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Authors

Park, Jungjae

Wickramasinghe, Saumya

Lönnerdal, Bo

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Original Research

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

Jungjae Park [1], Saumya Wickramasinghe [2], David A. Mills [2], Bo L. Lönnerdal [1], Peng Ji [1,*]

[1] *Department of Nutrition, University of California Davis, CA, United States;* [2] *Department of Food Science and Technology, University of California Davis, CA, United States*

A B S T R A C T

Background: Infant formula in the United States 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 prebiotic or synbiotic supplementation on iron homeostasis and trace mineral bioavailability.
Methods: Twenty-four piglets were stratified and randomly assigned to treatments on postnatal day 2. Piglets were individually housed and received an iron-adequate milk diet (AI), a high-iron milk diet (HI), HI supplemented with 5% inulin (HI with a prebiotic [HIP]), or HIP with an oral gavage of *Ligilactobacillus agilis* YZ050, an inulin-fermenting strain, every third day (HI with synbiotic [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 postnatal day 29. Data were analyzed using mixed model analysis of variance with repeated measures whenever necessary.
Results: Diet did not affect growth. HI increased hemoglobin, hematocrit, and serum iron compared to AI. Despite marginal adequacy, AI upregulated iron transporter genes and maintained satisfactory iron status in most pigs. HI upregulated hepcidin gene expression 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 the expression of iron regulatory genes. HI lowered zinc (Zn) and copper (Cu) in the duodenum and liver compared to AI, whereas HIP and HIS further decreased Zn and Cu in the liver and diminished colonic and fecal trace minerals.
Conclusions: Early-infancy excessive iron fortification causes iron overload and compromises Zn and Cu absorption. Inulin decreases trace mineral absorption likely by 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 mo 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 (Fe) concentration significantly differs between breast milk and infant formula. For healthy term infants, Fe from endogenous stores and breast milk generally meet the requirements ≤6 mo of age [2]. However, most infant formulas in the United States are fortified with Fe at a concentration of 12 mg/L, which is 30–60 times higher than the Fe content of breast milk and is still higher even when

Abbreviations: AI, iron-adequate milk diet; BW, body weight; Cu, copper; DM, duodenal mucosa; *DMT1*, divalent metal transporter 1; Fe, iron; *FPN*, ferroportin; gDNA, genomic DNA; *HAMP*, hepcidin; Hb, hemoglobin; Hct, hematocrit; HI, high-iron milk diet; HIP, high-iron milk diet with a prebiotic; HIS, high-iron milk diet with a synbiotic; IDA, iron deficiency anemia; Mn, manganese; PD, postnatal day; ROI, region of interest; *TFR1*, transferrin receptor 1; TIBC, total iron-binding capacity; UCD, University of California, Davis; Zn, zinc.

* Corresponding author. *E-mail address:* penji@ucdavis.edu (P. Ji).

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accounting for the low bioavailability of formula Fe [3]. It is worth noting that the low bioavailability of formula Fe—a long-standing rationale supporting the current fortification dose—was determined in Fe-replete infants at 6–7 mo of age when solid foods, including cooked vegetables, cereals, meat, and eggs, had been introduced for 1–3 mo [4]. The study did not control for dietary factors that might inhibit Fe absorption, nor did it adjust for infants' pre-existing Fe status, another variable that could significantly modulate absorption.

Extensive research has been conducted to study the effectiveness of prophylactic Fe in preventing anemia in infants and young children. However, much less research has explored the developmental consequence of excessive Fe intake during infancy. Evidence drawn from a few studies demonstrated adverse effects of Fe supplementation or feeding an Fe-fortified formula on cognitive development, linear growth, and risk of infections in Fe-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 (HAMP)–ferroportin (FPN) axis, the systemic mechanism controlling Fe absorption and homeostasis, is functionally immature in the early postnatal stage [10,11]; Fe oversupplementation through oral drops resulted in brain and body Fe overload in suckling piglets [11]. Few studies have evaluated Fe and other trace mineral metabolism in response to excessive Fe intake from a fortified formula in early infancy, and it is unclear what the safety range for lowering formula Fe 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 breastfed infants [12–15]. Additionally, Fe 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 Fe-fortified biscuits had significantly less fecal *Lactobacilli* and greater counts of enterobacteria, which may consist of enteropathogens and is believed to contribute to heightened intestinal inflammation [17]. Given the vital role of the *Lactobacillus* species in gut health and development, there is increased interest in using prebiotics, probiotics, and synbiotics to restore beneficial commensal communities in formula-fed infants [18,19]. Long-chain inulin, a nondigestible 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 Fe bioavailability in anemic pig and Fe-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 Fe 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, whereas the combined use of prebiotics and probiotics manifested a more pronounced influence on gut microbiota [30,31]. Zhu et al. [32] (2020) isolated *Ligilactobacillus agilis* YZ050 (*L. agilis* YZ050) from feces of milk-fed calves. *L. agilis* YZ050 displayed the activity to catabolize 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 biotics, gut microbiota, and trace minerals [33,34]. However, the application of inulin and inulin-consuming probiotics in Fe-fortified formula and their impact on trace mineral absorption have not been comprehensively evaluated. In the current study, we used milk-fed neonatal piglets as a preclinical model for formula-fed infants. The objective was to determine how Fe fortification and inulin or synbiotic supplementation of inulin and *L. agilis* YZ050 modulate Fe and trace mineral metabolism in early infancy.

Methods

Study design and animal management

The study protocol was approved by the Institutional Animal Care and Use Committee 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 1 of 4 dietary treatments (n = 6/treatment) at postnatal day (PD) 2. The experiment was conducted in a temperature-controlled room (25°C) with a preset 12-h shift of light–dark cycle (light on 07:00–19:00) in the Cole A Facility at UCD. From PD 2–29, piglets were housed in individual cages that had a heating pad, a stationary feeding bowl, and an enrichment toy. Piglets in each of the 4 dietary treatments received an Fe-adequate milk diet (AI) replacer (60 mg Fe/[kg milk solids] supplemented with 5% [wt/wt milk solid] maltodextrin), a high-Fe milk diet (HI) replacer (480 mg Fe/[kg milk solids] supplemented with 5% maltodextrin), HI supplemented with 5% inulin (Orafti IPS, BENEO) as a prebiotic (HI with a prebiotic [HIP]), or HIP and an oral gavage of *L. agilis* YZ050 (5 mL, 2.5×10^9 CFUs/mL; inoculants were prepared by Dr. David Mills laboratory in the Department of Food Science and Technology at UCD) once every 3 d during the study (HI with a synbiotic [HIS]). This strain was shown to use inulin as a substrate [32,35]. The AI, HI, and HIP replacer solids were prepared by mixing a basal milk replacer (NutraStart Liqui-Wean without trace mineral premix; Milk Specialties Global) that was free of trace mineral supplements with a mineral premix prepared in the laboratory and maltodextrin or inulin. The basal milk replacer was analyzed for dry matter, crude fat, crude protein, and minerals at Cumberland Valley Analytical Service. The ingredient composition of the experimental diets and nutrient profile of the basal milk replacer are listed in Table 1 and Supplemental Table 1, respectively. The milk replacer powder was reconstituted with deionized water following the manufacturer's instructions. Piglets were weighed every other day, and their daily liquid milk allowance was calculated as 270 mL/(kg·BW) based on the most recent BW. The liquid milk was prepared every morning and stored in individual containers for each piglet. It was then automatically delivered from the 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,

TABLE 1
Feed ingredients and mineral premix compositions of experimental diets.

Item	AI	HI	HIP	HIS
Ingredient, % diet solid				
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
Mineral premix, mg/kg diet solid				
Zinc sulfate ($ZnSO_4 \cdot 7H_2O$)	373.79	373.79	373.79	373.79
Copper sulfate ($CuSO_4 \cdot 5H_2O$)	76.05	76.05	76.05	76.05
Manganese sulfate ($MnSO_4 \cdot H_2O$)	76.76	76.76	76.76	76.76
Calcium iodate ($Ca(IO_3)_2$)	2.96	2.96	2.96	2.96
Cobalt sulfate ($CoSO_4 \cdot 7H_2O$)	4.76	4.76	4.76	4.76
Sodium selenite (Na_2SeO_3)	0.66	0.66	0.66	0.66
Ferrous sulfate ($FeSO_4 \cdot H_2O$)	121.86	1401.43	1401.43	1401.43

Abbreviations: AI, iron-adequate milk diet; HI, high-iron milk diet; HIP, high-iron milk diet with a prebiotic; HIS, high-iron milk diet with a synbiotic.
[1] The 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 (4409 IU), and vitamin E (110 IU).

which were adjusted for mealtime and duration every few days to accommodate the changes in the volume of the daily milk allowance. Sometimes, the bending of the suckling tubes led to a small amount of milk being left in the containers. The left-over milk was emptied into the feeding bowl to ensure complete consumption of the daily milk allowance calculated based on BW.

Data and sample collection

Milk intake and BW 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 07:00 and 19:00 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) that was validated in a previous study [36]. Fecal samples were obtained using a rectal swab, and blood samples were collected from the jugular vein through venipuncture into sodium heparin vacutainers (Becton Dickinson) 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 Drabkin's reagent (Sigma-Aldrich) following the manufacturer's instructions with modification (250 μL sample mixture) for detection using a microplate reader (BioTek Synergy HTX). Absorbance was determined at 540 nm, and Hb concentration was calculated based on a standard curve. Hematocrit (Hct) was determined using a micro-Hct centrifuge.

Analysis of Fe and trace mineral status

Plasma Fe, total Fe-binding capacity (TIBC), and transferrin saturation were measured and calculated using the Pointe Fe/TIBC assay kit (Pointe Scientific). Paraffin-embedded coronal sections of the duodenum and proximal colon (6 μm) were stained for ferric Fe with Prussian blue stain containing 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 magnifications. Crypt-villus areas were manually traced as regions of interest (ROIs) using ImageJ (NIH). The percentage of Fe-stained area within the ROI was quantified using ImageJ [37]. The concentrations of Fe 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) in screw-top glass vials for ≤120 h. The samples were placed on a heating plate and boiled at 100°C until minimal liquid was left. The samples were diluted with ultrapure water to a final volume of 5 mL and analyzed using inductively coupled plasma optical emission spectroscopy (iCAP 6300, Thermo Electron) to determine concentrations of 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, and 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 for pH measurements. pH was measured using a standard pH meter (Orion Research) with a micro combination electrode (Thermo Fisher Scientific).

Gene expression analysis

mRNA expression of genes was determined using real-time qRT-PCR. Samples (~100 mg) from duodenal mucosa (DM), ileal mucosa, colon tissue, and liver were homogenized using TissueLyser II (QIAGEN), and total RNA was extracted using TRIzol reagent (Invitrogen). The concentration and purity of the total RNA were assessed using a NanoDrop One (Thermo Fisher 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). qRT-PCR was performed in a 20 μL reaction following the protocol of PowerUP SYBR Green Master Mix (Applied Biosystems) on a QuantStudio 3 Real-Time Polymerase Chain Reaction System (Applied Biosystems). The 20 μL reaction contained 10 μL 2X SYBR Green Master Mix, 6 μL cDNA template, 1.5 μL of each primer, and 1 μL nuclease-free water. Expressions of target genes *FPN*, transferrin receptor-1 (*TFR1*), divalent metal transporter 1 (*DMT1*), duodenal cytochrome B, and Zn transporter 14 were analyzed in intestinal

mucosa and colon samples; *DMT1*, *FPN*, *TFR1*, and *HAMP* expressions were determined in the liver; and *TNF-α* and *IL-6* expressions were determined in the ileum. Ribosomal RNA 18S was used as the housekeeping gene. The expressions of the target genes were normalized against that of the housekeeping gene, and relative expression was calculated using the comparative cycle threshold (Ct) method ($2^{-\Delta\Delta Ct}$ method). Primer pair sequences are listed in Supplemental Table 2.

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 qRT-PCR following a standard curve method. To prepare the standard samples, *L. agilis* YZ050 was grown to 3 $\times 10^9$ CFUs/mL in de Man-Rogosa-Sharpe broth at 37°C for 48 h. A pellet of *L. agilis* YZ050 was harvested after centrifugation, and genomic DNA (gDNA) was extracted using the Quick-DNA Fecal/Soil Microbe Kit (Zymo Research). The concentration and purity of the gDNA sample were determined using a NanoDrop One (Thermo Fisher Scientific). A total of 9 standard samples were prepared after 10-fold serial dilutions of the gDNA sample in Tris-HCl buffer (10 mM, pH 8.0). The gDNA standards correlated with 10^9, 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, and 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 (see Supplemental Table 2 for primer sequences). 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 nonspiked samples. The reaction efficiency was verified to align with the normal range of 0.9–1.1. The expression of the *Sir2* gene in the DNA isolated from experimental and standard samples was determined 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). Homoscedasticity plot and Q-Q plot were used to assess equal variance and normal distribution. Data fit for both assumptions were analyzed using 1- or 2-way analysis of variance. The model included the fixed effects of treatment and day and the treatment by day interactions with 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 toward significance were declared at $P < 0.05$ and $P < 0.1$, respectively.

Results

Growth and diarrhea

BW and growth were not affected by the dietary treatment (Figure 1A). As liquid milk was provided at 270 mL/(kg•BW) daily, there was no 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, whereas no significant difference was observed in any other pairwise comparison (Figure 1B 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 Fe deficiency anemia

The AI pigs had the lowest serum Fe, which was significantly lower than that of HI and HIS pigs on PD 8 and 15 or that of HIS pigs on PD 22 ($P < 0.05$, Figure 2A). Similarly, transferrin saturation was the lowest in the AI pigs during the study; the difference was significant between the AI and HI pigs on PD 8 and 15 ($P < 0.05$, Figure 2B). TIBC should be reversely associated with circulating Fe. Indeed, it was highest in the AI pigs during the study; the differences between the AI and HI and HIP pigs were significant on PD 8 and 15 ($P < 0.05$, Figure 2C). Both Hb and Hct were significantly lower in the AI group than in the other groups on PD8, 15, and 29 ($P < 0.05$, Figure 2D and E, respectively), whereas no pig was anemic (Hb <110 g/L) during the study except the one that died suddenly.

Body Fe status

Prussian blue staining showed a pronounced nonheme Fe deposition in the duodenal (Figure 3A) and colon mucosa (Figure 3B) crypt-villus areas in all 3 groups fed the high-Fe diets compared with negligible amounts in the AI group ($P < 0.05$, Figure 3C and D) when the percentage of Fe-stained area within the ROI was quantified. Compared with the HI group, the HIP and HIS groups had significantly lower mucosal Fe in the duodenum ($P < 0.05$, Figure 3C) but not in the colon. Furthermore, high-Fe milk resulted in Fe overload in the liver ($P < 0.0001$, Table 2) and duodenum ($P < 0.01$, Table 2), and increased iron concentration in colon digesta ($P < 0.0001$, Table 2). Both the HIP and HIS treatments decreased Fe accumulation in the liver ($P \leq 0.05$), duodenum ($P \leq 0.05$), and colon digesta ($P \leq 0.001$) compared with the HI. A similar trend was observed in fecal Fe concentration (Table 3). Compared with the minimal fecal Fe in pigs fed the AI, the high-Fe diets significantly increased Fe excretion in feces on PD 8, 15, and 22 ($P < 0.05$), although both the HIP and HIS treatments lessened increase compared with the HI. On PD 29, fecal Fe 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, and Mn) across feces (Table 3), colon, duodenum, and liver (Table 2) was investigated. Notably, the HI group consistently

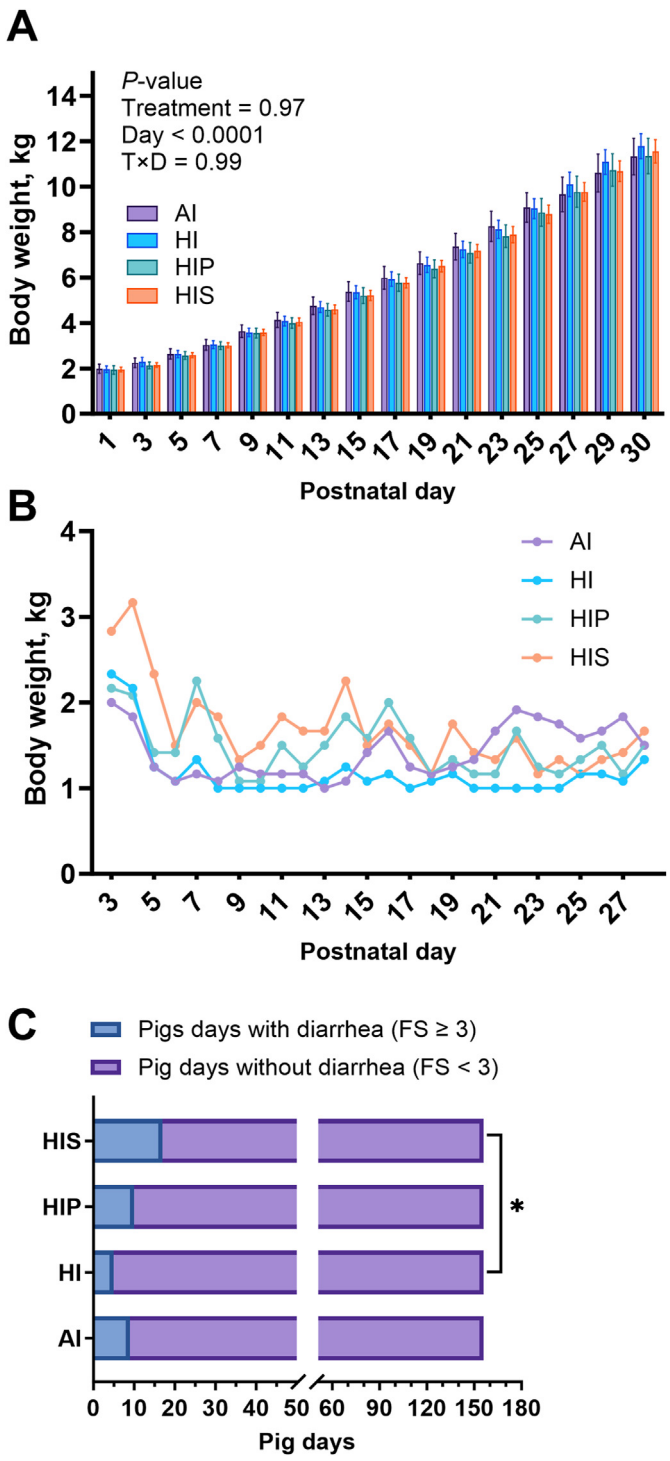

FIGURE 1. Effects of dietary treatments on body weight, fecal consistency, and frequency of diarrhea. (A) Body weight was recorded every other day throughout the study. (B) Fecal consistency was scored twice daily using a 5-scale scoring system (1 = normal firm and shaped feces, 2 = soft and shaped feces, 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). (C) Frequency of diarrhea was calculated as total pig days with a fecal score ≥3. Data show least squares means ± SEM. *Statistical significance at $P < 0.05$. AI, iron-adequate milk diet; HI, high-iron milk diet; HIP, high-iron milk diet with a prebiotic; HIS, high-iron milk diet with a synbiotic; T×D, treatment by day; FS, fecal consistency score.

exhibited higher Fe concentrations in feces ($P < 0.001$), colon ($P < 0.0001$), and liver ($P < 0.0001$) compared with the AI, HIP, and HIS groups. The Fe concentration of the HI group was only higher than that of the AI group in the duodenum ($P < 0.01$), where the AI group had substantially lower Fe concentration than groups fed high-Fe diets. Variations in Fe concentrations were noted with time, and distinct trends emerged across the study duration. Additionally, comparisons between organs on the same day highlighted differences in Fe 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 the duodenum, where AI piglets had higher Zn concentration than HI piglets ($P < 0.05$). In feces, HIP and HIS piglets showed significantly lower Zn concentrations 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 ($P < 0.05$), whereas HIS piglets displayed lower Zn concentrations 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 in which HIP and HIS piglets had lower Cu concentrations than AI and HI piglets in feces and tissues. In feces, HIP piglets had lower Cu concentrations than AI piglets on PD 29 ($P < 0.01$) and HI piglets on PD 22 ($P < 0.05$) and PD 29 ($P < 0.01$) and were similar to that of 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 ($P < 0.001$), whereas HI piglets had slightly higher concentrations than HIP and HIS piglets but lower concentrations than AI piglets. In colon, although 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 trends 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 concentrations 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, which should be further investigated. This should provide valuable insights for further research and potential applications in dietary Fe and prebiotic related interventions.

Gene expression of Fe regulatory proteins and inflammatory markers

The mRNA expression of ferric reductase (duodenal cytochrome B) was not affected in DM but was higher in the colon of HIS piglets than HIP and AI piglets ($P < 0.05$, Figure 4A and F). The relative expression of *DMT1* and *TFR1* in DM and colon of AI pigs was 2–4-fold higher than that in the 3 groups fed the high-Fe diets ($P < 0.05$, Figure 4B, D, G, and I). However, the mRNA expression of Zn transporter 14 was not affected by the treatment in either the DM or colon (Figure 4C and H). The expression of

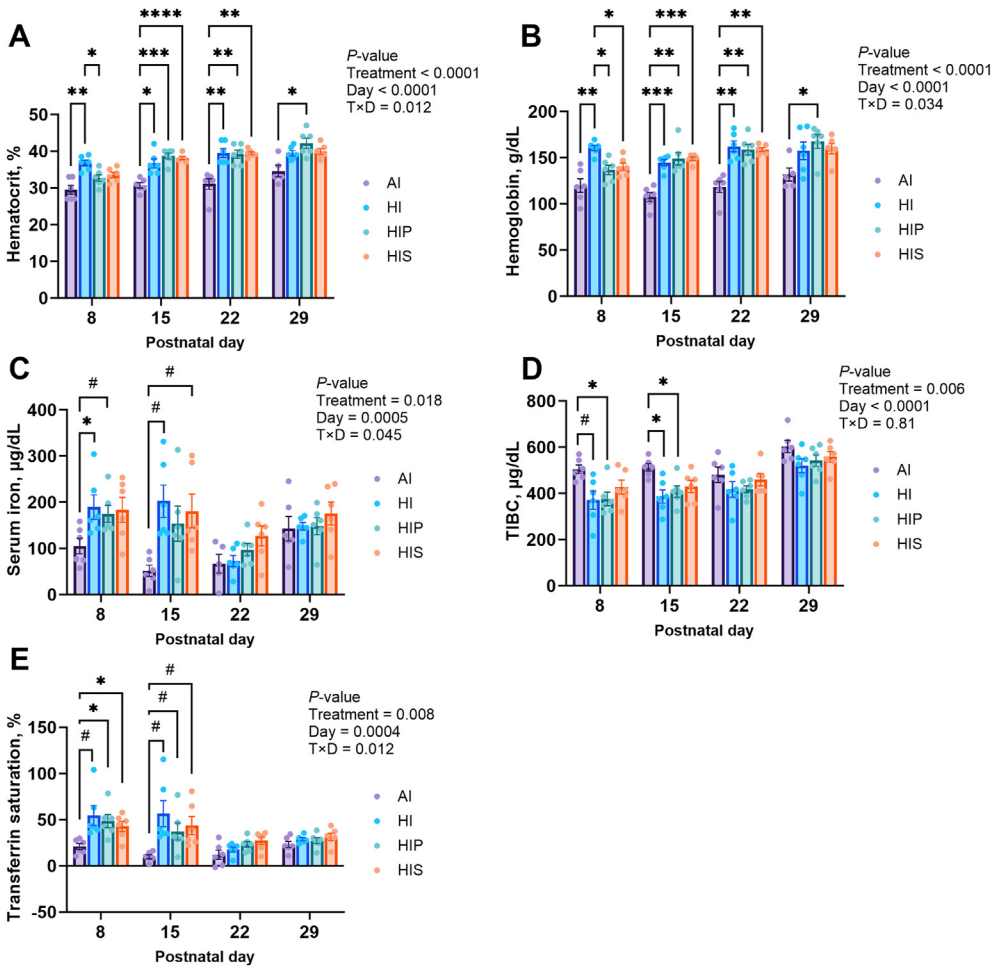

FIGURE 2. Effects of dietary treatment on (A) hematocrit, (B) hemoglobin, (C) serum iron level, (D) total iron-binding capacity (TIBC), (E) and transferrin saturation. Data show least squares means ± SEM. #Trend toward significance ($P < 0.1$); statistical significance at *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$. AI, iron-adequate milk diet; HI, high-iron milk diet; HIP, high-iron milk diet with a prebiotic; HIS, high-iron milk diet with a synbiotic; T×D, treatment by day.

FPN in the DM and liver was not affected by the treatment (Figure 4E and N), but its expression in colon was significantly higher in AI group than in the other groups ($P < 0.01$, Figure 4J). The AI diet significantly increased *TFR1* expression in liver ($P < 0.001$, Figure 4M), whereas feeding high-Fe milk dramatically increased *HAMP* in the liver compared with the AI group regardless of inulin or synbiotic supplementation ($P < 0.04$, Figure 4O). In the ileal mucosa, *IL6* ($P = 0.07371$, Figure 4K) and *TNFA* ($P = 0.724$, Figure 4L) were unaffected by treatment.

Enteric colonization of *L. agilis* YZ050

The HIS pigs received an average of 1.3×10^{10} CFUs/5 mL of *L. agilis* YZ050 every 3 d 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 (Supplemental Figure 1). *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 ($P < 0.05$).

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 piglets than AI piglets on PD29 ($P < 0.05$, Supplemental Figure 2). The difference was statistically significant but numerically small and thus its biological importance is uncertain.

Discussion

We used a neonatal pig model to investigate the impacts of Fe fortification, inulin, and synbiotic supplementation in a cow milk-based formula on trace mineral metabolism. According to the Swine National Research Council (2012), milk-fed young piglets should receive 50–150 mg Fe/kg of milk solid to meet the requirement [39]. When fed the AI, which provided Fe at the lower end of this recommendation (60 mg/kg of milk solid), 5 of 6 piglets maintained a satisfactory Fe level and nonanemic status (defined as Hb >110 g/L in infants or Hb >100 g/L in piglets).

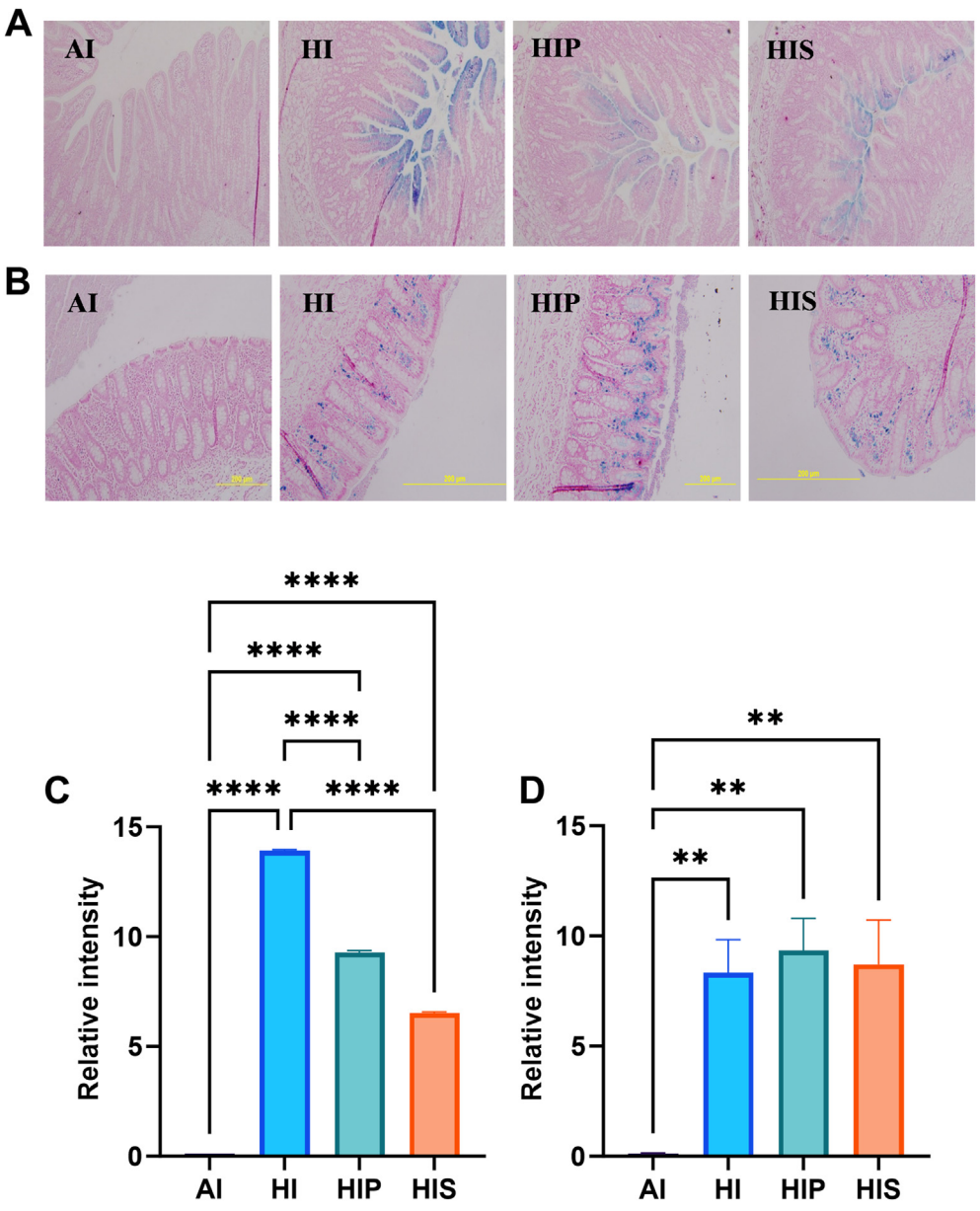

FIGURE 3. Effect of dietary treatment on iron accumulation in duodenal and colonic mucosa. Prussian blue-stained sections of (A) duodenum and (B) colon tissues from iron-adequate milk diet (AI), high-iron milk diet (HI), HI with a prebiotic (HIP), HI with a synbiotic (HIS) piglets. Relative intensity of Prussian blue-stained area (iron) in (C) duodenum and (D) colon of piglets from each treatment group (n = 6/treatment). The histograms display levels in arbitrary units of relative intensity of stained areas. Data show least squares means ± SEM. Statistical significance at $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

This was likely achieved through upregulation of intestinal Fe absorption indicated by the higher gene expression of Fe transporter (*DMT1*) in the duodenum and colon. Meanwhile, the extremely low hepatic expression of *HAMP* also facilitates basolateral Fe efflux from enterocytes. The nearly nondetectable level of Fe in the duodenal and colon mucosa of the AI pigs corroborated the enhanced Fe 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 Fe supplement through oral drops [11]. The transcriptional changes of *DMT1* and *HAMP* indicate a negative feedback regulation of intestinal Fe uptake in response to lower Fe intake. This implies that the absorption rate of Fe 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 iron deficiency anemia (IDA) with marginally adequate dietary intake.

Few studies have evaluated feeding a formula fortified with the lowest recommended Fe level in young infants. In a randomized controlled trial, Björmsjö et al. [40] found that healthy term infants fed a low-Fe formula (2 mg/L) until 6 mo of age, regardless of lactoferrin supplementation, did not have an increased risk of Fe deficiency or IDA compared with those fed a formula containing 8 mg Fe/L. This result is consistent with the finding of an earlier study that showed a formula containing 1.6 mg Fe/L being sufficient to meet requirements ≤6 mo of age

TABLE 2
Trace mineral concentrations in colon digesta, duodenum, and liver on postnatal day 29.

Site	Trace mineral	Day	Trace mineral concentration,[1] μmol/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 ± 0.83	8.64 ± 0.9	4.54 ± 0.29	5.67 ± 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 ± 0.03	0.15 ± 0.02	0.20 ± 0.03	0.21 ± 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 ± 0.04	0.33 ± 0.02	0.32 ± 0.02	0.33 ± 0.01	0.5351

Abbreviations: AI, iron-adequate milk diet; HI, high-iron milk diet; HIP, high-iron milk diet with a prebiotic; HIS, high-iron milk diet with a synbiotic.
[1]Data are presented as least squares means ± SEM. Multiple comparisons were performed across treatments. Values sharing no common superscript are significantly different (adjusted $P < 0.05$).

TABLE 3
Effect of dietary treatments on trace mineral concentrations in fecal samples collected on postnatal days 8, 15, 22, and 29.

Trace mineral	Day	Trace mineral concentration[1], μmol/g				*P* value		
		AI	HI	HIP	HIS	Treatment × 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 ± 21.68	78.07 ± 18.32	35.60 ± 9.65	41.94 ± 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 ± 19.95	96.08 ± 17.12	43.37 ± 2.13	47.69 ± 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 ± 1.05	9.09 ± 3.11	4.87 ± 1.5	6.47 ± 0.81	0.5241	0.0059	0.0005
	15	11.84 ± 2.63	13.82 ± 1.38	8.64 ± 0.66	8.64 ± 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 ± 13.73	48.07 ± 12.27	22.90 ± 5.23	20.29 ± 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 ± 10.98	50.93 ± 13.44	19.94 ± 1.18	21.25 ± 2.24			
	29	$60.39^{a} \pm 7.6$	$36.25^{ab} \pm 8.89$	$22.56^{b} \pm 1.92$	$23.59^{b} \pm 1.11$			

Abbreviations: AI, iron-adequate milk diet; HI, high-iron milk diet; HIP, high-iron milk diet with a prebiotic; HIS, high-iron milk diet with a synbiotic.
[1]Data are presented as least squares means ± SEM. Multiple comparisons were performed across treatments within each sampling day. Values sharing no common superscript letters are significantly different (adjusted $P < 0.05$).

[41]. The altered gene expression of Fe 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 Fe, although more well-powered randomized controlled trials are required to define small differences and fine-tune the optimal Fe level.

HAMP acts as the master regulator of systemic Fe homeostasis, inhibiting FPN-mediated intestinal Fe release when body Fe status is adequate, thus preventing Fe overload [42]. The consumption of high-Fe milk, regardless of inulin or synbiotic supplementation, led to a dramatic increase in *HAMP* expression in the liver and significant Fe deposition in the mucosa of the duodenum and colon. These findings support the well-established role of HAMP. However, the significant accumulation of hepatic Fe, 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 HAMP–FPN axis. We and others have observed that, despite the pronounced upregulation of HAMP in response to excessive Fe intake, FPN expression in enterocytes was resistant to HAMP-induced degradation and its inhibitory effect on Fe efflux in suckling piglets and mouse pups, respectively [10,11]. However, intact HAMP–FPN regulation was observed in the gut of weaned mice [10].

Developmental maturation of systemic Fe regulation was also reflected by the temporal changes in serum Fe in the current study: high Fe intake significantly increased serum Fe concentration, which would have otherwise remained stable if HAMP regulation had been effective, during the first 2 wk after birth. In contrast, serum Fe concentration was unaffected by dietary treatment during the following 2 wk, implying the functional

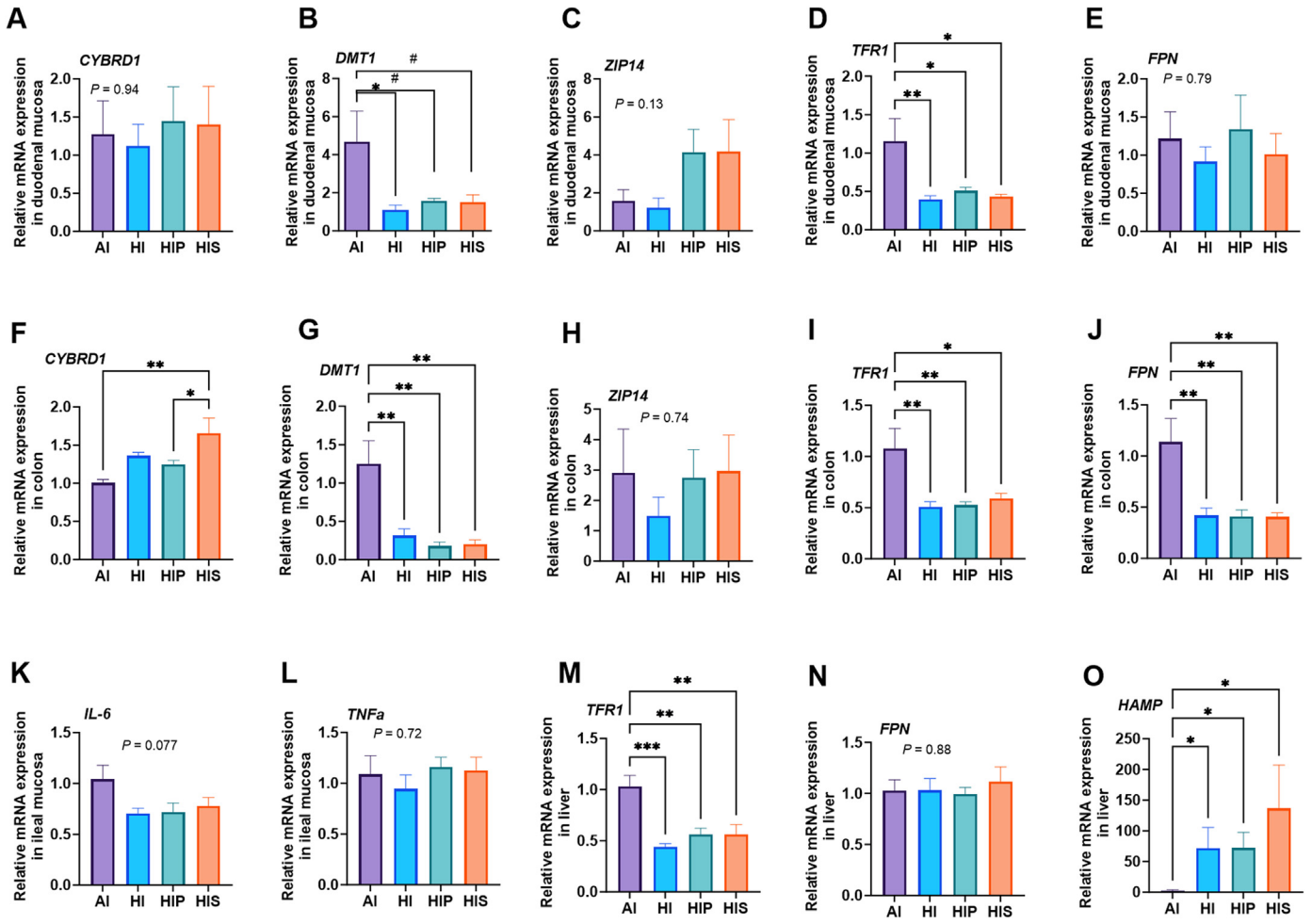

FIGURE 4. Effects of dietary treatment on the mRNA expressions of genes encoding iron regulatory proteins and inflammatory cytokines. Relative mRNA expressions of duodenal cytochrome B (*CYBRD1*), divalent metal transporter 1 (*DMT1*), zinc transporter 14 (*ZIP14*), transferrin receptor-1 (*TFR1*), and ferroportin (*FPN*) in the (A–E) duodenal mucosa and (F–J) colon tissue; (K, L) *IL-6* and *TNF-α* in the ileal mucosa; and (M–O) *TFR1*, *FPN*, and hepcidin *(HAMP)* in the liver. Data show least squares means ± SEM. #Trend toward significance ($P < 0.1$); statistical significance at *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. AI, iron-adequate milk diet; HI, high-iron milk diet; HIP, high-iron milk diet with a prebiotic; HIS, high-iron milk diet with a synbiotic.

activity of the HAMP–FPN axis. The immature HAMP–FPN axis may facilitate excess Fe absorption and hepatic deposition, whereas more Fe would be sequestered in the intestinal lining when FPN-mediated Fe efflux becomes sensitive to the inhibitory effect of HAMP. Fe oversupplementation via oral drops was found to significantly elevate Fe load in the liver and the developing brain [11].

Increased colonic Fe absorption through a HAMP-independent route may also contribute to hepatic Fe overload. Although the large intestine was shown to account for only 5% of the entire intestinal Fe absorption in weaned pigs fed a solid diet [43], intracecal Fe infusion at a high dose (20 mg/kg BW) led to a sustained large increase of Fe in the portal blood in growing pigs, presumably resulting from increased colonic epithelial permeability [44]. In the current study, considering the abundant expression of Fe transporters and marked Fe accumulation in the colon mucosa, substantial colonic Fe absorption cannot be excluded in pigs fed the high-Fe milk. Together, the current piglet model demonstrates significant body Fe accumulation and elevated colonic Fe flux resulting from excessive dietary Fe exposure during the early postnatal period. This highlights the critical need to evaluate the risk of Fe 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 on the elevation of colonic Fe 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 ≤10% monosaccharides, disaccharides, 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 breastfed 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 Fe bioavailability in anemic pig and Fe-deficient rat models through a mechanism that is not fully understood [26–28]. In a study of anemic pigs, Yasuda et al. [26] found that supplementing 4% inulin in a corn

and soybean meal-based diet significantly increased total soluble Fe in the colon digesta and Hb repletion efficiency, indicating greater Fe bioavailability. These findings align with the hypothetical model that inulin fermentation acidifies the gut lumen and facilitates ferric reduction and subsequent uptake via DMT1. However, neither the pH of colon digesta nor intestinal or colonic Fe 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 (Supplemental Figure 2). Tako et al. [22] observed higher mRNA expression of Fe transporters (e.g., *DMT1* and transferrin receptor, presumably *TFR1*) within the duodenum and colon of inulin-supplemented pigs, which displayed greater Hb repletion efficiency. 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 Fe status via the Fe responsive element–Fe responsive protein system, an upregulation should have indicated a lower cellular Fe 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 Fe modulatory effect of inulin in formula-fed infants or young animals in the context of dietary Fe overconsumption. Contrary to the previous findings, inulin supplementation in the high-Fe milk (HIP and HIS) not only did not affect Hb, Hct, or total serum Fe but also reduced hepatic Fe accumulation compared with the HI group. These results suggest that inulin supplementation may have reduced Fe absorption and mitigated Fe overload caused by the high-Fe formula. Since mRNA expression of the Fe 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 Fe accumulation in inulin-fed pigs.

The reduction in Fe absorption would have increased the intestinal flow of unabsorbed Fe. Surprisingly, both HIP and HIS pigs had lower colonic and fecal Fe 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 Fe levels in the inulin-fed pigs resulted from increased bowel movement (shortened colonic transit time) and stool frequency. These factors reduce colonic Fe 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 5 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 6 studies involving 86 subjects who consumed $\geq$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-Fe milk may indicate an antagonistic effect of excessive Fe 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 Fe 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 3 groups fed the high-Fe milk. This finding suggested that inulin supplementation at 5% of milk solids Fe-independently reduced the bioavailability of these trace minerals, likely by decreasing colonic absorption.

The abundance of *L. agilis* YZ050 in feces collected on PD 15 and 29 (1 or 2 d after the most recent inoculation) reached 10^5 CFUs/g feces (Supplemental Figure 1). This indicates moderate 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 Fe absorption in infants and children with Fe deficiency 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 Fe and other trace minerals in the context of excessive Fe intake.

In conclusion, using a piglet model, we showed that feeding a high-Fe formula in early infancy led to body Fe overload and decreased Zn and Cu bioavailability. This effect is possibly due to the antagonistic effect of excess Fe on duodenal absorption of Zn and Cu. Moreover, feeding marginally AI triggered the upregulation of genes involved in Fe absorption and maintained satisfactory Fe status in most piglets, highlighting the opportunity to lower Fe fortification in infant formula. Considering that one AI pig developed anemia, it is imperative for future studies to evaluate the safety range of Fe reduction in a fortified formula. Instead of enhancing Fe absorption, as observed in other studies, inulin supplementation (5% of milk solids) in Fe-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 future studies is warranted to directly analyze the bioavailability and fecal excretion of trace minerals.

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Author contributions

The authors' responsibilities were as follows – PJ: designed research; JP: conducted research and sample analysis; JP, PJ: performed data analysis; SW, DAM: provided probiotic inoculants; JP, PJ: wrote the manuscript; and all authors: edited and approved the final manuscript.

Conflict of interest

The authors report no conflicts of interest.

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Data availability

All data are reported in the article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cdnut.2024.102147.

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