrayna, 2015). Over the years, industry practices have evolved to enhance productivity. The trend toward shorter suckling periods, now often 21 to 28 days instead of the traditional 42 days, aims to maximize sow productivity but can result in challenges such as diarrhea, increased mortality, and slower growth after early weaning (Suiryanrayna, 2015).

The transition from a liquid to solid diet is particularly stressful for the gastrointestinal tract, which is sensitive to dietary changes (Myrlene et al., 2017). Disruption of the gut microbiota during this period, known as dysbiosis, can lead to damage of the gut lining facilitated by pathogenic bacteria (Wang et al., 2019). To mitigate these effects and improve performance, antibiotics have historically been employed. Studies have demonstrated that antibiotic use in nursery pigs can enhance weight gain and feed efficiency (Doyle et al., 2001). However, the repeated use of antibiotics raises concerns about the development of resistant pathogenic strains. In response to these concerns, research efforts have increasingly focused on exploring alternative approaches to controlling pathogenic bacteria in post-weaning piglets.

Iron is essential for the proper metabolic function of both microorganisms and mammals. In bacteria, iron plays critical roles in DNA replication, transcription, metabolism, and energy generation through respiration (Frawley and Fang, 2014). Similarly, in mammals, iron is indispensable for vital cellular and tissue functions, as it is a key component of hemoglobin, myoglobin, and various enzymes involved in oxygen transport and utilization (Valerio, 2007). Supplementing iron in pigs is crucial to prevent iron deficiency as it can lead to impaired immune response, infection risk and retarded growth (Abbas et al., 2022). Years of genetic selection favoring large litter size and birth weight have led to sows having limited iron supply in colostrum to meet the growing demands of piglets. Also, piglets are born with limited hepatic iron reserves in their bodies. To avoid iron deficiency in suckling piglets, iron supplementation is commonly administered to piglets in the form of intramuscular injections (Szudzik et al., 2018). Similarly, due to rapid growth and expansion of blood volume in nursery pigs, iron must be supplied in diet.

Ferrous sulfate, commonly used as an iron supplement for both humans and farm animals, contains high iron content but is often associated with adverse reactions when taken orally. Human

studies have reported incidents of nausea, vomiting, and abdominal pain (Graczykowski and Dobek, 2011). In animal studies, particularly with post-weaning pigs, increased inclusion of iron sulfate in the diet has been linked to elevated incidents of diarrhea, proliferation of pathogenic bacteria, and decreased growth performance (Lee et al., 2008). This correlation suggests that intestinal bacteria have a significant requirement for iron and often compete for this mineral source in the gut to support their growth. Further insights from *in vitro* studies indicate that pathogenic bacteria exhibit a high capacity for iron uptake and are particularly responsive to changes in available iron concentration (Skaar, 2010). Such alterations in iron availability can trigger shifts in the adhesion and invasion of pathogenic bacteria to host cells (Kortman et al., 2012). These findings underscore the complex interplay between iron supplementation, gut microbiota, and host-pathogen interactions, highlighting the need for careful consideration when selecting iron supplements for both human and animal use.

Polysaccharide iron complexes (PIC), as an alternative dietary iron source, provide high iron absorption and bioavailability due to its chelating effect (Jing et al., 2022). The ferric iron is chelated with the polysaccharide in the complex, which serves as a carrier for a more stable and soluble structure for iron delivery to the mucosa in duodenum and jejunum (Jing Y et al., 2022) (Byrne L et al., 1981). Furthermore, polysaccharide iron complexes inclusion in diet not only did not result in adverse effects in gut like that of iron sulfate, but it was also associated positively with animal growth. A previous study showed polysaccharide iron complex as supplement to the broiler chicken resulted in higher growth performance and lower pathogenic bacteria loads. It was suggested that due to higher iron bioavailability, the SQM® iron source was less available to the pathogenic bacteria; thus, reducing chances of getting acquired by the pathogenic bacteria (Garrett and McNaughton, 2019).

The hypothesis of this study was that a PIC supplement as SQM® iron (QualiTech) has positive effects on growth performance, increases gut integrity, and improves the gut microbiome profile in nursery pigs. The objective of this study was to evaluate the effect of SQM® iron on growth performance, gut integrity, and gut microbiome in nursery pigs when compared with dietary ferrous sulfate supplement.

4.3 Materials and methods

Animal use protocol F22-009 (AC11741) was reviewed and approved by the University of Manitoba Animal Care committee and pigs were handled according to the guidelines described by the Canadian Council on Animal Care (CCAC, 2009).

4.3.1 Feed preparation

The diets were provided as mash, formulated in a two-phase feeding program (Table 4.1) and prepared at Glenlea Research Station Feed Mill at the University of Manitoba. The iron levels in the feed ingredients and in the final mash feeds were analyzed prior to the experiment. Iron was supplemented to the feeds with a trace mineral premix (supplemented with SQM® iron or ferrous sulfate), which was provided by QualiTech. The SQM® iron contained polysaccharide iron complexes (SQM®iron, QualiTech), and the inorganic iron contained iron (ferrous) sulfate. The iron content in diets was set to meet or exceed the dietary iron requirement for nursery pigs (NRC, 2012).

Table 4. 1 Composition and nutritional values of experimental diets, as-fed basis (g/kg).

	d 0 - 14	d 14 - 28

Ingredients, kg	SQM®[9]	FS[10]	SQM®	FS
Corn	346.77	346.77	571.43	571.43
Soybean meal	220	220	226	226
Dried whey powder	110	110	35	35
Oat groats	100	100	0	0
Wheat	75	75	50	50
Hamlet HP300[1]	45	45	15	15
NUPRO[2]	35	35	10	10
Barley	0	0	25	25
Canola meal	0	0	25	25
Oil	33	33	6.5	6.5
Limestone	10	10	10	10
Dicalcium phosphate	7	7	8	8
Salt	4.4	4.4	4.5	4.5
Lysine HCL	4	4	4.25	4.25
dl-Methionine	2.57	2.57	2.13	2.13
Vitamin premix[3]	1.5	1.5	1.5	1.5
SQM®premix[4]	2	0	2	0
Ferrous sulfate premix[5]	0	2	0	2
L-Threonine	1.6	1.6	1.74	1.74
Choline chloride (70%)	0.8	0.8	0.5	0.5
L-Tryptophan	0.7	0.7	0.64	0.64
L-Valine	0.56	0.56	0.71	0.71
Phytase[6]	0.1	0.1	0.1	0.1
Total	1000	1000	1000	1000
Analyzed iron level mg/kg	244.61	239.53	275.94	278.1
Calculated nutrients				
Dry matter %	89.35	89.35	87.28	87.28
Crude protein %	20.97	20.97	18.79	18.79
Crude fat %	6.21	6.21	3.65	3.65
Crude fibre %	1.94	1.94	2.29	2.29
Available calcium %	0.81	0.81	0.79	0.79
Available phosphorus %	0.75	0.75	0.69	0.69
SID[7] Lysine %	1.35	1.35	1.2	1.2
SID[7] Methionine %	0.53	0.53	0.46	0.46
SID[7] Threonine %	0.88	0.88	0.78	0.78

SID[7] Tryptophan %	0.28	0.28	0.24	0.24
NE[8] Swine Calc Mcal/kg	2.58	2.58	2.44	2.44
SID[7] Lys/NEc Ratio	5.23	5.23	4.92	4.92

[1]Hamlet HP3001: from Hamlet Protein.
[2]NUPRO2: from Alltech.
[3]The vitamin premix (DSM Nutritional Products Canada Inc. Ayr, ON) provided the following quantities of vitamins per kilogram of diets: vitamin A, 9,000,000 IU; vitamin D3, 1,166,800 IU; vitamin E, 90,000 IU; vitamin B12, 36,670 μg; biotin, 200000 μg; menadione, 2,667 mg; thiamine, 2,667 mg; riboflavin 7350mg; pantothenic acid, 30,000 mg; pyridoxine, 3,000 mg; ascorbic acid, 66,667 mg; niacin 33,333 mg; folic acid, 1,113 mg.
[4]The trace mineral premix with SQM® iron (QualiTech, Inc., MN) contains iron polysaccharide complex, roughage products, zinc sulfate, manganese sulfate, copper sulfate, sodium selenite, ethylenediamine dihydriodide, and mineral oil. The premix provided the following quantities of minerals in both organic and inorganic diet per kg: Manganese,75mg; Zinc,130mg; Iron,100 mg; Copper,25 mg; Iodine,12.5mg; Selenium,0.3 mg.
[5]The trace mineral premix with ferrous sulfate (QualiTech, Inc., MN) contains roughage products, zinc sulfate, ferrous sulfate, manganese sulfate, copper sulfate, sodium selenite, ethylenediamine dihydriodide, and mineral oil. The premix provided the following quantities of minerals in both organic and inorganic diet per kg: Manganese,75mg; Zinc,130mg; Iron,100 mg; Copper,25 mg; Iodine,12.5mg; Selenium,0.3 mg.
[6]Phytase: from AB vista.
[7]Standardized ileal digestible.
[8]Net energy.
[9]Polysaccharide iron complex.
[10]Ferrous sulfate.

4.3.2 Experimental design

A total of 320, TN70 mix sex piglets were obtained from the swine barn of the Glenlea Research Station at the University of Manitoba. The pigs were housed in 32 pens with 10 pigs per pen. Two rooms were assigned as clean rooms, and two rooms were assigned as dirty rooms. The clean rooms were sanitized and disinfected and were gently washed with an unpressurized hose weekly during the experiment, whereas the dirty rooms remained unwashed for the duration of the experiment. PCR tests were conducted on the fecal samples collected in various locations (gestation, farrow, nursery sections) within the Glenlea Swine Barn to confirm the presence of pathogenic bacteria in the chosen pens (Table 4.2). The genes of interest were *E.Coli O157*,

Campylobacter jejuni, *Clostridium perfringen*, and *Salmonella enterica*. The quantification of pathogenic bacteria was calculated as $2^{\wedge\Delta Ct}$ where ΔCt = Ct (bacteria of interest) – Ct (Universal bacterial gene). The area of confirmed pathogenic bacteria was targeted for fecal collection into 5-gallon plastic buckets and stored in a freezer one day prior to the experiment. To ensure equal application of bacteria to each dirty pen, one scoop from each fecal containing bucket was taken into an empty bucket and mixed with room temperature tap water (1 part fecal to 1 part water) to make a slurry. Experimental pens in the dirty rooms were inoculated with fecal matter slurry. The pens were randomly allocated to two dietary treatments in two sanitary conditions within the clean and dirty rooms (8 pens per treatment): 1) diet supplemented with SQM® iron in clean sanitary conditions; 2) diet supplemented with SQM® iron in dirty sanitary conditions; 3) diet supplemented with ferrous sulfate in clean sanitary conditions; 4) diet supplemented with ferrous sulfate in dirty sanitary conditions. The piglets were farrowed on day 21 then randomly assigned to the experimental pens. The moving date was set as day 0 and the starting date was set as day 1. The pigs were fed with phase I diet from day 0 to day 14 and phase II diet from day 15 to day 28. On day 28th, one pig was randomly selected from each pen and transported to the T.K. Cheung Centre for Animal Science Research and then euthanized for sample collection.

Table 4. 2 Relative quantification of pathogenic bacteria from ground fecal samples collected in various sites from Glenlea Swine Barn

Sites	*E.Coli_O157*	*Campylobacter jejuni*	*Clostridium perfringens*	*Salmonella enterica*
1	3.842E-05	5.2332E-05	1.7725E-05	4.1149E-08
2	3.6399E-05	6.116E-05	3.1255E-06	x^2
3	3.7886E-06	1.1247E-05	1.4028E-06	3.0342E-08
4	1.9166E-05	0.00015804	2.7281E-06	3.9814E-08
5	1.6272E-05	6.4321E-05	2.2564E-05	3.4348E-08
6	9.2513E-06	5.2829E-05	3.1134E-05	9.6074E-08
7	2.7393E-05	0.00013249	4.36E-06	0.00017438

8	1.1938E-05	3.9847E-05	0.0001394	5.9092E-05
9	3.4198E-05	6.3055E-05	3.6448E-05	2.4347E-07
10	4.5842E-06	7.317E-05	1.2307E-05	2.6132E-08
11	1.3303E-05	1.652E-05	2.8058E-05	X[2]
12	3.1522E-06	3.7986E-05	7.0996E-07	3.5266E-08
13	3.486E-05	0.00014696	1.7319E-06	6.5125E-08
14	3.5517E-05	0.00020733	3.9996E-05	2.6121E-08
15	8.1999E-06	2.3884E-05	2.683E-06	6.398E-05
16	3.5623E-05	6.0296E-05	5.3793E-06	0.00036449
17	7.2003E-06	3.8125E-05	1.0741E-05	X[2]
18	1.116E-05	5.9293E-05	8.1983E-06	1.1652E-08
19	1.094E-05	7.3665E-05	9.7959E-06	4.2112E-08
20	1.2423E-05	6.3562E-05	2.6944E-05	7.242E-08
21	5.4693E-06	6.7544E-05	6.8482E-06	1.0608E-08

[1]Numeric values obtained by qPCR and calculated by ΔΔCt method.

[2]x is defined as data values not observed in the sample analysis.

4.3.3 Animals and management

The pigs were weighted on day 0 and distributed into the floor pens of identical size 6ft × 5ft (30 Sq Ft). The pens were separated by plastic partitioning board and the floor was slatted metal coated with plastic. Pigs were allowed *ad libitum* access to feed and drinking water (water nipples) during the experiment. Pigs' health condition was monitored daily. The temperature curve started at 27°C and decreased all the way to 22°C by day 28 in the nursery. The lights were turned off from 5 pm to 5 am automatically in nursery rooms.

4.3.4 Growth performance and organ weights

Feed intake and body weight were collected weekly to calculate average daily gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR, feed/gain). Pig body weight and organ weigh (heart, liver, kidney) were recorded. The relative organ weight was calculated as follows:

Relative organ weights (%) = Organ weight (g) /pig BW (g) × 100 %

4.3.5 Fecal scores

Fecal scores were recorded weekly at 9:30 am throughout the experiment. The fecal consistency score was determined based on 0 to 3 score scale described in a previous study (Pérez-Calvo et al., 2019). The rating of fecal score was done by the same person throughout the experiment. A brief description of the scale is as follows:

0. Normal: well-formed solid feces

1. Soft feces: formed soft feces

2. Mild diarrhea: fluid feces with yellowish color

3. Severe diarrhea: watery and projectile feces

4.3.6 Fecal iron analysis

Fresh floor fecal samples were collected on d 0, d14, and d21. The samples were flash frozen by liquid nitrogen and were stored in a -80°C freezer before transported in a sealed polyurethan foam box to the Central Testing Laboratory Ltd. (Winnipeg, Manitoba). In the lab, samples were thawed, and oven dried at 55 °C for 16 hours. Then the samples were ground using Retsch ZM200 grinder before being further dried at 135°C for 2 hours to obtain a moisture value and to produce the *as is* and the 100% dry matter basis. Finally, the samples were weighted and digested in acid to obtain an in-solution form suitable for Inductively Coupled Plasma Optical Emission Spectroscopy. Final readings of iron content were measured by the optical emission spectroscopy at wavelength of 238/204 nm.

4.3.7 Transepithelial electric resistance (TEER) and permeability measurement

Mid jejunum was located 4 meters from the stomach-duodenum junction. Jejunum samples were rinsed with phosphate buffered saline (PBS) solution immediately after sample collection and sealed in a 15 mL tube emersed in an icebox and transported to lab where transepithelial resistance (TEER) and Paracellular flow with fluorescein isothiocyanate-dextran (FITC-dextran) of jejunum samples were determined using Ussing chamber. The Ussing chambers contained pairs of voltage (Ag/AgCl pellet) and current (Ag wire) electrodes enclosed in 3% agar bridges and filled with Kreb-Ranger bicarbonate (KRB) buffer (in $mmol \cdot L^{-1}$: 154 Na^{+}, 6.3 K^{+}, 137 Cl^{-}, 0.3 H_2PO_4, 1.2 Ca^{2+}, 0.7 Mg^{2+}, 24 HCO^{3-} - pH 7.4 with 1$\mu mol \cdot L^{-1}$ of indomethacin). Five milliliters of KRB buffer solution mixed with 10 $mmol \cdot L^{-1}$ D- glucose was added to the serosal chambers. Five milliliters of KRB buffer solution mixed with 10 $mmol \cdot L^{-1}$ D-glucose was added to the mucosal chambers. A mix of 95% O2 and 5% CO2 was added continuously to the serosal and mucosal chambers. A water-jacketed reservoir was used to keep the temperature of the chambers at 37°C by using a water-jacketed reservoir. The possible potential difference existing between the mucosal and serosal chambers was offset before tissue was mounted. Serosal and longitudinal muscle layers were stripped off using micro-forceps before the tissues were mounted in Ussing chambers by using a tissue slider with an aperture of 1 cm^2. The tissues were left to equilibrate for 10 minutes followed by the recording of TEER for 1 hour. The data were calculated by the average of the data points for each tissue sample for the 1-hour duration. After equilibration, 0.1 $mg \cdot mL^{-1}$ of FITC- D4 (molecular weight 4 kDa; Sigma-Aldrich Co.) was added to the mucosal side and after 1 hour, 1 mL of the sample were obtained from the serosal side to measure intestinal permeability. The KRB samples (100 µL) from the serosal side were transferred to 96-well plates. The fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 528 nm using a Bio-Tek PowerWave HT Microplate Scanning Spectrophotometer (BIO-TEK

Instruments, Inc., Winooski, VT). The concentrations of FITC-D4 in the KRB buffer ($ng \cdot mL^{-1}$) were calculated based on a standard curve ($R^2 = 0.99$). The FITC-D4 flux was measured for 1 h using a slide that has 1 cm^2 of well surface area and was expressed as $\mu g \cdot cm^{-2} \cdot h^{-1} \cdot mL^{-1}$.

4.3.8 Gene expression

Mid jejunum was located 4 meters from stomach-duodenum junction. Jejunum samples were rinsed with PBS solution before flash frozen in liquid nitrogen and stored in -80 ^oC freezer. Frozen jejunum samples were pulverized, and then total RNA was extracted from the jejunum samples using Trizol reagent (Invitrogen, Carlsbad, CA, U.S.) according's manufacturer's instructions. The extracted RNA's quantity was measured by Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Ottawa, ON, Canada) and the RNA quality was checked by 1% agarose gel electrophoresis. Complementary DNA was synthesized from RNA using iScriptTM cDNA synthesis kit (Bio-Rad, Mississauga, ON, Canada) and quantitative Real-time PCR of genes were determined by iQ™ SYBR®Green Supermix (Bio-Rad) in a CFX ConnectTM Real-Time PCR Detection System (Bio-Rad). The qPCR was performed to quantify nutrient transporter genes and tight junction proteins. Cyclophilin-A (CYCA) gene was used as the housekeeping gene. Relative gene expression of nutrient transporters was calculated by using the $2^{-\Delta\Delta CT}$ method and were reported as the fold change from the control value (Livak, K. J., and T. D. Schmittgen. 2001). The relative data were expressed as a ratio of the target gene to the Clean organic gene, using the formula $2^{-\Delta\Delta Ct}$ where $\Delta\Delta Ct = (Ct_{target} - Ct_{cyca})$ treatment $- (Ct_{Clean\ organic} - Ct_{cyca})$. Primers for the nutrient transporters and tight junction proteins used for this procedure are included in Table 4.3.

Table 4. 3 Primer sequence for Real-time quantitative PCR analysis for the gene expression of nutrient transporters and tight junction proteins

Genes	Sequence (5' to 3')		Genbank accession number
CAT-1[1]	FP[2]	AACTGGGTTTCTGCCAGAGG	NM_001145490.1
	RP[3]	AACCCATGATGCAGGTGGAG	
B^0AT-1[1]	FP	GCTCTACAGTGTTTGGAACCC	XM_419056
	RP	AAACTAGGCACACCAGCGAT	
EAAC-1[1]	FP	GATTGTTCTGAGCGCTGTCG	XM_424930.5
	RP	ACCAAAGGCATCTCCCAAG	
SGLT-1[1]	FP	ATGCTGCGGACATCTCTGTT	NM_001293240.1
	RP	TCCGTCCAGCCAGAAAGAAT	
PepT-1[1]	FP	CTTTGGCTACCCCTTGAGCA	NM_204365
	RP	AAAGTTGTCATCCCACCGCA	
ZO-1[1]	FP	TATGCACAAGGAGGTCAGCC	XM_015278981.1
	RP	TTGGCCGAAGCATTCCATCT	
CLDN-1[1]	FP	GGTATGGCAACAGAGTGGCT	NM_001013611.2
	RP	CAGCCAATGAAGAGGGCTGA	
xCT[1]	FP	TGAGCTGGGAACGTGCATTA	XM_426289.5
	RP	AGGGCGAATAACCAGCAGTT	
MG[1]	FP	AAGAACCTCTGCAACCTCCG	XM015273018.1
	RP	TCTCCGTCCACCCTATAGC	
CDH-1[1]	FP	GGCAAGCCGTTTACCACATC	NM_001039258.2
	RP	ATAATCCAGGCCCTTGGCTGT	
Ferroportin-1[1]	FP	CTCTATGGAAACAGCCTTCTC	XM_003359590.1
	RP	AGGATGACCGAAACATTCTG	
DMT-1[1]	FP	AGGATCTAGGGCATGTGGTG.	NC_038253.2
	RP	CCACAGTCCAGGAAGGACAT	
PCNA[1]	FP	GCCATGGGCGTCAACCTAAA	NM_204170.2
	RP	AGCCAACGTATCCGCATTGT	

[1]CAT-1: Cationic amino acid transporter; B^0AT-1: Neutral amino acid transporter; EAAC-1: Excitatory amino acid transporter 1: SGLT-1: Na+-glucose cotransporter: PepT-1: Peptide transport; ZO-1: Zonula occudens-1; CLDN-1: Claudin-1: xCT: cysteine/glutamate antiporter; MG: maltase-glucoamylase; CDH-1: cadherin 1; Ferroportin 1; DMT1: Divalent metal transporter; PCNA: proliferating cell nuclear antigen.
[2]FP: forward primer
[3]RP: reverse primer

Fresh fecal samples were collected on day 0, 14, and 21. Fecal samples were frozen immediately after in liquid nitrogen and stored at -80 °C freezer. DNA materials were isolated from the fecal samples using PureLink™ Genomic DNA Mini Kit (Invitrogen, Thermo Fisher Scientific). The extracted DNA samples were measured by Nanodrop 2000 spectrophotometer to test the concentration. Then the quality of the DNA was visually checked by 1% agarose gel electrophoresis. Universal bacteria gene was used as a housekeeping gene to normalize target gene transcript level. Once the concentration of the DNA samples was confirmed, each sample was diluted to the concentration of 1 ng/µl. For each sample well, 2ul of DNA solution from each sample was mixed with SsoAdvanced Universal SYNR Green Supermix (10 µL), forward and reverse primers (1 µL each), and double distilled water (6 µL). qPCR plate was then loaded into the real-time PCR instrument. The protocol was set at (1) polymerase activation and DNA denaturation (3 minutes at 98 °C); (2) amplification and quantification 40 cycles (5s at 98 °C, 20s at 60 °C); (3) melting curve analysis (65-95 °C). The gene expression in the bacteria, Acetate-CoA Butyrate-CoA Transferase, and Bisulfite Reductase were reported as the fold change from the control value. Primers used for this procedure are included in Table 4.4.

Universal bacteria control was used as housekeeping gene. The relative levels of genes on d0, 14, and 21 were expressed as a ratio of the target gene to the control using the formula: $2^{-\Delta\Delta Ct}$, and ΔΔCt was calculated as: ΔΔCt = (Ct sample − Ct universal bacteria gene) treatment − (Ct clean inorganic sample − Ct universal bacteria gene) control (Livak, K. J., and T. D. Schmittgen. 2001). Furthermore, the changes in relative gene expression of the pathogenic bacteria for the specific time periods of d0-14 and d0- 21 were also considered. The gene expression of d 0-14, d 0-21 were based on the change in ΔCt between d14 to d0 and d21 to d0. The ΔΔCt were expressed as a ratio of the target gene to the Clean organic gene using the formula $2^{-\Delta\Delta Ct}$, where ΔCt of day

0, 14, and 21 were calculated by subtracting from the control gene, and ΔΔCt was calculated as $\Delta\Delta Ct = (\Delta Ct_{target} - \Delta Ct\ d0) - (\Delta Ct_{clean\ inorganic} - \Delta Ct\ d0)$. The relative gene expression of Acetate-CoA Butyrate-CoA Transferase, and Bisulfite Reductase were calculated in the same manner.

Table 4. 4 Primer sequence for Real-time quantitative PCR analysis for the quantification of bacteria and the gene expression of enzymes

Gene		Sequence (5' to 3')	References
Escherichia coli O157:H7 strain TT12A	FP[1]	TCAACAGTCTTGTACAAGTCCAC	(Garrido-Maestu et al., 2020)
	RP[2]	GTT TCGCCACATCACGGTAGC	
Clostridium perfringens strain NH-11	FP	GCACAAGCAGTGGAGT	(Matsuki et al., 2002)
	RP	CTTCCTCCGTTTTGTCAAC	
Campylobacter jejuni H660	FP	CTTTGCACGCAGGAGGTAA	(Vizzini P, et al., 2021)
	RP	ATGGTGGGCCTAACAAGACT	
Salmonella enterica (strain P201982)	FP	GGCTAATTTAACCCGTCGTCAG	(Garrido-Maestu et al., 2018b)
	RP	GTTTCGCCACATCACGGTAGC	
Acetate-CoA butyrate-CoA transferase	FP	GAACGGAGCMTGYYTVCAGC	(Lu-Lu Wang, et al., 2017)
	RP	ATCCACAAACGCATCBACRTAC	
Bisulfite reductase	FP	GGCGTGGCGCACTTCCATA	Primer BLAST
	RP	TGGAGCCGTGCATGTTGGTCA	

[1]FP: forward primer
[2]RP: reverse primer

4.3.9 Serum d-lactate and diamine oxidase

Blood samples were drawn from jugular vein then centrifuged to obtain serum samples (Thermo Fisher Scientific) and stored in -80 °C freezer on the same day of collection. For the d-lactate samples, serum samples were deproteinized by protocatechuic acid and neutralized by potassium hydroxide to a pH range of 6.5 – 8 prior to using the kit for further analysis to avoid reading interference from serum proteins, as instructed by the manufacturer's protocol (deproteination protocol, Abcam). D-lactate data were then analyzed by following the instructions of Abcam D-lactate Assay Kit (ab83429). Diamine Oxidase data were analyzed by following the instructions of Abcam Diamine Oxidase Assay Kit (ab241004).

4.3.10 Colon digesta microbiota

Colon digesta was collected and flash frozen in liquid nitrogen to be stored in -80 ^{o}C freezer. Genomic DNA was extracted from the digesta samples using QIAamp Fast DNA Stool Mini Kit (QIAGEN, Toronto, Canada). Quantity of the extraction were determined by Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA), and quality was determined by 1.0% agarose gel electrophoresis. DNA samples were then sent to Genome Quebec (Montreal, Quebec, Canada) for 16S rRNA amplicon sequencing at the V4 region. 515F (GTGYCAGCMGCCGCGGTAA) was set as the forward primer and 806R (GGACTACNVGGGTWTCTAAT) as the reverse primer. Illumina MiSeq PE 300bp paired-end sequencing with 10M reads was performed to obtain complete sequences of the V4 region. The two adaptors used were (ACACTCTTTCCCTACACGACGCTCTTCCGATCTA) and (GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT), and a 12 bp index was used for single multiplexing. The data sequences obtained from the sequencing were analyzed by QIIME2 (version 2021.11) as previously described by Xia and Sun (2023). The initial reads were filtered

and denoised by DADA2 (Callahan et al., 2016), which generates unique amplicon sequence variants (ASVs). Bacterial features were classified using the SILVA reference database classifier (version 138) with a 99% sequence similarity threshold. QIIME2 was used to generate alpha and beta diversities based on the ASV feature data and analysis of similarities (ANOSIM, Clarke, 1993) and permutational multivariate analysis of variance (PERMANOVA, with 999 Monte Carlo permutations. Compositional data at the various taxa levels was analyzed using the Kruskal–Wallis test. Discriminant analysis (LDA) and effect size (LEfSe) were generated using QIIME2 (Segata et al., 2011) and included taxa with average relative abundances greater than 0.01%, which were deemed significant in the LEfSe analysis. Total Sum Scaling (TSS) normalization (Robinson et al., 2010) was used for LEfSe analysis. Discriminant analysis was also conducted following the DESeq2 method by Love et al. (2014) using Relative Log Expression (RLE) normalization of the data (Abbas et al., 2022). Data visualization was performed using the ggplot2 package in R (version 4.22) and MicrobiomeAnalyst 2 (Chong et al., 2020). The predicted genes and their function were aligned to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, and the differences between pig groups were compared through the software STAMP http://kiwi.cs.dal.ca/Software/STAMP (Parks and Beiko, 2010). Two-side Welch's t-test and Benjamini-Hochberg false discovery rate (FDR) correction were used in two-group analysis and all other microbiota data analysis.

4.3.11 Statistical analyses

The experiment was analyzed as complete randomized design (CRD) and pens were considered as the experimental units. All parameter, including growth performance, organ weight, fecal score, Ussing chamber TEER and transcellular flow, fecal iron content, acetate-CoA butyrate-CoA

transferase, bisulfite reductase, serum d-lactate, serum diamine oxidase, quantification of bacteria in fresh fecal samples, and gene expression of nutrient transporters and tight junction proteins from jejunum samples were analyzed using PROC MIXED followed by the Tukey's multiple comparison test (SAS studio 3.81; SAS Inst. Inc., Cary, NC) with the model: $Yij = \mu + li + sj + li \times si + eij$, where μ is the total means, li is the iron source effect, si is the sanitary conditions effect; eij is residual error of the model. *P* value of 0.05 was used to declare significance and a trend was discussed at $0.05 < P < 0.1$.

4.4 Results

4.4.1 Growth performance and relative organ weights

The effects of iron sources and sanitary conditions on growth performance parameters are presented in Table 4.5. No significant interactions between iron sources and sanitation conditions were observed for average daily gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (feed: gain, FCR) ($P > 0.10$). Piglets receiving SQM® iron exhibited significantly higher ADG compared to those receiving ferrous sulfate, irrespective of hygiene conditions during Phase 1 (days 0-14, $P < 0.05$). However, ADG did not differ between piglets receiving different iron sources during Phase 2 (days 14-28, $P > 0.05$). There was, however, a trend towards higher ADG in piglets fed SQM® iron compared to those fed ferrous sulfate over the entire growth period (days 0-28, $P = 0.0908$). Significant sanitary conditions effects on ADG were not seen till Phase 2 (days 14-28). In this phase and for the overall period (days 0-28), piglets reared in clean conditions showed higher ADG compared to those in dirty conditions, regardless of iron sources ($P < 0.05$). Piglets also tended to have a higher ADFI in phases 1 ($P = 0.0627$) and phase 2 ($P = 0.0886$) when fed SQM® iron compared with piglets fed ferrous sulfate. For the overall growth period, ADFI

was significantly higher ($P < 0.05$) for piglets fed SQM® iron than piglets fed ferrous sulfate. There was no effect of sanitary conditions on the ADFI by piglets for the two growth phases and overall period. FCR was not affected by iron sources ($P > 0.05$). FCR for phase 1 was also not affected by sanitary conditions ($P > 0.05$). However, FCR in phase 2 and for the overall period were significantly lower for piglets raised under clean conditions compared to the piglets raised under dirty conditions ($P < 0.05$). Despite growth performance differences, there were no significant effects of iron source or sanitary conditions on the organ weight indexes of heart, liver, and spleen (Table 4.6, $P > 0.05$).

Table 4. 5 Effects of differing iron sources and sanitary conditions on growth performance of weaned pigs

	Clean Conditions		Dirty Conditions			*P*-values		
Items[4]	Organic[1]	Inorganic[2]	Organic	Inorganic	SEM[5]	Condition	Diet	C×D
ADG, kg/d								
d 0-14	0.170	0.158	0.169	0.155	0.001	0.762	0.041	0.920
d 14-28	0.521	0.503	0.439	0.429	0.002	<0.0001	0.318	0.759
d 0-28	0.345	0.326	0.303	0.293	0.001	<0.0001	0.091	0.598
ADFI, kg/d								
d 0-14	0.245[a]	0.229[b]	0.243[ab]	0.240[ab]	0.001	0.373	0.062	0.166
d 14-28	0.744[a]	0.723[ab]	0.725[ab]	0.688[b]	0.003	0.118	0.089	0.629
d 0-28	0.494[a]	0.470[ab]	0.485[ab]	0.463[b]	0.002	0.382	0.017	0.946
FCR, kg/kg								
d 0-14	1.465	1.455	1.453	1.549	0.007	0.346	0.318	0.220
d 14-28	1.428	1.439	1.664	1.608	0.006	<0.0001	0.504	0.318
d 0-28	1.435	1.439	1.603	1.586	0.004	<0.0001	0.794	0.676

[1]Diet was supplemented with iron polysaccharide complex (QualiTech, Inc., MN) as the iron source.
[2]Diet was supplemented with ferrous sulfate as the iron source.
[a,b]means in the same row with different superscripts differ ($P < 0.05$).
ADG, average daily gain; ADFI, average daily feed intake; FCR, feed conversion ratio.
[3]Standard error of the mean.

Table 4. 6 Effects of differing iron sources and sanitary conditions on relative organ weight of post-weaned pigs measured at the end of phase 2 feeding

	Clean conditions		Dirty conditions			*P* -values		
Items	Organic[1]	Inorganic[2]	Organic	Inorganic	SEM[3]	Condition	Diet	C×D
Relative organ wt, (% BW)								
Heart	0.509	0.528	0.496	0.515	0.002	0.372	0.184	1.000
Liver	3.759	3.599	3.669	3.494	0.019	0.37	0.129	0.945
Spleen	0.203	0.229	0.213	0.193	0.003	0.488	0.868	0.226

[1]Diet was supplemented with iron polysaccharide complex (QualiTech, Inc., MN) as the iron source.
[2]Diet was supplemented with ferrous sulfate as the iron source.
[3]Standard error of the mean.

4.4.2 Fecal iron concentration and fecal scores

The effects of iron source and sanitary conditions on fecal iron (dry matter basis, mg/kg) are summarized in Table 4.7. Feces were collected at d0, d14 and d21. There were no iron source x sanitary conditions interaction effects on fecal iron and fecal moisture contents ($P > 0.10$). There was, however, a significant effect of iron source on the fecal iron content on d 21. At this sampling day, the fecal iron concentration (based on dry matter) was higher in the group fed with SQM® iron under clean condition compared with other treatment groups ($P < 0.05$).

The effects of iron source and sanitary conditions on fecal scores of post-weaned pigs are presented in Table 4.7. There were no sanitary conditions or iron source x sanitary conditions interaction effects on fecal scores ($P > 0.05$) at the various days reported. There was, however, on d 27, a significantly lower fecal score of piglets fed SQM® iron than fecal score of piglets fed ferrous sulfate and raised under dirty conditions ($P < 0.05$).

Table 4. 7 Effects of differing iron sources and sanitary conditions on fecal iron concentration based on dry matter and fecal scores of post-weaned pigs

	Clean conditions		Dirty conditions			*P*-values		
Items[3]	Organic[1]	Inorganic[2]	Organic	Inorganic	SEM[3]	Condition	Diet	C ×D
Fecal iron (based on dry matter, mg/kg)								
d 0	2524.09[ab]	2700.40[a]	2347.98[b]	2462.49[ab]	19.734	0.074	0.203	0.784
d 14	1219.7	1254.23	1214.61	1251.09	9.038	0.936	0.493	0.985
d 21	1384.99[a]	1171.30[b]	1221.79[b]	1143.87[b]	9.456	0.086	0.011	0.215
Fecal Scores								
d 3	1.00	0.88	1.13	1.38	0.028	0.059	0.696	0.247
d 7	2.13	2.38	2.00	2.25	0.03	0.461	0.146	1.000
d 14	2.00	2.25	2.00	1.88	0.038	0.391	0.774	0.391
d 21	1.00	1.13	1.25	1.13	0.021	0.308	1.000	0.308
d 27	0.13[b]	0.38[ab]	0.25[b]	0.88[a]	0.036	0.133	0.039	0.361

[1]Diet was supplemented with iron polysaccharide complex (QualiTech, Inc., MN) as the iron source.
[2]Diet was supplemented with ferrous sulfate as the iron source.
[a,b]means in the same row with different superscripts differ significantly ($P < 0.05$)
[3]Standard error of the mean.

4.4.3 Serum d-lactate, diamine oxidase (DAO) and gut permeability

Table 4.8 summarizes the effects of differing iron sources and sanitary conditions on two serum indicators of gut health, i.e., D-lactate and diamine oxidase and on two gut permeability indicators, i.e., transepithelial Electrical resistance (TEER) and fluorescein isothiocyanate (FITC) flux. There were no significant effects of differing iron sources or sanitary conditions on the gut health and gut permeability indicators measured ($P > 0.05$); however, a significant interaction was observed where SQM® iron resulted in higher TEER value under clean condition over dirty condition ($P < 0.05$). Also, there was a trend of higher serum d-lactate value under dirty conditions ($P = 0.051$).

Table 4. 8 Effects of differing iron sources and sanitary conditions on serum d-lactate, DAO activity, jejunum paracellular flow (FITC-D flux)[4], and transepithelial resistance (TEER)[5] of post-weaned nursery pigs measured at the end of phase 2 feeding

	Clean Conditions		Dirty Conditions			*P*-values		
Items	Organic[1]	Inorganic[2]	Organic	Inorganic	SEM[6]	Condition	Diet	C × D
DAO[3] activity (mU/mL)[7]	2.915	1.539	2.118	2.156	0.074	0.831	0.120	0.101
D-lactate (nmol/mL)	0.093	0.090	0.112	0.168	0.004	0.051	0.265	0.218
FITC-D flux $\mu g \cdot cm^{-2}.h^{-1}.mL^{-1}$	3939	3389	3780	4259	106.823	0.549	0.952	0.387
TEER (Ohm/cm^2)	62.947[a]	47.985[ab]	36.270[b]	51.646[ab]	1.116	0.079	0.974	0.023

[1]Diet was supplemented with iron polysaccharide complex (QualiTech, Inc., MN) as the iron source.
[2]Diet was supplemented with ferrous sulfate as the iron source.
[3]Diamine Oxidase.
[4] Fluorescein-5-isothiocyanate-Dextran.
[5]Transepithelial electrical resistance
[6]SEM: calculated by dividing standard deviation by square root of the sample size (n = 32).
[a,b]means in the same row with different superscripts differ significantly ($P < 0.05$).
[7]The unit is defined as one unit of diamine oxidase is the amount of enzyme that generates 1.0 µmol of H_2O_2 per min. at pH 7.4 at 37 °C.

4.4.4 Relative gene expression of nutrient transporters and tight junction proteins in jejunum

As shown in Table 4.9, there were no significant effects from differing iron sources nor sanitary conditions nor interaction on the nutrient transporters and tight junction proteins studied except for a tendency for the relative gene expression of PepT -1 to be higher in the piglets fed with the SQM® iron than in the piglets fed with the ferrous sulfate ($P = 0.088$).

Table 4. 9 Effects of differing iron sources and sanitary conditions on the relative gene expression ($2^{-\Delta\Delta Ct}$) of nutrient transporters and tight junction proteins in post-weaned pig jejunum samples measured at the end of phase 2 feeding

	Clean Conditions		Dirty Conditions			*P* Value		
Items	Organic[1]	Inorganic[2]	Organic	Inorganic	SEM[3]	Condition	Diet	C × D
CAT-1	1.060	1.692	1.969	1.183	0.092	0.704	0.884	0.186
BOAT-1	1.076	1.731	2.185	1.113	0.119	0.718	0.759	0.209
EAAC-1	1.898	1.443	3.529	3.776	0.245	0.151	0.939	0.796
xCT	1.694	4.446	6.511	1.795	0.507	0.709	0.735	0.204
SGLT-1	1.037	1.094	1.458	0.780	0.053	0.860	0.308	0.229
PepT-1	1.074	0.784	1.059	0.687	0.033	0.766	0.088	0.828
DMT1	1.379	1.095	0.800	0.753	0.047	0.093	0.536	0.657
MG	1.122	0.907	1.022	0.803	0.046	0.695	0.405	0.993
ZO-1	2.000	3.221	2.856	1.877	0.188	0.811	0.906	0.288
CDH-1	1.388	1.192	1.018	0.782	0.053	0.206	0.480	0.948
CLDN-1	1.572	4.110	9.364	7.871	0.629	0.111	0.883	0.570
Ferroportin	1.091	0.771	1.078	0.938	0.032	0.666	0.203	0.615

PCNA	1.173	1.647	1.166	0.824	0.080	0.370	0.886	0.378

[1]Diet was supplemented with iron polysaccharide complex (QualiTech, Inc., MN) as the iron source.
[2]Diet was supplemented with ferrous sulfate as iron source.
[3] SEM: calculated by dividing standard deviation by square root of the sample size (n = 32).
CAT-1, cationic amino acid transporter; B^0AT-1, neutral amino acid transporter; xCT, cysteine/glutamate antiporter; SGLT-1, sodium-dependent glucose transporter 1; PepT-1, peptide transporter 1; MG, maltase-glucoamylase; ZO-1, zonula occludens; CDH-1, cadherin 1; CLDN-1, claudin 1; DMT1, divalent metal transporter 1; PCNA, proliferating cell nuclear antigen; EAAC-1, excitatory amino acid carrier 1.

4.4.5 Relative quantification of pathogenic bacteria

The effects of iron source and sanitary conditions on the relative quantification of several pathogenic bacteria in the fecal samples are summarized in Table 4.10.

For the relative quantification of *0157 E. coli*, there was a significant interaction on d14, where SQM® iron under clean conditions resulted in a higher value than rest of the treatments ($P < 0.05$). For C*lostridium perfringens*, there was also a significant interaction effect in the relative quantification of this bacterium from d0 to d21. For this period, SQM® iron treatment under clean conditions resulted in significantly higher values than rest of the treatments ($P < 0.05$). There was also a trend ($P = 0.057$) for an interaction effect when comparing changes in the relative quantification from d0 to d14. In this case, SQM® iron treatment under clean conditions resulted in higher values than the other treatments. For the relative quantification of *Campylobacter Jejuni*, there was a trend for an interaction effect ($P = 0.076$) on d14, where SQM® iron treatment under clean conditions had higher values than the ferrous sulfate treatment under dirty conditions. Sources of iron nor sanitary conditions nor interactions of these two factors were detected in the relative gene expression of *ETEC E. coli* and *Salmonella spp* among treatment groups ($P > 0.05$).

Table 4. 10 Effect of diets supplemented with iron sources and sanitary conditions on the relative quantification of bacteria in the fecal samples of weaned pig

	Clean conditions		Dirty conditions			*P* Value		
Items	Organic[1]	Inorganic[2]	Organic	Inorganic	SEM[3]	Condition	Diet	C × D
Campylobacter Jejuni								
d 0	26.570	1.894	11.846	6.145	1.756	0.602	0.138	0.348
d 14	7.016[a]	2.740[ab]	2.276[b]	3.742[ab]	0.274	0.240	0.374	0.076
d 21	5.268	1.907	0.495	4.414	0.354	0.576	0.890	0.080
d 0-14	3.608	2.740	0.423	3.010	0.197	0.202	0.447	0.132
d 0-21	2.709	1.907	0.092	3.550	0.235	0.717	0.327	0.121
Clostridium perfringens								
d 0	0.006075	89904255	57772093	2.3825E8	9546559	0.067	0.018	0.409
d 14	776910	374858	169428	3114205	228728	0.417	0.334	0.207
d 21	7325756	5792586	5190685	26545191	2084837	0.437	0.408	0.340
d 0-14	2.729E8[a]	374858[b]	134848[b]	194.59[b]	12109350	0.056	0.056	0.057
d 0-21	2.5732E9[a]	5792586[b]	4131268[b]	1658.63[b]	86125606	0.013	0.013	0.014
ETEC E. coli								
d 0	15.167	1.273	2.068	2.324	1.114	0.347	0.289	0.271
d 14	0.797	1.617	2.557	3.223	0.205	0.153	0.521	0.947
d 21	2.204	1.182	2.039	3.290	0.172	0.327	0.907	0.253
d 0-14	0.224	1.617	1.727	1.523	0.118	0.301	0.382	0.243
d 0-21	0.619	1.182	1.377	1.555	0.091	0.280	0.477	0.710
O157 E. coli								
d 0	11.340	5.740	1.183	3.239	0.999	0.272	0.756	0.504
d 14	40.417[a]	1.763[b]	3.165[b]	14.455[b]	2.082	0.306	0.255	0.043
d 21	1.937	1.740	1.075	5.365	0.303	0.428	0.243	0.202
d 0-14	11.331	21.784	8.922	7.688	1.068	0.183	0.452	0.341

d 0-21	1.821	1.739	1.931	6.412	0.362	0.253	0.292	0.275
Salmonella spp.								
d 0	2.854	1.161	1.425	1.429	0.205	0.602	0.449	0.447
d 14	1.632	1.401	1.196	3.094	0.187	0.557	0.437	0.323
d 21	2.447	2.262	2.430	7.913	0.511	0.338	0.367	0.335
d 0-14	2.280	1.401	4.676	3.766	0.257	0.112	0.543	0.992
d 0-21	3.419	2.262	9.498	9.631	0.723	0.111	0.901	0.876

[1]Diet was supplemented with iron polysaccharide complex (QualiTech, Inc., MN) as the iron source.
[2]Diet was supplemented with ferrous sulfate as the iron source.
[3] SEM: calculated by dividing standard deviation by square root of the sample size (n = 32)
[a,b]means in the same row with different superscripts differ significantly ($P < 0.05$).

4.4.6 Relative gene expression of enzymes: Acetate-CoA Butyrate-CoA Transferase (ABT) and Bisulfite Reductase

As shown in Table 4.11, the piglets fed with the ferrous sulfate had a higher change of ABT gene relative gene expression than the piglets fed with the SQM® iron ($P < 0.05$) for the period d0-14. In addition, ferrous sulfate treatment resulted in a higher change in the relative ABT gene expression than SQM® for the period d0-21 under clean conditions ($P < 0.05$); Ferrous sulfate treatment under clean conditions also resulted in a higher change in the ABT relative gene expression than the ferrous sulfate treatment under dirty conditions. The relative gene expression of ABT was however not affected by iron source nor sanitary conditions on d0, d14, and d21 ($P > 0.05$).

As shown in Table 4.12, there was a significant effect of iron source, sanitary conditions and interaction of these two factors on the relative gene expression of bisulfite reductase ($P < 0.05$) for the period d0-21. Ferrous sulfate treatment resulted in higher change of relative bisulfite reductase gene expression than SQM® for the period d0-21 under clean conditions ($P < 0.05$); Ferrous sulfate treatment under clean conditions also resulted in higher change of bisulfite reductase relative gene expression than the ferrous sulfate treatment under dirty conditions. For the other periods, i.e., d0, d14, d21 and d0-14, there were no significant differences among treatments ($P > 0.05$).

Table 4. 11 Effects of differing iron sources and sanitary conditions on the relative gene expression of Acetate-CoA Butyrate-CoA, Transferase in the fresh fecal samples of weaned pigs

	Clean Conditions		Dirty Conditions			*P* Value		
Items	Organic[1]	Inorganic[2]	Organic	Inorganic	SEM[3]	Condition	Diet	C × D
Acetate-Coa Butyrate-Coa, Transferase								
d 0	0.561	0.691	0.956	0.761	0.032	0.198	0.855	0.366
d 14	1.822	1.399	1.400	1.416	0.039	0.356	0.354	0.317
d 21	1.006	1.504	0.847	0.744	0.061	0.186	0.563	0.382
d 0 – 14	10.127	27.548	7.174	34.034	0.517	0.866	0.042	0.653
d 0 – 21	3.925[b]	26.860[a]	3.049[b]	5.177[b]	0.792	0.018	0.009	0.028

[1]Diet was supplemented with iron polysaccharide complex (QualiTech, Inc., MN) as the iron source.
[2]Diet was supplemented with ferrous sulfate as the iron source.
[3] SEM: calculated by dividing standard deviation by square root of the sample size (n = 32)
[a,b]means in the same row with different superscripts differ significantly ($P < 0.05$).

Table 4. 12 Effects of differing iron sources and sanitary conditions on the relative gene expression of bisulfite reductase in the fresh fecal samples of weaned pigs

	Clean Conditions		Dirty Conditions			*P* Value		
Items	Organic[1]	Inorganic[2]	Organic	Inorganic	SEM[3]	Condition	Diet	C × D
Bisulfite reductase								
d 0	3.419	0.658	0.384	0.745	0.244	0.295	0.393	0.268
d 14	2.232	3.309	2.508	6.507	0.371	0.414	0.236	0.492
d 21	1.280	1.244	0.416	0.751	0.057	0.043	0.643	0.566
d 0 – 14	0.137	0.784	0.459	0.709	0.046	0.635	0.093	0.448
d 0 – 21	0.669[b]	2.518[a]	0.649[b]	0.699[b]	0.065	0.019	0.016	0.021

[1]Diet was supplemented with iron polysaccharide complex (QualiTech, Inc., MN) as the iron source.
[2]Diet was supplemented with ferrous sulfate as iron source.
[3] SEM: calculated by dividing standard deviation by square root of the sample size (n = 32)
[a,b]means in the same row with different superscripts differ significantly ($P < 0.05$).

4.4.7 Gut microbiota

4.4.7.1 Gut microbiota composition

In the current study, 3,357,20 qualified genetic sequences reads in total were obtained. After quality control with DADA2, an average of 10,491 genetic sequences reads per sample and 2,771 Amplicon sequence variants (ASVs) in total were identified. The top phyla in the colon digesta across treatments were by far Firmicutes (>80%) and to a less extent Bacteroidota (>16%) and Actinobacteriota (<2%) (Figure 4.1). Firmicutes and Bacteroidota were significantly different ($P < 0.05$, Figure 4.2) between pigs fed ferrous sulfate and raised under dirty conditions than in pigs fed SQM® iron and raised under clean conditions. There were no other significant phyla differences among the other treatments.

Most abundant families across all treatments, included, *Lactobacillaceae* (>38%), *Prevotellaceae* (<15%), *Lachnopiraceae* (<14%) and *Ruminococcaceae* (>6%) (Figure 4.3). There were no significant differences of *Lactobacillaceae* and *Ruminococcaceae* among treatments (Figure 4.4). There was, however, a higher relative abundance of *Prevottelaceae* in pigs fed ferrous sulfate and raised in dirty conditions than in the organic_clean group. The relative abundance of *Lachnopiraceae* was significantly higher in the inorganic_clean group compared to the organic_dirty group ($P < 0.05$, Figure 4.4). The most abundant genus across all treatments was *Lactobacillus* (>38%) (Figure 4.5). Other top genera were *Prevotella* (< 8%), *Streptococcus* (< 5%) and *Blautia* (< 4%). There were no significant differences of these genera across all treatments ($P > 0.05$). There was, however, a tendency ($P = 0.0936$) for a higher relative abundance of *Prevotella* in the inorganic_dirty group compared to the organic_clean group (Figure 4.6). Compositional differences at the phylum, family and genus, however, disappear when False Discovery Rate (FDR) adjusted *P* values were considered (Table 4.13).

Figure 4. 1 Stacked bar plot showing the relative abundance of bacterial phyla in the colon digesta of d28 weaned piglets fed either ferrous sulfate or SQM® iron and reared under clean or dirty conditions

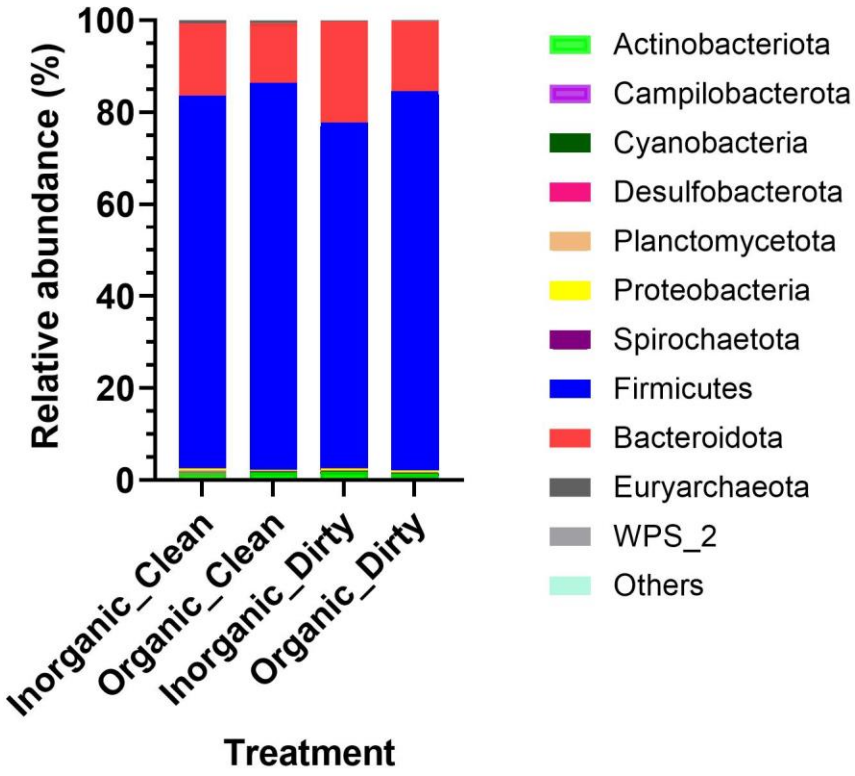
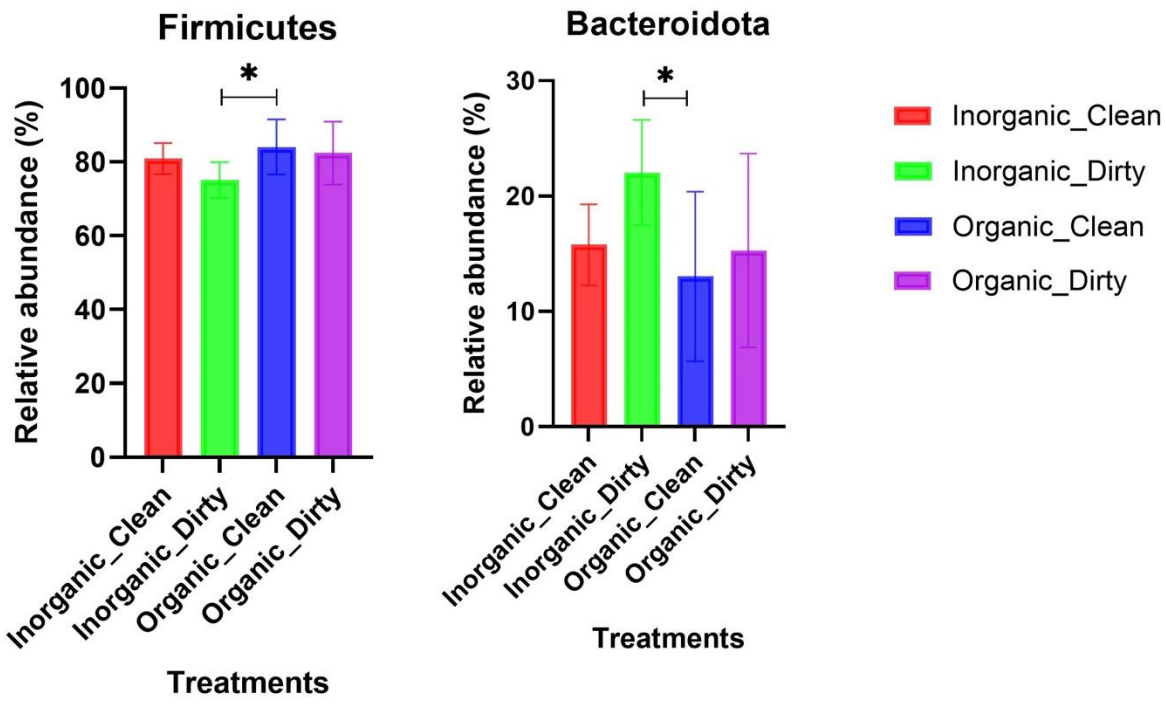


Figure 4. 2 Bar plots showing the relative abundance of bacterial phylum in the colon digesta of d28 weaned piglets fed either ferrous sulfate or SQM® iron and reared under clean or dirty conditions



[a, b, c] Means without a common superscript or * are different ($P < 0.05$).

Figure 4. 3 Stacked bar plot showing the relative abundance of the top 15 bacterial families in the colon digesta of d28 weaned piglets fed either ferrous sulfate or SQM® iron and reared under clean or dirty conditions

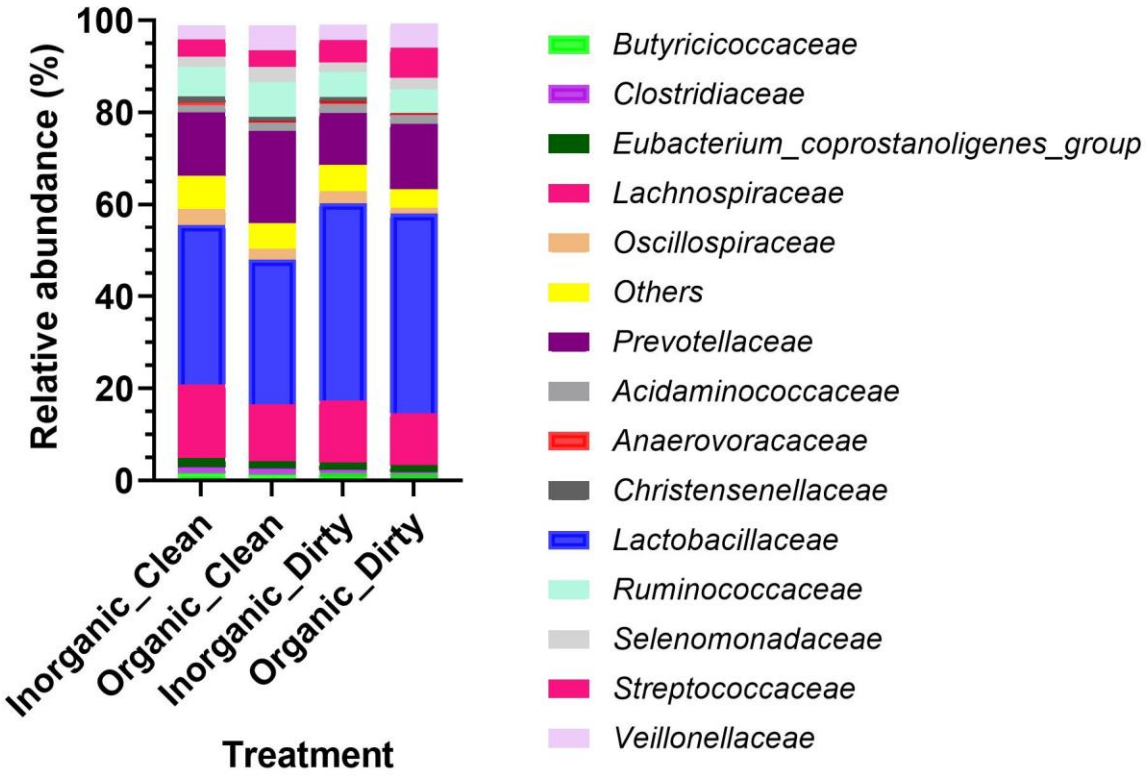
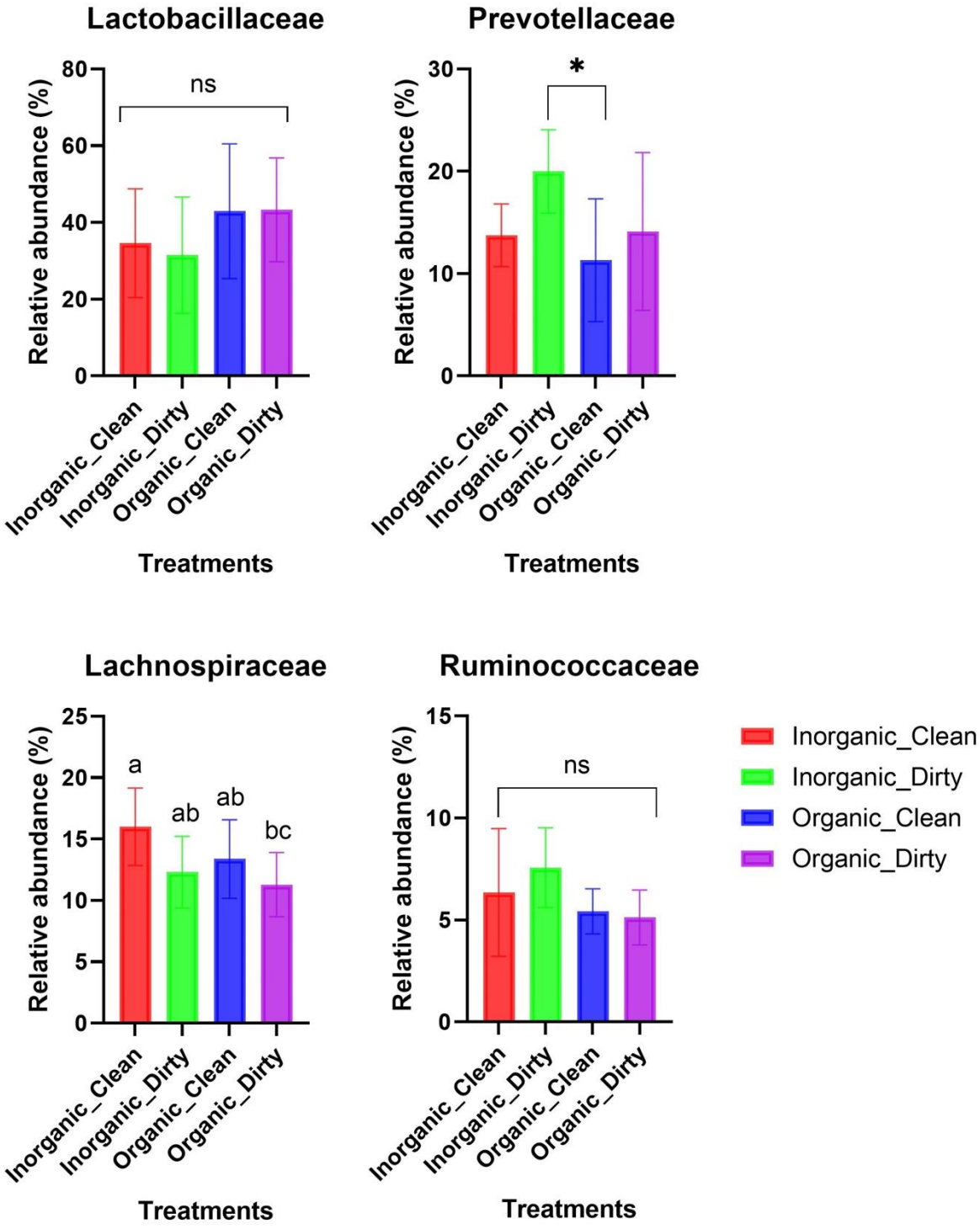


Figure 4. 4 Bar plots showing the relative abundance of bacterial families in the colon digesta of d28 weaned piglets fed either ferrous sulfate or SQM® iron and reared under clean or dirty conditions



. [a, b, c] Means without a common superscript or with * are different ($P < 0.05$).

Figure 4. 5 Stacked bar plot showing the relative abundance of the top 15 bacterial genera in the colon digesta of d28 weaned piglets fed either ferrous sulfate or SQM® iron and reared under clean or dirty conditions

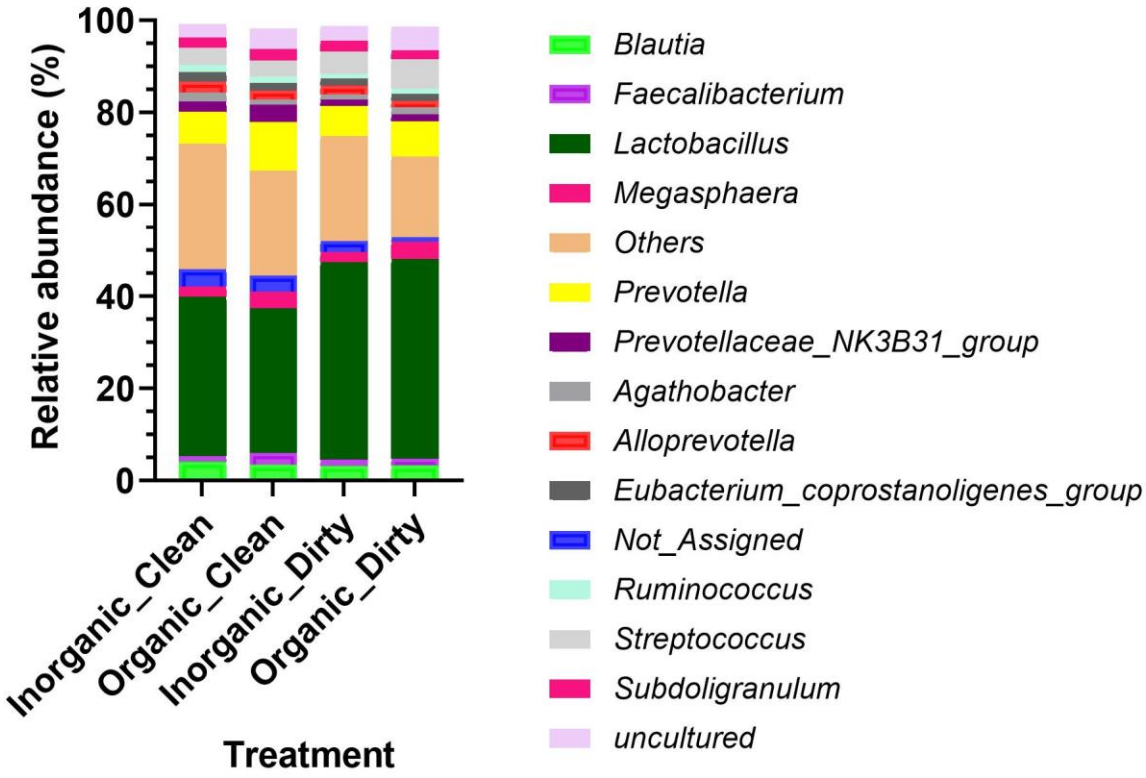
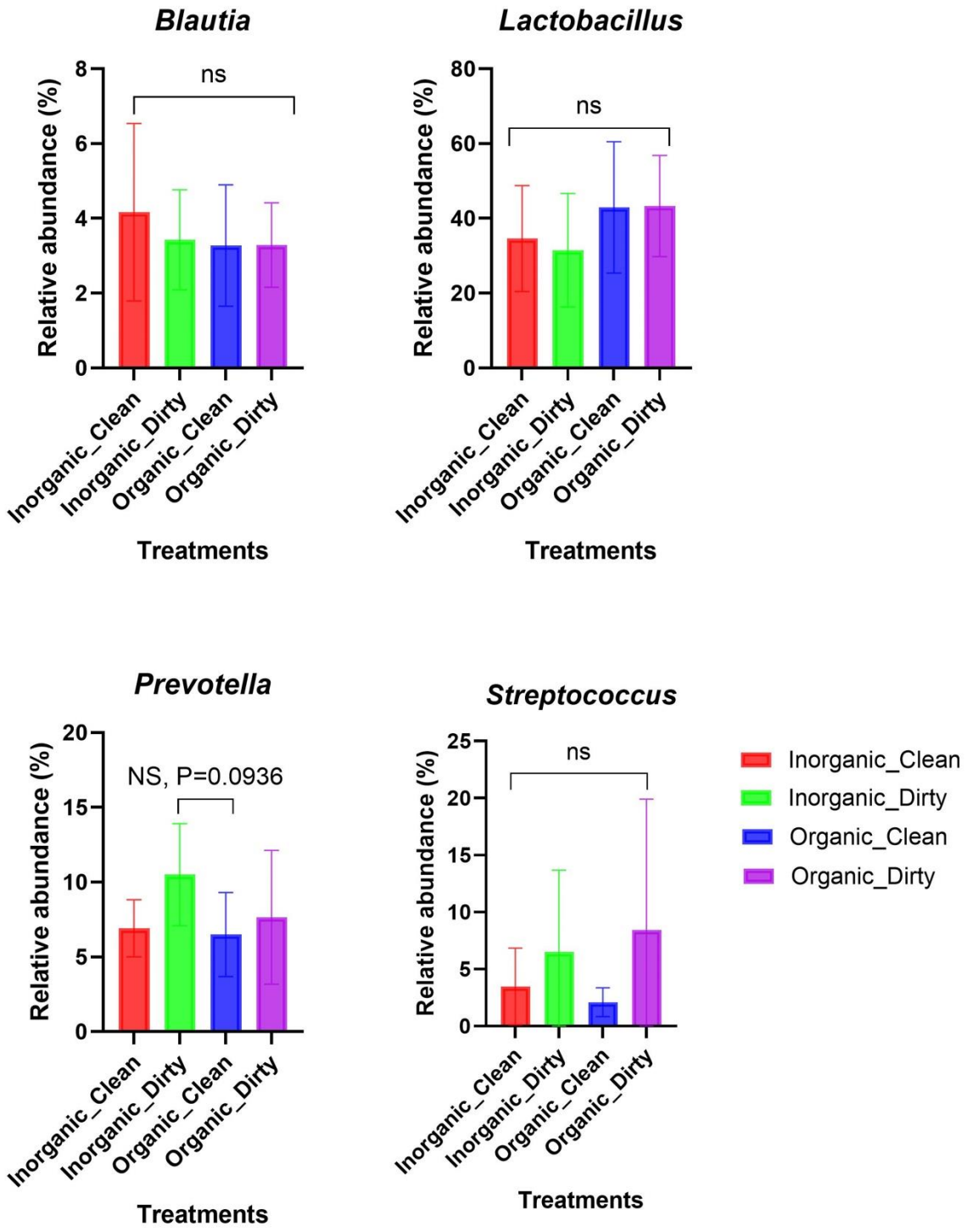


Figure 4. 6 Bar plots showing the relative abundance of bacterial genera in the colon digesta of d28 weaned piglets fed either ferrous sulfate or SQM® iron and reared under clean or dirty conditions



[a, b, c] Means without a common superscript or * are different ($P < 0.05$).

Table 4. 13 Statistics, *P* values and FDR adjusted *P* values for iron sources and sanitation conditions on microbiota at the phylum, family and genus level

Taxonomic level	**Taxonomy**	***P* values**	**FDR[3]**	**Statistics**
Phylum	Firmicutes	0.022823	0.16077	9.5483
Phylum	Bacteroidota	0.026795	0.16077	9.196
Phylum	Euryarchaeota	0.073944	0.26788	6.9367
Family	Clostridia_UCG__014	0.003646	0.13225	13.515
Family	Sutterellaceae	0.00575	0.13225	12.538
Family	Prevotellaceae	0.020308	0.31139	9.804
Family	Lachnospiraceae	0.044001	0.37794	8.0994
Family	Ruminococcaceae	0.047503	0.37794	7.929
Family	Christensenellaceae	0.057904	0.37794	7.4866
Family	Peptostreptococcaceae	0.061162	0.37794	7.3638
Family	Selenomonadaceae	0.07002	0.37794	7.0597
Genus	*Clostridia_UCG__014*	0.003646	0.20508	13.515
Genus	*Acidaminococcus*	0.004565	0.20508	13.034
Genus	*Sutterella*	0.00575	0.20508	12.538
Genus	*NK4A214_group*	0.018003	0.39877	10.067
Genus	*Ruminococcus_gauvreauii_group*	0.018898	0.39877	9.9613
Genus	*Shuttleworthia*	0.022361	0.39877	9.5931
Genus	*uncultured*	0.038766	0.42236	8.3807
Genus	*Lachnospiraceae_NK4A136_group*	0.03893	0.42236	8.3713
Genus	*Terrisporobacter*	0.039473	0.42236	8.3406
Genus	*Faecalibacterium*	0.046961	0.45680	7.9545
Genus	*Lachnospiraceae_ND3007_group*	0.055762	0.47659	7.571
Genus	*Christensenellaceae_R__7_group*	0.057904	0.47659	7.4866
Genus	*Prevotella*	0.076662	0.50174	6.8551
Genus	*Methanosphaera*	0.079037	0.50174	6.7861

[1]Mann-Whitney/Kruskal-Wallis method

[2]Only significant taxa are presented ($P < 0.05$) or taxa with a trend for significance ($0.05 \leq P < 0.1$).
[3]False discovery rate P value

4.4.7.2 Alpha and beta diversity

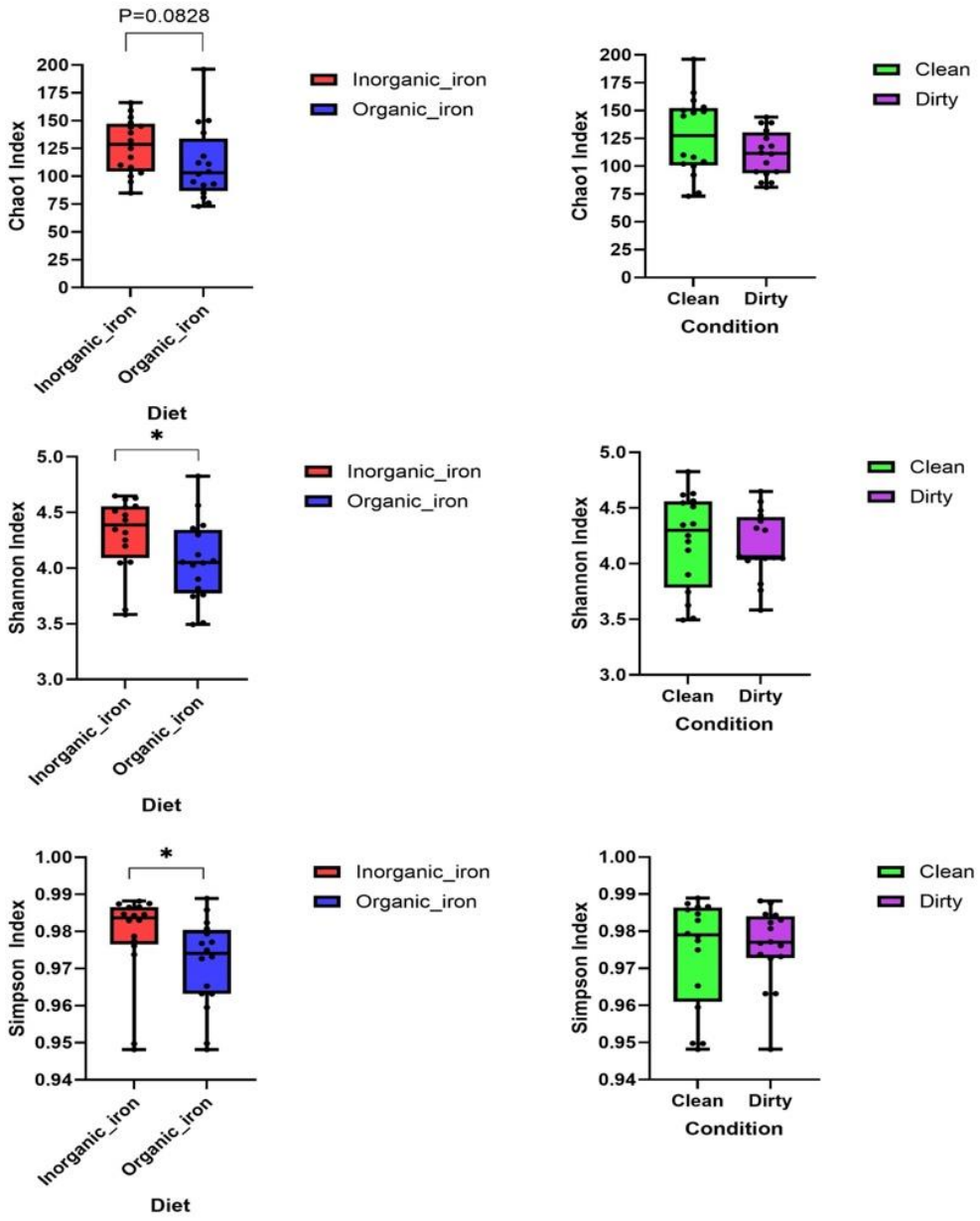
Alpha diversity results, including, Chao1, Shannon and Simpson indexes are summarized in Figure 4.7A and 4.7B. There was no effect of sanitary conditions on the alpha diversity indexes studied. On the other hand, Shannon and Simpson indexes were significantly higher in pigs fed ferrous sulfate than SQM®iron. Chao1 was not significantly different between the two diets but there was a trend for a higher Chao1 with the ferrous sulfate source than with the SQM® iron ($P = 0.0828$, Figure 4.7A). When looking into the pairwise comparisons between treatments, the three alpha diversity indexes were significantly higher for the inorganic_clean group compared with the organic_dirty group ($P < 0.05$, Figure 4.7B), however, this difference was not significant when FDR adjusted P values were considered (Table 4.14).

Beta diversity PcoA plots are presented in Figure 4.8, including, Bray Curtis dissimilarity plot (Figure 4.8A), The Unweighted Unifrac distances plot (Figure 4.8B), and the Weighted Unifrac distances plot (Figure 4.8C). According to Bray Curtis, there were no significant beta diversity differences among treatments (PERMANOVA, $P > 0.05$ and ANOSIN, $P > 0.05$, Table 4.15). There were, however, significant treatment effects on the beta diversity based on Unweighted (PERMANOVA, $P < 0.05$, ANOSIM, $P < 0.05$) and weighted Unifrac distances (PERMANOVA, $P < 0.05$, ANOSIM, $P < 0.05$, Table 4.15). There was a significant effect of sanitary condition on beta diversity based on Unweighted ($P < 0.05$) and a significant effects of iron source based on Weighted Unifrac distances ($P < 0.05$). Beta diversity significantly differed

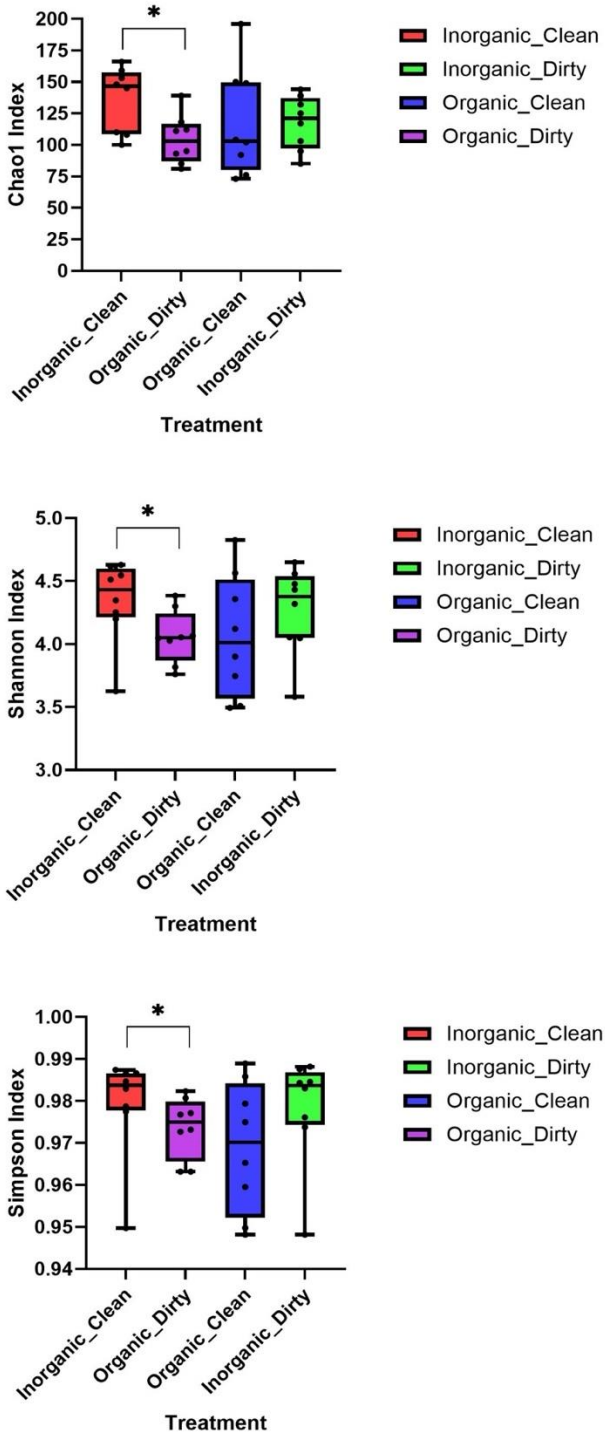
between organic_clean vs. organic dirty, organic_clean vs. inorgnic_dirty and inorganic_clean vs. organic_dirty.

Figure 4. 7 Alpha diversity as indicated by Chao1, Shannon and Simpson index in d28 weaned piglet's colon digesta fed either ferrous sulfate or SQM® iron and reared under clean or dirty conditions

(A)



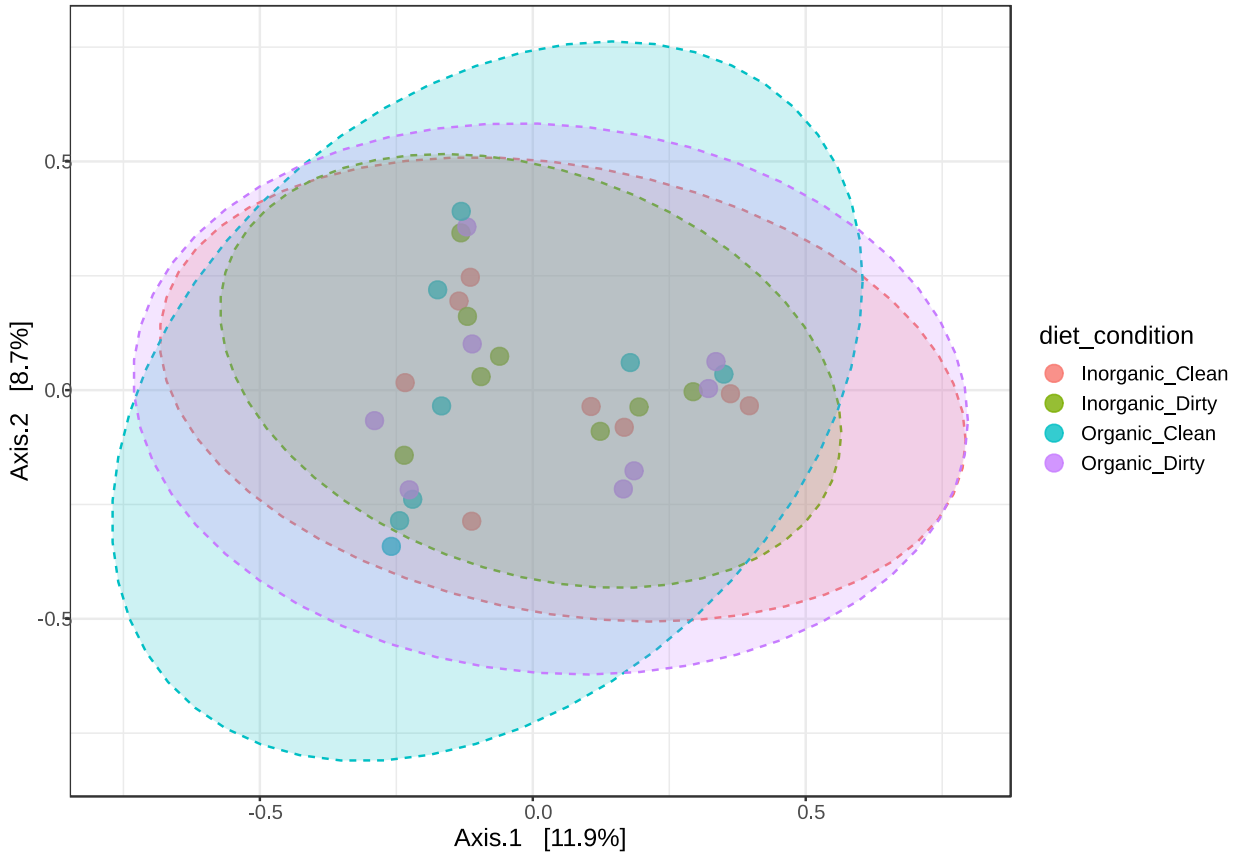
(B)



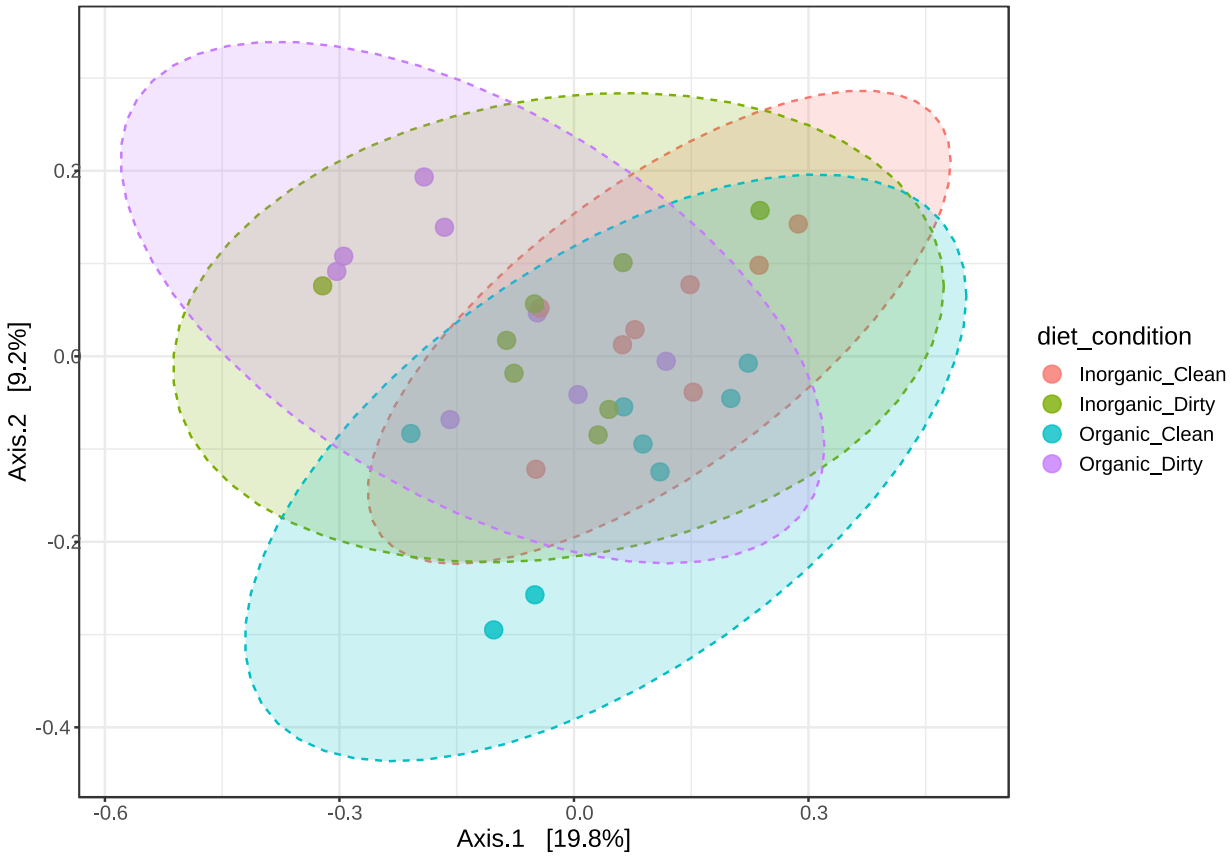
(A) iron source × sanitary conditions (B) Pairwise comparisons of alpha diversity indexes.
[a, b, c] Means without common superscript or * are different ($P < 0.05$).

Figure 4. 8 Principal coordinate analysis (PCoA) plot of beta diversity of microbiota in d28 weaned piglet's colon digesta based on Bray-Curtis dissimilarities

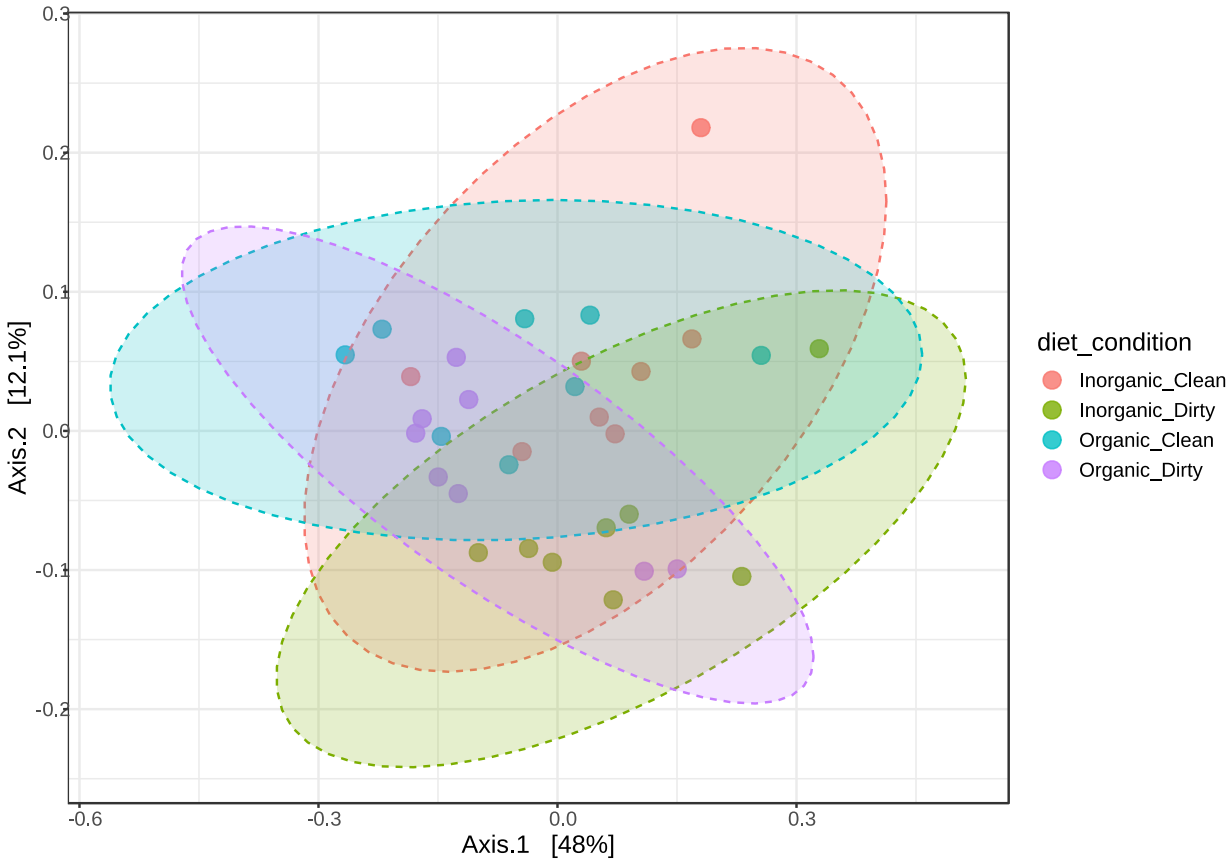
(A)



(B)


0.2
0.0
-0.2
-0.4
Axis.2 [9.2%]
-0.6
-0.3
0.0
0.3
Axis.1 [19.8%]
diet_condition
Inorganic_Clean
Inorganic_Dirty
Organic_Clean
Organic_Dirty

(C)



(A) Unweighted Unifrac distances (B) Weighted Unifrac distances (C) Axes represent the two dimensions explaining the greatest proportion of variances in the microbial communities for each analysis.

Table 4. 14 Statistics, *P* values and FDR adjusted *P* values for iron source and sanitation conditions on alpha diversity indexes Chao1, Shannon and Simpson indexes

Index	Treatment	F	*P* value	FDR
Chao1	diet	174.5	0.082888	0.082888
	condition	164	0.18079	0.18079
	Organic_Clean vs Inorganic_Clean	20	0.2345	0.35175
	Organic_Clean vs Organic_Dirty	34	0.87848	0.87848
	Organic_Clean vs Inorganic_Dirty	30	0.87848	0.87848
	Inorganic_Clean vs Organic_Dirty	52	0.037918	0.22751
	Inorganic_Clean vs Inorganic_Dirty	48	0.1049	0.31469
	Organic_Dirty vs Inorganic_Dirty	19.5	0.20657	0.35175
Shannon	diet	181	0.046815	0.046815
	condition	143	0.58958	0.58958
	Organic_Clean vs Inorganic_Clean	21	0.27863	0.55726
	Organic_Clean vs Organic_Dirty	31	0.95913	0.95913
	Organic_Clean vs Inorganic_Dirty	24	0.4418	0.6627
	Inorganic_Clean vs Organic_Dirty	51	0.049883	0.2993
	Inorganic_Clean vs Inorganic_Dirty	37	0.64538	0.77445
	Organic_Dirty vs Inorganic_Dirty	17	0.13038	0.39114
Simpson	diet	185	0.031842	0.031842
	condition	137	0.75205	0.75205
	Organic_Clean vs Inorganic_Clean	20	0.2345	0.469
	Organic_Clean vs Organic_Dirty	28	0.7209	0.86508
	Organic_Clean vs Inorganic_Dirty	23	0.38228	0.57343
	Inorganic_Clean vs Organic_Dirty	52	0.037918	0.22751
	Inorganic_Clean vs Inorganic_Dirty	34	0.87848	0.87848
	Organic_Dirty vs Inorganic_Dirty	16	0.1049	0.31469

FDR = False discovery rate adjusted *P* value

Table 4. 15 Statistics, *P* values and FDR adjusted P values of PERMANOVA and ANOSIM analysis of beta diversity indexes according to Bray Curtis dissimilarities, Unweighted Unifrac distances (UWUniFrac)and Weighted Unifrac distances (WUniFrac)

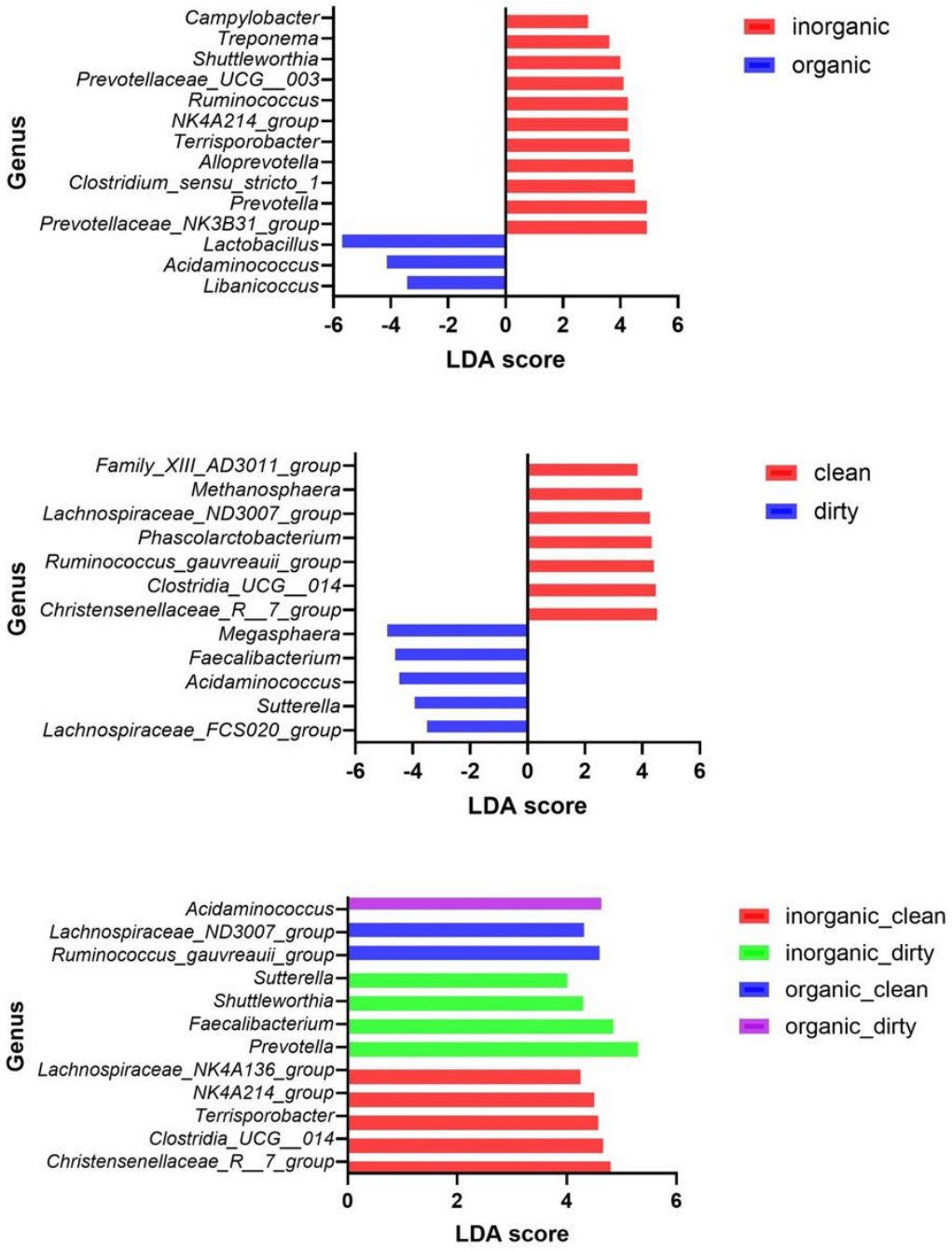
Test	Index	Pairwise PERMANOVA	F value	R square	*P* value	FDR
	Bray Curtis	diet	1.075	0.035	0.283	0.283
PERMANOVA	0.519	condition	0.864	0.028	0.755	0.755
ANOSIN	<0.666	Organic_Clean vs Inorganic_Clean	1.045	0.069	0.363	0.565
		Organic_Clean vs Organic_Dirty	0.974	0.065	0.451	0.565
		Organic_Clean vs Inorganic_Dirty	0.957	0.064	0.553	0.565
		Inorganic_Clean vs Organic_Dirty	0.982	0.066	0.457	0.565
		Inorganic_Clean vs Inorganic_Dirty	0.923	0.062	0.565	0.565
		Organic_Dirty vs Inorganic_Dirty	1.054	0.070	0.321	0.565
	UWUniFrac	diet	1.264	0.040	0.157	0.157
PERMANOVA	0.003	condition	2.660	0.081	0.005	0.005
ANOSIN	<0.001	Organic_Clean vs Inorganic_Clean	1.378	0.090	0.097	0.116
		Organic_Clean vs Organic_Dirty	2.402	0.146	0.010	0.030
		Organic_Clean vs Inorganic_Dirty	1.541	0.099	0.028	0.056
		Inorganic_Clean vs Organic_Dirty	2.556	0.154	0.006	0.030
		Inorganic_Clean vs Inorganic_Dirty	1.498	0.097	0.044	0.066
		Organic_Dirty vs Inorganic_Dirty	1.130	0.075	0.310	0.310
	WUniFrac	diet	3.455	0.103	0.011	0.011
PERMANOVA	0.03	condition	1.957	0.061	0.098	0.098
ANOSIN	<0.012	Organic_Clean vs Inorganic_Clean	1.394	0.091	0.217	0.260
		Organic_Clean vs Organic_Dirty	0.940	0.063	0.377	0.377
		Organic_Clean vs Inorganic_Dirty	2.894	0.171	0.042	0.084
		Inorganic_Clean vs Organic_Dirty	2.760	0.165	0.027	0.084
		Inorganic_Clean vs Inorganic_Dirty	2.021	0.126	0.060	0.090
		Organic_Dirty vs Inorganic_Dirty	3.084	0.181	0.037	0.084

FDR = False discovery rate adjusted *P* value

4.4.7.3 Differential analysis

To identify differential features, in particular, bacterial taxa that are differentially abundant between treatments, Linear discriminant analysis Effect Size (LEfSe) was performed (Figure 4.9). LDA score of 2 or -2 and FDR adjusted P-value cutoff of 0.1 were chosen. Under these criteria, there were no diet or diet x condition differences. There was however, two genera that were distinct for the pigs raised under dirty conditions, i.e., *Sutterella* ($P < 0.05$) and *Acidaninococcus* ($P = 0.0913$), while *Clostridia_UGC_014* was associated with pigs raised in a clean environment ($P = 0.0643$) (Figure 4.9, Appendix 1). Another differential analysis method was also used, i.e., DESeq2 (Figure 4.10 A-F, Appendix 2). Similarly, to the LEfSe results, there were no diet differences. On the other hand, sanitary conditions had significant effects when considering the FDR-adjusted P values. For example, *Anaerostipa* ($P <0.0001$, Figure 4.10A) and *Sutterella* like in the LEfSe analysis ($P < 0.0001$) were significantly associated with the clean and dirty environment, respectively. Other significant differential genera, based on FDR adjusted P-value, included, *NK4A214* (inorganic_clean, Figure 4.10C), *Ruminococcus* (organic_clean, Figure 4.10D), *Shutleworthia* (inorganic_dirty, Figure 4.10E) and similarly to LEfSe analysis, *Acidaninococcus* (organic_dirty, Figure 4.10F).

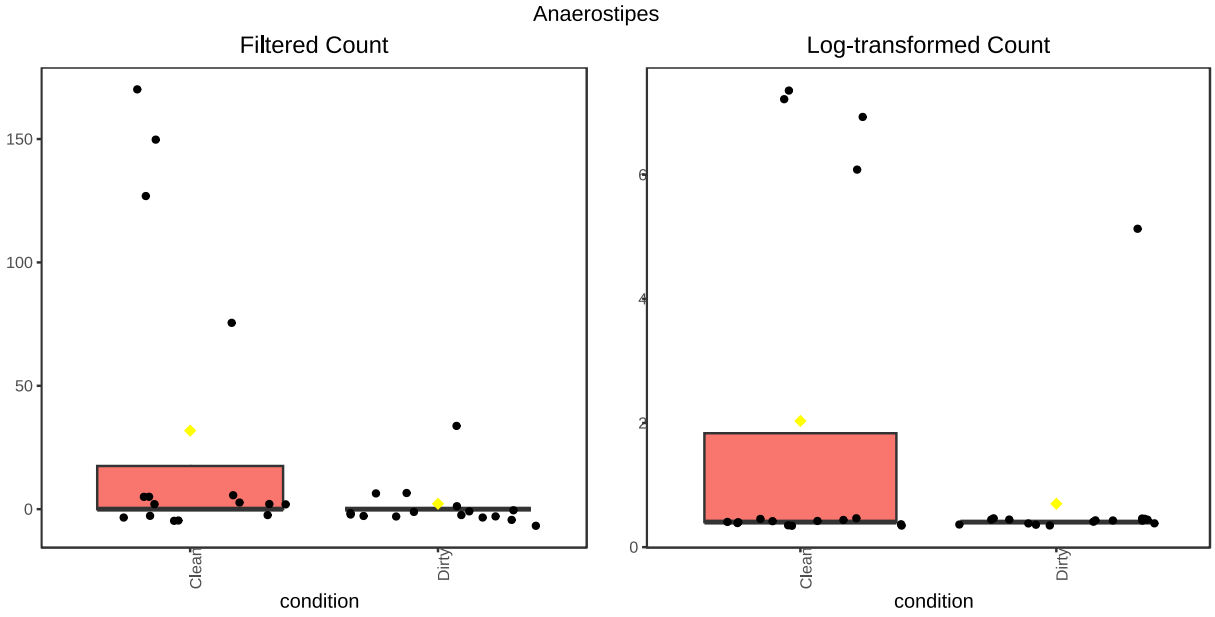
Figure 4. 9 Histogram of a linear discriminant analysis (LDA) score (-2< threshold >2) in colon digesta of d28 weaned piglets reared fed with ferrous sulfate or SQM® iron and under clean or dirty conditions



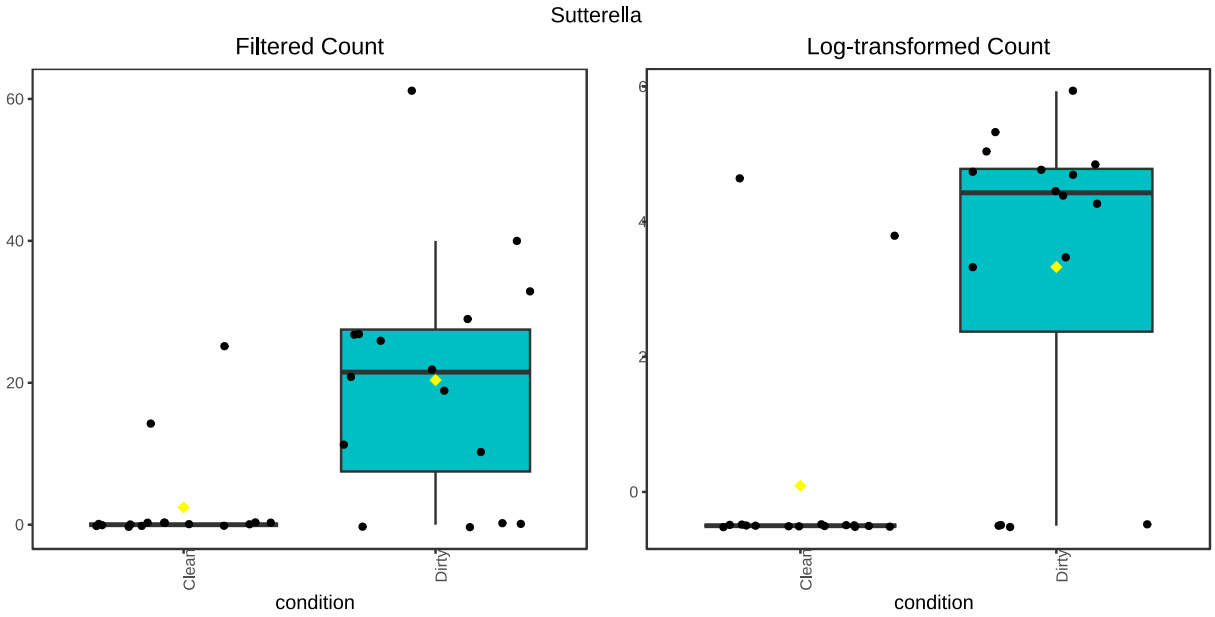
(Top) diets (middle) sanitary conditions (bottom) diets × sanitary conditions

Figure 4. 10 Box plots of differentially abundant genera in colon digesta of d28 weaned piglets fed ferrous sulfate and SQM® iron sources and reared under clean or dirty conditions (A-F)

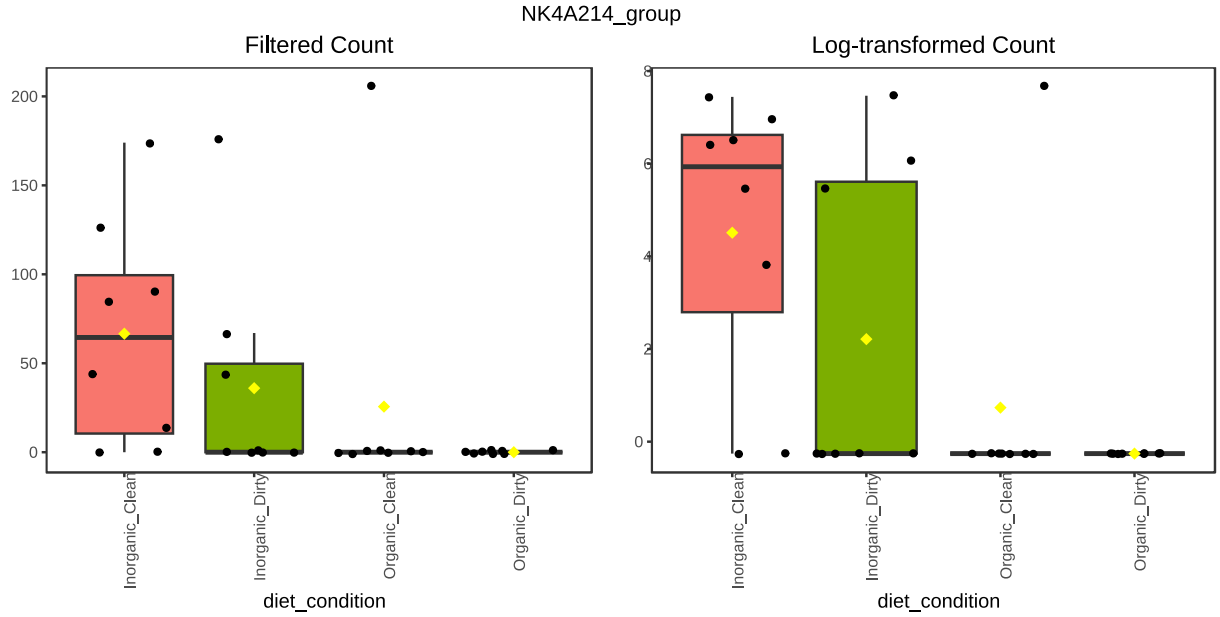
(A)



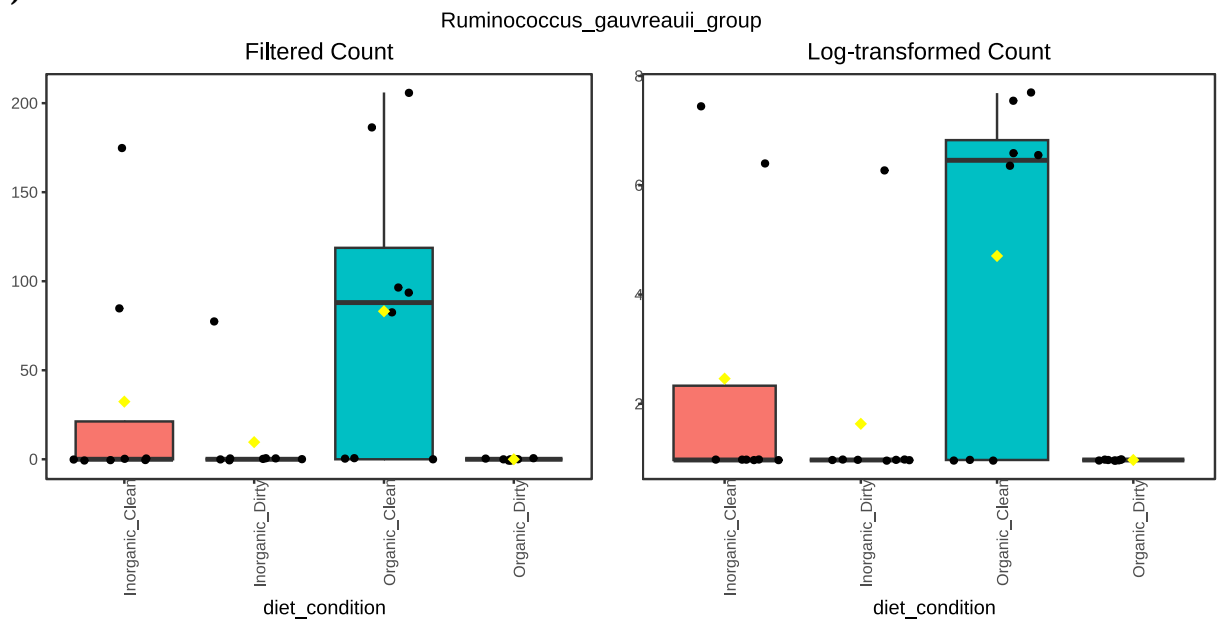
(B)



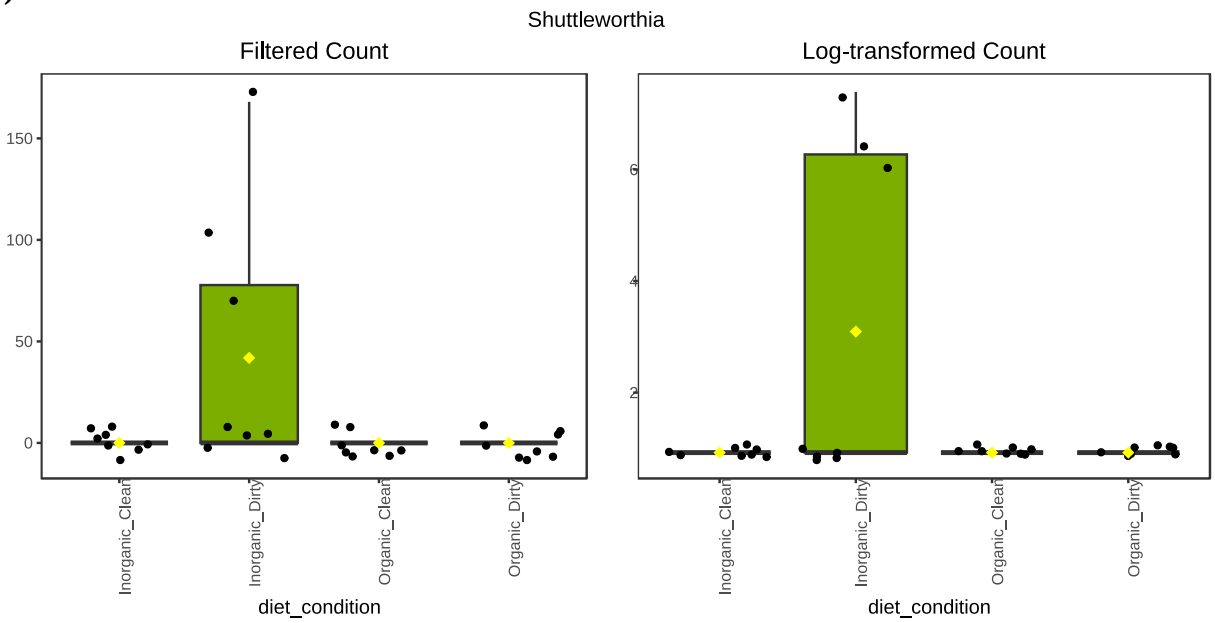
(C)


NK4A214_group
Filtered Count
Log-transformed Count
200
150
100
50
0
8
6
4
2
0
Inorganic_Clean
Inorganic_Dirty
Organic_Clean
Organic_Dirty
diet_condition

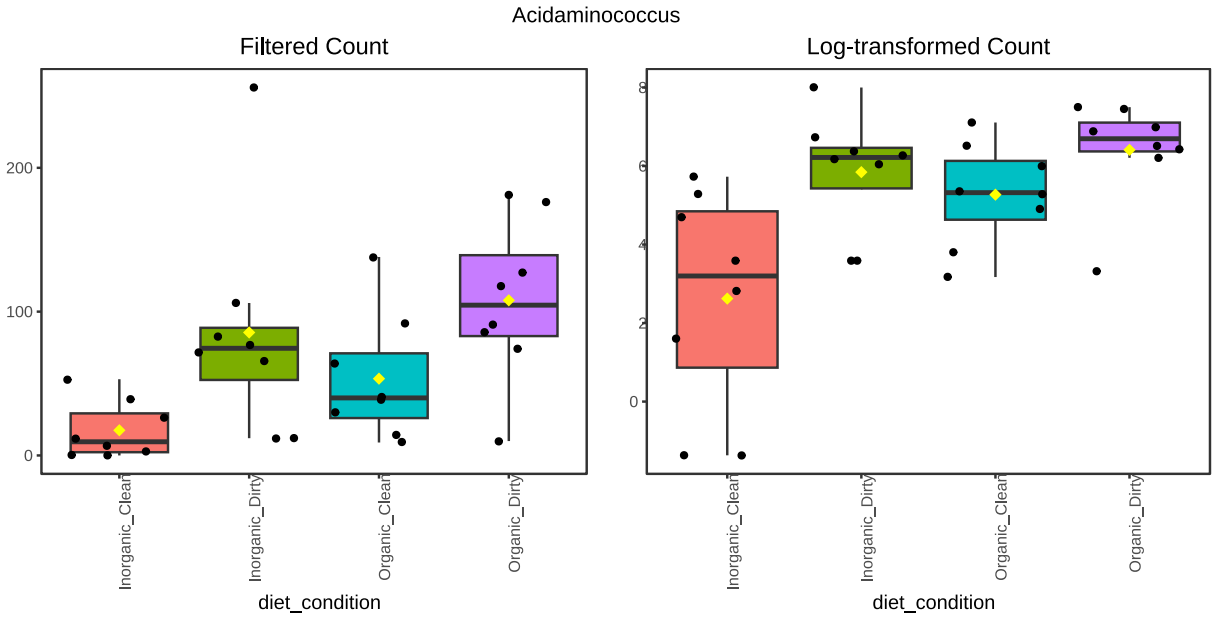
(D)


Ruminococcus_gauvreauii_group
Filtered Count
Log-transformed Count
200
150
100
50
0
8
6
4
2
Inorganic_Clean
Inorganic_Dirty
Organic_Clean
Organic_Dirty
diet_condition

(E)


Shuttleworthia
Filtered Count
Log-transformed Count
150
100
50
0
6
4
2
Inorganic_Clean
Inorganic_Dirty
Organic_Clean
Organic_Dirty
diet_condition
diet_condition

(F)

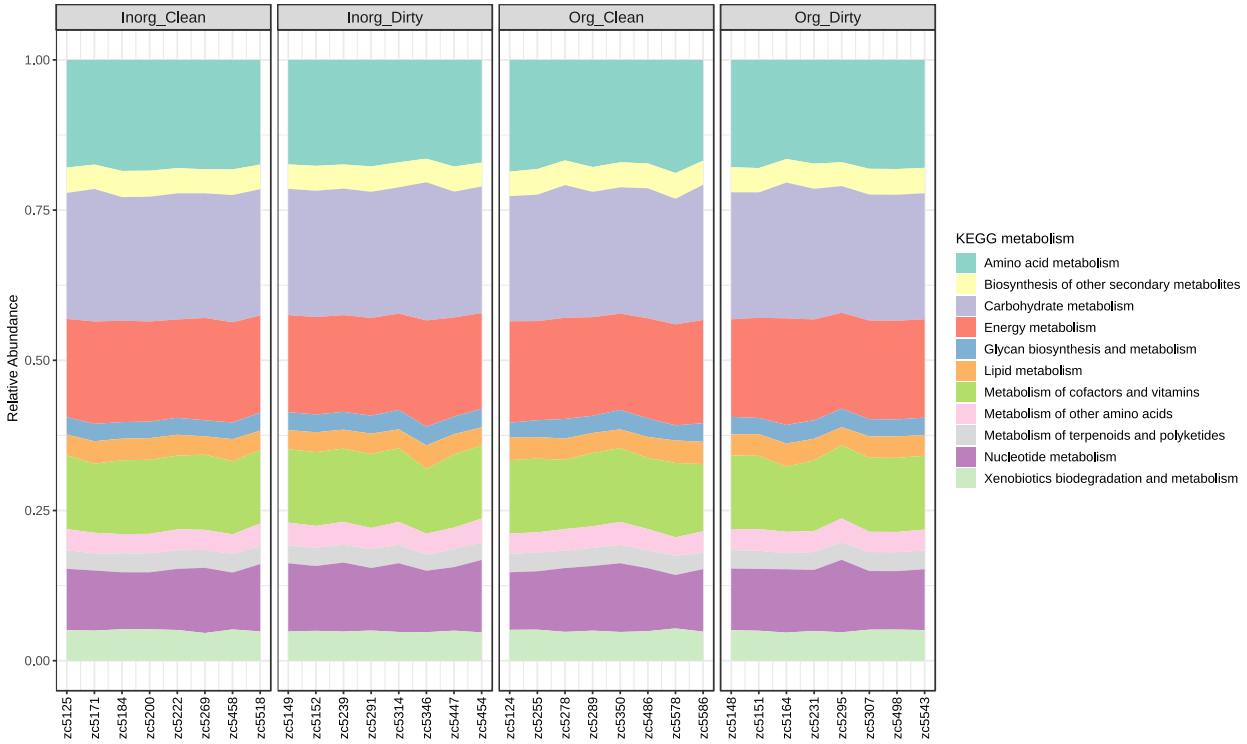

Acidaminococcus
Filtered Count
Log-transformed Count
200
100
0
8
6
4
2
0
Inorganic_Clean
Inorganic_Dirty
Organic_Clean
Organic_Dirty
diet_condition
diet_condition

4.4.7.4 Functional Analysis

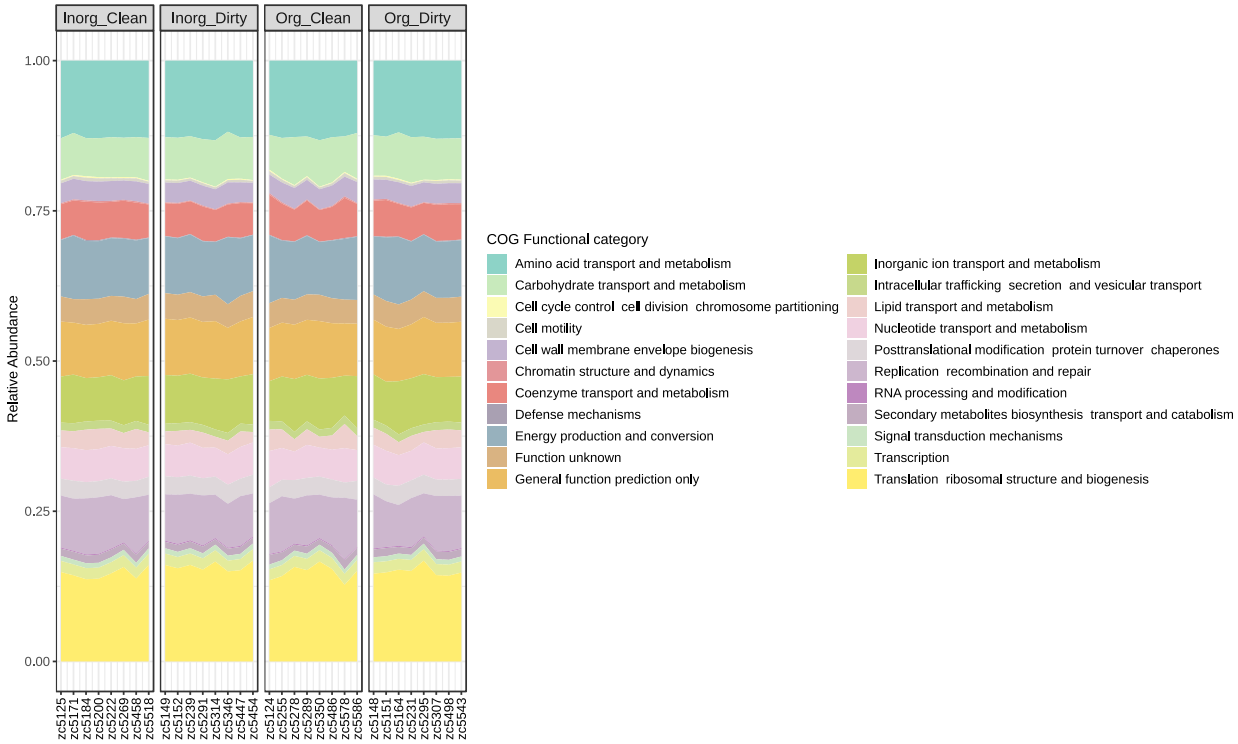
The R package, Tax4FUN was used to evaluate the functional profiles of the pig colon microbiota for the pigs fed either inorganic or SQM® iron sources and raised under clean or dirty conditions. The results indicated that 11 pathways, including carbohydrate, amino acids, and energy metabolism were not different between the 4 pig groups (Figure 4.11A) apart from some metabolism differences between inorganic_clean and inorganic_dirty groups. Amino acid and carbohydrate transport and metabolism were the two most dominant Clusters of Orthologous Groups (COG) functional categories for all pig groups (Figure 4.11B). Coenzyme and lipid transport and metabolism as well as replication recombination and repair were significantly higher in the inorganic_clean group compared to the inorganic_dirty group (Figure 4.12). On the other hand, inorganic ion transport and metabolism, translation ribosomal structure and biogenesis, and carbohydrate transport and metabolism were higher in the inorganic_dirty group compared to the inorganic_clean group.

Figure 4. 11 Predicted functionality of colon digesta microbiota of pigs fed ferrous sulfate or SQM® iron sources and reared under clean or dirty conditions

(A)

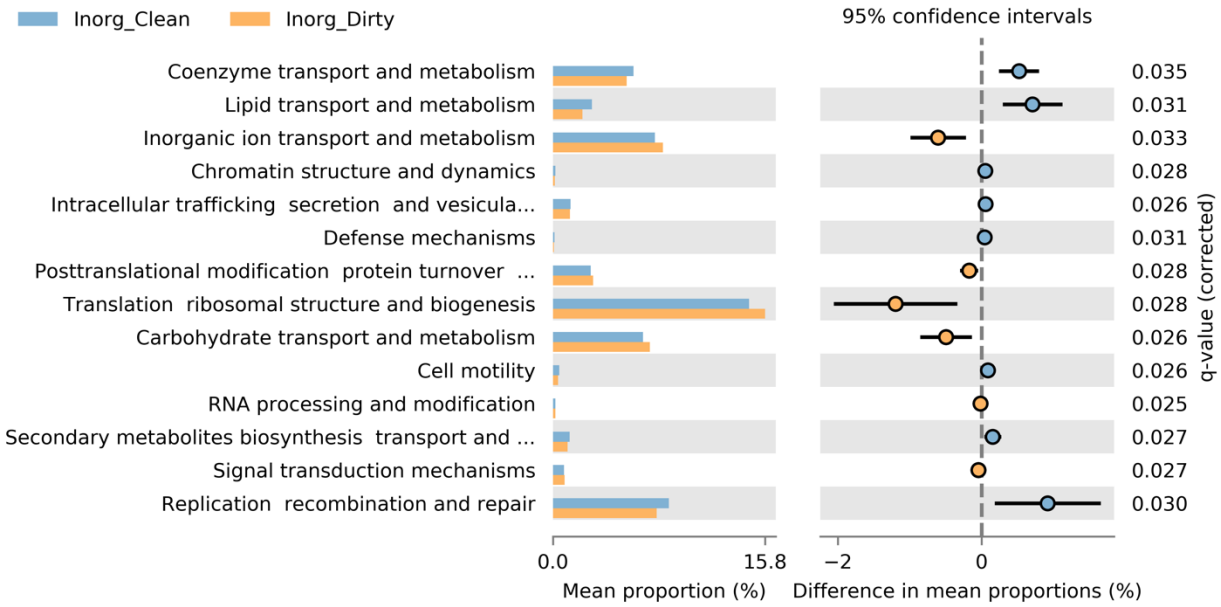


(B)



(A) KEGG metabolism (B) COG functional pathways.

Figure 4. 12 Predicted functionality of colon digesta microbiota of d28 weaned piglets related to iron source and sanitary conditions in terms COG functional pathways



Blue and orange represent the post weaning group fed ferrous sulfate and raised under clean or dirty conditions, respectively. The samples are represented on the X-axis and separated based on metadata (ferrous sulfate at two sanitary conditions). The relative abundances of the various metabolism categories across all the samples are represented on the Y-axis. Q-values are FDR adjusted *P* values. All the other treatments were not significant (FDR adjusted $P > 0.05$, figures not shown).

4.5 Discussion

4.5.1 Growth performance and relative organ weight

We hypothesized that SQM® iron and clean sanitary conditions would result in higher growth performance in weaned piglets. SQM® iron treatment had a strong trend of higher ADFI ($P = 0.062$), and a significantly higher ADG ($P < 0.05$) in the feeding stage I (d0 to d14). However, the grow promoting effect of SQM® iron decreased from d14 to d28.

In our results, clean sanitary conditions were associated with higher ADG and improved FCR than dirty conditions. Previous studies also found that poor sanitation conditions decreased feed intake by 10 % and decreased growth performance by as much as 20% and the decrease in performance was due to immune system stimulation from exposure to stressors imposed by uncleaned housing conditions, which affected animal performance (Adewole et al., 2016; Le floc'h et al., 2014). Visceral organs represent a large portion of the animal host's energy spending as these organs are associated with metabolically expensive processes, for example, protein synthesis and degradation (Nobel et al., 1999). Lacking a difference in relative organ weight among the treatments suggested that animals were anatomically equipped with similar metabolic rates regardless of sources of iron supplements or sanitary conditions.

4.5.2 Fecal iron excretion and fecal scores

In our study, sanitary conditions did not affect the iron absorption as evident in the fecal iron concentration on the dry matter basis. Furthermore, fecal iron contents were similar between the two iron sources on d14 suggesting that iron absorption by the pigs was similar. However, on d21, SQM® iron was associated with a higher fecal iron content suggesting a lower absorption

from d14 to d21. Polysaccharide iron complexes were associated with higher bioavailability (Jing et al., 2022). However, many human studies found that inorganic iron sources, compared to polysaccharide iron complexes, resulted in greater increase in hemoglobin concentration in host (Moe et al., 2019; Powers et al., 2017). Despite higher fecal iron content on d21 among SQM® iron treatment groups, no signs of iron deficiency anemia nor associated symptoms were observed among the pigs. Iron deficient pigs were found to have smaller liver relative organ weight (Knight et al., 2018), which were not observed in our study. This could be explained by higher analyzed feed iron level for both diets at both feeding stages over the baseline level recommended for the weaned piglets (Williams et al., 2020).

Regarding the fecal score, a significantly reduced fecal score was obtained on d27 based on the numeric values in SQM® iron treatment groups. However, the improvement in fecal score, when taking into the consideration the rating scheme, was deemed limited. A previous study on weaned piglets linked poor sanitary conditions to higher fecal scores (Pastorelli, 2012). In our study, sanitary conditions had no effect on the fecal consistency throughout the experiment and iron sources influenced the fecal scores only on d27. The numeric values that were used to analyze fecal scores were significant, but the difference in the numeric values between treatments was less than one. Since fecal scores were rated based on a scale of 0 to 3, the improvement in fecal conditions under SQM® iron treatment was not drastic.

4.5.3 Gut integrity and permeability

In the measures of guy integrity and permeability, there was a trend that associates sanitation conditions to some of the measurements while iron sources had no effect.

We measured the gut permeability and integrity through analyzing jejunum transepithelial electrical resistance (TEER), which measured electric resistance of epithelial cells. A higher TEER value indicates lower gut permeability; We also analyzed FICT florescence, which is a measure of jejunum paracellular flow. A higher FITC value indicates higher jejunum epithelial permeability. Functioning tight junction proteins build strong gut barrier functions and integrity (Assimakopoulos, 2011). A previous study on iron glycine chelates, another organic iron source, found increased abundance of tight junction protein ZO-1; however, the gene expression of transmembrane proteins such as claudin-1 and cadherin which determine junctional permeability was not different among treatments (Sun et al., 2022; Zhao et al., 2021). Our study showed that dietary source and sanitation conditions alone had no effect on TEER and FITC florescence values. However, there was an interaction effect that clean sanitary conditions resulted in higher TEER values than dirty sanitary conditions with the SQM® iron treatment.

As a cytoplasmic enzyme found mostly in the villus epithelial cells in the small intestine, serum DAO has inverse relationship with small intestine permeability. Higher intestinal mucosal barrier damage due to increased production of inflammatory cytokines was associated with higher DAO level, which catalyzes histamine oxidation (Shi et al., 2022). Neither iron sources nor sanitation conditions in our study influenced the serum concentration of DAO. D-lactate, derived from microbial fermentation in the gut can be significantly elevated in the blood in response to infection, ischemia, and trauma. Higher elevation of serum d-lactate is likely due to impaired intestinal barrier function (Ewaschuk et al., 2005). Iron sources had no effect on d-lactate which was consistent with findings in a previous study comparing effects of organic iron and ferrous sulfate on weaned piglets (Sun et al., 2022). There was a strong tendency for a higher d-lactate in serum of pigs raised under dirty condition in our study, which was indicative of a decreased

intestinal barrier permeability ($P = 0.051$). Unclean sanitary conditions have been used as a model to initiate a low-grade inflammatory response in pigs as it stimulated immune response from stressors (Adewole et al., 2016).

The effects of iron sources and sanitary conditions on the relative gene expression of Acetate-coA butyrate-CoA transferase was examined. Butyrate is essential for maintaining the structure of the large and small intestine and is the main energy source for gut colonocytes (Hamer et al., 2008). Acetate-coA butyrate-CoA transferase is an enzyme involved in the formation of butyrate from butyryl CoA and acetate in butyrate producing bacteria (Sato et al., 2016). Thus, gut butyrate production has implication on gastrointestinal health. Piglets fed with the ferrous sulfate had higher relative gene expression of acetate-CoA butyrate-CoA transferase than the piglets fed with the SQM® iron for the time periods d0-d14 and d0-d21. A higher gene expression of this enzyme may indicate higher presence of butyrate producing bacteria from ferrous sulfate treatment. This could be explained as many of the butyrate producing bacteria has iron requirement, and iron restrictive effect SQM® iron in colon would have resulted in lowered bacterial access to iron. In return, this may have resulted in lower butyrate production. In addition, the linear discriminant analysis (LDA) on the microbiota analysis from the d28 colon digesta supported this argument with presence of higher relative abundance of butyrate producing bacteria *Lachnospiraceae_NK4A36_group*, among the ferrous sulfate treatment groups.

The effects of iron sources and sanitary conditions on the relative gene expression of bisulfite reductase were also investigated. Bisulfite reductase catalyses the reduction of sulfite to hydrogen sulfide in specific microbial populations involved in the sulfur metabolism. Hydrogen sulfide can be either toxic or exhibit anti-inflammatory proprieties in the gut depending on the amounts being produced (Teigen et al., 2023, Buret et al., 2022). At lower concentration, hydrogen

sulfide can prevent the adherence of microbiota biofilms to the epithelium, inhibiting invasive pathogenic bacteria and protecting mucus layers. At higher concentration, it disrupts mucus production leading to increased gut inflammation (Buret al., 2022). Excess hydrogen sulfite also inhibits butyrate oxidation, a main fuel source of colonic epithelial cells, thus compromising gut barrier function and increasing gut inflammation (Babidge et al., 1998; Fagundes et al., 2024; Singh et al., 2015). Therefore, quantifying relative gene expression of bisulfite reductase can be indicative of gut health status. There was a significant effect of iron sources, sanitary conditions and interaction on bisulfite reductase when comparing the change of gene expression from d21 to d0. Ferrous sulfate treatment resulted in higher relative gene expression of bisulfite reductase than SQM® treatment under clean conditions ($P < 0.05$). The reason why clean conditions resulted in higher relative gene expression of bisulfite reductase than dirty conditions remain unknown. The colon digesta analysis of inorganic_clean (ferrous sulfate treatment in clean condition) through the linear discriminant analysis revealed that *Terrisporobacter* on the genus level was associated with an impactful LDA score (LDA > 4). Genome-based metabolic and phylogenomic analysis of certain *Terrisporobacter* species showed sulfur reducing potential (Böer et al., 2023).

4.5.4 Nutrient absorption capacity and gut health

The integrity of intestinal epithelium is closely related to the digestion and absorption of nutrients (Sun et al., 2022). Nutritional changes during weaning are often associated with changes in gut physiology and expression patterns of digestive enzymes and nutrient transporters (Wellington et al., 2021). To evaluate the effects of differing iron sources and sanitary conditions on nutrient absorption capacities and epithelial barrier function, the relative gene expression of various nutrient transporters was considered (Table 4.9). Also, to gain further insight into iron

homeostasis in the pigs fed the two different iron sources and raised under either clean or dirty conditions, the relative gene expression of divalent metal transporter 1 (DMT-1) and Ferroportin-1 were measured (Table 4.9). Proliferating Cell Nuclear Antigen (PCNA) is essential for nucleic acids metabolism as it takes park in DNA repair and replication. The relative gene expression of PCNA for piglets fed with inorganic or SQM® iron and raised under clean or dirty conditions was also compared. In our study, there were no effects of either iron sources or sanitary conditions on these transporters except for a tendency for a higher expression of PepT-1 on pigs fed the SQM® iron compared to the pigs fed the ferrous sulfate ($P = 0.088$) and a trend of higher DMT-1 under clean conditions ($P = 0.093$).

Sun et al. (2022) found that iron glycine chelates not only elevated the expression levels for the cationic amino acid transporter 1 (CAT-1) in the duodenum and jejunum but also elevated the expression levels of glucose transporter-2 (GLUT2) and sodium/glucose co-tranporter-1 (SGLT-1) in the ileum. In our study, however, no effect of SQM® iron was observed on the nutrient transporters. Differences in the effect of organic iron on the nutrient transporters may be due to the sources of organic iron structure which can vary significantly and exhibits distinct physiochemical properties.

4.5.5 Relative quantification of pathogenic bacteria

We hypothesized that SQM® iron would potentially limit iron availability in the colon to the pathogenic bacteria. However, the results showed no inhibitory effects of SQM® iron on pathogenic bacteria. This could be explained by the potential interference of free available iron in the feed. Trace mineral supplementation in the diet accounted for 100 mg/kg whereas the analyzed iron level in the feed was around 240 mg/kg for stage one diet (d0 – 14) and 276 mg/kg for stage

two (d14 – 28). Non-heme iron from feedstuff may have released free iron through digestion, which may have lessened the effect of iron restrictive properties of SQM® iron supplement to the pathogenic bacteria. This could also explain the observation on the variations in the fecal consistencies within the SQM® iron treatment pens which showed no improvement compared to ferrous sulfate treatment until d27 where limited improvement in fecal score among of SQM® iron treatment groups was observed.

4.5.6 Gut microbiota composition

Ferrous sulfate has been associated with changes in the gut microbiota including favouring iron requiring pathogenic bacteria (e.g., Pajarillo et al., 2021; Sun et al., 2024) whereas the effects of SQM® iron on the pig colon microbiota had not been investigated. Gut microbiota plays an important role in swine physiology and homeostasis, especially in energy harvest, nutrient digestion, and intestinal health, and is likely to affect growth and feed efficiency (McCormack et al., 2017).

In agreement with other studies, Firmicutes and Bacteroidotes were the most abundant phyla in the colon microbiota of pigs in the current study (Lamendella et al., 2011; Ma et al., 2022; Wang et al., 2019, 2021; Yang et al., 2017). Our study showed that relative abundance of Firmicutes/Bacteroidota ratio was significantly lower in pigs fed ferrous sulfate and raised under dirty conditions than in pigs fed SQM® iron and raised under clean conditions. This was in accordance with study from Ma et al. (2022) who found a higher abundance of Firmicutes when weaned pigs were fed ferrous glycine chelate (an organic iron source) compared to the control pigs supplemented with ferrous sulfate source. Zeng et. (2023) also found that piglets when

supplemented with another organic iron source, i.e., yeast iron, exhibited higher abundance of Firmicutes compared to pigs supplemented with ferrous sulfate.

The most abundant families across all treatments in our study were *Lactobacillaceae*, *Prevotellaceae*, *Lachnopiraceae* and *Ruminococcaceae*. We did not find any treatment effects on the relative abundance of *Lactobacillacea*e or *Ruminococcaceae* except a higher relative abundance of *Prevotellaceae* in ferrous sulfate treatment groups. In our study, *Clostridiaceae* was also higher in pigs fed the SQM® iron but *Lachnospiraceae* and *Spirochaetaceae* were lower than in pigs fed the ferrous sulfate, which is different from the results in a previous study by Ma et al. (2022). These authors reported a significant lower abundance of *Lactobacillaceae*, *Streptococcacea*e and a higher abundance of *Clostridiaceae* in pigs fed with ferrous glycine chelate than control pigs fed iron sulfate for 14 d old pigs. It is possible that the source of iron differences between our study and Ma et al (2022) and the pig's age may be behind different gut microbiota responses.

The most abundant genus across all treatments was *Lactobacillus* (>38%). Other abundant genera were *Prevotella*, *Streptococcus* and *Blautia*. In general, these results are in agreement with other studies with weaned pigs (Chen et al., 2017; Greese et al., 2017; Ke et al., 2019). However, some variations in the microbiota composition among studies exist. Differences in pig's age, breed and genotype may be behind differences in microbiota composition and diversity (Gardiner et al., 2020; Wang et al., 2019). Ma et al (2022) found higher *Clostridium_sensu_stricto_1* and *Blautia* and a lower *Lactobacillus* and *Streptococcus* in pigs fed with ferrous glycine chelate compared to the control pigs fed ferrous sulfate for 14 d pigs. These authors found that *Clostridium_sensu_stricto_1* was also increased in 28 d pigs, but *Blautia* was lower than pigs fed ferrous sulfate. Zeng et al (2023) also found higher abundance of *Blautia* and a lower abundance

of *Prevotella* in piglets fed yeast iron than the control group and no significant differences in the abundance of pathogenic bacteria such as *Escherichia-Shigella* and *Clostrididium*, nor any commensal bacteria such as *Bifidobacterium*, *Roseburia*, and *Ruminococcus* was found between piglets fed yeast iron and control pigs. Again, the sources of organic irons and pigs's ages used may be behind these differences.

Iron sources, in our study, had no effects on the relative abundances of major genera and family when FDR adjusted *P* values were considered. The results of LEfSe analysis in our study revealed that colon digesta of d28 weaned piglets in the SQM® group were enriched with *Lactobacillus* (LDA score = 5.7) and the *P* value was 0.05; however, the adjusted P value (FDR) was not significant (*P* = 0.66). FDR adjusted p values in microbiota data analysis accounts for multiple simultaneous hypothesis testing which increases the likelihood of false positives. FDR adjustment helps control the rate of false discoveries while maintaining statistical power making the results more reliable and should therefore be considered instead of unadjusted P values. (Benjamin and Hochberg, 1995).

Montagne et al. (2010) found that when compared with the clean conditions, the feces of piglets housed in the poor conditions contained significantly more *Lactobacillus* and *Enterobacteria*. Mulder et al. (2009) also found that pigs housed in a natural outdoor environment showed a dominance of Firmicutes, in particular *Lactobacillus*, whereas animals housed in a hygienic indoor environment had reduced *Lactobacillus* and higher numbers of Proteobacteria, including potentially pathogenic phylotypes. Luise et al. (2021) and Schmidt et al. (2011) also reported gut microbiota differences among pigs reared under different farm conditions and different sanitation/ hygiene protocols suggesting that sanitary conditions can modulate the gut microbiota. Dirty sanitary conditions favoured *B. choerinum* while clean conditions favoured a

Clostridium related phylotype, which correlated with cytokine expression in blood and intestine (Le Floc'h., 2014). Contrary to previous studies, sanitary conditions in our study did not affect gut microbiota composition. This could be due to differences in breeds (Wang et al., 2021) or pig's age.

4.5.7 Alpha and beta diversity

At the feature level, there was a trend for iron sources effect (P = 0.0828) on species richness in pigs fed ferrous sulfate under clean conditions than in pigs fed SQM® iron and raised under dirty conditions. Ferrous sulfate treatment resulted in higher Simpson index and higher Shannon index over the SQM® iron treatment ($P < 0.05$). In addition, species richness, dominance and evenness were higher in pigs fed ferrous sulfate and raised in clean conditions than pigs fed SQM® iron and raised in dirty conditions. A higher Shannon index indicates higher alpha diversity whereas a higher Simpson index indicates lower alpha diversity. Opposite trends in response for the Shannon and Simpson index is not contradictory because Simpson index focuses on species dominance whereas Shannon index focuses on richness which accounts for number of species in a population (Dejong, 1975; Nagendra H., 2002). Our results suggested that SQM® iron resulted in a smaller number of bacterial species in the microbiota, but the gut microbiota was more evenly distributed compared to ferrous sulfate treatment.

At the feature level, the beta-diversity measures investigated results depended on the matrix used. When only ASVs counts were considered, i.e., beta-diversity based on Bray-Curtis dissimilarities, there were no difference in iron sources or sanitation conditions treatment in beta diversity, which indicated that microbial communities were not significantly different from each other despite different treatments. This is in accordance with previous study where dietary

supplementation with a ferrous glycine chelate had no significant effect on microbial diversity based on Bray-Curtis among d 28 old-weaned piglets (Ma et al, 2022).

A significant difference in beta diversity was reported when the analysis was based on Unweighted and weighted Unifrac distances. Differences in beta diversity could be due to the factors considered in the analysis. Unweighted Unifrac considered phylogenetic distances between observed bacteria and the presence/absence of ASVs. According to these Unifrac distances, there were significant diversity differences between the two sanitary conditions and no effect of iron sources. When the phylogenetic distances and the abundance of ASVs were considered (Weighted Unifrac), there were diversity differences between the two iron sources treatments.

4.5.8 Differential analysis

To identify differential features, in particular genera that were differentially abundant between treatments two approaches were used, i.e., LEfSe and DESeq2. Differential genus results were dependent on the statistical method used. When considering FDR adjusted P values, LEfSe and DESeq2 showed no effect between iron sources. Ma et al (2022), however, found differential genera associated with the iron sources fed to pigs. In their study, *Olsenella*, *Prevotella* and *Slackia* decreased while *Tezzerella* and *Sutterella* increased in ferrous glycine chelate fed pigs. Differences in findings could be due to differences in breed and iron sources.

In terms of sanitary conditions, LEfSe found two genera enriched for the pigs raised under dirty conditions, i.e., *Sutterella* and *Acidaninococcus* while *Clostridia_UCG-014* was strongly associated with pigs raised in clean conditions. With the DESeq2 method, *Sutterella*, a pathogenic bacterium associated with diarrhea, was also found to be distinct for pigs raising under dirty conditions while *Anaerostipes* was distinct for pigs raised in clean conditions (Lv et al., 2017).

Anaerostipes, is a beneficial bacteria known to be butyrate producers. Butyrate serves as the main energy source for colonocytes and therefore has various beneficial effects on gut health (Bui et al., 2014). Additionally, these bacteria are involved in the fermentation of dietary fibers and complex carbohydrates (Ye et al., 2022). Other significant differential genera based on FDR adjusted P values included *NK4A214* (inorganic_clean), *Ruminococcus* (organic_clean), *Shutleworthia* (inorganic_dirty) and similarly to LEfSe analysis, *Acidaninococcus* (organic_dirty). Ma et al (2022) found that the relative abundances of bacteria such as *Olsenella* (14 d piglets), *Slakia* (14 d), *Blautia* (28 d), *Ruminococcus_gauvreaui_group* (28 days), *Prevotell*a (28 days) and *Desulfovibrio* (28 days) were significantly lower in the feces of pigs fed with organic iron source compared to the pigs fed with ferrous sulfate. Differences between studies could be due to several biological and environmental differences such as pig breeds, age and diets among others (Gardiner et al., 2020). Moreover, the statistical approach for the differential analysis could also be behind the difference in results. LEfSe and DESeq2 analysis are two very popular statistical approaches for differential analysis of microbiota data often reported in the literature. Furthermore, LEfSe and DESeq2 analysis differ in their effectiveness in controlling false discovery rate (e.g., Gauthier et al., 2010, Segata et al., 2011). Moreover, to improve test robustness, these statistical approaches were based on different data normalization methods, e.g., Total Sum Scaling (TSS) for LEfSe and Relative Log Expression (RLE) for DESeq2. These differences would further affect differential analyses results (Weiss et al., 2017). This emphasized the importance to consider the statistical methods used in differential analysis when comparing different studies (Cho et al., 2023).

4.5.9 Predicted functionality

Predicted microbiota functionality did not differ among treatments except for some metabolism differences between inorganic_clean and inorganic_dirty groups. However, no difference in nutrient transporters between these two groups were found. Carbohydrate and amino acid metabolism dominated the metabolism of pigs regardless of iron sources and sanitation conditions used according to the KEGG annotation. Some organic iron sources such as ferrous glycine chelate stimulated the propanoate and butanoate metabolisms by increasing the abundance of short-chain fatty acid producing bacteria such as *Tezzerella* (Ma et al., 2022). In addition, ferrous glycine chelates also enhanced the amino acid metabolism, carbohydrate metabolism, and metabolism of cofactors and vitamins in 14 d piglets (Ma et al., 2022). However, no significant changes in the metabolic functions of KEGG for 28 d piglets were observed in the same study. These results indicated that pig age had a significant effect on the predicted microbiota functionality results regardless of iron sources treatment.

4.6 Conclusion

Compared to ferrous sulfate, SQM® iron was associated with higher fecal iron concentration on the dry matter basis. SQM® iron supplementation improved growth performance of weaned pigs. When phylogenetic information was considered, microbial structure was different between pigs raised under clean and dirty conditions. Despite of richness and microbial structure differences between diets and sanitary conditions, the predicted microbiota functionality did not differ between iron sources. Overall, SQM® iron could serve as an alternative iron source to nursery pigs with improved growth performance.

CHAPTER 5 GENERAL DISCUSSION AND CONCLUSION

5.1 General discussion

In our study, SQM® iron improved growth performance of weaned piglets at least in the first growth phase (d0-14). In the second growth phase (d14-28); however, we did not see a significant growth improvement with the SQM® iron. Dirty sanitation conditions can compromise the piglet's immune function and gut integrity, making them more susceptible to diarrhea-causing pathogens as well as reducing growth performance (Tang et al., 2022). In agreement with this, we found that mildly challenged dirty sanitation conditions were the main influencer that negatively correlated with ADG and FCR from d14 to d28.

We found higher fecal iron content in feces at d21 with the SQM® which would be an indication of lower iron absorption. In addition, organ weights (heart, liver, and spleen) were not affected by the iron source suggesting that SQM® iron did not compromise the development of these organs. Furthermore, fecal scores improved under SQM® iron treatment on d27; however, the improvement in scale was negligible. SQM® iron did not result in lower relative gene expression of pathogenic bacteria. Over supply of iron from non-heme iron in feedstuff could have been the source of interference to the iron restrictive effect of SQM® iron.

Several indicators of gut health were investigated in our study. These included serum indicators such as D-lactate and diamino oxidase as well as measurements of gut barrier functions including gut permeability measurements and expression of tight junction proteins. Although no differences were found on the above serum markers, there was a higher TEER values with the SQM® iron source under clean conditions. A higher TEER is indicative of an improved epithelial barrier integrity, even though the relative gene expression of tight junction proteins, such as, ZO-

1, CHD-1 and CLDN-1 were not affected by either iron source or sanitary conditions in the same jejunum samples. A strong trend was observed in higher gene expression of serum d-lactate in dirty sanitation conditions on d28 weaned pigs. Produced from bacterial fermentation in the GI track, higher serum d-lactate is associated with damaged intestinal mucosa (Sun et al., 2022). This indicated that lower gut health was influenced by dirty sanitation conditions. Future investigation on gut health on the sanitation conditions and iron sources could be through measurements of villus heigh and crypt depth of duodenum and jejunum (Nguyen et al., 2021).

To evaluate the effects of iron source and sanitary conditions on nutrient digestion and absorption capacities, gene expression of several nutrient transporters was investigated. Apart from a trend for a higher expression of peptide transporter 1 (PepT-1) and divalent metal transporter 1 (DMT-1) in jejunum samples from the SQM® iron treatment, no other differences in the transporters were observed. Ma et al. (2022) found a higher expression of this transporter in pigs fed with ferrous glycine chelate; however, the reason for a higher DMT-1 and PepT-1 were unknown and warrants investigation.

Since majority of the dietary iron was not absorbed in the duodenum, microbial composition of the gut is heavily influenced by the unabsorbed iron. Lower iron bioavailability of iron from ferrous sulphate for pigs could mean higher available iron in the colon for bacterial sequestration (Abbaz et al., 2022). Excess iron can foster the growth of pathogenic bacteria and result in intestinal inflammation, while iron deficiency may hinder bacterial cell growth and diminish microbial diversity (Chen et al., 2020). Consequently, maintaining a well-balanced iron status is crucial for preserving the ecological equilibrium of the intestinal tract and promoting overall health. This is particularly true in the colon where the highest numbers of bacteria reside within the gut. Furthermore, the efficiency of colonic iron absorption is only about 14% that of the

duodenum and the expression of several critical genes in iron absorption pathway, including duodenal cytochrome b reductase (Dcytb), DMT-1, transferrin receptor (TfR), and ferritin, are lower (not ferroportin) in the colon than in the duodenum (Yilmaz and Li, 2011). This lower iron absorption in the colon, often translates in higher available iron to the colon microbiota, which could be beneficial to commensal bacteria, but it could also exacerbate the effects of weaning and ferrous sulfate on promoting dysbiosis and favor pathogenic bacteria (Gardiner et al., 2020; Wang et al., 2019, 2021; Knight et al., 2019). We investigated the effects of SQM® iron on the gut microbiota composition and functionality. The result showed that iron sources and sanitation conditions modulated colon microbiota. Higher alpha diversity was observed in ferrous sulfate treatment than SQM® iron while sanitary conditions had no effect on these measures. In terms of beta diversity, when phylogenetic information was considered, microbial structure was different between pigs raised under clean and dirty conditions. Some beneficial butyrate producing bacteria such as *Anaerostipes* were differentially expressed in pigs raised in clean conditions (Bui et al., 2014). Others such as *Sutterella* which is a pathogenic bacterium were expressed in dirty conditions (Derqaoui et al., 2022). Both Zeng et al. (2023) and Ma et al. (2022) found that organic iron sources reduced the relative abundance of *Prevotella*. We also observed a trend for a lower abundance of this bacterium with the SQM® iron source, however, it was not significant (P = 0.0938).

Unabsorbed dietary iron at duodenum from inorganic sources have been reported to disrupt intestinal microbiota by enriching pathogenic bacteria such as coliform bacillus and reducing beneficial bacteria such as *Bifidobacterium* spp. in weaned pigs (Lee et al., 2008, Li et al., 2016). Furthermore, iron uptake by the pathogenic bacteria is associated with the virulence of pathogens in the case of certain gram-negative bacteria, such as *Salmonella*, *Shigella* and pathogenic *E. coli*,

which require iron for virulence and colonization (Kortman et al., 2014). Thus, a more restrictive iron source against bacterial sequestration would be associated negatively with virulence of pathogenic bacteria.

SQM® iron in our study did not lower loads of pathogenic bacteria. *Lactobacillus* is a beneficial bacterium with a low iron requirement (Huynh et al., 2022). A lower iron availability in the colon would promote growth for *Lactobacillus* over iron requiring pathogenic bacteria; however, excess unabsorbed free iron released from the basal diet during digestion would sabotage the iron restrictive effect from SQM® iron. That could explain a higher relative abundance of *Lactobacillus* in pigs fed SQM® iron numerically albeit lacking a statistical significance.

Previous studies on the effect of different organic iron on the abundance of gut pathogenic bacteria yield different result. Zeng et al. (2023) found that feeding pigs with organic yeast iron also did not result in differences in the abundance of pathogenic bacteria such as *Escherichia-Shigella* and *Clostridium*, nor a difference in the abundance of commensal bacteria such as *Bifidobacterium*, *Roseburia*, and *Ruminococcus*, which is consistent with our finding of lack of compositional differences in the commensal bacteria in the colon digesta microbiota. Gao et al. (2020) showed that a soluble soybean polysaccharide-iron complexes exhibited inhibitory effects on pathogenic bacteria like *E. coli* and S. *aureus*, while enhancing the growth of commensal bacteria such as *B. licheniformis*. Differences between studies may have to do with the iron source used, iron levels supplemented, iron contents of the basal diets and pig related factors such as age, genotype and pigs iron status.

We did see, however a lower of microbiota diversity in colon digesta with the SQM® iron over ferrous sulfate treatment. As pigs age, gut diversity increases. Higher diversity in microbiota is often associated with higher resiliency and a healthier gut, which support immune function,

resist disease, and regulate inflammation. (Belkaid and Hand, 2014; Dogra et al, 2020) Nevertheless, beta diversity was not affected by the iron sources (Bray-curtis) and even the significant results from the differential analysis need to be seen into perspective as the genera identified by either LEfSe or DEseq2 were present at very low relative abundances.

Even though alpha diversity significantly differs among different iron sources treatments and microbiota population structures differed under different sanitary conditions, the predicted microbiota functionality did not differ among treatments with some exceptions. Ferrous sulfate treatment under clean conditions differs from the same iron source treatment under dirty conditions. Coenzyme and lipid transport and metabolism as well as replication recombination and repair were significantly higher in the inorganic_clean group compared to the inorganic_dirty group. In addition, inorganic ion transport and metabolism, translation ribosomal structure and biogenesis and carbohydrate transport and metabolism were higher in the ferrous sulfate under dirty conditions compared to the ferrous sulfate under clean conditions. Also predicted inorganic ion transport differences are not clear as we did not see any differences in the expression of DMT-1 or ferroportin-1 under different sanitary conditions. Although, Tax4FUN or PICRUST are valuable tools for generating hypotheses about functional potential, they need to be seen with caution though as they are predictions and not real functional metabolism measures. The limitation of 16S rRNA analysis has lowest resolute on the genus level. In another words, it cannot accurately identify taxa on the species level. Bacteria identical on the genus level can be commensal or pathogenic at the species level (Muhamad Rizal et al., 2020; Van Rossum et al., 2020) Therefore, in the future, it would be more precise to conduct metagenome analysis or metabolomics studies to gain further insight into metabolism differences in microbiota between iron sources and sanitary conditions.

Besides predicted functionality we also investigated the expression of two enzymes associated with the butyrate and sulphur metabolism by bacteria in the feces. A higher change in the relative gene expression of Acetate-CoA Butyrate-CoA Transferase was observed from d0 to d21 in the ferrous sulfate treatment under clean conditions. Acetate-CoA Butyrate-CoA Transferase is related to butyrate producing bacteria such as *Faecalibacterium* or species within the Clostridia class such as *C. butyricum* and *Eubacterium rectale* (Fu et al., 2019*)*. We observed a higher abundance of *clostridium-sensu-stricto-1*, a butyrate producing bacterium, with ferrous sulfate treatment. It is possible that this bacterium in part was responsible for a higher expression of this enzyme in pigs fed ferrous sulfate.

The effects of iron sources and sanitary conditions on the relative gene expression of bisulfite reductase was also investigated. Ferrous sulfate treatment was associated with higher changes of relative gene expression of bisulfite reductase under clean condition from d0 to d21 in fecal samples. An overgrowth of H_2S-producing bacteria could be explained by the reduction on the population of butyrate-reducing bacteria (Murros, 2022). Nevertheless, we did not observe any significance in the abundance of H_2S-producing bacteria between iron sources or sanitary conditions and a higher change in the relative gene expression of Acetate-CoA Butyrate-CoA Transferase was observed from d0 to d21 in the same treatment. This indicated that iron sources and sanitation condition did not result in lower gut health due to overgrowth of H_2S-producing bacteria.

5.2 General conclusion

Despite higher fecal iron content on dry matter basis from SQM® iron supplementation, different iron sources did not affect relative organ weight nor on jejunum nutrient transporter expression. Furthermore, predicted functionality in microbiota analysis was also not affected by iron sources.

Different iron treatments also did not significantly affect gastrointestinal integrity and permeability in jejunum and did not alter fecal microbiota composition on d 28. SQM® iron improved ADG in the phase 1 (d 0 to 14) and overall ADFI (d 0 to 28). Overall, SQM® iron could serve as an alternative iron source to nursery pigs with improved growth performance.

CHAPTER 6 FUTURE DIRECTIONS

Future directions include:

1) Investigate SQM® iron supplementation and interaction of probiotics supplementation in nursery pigs on growth performance and gut health.

2) Investigate the effects of SQM® iron on markers of iron metabolism including, expression of soluble transferrin receptor, serum iron and iron content of organs such as heart, liver and spleen as well as the expression of hepcidin in pigs.

3) Investigate gut morphology responses, such as, villus height and crypts depth of jejunum and duodenum to SQM® iron.

4) Investigate the effects of SQM® iron on the virulence traits of pathogenic bacteria at the host-microbe interface using gut epithelial cells, such as, Caco-2 or IPEG J2 cells and look at bacterial responses in terms of bacterial growth, epithelial adhesion, invasion, translocation and epithelial damage.

5) Investigate the effects of SQM® iron on nursery pigs with Metagenomic sequencing to gain comprehensive understanding in pathogens on the genetic level.

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LIST OF APPENDENCES

Appendix 1

(A)

Treatment	Genus	Pvalues	FDR	LDAscore
Diet	*NK4A214_group*	0.00636	0.63407	4.26
Diet	*Terrisporobacter*	0.02121	0.63407	4.33
Diet	*Prevotellaceae_NK3B31_group*	0.03138	0.63407	4.92
Diet	*Alloprevotella*	0.03481	0.63407	4.44
Diet	*Treponema*	0.03556	0.63407	3.61
Diet	*Lactobacillus*	0.05002	0.65753	-5.7
Diet	*Acidaminococcus*	0.0522	0.65753	-4.14

(B)

Treatment	Genus	Pvalues	FDR	LDAscore
Condition	*Sutterella*	0.00041	0.04397	-3.93
Condition	*Clostridia_UCG__014*	0.0012	0.06439	4.47
Condition	*Acidaminococcus*	0.00256	0.09137	-4.47
Condition	uncultured	0.00529	0.13106	-4.95
Condition	*Lachnospiraceae_ND3007_group*	0.00622	0.13106	4.27
Condition	*Christensenellaceae_R__7_group*	0.00735	0.13106	4.52
Condition	*Ruminococcus_gauvreauii_group*	0.01071	0.1637	4.4
Condition	*Phascolarctobacterium*	0.01669	0.20495	4.33
Condition	*Lachnospiraceae_FCS020_group*	0.01724	0.20495	-3.5
Condition	*Methanosphaera*	0.0202	0.20917	3.98
Condition	*Megasphaera*	0.0215	0.20917	-4.88

(C)

Treatment	Genus	Pvalues	FDR	LDAscore
Diet_condition	*Clostridia_UCG__014*	0.00365	0.20508	4.66
Diet_condition	*Acidaminococcus*	0.00403	0.20508	4.63
Diet_condition	*Sutterella*	0.00575	0.20508	4.01
Diet_condition	*NK4A214_group*	0.01882	0.39877	4.5
Diet_condition	*Ruminococcus_gauvreauii_group*	0.0189	0.39877	4.6
Diet_condition	*Shuttleworthia*	0.02236	0.39877	4.3
Diet_condition	uncultured	0.03877	0.42236	5.06
Diet_condition	*Lachnospiraceae_NK4A136_group*	0.03893	0.42236	4.25
Diet_condition	*Terrisporobacter*	0.03947	0.42236	4.57
Diet_condition	*Faecalibacterium*	0.04696	0.4568	4.85
Diet_condition	*Lachnospiraceae_ND3007_group*	0.05576	0.47659	4.31
Diet_condition	*Christensenellaceae_R__7_group*	0.0579	0.47659	4.8

Appendix 2

Treatment	*Genus*	log2FC	lfcSE	Pvalues	FDR
Condition	*Anaerostipes*	22.357	2.932	2.4383E-14	2.609E-12
Condition	uncultured	-1.0377	0.22976	6.2882E-06	0.00033642
Condition	*Sutterella*	-4.087	1.1275	0.00028927	0.010317
IC vs ID	*Shuttleworthia*	23.248	4.1444	2.0287E-08	2.1708E-06
OC vs ID		25.777	4.1445	4.9828E-10	5.3316E-08
OD vs ID		25.627	4.1445	6.2752E-10	2.2382E-08
IC vs OD	*NK4A214_group*	-23.104	2.5345	7.7972E-20	8.343E-18
OC vs OD		-21.034	2.5384	1.1691E-16	1.2509E-14
OD vs ID		22.185	2.5357	2.1496E-18	2.3001E-16
IC vs OD	*Ruminococcus_gauvreauii_group*	-22.532	3.1621	1.0361E-12	5.5429E-11
OC vs OD		-24.003	3.1614	3.1368E-14	1.6782E-12
OD vs ID		21.139	3.1648	2.3964E-11	1.2821E-09
IC vs ID	*Acidaminococcus*	2.5275	0.78318	0.0012503	0.066891
IC vs OD		3.0358	0.78287	0.00010538	0.0028188
IC vs OD	uncultured	1.3707	0.3241	2.3429E-05	0.00083562
OC vs OD		1.125	0.32407	0.00051779	0.018468