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Iron Prophylaxis in Infants: Excess Iron Intake and Inulin Supplementation Modulate Trace Mineral Absorption, Gut Microbiota, and Resilience to Enteric Infection in Piglet Models

By

JUNGJAE PARK
DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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of the

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DAVIS

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2024

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ABSTRACT

Early-life nutrition significantly impacts neonatal health and development, where nutrient deficiencies or excesses can lead to various health complications. This is particularly true for iron nutrition. Exclusively breast-fed infants are susceptible to iron deficiency, prompting the use of iron-fortified diets. However, excessive iron exposure poses risks, including disruption of the gut microbiome. We investigated the complex interactions between dietary iron levels, gut microbiota, and specific dietary interventions in neonatal and pathogenic *Escherichia coli* (*E. coli*)-challenged weaned pigs, offering insights applicable to both animal and human health and nutrition.

Using a piglet model as a translational model for human infants, we explored the impact of high iron fortification and inulin or synbiotic supplementation on iron homeostasis and trace mineral bioavailability to better understand the biological functions of iron, the regulation of iron homeostasis, and the health consequences of iron imbalance in early infancy. Twenty-four piglets were randomly assigned to four treatments, receiving either iron-adequate milk (AI), high-iron milk (HI), HI milk with 5% inulin (HIP), or HIP milk with an oral gavage of *Ligilactobacillus agilis* YZ050 (HIS). The study revealed that HI fortification increased hemoglobin (Hb), hematocrit (Hct), and serum iron levels but also caused hepatic tissue iron deposition and concurrently reduced zinc (Zn) and copper (Cu) absorption. Inulin supplementation in HIP and HIS diets attenuated hepatic iron overload and decreased colonic and fecal iron levels, suggesting that inulin has the potential to modulate systemic iron utilization and mitigate some of the adverse effects of excessive iron fortification.

Further analysis focused on the impact of iron fortification and inulin supplementation, with or without *Ligilactobacillus agilis* YZ050 (*L. agilis* YZ050), on the gut microbiome of piglets. Using 16S rRNA sequencing, the study found that both dietary iron and inulin significantly

influenced colonic and fecal microbiota diversity, with inulin exerting a more pronounced effect than iron. Inulin supplementation led to increased relative abundances of beneficial bacterial families such as *Lachnospiraceae* and *Prevotellaceae*, while decreasing *Bacteroidaceae* and *Ruminococcaceae*. The addition of *L. agilis* YZ050 did not produce significant changes beyond those caused by inulin alone. These results suggest that inulin supplementation plays a critical role in modulating gut microbiota composition, potentially through inulin fermentation and cross-feeding activities among gut bacteria, thereby mitigating the adverse effects of high iron intake on gut health.

To further investigate the implications of dietary iron on health, we examined how host iron status and dietary iron supplementation impact disease resilience and susceptibility to intestinal infection and gut microbiota composition using a weanling pig model with enterotoxigenic *E. coli* (ETEC) as an infection model. Thirty-two pigs were assigned to four treatments: an adequate iron diet without ETEC challenge (CON), low iron diet (LOI), adequate iron diet (COI), or high iron diet (HII) with ETEC challenge. The study found that LOI pigs experienced more days with diarrhea and had lower body weight gain compared to the other groups, indicating that iron deficiency potentially exacerbates disease severity. HII diet was associated with higher Hb and Hct levels. Furthermore, the gut microbiota analysis revealed distinct shifts in microbial composition associated with dietary iron levels and ETEC infection. These shifts included increased abundance of *Peptostreptococcaceae* in CON and HII pigs, and higher levels of *Veillonellaceae* and *Megasphaera* in LOI pigs, suggesting that dietary iron influences gut microbiota.

In conclusion, this dissertation contributes to understanding the intricate relationships between dietary components such as iron and inulin, gut microbiota, and health outcomes in

neonatal and weaned pigs. The use of a piglet model provides valuable insights into the physiological and microbiological impacts of dietary interventions, offering a translational approach to improving infant nutrition and managing iron-related health issues. The findings highlight the importance of balancing iron intake to prevent both deficiency and overload, while highlighting the potential benefits of inulin supplementation in mitigating some of these adverse effects by affecting iron utilization and modulating gut microbiota composition. Additionally, maintaining adequate iron status is crucial for reducing susceptibility to ETEC infection and promoting better health outcomes. These insights inform evidence-based strategies for dietary iron optimization in both animal and human contexts.

Keywords: gut microbiota, iron fortification, iron overload, iron supplementation, infant formula, inulin, prebiotic, pig model, trace mineral

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CHAPTER 1

Literature Review on Iron Nutrition in Early Life

ABBREVIATIONS:

ATP: adenosine triphosphate

B. infantis: *Bifidobacterium infantis*

CP: ceruloplasmin

Cu: copper

CuZn-SOD: copper/zinc-dependent superoxide dismutase

DMT1: divalent metal transporter 1

DNA: deoxyribonucleic acid

DRI: dietary reference intake

DYCTB1: duodenal cytochrome b1

E. coli: *Escherichia coli*

ETEC: enterotoxigenic *Escherichia coli*

Fe: iron

Fe³⁺: ferric iron

Fe²⁺: ferrous iron

FeSO₄: ferrous sulfate

FPN: ferroportin

Hb: hemoglobin

HH: hemochromatosis

HMO: human milk oligosaccharides

IRE: iron-responsive element

IRP: iron-regulatory protein

LF: lactoferrin

mRNA: messenger ribonucleic acid

RBC: red blood cell

ROS: reactive oxygen species

UTR: untranslated regions

TF: transferrin

TFR: transferrin receptor

Zn: zinc

1.1. Biological Functions of Iron and Regulation of Iron Homeostasis in Early Life

1.1.1. Biological Functions of Iron

Iron is an essential nutrient for humans and almost all living organisms and is required for energy production, oxygen transport, and cell proliferation (1-4). There are many iron-containing proteins essential for biological functions and iron serves as a cofactor for numerous enzymes involved in essential physiological functions, including oxygen transport, energy production, deoxyribonucleic acid (DNA) synthesis, neurotransmitters synthesis, and overall growth and development (4-6). Hemoglobin (Hb), the oxygen-carrying protein in red blood cells (RBCs), relies on iron for its structure and function, highlighting the critical role of iron in facilitating cellular respiration (7). Hb is one of hemoproteins and oxygen transporters in RBCs (8) that bind to oxygen from lungs and transports it to areas that require it for aerobic metabolic processes (1). Moreover, iron participates in electron transport chains and catalytic reactions within mitochondria, contributing to adenosine triphosphate (ATP) production and cellular metabolism (9). Additionally, iron plays an important role in mediating immune responses. Insufficient iron intake to meet basic metabolic requirements leads to deficiency. Studies have shown that iron deficiency (ID) and iron deficiency anemia (IDA) have detrimental effects on immunity (10), where individuals with IDA exhibit signs of impaired cell-mediated immune response and iron-deficient neutrophils, as demonstrated by reduced bactericidal capacity (11).

1.1.2. Regulation of Iron Homeostasis

The molecular mechanisms that regulate iron homeostasis are mostly investigated in human adults and through adult animal models. Dietary iron absorption and body iron homeostasis are subjected to the control of two main mechanisms at cellular and systemic levels, respectively (12, 13). The hormone hepcidin is considered the master regulator of systemic iron balance through

its interaction with the only known mammalian iron exporter, ferroportin (FPN) (13, 14). Hepcidin controls FPN activity by binding to it and inducing its internalization and degradation (14, 15). When the body has adequate iron, the homeostatic mechanisms ensure balanced absorption, transport, and storage. In an iron-replete state, hepcidin levels are maintained at a moderate level, allowing for controlled iron absorption and distribution (9). High hepcidin inhibits ferroportin-mediated iron export from enterocytes, hepatocytes, and macrophages, reducing systemic iron availability (14-16). Hepcidin expression is influenced by several factors, including iron levels (increased by overload and decreased by deficiency), inflammation, and erythropoietic activity (14, 15). While the hepcidin-FPN axis is a defined mechanism that regulates iron homeostasis in adults (13, 17), there is discussion on whether or not this mechanism is present in early infancy to regulate iron absorption (18). Despite vast amount of research on iron metabolism in adults, there are still questions around how iron is regulated in the early infancy. Understanding the nuances of iron regulation during infancy is crucial for optimizing iron supplementation strategies and mitigating the risk of iron-related disorders.

Cellular iron balance is primarily controlled by iron-regulatory proteins (IRPs), which bind to iron-responsive element (IRE) sequences that are present at the 3' or 5' untranslated regions (UTRs) of messenger ribonucleic acid (mRNA) sequences and modulate mRNA stability and translational activity (13, 17), thereby regulating the expression of genes involved in iron metabolism. Under iron-deficient conditions, IRPs bind with high affinity to IREs. When IRPs bind to IREs located in the 5' UTR of mRNAs, such as those encoding for ferritin and FPN, this binding inhibits translation initiation, leading to decreased protein production. Conversely, when IRPs bind to IREs in the 3' UTR of mRNAs, such as the TFR mRNA, this binding stabilizes the mRNA, preventing its degradation and thus increasing translation and production of TFR, which

is critical for iron uptake. The key iron transporters including divalent metal transporter 1 (DMT1), FPN, and transferrin receptor (TFR) are subjected to regulation by the IRP-IRE mechanism, and they orchestrate iron absorption, transportation, storage, and utilization in response to changes in cellular iron levels (14, 15, 19).

Regulation of enteric iron absorption begins in the intestinal lumen, where dietary iron, primarily in the ferric form (Fe^{3+}), must be reduced to its ferrous state (Fe^{2+}) for absorption (20). This reduction is mediated by DYCTB1, a Fe^{3+} reductase enzyme located on the apical surface of enterocytes, making it suitable for uptake into the cells (21). Once iron is in its Fe^{2+} form, DMT1 facilitates its passage across the enterocyte apical membrane. DMT1, a highly specific transport protein, is crucial for iron absorption (13, 22). Following uptake by DMT1, iron enters the enterocyte cytoplasm, where it faces one of two fates: storage as ferritin or export to the bloodstream (13).

For systemic distribution, iron is exported from the enterocyte to the bloodstream via FPN (13, 23). Ferroportin is expressed on the basolateral membrane of enterocytes, macrophages, and hepatocytes (23). It releases iron from the cells into the circulation, allowing it to be utilized elsewhere in the body. Once outside the cell, Fe^{2+} must be oxidized back to its Fe^{3+} form to bind transferrin (TF), the primary iron transport protein in the blood (22, 24). This oxidation is carried out by hephaestin, a multicopper oxidase closely associated with FPN on the enterocyte membrane (25). In the bloodstream, TF binds Fe^{3+} for transport and distributes iron to tissues that require it (26). Cells take up the TF-Fe complex through receptor-mediated endocytosis (26). Once inside the acidic environment of the endosome, iron dissociates from TF and is utilized for various cellular processes (26). Within cells, ferritin efficiently stores excess iron, preventing oxidative damage by sequestering iron in a non-reactive form (27).

In addition to the two main regulation mechanisms, the presence of lactoferrin (LF) may also affect iron absorption in early life. Human milk has a high concentration of LF, an iron-binding protein present in milk that has been suggested to contribute to the high iron bioavailability from milk (28). LF has a high affinity for iron and is not easily digested by the infant's gastrointestinal system, providing an efficient mechanism for iron absorption by binding to its receptor located in the small intestine (28, 29).

1.1.3. Oxidative Stress

While iron is essential for various biological and physiological processes, excessive iron accumulation can lead to direct iron toxicity, posing serious health risks (30, 31). Iron-mediated oxidative stress arises from the generation of reactive oxygen species (ROS) through Fenton chemistry, wherein free iron catalyzes the conversion of hydrogen peroxide into highly reactive hydroxyl radicals (32, 33). Excessive ROS production can overwhelm cellular antioxidant defenses, leading to oxidative damage to lipids, proteins, and DNA, and subsequent tissue dysfunctions and cell death (34). This type of cell death, termed ferroptosis (9), is implicated in many degenerative diseases (34). The liver, heart, and pancreas are particularly vulnerable to iron-induced oxidative damage (35). Iron overload is prevented through the action of hepcidin (31). Mutations in the hepcidin pathway are the cause of hereditary hemochromatosis (HH), an iron overload disease. The pathology of HH clearly exhibits the harm of iron toxicity on biological systems. During HH, iron accumulates in the liver, the iron storage organ, but once iron overload is reached it initiates liver fibrosis, then cirrhosis and loss of liver function, eventually leading to complications and death if untreated (36, 37). In HH patients, liver fibrosis is believed to result from iron-induced oxidative stress (38).

Infants are especially susceptible to iron toxicity and iron-induced oxidative damage due

to their high demand of iron for rapid growth rate, with their immature antioxidant systems and iron regulation and limited capacity for iron excretion and higher vulnerability to oxidative stress (39), highlighting the importance of maintaining optimal iron levels during infancy.

1.2. Iron Requirements, Iron Deficiency and Overload, and the Practice of Iron Supplementation in Infancy.

Iron is one of the essential trace minerals and plays a pivotal role in various physiological and cellular processes that are crucial for growth, development, and overall health of both humans (18, 40) and animals (41).

1.2.1. Iron Requirements

Infants require iron to support rapid growth (40, 42). Term infants are usually born with 75 mg of elemental iron/kg of BW, with 75% found in hemoglobin, 10% in tissue protein iron, and 15% as stored iron (43). For healthy infants born at term, iron stores in the liver and iron from suckling breast milk, about 0.2 to 0.4 mg Fe/L (44), is adequate to support healthy growth and development up to 4 – 6 months of life (18, 40, 45). However, as the majority of fetal iron is acquired in the third trimester of pregnancy, preterm infants are born with lower iron stores and have higher risk of ID and IDA (46, 47). Likewise, infants born from ID mothers are at increased risk of ID because of limited body iron stores and the low iron concentration in breast milk (48, 49). Understanding the normal range and requirements of iron in infancy, particularly within the first 6 months, is fundamental to ensuring optimal growth and development.

The dietary reference intake (DRI) for iron for infants during 0 – 6 months is 0.3 mg per day, based on the amount provided in breast milk (50). The recommendation for a normal term infant to meet this requirement and maintain proper stores is to provide 1 mg/(kg·day) of iron

during this period to a maximum of 15 mg/day (51). The requirement for preterm infants ranges from 2 mg/(kg·day) for infants smaller than 2500 g at birth to 4 mg/(kg·day) for infants smaller than 1500 g at birth (52). Infants at about 6 months of age absorb ~50% of iron from breast milk (53). Therefore, breastfed infants around this age will normally ingest ~0.3 mg Fe/day, but actually absorb 0.15 mg Fe/day (50). Infants at about 6 months of age will absorb ~10% of iron from ferrous sulfate (FeSO_4) typically used in infant formulas regardless of concentration (54). For an infant under 11 months of age expected to consume about 1 L of formula per day, giving them formula containing 10 – 12 mg Fe/L like in the U.S. will allow them to absorb up to 1.2 mg Fe/day (18), which is about 10 times the amount absorbed from breast milk. Iron-fortified infant formula that contains up to more than 30 times of iron as in breast milk is commonly given to infants at 0 to 6 months of age (55). Because the recommended intake of iron to maintain and meet growing demands is just 1 mg/(kg·day) of iron for healthy infants in the first 6 months (56), there is concern that infants who consume these formulas are at risk of dietary iron overexposure. In contrast to the pressing need for iron treatment in developing countries, concern over the risk of dietary iron overexposure in early childhood is growing in high-income regions (57).

1.2.2. Clinical Evaluation of Iron Status

A systematic review by Garcia-Casal et al. evaluated serum or plasma ferritin concentrations as recommended biomarkers by the WHO for assessing iron status (58). They examined plasma or serum ferritin for its correlation with body iron status and established cutoff points in various populations. Among the 298 studies assessed, 72 focused on iron deficiency and 36 on iron overload, all being observational. In healthy individuals, ferritin concentration averaged 15 $\mu\text{g/L}$ when bone marrow iron content was 0, rising to 70 $\mu\text{g/L}$ with higher bone marrow iron levels (58). As ferritin is an acute phase protein, non-healthy populations showed a wider range of

variation, with mean ferritin concentrations of 82 µg/L for iron depletion and 381 µg/L for iron sufficiency, depending on pathology. Although limited, data suggested a cutoff close to 500 µg/L for iron overload (58).

Accurate assessment of iron status is crucial for identifying and addressing iron deficiency or overload effectively. The clinical evaluation involves measuring various biomarkers to diagnose these imbalances in infants and young children. This includes measuring serum iron, serum ferritin, transferrin saturation, and hematological parameters such as hemoglobin (59). Most patients with IDA present with low plasma or serum iron concentrations and increased iron-binding capacity (60). In IDA patients, the serum iron concentration is often less than 320 µg/L, and mean TF saturation is below 15% (61). While serum iron and TF saturation are biomarkers for identifying IDA, they do not provide sensitive specificity to distinguish IDA from other forms of anemia in less severely anemic patients (60). A more reliable marker to differentiate IDA from other forms of anemia is ferritin (62). Serum ferritin reflects body iron stores (63). However, ferritin levels can increase in response to infection, inflammation, adiposity, and alcohol consumption, limiting its utility as a biomarker (64). Serum TFR concentration is a sensitive marker for mild iron deficiency (65). However, although increased serum TFR is observed in ID and IDA patients, the TFR assay is not widely available, and standard values have not been established for infants and young children (51, 63). Hb concentration <110 g/L is considered the cutoff for IDA in humans (65). However, Hb concentration is not a specific measure for iron status and only detects more severe ID (63), and signs of IDA usually do not manifest until Hb levels fall below 80 g/L due to the body's compensatory mechanisms for assuring oxygen delivery (66, 67). No single measurement is complete indicative of iron status in infants or young children. Children with TF saturation below 16% are considered iron deficient (65), while presenting with anemia when Hb concentration is

approximately 110 g/L (68). However, measurement of Hb concentration alone lacks specificity and sensitivity because genetic disorders and chronic infections that limit erythropoiesis or result in chronic hemolysis can cause low Hb concentrations (51). Therefore, Hb concentration combined with one or more measurements for iron status (e.g. serum ferritin or TFR) is necessary to detect ID or IDA (51). Therefore, combining multiple biomarkers remains crucial for the accurate diagnosis and assessment of iron deficiency or overload in infants and young children.

1.2.3. Iron Deficiency

ID remains the most prevalent micronutrient deficiency and explains about 50% of anemia globally (69). Infants and young children are especially susceptible to ID and IDA (70). The high prevalence of IDA among American infants was first recognized as early as the 1930s (71) and it remains a significant concern worldwide, particularly among children under the age of 5, approximating 43% in 2011 (72). In regions with limited resources, such as central and west Africa, this figure rises significantly, reaching up to 70%. However, in high-income regions, the prevalence drops to below 10% (72). Stored iron depletes in normal term infants at about 4-6 months of age and even earlier in preterm infants (73). Unfortunately, human milk does not provide enough iron to sustain a healthy term infant for more than 6 months (44). Because breast milk provides only 0.2 to 0.4 mg/L iron that can sustain an exclusively breast-fed healthy term infant for only up to the first 6 months (44), rapidly growing infants will eventually need exogenous sources of iron to meet their growing requirements. IDA can impair the delivery of oxygen to tissues, leading to symptoms such as weakness, fatigue, difficulty concentrating, shortness of breath, and palpitations, and is ultimately associated with significant morbidity and early mortality (74). Children under 5 years of age are vulnerable to detrimental effects of IDA, which have shown to cause delay in mental, motor, and emotional development (74). In a study on early-life

neurobehavioral development, 2601 children aged 6 to 24 months participated in blood tests and neurobehavioral assessments (75). Results revealed that approximately a quarter of the children were anemic, with another quarter of them diagnosed with IDA (75). Comparing children with IDA to non-anemic children, those with IDA exhibited significantly lower developmental scores across total neurobehavioral development, as well as in gross motor and adaptability development (75). Infants suffering from ID may suffer adverse effects on long-term cognition and behavior because critical phases of brain development during infancy are adversely affected by ID (76). Maternal iron deficiency can also lead to lower iron stores in the newborn (77-79). In a cohort study of 180 mother-infant pairs, infants born to anemic mothers and non-anemic mothers were assessed for Hb and ferritin levels at birth and 14 weeks post-birth. Infants born to anemic mothers had significantly lower hemoglobin and ferritin levels compared to those born to non-anemic mothers (77). This highlights the impact of maternal anemia on the iron status of exclusively breast-fed infants and underscores the need for timely interventions to address maternal iron deficiency during pregnancy. Thus, sufficient iron intake is imperative for infants.

1.2.4. Iron Prophylaxis

Iron prophylaxis refers to preventive measures that address ID and IDA. Given the significance of maintaining adequate iron levels in infancy, implementing iron prophylaxis strategies is a standard practice. In early life, iron supplements, such as iron drops, multi-nutrient packets (MNPs), fortified formula, and fortified complimentary foods, are effective at preventing or treating ID in most infants (80, 81). The same rationale backs the American Academy of Pediatrics' (AAP) recommendations that exclusively breast-fed infants receive iron supplements beginning at 4 months, and that formula-fed infants receive iron-fortified formula (82). In the United States, iron-fortified formulas contain iron concentrations ranging from 10 to 12 mg/L (83).

Routine iron supplementation practices providing large amounts of exogenous iron at an early age, though effective in ID prevention, have not been fully investigated for their long-term health impact. Studies have reported adverse effects of iron supplementation in iron-replete infants on growth, cognitive development, gut microbiota, and risk of infections (30, 39). The WHO recommends universal iron supplementation for groups of high risk of ID and IDA, like preterm infants or those with low birth weight (80). Understanding the rationale behind iron prophylaxis aids in optimizing iron status and mitigating the risk of deficiency- and overload-related health complications during early development.

1.2.5. Iron Overload

To prevent ID and IDA, iron supplements in forms of oral drops, iron-fortified formula, and complementary foods are beneficial and mostly effective in infants and young children, especially those who live in resource-limited areas and/or have poor iron status (84). However, many infants in the USA and other developed countries are iron-replete, yet receiving exogenous iron at doses that far exceed their needs (45, 85). Most infant formulas in the United States are fortified with iron at a concentration of 12 mg/L, which is 30 to 60 times higher than the iron content of breast milk and is still higher even when accounting for the low bioavailability of formula iron (86) and provide 7 times more bioavailable iron after adjusting for the low absorption rate of formula iron (87). While nutritional iron overload has not been defined, there are concerns about infants accumulating excessive iron from consuming iron-fortified formula. Additionally, the mechanisms for regulating iron absorption and export in infants seem to be immature around this age (18). HH (88) and thalassemia (an inherited blood disorder) (89) exert significant health and developmental consequences that extend beyond immediate detrimental effects and lead to long-term complications if left unmanaged (90). Excess iron can deposit in organs like the liver,

heart, and pancreas, leading to conditions like cirrhosis and diabetes (91). In infants and children, iron overload may cause early liver disease, growth delays, and oxidative stress (18). Excessive iron levels present significant toxicity risks due to increased free radical production (33). To counter this, hepcidin production is upregulated, inhibiting FPN and preventing further iron absorption from the intestine (16). Hepcidin also inhibits the release of stored iron from macrophages, reducing the availability of circulating iron (92). DMT1 expression decreases to limit intestinal iron absorption. As circulating iron accumulates, TF becomes saturated, restricting its capacity to bind additional iron (26). Meanwhile, ferritin synthesis is upregulated, sequestering excess iron in a non-reactive form to minimize oxidative damage (19). In infancy, these regulatory mechanisms are crucial because the developing antioxidant systems in neonates make them particularly susceptible to oxidative stress. Therefore, efforts of investigating iron absorption and homeostasis are imperative to prevent iron overload during this critical growth phase.

1.3. Health and Developmental Consequences of Iron Imbalance in Infancy

1.3.1. Effect of Iron Imbalance on Growth and Development

ID can adversely affect health, growth, and development in infants and young children (18, 70, 93-96). ID can hinder cellular metabolism and oxygen transport, thereby compromising tissue growth and neurodevelopmental processes critical for overall growth trajectory (97). IDA affects neurodevelopment, causing delays in motor skills, language acquisition, and cognitive function (97). Moreover, children with IDA often exhibit behavioral issues such as irritability and poor attention span (97). There is also growing concern of dietary iron overexposure because it can lead to increased risks of poor growth, inflammation, chronic diarrhea, and alterations to the gut microbiome (39, 86, 93, 95). Excess colonic iron, due to low iron absorption at the duodenum of

the small intestine (98), is thought to favor the growth of pathogenic bacteria at expense of beneficial bacteria in the host and the development of pathogens can cause inflammation, in which hinders absorption of iron and other nutrients (99). In addition, excessive iron accumulation causes multitudes of health problems because the mammalian body has no mechanisms to dispose of stored excess iron (12).

1.3.2. Interactions between Iron and Other Trace Minerals

Adequate intake of various trace minerals is crucial for both growth and long-term health (100, 101). However, an excess of iron in the body may interfere with the absorption and metabolic processes of other essential trace minerals such as zinc, copper, and manganese by competing for membrane transporters (101). Iron and zinc compete at the site of absorption (102, 103), and high iron levels may inhibit zinc absorption and vice versa (102). In a secondary analysis of a randomized, placebo-controlled trial conducted with infants of 6 to 12 months of age, infants who received iron supplementation (10 mg of FeSO_4) for 6 months had lower serum zinc compared to those without iron supplementation (9.7 vs. 10.5 $\mu\text{mol Zn/L}$, respectively), while this change was only observed in iron-replete infants (94). However, a study in non-anemic Kenyan infants did not find an effect on serum zinc or zinc absorption after 3 months of home iron fortification with a micronutrient powder containing 5 mg zinc and 12.5 mg of iron (104). A study in suckling rats reported the antagonistic effect of excess iron supplementation on other trace minerals including zinc, copper, and manganese status (105). The status of other trace minerals can significantly affect iron uptake and metabolism. For example, the binding of iron to transferrin requires the oxidation of ferrous iron to its ferric form. This reaction is catalyzed by ceruloplasmin (CP), a copper-carrying protein. Copper deficiency has been shown to reduce CP levels and is associated with IDA (106, 107). A study involving 214 healthy, term, breast-fed infants from Sweden and

Honduras investigated the impact of iron supplementation on copper status. Infants were randomized into three groups including iron supplementation (1 mg/(kg·d)) from 4-9 months of age, iron supplementation from 6-9 months, and a placebo group (108). Plasma samples were analyzed for copper concentration and activity of copper/zinc-dependent superoxide dismutase (CuZn-SOD) at 4, 6, and 9 months of age (108). The results showed a significant increase in plasma Cu with age, with Honduran infants exhibiting higher levels than their Swedish counterparts at 9 months. Infants receiving iron supplements from 4-9 months had lower CuZn-SOD activity compared to those on placebo at 9 months, suggesting a negative effect on copper status (108). These findings highlight potential interactions in trace mineral absorption and metabolism (108). Therefore, it is important to monitor the status of other trace minerals when providing iron or other micronutrient supplements to infants and young children.

1.3.3. Effects of Iron Status on Host Gut Microbiota

While the effects of dietary iron on human cells and tissues have been extensively researched, there is still a gap in knowledge regarding the influence of varying iron levels on the human gut microbiome. Research has suggested that iron homeostasis significantly influences the gut microbiome and vice versa, highlighting how alterations in gut microbial communities can impact iron absorption and metabolism (18, 109). ID has been shown to alter the gut microbiome by reducing the abundance and diversity of beneficial bacteria, making ID individuals more susceptible to enteric infections (110, 111). ID conditions favor the proliferation of certain bacterial taxa capable of utilizing their iron acquiring mechanisms, such as siderophores, to overcome the iron limitations and allow them to thrive even in ID environment or hosts (112). Consequently, the relative abundance of iron-responsive bacteria may increase, while iron-dependent taxa may decline, reshaping the gut microbial community structure (110). ID impairs innate immunity,

decreasing the activity of immune cells like macrophages and neutrophils, making the body more susceptible to enteric pathogens (113). In addition, ID may affect gut epithelial integrity, increasing permeability and facilitating the translocation of pathogens (114, 115). Conversely, iron overload can also perturb gut microbiota composition and function by promoting growth of iron-utilizing bacteria and pathogenic taxa (114, 116), due to a nutrient-rich niche for pathogens that compete effectively with commensal bacteria (84). Iron overload can promote the growth of harmful bacteria such as *Salmonella* and *Escherichia coli* (*E. coli*) by providing excess free iron, which is essential for their metabolism and survival (99). Furthermore, iron overload-induced oxidative stress and inflammation can adversely affect gut microbial communities and compromise intestinal barrier function in iron overload individuals, creating an environment conducive to pathogen colonization and virulence (77) and disrupting ecological balance and promoting dysregulated host-microbiota interactions (117, 118). Additionally, iron overload-induced immune dysregulation, characterized by impaired antimicrobial defense mechanisms and exaggerated inflammatory responses, further heightens the risk of enteric bacterial infections (119). Considering the important role of gut microbiota in regulating gut health, it is crucial to investigate the influence of iron supplementation and fortification on the gut microbiome in infants and young children. Further research is needed to fully comprehend these interactions and develop evidence-based guidelines for iron supplementation and fortification in infants for managing iron-related disorders and optimizing gut health in infancy and early life.

Iron status exerts profound effects on gut microbiota composition and function, influencing susceptibility to enteric bacterial pathogen infections, as iron is essential for the growth of many pathogens (84, 120). Iron from fortified foods is typically poorly absorbed (~10%), resulting in a significant amount of unabsorbed iron passing into the colon (98, 121). Iron fortification has shown

to reduce the abundance of *Lactobacillus* in both animal (122) and human (99) studies. Both *Lactobacillus* (123-125) and *Bifidobacterium* (126) require minimal or no iron for their survival in the gut and tend to thrive in a low iron environment (123, 127). Therefore, they tend to react to changes in iron homeostasis easily. Gut microbiomes of iron-replete infants may be more affected by excess iron.

Since commensal gut bacteria are essential for health and development, disrupting their colonization may explain some adverse outcomes of iron supplementation (109, 128). The increase in abundance of potential pathogens may lead to intestinal inflammation (129), and hinder the absorption of iron (114) and other micronutrients. Therefore, further study is needed to investigate the efficacy of iron supplementation and fortification on the gut health and the complex relationship between iron availability and gut microbiota and gut health in infants.

1.3.4. Effects of Iron Supplementation on Risk of Enteric Infections

The safety of iron supplementation and fortification in infants and young children has been questioned over the concerns of adverse effects from dietary iron overexposure (39, 86). As either systemic iron reduction or promotion is associated with alterations in the gut microbiome, oral iron supplementation can directly impact microbial composition in the gut, often resulting in a reduction in beneficial bacteria and an increase in the growth of enteropathogens (110). These concerns have been confirmed in multiple studies. These studies have shown that excess colonic iron levels facilitate the growth of pathogenic bacteria such as *Salmonella*, *Clostridium*, and pathogenic *E. coli* at the expense of beneficial counterparts like lactobacilli and bifidobacteria in 6-month-old Kenyan infants, who received MNP containing 2.5 mg of iron daily for 4 months (96). In a mouse study that investigated the impact of dietary iron on the microbiota, dietary iron deprivation was associated with increased abundance of the *Lactobacillus* and reduced abundance

of *Bacteroides* and *Roseburia* compared to the iron-replete group (130). Moreover, a systematic review concluded that iron provision is associated with a higher risk of diarrhea in children (131). A weanling pig study further demonstrated the impact of iron levels on diarrhea incidence and microbial balance. Increasing doses of iron (0, 50, 100, and 250 mg ferrous sulfate/kg body weight) led to a linear increase in diarrhea incidence, higher coliform numbers, and reduced ratios of beneficial lactobacilli and bifidobacteria to enterobacteria (132). These findings suggest that iron fortification may contribute to a more pathogenic gut microbiota profile and increased gut inflammation and diarrhea incidence.

Enterotoxigenic *E. coli* (ETEC) stands out as a leading cause of enteric infection and diarrheal illness, contributing to up to 20% of diarrheal cases in resource-limited communities (133), where studies performed between 2010–2016 showed that ETEC emerged as the predominant pathogen responsible for traveler's diarrhea, constituting an average of 42% and 28% of cases among travelers to Latin America and Asia, respectively (134). Diarrheal diseases remain a significant public health concern globally, particularly among children under 5 years of age in developing countries (135-137). Early-life infection with ETEC not only poses immediate health risks but also raises concerns regarding its potential long-term impact. Recent studies suggest that early-life infections, such as ETEC-induced diarrhea, may compromise neuronal development, potentially affecting cognitive function and overall neurobehavioral outcomes in children (138, 139). The extent to which host resilience to ETEC infection is influenced by factors such as iron status and dietary iron content remains unclear. Iron is an important micronutrient for both microbial growth and host physiology (140, 141), raising questions about its role in shaping the host response to ETEC infection and the composition of the gut microbiota in young children. Understanding the complex interactions between iron status, gut microbiota composition, and

susceptibility to enteric infections is crucial for elucidating the pathogenesis of diarrheal diseases and developing effective preventive measurements.

1.4. Effects of Prebiotic Supplementation on Host Iron Homeostasis and Gut Microbiome

Understanding the effects of prebiotics supplementation on host iron homeostasis and gut microbiome is essential for elucidating their potential beneficial applications in managing iron-related health problems and promoting gut health. The imbalance in gut microbiota caused by excessive dietary iron or iron fortification highlights the need for supplementation with prebiotics to modulate the gut microbiota and mitigate the adverse effects of iron fortification (120). In particular, the bifidogenic and *Lactobacillus*-promoting properties of prebiotics are crucial, especially for formula-fed infants who typically exhibit lower levels of lactobacilli and bifidobacteria compared to breast-fed infants (142, 143). Breast milk supports healthy gut microbiota development by providing human milk oligosaccharides (HMOs) that preferentially craft the infant gut so that it is dominated by commensal *B. infantis*, which has been shown to suppress pathogen proliferation and improve mucosal barrier integrity, preventing inflammation and diarrhea (144-147). Therefore, a common method to modulate the gut microbiome to resemble that of breast-fed infants and to regain balance in the gut microbiome is dietary use of prebiotics (148-151). Studies and reviews have highlighted the ability of non-digestible polysaccharides, including inulin, fructooligosaccharides (FOS), and galactooligosaccharides (GOS) (152, 153), that modulate the gut microbiota by selectively promoting the survival and growth of specific microbial population, particularly beneficial bacteria like bifidobacteria and lactobacilli (120, 153, 154). Prebiotics exert their effects through several mechanisms, including the production of short-chain fatty acids (SCFAs) during the fermentation process, modulation of intestinal pH, and

enhancement of mucosal barrier function (152, 153). Non-digestible polysaccharides reach the colon intact, where they serve as a substrate for fermentation by gut microbiota (155), and the production of SCFAs, such as acetate, butyrate, and propionate, confers several benefits to gut health (156) by providing energy to colonocytes, exerting anti-inflammatory effects, enhancing immune function, maintaining colonic mucosal integrity by acting on the metabolism, proliferation, and differentiation of different epithelial cell types and contributing to a balanced and resilient gut microbiome (153). It has been demonstrated that dietary prebiotics can improve iron deficiency in Kenyan infants who received 3.5 mg iron supplements with prebiotics (3 g or 7.5 g of GOS and FOS), not only resulting in preventive effects against ID or IDA, but also promoting the relative abundance of beneficial bacteria such as *Bifidobacteriaceae* and *Lactobacillaceae* while relatively lowering the abundance of pathogenic bacteria (120). This has been confirmed in animal studies showing that dietary prebiotics can improve iron utilization in young pigs and potentially shift intestinal bacterial populations to those that may account for improved iron utilization (157, 158). In a pig study, while enhancing beneficial bacteria like lactobacilli and bifidobacteria and suppressing less desirable bacteria such as *Clostridium spp.* and *Enterobacteriaceae*, inulin supplementation of 2 or 4% of total diet improved hemoglobin concentrations and the overall hemoglobin repletion efficiency by 28% and 15%, respectively, indicating improved utilization of intrinsic iron in the diet by young pigs (158).

Inulin is a plant $\beta(2\rightarrow1)$ fructan with a degree of polymerization ranging from 2 to 60 or more (159) and enzymatic hydrolysis of inulin with endoinulinase is known to produce α -D-glucopyranosyl- $[-\beta$ -D-fructofuranosyl] n 1- β -D-fructofuranoside ($G_{py}F_n$) and β -D-fructopyranosyl- $[-\beta$ -D-fructofuranosyl] n 1- β -D-fructofuranoside ($F_{py}F_n$) molecules, in which the DP varies from 2-7 (160). Both *in vivo* and *in vitro* studies have demonstrated that inulin and its oligosaccharides

selectively stimulate the growth of beneficial bacteria such as lactobacilli and/or bifidobacteria, both of which are considered to be beneficial to the host (156, 161). Additionally, studies suggest that inulin supplementation could aid in managing iron homeostasis, further highlighting its potential benefits in iron-related health problems and the health (157, 158, 162), as the beneficial effects of inulin and oligofructose on mineral bioavailability have been well documented in animals (158, 163, 164). In a study with 5-week-old anemic pigs, 4% inulin supplementation for 6 weeks resulted in higher expressions of iron transporters, such as DMT1, DCYTB1, FPN, ferritin, TFR, and mucin genes in addition to increased *Lactobacillus* and *Bifidobacterium* populations (158).

1.5. Pig as a Transitional Model for Human Infants

The pig serves as an exceptional translational model for studying various aspects of human development, physiology, and anatomy, particularly in the context of early-life nutrition and gastrointestinal health (165). Furthermore, the availability of well-characterized pig breeds and genetic tools allows for the generation of genetically modified pig models to study specific aspects of nutrition, metabolism, and gastrointestinal physiology relevant to human health (165). Moreover, pigs are omnivorous like humans, allowing for the investigation of dietary interventions and their effects on gut microbiota composition and host health (165).

Pigs undergo postnatal growth and development stages that parallel those of human infants, including the transition from a liquid to solid diet and the development of gastrointestinal and immune systems (165). Studying pig development provides valuable insights into nutrient requirements, metabolic adaptations, and gut microbiota establishment during early life, informing strategies for optimizing nutrition and gut health in human infants (165). Additionally, pigs exhibit

similar neurobehavioral development and immune maturation, allowing for comparative studies to elucidate the impact of nutritional interventions on overall health (165).

Unlike rodent models, pigs have a digestive system structure similar to humans, including comparable small intestine length and anatomy, and similar nutrient absorption mechanisms, facilitating the investigation of nutrient digestion, absorption, and gut barrier function during early development (165). The pig's size and organ development are more comparable to human infants, providing realistic scaling of doses and interventions. The neonatal pig brain grows rapidly after birth, similar to human infants (165).

Pigs exhibit physiological similarities to humans in various organ systems, including the gastrointestinal tract, liver, and pancreas (165). Notably, the structure and function of the pig gastrointestinal tract closely resemble those of humans, with similar digestive enzymes, pH, transit time, nutrient transporters, and gut microbial composition (165, 166). This similarity is particularly important when studying nutrient absorption and gut microbiota interactions.

A neonatal piglet model offers a promising opportunity to elucidate iron metabolism in early-life development and the impact of dietary prebiotics and probiotics because of the pig's similarity in nutritional requirements, especially for trace minerals and enteric microbiota composition to humans (165). Next-generation sequencing (NGS) has expanded our understanding of the roles that gut microbiomes play in human and animal health and diseases. Given that pigs serve as an important biomedical model for human diseases, the pig gut microbiome has drawn increasing attention. Modulation of the pig gut microbiome via probiotics and/or prebiotics to maintain or introduce a healthy microbiome has been a promising means of preventing pathogens and promoting beneficial bacteria abundances (166, 167). Therefore, the neonatal piglet model serves as an excellent translational model for the proposed studies.

1.6. Research Objectives

Early-life iron deficiency and overload pose significant health and developmental consequences, impacting growth, development, and immune function in both humans and pigs during early life. However, the mechanisms underlying these effects, particularly concerning iron homeostasis, gut microbiota modulation, and susceptibility to enteric infections in early life, remain incompletely understood. This dissertation seeks to fill these gaps by investigating 1) how iron deficiency and overload affect gut health and development, 2) beneficial effects of inulin and synbiotic supplements to improve iron homeostasis in neonatal piglets, and 3) how changes in dietary iron consumption levels modulate gut microbiota to influence susceptibility to enteric infections, such as ETEC, in weaned pigs. As a superior translational model due to physiological and anatomical similarities with humans, pigs were used in the studies to gain valuable insights and to bridge the gap between preclinical studies and clinical applications.

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CHAPTER 2

Iron Fortification and Inulin Supplementation in Early Infancy: Evaluating the Impact on Iron Metabolism and Trace Mineral Status in a Piglet Model

ABBREVIATIONS:

AI: iron adequate milk diet

cDNA: complementary deoxyribonucleic acid

CFU: colony-forming unit

DM: duodenal mucosa

DMT1: divalent-metal transporter 1

CYBRD1: duodenal cytochrome B

FPN: Ferroportin

gDNA: genomic DNA

HAMP: hepcidin

Hb: hemoglobin

Hct: hematocrit

HI: high iron milk diet

HIP: high iron milk diet with prebiotic

HIS: high iron milk diet with synbiotic

IACUC: International Animal Care and Use Committee

ID: iron deficiency

IDA: iron deficiency anemia

IL6: interleukin 6

IM: ileal mucosa

IRE: iron responsive element

IRP: iron responsive protein

mRNA: messenger RNA

MRS: de Man, Rogosa, and Sharpe

NRC: National Research Council

PD: postnatal day

qPCR: quantitative polymerase chain reaction

RCT: randomized controlled trials

RNA: ribonucleic acid

RN18S: ribosomal RNA 18S

ROI: region of interest

RT-qPCR: reverse transcription qPCR

TFR1: transferrin receptor

TIBC: transferrin binding capacity

TNFA: tumor necrosis factor- α

Tris-HCl: tris (hydroxymethyl) aminomethane

TSAT: transferrin saturation

UCD: University of California, Davis

ZIP14: zinc transporter 14

ABSTRACT

Background

Infant formula in the U.S. contains abundant iron, raising health concerns about excess iron intake in early infancy.

Objectives

Using a piglet model, we explored the impact of high iron fortification and pre- or synbiotic supplementation on iron homeostasis and trace mineral bioavailability.

Methods

Twenty-four piglets were stratified and randomly assigned to treatments on postnatal day (PD) 2. Piglets were individually housed and received an iron-adequate milk (AI), a high-iron milk (HI), the HI milk supplemented with 5% inulin (HIP), or the HIP milk with an oral gavage of *Ligilactobacillus agilis* YZ050, an inulin-fermenting strain, every third day (HIS). Milk was provided in 14 meals daily, mimicking formula feeding in infants. Fecal consistency score and body weight were recorded daily or every other day. Blood and feces were sampled weekly, and tissues collected on PD 29. Data were analyzed using mixed model ANOVA with repeated measures whenever necessary.

Results

Diet did not affect growth. High-iron milk increased hemoglobin, hematocrit and serum iron compared to AI. Despite marginal adequacy, AI milk upregulated iron transporter genes and maintained satisfactory iron status in most pigs. High-iron milk upregulated hepcidin gene in liver, caused pronounced tissue iron deposition, and markedly increased colonic and fecal iron. Inulin supplementation, regardless of *L. agilis* YZ050, not only attenuated hepatic iron overload but also decreased colonic and fecal iron without altering pH or iron regulatory genes. The HI milk lowered

zinc (Zn) and copper (Cu) in duodenum and liver compared to AI, while HIP and HIS further diminished Zn and Cu in liver and decreased colonic and fecal trace minerals.

Conclusion

Early-infancy excessive iron fortification causes iron overload and compromises Zn and Cu absorption. Inulin decreases trace mineral absorption likely through enhancing gut peristalsis and stool frequency.

Keywords: infant formula, inulin, iron overload, iron fortification, pig model, prebiotic, trace mineral

INTRODUCTION

In the United States, the average national breastfeeding initiation rate was 83.2% in 2019, but only 24.9% and 55.8% of infants received exclusive and any breastfeeding, respectively, at 6 months of age (1). A substantial number of infants receive formula as a supplement in varying amounts or as their primary source of nutrients during early infancy. The iron concentration significantly differs between breast milk and infant formula. For healthy term infants, iron from endogenous stores and breast milk generally meet the requirements up to 6 months of age (2). However, most infant formulas in the United States are fortified with iron at a concentration of 12 mg/L, which is 30 to 60 times higher than the iron content of breast milk and is still higher even when accounting for the low bioavailability of formula iron (3). It is worth noting that the low bioavailability of formula iron – a long-standing rationale supporting the current fortification dose – was determined in iron-replete infants at 6 – 7 months of age when solid foods, including cooked vegetables, cereals, meat, and eggs, had been introduced for 1 to 3 months (4). The study did not control for dietary factors that might inhibit iron absorption, nor did it adjust for infants' pre-existing iron status, another variable that could significantly modulate absorption.

Extensive research has been conducted to study the effectiveness of prophylactic iron in preventing anemia in infants and young children. However, much less research has explored the developmental consequence of excessive iron intake during infancy. Evidence drawn from a few studies demonstrated adverse effects of iron supplementation or feeding an iron-fortified formula on cognitive development, linear growth, and risk of infections in iron-replete infants (5-9), although the mechanisms underlying the adverse effects are not fully understood. Using suckling animal models, we and others have shown that the hepcidin-ferroportin axis, the systemic mechanism controlling iron absorption and homeostasis, is functionally immature in the early

postnatal stage (10, 11); iron over-supplementation through oral drops resulted in brain and body iron overload in suckling piglets (11). Few studies have evaluated iron and other trace mineral metabolism in response to excessive iron intake from a fortified formula in early infancy, and it is unclear what the safety range for lowering formula iron without negative consequences is.

The gut microbiota of formula-fed infants has been found to harbor a lower number of *Bifidobacterium* and *Lactobacillus*, which are present as either dominant bacteria or with high abundance in breast-fed infants (12-15). Additionally, iron fortification was shown to significantly decrease the relative abundance of *Lactobacillus* in the fecal microbiome of suckling rats (16). A similar finding was observed in a randomized controlled clinical trial: Ivorian children who received iron-fortified biscuits had significantly less fecal lactobacilli and greater counts of enterobacteria which may consist of enteropathogens and is believed to contribute to the heightened intestinal inflammation (17). Given the vital role of *Lactobacillus* species in gut health and development, there is increased interest in using pre-, pro-, and synbiotics to restore beneficial commensal communities in formula-fed infants (18, 19). Long-chain inulin, a non-digestible polysaccharide that is comprised of β -(2,1) glycosidic linked fructose residues, has been well-characterized for its prebiotic properties, particularly its capacity to stimulate the growth of bifidobacteria and lactobacilli (20-25). In addition, dietary supplementation with inulin or the mixture of long-chain inulin and oligofructose was shown to enhance iron bioavailability in anemic pig and iron-deficient rat models through a mechanism that is not yet fully understood (26-28). However, clinical trials yielded mixed results regarding the impact of inulin on iron absorption in infants and young children (29). A growing number of probiotics have been tested in various settings of formula feeding showing inconsistent outcomes for safety and efficacy, while the combined use of pre- and probiotics manifested a more pronounced influence on gut microbiota

(30, 31). Zhu et al. (2020) isolated *Ligilactobacillus agilis* YZ050 (*L. agilis* YZ050) from feces of milk-fed calves. *L. agilis* YZ050 displayed capacity of catabolizing inulin via an extracellular fructosidase, resulting in release of fructose, which could cross-feed lactic acid bacteria that are unable to ferment inulin (32). There is collective evidence suggesting intricate interplays between probiotics, gut microbiota, and trace minerals (33, 34). However, the application of inulin and inulin-consuming probiotics in iron-fortified formula and their impact on trace mineral absorption has not been comprehensively evaluated. In the current study, we used milk-fed neonatal piglets as a preclinical model for formula-fed infants. The objective is to determine how iron-fortification and inulin or synbiotic supplementation of inulin and *L. agilis* YZ050 modulate iron and trace mineral metabolism in early infancy.

MATERIALS AND METHODS

Study design and animal management. The study protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California, Davis (UCD). Twenty-four neonatal piglets (equal number of females and males) were stratified by birth body weight (BW) and sex and randomly assigned to one of the four dietary treatments (n = 6/treatment) at postnatal day (PD) 2. The experiment was conducted in a temperature-controlled room (25°C) with a pre-set 12 hour shift of light-dark cycle (light on 0700-1900 h) in the Cole A Facility at UCD. From PD 2 to 29, piglets were housed in individual cages that had a heating pad, a stationary feeding bowl, and an enrichment toy. Piglets in each of the four dietary treatments received an iron-adequate milk replacer (AI, 60 mg iron/(kg milk solids) supplemented with 5% (wt/wt milk solid) maltodextrin, a high-iron milk replacer (HI, 480 mg iron/(kg milk solids) supplemented with 5% maltodextrin, the HI milk supplemented with 5% inulin (Orafti® IPS, Beneo) as prebiotics

(HIP), or the HIP milk and an oral gavage of *L. agilis* YZ050 (5 mL, 2.5×10^9 CFU/mL; inoculants were prepared by Dr. David Mills laboratory in the Department of Food Science and Technology at UCD) once every 3 days during the study (HIS). This strain was shown to use inulin as a substrate (32, 35). The AI, HI, and HIP milk replacer solids was prepared by mixing a basal milk replacer (NutraStart Liqui-Wean without trace mineral premix, Milk Specialties Global, MN) that was free of trace mineral supplements with a mineral premix prepared in lab and maltodextrin or inulin. The basal milk replacer was analyzed for dry matter, crude fat, crude protein, and minerals at Cumberland Valley Analytical Service (Waynesboro, PA). The ingredient composition of experimental diets and nutrient profile of the basal milk replacer are listed in **Table 2.1.**, respectively. The milk replacer powder was reconstituted with deionized water following the manufacturer's instruction. Piglets were weighed every other day, and their daily liquid milk allowance was calculated as 270 mL/(kg·BW) based on the most recent body weight. The liquid milk was prepared every morning and stored in individual containers for each piglet. It was then automatically delivered from milk container to the corresponding feeding bowls inside each cage in 14 meals per day through polyethylene tubes. The automation of milk delivery was controlled by timer-regulated individual pumps, which were adjusted for meal time and duration every few days to accommodate the changes in the volume of daily milk allowance. Sometimes, the bending of the suckling tubes led to a small amount of milk being left in the containers. The leftover milk was emptied into the feeding bowl to ensure complete consumption of daily milk allowance calculated based on body weight.

Data and sample collection. Milk intake and body weight were recorded daily and on alternate days, respectively. Feeding response was assessed once daily at the first meal using a 4-scale scoring system (1 = pig approaches the feeding bowl immediately at the start of milk delivery and

finishes the meal within 1 min after delivery, 2 = pig takes more than 1 min after delivery to finish the meal, 3 = pig stops drinking milk at a certain point of delivery, 4 = pig ignores milk delivery). Fecal consistency was scored twice daily by a well-trained researcher at 0700 and 1900 h using a 5-scale scoring system (1 = normal firm and shaped feces, 2 = soft and shaped feces, 3 = mild diarrhea with mushy and loose stools, 4 = severe diarrhea with feces appearing as slurries on the floor, and 5 = severe diarrhea with watery stools), which had been validated in previous research (36). Fecal samples were obtained using a rectal swab, and blood samples were collected from the jugular vein through venipuncture in sodium heparin vacutainers (Becton, Dickinson and Company, Franklin Lakes, NJ) before the first meal on PD 2 (baseline), 8, 15, 22, and 29. All piglets were euthanized on PD 29, and sampled for mucosa from intestinal segments, intestinal tissues, colon contents, and peripheral organs. After euthanasia, tissue samples were snap-frozen in liquid nitrogen and subsequently transported back to the laboratory for storage at -80°C.

Hematological indices. Hemoglobin (Hb) was measured using the Drabkin's reagent (Sigma-Aldrich, St. Louis, MO) following the manufacturer's instruction with modification (250 µL sample mixture) for detection using a microplate reader (BioTek Synergy HTX, Winooski, VT). Absorbance was determined at 540 nm and Hb concentration was calculated based on a standard curve. Hematocrit (Hct) was determined using a microhematocrit centrifuge.

Analysis of iron and trace mineral status. Plasma iron, total-iron binding capacity (TIBC), and transferrin saturation were measured and calculated using the Pointe iron/TIBC assay kit (Pointe Scientific, Canton, MI). Paraffin-embedded coronal sections of the duodenum and proximal colon (6 µm) were stained for ferric iron with Prussian blue stain contained 5% potassium ferrocyanide and 5% hydrochloric acid and counter-stained with 0.1% nuclear fast red solution (Sigma-Aldrich). Slides were digitally imaged by an Olympus BX51 microscope at 10× and 20× objective

magnification. Crypt-villus areas were manually traced as regions of interest (ROI) under ImageJ. The percentage of iron-stained area within ROI was quantified using ImageJ (37). The concentrations of iron and other trace minerals in intestinal tissues, liver, colon content, and feces were analyzed as previously described (11). Briefly, samples (50 – 200 mg) were digested in 4 mL of 16 mol/L nitric acid, Ultrex II, J.T. Baker™ (Fisher Scientific, Hampton, NH) in screw-top glass vials for up to 120 hours. The samples were placed on a heating plate and boiled at 100°C until minimal liquid left. The samples were diluted with ultrapure water to a final volume of 5 ml and analyzed by inductively coupled plasma optical emission spectroscopy (ICP-OES) (iCAP 6300, Thermo Electron, MA) to determine concentrations of iron (Fe), zinc (Zn), copper (Cu), and manganese (Mn).

pH in fecal samples and colon digesta. pH of fecal samples from PD 2, 8, 15, 22, 29 and proximal colon digesta were measured following standard operating procedures as previously described (38). Samples of feces and colon digesta (100 mg) were diluted and homogenized in 1 ml of phosphate-buffered saline solution (PBS) for pH measurements. pH was measured by a standard pH meter (Orion Research Inc., Boston, MA) with a micro combination electrode (Fisher Scientific, Lenexa, KS).

Gene expression analysis. The mRNA expression of genes was determined using reverse transcription quantitative PCR (RT-qPCR). Samples (~100 mg) from duodenum mucosa (DM), ileum mucosa (IM), colon tissue, and liver were homogenized by TissueLyser II (QIAGEN, Hilden, Germany), and total RNA was extracted using TRIzol reagent (Invitrogen, Waltham, MA). Concentration and purity of the total RNA were assessed using NanoDrop One (ThermoFisher Scientific). Total RNA (1.5 µg) was used as a template for cDNA synthesis using the High-Capacity Reverse Transcription kit with RNase inhibitor (Applied Biosystems, Foster City, CA).

RT-qPCR was performed in 20 μ L reaction following the protocol of PowerUP SYBR Green Master Mix (Applied Biosystems) on a QuantStudio 3 Real-Time PCR System (Applied Biosystems). The 20 μ L reaction contains 10 μ L 2X SYBR Green Master Mix, 6 μ L cDNA template, 1.5 μ L of each primer, and 1 μ L nuclease-free water. Target genes encoding ferroportin (*FPN*), transferrin receptor-1 (*TFR1*), divalent-metal transporter (*DMT1*), duodenal cytochrome B (*CYBRD1*), zinc transporter 14 (*ZIP14*) were analyzed in intestinal mucosa and colon samples, and *DMT1*, *FPN*, *TFR1*, and hepcidin (*HAMP*) were determined in the liver, and tumor necrosis factor- α (*TNFA*) and interleukin 6 (*IL6*) were determined in the ileum. Ribosomal RNA 18S (*RN18S*) was used as the housekeeping gene. The expression of target genes was normalized against the housekeeping gene and calculated for relative expression using the comparative $2^{-\Delta\Delta CT}$ method.

Quantification of fecal *L. agilis* YZ050 abundance. The abundance of *L. agilis* YZ050 in fecal samples was determined based on the expression of the strain-specific *Sir2* gene analyzed via RT-qPCR following a standard curve method. To prepare the standard samples, *L. agilis* YZ050 was grown to 3×10^9 CFU/mL in De Man-Rogosa-Sharpe (MRS) broth at 37°C for 48 hours. A pellet of *L. agilis* YZ050 was harvested after centrifugation and extracted for genomic DNA (gDNA) using the Quick-DNA Fecal/Soil Microbe Kit (Zymo Research, Irvine, CA). The concentration and purity of gDNA were determined using the NanoDrop One (ThermoFisher Scientific). A total of 9 standard samples were prepared after the serial 10-fold dilutions of the gDNA sample in Tris-HCl buffer (10 mM, pH 8.0). The gDNA standards correlate to 10^9 , 10^8 , 10^7 , 10^6 , 10^5 , 10^4 , 10^3 , 10^2 , 10^1 *L. agilis* YZ050 cells/mL.

The primer pair designed to target the *Sir2* gene of *L. agilis* YZ050 was provided by Dr. David Mills's laboratory. To validate the specificity and efficiency, a qPCR analysis was conducted using fecal DNA samples from AI pigs as well as the same samples spiked with the

DNA isolated from *L. agilis* YZ050 (1×10^7 cells/mL). The specificity was confirmed by the presence of a single peak in the melting curve of the spiked samples, whereas no peak was detected in the non-spiked samples. The reaction efficiency was verified to align with the normal range of 0.9 to 1.1. The expression of *Sir2* gene was determined in the DNA isolated from experimental and standard samples through the SYBR Green-based qPCR analysis. A standard curve that correlates Ct values and the cell concentrations of the standard samples was used to calculate the abundance of *L. agilis* YZ050 in feces and colon digesta.

Statistical analysis. Statistical analysis and data visualization were conducted using GraphPad Prism 8 (GraphPad, San Diego, CA). Homoscedasticity plot and Q-Q plot were used to assess equal variance and normal distribution. Data fit both assumptions were analyzed using one- or two-way ANOVA. The model included the fixed effects of treatment and day and the treatment by day (T×D) interactions and subject as a random term. For variables measured over time, repeated measurement was used in the model. Tukey's test was used to adjust for multiple comparisons. *HAMP* mRNA expression data in the liver exhibited unequal variances and significant skewness, thus it was analyzed using the Kruskal-Wallis test for the main effect of treatment, followed by Dunn's test for multiple comparisons. The frequency of diarrhea was calculated as the total pig days with a fecal score ≥ 3 for each treatment group. Data of pairwise comparisons were analyzed using Fisher's exact test. Statistical significance and a trend towards significance were declared at $P < 0.05$ and $P < 0.1$, respectively.

RESULTS

Growth and diarrhea. Body weight and growth were not affected by the dietary treatment (**Fig. 2.1. A**). As liquid milk was provided at 270 mL/(kg·BW) daily, there was not significant treatment effect on milk consumption (data not shown). Piglets were healthy and active during the study with the exception that one AI piglet suddenly died on PD 27. The exact cause of sudden death was unclear but cannot exclude anemia which was detected with a Hb of 90 g/L on PD 22. The HIS pigs had the highest frequency (17 pig days) of diarrhea with a fecal score ≥ 3 . It was significantly higher than that of HI pigs, while no significant difference was observed in any other pairwise comparisons (**Fig. 2.1. B and C**). Pigs from all treatments were eager to eat and had a low feeding response score throughout the study (data not shown).

Risk of iron deficiency anemia. The AI pigs had the lowest serum iron which was significantly lower than that of HI and HIS pigs on PD 8 and 15 or compared with HIS on PD 22 ($P < 0.05$, **Fig. 2.2. A**). Similarly, transferrin saturation was the lowest in the AI during the study; the difference was significant between the AI and HI on PD 8 and 15 ($P < 0.05$, **Fig. 2.2. B**). The TIBC should be reversely associated with circulating iron. Indeed, it was highest in the AI during the study; the differences between the AI and HI and HIP were significant on PD 8 and 15 ($P < 0.05$, **Fig. 2.2. C**). Both Hb and Hct were significantly lower in the AI group than in the other groups on PD8, 15, and 29 ($P < 0.05$, **Fig. 2.2. D and E**, respectively), while no pig was anemic ($\text{Hb} < 110 \text{ g/L}$) during the study except the one that died suddenly.

Body iron status. Prussian blue staining showed a pronounced non-heme iron deposition in the duodenal (**Fig. 2.3. A**) and colon mucosa (**Fig. 2.3. B**) crypt-villus areas in all three groups fed the high-iron diets compared to negligible amounts in the AI group ($P < 0.05$, **Fig. 2.3. C and D**) when the percentage of iron-stained area within ROI was quantified. In comparison with the HI group,

HIP and HIS groups had significantly lower mucosal iron in the duodenum ($P < 0.05$, Fig. 2.3. C), but not in the colon. Furthermore, high-iron milk resulted in iron overload in liver ($P < 0.0001$, **Table 2.2.**) and duodenum ($P < 0.01$, Table 2.2.), and increased ($P < 0.0001$, Table 2.2.). Both the HIP and HIS treatment decreased iron accumulation in liver ($P \leq 0.05$), duodenum ($P \leq 0.05$), and colon digesta ($P \leq 0.001$) compared to the HI. A similar trend was observed in fecal iron concentration (**Table 2.3.**). Compared to the minimal fecal iron in pigs fed the AI, the high-iron diets significantly increased iron excretion in feces on PD 8, 15, and 22 ($P < 0.05$), although both HIP and HIS treatment lessened increase compared to the HI. On PD 29, fecal iron concentration was significantly higher ($P < 0.05$) in the HI group than in the other groups which were not different from each other.

Trace mineral status. The impact of the treatments on trace mineral status (Fe, Zn, Cu, Mn) across feces (Table 2.3.), colon, duodenum, and liver (Table 2.2.) was investigated. Notably, the HI group consistently exhibited higher iron concentrations in feces ($P < 0.001$), colon ($P < 0.0001$), and liver ($P < 0.0001$) compared to the AI, HIP, and HIS groups, while HI group was only higher than AI group in duodenum ($P < 0.01$), where AI had substantially lower iron than groups fed high-iron diets. Variations in iron concentrations were noted with time, and distinct trends emerged across the study duration. Additionally, comparisons between organs on the same day highlighted differences in iron concentrations.

Notable trends were found for the effect of treatments on Zn status. There was a distinct pattern between AI and HI piglets where they had similar Zn status in feces and tissues except for in duodenum where AI piglets had higher Zn concentration than HI piglets ($P < 0.05$). In feces, HIP and HIS piglets showed significantly lower Zn concentration than the AI piglets on PD 15 ($P < 0.01$) and PD 29 ($P < 0.001$) and HI piglets on PD 15 ($P < 0.05$). HIP piglets displayed lower Zn

concentrations than AI piglets in liver ($P < 0.05$), colon ($P < 0.05$), and feces on PD 15 ($P < 0.01$) and PD 29 ($P < 0.001$) and HI piglets in colon ($P < 0.05$), and feces on PD 15 ($P < 0.05$), while HIS piglets displayed lower Zn concentration than AI piglets in liver ($P < 0.01$) and feces on PD 15 ($P < 0.01$) and PD 29 ($P < 0.01$) and HI piglets in feces only on PD 15 ($P < 0.05$).

For Cu status, there was a distinct pattern where HIP and HIS piglets had lower Cu concentrations compared to AI and HI piglets in feces and tissues. In feces, HIP piglets had lower Cu concentrations compared to AI piglets on PD 29 ($P < 0.01$) and HI piglets on PD 22 ($P < 0.05$), PD 29 ($P < 0.01$) and was similar to HIS piglets throughout the study. In duodenum, AI piglets had higher Cu concentration than HI piglets ($P < 0.05$). In liver, AI piglets had higher Cu concentrations than HIP ($P < 0.01$) and HIS piglets ($P < 0.001$), while HI piglets had slightly higher concentration than HIP and HIS piglets, but lower concentration than AI piglets. In colon, though HIP and HIS piglets had lower Cu concentrations than AI and HI piglets, there was no significant difference found among treatments.

Fecal and tissue Mn showed similar trend as fecal and tissue Cu, except in liver and duodenum where no significant differences were found between treatments. In feces, HIP and HIS piglets had lower Mn concentration than AI piglets on PD 15 ($P < 0.001$) and 29 ($P < 0.05$) and HI piglets on PD 15 ($P < 0.05$). In colon, HIP ($P < 0.001$) and HIS piglets ($P < 0.01$) had lower Mn concentrations than AI and HI piglets.

Overall, the findings emphasize the complex interaction between treatments, sites, and time in influencing trace mineral status, emphasizing the complex interplay of trace mineral metabolism in pigs that should be further investigated. This should provide valuable insights for further research and potential applications in dietary iron and prebiotic related interventions.

Gene expression of iron regulatory proteins and inflammatory markers. The mRNA expression of ferric reductase (*CYBRD1*) was not affected in DM but was higher in the colon of HIS piglets than HIP and AI piglets ($P < 0.05$, **Fig. 2.4. A and F**). The relative expression of *DMT1* and *TFR1* in DM and colon of AI pigs were 2 – 4 fold higher than the three groups fed the high-iron diets ($P < 0.05$, **Fig. 2.4. B, D, G, and I**). However, the mRNA expression of *ZIP14* was not affected by treatment in either DM or colon (**Fig. 2.4. C and H**). The expression of *FPN* in DM and liver was not affected by treatment (**Fig. 2.4. E and N**), but its expression in colon was significantly higher in AI group than the other groups ($P < 0.01$, **Fig. 2.4. J**). The AI diet significantly increased *TFR1* expression in liver ($P < 0.001$, **Fig. 2.4. M**), while feeding a high-iron milk dramatically increased *HAMP* in the liver compared to the AI group regardless of inulin or synbiotic supplementation ($P < 0.04$, **Fig. 2.4. O**). In the IM, *IL6* ($P = 0.07371$, **Fig. 2.4. K**) and *TNFA* ($P = 0.724$, **Fig. 2.4. L**) were unaffected by treatment.

Enteric colonization of *L. agilis* YZ050. The HIS pigs received an average of 1.3×10^{10} CFU/5 mL of *L. agilis* YZ050 every 3 days via oral gavage. The abundance of *L. agilis* YZ050 in feces was extrapolated based on *Sir2* gene expression. The abundance of *L. agilis* YZ050 was nearly undetectable in all groups on PD 2 (baseline) suggesting this strain is not indigenous in pig gut (data not shown). *L. agilis* YZ050 was only detected in the fecal samples of HIS piglets with an abundance of 10^5 cells/g feces on PD 15 and 29 ($P < 0.05$, data not shown).

pH of colon content and feces. Dietary treatments did not affect pH of colon digesta or fecal samples collected on PD 15 and 29 with the exception of fecal pH being lower in HIP than AI on PD29 ($P < 0.05$, data not shown). The difference is statistically significant but numerically small and thus its biological importance is uncertain.

DISCUSSION

We used a neonatal pig model to investigate the impacts of iron fortification, inulin, and synbiotic supplementation in a cow milk-based formula on trace mineral metabolism. According to the Swine NRC (2012), milk-fed young piglets should receive 50 – 150 mg iron/kg of milk solid to meet the requirement (39). When fed the AI milk that provided iron at the lower end of this recommendation (60 mg/kg of milk solid), five out of six piglets maintained a satisfactory iron level and non-anemic status (defined as Hb > 110 g/L in infants or Hb > 100 g/L in piglets). This was likely achieved through upregulation of intestinal iron absorption indicated by the higher gene expression of iron transporter (*DMT1*) in the duodenum and colon. Meanwhile, the extremely low hepatic expression of hepcidin (*HAMP*) also facilitates basolateral iron efflux from enterocytes. The nearly nondetectable level of iron in the duodenal and colon mucosa of the AI pig corroborated the enhanced iron export to the systemic circulation. Similar changes in *DMT1* and *HAMP* expression were observed in sow-reared piglets that received no or a low-dose iron supplement through oral drops (11). The transcriptional changes of *DMT1* and hepcidin indicates a negative feedback regulation of intestinal iron uptake in response to lower iron intake. This implies that the absorption rate of iron from infant formula may vary inversely with its level of fortification. Despite this, one AI piglet developed anemia during the study, indicating an elevated risk of IDA with marginally adequate dietary intake.

Few studies have evaluated feeding a formula fortified with the lowest recommended iron level in young infants. In a randomized controlled trial (RCT), Björmsjö et al. (2020) found that healthy term infants fed a low-iron formula (2 mg/L) to 6 months of age, regardless of the lactoferrin supplementation, did not have an increased risk of ID or IDA compared to those fed a formula containing 8 mg iron/L (40). This result is consistent with the finding of an earlier study

that showed a formula containing 1.6 mg iron/L being sufficient to meet requirements up to 6 months of age (41). The altered gene expression of iron regulatory proteins observed in AI pigs provides a plausible molecular mechanism that corroborates these clinical findings. Both lines of evidence underscore an opportunity for moderate reduction of formula iron, although more well-powered RCTs are required to define small differences and fine-tune the optimal iron level.

Hepcidin acts as the master regulator of systemic iron homeostasis, inhibiting ferroportin-mediated intestinal iron release when body iron status is adequate, thus preventing iron overload (42). The consumption of high-iron milk, regardless of inulin or synbiotic supplementation, led to a dramatic increase in *HAMP* expression in the liver and significant iron deposition in the mucosa of the duodenum and colon. These findings support the well-established role of hepcidin. However, the significant accumulation of hepatic iron, particularly in the HI pigs, suggests a contrasting perspective. One possible explanation for these seemingly contradictory outcomes could be attributed to the developmental changes in the functional maturity of the hepcidin-ferroportin axis. We and others have observed that, despite the pronounced upregulation of hepcidin in response to excessive iron intake, ferroportin expression in enterocytes was resistant to hepcidin-induced degradation and its inhibitory effect on iron efflux in suckling piglets and mouse pups, respectively (10, 11). However, intact hepcidin-ferroportin regulation was observed in the gut of weaned mice (10).

Developmental maturation of systemic iron regulation was also reflected by the temporal changes in serum iron in the current study: high iron intake significantly increased serum iron concentration, which would have otherwise remained stable if hepcidin regulation had been effective, during the first two weeks after birth. In contrast, serum iron concentration was unaffected by dietary treatment during the following two weeks, implying the functional activity

of the hepcidin-ferroportin axis. The immature hepcidin-ferroportin axis may facilitate excess iron absorption and hepatic deposition, whereas more iron will be sequestered in intestinal lining when ferroportin-mediated iron efflux becomes sensitive to the inhibitory effect of hepcidin. Iron over-supplementation via oral drops was found to significantly elevate iron load in the liver and the developing brain (11).

Increased colonic iron absorption through hepcidin-independent route may also contributed to hepatic iron overload. Although the large intestine was shown to account for only 5% of whole intestinal iron absorption in weaned pigs fed a solid diet (43), intracecal iron infusion at a high dose (20 mg/kg body weight) led to a sustained large increase of iron in the portal blood in growing pigs, presumably resulting from increased colonic epithelial permeability (44). In the current study, considering the abundant expression of iron transporters and marked iron accumulation in the colon mucosa, substantial colonic iron absorption cannot be excluded in pigs fed the high-iron milk. Together, the current piglet model demonstrates significant body iron accumulation and elevated colonic iron flux resulting from excessive dietary iron exposure during the early postnatal period. This highlights the critical need to evaluate the risk of iron overload in term infants who transition to a fortified formula as their main nutrient source during early infancy in the United States. Additionally, particular emphasis should be placed to the elevation of colonic iron levels and its potential impact on gut microbiota and the associated risk of enteric infection in clinical trials.

The inulin product used in this study was extracted from chicory root, comprising approximately 90% long-chain fructan (inulin) and up to 10% mono-, di- and oligosaccharides. Numerous studies have reported the prebiotic properties of inulin, particularly its effects on promoting the growth of bifidobacteria and lactobacilli (45, 46). Lactobacilli were shown to

present at lower counts in the gut microbiota of formula-fed infants compared to their breast-fed counterparts (12-14), meriting inulin supplementation in infant formula. In addition to its bifidogenic effect, studies have demonstrated an enhancing effect of supplementing inulin or the mixture of long-chain inulin and oligofructose on iron bioavailability in anemic pig and iron-deficient rat models through a mechanism that is not fully understood (26-28). In a study with anemic pigs, Yasuda et al. (2006) found that supplementing 4% inulin in a corn and soybean meal-based diet significantly increased total soluble iron in the colon digesta and hemoglobin repletion efficiency, indicating greater iron bioavailability (26). These findings align with the hypothetical model that inulin fermentation acidifies gut lumen and facilitates ferric reduction and subsequent uptake via DMT1. However, neither the pH of colon digesta nor intestinal or colonic iron absorption, which was directly measured through stable isotopes in the following study, was altered by inulin supplementation (26, 43). Similarly, in the current study, milk replacer supplemented with 5% inulin did not alter colon or fecal pH (data not shown). Tako et al. (2008) observed higher mRNA expression of iron transporters (e.g. *DMT1* and transferrin receptor, presumably *TFR1*) within duodenum and colon of inulin-supplemented pigs, which displayed greater hemoglobin repletion efficiency (22). However, caution should be exercised when interpreting results of increased gene expression. As the mRNA abundance (stability) of *DMT1* and *TFR1* is inversely regulated by cellular iron status via the IRE-IRP system, an upregulation should have indicated a lower cellular iron status in the duodenum and colon of inulin-fed pigs, resembling the findings observed in the AI pigs in the current study.

Few studies have evaluated the iron modulatory effect of inulin in formula-fed infants or young animals in the context of dietary iron over-consumption. Contrary to the previous findings, inulin supplementation in the high-iron milk (HIP and HIS) not only did not affect hemoglobin,

hematocrit, or total serum iron but also reduced hepatic iron accumulation compared to the HI group. These results suggest that inulin supplementation may have reduced iron absorption and mitigated iron overload caused by the high-iron formula. Since mRNA expression of the iron transporters (*DMT1* and *TFR1*) in duodenum and colon did not differ between inulin-supplemented pigs and HI pigs, it is unlikely that absorption capacity was affected by inulin or that it explained the lower hepatic iron accumulation in inulin-fed pigs.

The reduction in iron absorption would have increased the intestinal flow of unabsorbed iron. Surprisingly, both HIP and HIS pigs had lower colonic and fecal iron concentrations than the HI group. Inulin fermentation is believed to promote the production of gas and short-chain fatty acids, potentially enhancing colon peristalsis through mechanical and chemical stimulation, respectively (47, 48). Therefore, it is reasonable to speculate that the concurrent low intestinal, fecal, and hepatic iron levels in the inulin-fed pigs resulted from increased bowel movement (shortened colonic transit time) and stool frequency. These factors reduce colonic iron absorption and dilute its concentration in digesta and feces. In support of this, a recent meta-analysis of 14 randomized controlled studies involving participants of all age groups identified an increase in stool frequency as a significant gastrointestinal effect of dietary supplementation with chicory-derived inulin-type fructan (46, 49). Although this increase was not statistically significant in the subgroup analysis of five studies conducted with formula-fed infants (46, 49), it should be noted that the doses of long-chain inulin contained in the prebiotic supplements varied across these studies (23, 49-52). Increasing stool frequency was also recognized as an outcome of consuming native chicory inulin, as concluded by the European Food Safety Authority after reviewing six studies involving 86 subjects who consumed at least 12 g of inulin per day (53).

Increased gut motility and stool frequency may also explain similar changes in other trace minerals: the lowest concentrations were found in the colon digesta, feces, and liver except for hepatic Mn concentration, which remained unaffected, in inulin-fed groups regardless of *L. agilis* YZ050 supplementation. The duodenum serves as an absorptive site for all trace elements measured in this study. The lower Zn and Cu levels in duodenal tissue of pigs fed the high-iron milk may indicate an antagonistic effect of excessive iron on Zn and Cu absorption. It is still debated whether these trace elements compete for shared absorptive pathways, such as DMT1 (54, 55). Clinical trials evaluating the impact of iron supplementation on the absorption of other trace elements during infancy have also yielded conflicting results (56, 57). It is worth noting that inulin supplementation (HIP and HIS) resulted in a more pronounced reduction of hepatic Zn and Cu levels, despite the similar levels of Zn and Cu in the duodenum of the three groups fed the high-iron milk. This finding suggested that inulin supplementation at 5% of milk solids iron-independently reduced the bioavailability of these trace minerals likely through decreasing colonic absorption.

The abundance of *L. agilis* YZ050 in feces collected on PD15 and 29 (1 or 2 days after the most recent inoculation) reached 10^5 CFU/g feces (data not shown). This indicates moderate to low colonization, although persistence cannot be assessed without a sample collected several days after the cessation of inoculation. Some studies have reported enhancing effects of probiotic lactic acid bacteria (e.g. *Lactiplantibacillus plantarum*, *Lactobacillus acidophilus* and *Limosilactobacillus reuteri*) on iron absorption in infants and children with ID or IDA (58, 59). However, in the current study, the lack of a significant difference in any measurements between the HIP and HIS groups suggested a minimal effect of *L. agilis* YZ050 on bioavailability of iron and other trace minerals in the context of excessive iron intake.

In conclusion, through a piglet model, we showed that feeding a high-iron formula in early infancy led to body iron overload and decreased Zn and Cu bioavailability. This effect is possibly due to the antagonistic effect of excess iron on duodenal absorption of Zn and Cu. Moreover, feeding a marginally iron-adequate milk triggered the upregulation of genes involved in iron absorption and maintained satisfactory iron status in most piglets, highlighting the opportunity to lower iron fortification in infant formula. Considering that one AI pig developed anemia, it is imperative for the future study to evaluate the safety range of iron reduction in a fortified formula. Instead of enhancing iron absorption, as observed in other studies, inulin supplementation (5% of milk solids) in an iron-fortified milk decreased hepatic and fecal concentrations of Fe, Zn, and Cu. We postulate that inulin may enhance gut motility and increase defecation frequency, leading to a shortened digesta transit time and decreased absorption of trace minerals. Utilizing an isotope-based analytical approach in the future studies is warranted to directly analyze the bioavailability and fecal excretion of trace minerals.

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TABLES AND FIGURES

Table 2.1. Feed ingredient and mineral premix compositions of experimental diets

Item	AI ²	HI	HIP	HIS
<i>Ingredient, % diet solid</i>				
Basal milk replacer ¹	94.93	94.80	94.80	94.80
Inulin	0.00	0.00	5.00	5.00
Maltodextrin	5.00	5.00	0.00	0.00
Mineral premix	0.07	0.20	0.20	0.20
<i>Mineral premix, mg/kg diet solid</i>				
Zinc sulfate (ZnSO ₄ ·7H ₂ O)	373.79	373.79	373.79	373.79
Copper sulfate (CuSO ₄ ·5H ₂ O)	76.05	76.05	76.05	76.05
Manganese sulfate (MnSO ₄ ·H ₂ O)	76.76	76.76	76.76	76.76
Calcium iodate (Ca(IO ₃) ₂)	2.96	2.96	2.96	2.96
Cobalt sulfate (CoSO ₄ ·7H ₂ O)	4.76	4.76	4.76	4.76
Sodium selenite (Na ₂ SeO ₃)	0.66	0.66	0.66	0.66
Ferrous sulfate (FeSO ₄ ·H ₂ O)	121.86	1401.43	1401.43	1401.43

¹The basal milk replacer was not supplemented with trace mineral premix. The basal milk replacer provided (minimum unit per kg solid) Lysine (498 g), vitamin A (22,046 IU), vitamin D (4,409 IU), and vitamin E (110 IU).

²AI, iron adequate milk diet; HI, high iron milk diet; HIP, high iron milk with prebiotic diet; HIS, high iron milk with synbiotic diet.

Table 2.2. Trace mineral concentrations in colon digesta, duodenum, and liver on PD 29.

Site	Trace mineral	Day	Trace mineral concentration ¹ , $\mu\text{mol/g}$				P-value
			AI	HI	HIP	HIS	
Colon digesta	Iron	29	23.35 ^c $\pm$ 5.92	138.50 ^a $\pm$ 16.57	51.92 ^{bc} $\pm$ 6.64	73.03 ^b $\pm$ 13.54	< 0.0001
	Zinc	29	46.97 ^a $\pm$ 3.04	47.73 ^a $\pm$ 4.81	23.50 ^b $\pm$ 1.6	29.94 ^{ab} $\pm$ 3.6	0.014
	Copper	29	7.69 $\pm$ 0.83	8.64 $\pm$ 0.9	4.54 $\pm$ 0.29	5.67 $\pm$ 0.59	0.0552
	Manganese	29	22.83 ^a $\pm$ 2.84	22.60 ^a $\pm$ 2.02	8.96 ^b $\pm$ 0.75	11.82 ^b $\pm$ 1.49	< 0.0001
Duodenum	Iron	29	3.47 ^b $\pm$ 0.43	15.37 ^a $\pm$ 1.48	12.31 ^a $\pm$ 2.59	13.77 ^a $\pm$ 3.20	0.0021
	Zinc	29	5.25 ^a $\pm$ 1.35	2.26 ^b $\pm$ 0.28	2.36 ^{ab} $\pm$ 0.3	2.61 ^{ab} $\pm$ 0.26	0.0336
	Copper	29	0.70 ^a $\pm$ 0.15	0.34 ^b $\pm$ 0.03	0.34 ^b $\pm$ 0.07	0.44 ^{ab} $\pm$ 0.03	0.0325
	Manganese	29	0.21 $\pm$ 0.03	0.15 $\pm$ 0.02	0.20 $\pm$ 0.03	0.21 $\pm$ 0.05	0.3676
Liver	Iron	29	6.31 ^c $\pm$ 0.93	24.75 ^a $\pm$ 1.87	15.09 ^{bc} $\pm$ 3.12	16.00 ^{ab} $\pm$ 2.38	0.0001
	Zinc	29	27.75 ^a $\pm$ 3.24	22.36 ^{ab} $\pm$ 2.58	16.68 ^b $\pm$ 1.33	16.09 ^b $\pm$ 1.44	0.0055
	Copper	29	11.40 ^a $\pm$ 1.58	7.95 ^{ab} $\pm$ 0.65	5.58 ^b $\pm$ 0.77	4.23 ^b $\pm$ 0.4	0.0002
	Manganese	29	0.28 $\pm$ 0.04	0.33 $\pm$ 0.02	0.32 $\pm$ 0.02	0.33 $\pm$ 0.01	0.5351

¹ Data are presented as means $\pm$ SEM. Multiple comparisons were performed across treatments. Values sharing no common superscript are significantly different (adjusted P < 0.05).

Table 2.3. Effect of dietary treatments on trace mineral concentrations in fecal samples collected on PD 8, 15, 22, and 29.

Trace mineral	Day	Trace mineral concentration ¹ , $\mu\text{mol/g}$				P-value		
		AI	HI	HIP	HIS	Treatment $\times$ Day	Treatment	Day
Iron	8	9.06 ^b $\pm$ 1.86	190.70 ^a $\pm$ 63.22	100.60 ^a $\pm$ 20.24	109.10 ^a $\pm$ 21.58	0.5046	< 0.0001	0.001
	15	9.28 ^b $\pm$ 1.01	187.24 ^a $\pm$ 51.32	84.45 ^{ab} $\pm$ 5.34	79.00 ^{ab} $\pm$ 19.68			
	22	9.71 ^c $\pm$ 1.75	251.20 ^a $\pm$ 24.8	149.60 ^b $\pm$ 20.94	174.60 ^b $\pm$ 13.60			
	29	88.89 ^b $\pm$ 44.42	289.10 ^a $\pm$ 34.28	116.20 ^b $\pm$ 14.36	157.60 ^b $\pm$ 12.44			
Zinc	8	87.94 $\pm$ 21.68	78.07 $\pm$ 18.32	35.60 $\pm$ 9.65	41.94 $\pm$ 5.44	0.0716	< 0.0001	0.02
	15	158.01 ^a $\pm$ 14.77	126.54 ^a $\pm$ 21.43	47.24 ^b $\pm$ 3.45	43.01 ^b $\pm$ 7.84			
	22	83.56 $\pm$ 19.95	96.08 $\pm$ 17.12	43.37 $\pm$ 2.13	47.69 $\pm$ 5.29			
	29	118.10 ^a $\pm$ 8.27	69.36 ^{ab} $\pm$ 21.59	51.48 ^b $\pm$ 4.37	55.42 ^b $\pm$ 3.11			
Copper	8	6.36 $\pm$ 1.05	9.09 $\pm$ 3.11	4.87 $\pm$ 1.5	6.47 $\pm$ 0.81	0.5241	0.0059	0.0005
	15	11.84 $\pm$ 2.63	13.82 $\pm$ 1.38	8.64 $\pm$ 0.66	8.64 $\pm$ 1.63			
	22	11.87 ^{ab} $\pm$ 1.02	13.15 ^a $\pm$ 1.16	8.45 ^b $\pm$ 0.29	8.79 ^b $\pm$ 1.01			
	29	17.93 ^a $\pm$ 1.64	14.50 ^{ab} $\pm$ 3.55	9.19 ^c $\pm$ 0.60	10.33 ^{bc} $\pm$ 0.50			
Manganese	8	54.34 $\pm$ 13.73	48.07 $\pm$ 12.27	22.90 $\pm$ 5.23	20.29 $\pm$ 2.6	0.0867	< 0.0001	0.1419
	15	87.63 ^a $\pm$ 5.09	62.68 ^a $\pm$ 13.30	18.86 ^b $\pm$ 1.47	17.41 ^b $\pm$ 3.23			
	22	43.41 $\pm$ 10.98	50.93 $\pm$ 13.44	19.94 $\pm$ 1.18	21.25 $\pm$ 2.24			
	29	60.39 ^a $\pm$ 7.6	36.25 ^{ab} $\pm$ 8.89	22.56 ^b $\pm$ 1.92	23.59 ^b $\pm$ 1.11			

¹ Data are presented as means $\pm$ SEM. Multiple comparisons were performed across treatments within each sampling day. Values sharing no common superscript are significantly different (adjusted P < 0.05).

Figure 2.1.

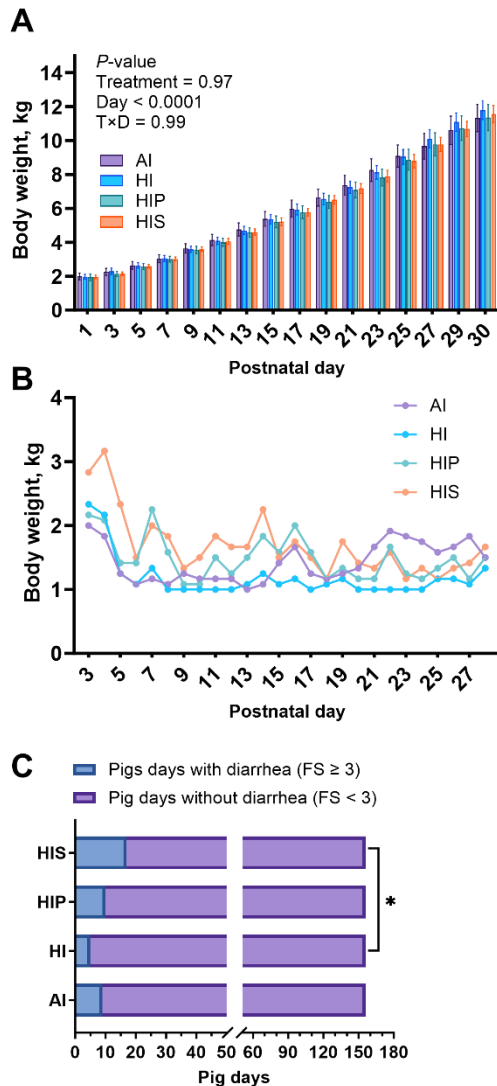


Figure 2.1. Effects of dietary treatments on body weight, fecal consistency, and frequency of diarrhea. Body weight (A) was recorded every other day throughout the study. Fecal consistency (B) was scored twice daily using a 5-scale scoring system (1 = normal firm and shaped feces, 2 = soft and shaped feces, 3 = mild diarrhea with mushy and loose stools, 4 = severe diarrhea with feces appearing as slurries on the floor, and 5 = severe diarrhea with watery stools). Frequency of diarrhea (C) was calculated as total pig days with a fecal score ≥ 3 . Data show LS means $\pm$ SEM.

* denotes statistical significance at $P < 0.05$

Figure 2.2.

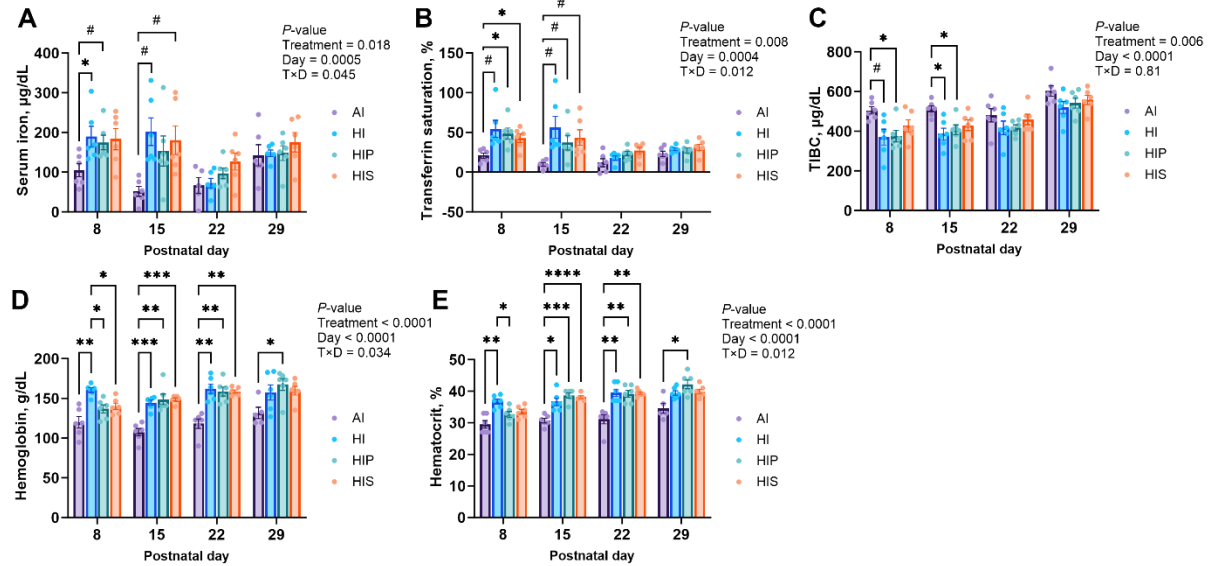


Figure 2.2. Effects of dietary treatment on hematocrit (A), hemoglobin (B), serum iron level (C), total iron-binding capacity (TIBC, D), transferrin saturation (E). Data show LS means $\pm$ SEM. #, *, **, ***, and **** denote a trend towards significance ($P < 0.1$), statistical significance at $P < 0.05$, 0.01, 0.001, and 0.0001, respectively.

Figure 2.3.

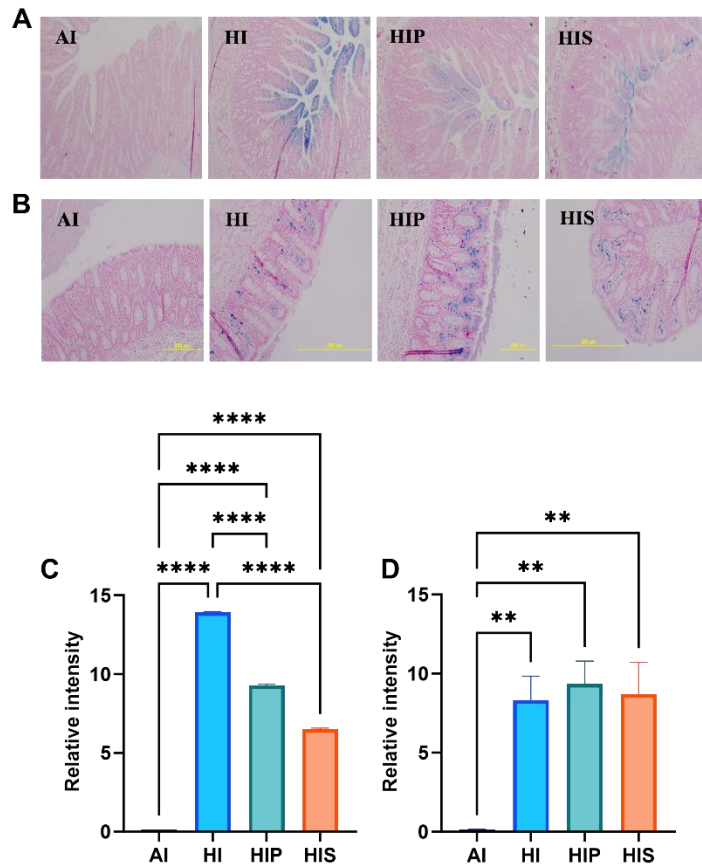


Figure 2.3. Effect of dietary treatment on iron accumulation in duodenal and colonic mucosa. Prussian blue stained sections of duodenum (A) and colon (B) tissues from AI, HI, HIP, HIS piglets. Relative intensity of Prussian blue-stained area (iron) in duodenum (C) and colon (D) of piglets from each treatment group (n = 6/treatment). The histograms display levels in arbitrary units of relative intensity of stained areas. Data show LS means $\pm$ SEM. *, **, ***, and **** denote statistical significance at $P < 0.05$, 0.01 , 0.001 , and 0.0001 , respectively.

Figure 2.4.

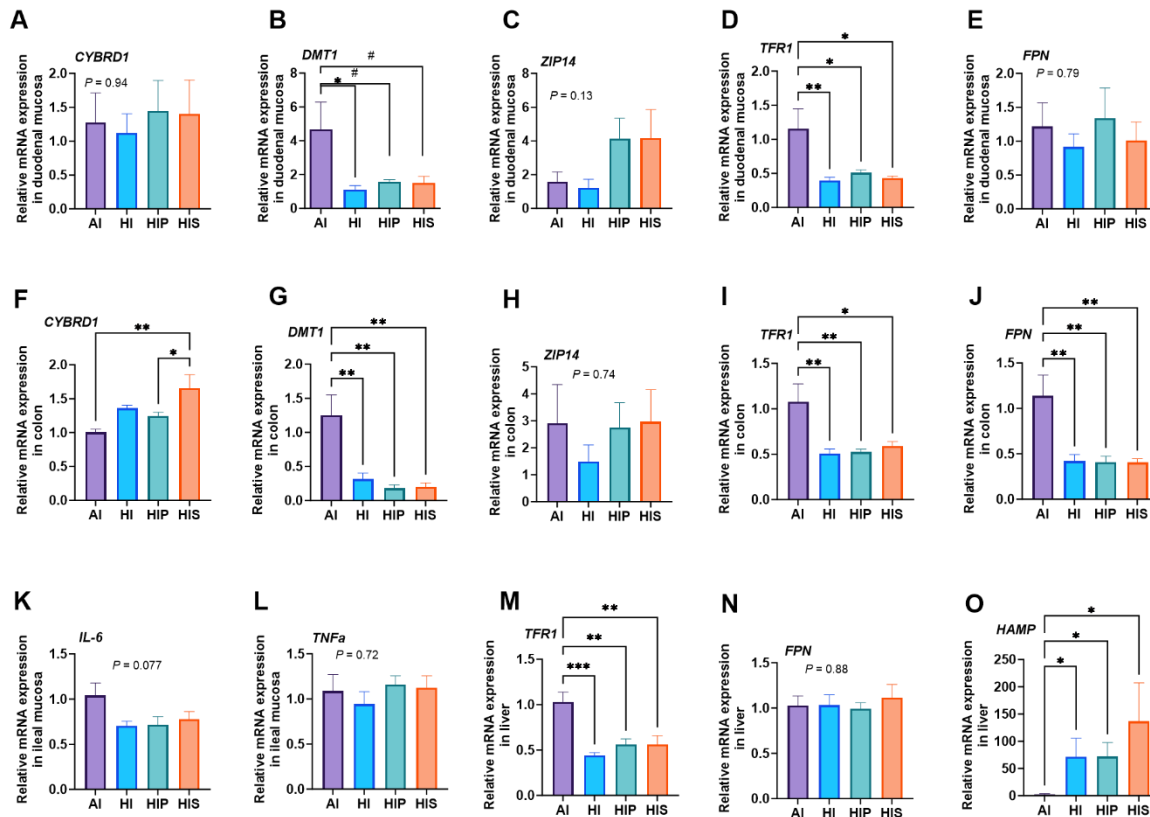


Figure 2.4. Effects of dietary treatment on the mRNA expressions of genes encoding iron regulatory proteins and inflammatory cytokines. Relative mRNA expressions of *CYBRD1*, *DMT1*, *ZIP14*, *TFR1*, and *FPN* in the duodenum mucosa (A – E) and colon tissue (F – J), IL6 and TNFA in the ileal mucosa (K and L), and TFR1, FPN, and HAMP in liver (M – O). Data show LS means $\pm$ SEM. #, *, **, and *** denote a trend towards significance ($P < 0.1$), statistical significance at $P < 0.05$, 0.01, and 0.001, respectively.

CHAPTER 3

Iron-Fortification and Inulin Supplementation in Early Infancy: Evaluating the Impact on the Gut Microbiota in a Piglet Model

ABBREVIATIONS:

AI: iron adequate milk diet

ASV: amplicon sequence variant

BW: body weight

SCFA: short-chain fatty acids

DNA: deoxyribonucleic acid

DP: degree of polymerization

E. coli: *Escherichia coli*

FC: fold change

Fe: iron

FOS: fructo-oligosaccharide

GIT: gastrointestinal tract

GOS: galacto-oligosaccharide

HI: high iron milk diet

HIP: high iron milk diet with prebiotic

HIS: high iron milk diet with synbiotic

HMO: human milk oligosaccharide

IACUC: International Animal Care and Use Committee

ID: iron deficiency

IDA: iron deficiency anemia

L. agilis YZ050: *Ligilactobacillus agilis* YZ050

MNP: micronutrient powder

mRNA: messenger

MRS: de Man, Rogosa, and Sharpe

PCoA: principal coordinates analysis

PCR: polymerase chain reaction

PD: postnatal day

qPCR: quantitative polymerase chain reaction

QIIME 2: Quantitative Insights Into Microbial Ecology 2

RCT: randomized controlled trials

RNA: ribonucleic acid

RT-qPCR: real-time

SCFA: short chain fatty acid

UCD: University of California, Davis

ABSTRACT

Background

Formula-fed infants display distinct gut microbiota profiles compared to breast-fed infants, primarily attributed to differences in nutrient composition between infant formula and human milk. Notably, the absence of human milk oligosaccharides (HMOs) in formula and differences in iron content contribute significantly to these alterations. Iron, essential for infant development, is often fortified in infant formulas to prevent iron deficiency (ID) but excessive fortification can lead to systemic iron overload and disrupt the gut microbiome. Inulin, a prebiotic compound, has shown potential in promoting beneficial bacteria growth, particularly lactobacilli and bifidobacteria. Despite the promise, limited research has explored the combined effects of pre- and synbiotic supplementation, along with iron fortification, on gut microbiome dynamics in formula-fed infants.

Objectives

This study aimed to determine the impact of excess iron fortification and inulin supplementation with or without *Ligilactobacillus agilis* YZ050 (*L. agilis* YZ050) on the gut microbiome of piglets.

Methods

Twenty-four piglets were stratified and randomly assigned to treatments on postnatal day (PD) 2. Piglets were individually housed and received iron-adequate milk (AI), high-iron milk (HI), HI milk supplemented with 5% inulin (HIP), or HIP milk with an oral gavage of *L. agilis* YZ050 every third day (HIS). Milk was provided in proportion to body weight and delivered to piglets in 14 meals daily, mimicking formula feeding in infants. Fecal consistency scores and body weight were recorded daily or every other day. Blood and feces were sampled weekly, and tissues were collected on PD 29. Data were analyzed using mixed model ANOVA with repeated measures whenever necessary. Microbial DNA extracted from colonic and fecal samples were analyzed for

microbiome through 16S rRNA sequencing. The diversity and relative abundance of taxa were analyzed using R statistics and QIIME 2.

Results

Both iron fortification and inulin supplementation significantly influenced colonic and fecal microbiota diversity, with dietary inulin exerting a more pronounced effect than dietary iron. The taxonomic profile of colonic and fecal microbiome was similar between HIP and HIS pigs, suggesting no impact of *L. agilis* YZ050 supplementation. In terms of diversity, pigs on HI diets, regardless of inulin or synbiotic supplementation, exhibited lower alpha diversity compared to those on the AI diet. Specifically, the Chao1 index in both colon and fecal samples was higher in the AI and HI pigs compared to those supplemented with inulin. Beta diversity analysis indicated a close clustering of samples from HIP and HIS pigs. These samples were notably separated from those on the AI and HI diets starting from PD 8 until PD 29. Additionally, marginal separation was observed between samples from AI and HI pigs on PD 15, 22, and 29, with a trend towards significance on PD 8. Inulin supplementation led to increased relative abundances of *Lachnospiraceae*, *Prevotellaceae*, *Veillonellaceae*, and *Selenomonadaceae*, and decreased *Bacteroidaceae*, *Ruminococcaceae*, and *Oscillospiraceae* at the family level, and increased abundances of *Prevotella*, *Megasphaera*, and *Lachnospiraceae_NK3A20_group*, while decreased *Bacteroides* and *Ruminococcus* at the genus level in both fecal and colonic microbiota.

Conclusions

Our study suggests that dietary inulin significantly influences colonic and fecal microbiota profiles in neonatal piglets, potentially through mechanisms such as utilizing inulin as the main energy source or cross-feeding activities of inulin substrates among gut bacteria, with inulin consumption emerging as the primary factor driving these changes and minimal effects of high doses of dietary

iron. While we did not directly assess short-chain fatty acid (SCFA) production and metabolites, it is plausible that inulin degradation by intestinal bacteria may lead to the production of SCFAs, impacting host colon health and benefiting overall gut microbiota composition. These findings highlight the need for further research to elucidate the complex interactions between dietary interventions, gut microbiota, and metabolites, particularly focusing on understanding the functional aspects of microbiota changes induced by inulin consumption during early life. Investigating these associations will provide valuable insights into the potential benefits of inulin as a dietary supplement and its role in promoting gut health and metabolic well-being.

Keywords: inulin, iron fortification, iron overload, *lactobacillus*, gut microbiota, pig model, prebiotic, probiotic, synbiotic

INTRODUCTION

Infants have the high rates of iron deficiency (ID) and iron deficiency anemia (IDA). While infant formula is fortified with iron to prevent ID or IDA, infant formula in the U.S. contains 10-60 times more iron than breast milk, causing concerns about its impact in early infancy (1-4). Fortified iron is poorly absorbed in the duodenum, approximately 10%, leading to abundant unabsorbed iron passing into the colon, where it may affect the host gut microbiome (5, 6). Indeed, excess iron fortification has shown a consistent impact on reducing the abundance of *Lactobacillus* in both animal (7) and human (8) studies. McMillen et al. investigated the impact of postnatal iron supplementation on the gut microbiome and metabolome in Sprague-Dawley rat pups, where they orally supplemented the rat pups with 10 mg/kg body weight (BW) of ferrous sulfate, ferrous bis-glycinate chelate, or vehicle control were administered from postnatal day (PD) 2–14 (7). Results showed that iron supplementation reduced microbiome alpha-diversity and altered short-chain fatty acids (SCFAs) production. When long-term effects of iron provision were assessed in a cohort supplemented with 200 mg iron/ kg diet until PD 21 and then weaned onto standard chow, it was found that iron provision led to a 10,000-fold reduction in *Lactobacillus* abundance in young adult rats that received iron supplementation in early life (7). In a 6-month randomized clinical study aimed to assess the impact of iron fortification on gut microbiota and inflammation in Ivorian children aged 6-14 years, children were assigned to receive either iron-fortified biscuits containing 20 mg Fe/d or non-fortified biscuits (8). Results showed that iron fortification did not improve iron status or anemia, rather it significantly altered the fecal microbiota, leading to increased profile dissimilarity and higher numbers of enterobacteria, along with decreased lactobacilli. In evidence, there was an increase in fecal calprotectin concentration, a marker of gut inflammation, which is correlated with the rise in enterobacteria. Furthermore, in a double-blind randomized controlled

trial (RCT) of iron in micronutrient powder (MNP) given to Kenyan infants, excessive colonic iron levels promoted the growth of pathogenic enterobacteria, such as *Salmonella*, *Clostridium difficile*, *Clostridium perfringens*, and pathogenic *Escherichia coli* (*E. coli*), potentially leading to intestinal inflammation and hindering the absorption of iron and other essential nutrients, at the expense of beneficial counterparts, such as lactobacilli and bifidobacteria (9). These findings suggest that iron fortification may contribute to a more pathogenic gut microbiota profile and increased gut inflammation. Given these concerns, research on the efficacy of iron fortification is needed to establish proper recommendations based on the complex relationship between iron metabolism, gut microbiota, and the health and development at early infancy.

Disruption of iron homeostasis has impact on the gut microbiome, resulting in host dysbiosis (10-12). Common methods to reshape the original state of microbial equilibrium within the gastrointestinal tract (GIT) in the host are the use of dietary prebiotics (13) and probiotics (14), and synbiotics (15). In a RCT with Kenyan infants, supplementing prebiotics, 7.5 g of galactooligosaccharides (GOS) with iron fortified MNP, alleviated the adverse effects of iron fortification on gut microbiota, showing an increase in relative abundance of beneficial bacteria, such as *Lactobacillus* and *Bifidobacterium*, and a reduction in pathogenic *E. coli*, compared to a group supplemented with the same MNP without GOS (10). Inulin, a non-digestible carbohydrate classified as a plant β (2-1) fructan, possesses a degree of polymerization (DP) ranging from 2 to 60 or more, making it a recognized prebiotic (16). Many studies have reported prebiotic properties of inulin (17-21) exert selective stimulation on the growth and activity of specific bacterial species within the gut, notably bifidobacteria and lactobacilli, thereby conferring health benefits. Furthermore, in the large intestine, prebiotics, including inulin, exert influence on various aspects of bowel functions and habits, including mild laxative effects (22), which may alter stool

consistency (23) and promote peristalsis (24), through bacterial fermentation (22), and it is suggested that most of the physiological changes induced by inulin in the host are mediated by bacterial metabolites, such as SCFAs (22, 25). In addition, dietary inulin supplementation (40 mg/kg diet) not only promoted enteric colonization of beneficial commensals and suppressed the less desirable bacteria, including *Clostridium spp.* and members of the *Enterobacteriaceae*, but also improved dietary iron utilization in young pigs (26, 27).

Lactobacilli play an important role in maintaining a healthy gut (28), with their functions extensively studied (29). Lactobacilli are highly responsive to changes in iron levels since they require minimal or no iron for their growth and survival (30-32). Studies have demonstrated that in iron-deficient rats, there is an increase in *Lactobacillus* abundance and a decrease in *Bacteroides* and *Roseburia*, whereas in iron-replete rats, the opposite occurs, with a decrease in *Lactobacillus* and an increase in *Bacteroides* (33). Given the sensitivity of lactobacilli to iron levels and the potential implications for gut health, the use of probiotic supplements has been suggested to preserve beneficial bacteria like *Lactobacillus* populations, particularly during periods of iron supplementation and vulnerable stages such as early infancy, where maintaining a healthy gut microbiome is essential for overall well-being (8). The bifidogenic and *Lactobacillus*-promoting effects of inulin and other pre-, pro- and synbiotics are of particular importance for formula-fed infants who tend to have fewer lactobacilli and bifidobacteria in their gut microbiota, which are either dominant or highly abundant in breast-fed infants (34-36). The difference in gut microbiota between breast-fed and formula-fed infants is mainly due to the presence of abundant and diverse oligosaccharides that are unique to human milk and are either absent or exist at a much lower level in cow milk (37). Human milk oligosaccharides (HMOs) are naturally occurring prebiotics that cannot be digested in human gut, rather metabolized by the gut microbiota, and shown to facilitate

the development of the gut microbiota and promoting the growth of beneficial gut bacteria and gut health (37-39). Therefore, prebiotic supplementation in infant formula is a promising strategy for reshaping the gut microbiome towards the profile of breast-fed infants. Dietary supplementation of a combination of probiotics with prebiotics they consume—defined as a synergistic synbiotic supplement (40)—appears to have a stronger impact on gut microbial communities. *Ligilactobacillus agilis* YZ050 (*L. agilis* YZ050) was previously identified as a strain that can efficiently utilize inulin as its main energy source (41). *L. agilis* YZ050 breaks down inulin externally and releases fructose monomers that can potentially be utilized as substrates by other gut bacteria (42). There is limited research on the effects of high iron fortification and the concurrent pre- and synbiotic supplementation on gut microbiome of formula-fed infants under good hygiene conditions (e.g. in the U.S.).

Our study employed the piglet as a preclinical model to investigate how HI fortification and the combined use of pre- and synbiotic affect gut microbiota in formula-fed infants. Piglets share physiological and developmental similarities with human infants (43), making them a valuable preclinical model to explore the effects of early-life iron overload and potential dietary interventions. In our previous publication (44), we showed that HI milk resulted in pronounced tissue iron deposition and a marked increase in colonic and fecal iron content. The introduction of inulin, irrespective of the presence of *L. agilis* YZ050, not only mitigated hepatic iron overload but also effectively reduced colonic and fecal iron levels without affecting pH or iron regulatory genes. Building upon these findings, we utilized 16S rRNA sequencing to investigate changes in the gut microbiome. This research addresses a critical gap in understanding the dynamics of early-life iron overload and its potential modulation through prebiotic and synbiotic interventions, shedding light on strategies to promote gut health and development in formula-fed infants.

MATERIALS AND METHODS

Study design and animal management. The study protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California, Davis (UCD). The details of study design, animal management, and sample collection have been described in Chapter 2. Briefly, twenty-four neonatal piglets (equal number of females and males) were stratified by birth BW and sex and randomly assigned to one of the four dietary treatments (n = 6/treatment) at postnatal day (PD) 2. From PD 2 to 29, piglets received iron-adequate milk replacer (AI, 60 mg iron/kg milk solids supplemented with 5% (wt/wt milk solid) maltodextrin, high-iron milk replacer (HI, 480 mg iron/kg milk solids supplemented with 5% maltodextrin, HI milk supplemented with 5% inulin (Orafit® IPS, Beneo) as prebiotics (HIP), or HIP milk and an oral gavage of *L. agilis* YZ050 (5 mL, 2.5×10^9 CFU/mL) once every 3 days during the study (HIS). This strain was shown to use inulin as a substrate (41, 42). Piglets were weighed every other day and fed 270 mL milk/kg BW·day according to the most recent body weight.

Data and sample collection. Fecal consistency was scored twice daily by a well-trained researcher at 0700 and 1900 h using a 5-scale scoring system (1 = normal firm and shaped feces, 2 = soft and shaped feces, 3 = mild diarrhea with mushy and loose stools, 4 = severe diarrhea with feces appearing as slurries on the floor, and 5 = severe diarrhea with watery stools). Fecal samples were obtained using a rectal swab on PD 2 (baseline), 8, 15, 22, and 29. All piglets were euthanized on PD 29 and sampled for colon contents. The samples were snap-frozen in liquid nitrogen and stored in -80°C until lab analysis.

Fecal and colon digesta DNA Extraction. Bacterial DNA was extracted from feces and proximal colon contents (~100 mg) using the Quick-DNA Fecal/Soil Microbe Kit (Zymo Research, Irvine, CA) following the manufacturer's instructions.

Library preparation and 16S rRNA sequencing. Microbiome Library Preparation and Analysis.

Microbial profiles in proximal colon digesta and fecal samples were determined using 16S rRNA sequencing. The microbiome library preparation was done following a procedure previously described (45). The 16S rRNA genes of bacterial DNA were amplified by PCR using a primer set that targets the V4 region (515 F: 5'-XXXXXXXXGTGTGCCAGCMGCCGCGG TAA-3'; 806 R: 5'-GGACTACHVGGGTWTCTAAT-3') and contains an 8 bp barcode (X) and Illumina adapter (GT). Each PCR reaction contained 2 µL DNA template, 9.5 µL nuclease-free water, 12.5 µL GoTaq 2×Master Mix (Promega, Madison, WI), 0.5 µL of each V4 forward and reverse primer (10 µM). The PCR was performed with the following protocol: 1) 94°C for 3 min for denaturation, 2) 94°C for 45 sec, 3) 50°C for 1 min, 4) 72°C for 1.5 min (Steps 2-4 were repeated for 35 times), 5) 72°C for 10 min for elongation. Each sample was amplified via PCR in duplicates. The DNA library was prepared by pooling PCR products of all extracted bacterial DNA samples from piglets. The volume of each sample added to the library was subjectively adjusted based on band intensity stained with SYBR Safe in a 2% agarose gel. The pooled sample was purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and then submitted to the UCD Genome Center for 250-bp paired-end sequencing on the Illumina MiSeq platform (Illumina, Inc. San Diego, CA).

Microbiome data processing, taxonomic classification, and visualization. The microbiome analysis was performed following a procedure previously described (46, 47). Data processing and statistical analysis were conducted using the Farm Cluster, a Linux-based supercomputing cluster for the College of Agricultural and Environmental Sciences at UCD, and R statistics software, respectively. Sabre (<https://github.com/najoshi/sabre>) was used for demultiplexing and trimming sequence reads by aligning sequences to its sample of origin based on the barcode and removing barcodes from each end of raw sequences. Reads not matching any barcode and sequences that fail

to meet the minimum quality thresholds were discarded. The sequences were then imported into Quantitative Insights Into Microbial Ecology 2 (QIIME 2; version 2022.2.0) for downstream filtering and bioinformatics analysis (48). DADA2 plugin was used to remove primers and lower quality reads and denoise the paired end reads (49). Taxonomic classification was assigned using the taxonomy-classifier plugin, the SILVA rRNA V4 database (version 138.1) (50). The amplicon sequence variant (ASV) table and phylogenetic tree were imported into R (version 4.2.3) using the phyloseq package for analysis of alpha-diversity, beta-diversity, relative abundance, and statistical significance.

Statistical analysis. Statistical analysis and data visualization were conducted using R statistics (version 4.2.3). The fecal microbiome analysis employed a statistical model that included treatments, days, and their interaction, while the colon microbiome analysis only included treatment as main effect. Analyses were carried out using the phyloseq package and visualized with the ggplot2 package and QIIME2 (51). Normality was assessed using the Shapiro-Wilk test, while homoscedasticity was checked using the Bartlett test for alpha diversity and relative abundance. Non-parametric tests, including the Kruskal-Wallis sum-rank test, Dunn's posthoc test, and Conover test, were utilized for relative abundance pairwise comparisons for main effects, employing the agricolae package. For beta diversity analysis, the Bray-Curtis dissimilarity was employed for principal coordinates analysis (PCoA). The statistical significance of beta diversity was determined using PERMANOVA with 999 replicate permutations, using the vegan package. Differential abundance analyses were performed using the DESeq2 package. P-values for relative and differential abundances were adjusted for multiple testing using the Benjamini–Hochberg correction to estimate method-specific false discovery rate. Statistical significance was declared at $P < 0.05$, with a trend towards significance at $P < 0.1$.

RESULTS

Diversity of colon microbiota. 16S rRNA sequencing detected average ASV reads of 37,310 after initial quality-filtering. Further filtering, which involved removing Archaea, chloroplast rDNA, and the phylum *Cyanobacteria*, as well as rarifying, resulted in a reduction of ASV reads to 10,869. Additionally, a minimum taxa prevalence threshold of 1.0% of total samples was applied. The phyloseq object underwent cleaning by eliminating taxa sequences that were not available, meaning poor classification from the SILVA database. This process also involved removing bacterial sequence counts that accounted for less than 1% of the total microbial composition. The phyloseq object of colon sample analysis had 1,301 sequences and 23 samples.

The α -diversity of colon microbiota were assessed using Chao1 (richness) and Shannon indices (richness and evenness). The results showed that the high-iron diet (HI, HIP, and HIS) pigs, regardless of inulin or synbiotic supplementation, decreased Shannon index ($P \leq 0.078$, **Fig. 3.1. A**) and Chao1 ($P \leq 0.021$, **Fig. 3.1. B**) compared to AI pigs. However, there were no significant differences in Chao1 or Shannon among HI, HIP, and HIS pigs.

The β -diversity is presented in PCoA plot based on Bray-Curtis distance (**Fig. 3.1. C**). Adonis pairwise tests revealed significant differences in β -diversity in all pair-wise comparisons ($P < 0.001$), except for the comparison between HIP and HIS ($P = 0.669$). This finding suggested both HI fortification and inulin supplementation modulated taxonomic diversity of the colonic microbiome, while *L. agilis* YZ050 inoculation had minimal effect on microbial diversity. The clear separation of AI and HI samples from samples of inulin-supplemented groups (HIP and HIS) on Axis 1 (25.64%) indicated a profound influence of inulin on the diversity of colonic microbiota. In contrast, AI and HI samples were separated to a less extent primarily along the Axis 2 (10.07%),

indicating a moderate effect of iron excess that modulates microbial diversity in a different manner compared to inulin ($P = 0.0312$).

The PCoA biplot analysis was conducted to provide visualization of dissimilarity in composition among samples and present important bacterial features (taxa) that significantly contribute to the separation of samples (**Fig. 3.1. D**). The analysis revealed the top five taxa, aiding in understanding the patterns and relationships among different treatment groups. The vectors show the direction in the ordination space toward which genus with the highest relative abundance in each group was considered the main contributor. The enrichment of *Bacteroides* was the most significant feature in colon microbiota that distinguishes non-inulin groups (AI and HI) from the inulin-supplemented groups (HIP and HIS) piglets. Conversely, the higher abundance of *Lachnospiraceae NK3A20 group* and *Selenomonadaceae* are key features in the two inulin-supplemented groups.

Relative abundance of colon microbial taxa. The colon microbiota were primarily dominated by the phyla of *Firmicutes* and *Bacteroidetes*, which collectively accounted for 97%, 98.6%, 96.9%, and 98.4% of microbial taxa in AI, HI, HIP, and HIS, respectively (**Fig. 3.2. A**). *Actinobacteriota* was less than 1% relative abundance in AI and HI and 2.6% and 1% in HIP and HIS, respectively, representing the third abundant phylum, while other phyla such as *Proteobacteria*, *Desulfobacterota*, *Euryarchaeota*, *Fusobacteriota*, and *Synergistota* combined made up less than 2% relative abundance. Dietary treatments significantly impacted *Firmicutes* ($P = 0.048$), where HIS pigs had higher relative abundance than AI and HI pigs, and *Bacteroidetes* ($P = 0.047$), where AI and HI pigs were higher than HIP pigs.

The relative abundance of families within *Firmicutes* (**Fig. 3.2. B**), *Bacteroidetes* (**Fig. 3.2. C**), and *Actinobacteriota* were analyzed. Seven *Firmicutes* families were significantly affected by

treatment ($P \leq 0.02$). AI pigs had higher abundance of *Christensenellaceae* and *Oscillospiraceae* compared to HI, HIP, and HIS pigs ($P < 0.05$). AI pigs had higher abundance of *Leuconostocaceae* than HIP and HIS pigs ($P < 0.05$). AI and HI pigs had higher abundance of *Ruminococcaceae* and *[Eubacterium]_coprostanoligenes_group* compared to HIP and HIS pigs ($P < 0.001$). Conversely, *Selenomonadaceae* and *Veillonellaceae* were more abundant in HIP and HIS pigs than AI and HI pigs ($P < 0.001$). Within *Bacteroidetes*, dietary treatment altered six families ($P \leq 0.03$), with the most significant changes observed in *Bacteroidaceae*, *Muribaculaceae*, and *Rikenellaceae* which were more abundant in AI and HI pigs compared to HIP and HIS pigs ($P < 0.0001$). *Rikenellaceae* were higher in AI pigs than HI pigs ($P < 0.05$). HIP and HIS pigs had higher abundance of *Prevotellaceae* than AI and HI pigs ($P < 0.01$), while HI pigs had the lower abundance compared to AI pigs ($P < 0.05$). For families of *Actinobacteriota* (**Fig. 3.2. D**), *Atopobiaceae* was most abundant in HIP pigs and was higher than that in AI ($P < 0.05$) and HI pigs ($P = 0.087$). *Bifidobacteriaceae* represented $< 1\%$ of relative abundance of total colonic taxa, highlighting a critical difference in gut microbiota between young pigs and human infants (52). The abundance of *Bifidobacteriaceae* was higher in HIP pigs compared to HI pigs ($P < 0.05$).

The top 10 abundant genera are visualized on a bar plot (**Fig. 3.2. E**). *Bacteroides*, *Colidextribacter*, and *Ruminococcus* were less abundant in HIP and HIS pigs than in AI and HI pigs ($P < 0.0001$). Conversely, *Megasphaera*, *Mitsuokella*, and *Prevotella* were more abundant in HIP and HIS pigs than in AI and HI pigs ($P < 0.0001$). *Lachnospiraceae_NK3A20_group* abundance of HIP and HIS pigs is higher compared to AI and HI pigs ($P < 0.01$). *Prevotella* abundance is lowest in HI pigs compared to HIP pigs ($P < 0.001$) and HIS pigs ($P < 0.01$), while did not have significant difference compared to AI pigs. *Weissella* abundance (not shown) is

highest in AI pigs compared to HI, HIP, and HIS pigs, while only significant difference was found compared to HIS pigs ($P = 0.035$).

Differential abundance of colon microbiota. The radar charts illustrate the $\log_2FC$ (fold change) of 8 families (**Fig. 3.3. A**) and 7 genera (**Fig. 3.3. B**) in the colon microbiota across treatment groups (HI, HIP, and HIS) compared to the AI group (reference group). Taxa were selected based on their actual abundance and statistical significance. At family level, *Prevotellaceae* ($P < 0.05$), *Veillonellaceae* ($P < 0.0001$), *Selenomonadaceae* ($P < 0.0001$), and *Lachnospiraceae* ($P < 0.1$) were more abundant in the colon of HIP and HIS pigs than AI and HI pigs. *Selenomonadaceae* ($P < 0.0001$) was more abundant in HI than AI pigs, while *Bacteroidaceae* ($P < 0.0001$) and *Oscillospiraceae* ($P < 0.1$) were less abundant in HIP and HIS pigs compared to AI pigs. The abundance of *Ruminococcaceae* was lower in HIS pigs compared to AI pigs ($P < 0.05$). At genus level, *Prevotella* ($P < 0.05$), *Mitsuokella* ($P < 0.0001$), *Megasphaera* ($P < 0.0001$), and *Lachnospiraceae_NK3A20_group* ($P < 0.0001$) were more abundant in HIP and HIS pigs than in AI and HI pigs. HI pigs also had more *Mitsuokella* ($P < 0.0001$) compared to AI pigs, while *Bacteroides* ($P < 0.05$) and *Ruminococcus* ($P < 0.001$) were less abundant in HIP and HIS pigs than in AI and HI pigs.

Diversity of fecal microbiota. 16S rRNA sequencing detected average ASV reads of 38,940 after quality-filtering. The sampling depth of ASV reads was 10,869 after the filtering and rarifying, with a minimum prevalence threshold set at 1.0% of total samples. The phyloseq object of fecal sample analysis had 1,301 sequences and 119 samples. The Shannon index of fecal microbiome exhibited no significant difference for the main effect of treatment ($P = 0.457$), a significant effect of time ($P < 0.0001$), and a significant treatment $\times$ day interaction ($P = 0.01$) (**Fig. 3.4. A**). The Chao1 index was significantly affected by the treatment $\times$ day interaction ($P < 0.0001$) (**Fig. 3.4.**

B). Chao1 index was higher in AI pigs than in HIS pigs on PD 8 ($P = 0.029$), HI pigs than in HIP pigs on PD 15 ($P = 0.091$), and AI pigs than in HIS pigs on PD 29 ($P = 0.065$). In addition, there was a trend in main effect of treatment $\times$ day interaction on Chao1 index, which was higher in AI and HI pigs compared to HIP and HIS pigs ($P \leq 0.068$). Additionally, the Chao1 index showed a continuous increase across all four treatment groups throughout the study ($P < 0.0001$). β -diversity presented in PCoA plots (**Fig. 3.4. C**) revealed a close cluster of HIP and HIS pigs samples throughout the study. These samples were clearly separated from AI and HI pigs starting on PD 8 until PD 29 (adjusted- $P \leq 0.01$). Furthermore, AI samples were marginally separated from HI samples along Axis 2 (12.3%), as confirmed by pairwise Adonis tests showing significant dissimilarity between the two groups on PD 15, 22, and 29 (adjusted- $P \leq 0.01$), with a trend towards significance on PD 8 (adjusted- $P = 0.073$). Overall, the β -diversity revealed a significant effect of inulin and, to a less extent, dietary iron on the diversity of the fecal microbiome whereas administration of *L. agilis* YZ050 had little effect on diversity of fecal microbiome.

The PCoA biplot analyses demonstrated associations between bacterial taxa and treatments in fecal samples across different time points (**Fig. 3.5. A-D**). Five large clusters were observed at the genus level of bacterial taxonomy, with specific taxa driving the separation at different time points. On PD 8, the genera of *Megasphaera*, *Fusicatenibacter*, and *Syntrophococcus* were the taxonomic signatures of fecal microbiome of inulin-fed piglets while *Bacteroides* and *Weissella* were the key taxonomic features of the AI and HI fecal microbiome. *Weissella* and *Megasphaera* had the most significant contribution to the separation of samples from inulin-fed piglets from those from the other two groups. On PD 15, *Megasphaera* and *Fusicatenibacter* remained the key features of the HIP and HIS fecal microbiome followed by *Blautia* and *Mitsuokella*. In contrast, *Weissella* was the only taxon that markedly distinguish the AI and HI fecal microbiome from those

of inulin-fed piglets. On PD 22, *Blautia* and *Lachnospiraceae* NK3A20 group became the most distinct features of the HIP and HIS fecal microbiome, while *Weissella* and *Ruminococcus* were the most significant taxonomic signatures that distinguish the AI and HI fecal microbiome from the other groups, respectively. On PD 29, fecal microbiome from inulin-fed piglets was featured with *Lachnospiraceae* NK3A20 group, *Catenisphaera* and *Selenomonadaceae* while *Bacteroides* was the key feature of the AI and HI fecal microbiome.

Relative abundance of fecal microbial taxa. Mirroring the colon microbiome, the fecal microbiome was dominated by phyla of *Firmicutes*, *Bacteroidetes*, and *Actinobacteriota*, accounting for 70.2% to 86.4%, 10.6% to 26.8%, and 0.6% to 5.2% of total microbial taxa, respectively, across treatments and sampling days (**Fig. 3.6. A**). The relative abundances of other phyla remained below 1%. At the phylum level, Kruskal-Wallis rank sum test revealed a significant treatment $\times$ day interaction effect on relative abundance of *Bacteroidetes*, *Firmicutes*, *Desulfobacterota*, *Euryarchaeota*, *Fusobacteriota*, *Proteobacteria*, and *Synergistota* ($P < 0.0001$). Furthermore, the HIS (on PD 8 and 15) and HIP (on PD 15) diet increased the abundance of *Proteobacteria* compared to the AI and HI diets ($P \leq 0.012$).

Families and genera within the three predominant phyla (*Firmicutes*, *Bacteroidetes* and *Actinobacteriota*) of the fecal microbiome were analyzed for their relative abundance. Within *Firmicutes* (**Fig. 3.6. B**), *Lactobacillaceae*, *Leuconostocaceae*, *Ruminococcaceae*, *Selenomonadaceae*, and *Veillonellaceae* were investigated due to their statistically significant differences. Despite the significant interaction effect on relative abundance of *Lactobacillaceae*, post-hoc analysis did not detect a significant difference among treatment in any sampling days, except for a trend on PD 8, where AI had higher abundance than HIS pigs ($P = 0.081$), and PD 15, where HI pigs had higher abundance than HIS pigs ($P = 0.09$). The abundance of

Leuconostocaceae was the highest in AI pigs, and present at a slightly lower level in HI pigs on PD 8 ($P = 0.73$), PD 15 ($P < 0.0001$), and PD 29 ($P < 0.05$) than in AI pigs, and much lower level in HIP and HIS pigs than in HI pigs on PD 22 ($P < 0.1$) and AI pigs ($P < 0.0001$) during the study. Despite variation across sampling days, *Ruminococcaceae* abundance was highest in HI followed by AI and HIP pigs, where on PD 8, HI pigs had higher in the abundance than HIP pigs ($P < 0.05$); on PD 15 and 22, HI pigs had higher than AI, HIP, and HIS pigs ($P < 0.05$); on PD 29, AI and HI pigs were higher than HIP and HIS pigs ($P < 0.01$). The abundance of *Selenomonadaceae* and *Veillonellaceae* were higher in HIS and HIP pigs compared to HI and AI pigs throughout the study ($P < 0.0001$). Within the phylum of *Bacteroidetes* (**Fig. 3.6. C**), all families presented in the Figure were significantly affected by the interaction effect ($P \leq 0.029$). *Bacteroidaceae* exhibited a higher relative abundance in AI pigs compared to HIP and HIS pigs ($P < 0.01$) on PD 15 and 22, and slightly higher in AI compared to HIP and HIS on PD 29 ($P < 0.1$). Additionally, it was higher in HI than in HIS pigs ($P < 0.05$) on PD 15 and higher than in HIP and HIS pigs on PD 22 and 29 ($P < 0.05$). *Prevotellaceae* showed a higher relative abundance in HIP compared to HI pigs ($P < 0.05$) on PD 15 and 29. *Rikenellaceae* displayed a higher trend in HI compared to AI, HIP, and HIS pigs ($P \leq 0.1$) on PD 8, while on PD 15, it showed a higher relative abundance in AI and HI than in HIP and HIS pigs ($P < 0.05$). By PD 22, *Rikenellaceae* exhibited a higher relative abundance in AI than in HI, HIP, and HIS pigs ($P < 0.001$). Large variations in relative abundance were observed for *Actinobacteriota* families (**Fig. 3.6. D**). *Bifidobacteriaceae* was much less abundant in the gut of piglets compared to human infants, representing 0.3 to 1.8% of total relative abundance on PD 2 and 8 and below 0.5% later in the study. *Atopobiaceae* exhibited a higher relative abundance in HIP compared to AI and HI pigs on PD 8, as well as in HI pigs on PD 29.

At the genus level (**Fig. 3.6. E**), while genera that accounts for at least 1% of total relative abundances were shown in the bar plot, *Bacteroides*, *Lachnospiraceae NK3A20 group*, *Lactobacillus*, *Megasphaera*, *Mitsuokella*, *Prevotella*, *Ruminococcus*, *Catenisphaera*, *Fusicatenibacter*, *Syntrophococcus*, and *Weissella* were investigated further in statistical analyses for significant differences across the treatment groups. For *Bacteroides*, on PD 8, HI pigs exhibited a higher relative abundance than HIS pigs ($P = 0.088$). On PD 15 and 22, AI pigs showed higher abundance compared to HIP and HIS pigs ($P < 0.001$), and HI was higher than HIS ($P < 0.05$). On PD 29, HI displayed higher abundance than HIP and HIS pigs ($P < 0.05$). *Lachnospiraceae NK3A20 group* had higher relative abundance in HIP and HIS compared to AI and HI pigs on PD 22 and 29 ($P < 0.01$). For *Lactobacillus*, AI pigs had a higher relative abundance compared to HIS on PD 8 ($P = 0.082$), and HI was higher than HIP pigs on PD 15 ($P = 0.083$). For *Megasphaera* and *Mitsuokella*, HIP and HIS had higher relative abundance than AI and HI pigs during the study ($P < 0.01$), except for *Mitsuokella* on PD 29, where there was no difference among treatment groups. Similarly, the relative abundance of *Fusicatenibacter*, *Mitsuokella*, and *Megasphaera* was significantly higher in inulin-fed groups compared to non-inulin groups during the study. However, the difference in *Fusicatenibacter* caused by inulin diminished on PD 29. For *Prevotella*, HIP was higher than AI pigs on PD 15 ($P \leq 0.1$), and HIS was higher than in AI and HI pigs on PD 29 ($P < 0.05$). *Ruminococcus* was much higher in abundance (1.7 – 9.9%) in AI and HI than HIP and HIS pigs in which the genus was minimally detected during the study ($P < 0.0001$). *Catenisphaera* was below 0.1% of total relative abundance in AI and HI pigs, while, after PD 8, inulin-supplementation gradually increased its abundance which reached 5.3 – 7.5% in HIP and HIS pigs, respectively, on PD 29. *Weissella* were more abundant in feces of the AI group compared to the HI and inulin-fed groups ($P < 0.05$) during the study.

Differential abundance of fecal microbiota. The differential abundance analysis showed temporal changes ($\log_2FC$) of the selected genera in comparison with their level in AI pigs (reference group, **Fig. 3.7. A-H**). The genera were selected based on their actual abundance and statistical significance. *Bacteroides* (Fig. 3.7. A) was significantly lower in HIS on PD 15 and 22 ($P < 0.001$), while *Ruminococcus* (Fig. 3.7. B) was lower in both inulin-fed groups throughout the study and tended to be higher in the HI group. Inulin supplementation increased *Prevotella* and *Lachnospiraceae_NK3A20_group* at various levels from PD 15 and significantly increased *Megasphaera* and *Mitsuokella* from PD 8 ($P < 0.0001$) with the exception of HIP pigs on PD 8 ($P < 0.1$). In contrast, inulin supplementation significantly decreased *Weissella* throughout the study ($P < 0.01$) except for a transient increase in HIP pigs on PD 8 ($P < 0.01$). The changes of these genera in HI pigs were either insignificant or at a moderate extent.

DISCUSSION

The present study shows that inulin supplementation in milk formula has dramatic impact on the gut microbiota composition. To a lesser extent, high-iron fortification modulated both diversity and profile of the gut microbiome. A modulatory effect of iron supplementation on gut microbiota of infants and in suckling animal model has been observed in other studies (4, 8, 9, 53), while, to our knowledge, this is the first study that evaluated the potential synergistic effect of high-iron fortification with concurrent prebiotic or synbiotic supplementation in milk formula. In a speculative manner, these shifts could be due to inulin's role as a substrate for utilization by various bacterial species and SCFA synthesis in the large intestine (54). Increases in SCFA levels in the large intestine was also observed in a rodent study when 10% of dietary wheat starch was replaced with 10% of dietary inulin (55). In a clinical trial where participants were administrated

inulin, GOS, or wheat dextrin twice daily in alternate weeks of a 6-week intervention period, inulin increased while GOS and wheat dextrin decreased butyrate in participants' stool samples (56). However, SCFAs were not analyzed in the current study, thus preventing us to further evaluate microbiota changes in relation to the SCFA profile.

Inulin consumption is widely recognized for promoting the growth of beneficial microbes like lactobacilli and bifidobacteria (54, 57). Surprisingly, in our study, the relative abundances of these microbes remained stable throughout the study, suggesting a potential equilibrium in their interactions within the gut microbiota. It worth to note that, in contrast to human infants' GIT, the GIT of suckling or milk-fed piglets is naturally habited with a considerably lower abundance of bifidobacteria, amounting to less than 1% of total bacteria or being even undetectable (58), which was also observed in the current study. However, while previous studies have demonstrated such promotional effects of inulin on specific beneficial microbes, our findings revealed broader alterations in fecal and colonic microbiota composition between inulin-fed and non-inulin-fed piglets as early as PD 8. These changes encompassed shifts in overall microbial diversity and the relative proportions of different bacterial taxa associated with inulin consumption. The early detection of these differences may be attributed to unique aspects of our study design, such as the specific diet composition and inulin dosage used. In the current study, inulin supplementation was found to enrich genera *Lachnospiraceae_NK3A20_group*, *Megasphaera*, and *Mitsuokella* that belong to *Firmicutes* families *Lachnospiraceae*, *Veillonellaceae*, and *Selenomonadaceae*, respectively. These genera or families were previously identified as inulin-responsive bacteria that have inulin binding capacity and are metabolically active by inulin in human gut (59). This is consistent with our findings from the PCoA biplot analysis that these genera are the key microbial features that contributed to distinguish samples from inulin-fed piglets. The *Lachnospiraceae*

family is known to generate SCFAs, particularly butyrate, which provides a potential intestinal defense to the host (60, 61) and host epithelial cells and enhances mucosal barrier functions (61, 62). Dou et al. conducted a study on piglets highlighting the significance of early-life microbial exposure in shaping growth and overall health and found at PD 7 that healthy piglets displayed distinct microbial profiles compared to diarrheic piglets at PD 7, characterized by higher abundance of bacterial families including *Lachnospiraceae*, *Prevotellaceae*, *Lactobacillaceae*, and *Ruminocacaceae* (63). This research highlights the potential role of *Lachnospiraceae* as a bacterial feature indicative of the health status of piglets and highlights avenues for further investigation into gut microbiota-mediated strategies for promoting piglet health and preventing digestive disorders. In line with our findings, a study involving weaned piglets supplemented with a diet containing 2% citrus pulp aimed to mimic prebiotic effects similar to those of inulin. Although citrus pulp primarily consists of pectic oligosaccharides with DP typically ranging from 3 to 8, which differs from the fructan structure of inulin (64), the study sought to investigate its potential impact on gut microbiota. Despite compositional differences, the supplementation led to increased levels of *Megasphaera*, a genus within the *Veillonellaceae* family (65). While not all dietary fibers share identical properties, this study suggests that citrus pulp supplementation may elicit responses in the gut microbiota akin to those observed with traditional prebiotics like inulin. Similarly, dietary supplementation of 10% long-chain purified chicory inulin (OraftiHP, Beneo, Belgium) increased fecal *Megasphaera* in 8-week-old pigs (66). *Megasphaera* species are acknowledged for their significant contributions to digestive health through the fermentation of dietary fibers and complex carbohydrates, thereby aiding in the digestion process and generating SCFAs such as butyrate, acetate, formate, and caproate (67). These SCFAs play a crucial role in supporting gut health and exerting anti-inflammatory properties (68), (69). In a study

characterizing gut microbial features associated with diarrheal cryptosporidiosis, it was found that infants affected by either subclinical or diarrheal *Cryptosporidium* infection had significantly lower level of *Megasphaera* in the gut microbiota compared to healthy individuals (70). The findings of the present study suggest that inulin supplementation plays a role in increasing *Megasphaera* levels, known for their digestive health benefits and SCFA production. This observation aligns with similar trends observed in infants with *Cryptosporidium* infection, indicating the potential relevance of inulin in promoting gut health across different contexts. *Prevotella*, a genus of the *Prevotellaceae* family, colonizes both human and pig guts and become more abundant in individuals consuming more plant-based food and less animal products (71). Enteric high abundance of this genus defines one of the major enterotypes in the human and pig gut (71, 72), characterized by a robust fiber-utilizing capacity for fermenting fibers such as fructooligosaccharides, sorghum arabinoxylan, and corn arabinoxylan, as well as high short-chain fatty acid (SCFA) production, especially propionate, found in in vitro human fecal fermentation samples (73). Consistent with a piglet study where the diet was modified by supplementation with 10% (w/w) long-chain purified chicory inulin (66) and a study supplementing 5% inulin to a corn-soybean basal pig diet (74), our findings in the inulin diet groups similarly revealed a notable increase in *Prevotella* abundance alongside a reduction in *Bacteroides* abundance. Within the *Bacteroidetes*, the *Bacteroidaceae* family was more abundant in gut of HI piglets compared to AI, HIP, and HIS piglets. An increase in gut *Bacteroidaceae* abundance was observed in a rodent study when germ-free rats were fed an iron-excess diet, suggesting an iron-rich gut lumen favors the colonization of *Bacteroidaceae* (75). Interestingly, dietary inulin supplementation led to a reduction in *Bacteroidaceae* levels in HIP and HIS piglets, potentially due to the concurrent increase in *Lachnospiraceae*, *Prevotellaceae*, *Veillonellaceae*, and *Selenomonadaceae*. Inulin is a

common non-digestible oligosaccharides and known to feed the intestinal microbiota (76), including various bacterial taxa such as *Firmicutes*, *Bacteroides*, and *Bifidobacterium* (77). However, the response of the microbiota to dietary fructans can vary, resulting in the expansion of *Actinobacteria*, *Firmicutes*, or *Bacteroides* (78). Sonnenburg et al. demonstrated that *Bacteroides* species exhibit differences in their ability to utilize fructans (79). While *Bacteroides thetaiotaomicron* shows minimal growth in inulin-rich environments, *Bacteroides caccae* thrive in such conditions due to the genetic and functional variances among *Bacteroides* species (79), which can predict their competitiveness in vivo in the presence of dietary fructans. The lower abundances of *Bacteroidaceae* and *Bacteroides* in inulin-fed diet groups may be attributed to the metabolic preferences and versatility of these taxa. They are known for their adeptness at processing diverse substrates such as proteins, host-derived glycans, and other complex molecules commonly found in the gut environment (80-82). Therefore, it is plausible that these bacteria prioritize the utilization of alternative substrates over inulin as a primary energy source, leading to reduced abundances in environments rich in inulin. Additionally, it is conceivable that other bacterial taxa, better suited to exploit inulin supplementation, may have outcompeted *Bacteroidaceae* in abundance. Moreover, these findings are consistent with previous research demonstrating an increase in *Bacteroides* abundance in an iron-supplemented group compared to an iron-deficient group (33). This association was observed in an in vitro gut microbiota fermentation study designed to replicate conditions similar to those found in the proximal colon of children (33). In an iron-rich gut environment, a pathobiont, *Bacteroides vulgatus* tends to thrive with enhanced virulence, making it one of the most abundant genera in the gut and contributing to inflammation (81, 83, 84), potentially implicated in Crohn's disease and ulcerative colitis, through the activation of NF-kappa B (NF-κB) inflammatory pathways in a human gut epithelial cell line

(Caco-2) (84). This is supported by a study conducted on four-week-old mice, where oral iron supplementation resulted in shifts in gut microbiota composition, with an increase in the abundance of *Bacteroides vulgatus*, suggesting that iron excess may promote the proliferation of pathogenic bacteria like *Bacteroides vulgatus*, potentially exacerbating inflammation and impacting gut health. These findings suggest that alterations in the gut microbiome induced by high-iron diet in HIP and HIS piglets may be less detrimental compared to the changes observed in HI piglets. While the increased abundance of *Bacteroides* in an iron-rich environment may be indicative of certain inflammatory processes, there were no detrimental effects on piglet growth, as evidenced by no differences in body weights among the treatment groups, as previously described (44). While there was a decrease in *Bacteroides* in HIP and HIS piglets, the dietary inulin supplementation might have offset the expected rise in *Bacteroides* associated with iron-rich diets, potentially by increasing the abundance of inulin-fermenting bacteria or reducing colonic and fecal iron levels and promoting other beneficial bacterial groups such as *Prevotella* and *Lachnospiraceae_NK3A20_group*. *Weissella* was increased only in the AI group, with an initial increase found in HIP piglets, although this trend did not persist throughout the study. Conversely, we began to see a significant decrease in *Weissella* abundance in the HIS group on PD 8. *Weissella* strains have garnered attention for their probiotic potential and their ability to ferment prebiotic fibers in the GIT (85-87). Their capacity to produce exopolysaccharides (EPS) with prebiotic properties, further contributes to their probiotic features, and *Weissella* strains have exhibited the ability to produce bacteriocins, hydrogen peroxide, and organic acids, all of which have known pathogen-inhibitory activities (85, 88). Despite *Weissella* being identified in AI piglets in our study, further investigation into its probiotic potential and beneficial attributes in the context of dietary interventions is warranted to better understand its impact on gut microbiota and host health.

Our results suggest limited colonization of the probiotic *L. agilis* YZ050 in HIS piglets. This finding raises questions about the colonization potential of specific probiotics in neonatal piglets. *L. agilis* YZ050, known for its fructosidase activity on inulin, may serve as a cross-feed substrate for bacteria unable to ferment inulin, as demonstrated in previous research (42, 89). Despite this, it may still have transient effects on the gut microbiota during early infancy. Probiotic strains often do not become established members of the fecal microbiota post-ingestion, requiring constant administration to maintain their effects (90). The addition of probiotics may not readily impact resident microbial populations in the context of a healthy microbiota (91). However, in cases of dysbiosis, the introduction of probiotics could potentially exert a significant influence on reshaping the microbiota (92). Overall, our data indicate that the impact of iron supplementation or probiotic on the gut microbiota of neonatal piglets appears to be less pronounced compared to the notable effects observed with dietary inulin supplementation.

As we explore deeper into understanding the distinct alterations in the gut microbiota composition in response to high-iron and dietary inulin supplementation, it becomes apparent that the gut microbiota is particularly vulnerable to disturbances during early life, especially before the microbial community stabilizes with maturation (93). Breast milk plays a vital role by supplying essential probiotics and prebiotics for the developing gut. Studies suggest that long-term health differences associated with formula feeding may stem from early gut microbiota disruption (94-96). The typical infant formula provides approximately thirty-fold more iron than breast milk (97, 98) and early introduction of formula disrupts the development of the gut microbiota, which has negative implications for infant health and development (96, 99). It is plausible that the consumption of infant formula with excessive iron content may lead to adverse health effects in infants by displacing breast milk-associated microbes in the developing gut, although exactly how

dietary iron supplementation alters the gut microbiota is unclear. In infant formula, we can consider how dietary prebiotic supplements such as inulin might play a crucial role in mimicking the benefits of breast milk for infant gut health. Inulin is a dietary fiber that selectively promotes the growth of beneficial bacteria in the gut, similar to breast-fed infants' gut microbiota (39). Furthermore, the addition of probiotics, such as *Lactobacillus* strains, to infant formula can contribute to a balanced gut microbiome, mirroring the probiotic-rich nature of breast milk (100). The combination of prebiotics (37) and probiotics (101) in infant formula could provide a means to support infant gut health, promote proper digestion, and strengthen the immune system, potentially mitigating some of the disparities between formula-fed and breast-fed infants (102). However, it is essential to conduct further research to understand the exact mechanisms through which these supplements affect the gut microbiota in early life and to ensure their safety and efficacy in infant nutrition.

While the effects of dietary inulin supplementation on microbial composition necessitate a deeper understanding of these complex interactions, our research extends beyond the neonatal piglet model, offering potential insights into the modulation of the gut microbiome during early infancy. These findings lay the groundwork for future research aimed at elucidating the underlying mechanisms of inulin and SCFAs on whole-body metabolism (metabolomics) and changes in functional aspects of microbiota (metagenomics) induced by iron overload and dietary interventions in early life. By providing a clearer understanding of the complex interplay between dietary components and gut microbiota, our study contributes to advancing knowledge in this field, with implications for optimizing infant nutrition.

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FIGURES

Figure 3.1.

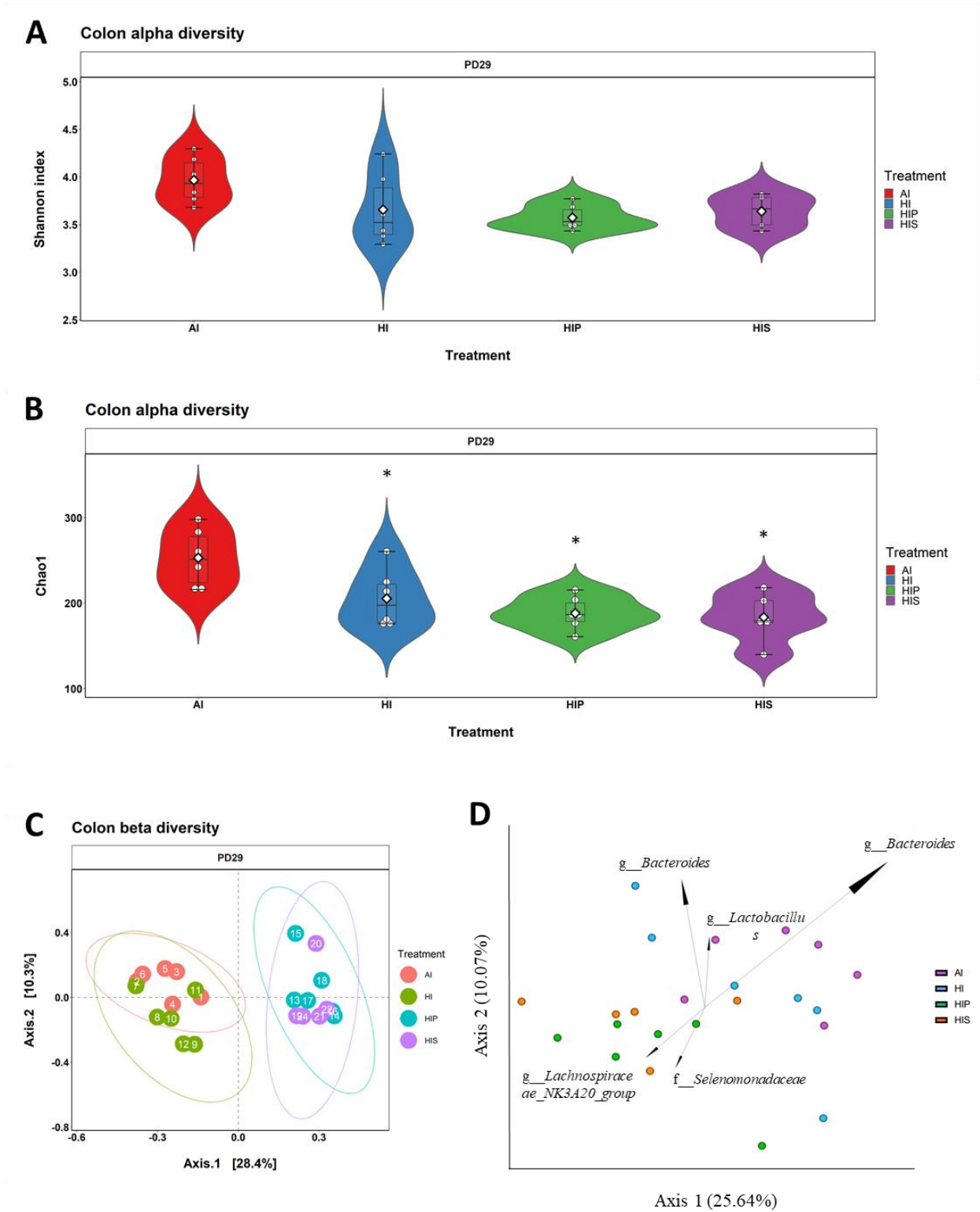
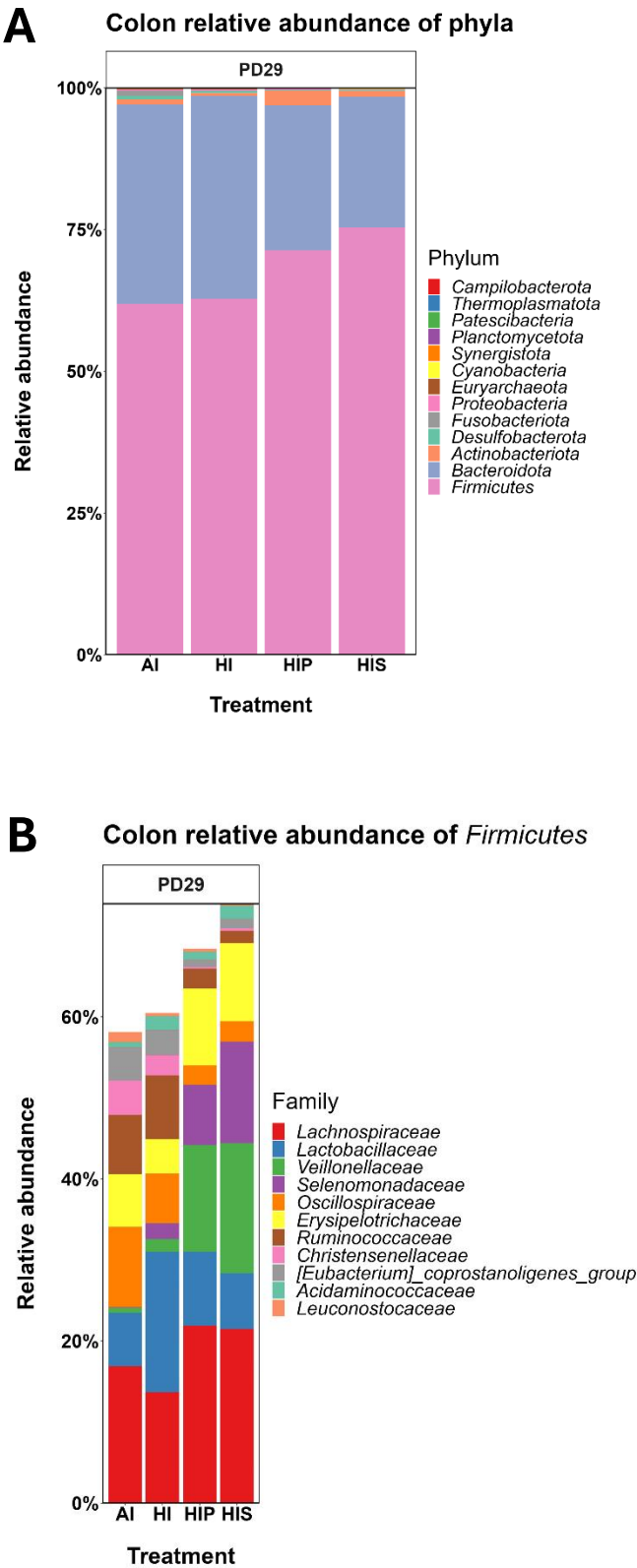


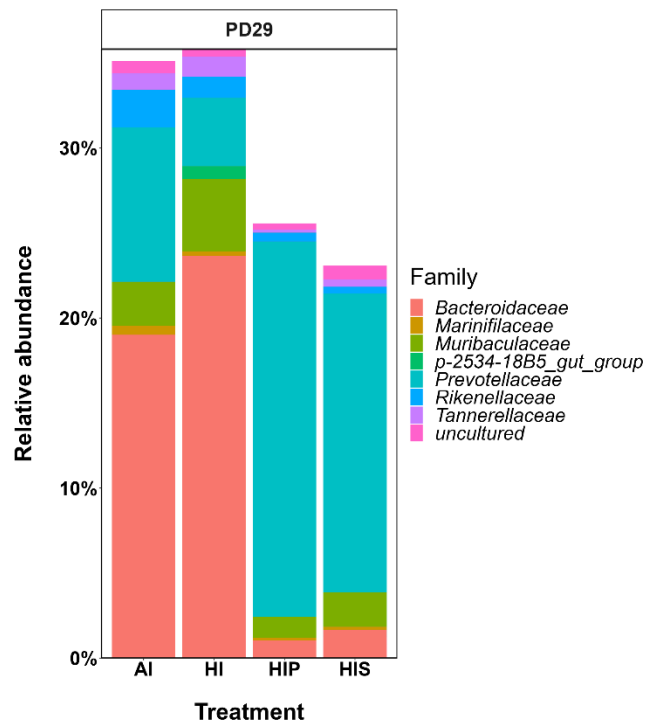
Figure 3.1. Diversity of colon microbiota

Alpha diversity, measured by Shannon (A) and Chao1 (B) indices, in colon content of piglets receiving AI, HI, HIP, or HIS diets on PD 29. Data are presented as mean (diamond) $\pm$ SEM. Principal Coordinate Analysis (PCoA) based on Bray-Curtis distances was used to assess beta diversity of colon content in neonatal piglets fed with AI, HI, HIP, or HIS diets on PD 29 (C). PCoA biplot was generated using QIIME 2 Emperor biplot (D), illustrating taxonomic annotations at the genus level, except for one arrow, annotated as *Selenomonadaceae* at the family level. Arrows in the biplot represent four genera and one family influencing sample positioning. The arrows effectively delineate differences between treatment groups in the colon digesta microbiome on PD 29. Each dot represents an individual piglet according to its treatment group. '*' denotes statistical significance at $P < 0.05$.

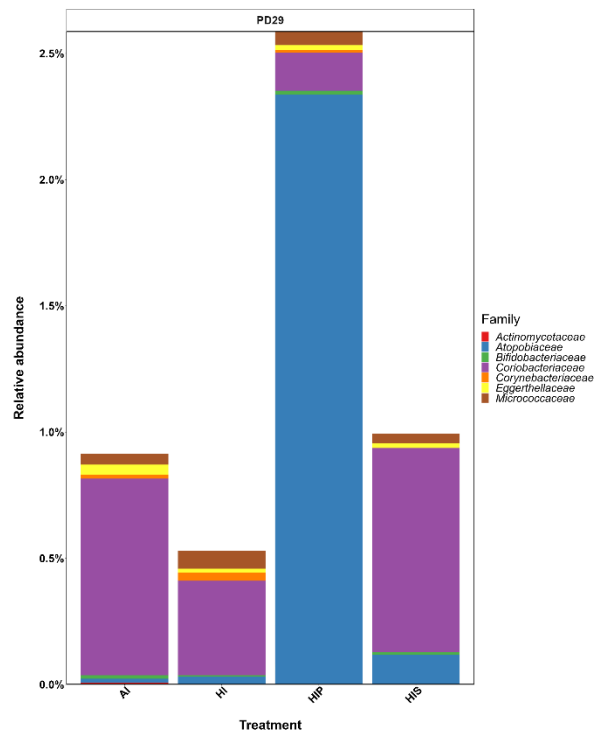
Figure 3.2.



C Colon relative abundance of *Bacteroidetes*



D Colon relative abundance of *Actinobacteriota*



E

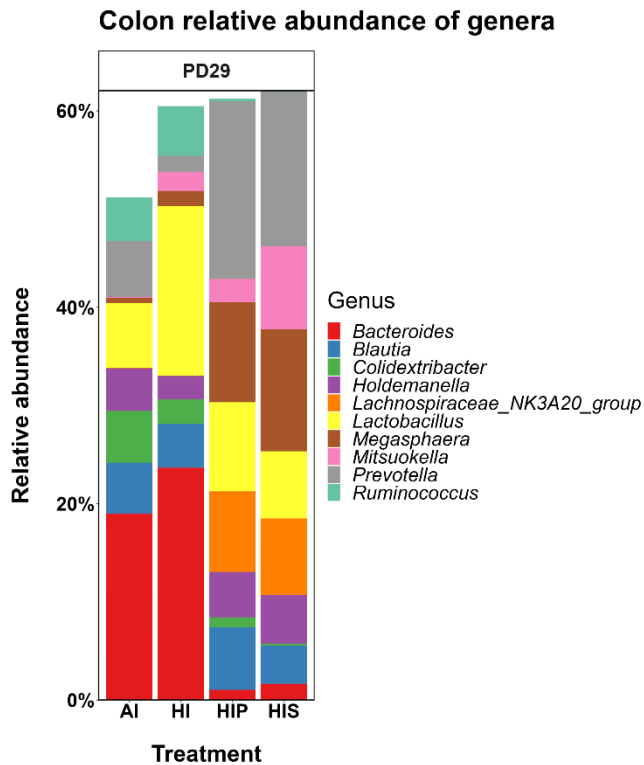


Figure 3.2. Relative abundance of colon microbiota

Relative abundances of gut microbiota at various taxonomic levels were examined: phylum (A), family (B-D), and genus (E). All phyla and *Bacteroidetes* were considered in the analyses, while only *Firmicutes* and genera constituting at least 1% of the total abundance were included in the analyses.

Figure 3.3.

A



B

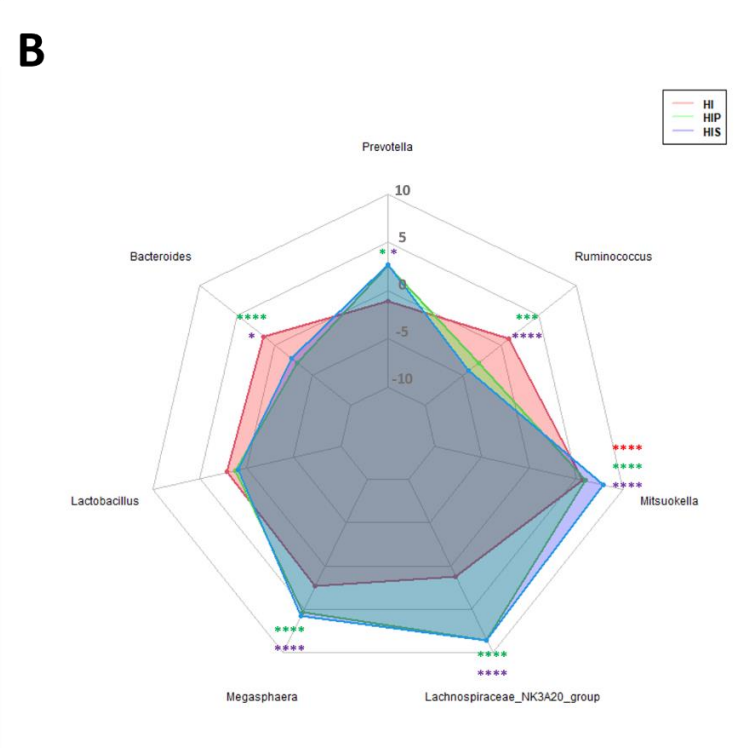


Figure 3.3. Differential abundance of colon microbiota

Fold changes (FCs) in microbial abundance in the colon across treatment groups (HI, HIP, and HIS) compared to the adequate iron group (AI). Radar charts depict the $\log_2$ FCs with a significance level of at least $P \leq 0.05$ in differential abundance of gut microbes at the genus and family levels in HI, HIP, and HIS pigs relative to AI pigs. Taxa were selected based on their actual abundance (top 8 genera and 7 families) and statistical significance. Symbols '#', '*', '**', '***', and '****' indicate a trend towards significance ($P < 0.1$), and statistical significance at $P < 0.05$, 0.01, 0.001, and 0.0001, respectively.

Figure 3.4.

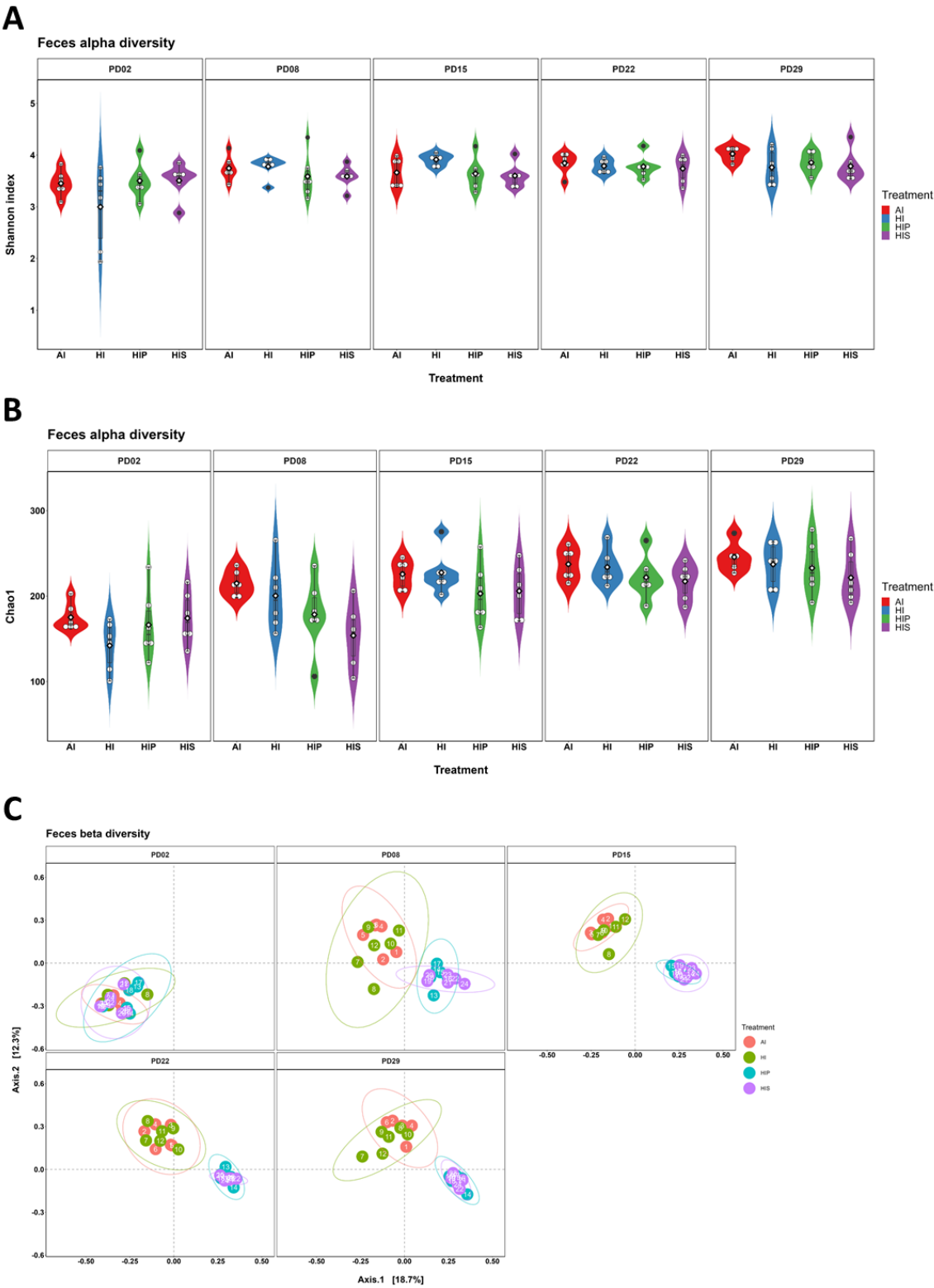
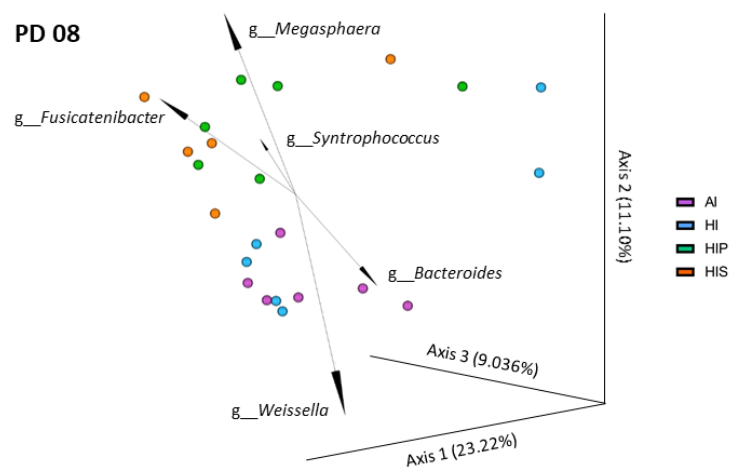


Figure 3.4. Diversity of fecal microbiota

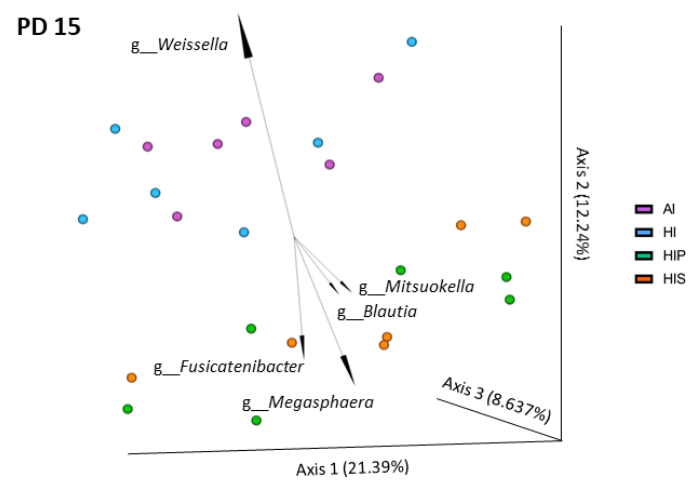
Alpha diversity, represented by Shannon (A) and Chao1 (B) indices, was assessed in fecal microbiota of piglets fed with AI, HI, HIP, or HIS from PD 2 to 29. The data are presented as mean (diamond) $\pm$ SEM. Beta diversity analysis was conducted using PCoA based on Bray-Curtis distances to examine the similarity of community composition and structure across fecal samples collected at PD 8, 15, 22, and 29 from piglets in each dietary group (C).

Figure 3.5.

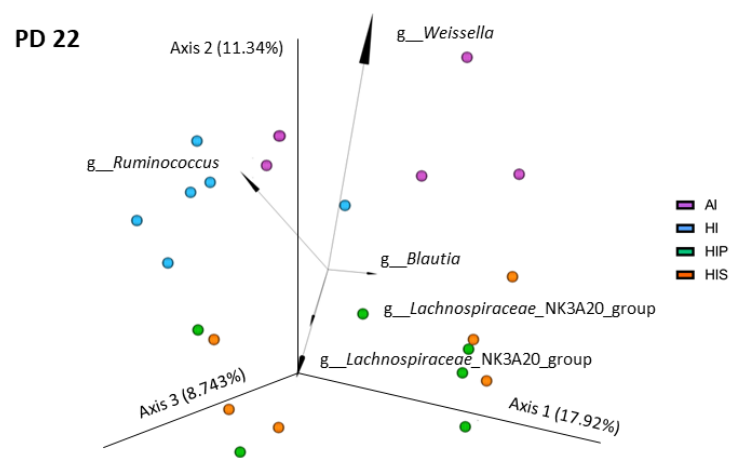
A



B



C



D

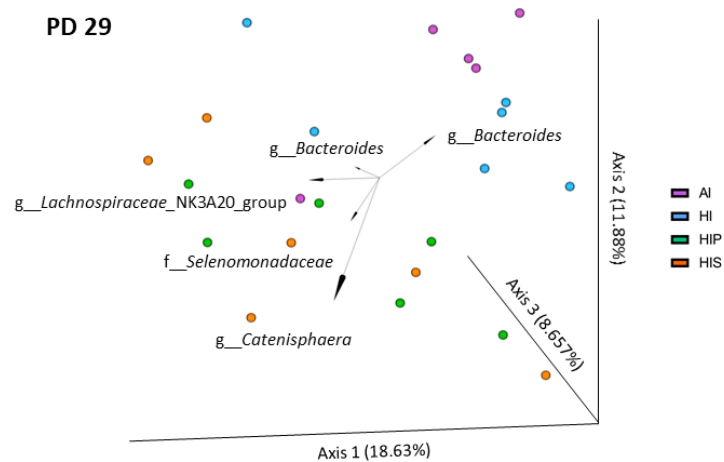
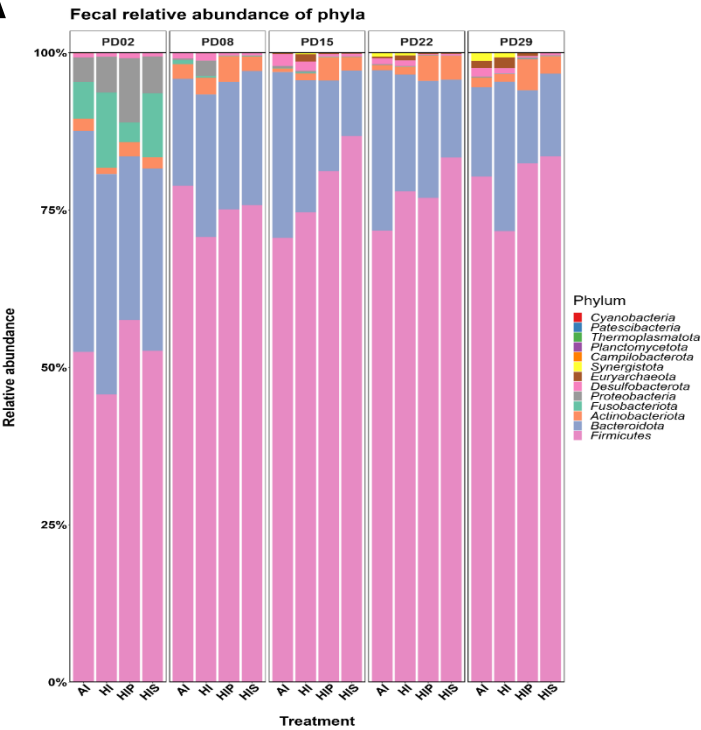


Figure 3.5.

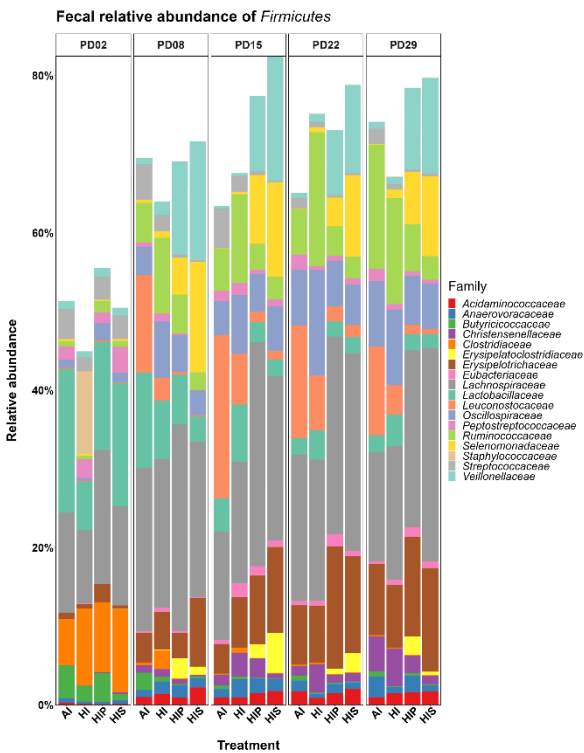
PCoA biplots were constructed to visualize the fecal microbiota composition of piglets fed with AI, HI, HIP, and HIS from PD 2 to 29, based on distance matrices generated using QIIME 2 Emperor biplot (A-B). Taxonomic annotations at the genus level were provided for most arrows, with one exception where *Selenomonadaceae* was annotated at the family level for PD 29. The biplot arrows highlight the top four genera driving sample differences on the plot, effectively illustrating distinctions between treatment groups in fecal microbiome composition throughout the study period. Each dot represents individual piglets according to their respective treatment group.

Figure 3.6.

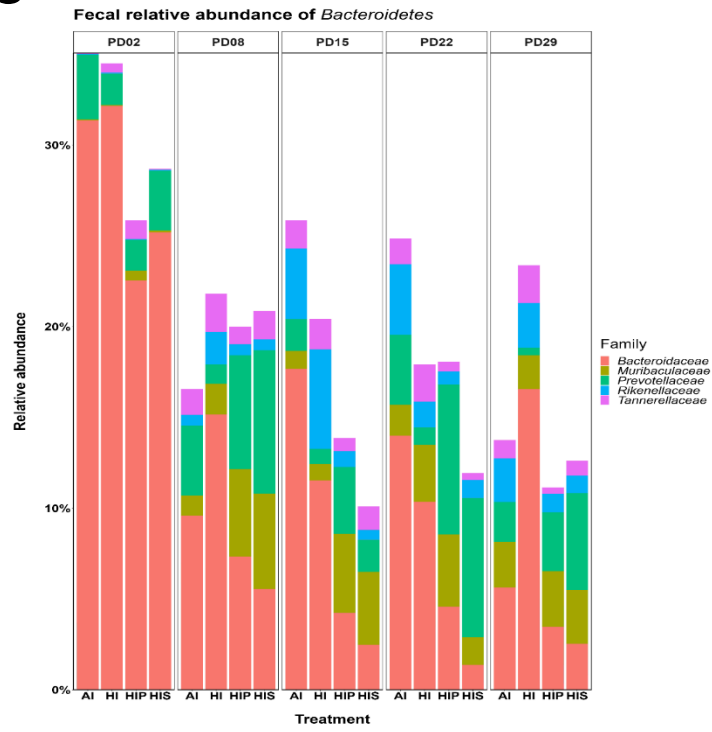
A



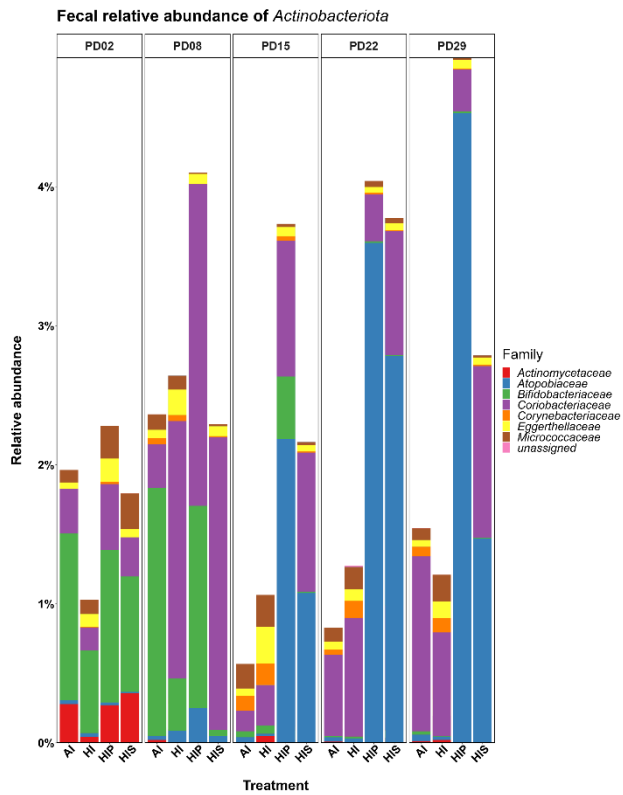
B



C



D



E

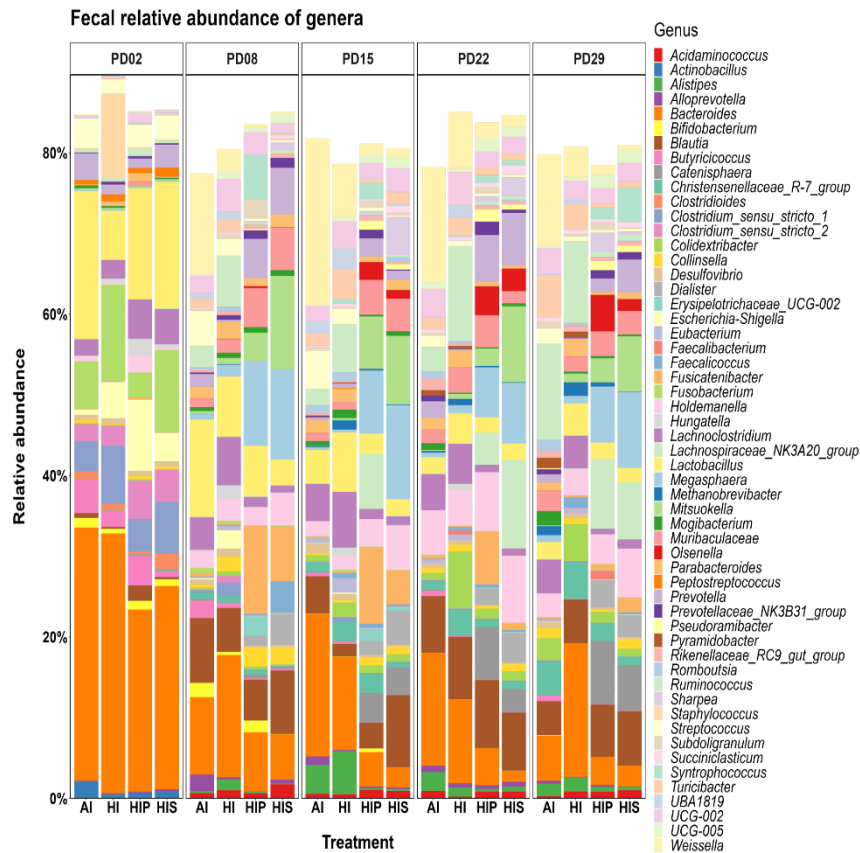


Figure 3.6. Fecal microbiota abundance

Relative abundances at different taxonomic levels were analyzed at phylum (A) and family (B-D), genus (E) levels. All phyla were considered in the analyses, while families and genera constituting at least 1% of the total abundance were included in the analyses.

Figure 3.7.

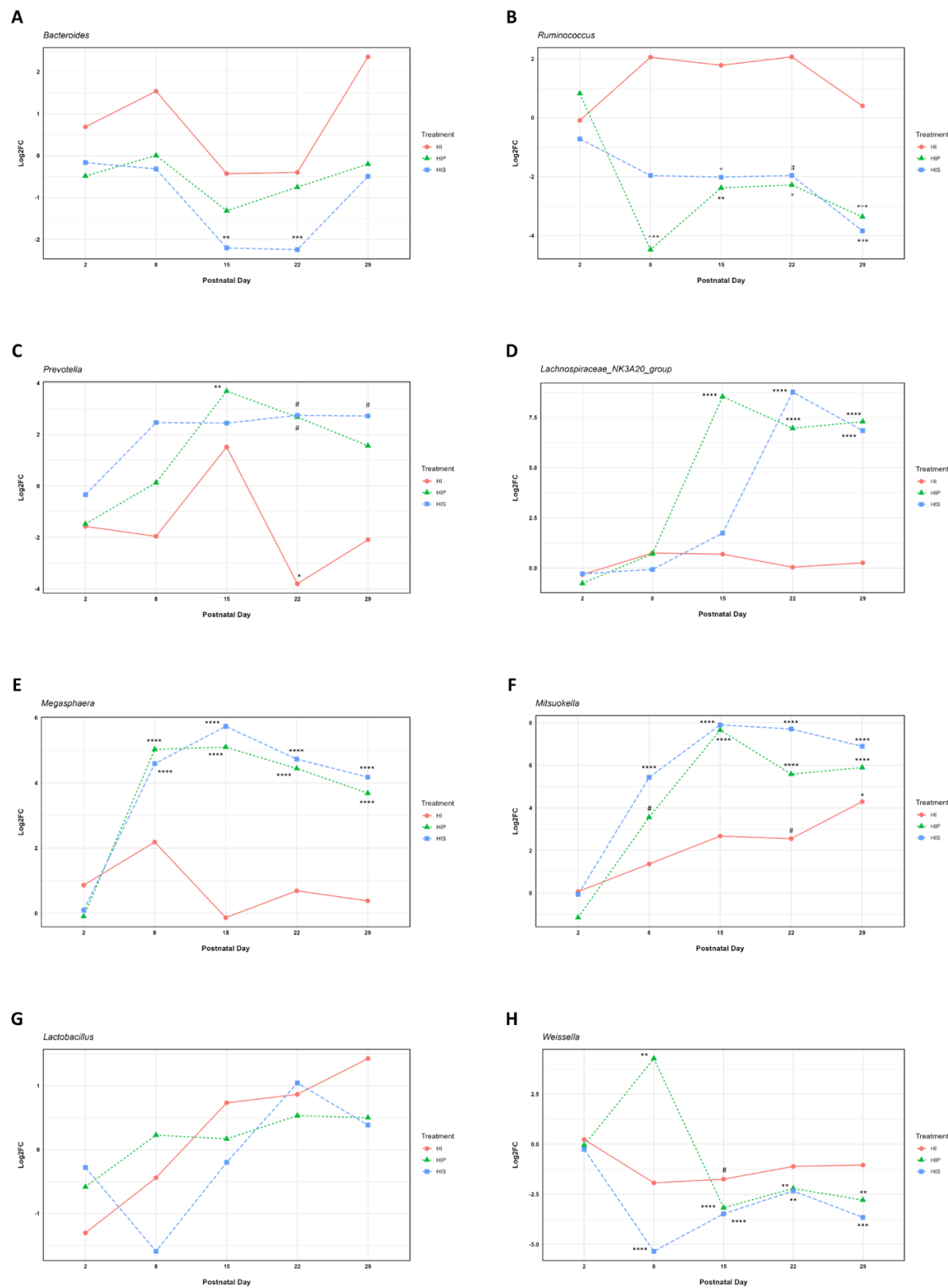


Figure 3.7. Differential abundance of fecal microbiota

To identify fecal microbiota with differential abundance over time between treatment groups, FCs in actual microbial abundance in fecal samples across treatment groups (HI, HIP, and HIS) were compared to the adequate iron group (AI). Time-course line graphs illustrate the $\log_2$ FCs with a significance level of at least $P \leq 0.05$ in differential abundance of gut microbes at the genus level in HI, HIP, and HIS pigs relative to AI pigs. Taxa were selected based on their actual abundance (top 8 genera) and statistical significance. Symbols such as #, *, **, ***, and **** denote a trend towards significance ($P < 0.1$) or statistical significance at $P < 0.05$, 0.01, 0.001, and 0.0001, respectively.

CHAPTER 4

Effects of dietary iron supplementation on gut microbiota and disease resilience of weaned pigs infected with enterotoxigenic *Escherichia coli*

ABBREVIATIONS:

ASV: amplicon sequence variant

BW: body weight

CON: Control iron diet

COI: Control iron diet with infection

DNA: deoxyribonucleic acid

E. coli: *Escherichia coli*

FC: fold change

Fe: iron

GIT: gastrointestinal tract

HII: High iron diet with infection

IACUC: International Animal Care and Use Committee

IBD: inflammatory bowel disease

ID: iron deficiency

IDA: iron deficiency anemia

IL-6: interleukin-6

LOI: Low iron diet with infection

MNP: micronutrient powder

mRNA: messenger

PCoA: principal coordinates analysis

PCR: polymerase chain reaction

qPCR: quantitative polymerase chain reaction

QIIME 2: Quantitative Insights Into Microbial Ecology 2

RCT: randomized controlled trials

RNA: ribonucleic acid

RT-qPCR: real-time

SCFA: short chain fatty acid

UCD: University of California, Davis

ABSTRACT

Background

Diarrhea remains a significant global health challenge, impacting individuals of all ages across all regions of the world. In areas where intestinal infections are prevalent, the question of iron supplementation for young children remains contentious. The interaction between dietary iron intake, intestinal iron levels, and their impact on the development of enteric pathogens, the composition of the gut microbiome, and the host's defenses against intestinal infections remains unclear. To explore this complex dynamic, an attenuated diarrheagenic *Escherichia coli* (*E. coli*) strain has been utilized as an intestinal infection challenge model. While enterotoxigenic *E. coli* (ETEC) serves as a prominent cause of infectious diarrhea, particularly affecting children under 5 years old in developing nations characterized by limited resources, poor sanitation, and high rates of micronutrient deficiencies, our primary focus lies in understanding intestinal infections and their association with diarrheal episodes and dietary iron intake in this vulnerable population around the world.

Objectives

Using a weanling pig-challenge model, this study aimed to investigate how host iron status and dietary iron supplementation impact the host susceptibility to ETEC infection and the host gut microbiota.

Methods

Thirty-two pigs were weaned at postnatal day (PD) 21, stratified by body weight (BW) and sex, and randomly assigned to four treatments including an adequate iron diet (250 mg/kg) without ETEC challenge (CON), and a low iron diet (125 mg/kg; LOI), an adequate iron diet (COI), or a high iron diet (750 mg/kg; HII) with ETEC challenge. On study day (d) 10, pigs were orally

inoculated with a 1×10^{10} cfu/dose of F18 ETEC once daily for 3 consecutive days. Pigs were euthanized on d 16 for tissue sampling. BW and feed intake were recorded on d 0 (baseline), 10, and 16. Fecal consistency was recorded daily using a 5-scale fecal score, and feces were sampled via rectal swabs on d 0, 10, 13, and 16. Fecal swabs were subjected to bacterial culture on MacConkey agar and Columbia Blood Agar verification of beta-hemolytic *E. coli*. Blood sampled on d 0, 10, 13, and 16 was analyzed for hemoglobin (Hb) and hematocrit (Hct). Intestinal tissue sections were stained for iron using Prussian blue. Gut microbiota in ileal and colonic digesta and feces were analyzed using 16S rRNA sequencing.

Results

ETEC was undetected in feces of all pigs on d 10 before inoculation but was detected in ETEC-inoculated pigs on d 13. By d 16, only 2 fecal samples from each ETEC-inoculated group were positive for the pathogen, which was accompanied by improved fecal scores. LOI pigs had more days with diarrhea (fecal score ≥ 3) as compared to CON and COI pigs ($P < 0.05$). LOI pigs had lower average daily BW gain ($P < 0.05$) than CON and HII pigs by d 16. Hb levels were consistently higher in HII pigs than COI and LOI pigs ($P < 0.001$). Additionally, HII pigs exhibited higher Hct levels compared to COI and LOI pigs on d 10 and 16 ($P < 0.05$). *Peptostreptococcaceae* abundance was highest in CON and HII pigs and lowest in LOI pigs across fecal, colon, and ileum microbiota ($P < 0.05$). *Veillonellaceae* and its genus *Megasphaera* were more abundant in fecal and colon microbiota of LOI pigs compared to HII pigs on both d 10 and 16. *Actinobacteroida* and *Bifidobacteriaceae* abundance were highest in LOI pigs and lowest in HII pigs on d 16, with *Bifidobacterium* showing similar trends. Within *Proteobacteria*, *Desulfobacterota* abundance was highest in LOI and HII pigs and lowest in CON pigs.

Conclusion

This study demonstrated that dietary iron supplementation and host iron status influence susceptibility to ETEC infection and gut health in weanling pigs. LOI pigs experienced more days of diarrhea and exhibited lower BW compared to the other groups, suggesting a potential role of iron in modulating disease severity. Moreover, higher Hb and Hct levels observed in HII pigs may indicate better overall health and resistance to infection. Maintaining iron replete status during ETEC infection alleviated diarrheal illness and mitigated BW reduction. Analysis of gut microbiota revealed distinct shifts in microbial composition, suggesting associations between dietary iron, ETEC, and specific bacterial taxa. Overall, these findings highlight the complex interplay between dietary iron, host susceptibility to infection, and gut microbial ecology, providing insights into potential strategies for improving gut health and disease resistance in pigs and potentially informing approaches to managing diarrheal diseases in humans, particularly in resource-limited settings.

Keywords: diarrhea, enterotoxigenic *Escherichia coli*, ETEC, gut microbiota, iron deficiency, iron fortification, iron overload, iron supplementation, pig model, young children

INTRODUCTION

Diarrheal diseases remain a significant public health concern globally, particularly among children under 5 years of age in developing countries (1-3). While iron fortification presents an effective strategy to combat iron deficiency (ID) and iron-deficiency anemia (IDA) in such regions (4, 5), the use of iron-containing micronutrient powders (MNPs) and supplements has been associated with a modest increase in diarrhea risk (6). Both in vitro and in vivo studies have suggested that this heightened risk stems from elevated colonic iron levels, which disrupt the gut microbiome by reducing the populations of beneficial barrier commensal bacteria like lactobacilli and bifidobacteria, while increasing the abundance of enteropathogenic *Escherichia coli* (*E. coli*) (6). In a 6-month randomized controlled trial (RCT) examining the effects of iron fortification in Ivorian children aged 6-14 years, those receiving iron-fortified biscuits (20 mg Fe/d) showed no improvement in iron status or anemia (7). Instead, the fortified biscuits altered fecal microbiota, increasing diarrhea incidence and enterobacteria while reducing lactobacilli and bifidobacteria, and elevating fecal calprotectin levels, a marker of gut inflammation (7). A weanling pig study further demonstrated the impact of iron levels on diarrhea incidence and microbial balance. Increasing doses of iron (0, 50, 100, and 250 mg ferrous sulfate/kg body weight (BW)) led to a linear increase in diarrhea incidence, higher coliform numbers, and reduced ratios of beneficial lactobacilli and bifidobacteria to enterobacteria (8). These findings suggest that iron fortification may contribute to a more pathogenic gut microbiota profile and increased gut inflammation. Moreover, a systematic review concluded that iron provision is associated with a higher risk of diarrhea in children (9). In Nepal, although there was a nonsignificant increase in diarrhea-related deaths among the iron-supplemented group (10), clinical trials in Bangladesh reported a significant rise in diarrhea incidence (11).

Enterotoxigenic *Escherichia coli* (ETEC) is a leading cause of enteric infection and diarrheal illness, contributing to up to 20% of diarrheal cases in resource-limited communities (12). In published studies performed between 2010–2016, ETEC emerged as the predominant pathogen responsible for traveler's diarrhea (TD), constituting an average of 42% and 28% of cases among travelers to Latin America and Asia, respectively (13). ETEC is estimated to cause approximately 220 million episodes of diarrhea globally, with around 75 million occurring in children under 5 years old. These episodes result in an estimated 18,700 to 42,000 deaths among children younger than 5 years, as reported by the Institute for Health Metrics and Evaluation (IHME) and the Maternal and Child Epidemiology Estimation Group (MCEE), respectively (14, 15). Early-life infection with ETEC not only poses immediate health risks but also raises concerns regarding its potential long-term impacts. Recent studies suggest that early life infections, such as ETEC-induced diarrhea, may compromise neuronal development, potentially affecting cognitive function and overall neurobehavioral outcomes in children (16, 17). Frequent diarrhea episodes during the first 2 years of life have been inversely associated with cognitive development and early school performance (16, 17). Checkley et al. estimated that diarrhea during early childhood increases the likelihood of being stunted at 24 months of age five-fold (18). Moreover, early childhood stunting has been linked to cognitive impairment (19).

The extent to which host resilience to ETEC infection is influenced by factors such as iron status and dietary iron content remains unclear. Iron is an important micronutrient for both microbial growth and host physiology (20, 21), raising questions about its role in shaping the host response to ETEC infection and the composition of the gut microbiota in young children. To address these critical knowledge gaps, this study utilized a pig model. The pig model offers several advantages, including physiological and anatomical similarities to humans and the ability to

closely mimic dietary conditions and infectious challenges relevant to early childhood (22). In addition, the ETEC infection model for post-weaning diarrhea is an established and reproducible intestinal infection model for diarrhea in pigs (23) and mice (24), and in vitro (24). By assessing the interplay between dietary iron levels, ETEC infection, and host gut health responses, this study aimed to provide valuable insights into the mechanisms underlying host resilience to intestinal infection in early childhood, while also investigating the implications of dietary iron supplementation for gut health and disease resistance through an analysis of shifts in gut microbiota composition.

MATERIALS AND METHODS

Study design and animal management. The study protocol was approved by the Institutional Animal Care and Use Committee at University of California Davis. Thirty-two crossbred (Duroc × Yorkshire × Landrace) weanling pigs (BW = 6.83 ± 0.67 kg; 19 gilts and 13 barrows selected from the Swine Teaching and Research Center of the UCD) were stratified by birth BW and sex and randomly assigned to one of the four dietary treatments (n=8/treatment) at postnatal day (PD) 21. The four treatments included: 1) control – iron-replete diet (250 mg elemental iron/kg) and no ETEC inoculation (CON), 2) low-iron diet (100 mg elemental iron/kg) and oral inoculation with ETEC (LOI), 3) iron-replete diet and oral inoculation with ETEC (COI), or 4) high-iron diet (750 mg elemental iron /kg) and oral inoculation with ETEC (HII). The ingredient composition and nutrient profile of both the experimental and basal diets, as well as the amounts of iron supplementation in the experimental diets, are listed in **Table 4.1.** and **Table 4.2.**, respectively.

Pigs were individually housed (pen size: 0.61 m x 1.22 m) in an environment control unit at the Cole A Facility at UCD. The experiment was conducted in a temperature-controlled room

(25°C) with a pre-set 12-h shift of light-dark cycle (light on 0700-1900 h). Pigs were fed *ad libitum* with free access to water throughout the experiment.

All piglets were subjected to genotyping for polymorphism in FUT1, a gene encoding an alpha 1,2-fucosyltransferase which modulates mucosal glycan moieties and host susceptibility to F18 ETEC adhesion (25, 26). Only pigs of susceptible genotypes were used in the study. Before weaning, feces were collected from sows and all their piglets to verify the absence of β -hemolytic *E. coli* (26). Neither selected piglets or their sows received *E. coli* vaccines or antibiotic treatment before weaning.

The experiment lasted for 17 days including 10 days for diet adaptation followed by 7 days post-ETEC inoculation (from the first day of the inoculation). On study day 10, pigs were orally inoculated with a 3-mL dose of F18 ETEC inoculum (1×10^{10} cfu/mL, prepared in phosphate-buffered saline (PBS)) for 3 consecutive days. CON pigs were sham inoculated with sterile PBS solution following the same protocol for inoculation as the infection groups.

Data and sample collection. BW were recorded weekly on d 0 (baseline), 10, and 16, and feed intake was recorded daily. Average daily gain (ADG) and average daily feed intake (ADFI) were measured for the period of pre-inoculation (d 0 to d 10) and post-inoculation (d 11 to d 17). Clinical observations (diarrhea and alertness scores) were recorded twice daily throughout the study. The alertness score of each pig was assessed visually with a score from 1 to 3 (1 = normal, 2 = slightly depressed or listless, and 3 = severely depressed or recumbent). Data are not reported, as all pigs had an alertness score of 1 during the study. Fecal consistency was scored twice daily at 0700 and 1900 h using a 5-scale scoring system (1 = normal firm and shaped feces, 2 = soft and shaped feces, 3 = mild diarrhea with mushy and loose stools, 4 = severe diarrhea with feces appearing as slurries on the floor, and 5 = severe diarrhea with watery stools). Severity of diarrhea is calculated as

average days with fecal score ≥ 3 . Fecal samples were collected from the rectum of pigs on d 0, 10, 13, and 16 using a fecal loop for the detection of β -hemolytic coliforms. Fecal samples were stored at -80°C for further analyses. Blood samples were collected from the jugular vein through venipuncture in sodium heparin vacutainers (Becton, Dickinson and Company, Franklin Lakes, NJ) before the first meal on d 0, 10, 13, and 16. Plasma samples were harvested through centrifugation ($1,500\times g$ at 4°C for 15 min), aliquoted and stored at -80°C until further analyses. Pigs were euthanized on d 16, and tissue samples, and ileal and distal colon contents were collected and stored at -80°C until further analyses.

Verification of β -hemolytic *E. coli* infection. Fecal samples were plated on MacConkey agar and Columbia Blood Agar to detect and enumerate total coliforms and hemolytic coliforms, respectively. All agar plates were incubated at 37°C for 24 hours in an air incubator. Visual assessment was employed to quantify populations of both total coliforms and β -hemolytic coliforms on the blood agar plates, with scores ranging from 0 (no bacterial growth) to 8 (heavy bacterial growth) denoting bacterial growth intensity. The ratio of scores between β -hemolytic coliforms and total coliforms was calculated. Any colonies raising doubt were subjected to further confirmation of β -hemolytic *E. coli* identity by PCR testing to verify F18 *E. coli* positivity (27).

Hematological indices. Hemoglobin (Hb) was measured in whole blood samples using Drabkin's reagent (Sigma-Aldrich, St. Louis, MO) with modifications. The sample mixture volume was reduced to 250 μL for detection using a microplate reader (BioTek Synergy HTX, Winooski, VT). Absorbance was determined at 540 nm and Hb concentration was calculated based on a standard curve. The modification had been previously verified and showed no difference in accuracy compared to the original method. Hematocrit (Hct) was determined using a microhematocrit centrifuge.

Microbiome analysis. Microbial profiles in ileal and proximal colon contents and fecal samples were determined using 16S rRNA sequencing. The microbiome library preparation was done following a procedure previously described (28). The 16S rRNA genes of bacterial DNA were amplified by PCR using a primer set that targets the V4 region (515 F: 5'-XXXXXXXXGTGTGCCAGCMGCCGCGG TAA-3'; 806 R: 5'-GGACTACHVGGGTWTCTAAT-3') and contains an 8 bp barcode (X) and Illumina adapter (GT). Each PCR reaction contained 2 µL DNA template, 9.5 µL nuclease-free water, 12.5 µL GoTaq 2×Master Mix (Promega, Madison, WI), 0.5 µL of each V4 forward and reverse primer (10 µM). The PCR was performed with the following protocol: 1) 94°C for 3 min for denaturation, 2) 94°C for 45 seconds, 3) 50°C for 1 min, 4) 72°C for 1.5 min (Steps 2-4 were repeated for 35 times), 5) 72°C for 10 min for elongation. Each sample was amplified via PCR in duplicates. The DNA library was prepared by pooling PCR products of all extracted bacterial DNA samples from pigs. The volume of each sample added to the library was subjectively adjusted based on band intensity stained with SYBR safe (Invitrogen Co., Carlsbad, CA) in a 2% agarose gel. The pooled sample was purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and then submitted to the UCD Genome Center for 250-bp paired-end sequencing on the Illumina MiSeq platform (Illumina, Inc. San Diego, CA).

The microbiome analysis was performed following a procedure previously described (29, 30). Data processing and statistical analysis were conducted using the Farm Cluster, a Linux-based supercomputing cluster for the College of Agricultural and Environmental Sciences at UCD, and R statistics software, respectively. Sabre (<https://github.com/najoshi/sabre>) was used for demultiplexing and trimming sequence reads by aligning sequences to its sample of origin based on the barcode and removing barcodes from each end of raw sequences. Reads not matching any

barcode and sequences that fail to meet the minimum quality thresholds were discarded. The sequences were then imported into Quantitative Insights Into Microbial Ecology 2 (QIIME 2; version 2022.2.0) for downstream filtering and bioinformatics analysis (31). DADA2 plugin was used to remove primers and lower quality reads and denoise the paired end reads (32). Taxonomic classification was assigned using the taxonomy-classifier plugin, the SILVA rRNA V4 database (version 138.1) (33). The amplicon sequence variant (ASV) table and phylogenetic tree were imported into R (version 4.2.3) using the phyloseq package for analysis of alpha-diversity, beta-diversity, relative abundance, and statistical significance.

Statistical analysis. All statistical analysis and data visualization were conducted using SAS (v.9.4) or GraphPad Prism 10.0 (GraphPad Software, Boston, Massachusetts), except for gut microbiome data analysis and data visualization, which are done using R statistics (R version 4.2.3). The model included the fixed effects of treatment and day, as well as their applicable interactions. Pig nested in the treatment was included as the random term. The statistical model for fecal, colon, and ileal microbiome analysis included treatments, days, and the interaction. The analysis was performed using the phyloseq package and visualized using the ggplot2 package and QIIME2 (34); <https://qiime2.org/>). Data were tested for homogeneity of variance using Bartlett's test and for normality using the Shapiro-Wilk test for alpha diversity and relative abundance. Non-parametric tests, specifically the Kruskal-Wallis sum-rank test, Dunn's posthoc test, and Conover test, were conducted using the agricolae package. Beta diversity metrics among treatment groups were assessed using the Bray-Curtis dissimilarity for principal coordinates analysis (PCoA). The statistical significance of beta diversity was determined using PERMANOVA with 999 replicate permutations, employing the vegan package. Pairwise post hoc comparison was performed using the pairwiseAdonis package. Differential abundance analyses were performed using the DESeq2

package in R. The frequency of diarrhea was calculated as the total pig days with a fecal score ≥ 3 for each treatment group. Data of pairwise comparisons were analyzed using Fisher's exact test. Statistical significance and a trend towards significance were declared at $P < 0.05$ and $P < 0.1$, respectively.

RESULTS

Growth and diarrhea. Prior to the ETEC challenge, pigs were healthy and had alertness levels at 1 (data not shown), with no significant differences in BW observed among the treatment groups on d 10 (**Fig. 4.1. A**). However, by d 16, pigs in the HII group showed higher BW compared to those in the COI group ($P < 0.05$), while no significant difference in BW was observed between CON and HII groups. Especially, HII pigs demonstrated the highest BW across all treatment groups. On the other hand, LOI pigs exhibited the lowest BW gain post-ETEC inoculation compared to CON ($P = 0.0191$), COI ($P = 0.1137$), and HII ($P = 0.0013$) pigs (**Fig. 4.1. B**). Diarrhea frequency was assessed (**Fig. 4.2. A**), and the average number of days with a fecal score above 2 was determined (**Fig. 4.2. B**). LOI pigs had the highest number of days with diarrhea (fecal score > 2), significantly higher than CON and HII pigs ($P < 0.05$), indicating a worsening infection.

β -hemolytic positive coliforms. COI, LOI, and HII pigs had higher ($P < 0.05$) percentage of β -hemolytic coliforms in feces on d 13 compared with CON, while there were not differences observed between COI, LOI, and HII pigs on d 13 and across the treatment groups on d 16. (**Fig. 4.3.**).

Hematological indices. There was significant interaction effect (Trt $\times$ Day) for Hb and Hct levels ($P \leq 0.02$). Both Hb and Hct levels were significantly higher in HII than LOI on d 10 (**Fig. 4.4. A-B**, $P < 0.05$), and there was no difference among the other groups. On d 13, both indices were

higher in CON compared to COI and LOI pigs ($P < 0.05$). The Hb level was not significantly different among treatments on d 16, but Hct was significantly higher in HII than COI on d 16 ($P < 0.05$).

Iron accumulation in duodenum. Prussian blue staining showed pronounced non-heme iron deposition in duodenal crypt-villus areas in CON and HII pigs compared to negligible amounts in the LOI pigs (**Fig. 4.5. A**). Consistently, quantification of the iron-stained area by ROI based analysis using ImageJ showed a significant increase in iron in CON and HII compared to LOI pigs ($P < 0.05$; **Fig. 4.5. B**).

Gut microbiota analysis. In the gut microbiome analysis, 16S rRNA sequencing yielded an average of 15,077 amplicon sequence variant (ASV) reads following quality-filtering procedures outlined in the Methods and Materials section. To ensure robust analysis, a prevalence threshold was set at or below 1.0% of total samples, leading to the removal of taxa with abundances lower than 1% of the total sample percentage. Subsequently, the phyloseq object underwent cleaning by excluding sequences corresponding to not available (N/A) phyla, bacterial sequence counts constituting less than 1% of the total microbial composition, and phyla such as *Cyanobacteria* and chloroplast rDNA.

Diversity and relative abundance of fecal microbiota. The phyloseq object of 64 fecal samples yielded 1540 taxa on d 10 and 1524 taxa on d 16. The α -diversity of the fecal microbiota was assessed using Chao1 (richness; **Fig. 4.6. A**) and Shannon indices (richness and evenness; **Fig. 4.6. B**). The results showed no significant differences across the four treatment groups for both treatment and time effects.

The PCoA plots based on Bray-Curtis distance display the β -diversity of the fecal microbiome (**Fig. 4.6. C**). Adonis pairwise tests revealed significant main effects of treatment and

time (day; $P < 0.001$) but not an interaction effect ($P = 0.68$) on β -diversity. Specifically, the fecal microbiome β -diversity of LOI pigs was significantly different from that of the other treatment groups, while there was no significant difference among the CON, COI and HII groups.

The relative abundance of gut microbial taxa was examined on d 10 and 16, separately. On d 10, at the phylum level (**Fig. 4.6. D**), *Desulfobacterota* and *Spirochaetota* abundances were higher in HII pigs compared to the other groups ($P < 0.05$) and to LOI pigs ($P < 0.05$), respectively. In the family of *Firmicutes* (**Fig. 4.6. E**), *Anaerovoracaceae* abundance was higher in CON pigs compared to LOI pigs ($P < 0.05$); *Clostridia*_UCG-014 abundance was higher in CON and COI pigs than in HII pigs ($P < 0.05$); *Clostridia*_vadinBB60_group abundance was higher in HII pigs than in CON and LOI pigs ($P < 0.05$); *Clostridiaceae* abundance was higher in COI pigs than in LOI pigs ($P < 0.05$); *Peptostreptococcaceae* abundance was higher in HII pigs than in LOI pigs ($P < 0.05$); *Selenomonadaceae* abundance was higher in LOI pigs than in CON pigs ($P < 0.05$); *Veillonellaceae* abundance was higher in LOI pigs than in COI and HII pigs ($P < 0.05$). In the family of *Bacteroidota* (**Fig. 4.6. F**), no notable differences in relative abundance were found. In the phylum *Proteobacteria* (**Fig. 4.6. G**), *Enterobacteriaceae*, the family containing *E. coli*, were higher in the order of LOI, HII, COI, and CON pigs, no notable differences in relative abundance were found. At the genus level (**Fig. 4.6. I**), *Megasphaera* abundance was higher in LOI pigs than in HII pigs ($P < 0.05$), whereas *Prevotella* abundance was higher in LOI pigs than in CON pigs ($P < 0.05$).

The phylum profile of the fecal microbiome on d 16 was similar to that on d 10 with *Firmicutes* being the dominant phylum followed by *Bacteroidota* (Figure 6D). The relative abundance of three phyla was significantly affected by treatment ($P \leq 0.046$). *Firmicutes* was the most abundant in COI followed by CON pigs, HII and the least abundant in LOI pigs ($P = 0.022$).

The difference was significant in comparisons between COI and HII or LOI pigs or between CON and LOI pigs ($P < 0.05$). *Actinobacteriota* was more abundant in LOI than HII pigs ($P < 0.05$), but was not different in any other comparisons. The relative abundance of *Desulfobacterota* was significantly higher in LOI and HII pigs compared to CON pigs ($P < 0.05$).

Among the *Firmicutes* families (Fig. 4.6. E), *Peptostreptococcaceae* abundance was highest in abundance and higher in CON and HII pigs than in LOI pigs ($P < 0.05$); *Veillonellaceae* abundance was highest and higher in LOI pigs than in HII pigs ($P < 0.05$). In the family of *Bacteroidota*, *Rikenellaceae* abundance was highest and higher in HII pigs than in CON pigs ($P < 0.05$). In the phylum *Proteobacteria* (Fig. 4.6. G), while *Enterobacteriaceae* were higher in order of LOI, CON, HII, and COI pigs, no notable differences in relative abundance were found. In the phylum *Actinobacteria* (Fig. 4.6. H), *Bifidobacteriaceae* abundance was highest and higher in LOI pigs than in CON, COI, and HII pigs ($P < 0.05$), while HII pigs had the lowest abundance. At the genus level (Figure 6I), *Bifidobacterium* abundance was highest and higher in LOI pigs than in CON, COI, and HII pigs ($P < 0.05$), where HII pigs had the lowest abundance; *Holdemanella* abundance was highest and higher in LOI pigs than in HII pigs ($P < 0.05$); *Megasphaera* abundance was highest and higher in LOI pigs than in HII pigs ($P < 0.05$); *Prevotellaceae_NK3B31* abundance was highest and higher in LOI pigs than in COI pigs ($P < 0.05$).

Diversity of colon microbiota. The phyloseq object of colon sample analysis has a total number of taxa at 1296 and 32 samples. α -diversity of the colon microbiota was assessed using Chao1 (Fig. 4.7. A) and Shannon (Fig. 4.7. B) indices. The results showed no significant differences across the four treatment groups.

For β -diversity, adonis pairwise tests revealed significant differences in β -diversity in pairwise comparisons ($P = 0.018$) (Fig. 4.7. C). The notable differences arose from the comparison

between CON and HII ($P = 0.134$), CON and LOI pigs ($P = 0.05$), COI and HII pigs ($P = 0.121$), and LOI and HII pigs ($P = 0.006$).

At the phylum level (**Fig. 4.7.D**), *Actinobacteriota* abundance was highest in LOI pigs and higher in LOI and COI pigs than in HII pigs ($P < 0.05$); *Spirochaetota* abundance was highest and higher in HII pigs than in LOI and COI pigs ($P < 0.05$); *Synergistota* abundance was highest and higher in HII pigs than in CON pigs ($P < 0.05$). No significant differences in abundances were observed for *Firmicutes*, *Bacteroidota* and *Proteobacteria*. In the family of *Firmicutes* (**Fig. 4.7.E**), *Christensenellaceae* and *Oscillospiraceae* abundance was highest and higher in HII pigs than in LOI pigs ($P < 0.05$); *Peptostreptococcaceae* abundance was highest in HII pigs and higher in CON, COI, and HII pigs than in LOI pigs ($P < 0.05$); *Peptostreptococcales-Tissierellales* abundance was highest and higher in HII pigs than in LOI and COI pigs ($P < 0.05$); *Veillonellaceae* abundance was highest in LOI pigs and higher in LOI and COI pigs than in HII pigs ($P < 0.05$). In the family of *Bacteroidota* (**Fig. 4.7.F**), *Rikenellaceae* abundance was highest and higher in HII pigs than in CON, LOI, and COI pigs ($P < 0.05$). In the family of *Actinobacteria*, *Bifidobacteriaceae* abundance was highest and higher in LOI pigs than in CON and HII pigs ($P < 0.05$); *Coriobacteriaceae* abundance was highest and higher in COI pigs than in HII pigs ($P < 0.05$). At the genus level (**Fig. 4.7.G**), *Bifidobacterium* abundance was highest and higher in LOI pigs than in CON and HII pigs ($P < 0.05$); *Christensenellaceae_R-7* abundance was highest and higher in HII pigs than in LOI pigs ($P < 0.05$); *Megasphaera* abundance was highest in LOI pigs and higher in LOI and COI pigs than in HII pigs ($P < 0.05$); *Rikenellaceae_RC9_gut* abundance was highest and higher in HII pigs compared to CON, LOI, and COI pigs ($P < 0.05$).

Diversity of ileal mucosal microbiota. The phyloseq object of fecal sample analysis has a total number of taxa at 343 and 30 samples. α -diversity of the colon microbiota was assessed using

Chao1 (**Fig. 4.8. A**) and Shannon (**Fig. 4.8. B**) indices. The results showed no significant differences across the four treatment groups. For β -diversity, PCoA plots based on Bray-Curtis distance were used (**Fig. 4.8. C**). Adonis pairwise tests revealed significant differences between CON and HII pigs ($P = 0.018$), LOI and HII pigs ($P = 0.135$), and COI and HII pigs ($P = 0.099$) in β -diversity. In the family of *Firmicutes* (**Fig. 4.8. D**), *Peptostreptococcaceae* abundance was highest and higher in COI and HII pigs than in LOI pigs ($P < 0.05$).

DISCUSSION

The safety of iron supplementation and fortification in infants and young children has been questioned due to the concerns for adverse effects resulting from iron overload (5, 35). These concerns have been confirmed in a double-blinded RCT in Kenyan infants showing that excessive colonic iron levels from MNP promoted the growth of pathogenic enterobacteria such as *Salmonella*, *Clostridium difficile*, *Clostridium perfringens*, and pathogenic *E. coli*, potentially exacerbating intestinal inflammation and impeding nutrient absorption, while depleting lactobacilli and bifidobacteria (36). In a study conducted by Kortman et al., the safety of untargeted oral iron supplementation in developing countries was questioned, with concerns raised about its potential to promote the growth of enteric pathogens while compromising beneficial commensals in the gut microbiota (37). To investigate this further, they conducted a series of experiments: 1) examining whether pathogenic bacteria exhibited a growth advantage over non-pathogenic bacteria in an iron-rich environment by testing the effect of ferric citrate on the growth of a selection of enteric bacteria in vitro, 2) assessing the adhesion of these bacteria to host epithelial cells, a crucial virulence characteristic, which can be followed by invasion into intestinal epithelial cells during the infectious process, and 3) whether enteric bacteria could breach the intestinal

epithelial barrier to cause systemic infection. Their results highlighted the possibility of excess luminal iron favoring the selection of enteric pathogens, especially *Salmonella typhimurium*, over commensals, thereby escalating the risk of infectious diseases. Building upon these findings, the current study sought to delve deeper into the impact of host iron availability on the prevalence of enteric pathogens in ETEC-challenged pigs.

As ETEC poses a significant threat to both young children (1-3) and pigs (28, 38), leading to the development of diarrhea and the potential for growth retardation during early life development, we studied the pathogenic ETEC-challenged pigs to explore the impact of varying iron concentrations in conjunction with ETEC infection on the gut microbiota as a translational model for young children in poor hygiene conditions or resource limited areas. Moreover, the relationship between iron supplementation, diarrhea risk, and alterations in the gut microbiome remains uncertain. Given that IDA (39, 40) and ETEC (41) leading to diarrhea are major contributors to morbidity and mortality among infants in developing nations, it is imperative to explore potential benefits and risks associated with iron supplementation, and addressing this challenge necessitates the development of dietary iron practices aimed at treating diarrhea and enhancing host resilience.

Iron plays a pivotal role in the growth and development of young children (42) and piglets (43), with both populations exhibiting high demands for this essential nutrient. Our study employed a control diet containing 250 mg/kg iron, ensuring adequate iron supply to prevent iron deficiency anemia (IDA) after weaning, as per the recommendation of 100-150 mg dietary iron/kg of diet by the National Research Council (NRC) (44). The LOI diet provided just enough iron to meet the requirements for normal growth, avoiding the onset of anemia or other clinical symptoms. The dietary iron dosages used in our study showed a dose-dependent effect on Hb and Hct levels

after a 10-day acclimation period. Hb concentrations of ≥ 100 g/L are indicative of adequate iron status in pigs, whereas levels ≤ 70 g/L signify anemia. By d 13 and 16, Hb and Hct levels in all ETEC-challenged pigs were significantly lower compared to CON pigs, suggesting compromised erythropoiesis during peak infection. Hypoferremia and reduced erythrocyte production in chronic intestinal infection patients primarily result from hepcidin induction and subsequent iron sequestration (45, 46). In a RCT with inflammatory bowel disease (IBD) patients, with upregulated serum levels of interleukin-6 (IL-6), in turn upregulated the expression of liver hepcidin, iron uptake decreased from the enterocytes and there was a reduced ability to take advantage of sufficient iron for effective erythropoiesis (45). However, in a mouse model of turpentine-induced chronic inflammation, anemia development involved a hepcidin-independent suppression of intramedullary erythropoiesis (47). This reduction in Hb and Hct levels on d 13 aligns with observations of hypoferremia and decreased erythrocyte production in individuals with chronic infections, primarily attributable to the induction of hepcidin and subsequent iron sequestration (48). Such mechanisms likely contribute to the observed outcomes in our study as well. Iron absorption primarily occurs in the duodenum in both humans and pigs (49). Our study revealed that pigs subjected to the LOI diet induced the smallest area stained for iron. The hematological changes observed across the treatment groups highlights the impact of varying levels of dietary iron supplementation on the pigs' physiological parameters.

The rapid growth rate of weaned pigs makes them particularly susceptible to iron deficiency (50, 51). This rapid growth likely contributed to the observed reductions in both hematological indices in all pigs. All ETEC-challenged pigs developed diarrhea post-infection. This aligns with previous research confirming the ability of the ETEC F18 strain to induce pathogenicity (52). ETEC-infected pigs exhibited reduced feed intake and weight gain,

experiencing severe diarrhea for approximately six days post-infection, consistent with the typical duration of diarrheal symptoms in weaned pigs (53). It is worth noting that the parameters measured to characterize the severity of infection, such as the percentage of β -hemolytic positive colonies and diarrhea score, are semi-quantitative and possess limited sensitivity and accuracy in capturing subtle changes (54). The prevalence of β -hemolytic positive coliforms decreased by day 16, meaning that most pigs recovered from diarrhea by the end of the study.

No significant differences were observed in growth performance of weaned pigs among the CON, HII, and COI groups. However, LOI pigs exhibited lower average daily BW gain, especially compared to CON and HII pigs. The HII diet tended to increase BW and average daily gain compared to the LOI diet. Surprisingly, the HII diet reduced the frequency of diarrhea and promoted the growth rate of ETEC-challenged pigs. Previous research suggested an association between iron supplementation and increased risk of diarrheal illness, particularly in populations with low prevalence of iron deficiency (55). However, LOI pigs exhibited a higher incidence of diarrhea post-ETEC infection. The increased average days with diarrhea (fecal score > 2) among LOI pigs suggest that suboptimal body iron status and being borderline anemic exacerbated infection severity. In previous research, iron-deficient rats were found to have substantially fewer sIgA- and IgM-containing cells in their intestinal mucosa compared to iron-sufficient conspecifics (56). Moreover, an iron-starved environment stimulated the expression of virulence genes (e.g., α -hemolysin, Shiga-like toxin 1, fimbrial adhesin), and siderophore genes of ETEC in vitro (57-60). Such an adaptation may serve as a survival strategy for the pathogen to compete for iron with host cells, potentially leading to prolonged diarrhea due to both impaired host mucosal immunity and enhanced pathogen virulence. In our study, higher dietary iron conferred a protective advantage, as evidenced by the reduced duration of diarrhea observed in the COI and HII pigs among the

ETEC-inoculated groups. Higher concentrations of ferrous sulfate (0.05 or 0.1 mM) were shown to repress the expression of the colonization factor antigen (CFA/I) in ETEC in vitro compared to those cultured on media without additional iron or with a lower concentration of ferrous sulfate (0.03 mM) (59). However, *E. coli* pre-incubated with 10 μ mol/L ferric citrate exhibited greater adhesion to the monolayer of epithelial cells in vitro compared to bacteria pre-incubated in iron-limiting medium (37). The growth of *E. coli* was significantly elevated by medium containing sera collected from individuals at 4 h after oral iron supplementation (2 mg iron/kg as ferrous sulfate) compared to cultures collected before oral iron supplementation (61). Infants who received intramuscular iron administration were associated with a higher risk of neonatal *E. coli* sepsis compared to a placebo group (62). In our pig model, iron excess did not have a clear effect on the severity of ETEC infection but promoted growth during infection.

In our study, we delved into the effects of varying dietary iron concentrations alongside ETEC infection on the gut microbiome of weaned pigs. The results revealed that different dietary iron concentrations, in conjunction with ETEC infection, significantly alter the composition of the gut microbiota in weaned pigs. The gut microbiota plays a pivotal role in reducing intestinal inflammation, thereby fostering a mutualistic relationship with the host (63). Diarrhea induced by ETEC infection can disrupt the gut microbiota, hampering the suppression of inflammation (64). Alpha diversity remained unchanged in microbial diversity and richness across fecal, colon digesta, and ileal mucosa samples of ETEC-challenged pigs compared to CON pigs. This finding contrasts with the results where fecal microbial diversity decreased over time following ETEC challenge (65). However, our alpha diversity results align with another study where microbial diversity and richness in feces were unaffected by ETEC challenge (66).

Firmicutes and *Bacteroidota* emerged as the predominant phyla in fecal, colon digesta, and ileal mucosa microbiota across the treatment groups throughout the experiment, consistent with expectations for weaned pigs (67, 68). However, no significant differences in abundances were observed in *Firmicutes* and *Bacteroidota* across the treatment groups in fecal, colon digesta, and ileal mucosa microbiota. Notably, ETEC infection and dietary iron supplementation exerted influence on the intestinal microbiota composition, though limited effects were observed for the ileal mucosal microbiota. Several common factors can be identified from the shifts observed in the gut microbiota of weaned pigs across different treatments and time points. The abundance of certain bacterial taxa varied with different levels of dietary iron supplementation. For instance, the abundance of *Peptostreptococcaceae*, a family of *Firmicutes*, was highest in CON and HII pigs and lowest in LOI pigs in fecal, colon, and ileum microbiota, suggesting a correlation between high iron diet and the composition of this bacterial family. Likewise, in a rodent study, the abundance of *Peptostreptococcaceae* significantly increased in groups fed a high-iron diet (2.9 g% ferric citrate and 0.51 g% iron content of total diet), regardless of the dietary fat content. This increase was observed compared to the control group, which consumed a standard rodent diet (CE-2), consisting of wheat, corn, and milo as the source of carbohydrates, with no ferric citrate supplementation (69). Additionally, in patients with colorectal cancers (CRC), there was a higher abundance of *Peptostreptococcus anaerobius* than in stool from individuals without CRC (70).

The higher abundance of *Veillonellaceae* and its genus *Megasphaera*, in fecal and colon microbiota of LOI pigs compared to HII pigs suggests that low intestinal iron availability favors the growth of these bacterial taxa and reduces growth of other bacterial taxa. This finding is supported by another study which showed a higher abundance of *Megasphaera* in the proximal colon of pigs fed with low-iron milk (2.72 mg Fe/L) compared to those received iron-adequate

milk (21.3 mg Fe/L) (71). While there is limited information on the specific role of *Veillonellaceae* and *Megasphaera* in response to iron availability in the gut, their higher abundance in low iron environments suggests a potential adaptation to such conditions. Further research is warranted to elucidate the mechanisms underlying the relationship between *Veillonellaceae*, *Megasphaera*, and dietary iron concentration in the gut microbiota of weaned pigs.

On d 16, *Actinobacteriota* levels were highest in LOI pigs and lowest in HII pigs, both in fecal and colon microbiota. Specifically, *Bifidobacteriaceae* abundance was higher in LOI pigs compared to CON, COI, and HII pigs. Moreover, *Bifidobacterium* levels followed a similar pattern, with LOI pigs showing the highest abundance and HII pigs displaying the lowest in fecal and colon microbiota. Bifidobacteria thrive in low-iron environments (72), requiring iron for growth and employing iron-sequestration mechanisms (73, 74). These mechanisms are crucial for their adaptation to low-iron conditions, facilitating iron transport and enhancing their fitness, and they may play a role in competing for iron in the gastrointestinal tract and potentially inhibiting enteropathogens (73, 74). Bifidobacterial species were shown to protect the host from enteropathogenic infection (75, 76). In a study investigating the impact of feeding practice on the gut microbiome of infants during the first 12 weeks of life, the gut of breast-fed infants was dominated by bifidobacteria and had a low abundance of *E. coli*. In contrast, infants receiving iron-fortified formula had lower counts of *Bifidobacteriaceae* and higher counts of *Bacteroides spp.* and *E. coli* (77). While the higher abundance of bifidobacteria in the LOI pigs suggests a potential protective role against enteropathogenic infections, its beneficial effects may not fully compensate for the aforementioned potential adverse effects of suboptimal iron status on growth, host immunity, and pathogen virulence.

Within *Proteobacteria*, neither the *Enterobacteriaceae* family nor the *Escherichia–Shigella* genera were significantly affected by iron treatment or ETEC infection. The relatively stable abundance of *Enterobacteriaceae* indicates several important aspects of gut microbiota dynamics in weaned pigs. Firstly, we can assume that *Enterobacteriaceae* are commensal colonizers of the pig gut, capable of persisting under various conditions irrespective of dietary iron concentrations or ETEC infection. Moreover, the stability of *Enterobacteriaceae* abundance over time, as evidenced by the lack of significant differences between d 10 and 16, suggests that their colonization dynamics remain relatively unaffected by changes in diet or exposure to ETEC. Interestingly, the consistent abundance of *Enterobacteriaceae* across different dietary iron concentrations implies that their proliferation may not be strongly influenced by iron availability in the gut environment. Instead, other factors such as substrate availability, host-microbe interactions, and microbial competition may play a more significant role. *Desulfobacterota*, observed to be most abundant in LOI and HII pigs and least in CON pigs, suggest a potential responsiveness to ETEC infection irrespective of iron availability. This phylum has been linked to immune response and gut inflammation (78, 79). Despite these associations, there remains a limited understanding regarding the precise role of *Desulfobacterota* within the gut ecology context.

Overall, the complex interplay between dietary iron concentration, ETEC infection, and temporal dynamics in shaping the gut microbiota composition of weaned pigs is highlighted. Further research is needed to elucidate the specific mechanisms underlying these interactions and their implications for host health and disease. Interactions between dietary iron concentration and ETEC infection may have contributed to the observed changes in the gut microbiota. For instance,

the abundance of certain bacterial taxa showed differential responses to varying levels of dietary iron supplementation in the presence of ETEC infection.

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TABLES AND FIGURES

Table 4.1. Basal feed ingredients and mineral premix of the experimental diets.

Ingredient	%
Corn, NRC, 2012	44.41
Whey, dried, NRC, 2012	15.00
SBM, 48 %, NRC, 2012	24.12
Limestone	1.30
Lysine HCL	0.41
DL-Met	0.15
Threonine	0.11
Soybean oil	2.00
Salt	0.40
Milk, Lactose NRC 2012	6.00
MCP	0.80
Soy protein concentrate	5.00
Vitamin premix	0.15
Mineral premix ¹	0.15
Total	100.00

Table 4.2. Iron composition of the experimental diets.

	Low-iron diet	Iron-replete diet	High-iron diet
Iron content, mg/kg	120	264	721

Figure 4.1:

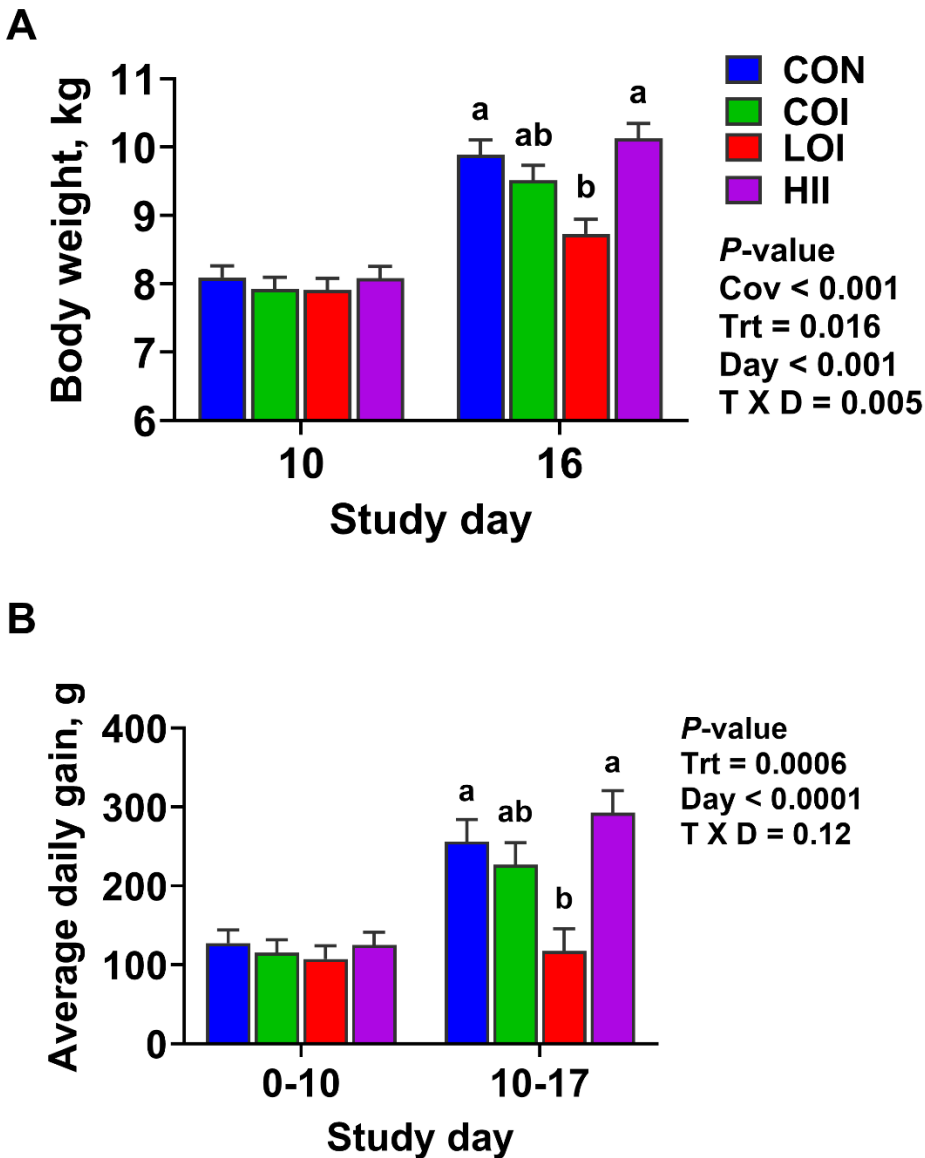


Figure 4.1. Effect of dietary iron concentration on vulnerability of ETEC on (A) body weight (BW) on d 10 and 16 and (B) average daily gain (ADG) from d 0 to 10 and d 10 to 17 in weaning pigs across the four treatment groups. Data show least square means $\pm$ SEMs, $n = 8/\text{treatment}$. Letters indicate significant differences between groups, with a difference denoting $P < 0.05$.

Figure 4.2.

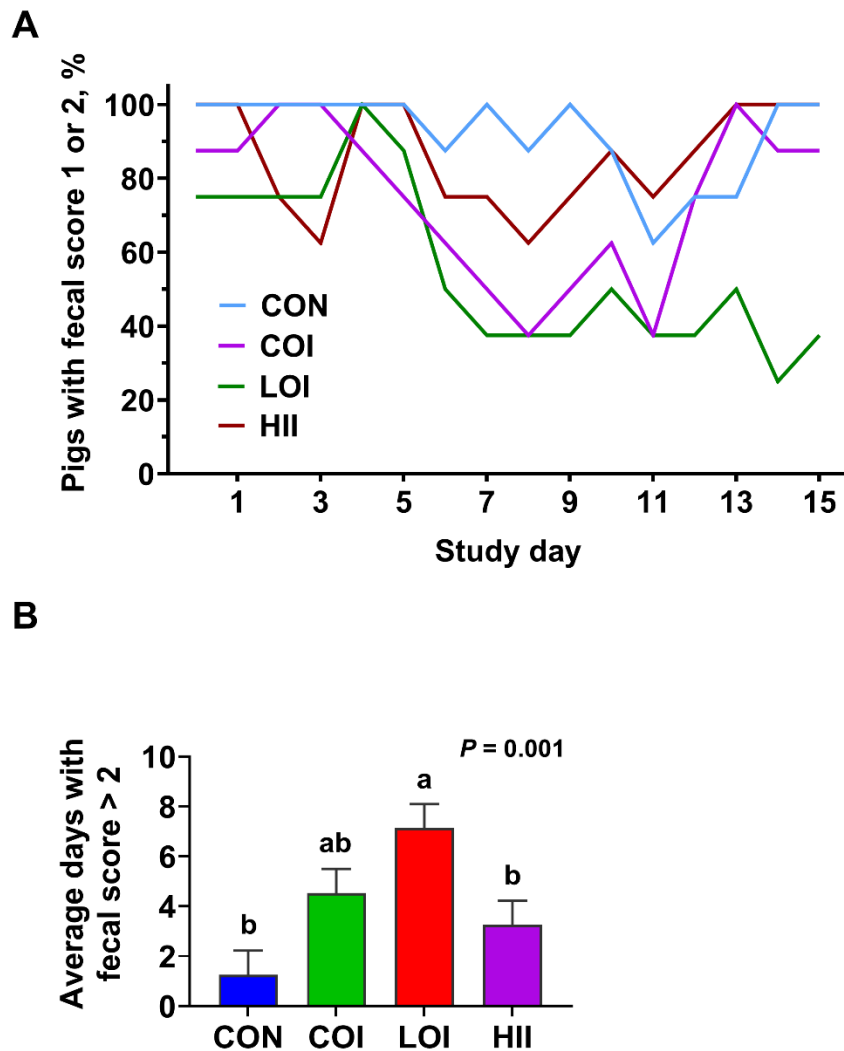


Figure 4.2. Effect of dietary iron concentration on vulnerability of ETEC coli infection on days with (A) fecal score of 1 or 2, % and (B) average days with fecal score < 2 in weaning pigs across the four treatment groups. Data show least square means $\pm$ SEMs, $n = 8/\text{treatment}$. Letters indicate significant differences between groups, with a difference denoting $P < 0.05$.

Figure 4.3.

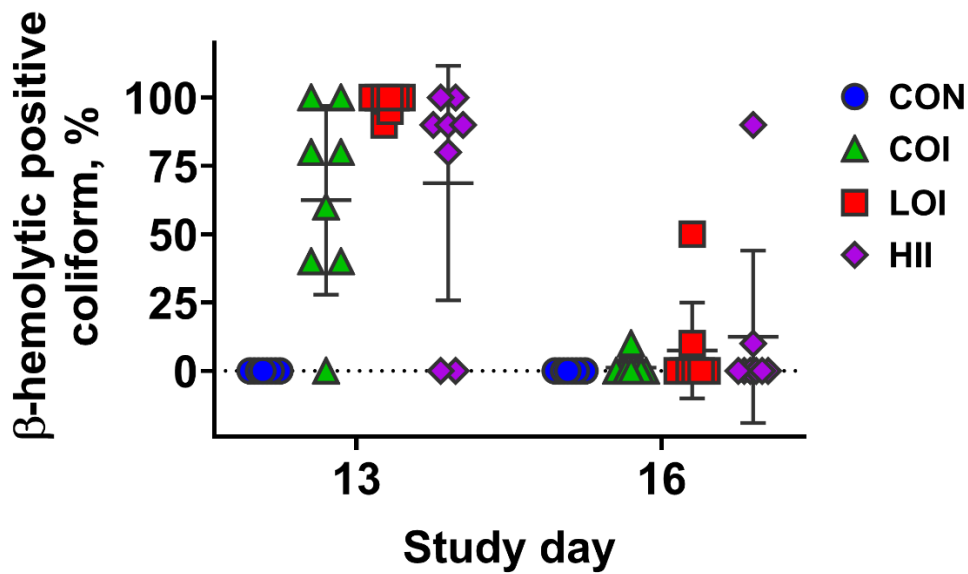
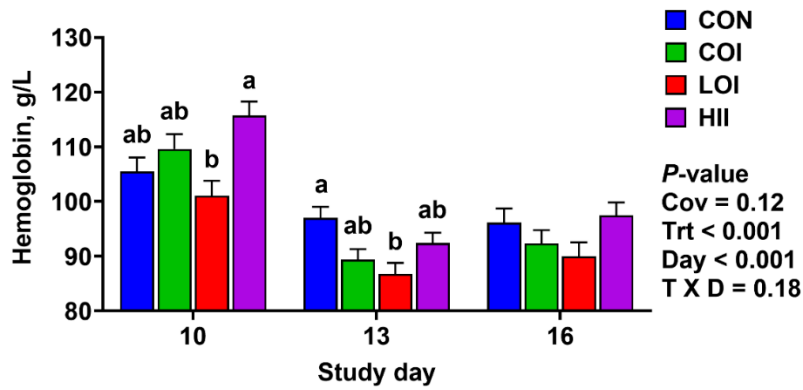


Figure 4.3. Percentage of positive β -hemolytic *E. coli* colonies. Fecal samples from days 13 and 16 were plated on Columbia Blood Agar to enumerate and detect total coliforms and hemolytic coliforms. Visual assessment was used to quantify the populations of β -hemolytic coliforms on the blood agar plates, with scores ranging from 0 (no bacterial growth) to 8 (heavy bacterial growth) indicating bacterial growth intensity. The ratio of scores between β -hemolytic coliforms and total coliforms was calculated.

Figure 4.4.

A



B

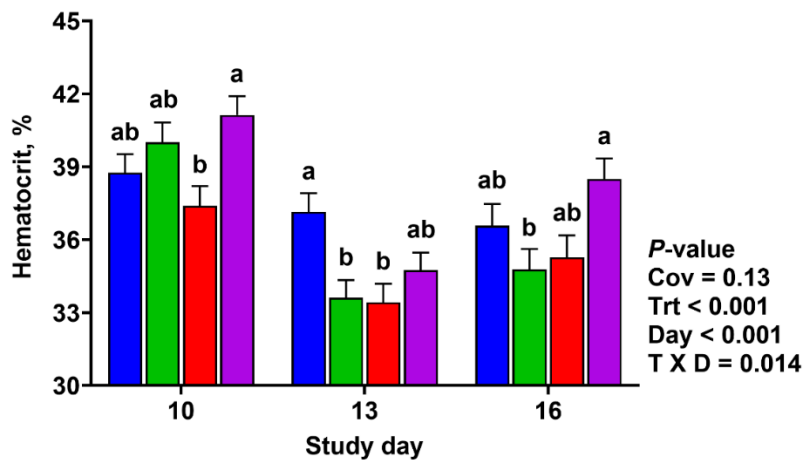
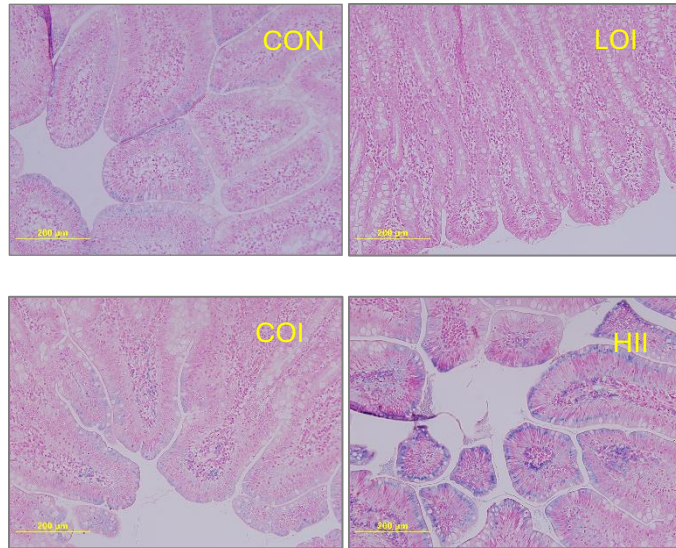


Figure 4.4. Effect of dietary iron concentration on vulnerability of ETEC infection on (A) hemoglobin (Hb) and (B) hematocrit (Hct) in weaning pigs across the four treatment groups. Data show least square means $\pm$ SEMs, $n = 8/\text{treatment}$. Letters indicate significant differences between groups, with a difference denoting $P < 0.05$.

Figure 4.5.

A



B

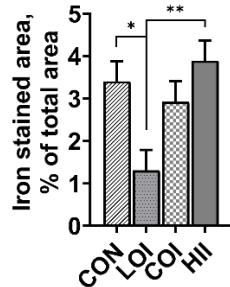
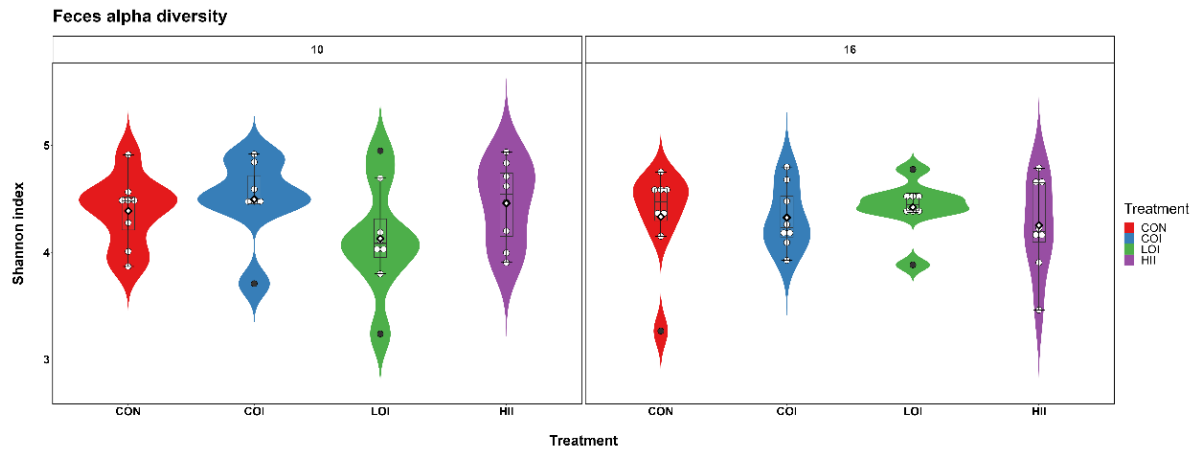


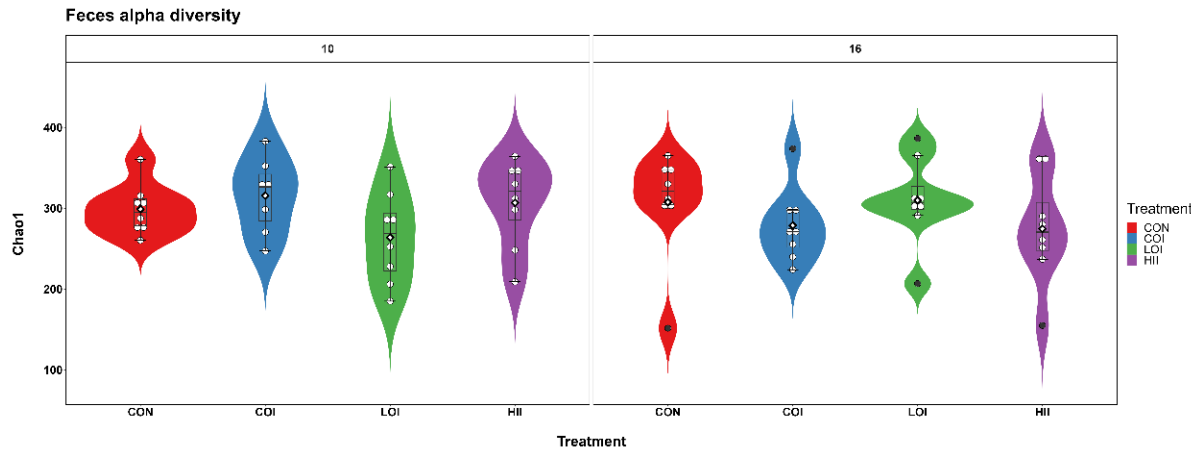
Figure 4.5. Effect of dietary iron concentration on vulnerability of ETEC infection on histomorphology of duodenum (A) and percentage of iron-stained area in duodenal mucosa (B) in weaning pigs across the four treatment groups. Data show least square means $\pm$ SEMs, $n = 8/\text{treatment}$. Statistical significance at * $P < 0.05$ and ** $P < 0.01$. (A) Histomorphology of the duodenum, the duodenum sections were stained with Prussian blue; photomicrographs are presented at 200 x magnification. Iron with blue area can be seen. (B) Percentage of iron-stained area in duodenal mucosa.

Figure 4.6.

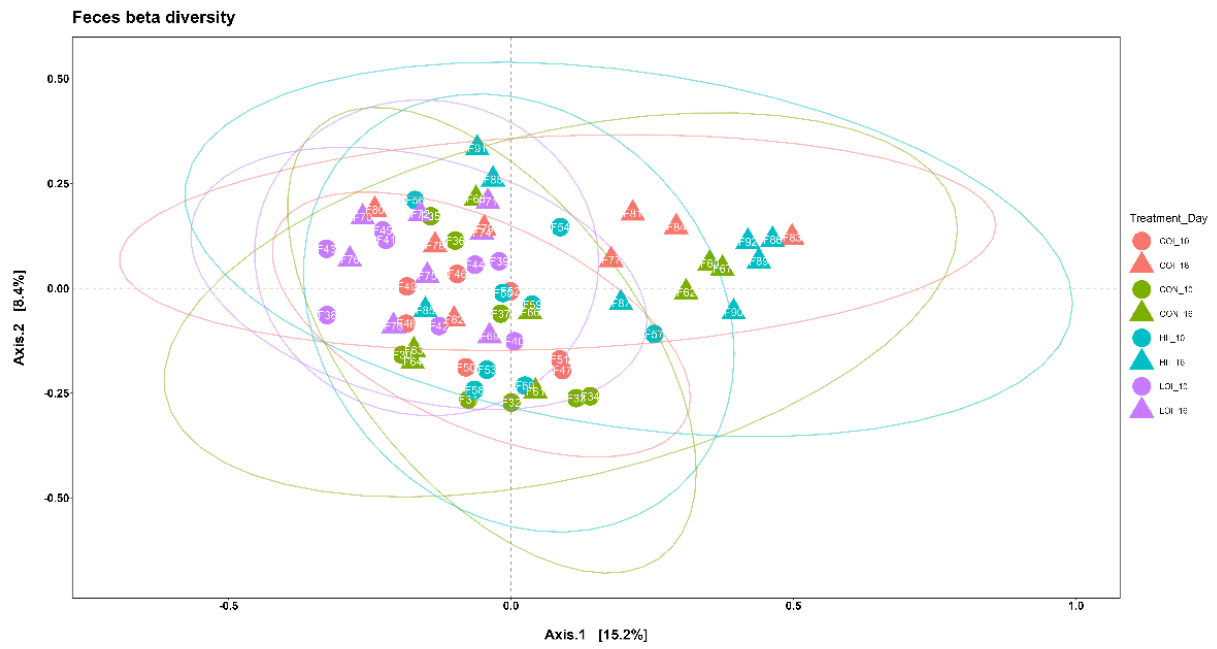
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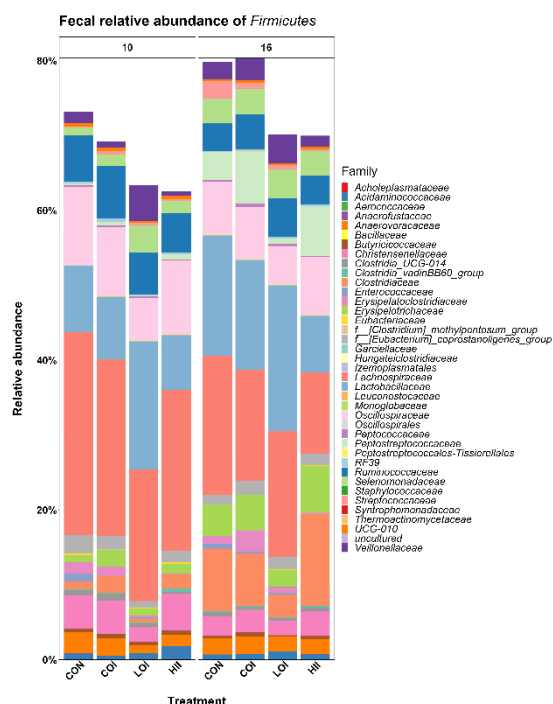
B



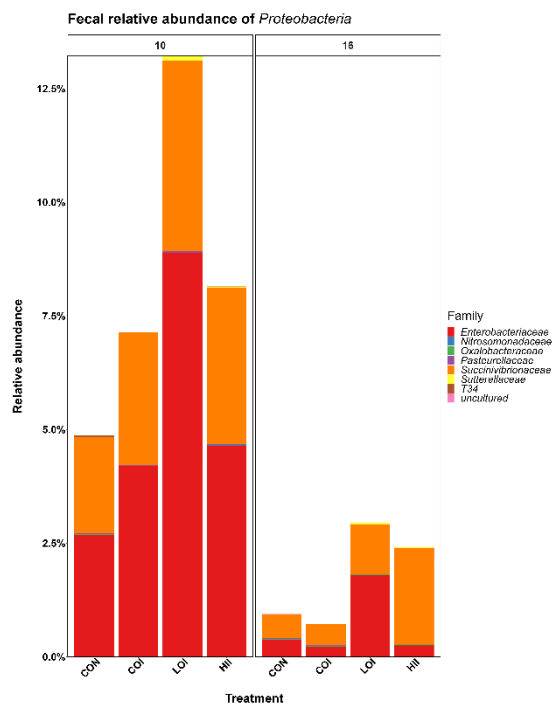
C



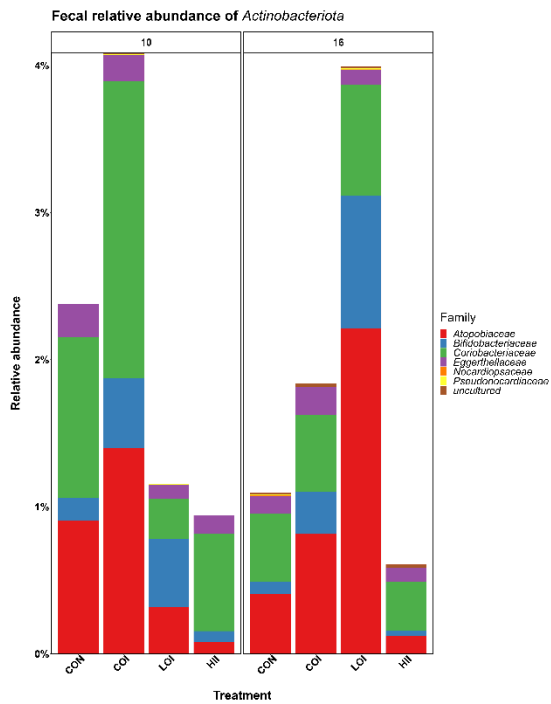
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G



H



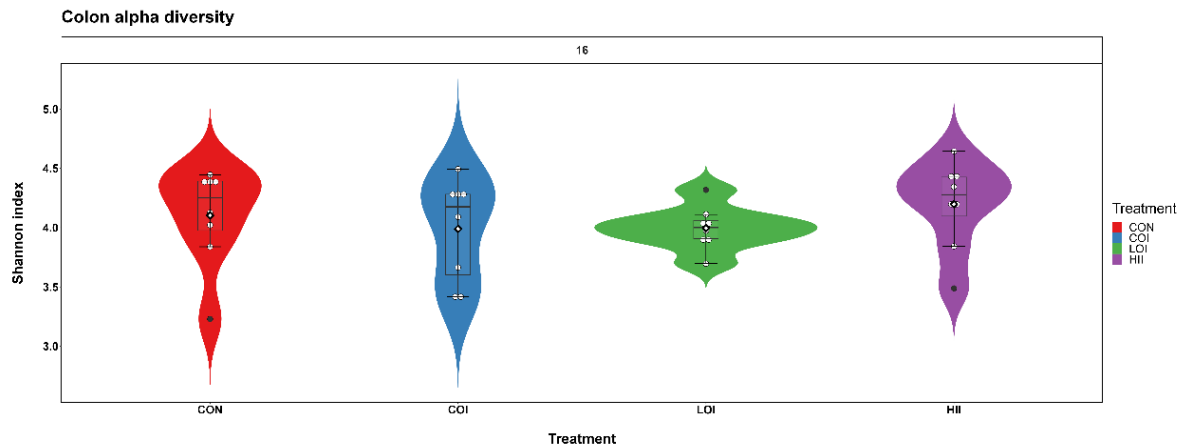
I



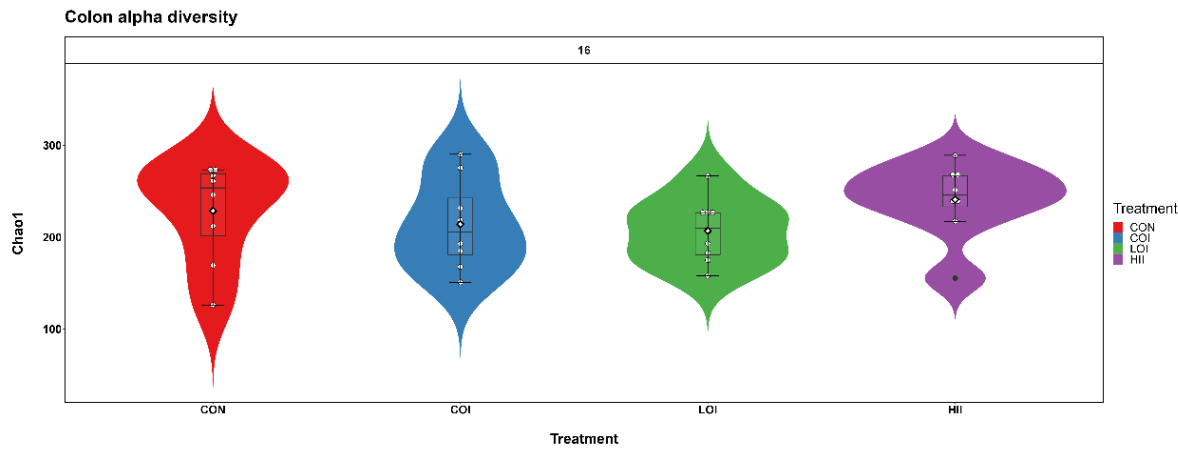
Figure 4.6. Alpha diversity, measured by Shannon (A) and Chao1 (B) indices, in feces of piglets receiving CON, LOI, COI, and HII diets on d 10 and 16. Data are presented as mean (diamond) $\pm$ SEM. Principal Coordinate Analysis (PCoA) based on Bray-Curtis distances was used to assess beta diversity of fecal microbiota in weaned-pigs fed with CON, LOI, COI, and HII diets on d 10 and 16 (C). Relative abundances of gut microbiota at various taxonomic levels were examined: phylum (D), family (E-H), and genus (I).

Figure 4.7.

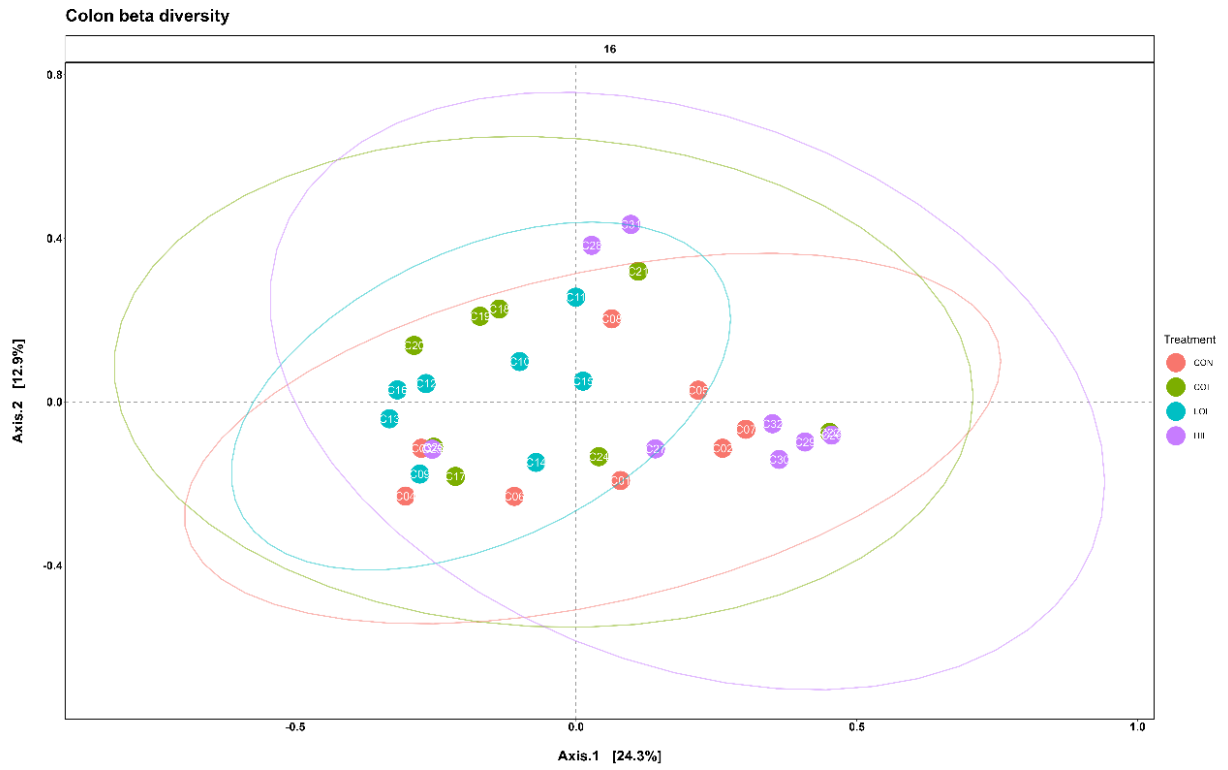
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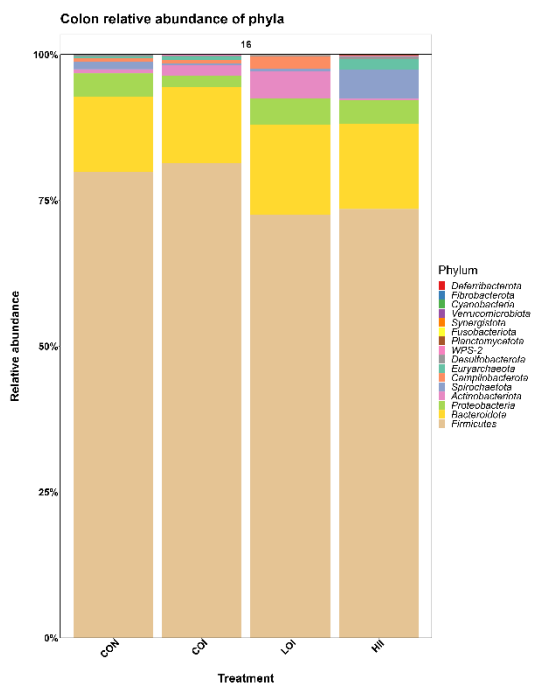
B



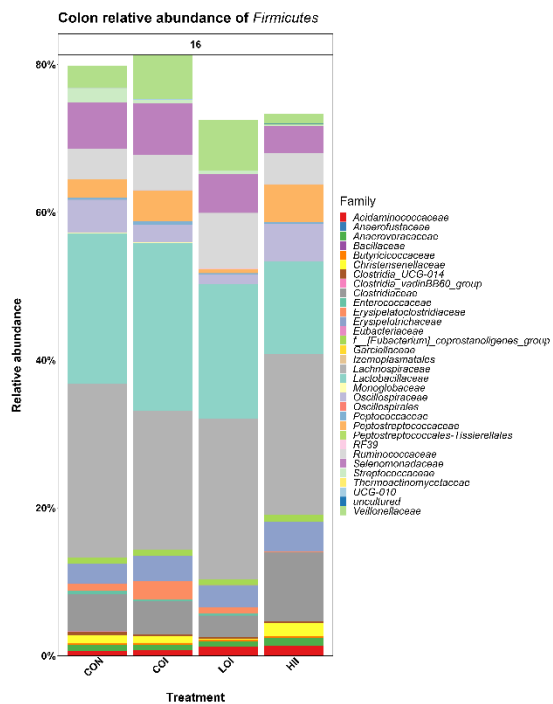
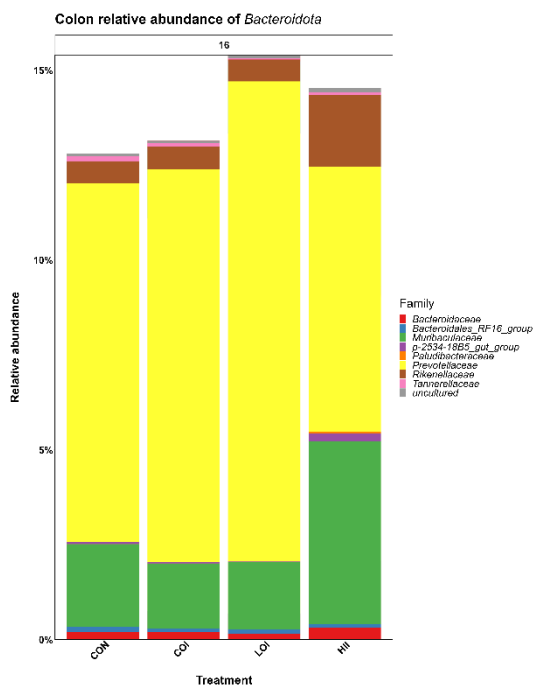
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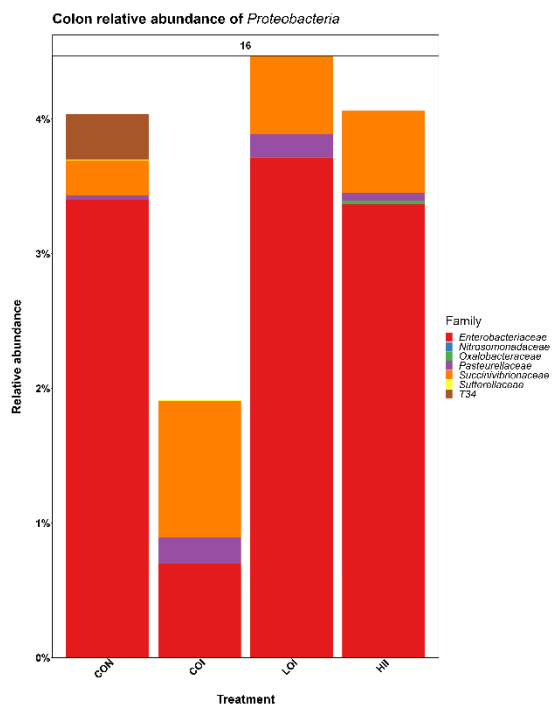
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**F**

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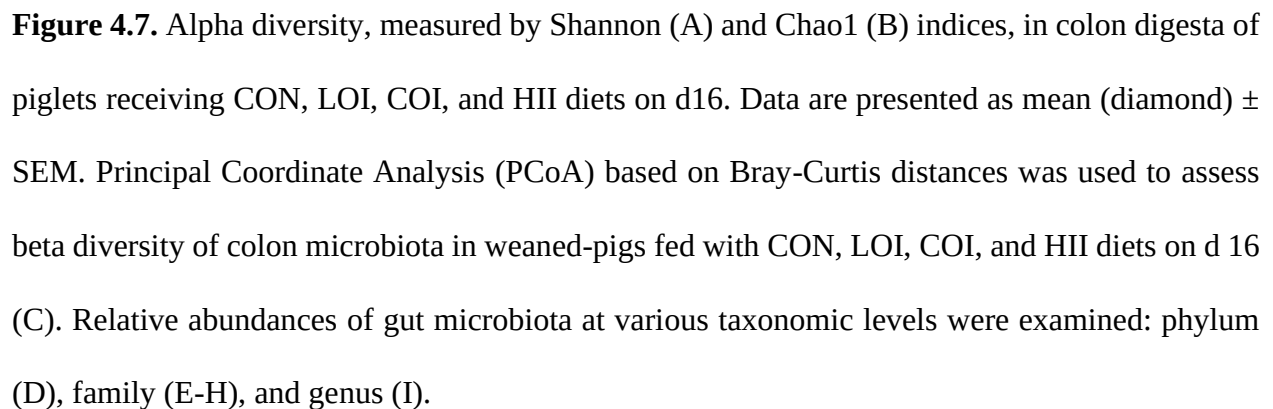
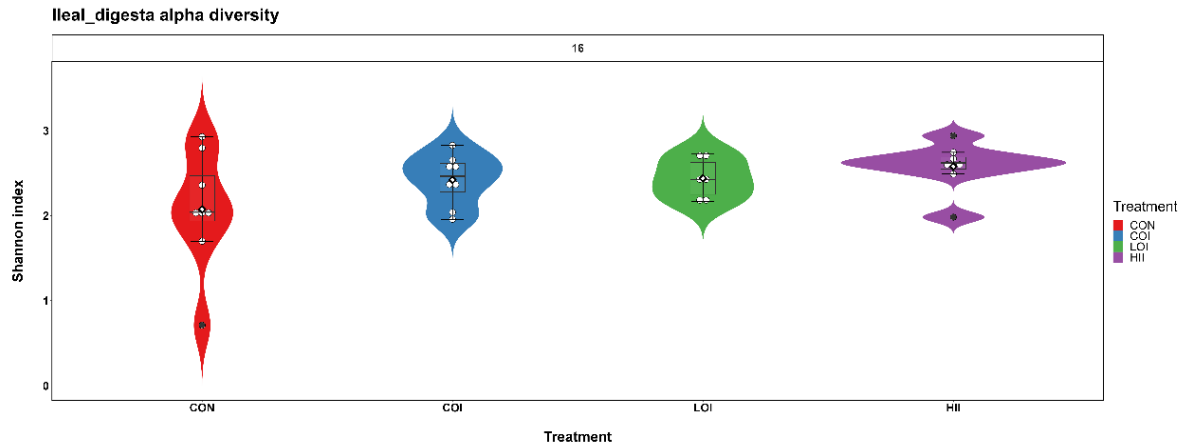
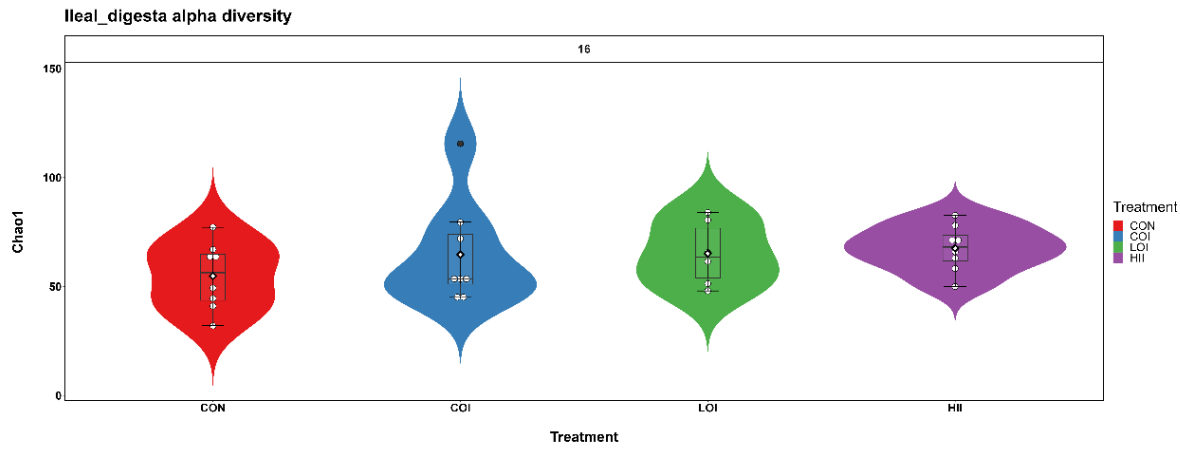


Figure 4.8.

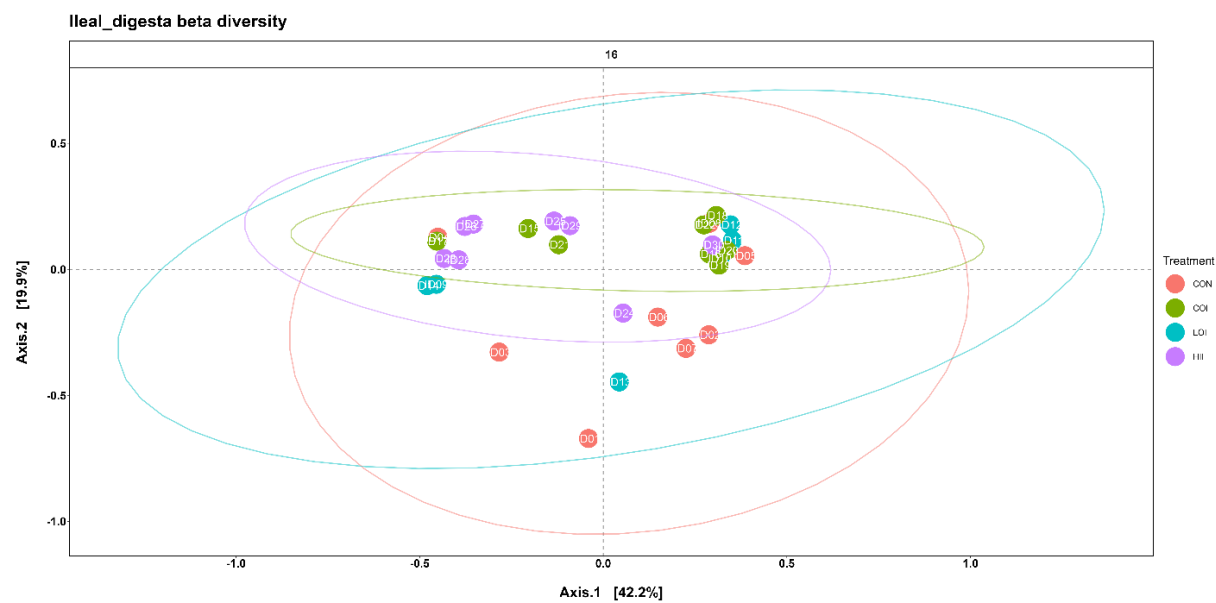
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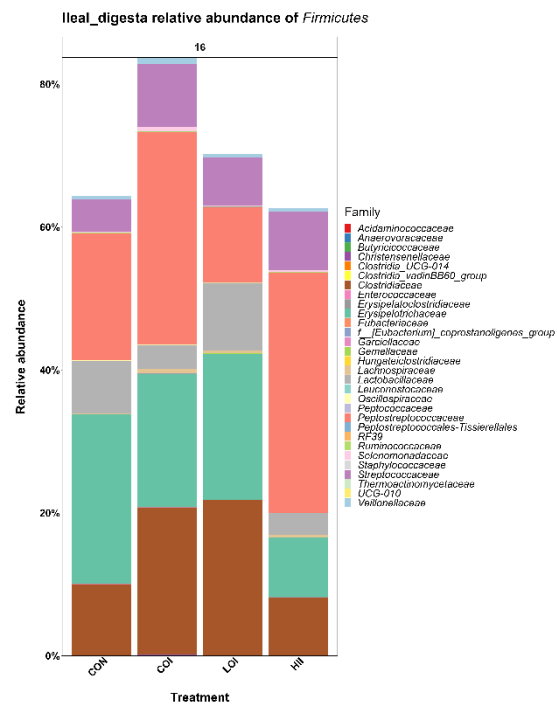
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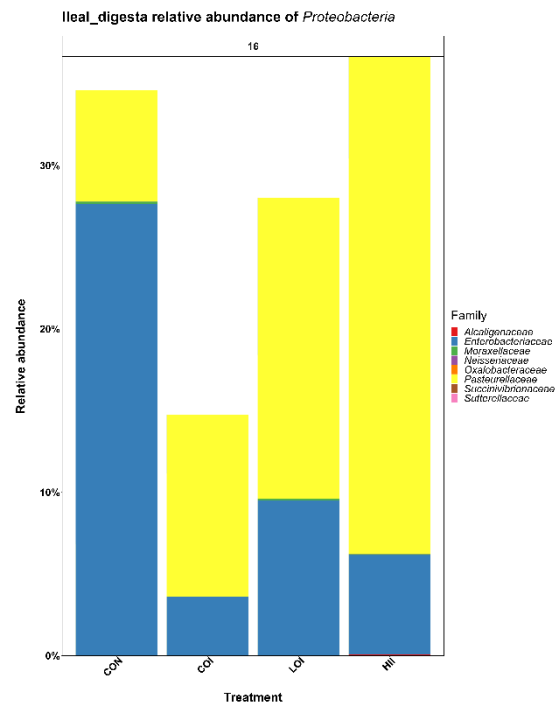
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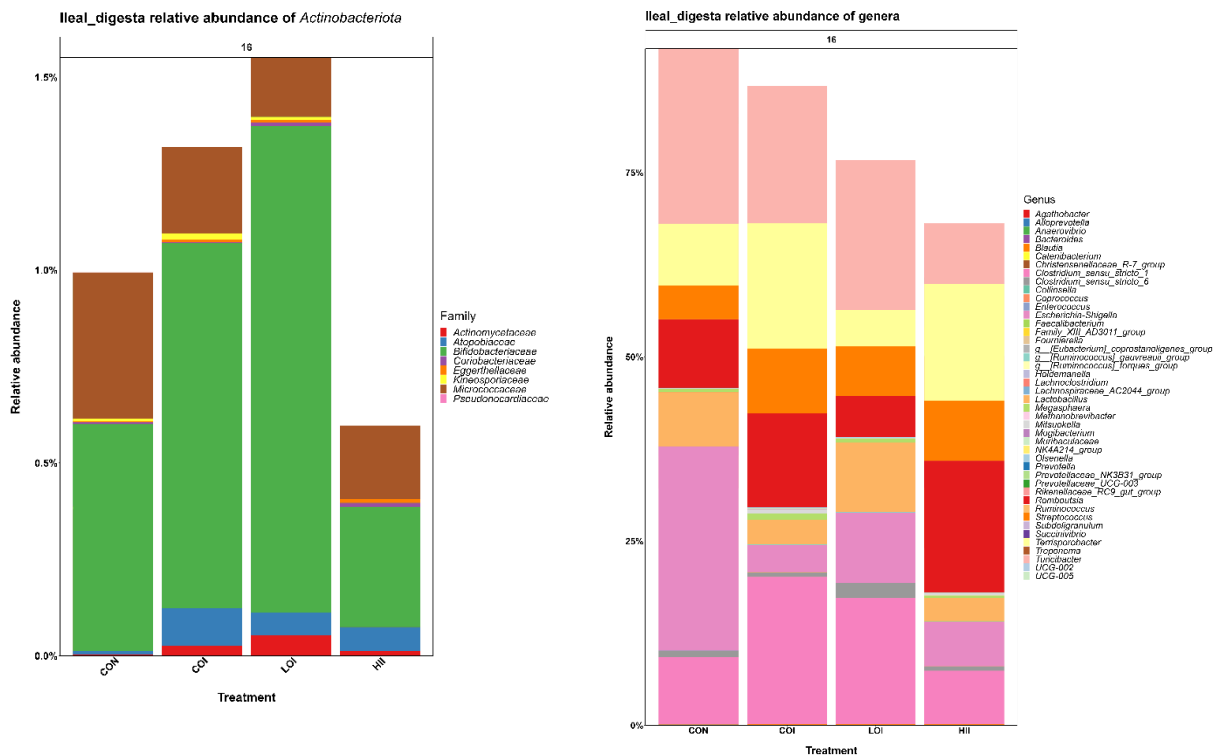


Figure 4.8. Alpha diversity, measured by Shannon (A) and Chao1 (B) indices, in ileal mucosa of piglets receiving CON, LOI, COI, and HII diets on d 16. Data are presented as mean (diamond) $\pm$ SEM. Principal Coordinate Analysis (PCoA) based on Bray-Curtis distances was used to assess beta diversity of ileal microbiota in weaned-pigs fed with CON, LOI, COI, and HII diets on d 16 (C). Relative abundances of gut microbiota at various taxonomic levels were examined: phylum (D), family (E-H), and genus (I).