Bioavailability of common iron fortificants

by

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M.B., B.S, Shanghai University of Traditional Chinese Medicine, China, 2008

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AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the
requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Food, Nutrition, Dietetics and Health
College of Health and Human Sciences

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2024

Abstract

Background: Anemia is recognized by the World Health Organization as a global concern of public health and a common comorbidity of many chronic diseases. Iron fortification is one of the most cost-effective approaches to combat iron deficiency anemia. It can improve the iron status of people with iron deficiency or at the risk of iron deficiency. However, it is challenging to achieve high iron bioavailability. Thus, we conducted preclinical and clinical systematic reviews with meta-analysis and two *in vitro* studies to upgrade previous iron formulations for the prevention and treatment of iron deficiency anemia.

Objective: To identify, evaluate, and summarize the evidence regarding the efficacy of common iron fortifications in preclinical studies and clinical trials. To assess the bioavailability of common iron fortifications in *in vitro* INFOGEST digestion/Caco-2 cell model.

Methods: In two systemic reviews, final included articles were collected after initial articles were scanned and sorted. The characteristics of included studies were summarized for the systematic review. The data was extracted for meta-analysis.

In two cell-culture studies, 20 rice or corn flour samples fortified with NaFeEDTA, ferrous fumarate, $FeSO_4$, FePP, or $FePO_4$ were digested with INFOGEST method. Subsequently, Caco-2 cells were treated with the iron digesta or iron supernatant in dual-chamber systems (dialysis membrane or insert membrane) or triple-chamber system (dialysis membrane and insert membrane) for 2 hours. The cell lysates were collected and iron bioavailability was examined by measuring ferritin concentrations.

Main Results: 42 studies were included in preclinical systemic review. The data from 17 rat experiments including 52 comparisons was analyzed with pairwise meta-analysis. The results of meta-analysis showed that the overall Hb effect of NaFeEDTA was superior to that of positive control ($FeSO_4$). Ferrous fumarate was equivalent to $FeSO_4$. FePP and $FePO_4$ were inferior to $FeSO_4$.

145 studies were included in clinical systemic review. 46 studies including 88 comparisons and 24107 participants (under 18 years old) contributed to the network meta-analysis in Hb. $FeSO_4$, NaFeEDTA, fumarate, and FePP were more effective than placebo. The rank order of Hb effect was NaFeEDTA> ferrous fumarate> FePP> $FeSO_4$>electrolytic iron> placebo.

In the first Caco-2 cell study (corn flour samples), the ferritin concentrations of NaFeEDTA, ferrous fumarate, and $FeSO_4$ in the classic dual-chamber system (dialysis membrane) were higher than those of NaFeEDTA, ferrous fumarate, and $FeSO_4$ in the alternative dual-chamber system (insert membrane) and the triple-chamber system. The ferritin concentrations of NaFeEDTA digesta and ferrous fumarate digesta were higher than those of NaFeEDTA supernatant and ferrous fumarate supernatant in classic dual-chamber system. NaFeEDTA had higher ferritin concentration than $FeSO_4$. The combination of NaFeEDTA and $FeSO_4$ at low weight ratio (2:11) had higher ferritin response than the combination of NaFeEDTA and $FeSO_4$ at high weight ratio (4:9) and the combinations of NaFeEDTA and fumarate.

In the second Caco-2 cell study (rice flour samples), the ferritin concentrations of high ratio FePP, $FePO_4$, and μFePP were significantly higher than those of low/zero ratio FePP, $FePO_4$, and μFePP.

Conclusions: The findings of systematic reviews and *in vitro* studies found that NaFeEDTA had higher bioavailability than $FeSO_4$. The dual-chamber system with dialysis membrane and digesta was a reliable iron bioassay. The combination of NaFeEDTA and $FeSO_4$ at low weight ratio had the highest bioavailability among the combination samples. The iron bioavailability of FePP and $FePO_4$ was improved with high molar ratio (1:0.3:5.5) citric acid and trisodium citrate. The results of our secondary studies and primary studies on iron bioavailability were consistent.

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Conclusions: The findings of systematic reviews and *in vitro* studies found that NaFeEDTA had higher bioavailability than $FeSO_4$. The dual-chamber system with dialysis membrane and digesta was a reliable iron bioassay. The combination of NaFeEDTA and $FeSO_4$ at low weight ratio had the highest bioavailability among the combination samples. The iron bioavailability of FePP and $FePO_4$ was improved with high molar ratio (1:0.3:5.5) citric acid and trisodium citrate. The results of our secondary studies and primary studies on iron bioavailability were consistent.

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Chapter 1

Nutritional Iron Deficiency Anemia and Iron Fortification: Progress and Challenge

1.1 Anemia and iron deficiency anemia

Anemia is a hematological system disease due to the decreased production or shortened survival of erythrocyte. Based on the number and morphology of red blood cells (RBCs), it can be classified into megaloblastic anemia, aplastic anemia, hemolytic anemia, hereditary anemia, and iron deficiency anemia (IDA). The largest proportion of anemia worldwide is IDA, which is caused by inadequate iron for hemoglobin synthesis. The etiology of iron deficiency anemia includes low iron intake (e.g., vegetarian, etc.), low iron absorption, blood loss, increased physiologic demand of iron (e.g., growth, pregnancy, etc.), disease (e.g., inflammation, infection, malaria, Crohn's diseases, etc.), surgery (e.g., gastreclomy, etc.) and special medicine and therapy (e.g., H_2 receptor blockers, erythropoietin therapy, etc.).[1–9]

IDA develops slowly. Usually, the gradual depletion of the iron in human body goes through 5 phases: normal, early negative iron balance (decreased iron store), iron depletion (severe decreased iron store), iron deficient erythropoiesis (decreased circulating iron and

erythron iron), and IDA (severe decreased circulating iron and erythron iron). Pre-IDA and early IDA may be present without any signs or symptoms, so they are often unrecognized. If signs and symptoms are present, patients may have pallor, fatigability, shortness of breath, dizziness, light-headedness, poor appetite, cold sensitivity, increased thirst, and decreased ability to concentrate. In severe cases, tachycardia, faintness, palpitations, tachypnea, glossitis, angular cheilitis, koilonychia (spoon-shaped and brittle nails), dysphagia (Plummer-Vinson syndrome), periorbital hyperpigmentation, and pica (pagophagia) can occur.[1–9]

The diagnosis of pre-IDA and IDA can be confirmed by blood tests. The major pathogenetic sign is the impaired hemoglobin (Hb) synthesis. The WHO criteria of Hb for IDA are as follows: hemoglobin<110 g/L (6 months old-4 years old), <115 g/L (5-11 years old), <120 g/L (males ages 12-14 years old, females over 12 years old), and <135 g/L (males over 15 years old). Other important criteria for iron status evaluation are serum iron (unbound iron) <10 μmol/L, serum ferritin (iron storage) <25 μg/L, hematocrit (volume percentage of RBC)< 37% (women) and <40% (men), transferrin saturation (cellular iron bind to transport protein) <16%, mean corpuscular volume (the size of RBCs)<80 fl, mean corpuscular hemoglobin (hemoglobin/RBC) <27 pg, and total iron binding capacity (ability to bind more iron)>70 μmol/L. When serum ferritin and transferrin saturation fall, the supply of iron in the bone marrow is unable to meet the requirement of hemoglobin production. Abnormal iron metabolism occurs along with increased total iron binding capacity and decreased hepcidin. The number of reticulocytes is low. The concentrations of Hb and hematocrit drop. Erythropoiesis is suppressed and immature erythroid cells are generated. The RBCs are microcytic and hypochromic.[1–11]

1.2 Epidemiology of anemia and iron deficiency anemia

In 2011, the World Health Organization (WHO) reported that 43% (273 million) children aged 6-59 months old, 38% (32 million) pregnant women and 29% (496 million) non-pregnant women had anemia worldwide. The global prevalence of severe anemia was 0.9-1.5% (9-19 million). Meanwhile, in Arica, 62% (85 million) children aged 6-59 months old, 46% (9

million) pregnant women and 38% (70 million) non-pregnant women had anemia. The prevalence of severe anemia was 1.5-3.6% (0.3-4.9 million). In America, 22% (17 million) children aged 6-59 months old, 25% (2.4 million) pregnant women and 17% (38 million) non-pregnant women had anemia. The prevalence of severe anemia was 0.2-0.5% (0.18-1.3 million). In Asia, 54% (97 million) children aged 6-59 months old, 49% (12 million) pregnant women and 42% (191 million) non-pregnant women had anemia. The prevalence of severe anemia was 1.1-1.9% (0.3-8.6 million). In Europe, 23% (48 million) children aged 6-59 months old, 26% (1.8 million) pregnant women and 29% (496 million) non-pregnant women had anemia. The prevalence of severe anemia was 0.3-0.6% (0.2-1.3 million).[12]

Children and women of childbearing age are particularly susceptible to IDA. Park et al. reported the national IDA prevalence of all age groups in Korea using Korean National Health and Nutrition Examination Survey (NHANES) and Korean National Health Information Database from 2002 to 2013. Approximate 1 million Korean were involved every year. The overall IDA prevalence was 3.81-4.73%. The IDA prevalence (10-18.2%) was highest in women aged 30–49 years old. The total expenditures of IDA treatment were $210,203 in 2002 and $596,256 in 2013.[13] In 2016, 12,403 Chinese pregnant women were investigated, and 19.8% pregnant women were diagnosed with anemia. 70% of these anemia pregnant women suffered from IDA.[14] In a systematic review on IDA prevalence in Nigeria (2013-2018), 32-45% pregnant women had IDA.[15] Habid et al. analyzed the 2018 data of Pakistani National Nutrition Survey and identified the IDA prevalences were 28.9% in children under 5 years old (n=17,814) and 18.4% in women aged 15-49 years old (n=22,114).[16] In most recent studies, Weyand et al. also evaluated the IDA prevalence among 12-21 years old females using the 2003-2020 data of USA NHANES database. 3,490 individuals were included. The IDA prevalence was 6.3% using a cutoff value of 120 mg/L hemoglobin, and was 27.1% using a cutoff value of 25 μg/L ferritin.[17]

1.3 Nutritional iron deficiency anemia and food fortification

Anemia can also be classified into nutritional anemia and non-nutritional anemia based on whether it is associated with nutritional deficiencies or disorders. Nutritional IDA means that anemia caused by physiological iron requirements cannot be met from food.[18] Taking supplementation is a good way to combat nutritional IDA. Oral iron supplementations are usually available over-the-counter. However, they have some gastrointestinal side effects including nausea, diarrhea, flatulence, constipation, and epigastric pain. Additionally, many patients feel uncomfortable with metallic taste, staining of the teeth, and the color of stool getting darker. Therefore, the tolerability of oral iron supplementations is often questioned, especially for children and pregnant women.[19] Food fortifications can alleviate these gastrointestinal side effects and discomfort.

Iron food fortification is defined as the practice of mixing iron with food. It is a safe, proven, and cost-effective diet strategy for the management of iron deficiency. In the 1920s, salt was firstly iodized in North American and European countries.[20] In the 1940s, bread started to be fortified with iron in USA and Britain.[21] Nowadays, iron fortification has been widely applied in cereal products, infant complementary food, formula, milk, drink, and condiments. While iron is one of the most difficult micronutrients to be fortified with food due to their interaction. The bioavailability of iron is reduced by the inhibitors (e.g., polyphenols, tannins, phytic acid, etc.) of iron absorption in food matrixes. Besides, the unacceptable change in sensory property is another challenge.

1.4 Food processing and extrusion

Many food processing methods such as coating, spraying, sonication, dusting, germination, parboiling, and extrusion can partially address these issues on iron bioavailability and sensory property. As one of the most commercial food processing methods, extrusion is an effective and sustainable food processing strategy to refine starchy material by pressure, temperature,

and mechanical shear force.[22] The initial extruded rice analogue was patented by Gorozpe in 1959.[23] With the development of extrusion technique in several decades, extruded rice has been widely used as an ideal food vehicle to deliver deficient micronutrients to human body. The ideal extruded rice fortification can improve the micronutrient retention and bioavailability, lower the post-processing loss, increase storage stability, and minimize sensory changes. Rice is the staple food for half of the world's people. Cooked rice containing 29% carbohydrate, 2% protein and 0.4% fiber can provide 130 calories of energy, while some antinutritional components pose the challenge to the micronutrient bioavailability.[24] Phytic acid and tannic acid can bind to the iron ions to form insoluble iron complexes, thus preventing iron absorption in the gastrointestinal tract. 200-300 mg of phytic acid and 3,000 mg of tannic acid were detected in 100 g of rice-base meal.[25] Extrusion can deactivate these antinutritional components by kneading and melting. In Albarracin's study, the concentration of phytic acid concentration could be reduced from 740.1 mg/100g raw rice to 163.5 mg/100g extruded rice, and iron bioavailability could be increased from 11.2% in raw rice to 14.5% in extruded rice.[26] Moreover, extrusion can ameliorate the sensory characteristics of iron fortifications. The results of multiple sensory observations indicated no significant difference between FePP-fortified extruded rice and unfortified natural rice.[27–29] It implied that their appearances were similar and the organoleptic properties of extruded rice fortified with FePP were acceptable.

1.5 Enhancers of iron absorption

Iron absorption can be defined as the transportation of dietary iron from the gastrointestinal lumen through the brush border membrane to be absorbed by enterocytes. Adding enhancers of iron absorption is another good approach to improve iron bioavailability. Foods, dietary compounds, endogenous components, and additives known as iron enhancers include sugar (e.g., fructose), meat, seafood, mucin, and organic acids (e.g., ascorbic acid, citric acid, lactic acid, etc.). In terms of food fortification, organic acids can reduce more insoluble Fe^{3+} to soluble Fe^{2+} in the gastrointestinal tract. They can also complex iron ions as chelators to

bind nonheme iron (ligands) with carboxyl and hydroxyl groups, thereby desorbing them from inhibitors in the stomach. Most iron-chelate complexes can remain soluble status due to the loose binding of iron, these iron ions can be released in the duodenum and absorbed by enterocyte. Other iron ions are tightly bonded to chelate, and these insoluble iron-chelate complex can be excreted.[30–32]

The type of organic acids also plays an important role in the improvement of iron absorption. In a Mexican clinical trial, the mean of iron absorption was significantly increased from 6.6% to 22.9% after a ^{58}Fe (2.7 mg)-ascorbic acid (25 mg) treatment.[33] Gillooly et al., conducted a human study in India. They tested the absorption of ^{55}Fe/^{59}Fe with multiple organic acids at a weight ratio of 3:1000, and found that citric acid, tartaric acid, and malic acid could significantly improve the iron absorption.[34] In a Caco-2 cell experiment on a variety of organic acids, tartaric acid had the highest iron uptake, which was increased by 43.1 folds than that without tartaric acid. Succinic acid was at the second place (increased by 17.8 folds). Citric acid, malic acid, oxalic acid, fumaric acid, and lactic acid also showed high iron uptake (increased by 3.2-5.7 folds).[35] Porres et al. reported that iron dialyzability could be increased by 12 folds when citric acid (6.25 mg/g wheat flour) was added to $Fe(NO_3)_3$ (279 mg Fe/g wheat flour) fortification. If phytase (0.285 PU/g wheat flour) and ascorbic acid (4.4 mg/ g wheat flour) were added to this Fe-citric acid fortified wheat flour, the iron dialyzability could be risen by 24 folds.[36] Moreover, the dose-response of chelation was found and might cause a change in iron metabolism. Cook et al. found a linear relationship between iron absorption and the molar ratio of ascorbic acid (25-1000 mg) and ^{55}Fe/^{59}Fe (4.1 mg) in human study. When the molar ratio was less than 7.5, the slope was larger, and vice versa.[37]

Naturally, organic acids in fruits are 4g tartaric acid per kg of grapes, 11g malic acid per kg of plums, and 29g citric acid per kg of currants, and lower in vegetables ranging from 1 to 8 g per kg of vegetable.[38] Boato et al. purchased juice products of apples, prunes, pears, oranges, white grapes, red grapes, and grapefruits from supermarkets. These juices were mixed with $FeCl_3$ additive (34.2 µg/ml juice) or Fe-fortified commercial infant cereal (540 µg/g cereal). The ferritin response of Caco-2 cells implied that the organic acids in

the juices of pears, apples, oranges, and white grapes could significantly improve the iron bioavailability of both $FeCl_3$ samples and infant cereal samples.[39] Hallberg et al. added 50 mg of ascorbic acid or 125 g of boiled cauliflower containing 65 mg of ascorbic acid to the $^{55}Fe/^{59}Fe$ fortified basal meal for 20 females. The iron absorption was significantly increased from 0.05 mg to 0.12 mg and from 0.18 mg to 0.58 mg, respectively.[40] These results suggested that consuming fruits or vegetables rich in organic acids was a healthy manner to enhance the nonheme iron absorption in human body.

1.6 Preclinical and clinical studies on iron bioavailability

In terms of iron bioavailability, it usually refers to the proportion of dietary iron used by human body. Its prerequisite is absorption. Although many large-scale intervention projects on iron fortification have been implemented in the world, nutritional iron deficiency anemia still remains one of the most serious public health issues due to the unsatisfactory iron bioavailability. Thus, increasing number of preclinical and clinical studies are being conducted.

Static digestion methods and cell-culture models are commonly employed in *in vitro* studies. Take INFOGEST digestion method and dual-chamber Caco-2 cell model as paradigms. Since 2014, an international academic association name INFOGEST refined and validated a static digestion method, which could mimic the oral and gastrointestinal digestion condition with the adjustments of pH value, transit time, enzyme activity, mechanical force, temperature, electrolyte composition, bile concentration, etc. It has been used to assess the digestibility and bioavailability of macronutrients and micronutrients in foods. Muleya et al., assessed the Fe^{57} bioavailability in cereals and legumes with modified INFOGEST method, in which pancreatin and bile concentrations were reduced. They thought that the iron bioavailability was food matrix-dependent.[41] Rebellato's team and Castro' team used INFOGEST method to measure the solubility of endogenous iron in meats and cruciferous vegetables, respectively. The results were 4.3 to 14 mg soluble iron per kg of meats and

6.3-16.7 mg soluble iron per kg of vegetables.[42;43]

Caco-2 cell model is a high-throughput *in vitro* platform for screening iron fortification candidates. Caco-2 cells can spontaneously differentiate and polarize to form tight junctions with apical and basolateral domes. This tight junction is called monolayer, which is very similar to enterocytes in morphology and function. Caco-2 cell monolayer has brush border enzymes and transport proteins. It is a catenary structure rather than a "single barrier". Digestion methods have usually been incorporated with Caco-2 cell model to evaluate iron uptake, iron permeability, iron transport mechanism, etc. As an enhancement of the dialysis tube, the dual-chamber system has been built for the iron bioavailability assessment. A silicon O ring is used to hold a piece of dialysis membrane covering the bottom of insert frame without insert membrane. Iron digesta is added to the upper chamber so that the low molecular weight iron can gradually and steadily transfer to the Caco-2 cells growing in the lower chamber.[44;45] In Puyfoulhoux's study, the iron bioavailability of spirulina was measured using digestion/Caco-2 cell model. It had a 6.5-fold ferritin level higher than meat. Spirulina was suggested as a good iron resource.[46] Stefos et al. used the INFOGEST digestion/Caco-2 cell model to assess the iron bioavailability in milk and yogurt acid whey. There was no significant difference in iron bioavailability between milk and yogurt, while 3-10 kDa yogurt fraction was superior to 0-3 kDa yogurt fraction in iron bioavailability.[47]

The use of *in vivo* small animal models, such as rat, piglet, chicken, rabbit, and mouse, has enabled scientists to access the new knowledge and applications about iron bioavailability. The implementation of clinical trials necessitates the interpretated results of *in vitro* cell experiments and *in vivo* animal experiments. Animal models are often classified into the depletion-repletion model and the preventative-prophylactic model. In the depletion-repletion model, animals are first fed the low iron diet to deplete iron stores and then fed with the intervention diets during the iron repletion period, which examines the efficacy of intervention for disease treatment. In the preventative-prophylactic model, animals are immediately fed with the targeted diets after weaning, which focuses on the disease prevention.[48;49] Malika et al. conducted a prophylactic-preventative rat experiment to assess the iron bioavailability of NaFeEDTA and $FeSO_4$. The results showed that the rats fed with

NaFeEDTA had the higher levels of iron, Hb, and total iron binding capacity.[50] In Hernandez's animal study, rats were fed with iron depleted diet for 10 days, and then fed with ferrous fumarate, ferric citrate, reduced iron or $FeSO_4$ fortified diets for 14 days. The ranking of iron bioavailability was $FeSO_4$, ferrous fumarate, ferric citrate, and reduced iron.[51]

Clinical trial is prospective biomedical study on human participants' response to the specific interventions such as supplement, food fortification and medical device. In general, double-blind randomized control clinical trials are carried out in one or multiple research centers to generate data on efficacy, dosage, duration, and safety for warranted scientific validity and reproducibility. Tchum et al. conducted a double-blind, cluster-randomized clinical trial in Ghanaian children aged from 6 to 35 months old. They reported that the Hb level, serum ferritin concentration, and anemia status could be significantly improved after a 5-month intervention of ferrous fumarate fortified meals.[52] In another double-blind randomized clinical trial, Zimmermann et al. recruited 330 Thai women aged from 18 to 50 years old. After 35-week treatment of wheat-based food fortified with electrolytic iron, hydrogen-reduced iron, or $FeSO_4$, the level of serum ferritin was increased, and the number of individuals with iron deficiency anemia was decreased.[53] From a methodological view, stable isotope iron technique is a particular assessment method of iron bioavailability. The rapid iron absorption is the main advantage. The duration of daily iron diet is usually 4-6 months, while the duration of isotope iron treatment is only 14 days. Over the past few decades, this technique has been used to study the iron absorption in a wide range of population groups and settings as a predictor or screening tool. Henare et al. conducted a randomized cross-over trial in 22 Zelanian young healthy women to evaluate the efficacy of iron–casein complex. They found that the iron bioavailability of iron–casein compound and $FeSO_4$ was equivalent after the isotope iron treatment. Iron–casein complex was considered as a promising fortificant.[54]

Chapter 2

Preclinical Perspectives of Common Iron Fortificants on Iron Bioavailability: A Systematic Review and Pairwise Meta-analysis

Abstract

Background: Systematic review with meta-analysis is an evidence-based approach that addresses specific research questions by evaluating all relevant and available literatures. In recent decades, preclinical studies on improving iron bioavailability to combat iron deficiency anemia have increased sharply, while no preclinical systemic reviews with pairwise meta-analysis on iron bioavailability have been published yet. Thus, this review is the first study in this field. It is pivotal to have a better knowledge of existing preclinical studies on iron bioavailability to guide our upcoming preclinical research.

Objective: To review and summarize the evidence regarding the effect of common iron fortifications in *in vitro* and *in vivo* animal studies and the relevant mechanisms on the regulations of iron absorption.

Methods: Final included articles were collected after initial articles were scanned and

sorted. The data was extracted for pairwise meta-analysis and relevant mechanisms were reviewed.

Main Results: 42 studies were included in this systemic review. The data from 17 rat experiments including 52 comparisons was analyzed with pairwise meta-analysis. The results of meta-analysis showed that the overall Hb effect of NaFeEDTA was superior to that of $FeSO_4$. Ferrous fumarate was equivalent to $FeSO_4$. FePP and $FePO_4$ were inferior to $FeSO_4$.

Conclusions: The meta-analysis results suggested that NaFeEDTA and ferrous fumarate could improve iron-deficient status. Iron absorption might be bidirectionally regulated by the iron regulatory protein (IRP)-iron responsive element (IRE) system and Hepcidin-Ferroportin interaction. However, the evidence level of meta-analysis results should be prudently applied as conditional recommendations due to the moderate and high heterogeneities, unsolvable biases, limited information, and insufficient data.

Keywords: Iron bioavailability, Iron fortification, Preclinical study, Meta-analysis, Iron regulation, IRE-IRP system, Hepcidin-ferroportin interaction

2.1 Background

2.1.1 Description of the condition

Anemia is recognized by the WHO as a major global concern of public health and a common comorbidity of many chronic diseases. Approximately 1.2 billion population worldwide is suffering from iron deficiency anemia, particularly among growing children, pregnant women, and the elderly.[55–57]. Food fortification is one of the most cost-effective ways to combat this disease. Iron deficiency in most people can be prevented and treated by iron fortification of staple food such as rice, wheat, corn, and maize, or drinks such as water, milk, and beverages, or condiments such as curry powder, soy sauce and salt. Especially, for infants and children, formulas and complementary foods can be fortified with iron. However, it is challenging to achieve high iron bioavailability and good organoleptic properties.

2.1.2 Description of the intervention

In theory, the more solubilized iron, the more iron can be absorbed and utilized.[58] The most common iron additives can be divided into three divisions in accordance with their water solubility and diluted-acid solubility: (1) Water-soluble iron compounds such as ferrous sulfate ($FeSO_4$) and sodium iron ethylene diamine tetraacetic acid (NaFeEDTA, Figure 1b); (2) Water-insoluble but diluted-acid-soluble iron compounds such as ferrous fumarate (FeC_4H_2O); (3) Water-insoluble and diluted-acid-insoluble iron compounds such as ferric orthophosphate ($FePO_4$) and ferric pyrophosphate (FePP) (Figure 2.1).[59;60] $FeSO_4$ is often used as an optimal positive control or a gold standard, since $FeSO_4$ can be dissolved in water to supply relatively high levels of bioavailable iron, but it has substandard organoleptic qualities by interacting with food.[61] NaFeEDTA is a chelate. It has a special structure, an octahedral complex with ferric iron in the center. It is formed when EDTA chelates ferric iron through six coordinate covalent bonds. It has high iron bioavailability without undesirable colors or flavors, but it is expensive. Ferrous fumarate is moderately soluble in hydrolysis, while the sensorial characteristics are also altered during catalyzes oxidations. As for $FePO_4$ and FePP, although they do not affect the sensorial properties of the food matrix, they have a low solubility and bioavailability.[59–65] The ideal products of iron fortifications should possess high iron bioavailability without altering the sensorial properties.

2.1.3 How the intervention might work

The intervention of iron fortificant refers to the addition of iron to food matrix. It aims to improve the nutritional iron status of people with iron deficiency or at the risk of iron deficiency by increasing the bioavailability of dietary iron intake. The efficacy of iron fortification in human body depend on multiple influencing factors such as iron varieties, iron concentrations, food matrixes, enhancers (e.g., citric acid, ascorbic acid, etc.), inhibitors (e.g., polyphenols, tannins, phytic acid, etc.), food processing approaches (e.g., precooking, extrusion, etc.), sensory properties, and physiological and pathological conditions.[66] Thus, there are diverse strategies to fortify food products to achieve high iron bioavailability. Selecting several cost-effective strategies based on existing studies as trade-off interventions among the above-mentioned influencing factors can address this subtitle question. In our systematic review, *in vitro* and *in vivo* animal studies were centered. Experimental partic-

ipants (P) were animals and cells. Interventions (I) were 4 common iron fortificants, FePP, $FePO_4$, ferrous fumarate, and NaFeEDTA. Comparisons (C) were positive controls (e.g. $FeSO_4$) or blank control. The primary outcomes (O) were hemoglobin and ferritin. This systematic review can assist scientists and health professionals to develop the nutritional interventions of common iron fortificants for the iron deficiency prevention and treatment.

2.1.4 Overview of iron metabolism

Iron comes in two natural forms of iron, heme (animal-based food) iron and nonheme (plant-based food) iron. Hemoglobin in animal-based food can be hydrolyzed by protease in gastrointestinal tract to heme iron and globin. Heme iron is a ring structure, which contain iron binding to the protoporphyrin. It is soluble and readily absorbed. Heme iron can be transported by heme responsive gene-1 protein (HRG-1P) and heme carrier protein (HCP1) across the brush border membrane. In the enterocyte, heme iron can be further hydrolyzed to ferrous iron and protoporphyrin by heme oxygenase.[67–72]

The common iron fortifications provide nonheme iron. Nonheme iron binding the components of food enter in gastrointestinal tract and then it is hydrolyzed by gastric acids, proteases, and other enzymes to free insoluble ferric iron and soluble ferrous iron. Some ferric iron can be reduced to ferrous iron by the catalyzation of reductases, mainly duodenal cytochrome b (DCYTB). Some ferric iron can generate ferric hydroxide and others remain ferric form. Subsequently, divalent metal transporter 1 (DMT1) can carry ferrous iron across the brush border membrane into the enterocyte through endocytosis.[67–72]

In addition, there is a cellular iron recycling pathway. The diferric-transferrin (Tf) complexes bind to the transferrin receptors (TfRs) on the cell membranes (e.g., intestinal or liver cells). The diferric-Tf-TfR complex is internalized by endocytosis, forming an endosome (vesicle). Protons are pumped into the endosome to reduce the pH value. In an acidic environment, the diferric-Tf-TfR complexs release ferric iron, which is reduced to ferrous iron by six-transmembrane epithelial antigen of the prostate 3 (STEAP 3). Similarly, ferrous iron can be carried by DTM1 to cross the endosomal membrane into enterocyte. The apotransferrin returns to the blood and the TfR returns to the cell membrane.[67–72]

Cytosolic ferrous iron binds to poly (rC)-binding protein 2 (PCBP2) and other substances

to play three main roles, the functional use in enterocyte, short-term storage as ferritin, and transportation into blood. Ferroportin transports ferrous iron with its oxidation to ferric iron by ferroxidases (hephaestin or ceruloplasmin) across the basolateral membrane so that ferric iron attaches to apotransferrin to form monoferric or diferric transferrin complex, which then can be transported in the blood for further tissue use or cellular iron recycle (Figure 2.2).[67–72]

2.1.5 Why it is important to do this review

Since some persistent doubts and barriers remain as how the iron fortifications have sufficient bioavailable forms without adverse sensory changes of food vehicle, many *in vitro* and *in vivo* animal studies have been conducted in recent decades to upgrade the products of iron fortification and verify some of the underlying regulatory mechanisms. In general, iron absorption is regulated by the conditions of cellular iron and body iron. Currently, IRP-IRE interactions in the cellular iron regulatory system and hepcidin-ferroportin complexes in the body iron regulatory system are two promising targets for the treatment of refractory iron disorders.[67] Exploring their molecular mechanisms with cell models and animal models can promote the development of iron treatments. In addition, there are no published preclinical systematic reviews with pairwise meta-analysis on iron bioavailability. Thus, this review is the first study in this field. This preclinical systematic review on both *in vitro* cell-culture studies and *in vivo* animal studies has been registered on the INPLASY (INPLASY202440031). The INPLASY has fewer restrictions than other systematic review registration platforms such as PROSPERO, which limits the preclinical systematic review in animal field only.

2.2 Objectives

The research and PICO questions posted in this systemic review were as follows: (1) In animal model, does iron fortification compared with control prevent or improve iron-deficient status and/or anemia status? (2) In cell model, does iron fortification compared with control improve the cellular iron absorption? (3) What are the relevant mechanisms on the regulations of iron absorption and how do they work? In this review, we identified, evaluated,

and summarized the findings of all relevant preclinical studies on common iron fortifications (FePP, $FeSO_4$, ferrous fumarate, and NaFeEDTA) and the understandings of two targeted iron regulatory mechanisms to provide evidence-based suggestions for further clinical trials. The process was schematically shown in Figure 2.3.

2.3 Methods

2.3.1 Criteria for considering studies (inclusion and exclusion criteria)

They must be preclinical studies involving *in vitro* studies or *in vivo* animal studies. The studies must be the primary research. Iron treatments must be given in the food matrix. At least one targeted iron fortificant with identified iron type and quantity was used. At least one desired outcome was reported. The manuscript must be peer-reviewed and in English. The clinical trials, observational studies, case reports, reviews, protocols, and secondary research were excluded. Theses, dissertations, book sections, meeting abstracts and proceedings were not included in this systemic review.

2.3.2 Types of outcomes

In animal experiments, Hb was the primary outcome in the assessment of iron deficient anemia status. In cell-culture experiments, ferritin was the primary outcome in the assessment cellular iron storage status. Secondary outcomes included but were not limited to plasma/serum iron, hematocrit, transferrin saturation, total iron binding capacity, iron solubility, hepatic iron, and bone marrow iron, which were the indicators in the assessment of early negative iron storage status and iron depletion status.[73]

2.3.3 Search strategy

We carried out the search strategy in 4 scientific databases (Pubmed, Web of Sciences Core Collection, Scopus, and EBSCO Agricola) on July 29, 2023. Snowball and citation search were used to extend the search strategy for additional articles using other academic search engines (e.g., Kansas state university libraries, Google Scholar, etc.).[74] In this search strategy, a special syntax was employed. The words in the quotation marks could not be separated. The asterisk indicated word variations. The search strategy followed the user

guides of the databases and was classified into 5 categories. Each category contained a series of search items.

(1) Category #1 contained 5 search items: "ferric pyrophosphate", "ferric orthophosphate", NaFeEDTA, "ferrous sulfate" and "ferrous fumarate", to ensure that the testing diet must be fortified with one targeted iron additive. (2) Category #2 contained 18 search items, which enabled iron status must be investigated. They were "iron status", "iron deficiency", "iron deficient", "iron depleted", "iron depletion", "iron stores", anaemia, anemia, anemic, anaemic, microcyt*, hypochrom*, "iron state", "iron balance", prophylactic, preventative, repleted, and repletion. (3) Category #3 including 13 search items: hemoglobin, haemoglobin, hematocrit, haematocrit, ferritin, "total iron binding capacity", "transferrin saturation", "serum iron", "plasma iron", solub*, "hepatic iron", "bone marrow iron" showed that at least one desired outcome was reported. (4) 5 search items, forti*, enrich*, food, diet, "micronutrient powder" in Category #4 indicated that iron additives must be presented in food. (5) Category #5 had only one search item (trial) to ensure that the included study must not be a clinical trial.

All relevant literatures were identified in "all fields or topic" using advanced search builder and Boolean operators (AND, OR, and NOT). All search items under one category were typed into one query search box with "OR" to get a query search result. All of query searches were combined with "AND" and "NOT" (#1 query AND #2 query AND #3 query AND #4 query NOT #5 query) into the final search box to obtain the initial articles. The filters were applied to specify publication types in peer-reviewed article and language in English.

2.3.4 Data managements, data extractions and quality assessments

Initial articles from 4 databases were imported into an online data manager, Zotero. After automatic duplicate/triplicate/quadruplicate merging, title- and abstract-reviewed articles were manually scanned. The remaining articles were thoroughly reviewed and further sorted to obtain the final included articles. The data and characteristics of included studies were extracted using Excel: author, year of publication, iron type, iron concentration, enhancer, animal type (species, gender) or cell line (strain), intervention duration, sample size, model

type (prophylactic–preventive method or depletion-repletion method), and outcome.

The quality assessments of the included studies were performed according to the modified checklist provided by previous researchers, Collaborative Approach to Meta-analysis and Review of Animal Data from Experimental Studies (CAMARADES) checklist, Systematic Review Center for Laboratory Animal Experimentation (SYRCLE), and the "Risk of bias" tools in the Cochrane Handbook adapted for *in vitro* studies and *in vivo* animal studies. The quality assessments were evaluated with three grades (high risk, unclear, and low risk) and further expressed with the total number of grade items.[75–78] The checklist comprised of 9 items: (1) Were there clear research purposes? (2) Was there relevant background information? (3) Were randomized allocation methods employed? (4) Were the investigators blinded? (5) Were dosage-response relationships investigated or the accepted fortification levels (e.g., licensed dosages) explained? (6) Did researchers explain the attritions (incomplete outcomes) and negative results? (7) Was the data clearly related to methodological design? (8) Did the conclusions and discussions match up with the findings? (9) Did the findings contribute to the current theories or future research/practices?[75–78]

2.3.5 Statistical analyses

Group-level data for pairwise meta-analysis was performed in ReviewManager (version 5.4, Cochrane's bespoke software) and STATA (version 18.0, USA) with the significance at p-value<0.05. In meta-analysis, the weighted mean difference (MD) and 95% confidence intervals (CIs) were used to assess the overall effect of the continuous outcome. Standard deviation (SD) could be calculated using the following formula: SD = SEM*sqrt (n), where n was the number of animals, if only the standard error of the mean (SEM) was reported.[79] Heterogeneity among studies was calculated with the I-squared, Chi-squared, and Tau-squared tests. The rough guides of I^2 are as follows: 0-40% I^2 suggested unimportant heterogeneity; 30-60% I^2 suggested moderate heterogeneity; 50-90% I^2 might delineated substantial heterogeneity; 75-100% I^2 delineated considerable heterogeneity. If the study had a high heterogeneity, a random-effect model would be used for pooling data. Otherwise, the random-effect model would be replaced by a fixed-effect model. In accordance with characteristics factors, sensitivity analysis, meta-regression, and subgroup analysis were conducted

to detect the source of high heterogeneity. Publication bias was assessed using the Egger's test and displayed in the funnel plot. A symmetrical funnel plot suggested the less possibility of publication bias, and vice versa.[76;80–86] The t test was used to compare the means between two groups. The certainty of evidence was assessed using the Grading of Recommendations Assessment, Development and Evaluation (GRADE) and GRADEProGDT online tool (https://www.gradepro.org/).[76;80–86]

2.4 Results

2.4.1 Description of search results

Our search yielded 1050 initial articles. 3 additional articles were added. After merging 394 iterations and screening 659 titles or abstracts, 541 articles were excluded. The full texts of 118 remaining articles were reviewed. 76 studies were further excluded. The number of final included articles was 42. The included studies were listed in Appendix A (page 191). The sorting and screening process was shown in Figure 2.4. The final included studies consisted of 34 animal studies and 8 cell-culture studies. In included studies, 17 rat experiments including 52 comparisons and 859 rats could be analyzed with pairwise meta-analysis. The rest of animal studies could not be involved in the meta-analysis due to the lack of outcome data at baseline or endline, different animal species, or the fact of not using $FeSO_4$ as a positive control. All included studies could contribute to the summary of data and characteristics. Although the excluded studies did not contribute to current secondary analysis, they could still be used to provide useful information and serve as examples in discussion section for better understanding. There were no sufficient experiments of other species for pairwise meta-analysis.

2.4.2 Characteristics and quality assessments of included studies

The characteristics of 42 studies with 121 comparisons were summarized in Table 2.1-2.6. Among these 42 studies, the food matrix of 30 studies was grain-based diets (71.4%), and others (n=12, 28.6%) were cheese, fruit juice, yogurt, milk, curry powder and cocoa powder. The predominant particle size of iron fortificants was regular size (20-30 μm, n=33, 78.6%).

7 micronized (0.3-3 μm, 16.7%) and 2 nano-particle (1-100nm, 4.8%) iron fortifications were investigated. The majority of animal studies (n=34) was performed on rats (n=32, 94.1%), in which the predominant species was male weaning Sprague-Dawley (SD) rats (n=12, 35.3%). 24 studies (70.1%) employed depletion (5-39 days)-repletion (10-90 days) models, and 11 studies (32.4%) employed prophylactic-preventative (21-60 days) models. One study conducted both depletion-repletion model and prophylactic-preventative model. Iron concentration (iron fortification level) of animal (n=34) and Caco-2 cell (n=8) studies ranged 10-250 mg Fe/kg food and 50 μmol Fe/L liquid-25 mg Fe/kg food, respectively.

The outcome effects of iron fortifications were summarized in Table 2.7-2.12. In cell-culture models, the percentages of non-inferiority effects between FePP and $FeSO_4$, ferrous fumarate and $FeSO_4$, NaFeEDTA and $FeSO_4$ were 55.6%, 85.7%, 80%, respectively. In animal models, the percentages of non-inferiority effects between $FePO_4$ and $FeSO_4$, FePP and $FeSO_4$, ferrous fumarate and $FeSO_4$, NaFeEDTA and $FeSO_4$ were 59.5%, 69.2%, 85.7%, 92.3%, respectively. In all studies, the percentages of non-inferiority effects between $FePO_4$ and $FeSO_4$, FePP and $FeSO_4$, ferrous fumarate and $FeSO_4$, NaFeEDTA and $FeSO_4$ were 59.5%, 65.7%, 85.7%, 88.9%, respectively (Table 2.13).

As shown in the table of quality assessments (Table 2.14), 20 studies did not report randomization. No studies reported blinding method. 15 studies did not investigate the dosage-response effects or explain the fortification levels. 5 studies did not and 4 studies might not interpret the attritions or the negative results. All studies presented clear research purposes, relevant background, methodological design, as well as good matching between conclusions, discussions, findings, current theories, and future practices.

2.4.3 Pairwise meta-analysis results

2.4.3.1 NaFeEDTA fortification versus $FeSO_4$ fortification

5 studies including 9 comparisons and 108 rats assessed final Hb value (g/L). Compared with rats receiving $FeSO_4$ fortification, rats receiving NaFeEDTA fortification had higher Hb levels (MD=9.23; 95%CI=0.41 to 18.06; p-value=0.04) (Figure 2.5) with moderate heterogeneity (I^2=55%) (Figure 2.5). The pooled MD and CI were not significantly changed after individual studies were successively swept in sensitivity analysis (Figure 2.6). No sig-

nificant publication bias was investigated by the Egger' test and the symmetrical funnel plot (Figure 2.7).

2.4.3.2 Ferrous fumarate fortification versus $FeSO_4$ fortification

3 studies including 4 comparisons and 56 rats assessed final Hb value (g/L). Compared with rats treated by $FeSO_4$ fortification, no significant difference in the overall effect was found among rats treated by ferrous fumarate fortification (MD=-0.25; 95%CI=-9.41 to 8.91; p-value=0.96) (Figure 2.8). A high heterogeneity was found (I^2=78%) (Figure 2.8). The pooled MD and CI were not significantly changed after individual studies were successively swept in sensitivity analysis (Figure 2.9). No significant publication bias was investigated by the Egger' test and the symmetrical funnel plot (Figure 2.10).

2.4.3.3 FePP fortification versus $FeSO_4$ fortification

5 studies including 16 comparisons and 248 rats assessed Hb gain (g/dL). Compared with rats consuming $FeSO_4$ fortification, rats consuming FePP fortification had lower Hb gain (MD=-0.92; 95%CI =-1.31 to -0.54; p-value<0.001) with a high heterogeneity (I^2=96%) (Figure 2.11). The pooled MD and CI were not significantly changed after individual studies were successively swept in sensitivity analysis (Figure 2.12). Three characteristic factors, particle size (regular or micronized), iron concentration (10-29 or 30-49 mg Fe/kg food), and intervention model (depletion-repletion model or prophylactic-preventative model) were analyzed with meta-regression. No influencing covariate of high heterogeneity source were found. There is no significant difference in the subtotal Hb effect between micronize FePP and $FeSO_4$, high iron concentration of FePP (30-49 mg Fe/kg food) and $FeSO_4$, and FePP and $FeSO_4$ in prophylactic-preventative model. The rest of subgroup analysis results showed that the subtotal Hb effects of FePP were significantly lower than those of $FeSO_4$. All subgroups had high heterogeneities (Figure 2.13, Figure 2.14, Figure 2.15). No significant publication bias was investigated by the Egger' test and the symmetrical funnel plot (Figure 2.16).

2.4.3.4 $FePO_4$ fortification versus $FeSO_4$ fortification

6 studies including 23 comparisons and 447 rats assessed Hb gain (g/dL). Compared with rats receiving $FeSO_4$ fortification, rats receiving $FePO_4$ fortification had lower Hb gain (MD=-1.85; 95% CI =-2.61 to -1.1; p-value<0.001) with a high heterogeneity (I^2=98%) (Fig-

ure 2.17). The pooled MD and CI were not significantly changed after individual studies were successively swept in sensitivity analysis (Figure 2.18). Three characteristic factors, particle size (regular or nano-particle), iron concentration (10-19, 20-29 or 30-39 mg Fe/kg food), and intervention model (depletion-repletion model or prophylactic-preventative model) were analyzed with meta-regression. Intervention model was an important covariate, while particle size and iron concentration were not important covariate. All subgroup analyses showed that the subtotal Hb effects of $FePO_4$ were lower than those of $FeSO_4$, while having high heterogeneities (Figure 2.19, Figure 2.20, Figure 2.21). The significant publication bias was investigated by the Egger' test and the asymmetrical funnel plot (Figure 2.22).

2.4.4 Certainty of evidence

The certainties of the NaFeEDTA, ferrous fumarate, and FePP meta-analysis evidence on Hb were evaluated as moderate level by the GRADE tool due to moderate or high heterogeneity. However, the certainty of FePP meta-analysis evidence on Hb was downgraded to low level due to high heterogeneity and significant publication bias (Table 2.15).

2.5 Discussion

2.5.1 Characteristics and quality assessments of included studies

The grain-based diets are usually chosen as the major food matrix of iron fortification. Their health benefits are partially ascribed to high levels of nature or fortified bio-actives. Although the high concentrations of inhibitors in grains such as polyphenols and phytic acid can reduce Fe bioavailability, the inhibitors can be almost degraded by some mature food processing technologies such as extrusion and precooking. Thus, grain-based food products with better nutritional profiles are commercially used not only as human staple food, but also as animal feed.[87–89]

Meanwhile, the significant influence on the absorption of iron fortificants has been demonstrated with the use of small particle size iron fortificants such as micronize-size and nano-size. It is because they have a larger surface area which can assist the iron fortificants to be more easily hydrolyzed in gastrointestinal tract. However, the toxicity of small-sized parti-

cles is a serious concern. For example, nano size can be readily translocated into body tissue, leading to excessive nonhematological iron uptake, agglomeration, and oxidative stress.[90–92]

In animals, SD rats are used as common animal subjects because of its calm temperament, low incidence of spontaneous tumors, and easy handling. Two animal models are often used: depletion-repletion model for disease treatment and preventative-prophylactic model for disease prevention.[48;49] In the present systemic review, the depletion-repletion rat model was chosen by a great number of laboratory technicians, implying that it had more appealing features for their research. Iron bioavailability studies using normal mouse models are rarely conducted because they are poor iron absorbers, while genetically modified mouse model is sometimes used to study iron disorder.[93] No large animal or non-human primate models on targeted iron fortificants were found.

In quality assessment table, blinding method was not used in preclinical studies. Probably because relatively small teams are often involved, one operator is usually responsible for intervention allocations, outcome assessments and data analyses. It might be the source of performance and detection biases. More than one third of the studies did not report two methods of reducing bias, dose-response relationship investigation or explanation of iron fortification levels, and randomized design. The former might prevent the proper application of research findings. The later led to selection biases, which might reduce the reproducibility and effectiveness of animal experiments. The attritions and the negative results might not be interpreted in a small number of studies. That was a cause of the publication bias and the reporting bias.[78;94;95] In addition, the sample sizes of some rat studies were too small. This increased the possibilities of statistical error and decreased the extrapolated value of results. No large-size studies were found.

2.5.2 Effects of iron fortifications, pairwise meta-analysis results, and Certainty of evidence

As illustrated in the table of iron fortification effects (Table 2.13), it was noted that NaFeEDTA had the largest amount of non-inferiority effect, while $FePO_4$ had the least amount. The results of the pairwise meta-analysis were consistent with the above findings. Although the equivalent effect occupied a dominant position in NaFeEDTA studies, the

overall effect (cumulative effect) of NaFeEDTA was superior to that of $FeSO_4$. The overall effect of ferrous fumarate was equivalent to that of $FeSO_4$. In addition, the overall effects of FePP and $FePO_4$ were inferior to that of $FeSO_4$. The indirect evidence of effect between these iron fortificants could not be estimated by the network meta-analysis due to the limitations in the number of studies.

Due to its excellent iron absorbability, NaFeEDTA, an iron-EDTA chelate is reviewed by the Joint WHO Expert Committee on Food Additives for government-recommended iron fortification strategies. EDTA chelates with ferric iron to form an iron-EDTA complex with the ferric ion located in the center by coordinate covalent bonds. EDTA can serve as a carrier to protect iron from the ligands of inhibitors in the stomach, thus allowing iron to be better released and absorbed in the duodenum.[62;96;97] In a SD rat experiment, the bioavailability of NaFeEDTA in bread was tested. NaFeEDTA was significantly more effective in Hb than $FeSO_4$.[98]

Ferrous fumarate is a ferrous complex. It benefits in the acid surroundings of the stomach and then can be absorbed in the more alkaline environment of the duodenum. Thus, it has a good solubility and absorbability. Moreover, it was found that the encapsulation technology could enhance the iron absorption of ferrous fumarate in gastrointestinal tract.[99;100] In the Caco-2 cell experiment conducted by Wortley et al., encapsulated ferrous fumarate in wheat-cereal vehicle had the higher ferritin response than $FeSO_4$.[101]

Although FePP and $FePO_4$, as two types of water-insoluble iron compounds discussed in this systemic review, did not have good effect in iron absorption, the nano-particle techniques including wet chemical methods, dry physical methods, and microbiological processes might improve iron bioavailability. Rohner et al. reported that the specific surface area of the regular size $FePO_4$, larger nano size $FePO_4$ (30.5 nm), and small nano size $FePO_4$ (10.7 nm) were 32.6, 68.6, 194.7 m^2/g, respectively. Their solubilities were 73%, 79%, and 85% of $FeSO_4$, respectively. The relative bioavailability value in rats were 61%, 70%, and 96% of $FeSO_4$, respectively. No toxicity was found in rats.[102]

However, as shown in the pairwise meta-analysis plots, high heterogeneities dominated in the inter-study variations. Similarly, in a clinical systemic review by Sabitova et al., all I^2

of their meta-analysis exceeded 95%.[103] High heterogeneity is therefore a common methodological limitation that posed interpretive challenges. Sensitivity analysis can determine whether the overall effect of included comparisons is robust based on the characteristics of studies, when the decisions are made to include them.[76] In this review, no comparisons showed that their inclusion and exclusion would significantly influence the overall effect. $FeSO_4$ meta-regression revealed that the intervention model was an influencing covariate for heterogeneity. However, even if the only one comparison using preventative-prophylactic model was separated from the rest of 23 comparisons using depletion-repletion model in further subgroup analysis, the heterogeneity and subtotal Hb effect of 23 depletion-repletion comparisons were not significantly changed. Thus, there was a weak association between intervention model and heterogeneity derived from this $FePO_4$ meta-regression, since too few preventative-prophylactic comparison was involved in.[104–106] Similarly, although FePP subgroup analyses suggested that FePP was equivalence to $FeSO_4$ in 2 prophylactic-preventative models and lower than $FeSO_4$ in 14 depletion-repletion models, it was also an underpowered correlation due to the small number of prophylactic-preventative comparisons.[106] In addition, FePP subgroup analysis also demonstrated that micronize FePP and high FePP concentration (30-49 mg Fe/kg food) were equivalent to $FePO_4$ in subtotal Hb effect. It implied that they had good iron bioavailability in rat studies.

The significant publication bias found in the results of pairwise meta-analysis indicated that the studies with positive findings were more likely to be published than the studies with negative findings. Additionally, the funnel plots had some outliers that were perhaps sources for the high heterogeneity as well. In our systemic review, the moderate and high heterogeneities might be primarily due to study design, including differences in animal strains, food matrix, particle size, doses of interventions, food processing strategies, randomizations, intervention durations, measurement methods, instruments, statistical covariate adjustments, etc. Thus, based on the results of GRADE assessments, we had moderate confidence in the overall effects of NaFeEDTA, ferrous fumarate, and FePP meta-analysis. However, there was a limited confidence in the overall effect of $FePO_4$ meta-analysis. Thus, conditional recommendations on the preclinical and clinical applications could be provided.[107] New preclinical

studies will be included to update the meta-analysis results on common iron fortifications every 3-5 years.

2.5.3 Relevant Mechanisms

2.5.3.1 Molecular basis of iron digestion and absorption

The gastrointestinal tract is the main locus of iron homeostatic regulation, which is accomplished by a plenty of well-unknown and unknown molecular mechanisms in response to alterations of cellular iron absorption and storage. Micheletto et al. stated that the intestinal absorption and ferritin expression of Ferro Fosfosoma (composed of FePP, phospholipid and modified starch) were higher than those of conventional FePP in both the Caco-2 model and the human follicle-associated intestinal epithelium model.[108] Perfecto et al. operated a well-designed mRNA-knockdown of DMT1 in Hutu-80 and Caco-2 cells. In both DMT1-knockdown cell lines treated with digested ferric ammonium citrate and nano-particle $FePO_4$, the reduced ferritin levels were discovered.[109] Laparra et al. examined the gene expressions of DMT1, DCYTB, and ferritin levels in a Caco-2/HT-29 co-culture cell model. Cells were treated with ferric fortificant and ascorbic acid at 50 μmol/L for 24 hours (sufficient iron uptake). The iron treatment significantly improved the gene expressions of DMT1, DCYTB and ferritin levels.[110] However, in another Caco-2 cell model, cells were treated by ferric nitrilotriacetic acid at 200 μmol/L for 72 hours (excessive iron uptake), the gene expressions of DMT1 and TfR were significantly decreased.[111] These observations were consistent with the fact that the iron regulatory genes and proteins played a crucial role in iron homeostasis. This regulation can not only ameliorate the decreased efficiency of iron absorption in the gastrointestinal track, but also protect the enterocyte from iron overload and toxicity.

2.5.3.2 Cellular iron regulation (IRE-IRP system)

Currently, two important themes on the iron regulation have been identified as the promising small-molecule medicine that may cure refractory iron deficiency anemia. The first is cellular iron regulation. The main regulator is iron regulatory proteins (IRPs). It can be divided into two isoforms, IRP 4Fe-S and IRP 3Fe-S. The former can be used for tricarboxylic acid (TCA) cycle. The later can bind to iron response elements (IREs), which are multiple stem-loop structures (30 nucleotides) located in specific untranslated mRNA re-

gions of proteins, including ferritin, transferrin receptors, DCYTB, ferroportin, and DMT1. IRE-IRP complex can alter the transcriptions of proteins. For example, when the level of cellular iron is low, ferritin can be increased by IRP 3Fe-S binding to IRE of ferritin gene. When the cellular iron is overloaded, IRP 3Fe-S binds to IRE of TfR gene, thus, preventing diferric-transferrin complexes from entering cell (Figure 2.23).[67–72]

The cellular high-iron conditions can repress the DMT1 transcriptions, while facilitating the ferroportin transcriptions, which is achieved by IRPs binding to the IRE motifs within the specific regions of DMT1 and ferritin mRNAs. Some preclinical studies probed into the correlation between DMT1, ferroportin and IRP-IRE system. For example, in a IRPs-deficient mice study, mice with low IRP expression had low iron uptake capacity since the DMT1 expression was down-regulated.[112] Similarly, the iron perturbations caused by DMT1 and ferroportin expressions were assessed in Caco-2 cells treated with desferrioxamine. Both DMT1 and ferroportin mRNA expressions were improved. However, the opposite also worked after iron treatment.[113] Thus,intracellular iron availability can be regulated by the IRP-IRE system, affecting the transcriptions of DMT1 and ferroportin, acting as a feed-back loop between cellular iron conditions and transmembrane iron transportations.

2.5.3.3 Body iron regulation (Hepcidin-Ferroportin interaction)

The second is body iron regulation. The main regulator of iron homeostasis at systemic level is hepcidin, a 25 amino acids peptide hormone produced by the liver in response to the high content of plasma iron. Hepcidin can restrict the iron delivery by interacting with ferroportin on the cell membrane, inducing its degradations and internalizations, so that ferrous iron cannot be transported from cell to blood. Hepcidin synthesis is regulated through TfR2/MAPK and SMAD/BMP pathways. When the iron is replete in the body, transferrin-$2Fe^{3+}$ binds to TfR1. The hemochromatosis protein (HFE) released from TfR1 can attach to TfR2. HFE-TfR2 complex can activate the mitogen-activated protein kinase (MAPK) signaling pathway. Meanwhile, bone morphogenic protein (BMP) 6 secreted from liver cells binds to hemojuvelin (HJV). The BMP6-HJV complex can trigger the phosphorylation of small mothers against decapentaplegic (SMAD) proteins, and activate SMAD signal pathway. Intracellular MAPK and SMAD signaling cascades enhance hepcidin tran-

scriptions by interacting with response elements in the promoter region of hepcidin mRNA. In contrast, within low plasma iron condition, TfR1 remains binding to HFE and prevents it from attaching to TfR2. In the meantime, less BMP6 is released from the liver. Then, the MAPK and SMAD signaling pathways are inactive and hepcidin transcription is inhibited (Figure 2.23).[67–72]

Han conducted a SD rat experiment to investigate the effect of rich iron diet and the hepcidin mRNA expression. They found that the levels of serum ferritin, transferrin saturation, serum iron, and hepcidin mRNA expression were increased, while the ferroprotin mRNA expression in duodenum was decreased.[114] Yeh et al. also examined the mRNA expressions of hepcidin and ferroportin in rat model. Rats were treated by Lipopolysaccharide (an iron chelator) or His-tagged recombinant hepcidin. Both treatments induced the increased hepcidin mRNA expressions and the decreased ferroportin mRNA expressions.[115] In a mice study, upstream stimulation factor 2 knockout reduced the hepcidin expression and iron overload level,[116] while over-expression of hepcidin mRNA in another mice model leaded to iron deficiency anemia.[117] Similarly, in normal mouse and rat models, iron overload increased hepcidin expression and iron deficiency decreased it. Also, poor dietary iron intake resulted in decreased hepcidin expression and increased ferroportin expression.[118–120] Taken together, there was a causal relationship between the hepcidin mRNA expressions, ferroportin mRNA expression and plasma iron level.

2.6 Conclusion

Based on the meta-analysis results, the evidence suggested that the overall effect of NaFeEDTA was superior that of $FeSO_4$. Ferrous fumarate was equivalent to $FeSO_4$, and FePP and $FePO_4$ were inferior to $FeSO_4$. The research questions raised in this systemic review could be partially answered. In the rat models, NaFeEDTA and ferrous fumarate could ameliorate iron-deficient anemia. In the cell models, we could put forward a reliable hypothesis that NaFeEDTA and ferrous fumarate might improve the cellular capacity of iron absorption. Iron absorption was bidirectionally regulated by the IRP-IRE system and Hepcidin-Ferroportin

interaction. However, the evidence of meta-analysis results should be prudently applied as conditional recommendations due to the moderate and high heterogeneities, unsolvable biases, limited information, and insufficient data. Nevertheless, this preclinical systemic review remains an important cornerstone of the iron fortification research. More high-quality preclinical studies and clinical trials are needed to elevate the evidence level of the meta-analysis results, optimize the existing conclusions, examine the safety and efficacy of iron fortifications, develop the theories pragmatically, explore new areas of the iron bioavailability mechanisms, and open innovative applications in the prevention, diagnosis, and therapy on iron disorder.

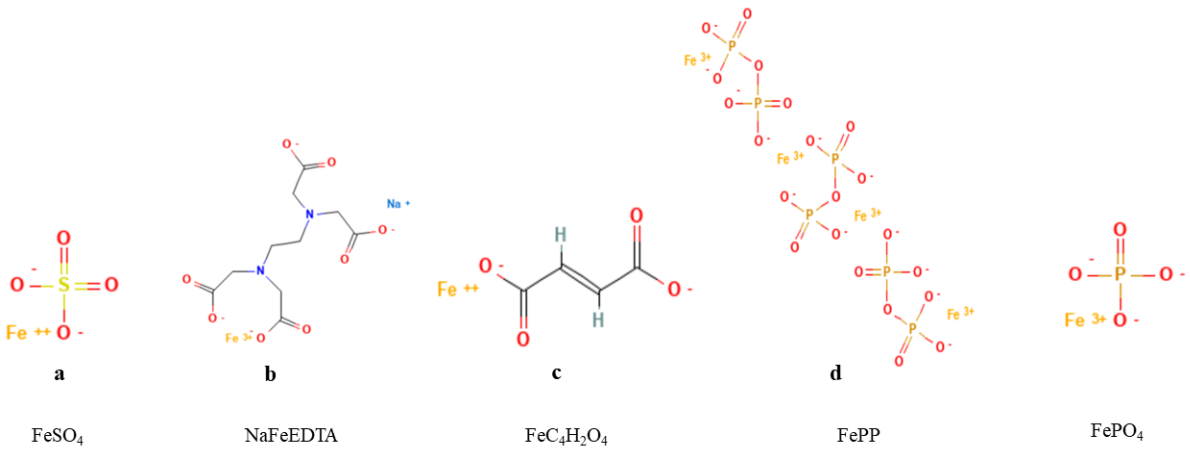


Figure 2.1: Chemical structures of iron fortificants from PubChem

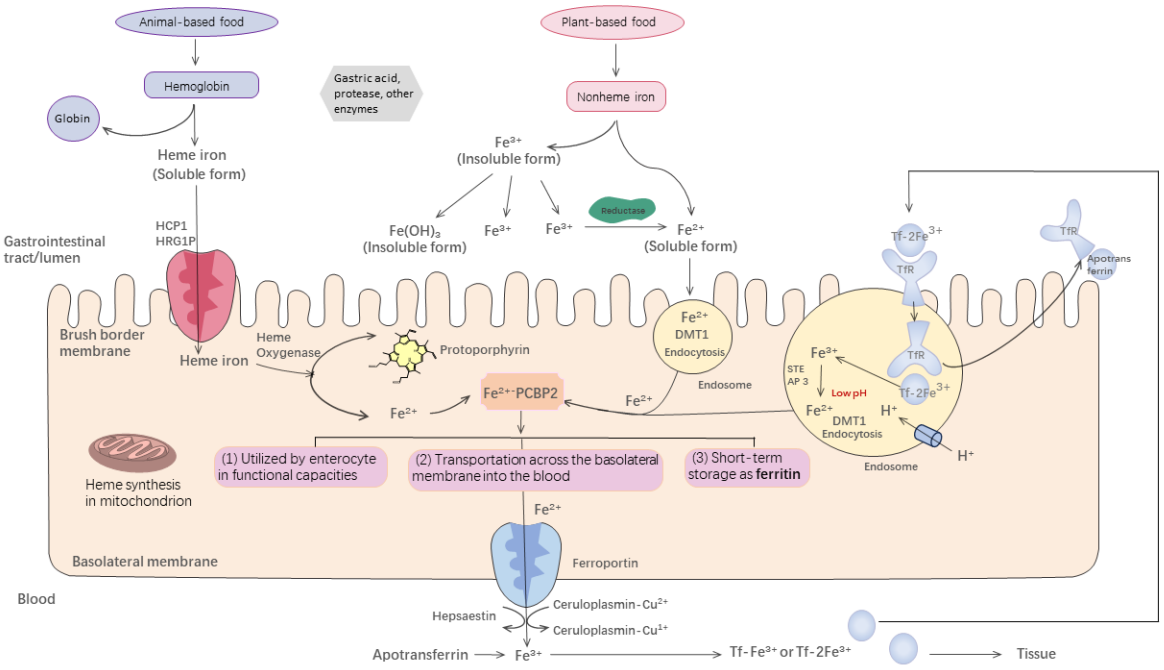


Figure 2.2: Mechanism of iron digestion, absorption, and transport

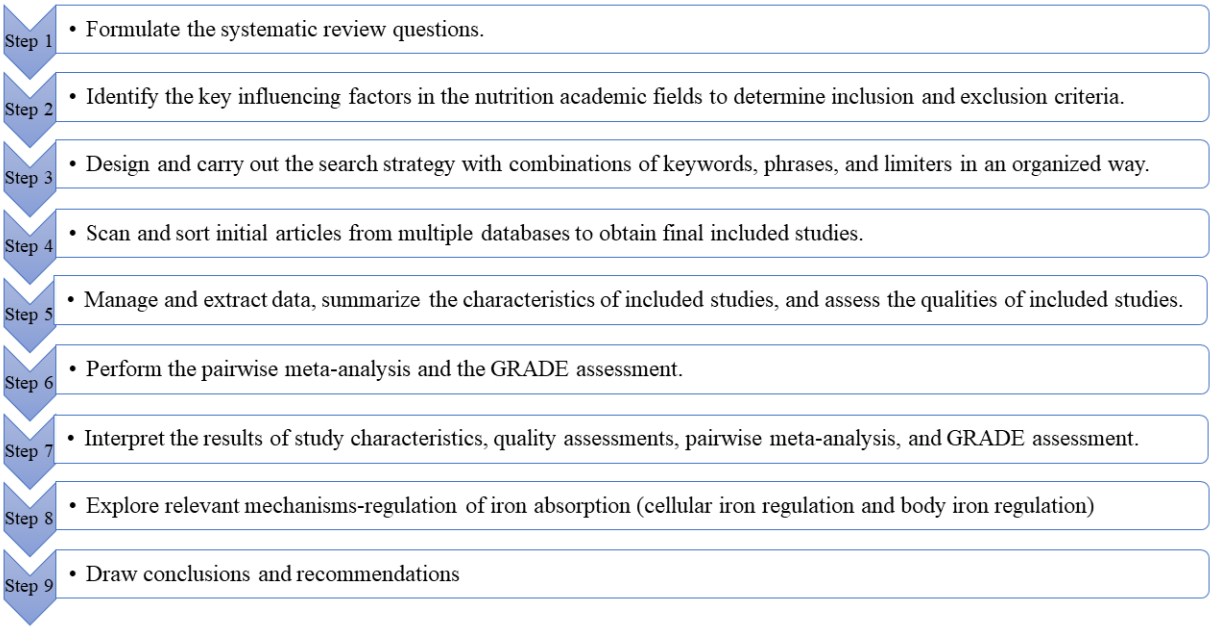


Figure 2.3: Flow diagram of steps in the preclinical systematic review process

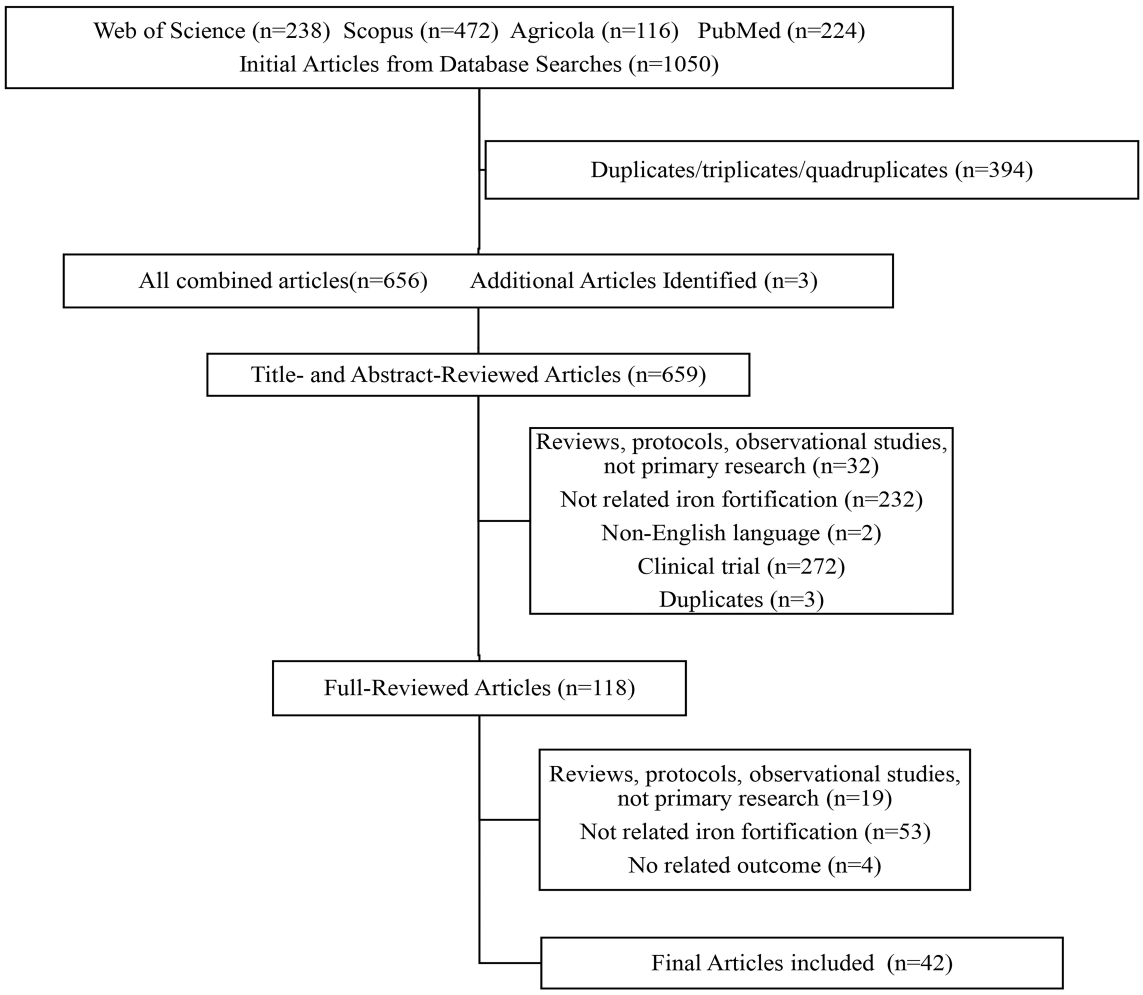


Figure 2.4: Flow diagram of scanning and sorting studies

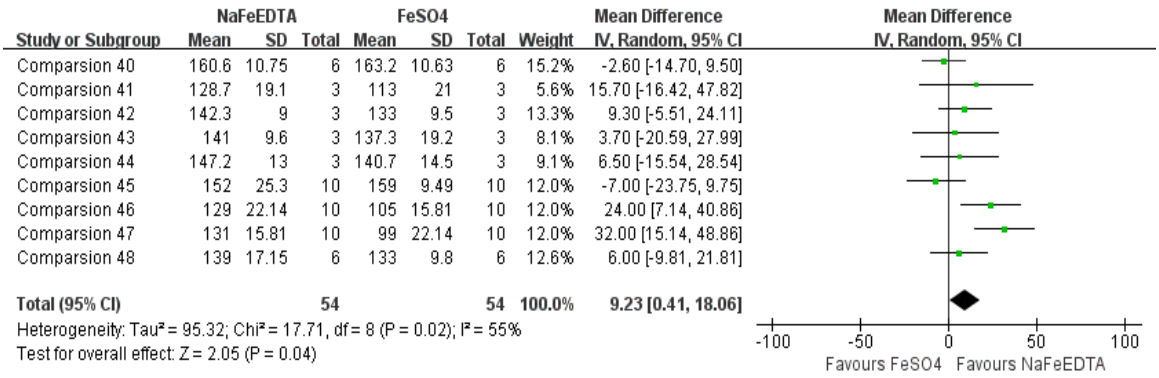


Figure 2.5: NaFeEDTA versus $FeSO_4$ forest plot

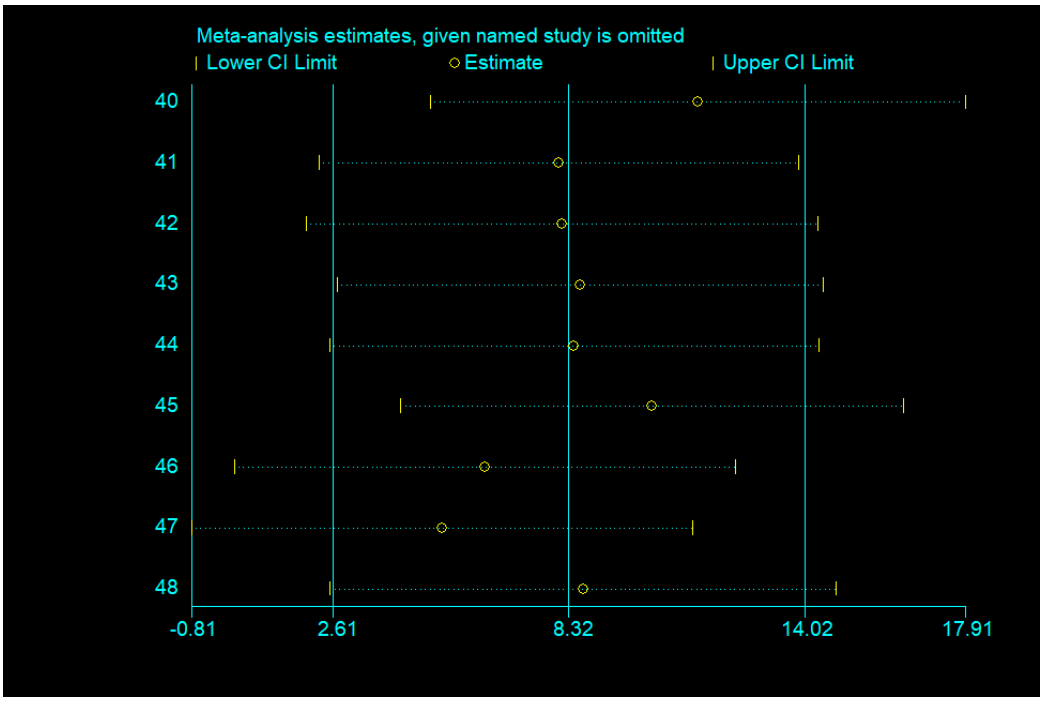


Figure 2.6: NaFeEDTA versus $FeSO_4$ sensitivity analysis plot

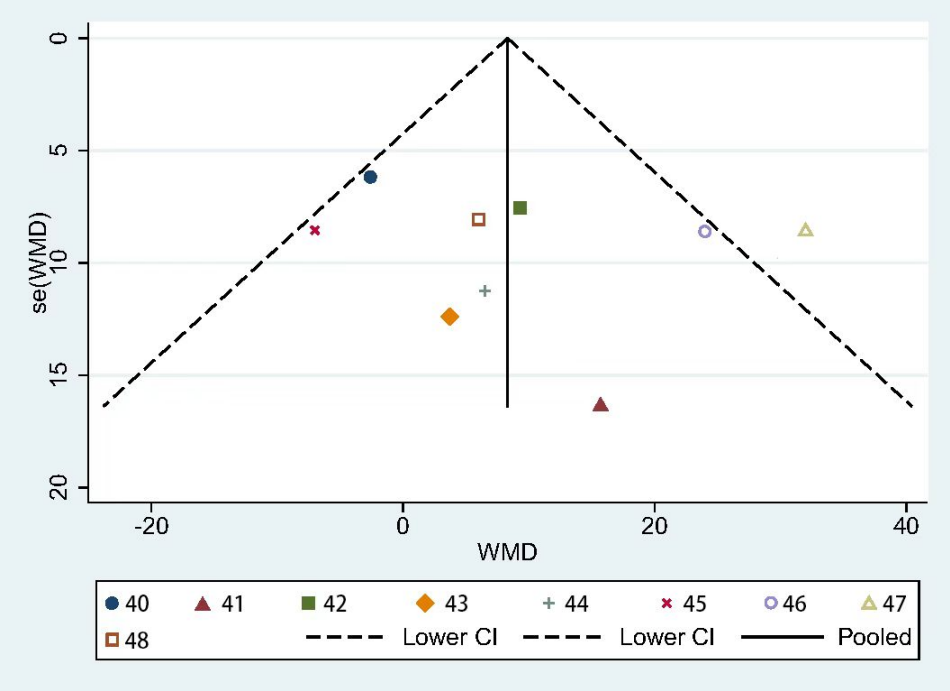


Figure 2.7: NaFeEDTA versus $FeSO_4$ funnel plot

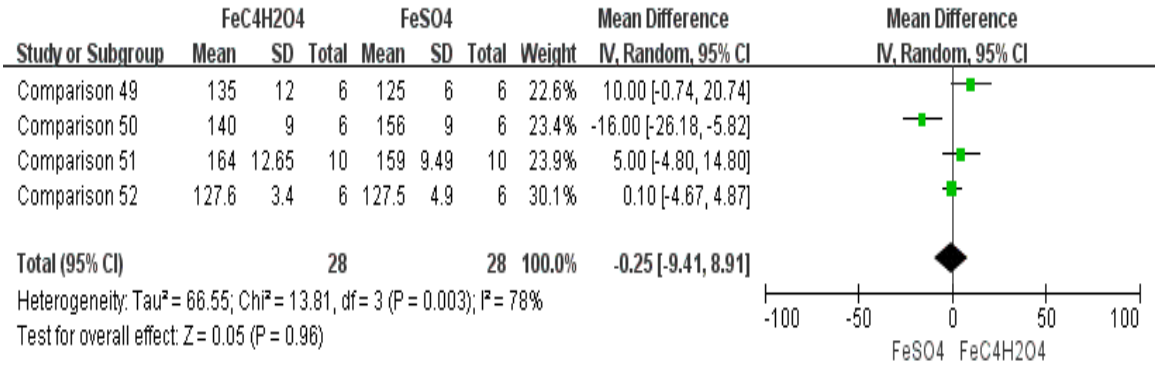


Figure 2.8: Ferrous fumarate versus $FeSO_4$ forest plot

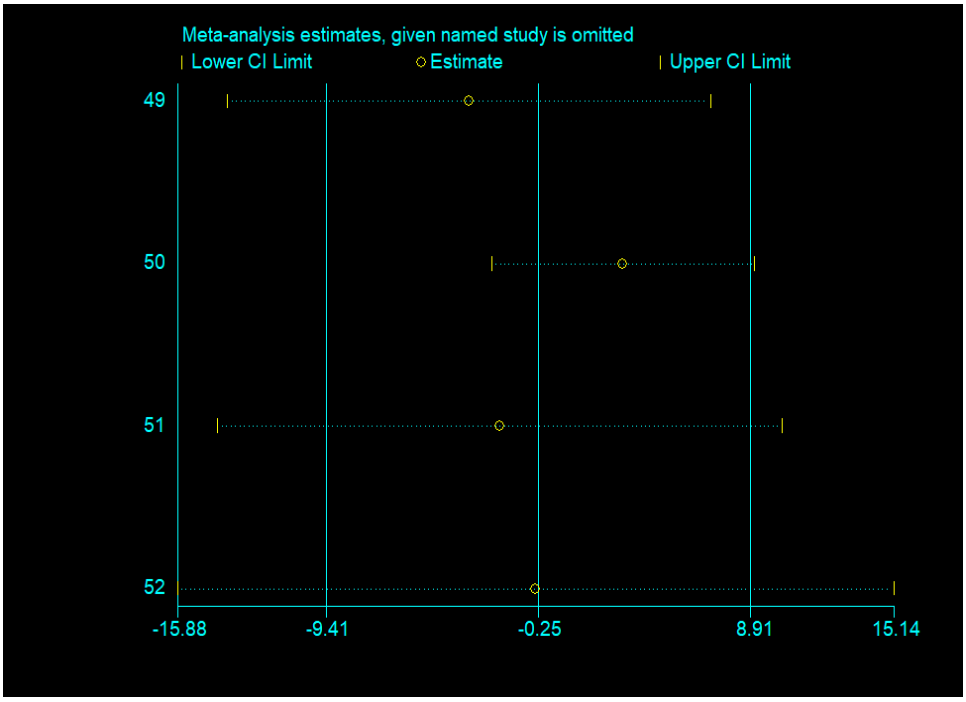


Figure 2.9: Ferrous fumarate versus $FeSO_4$ sensitivity analysis plot

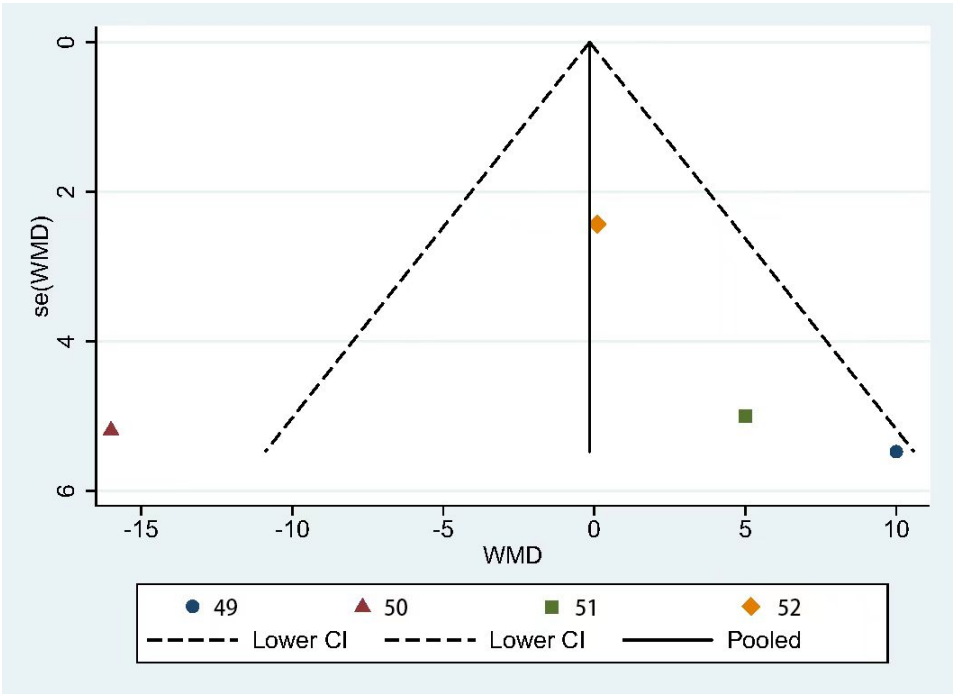


Figure 2.10: Ferrous fumarate versus $FeSO_4$ funnel plot

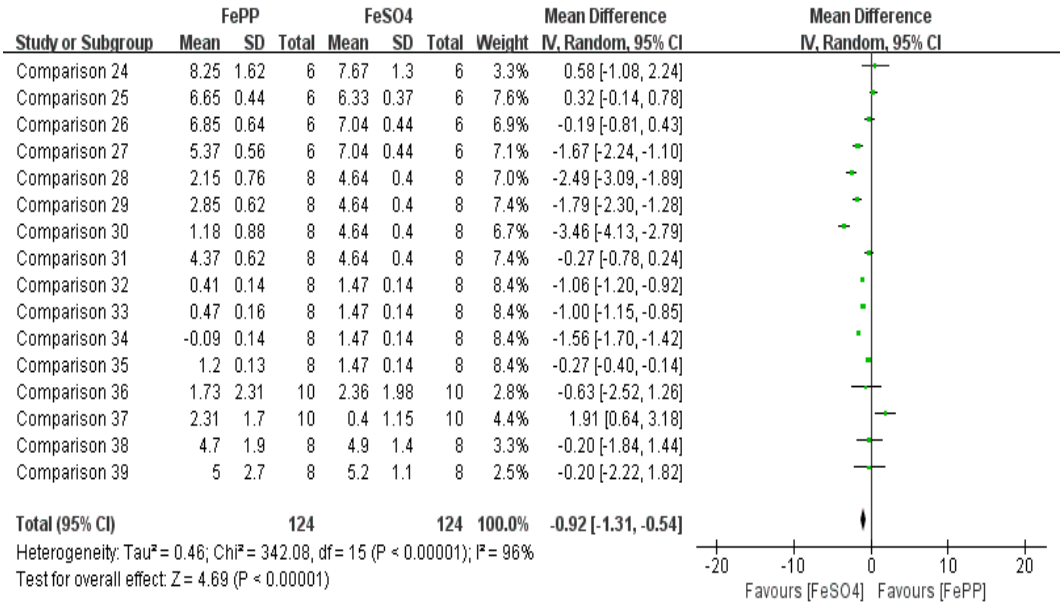


Figure 2.11: FePP versus $FeSO_4$ forest plot

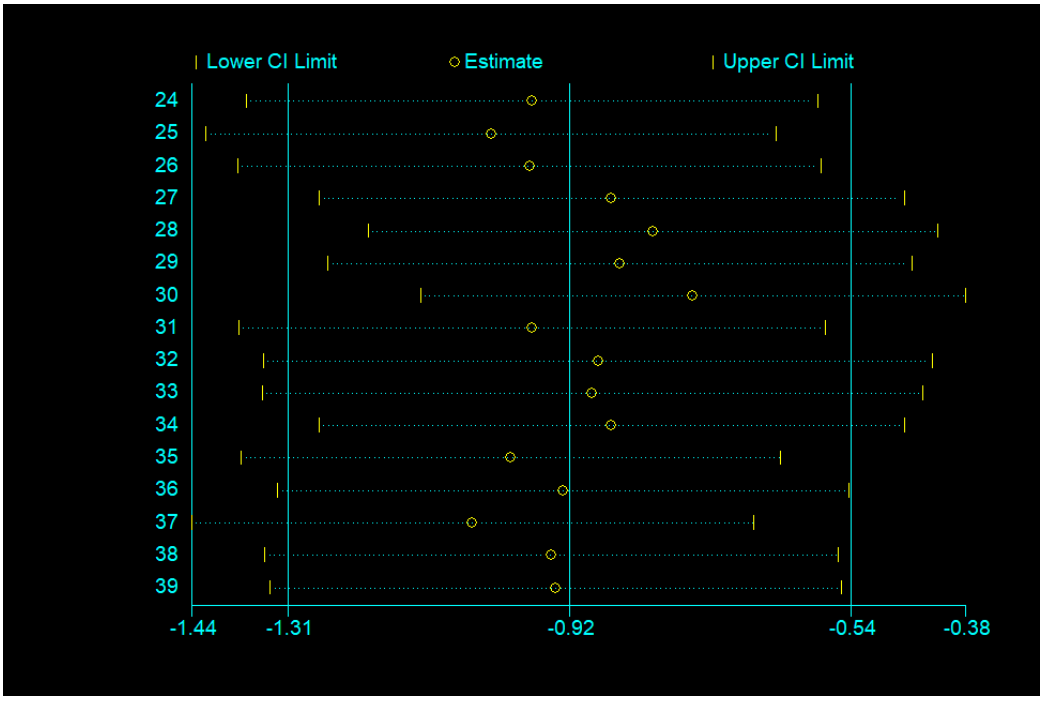


Figure 2.12: FePP versus $FeSO_4$ sensitivity analysis plot

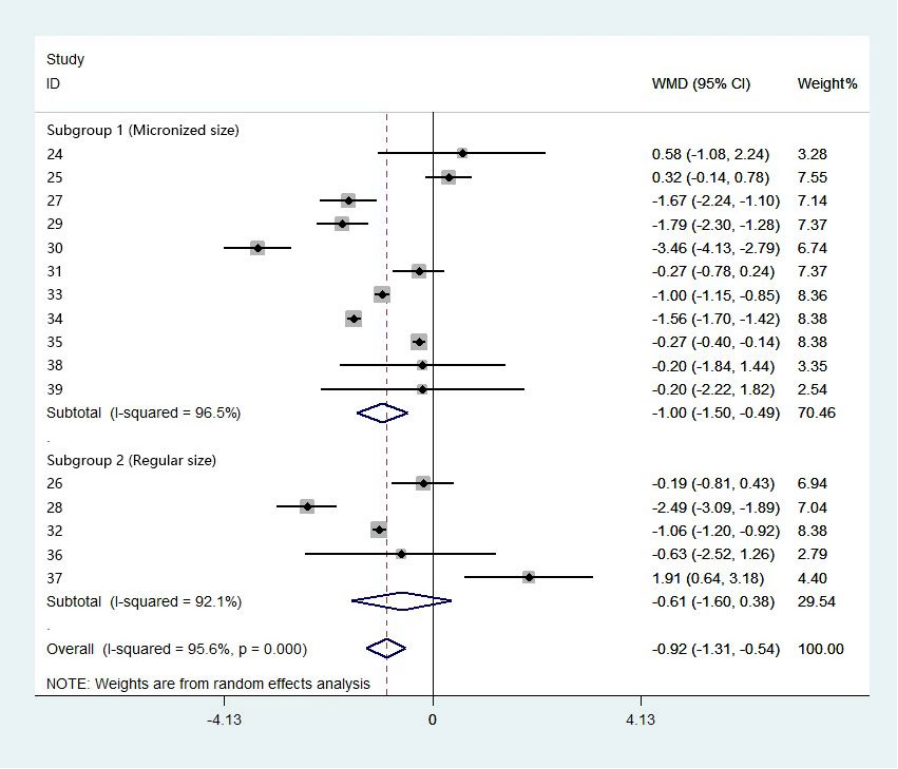


Figure 2.13: FePP versus $FeSO_4$ subgroup (particle size) forest plot

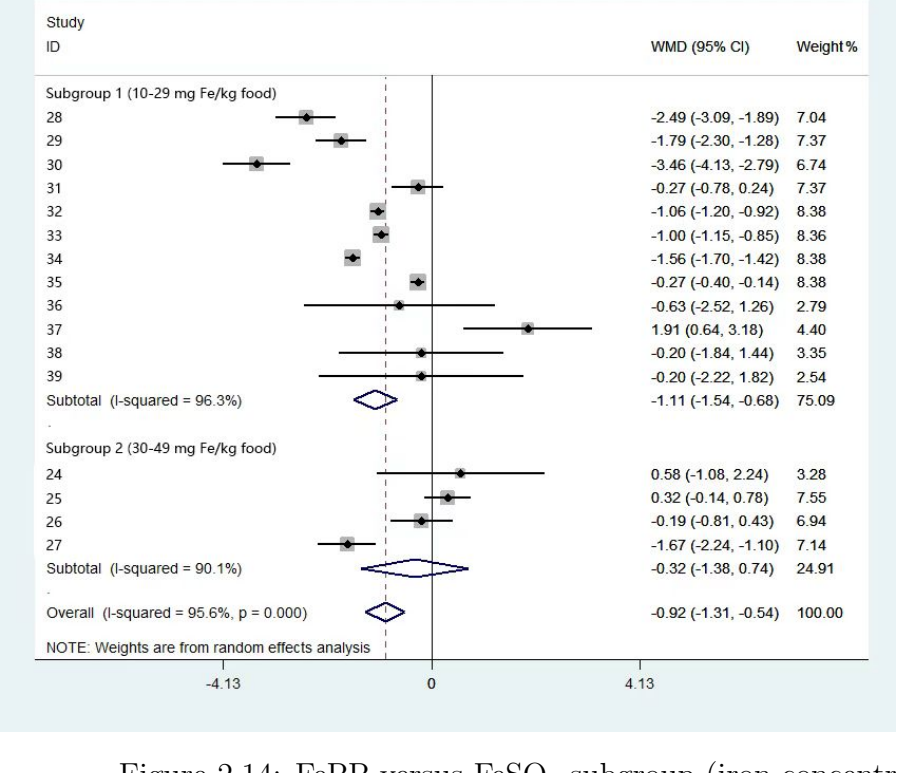


Figure 2.14: FePP versus $FeSO_4$ subgroup (iron concentration) forest plot

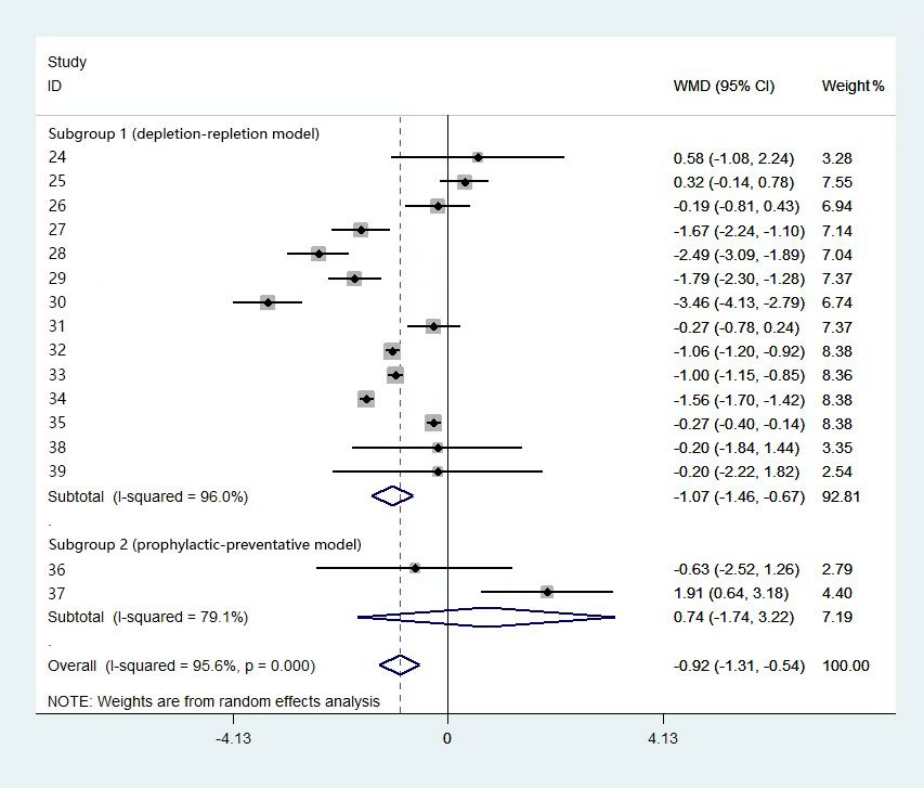


Figure 2.15: FePP versus $FeSO_4$ subgroup (intervention model) forest plot

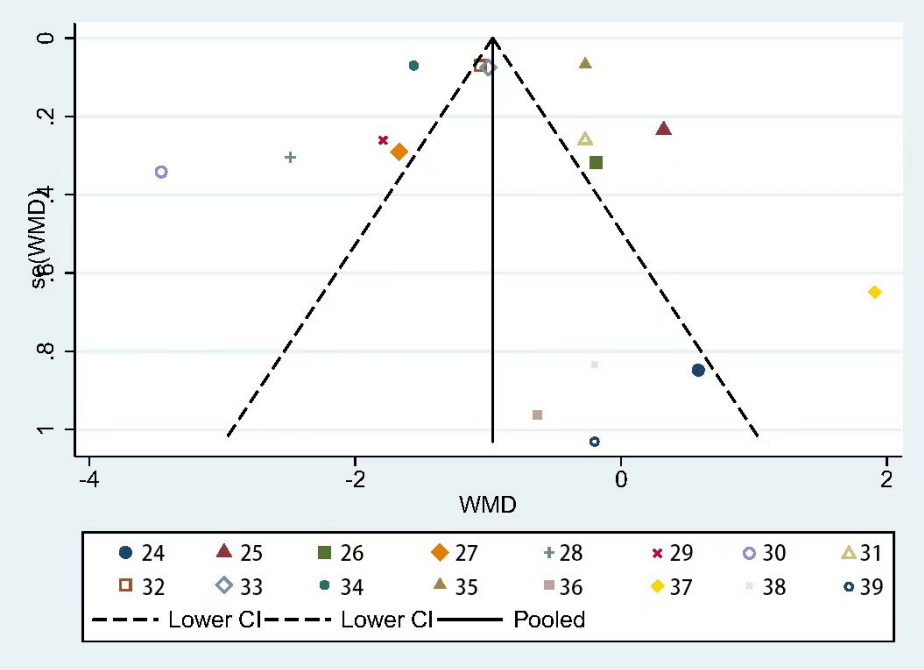


Figure 2.16: FePP versus $FeSO_4$ funnel plot

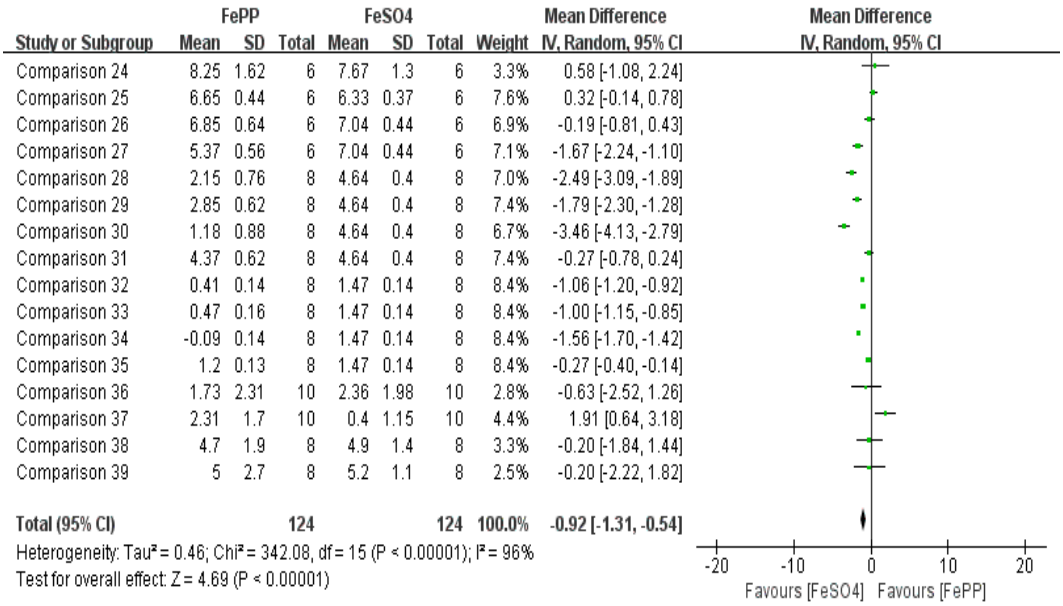


Figure 2.17: $FePO_4$ versus $FeSO_4$ forest plot

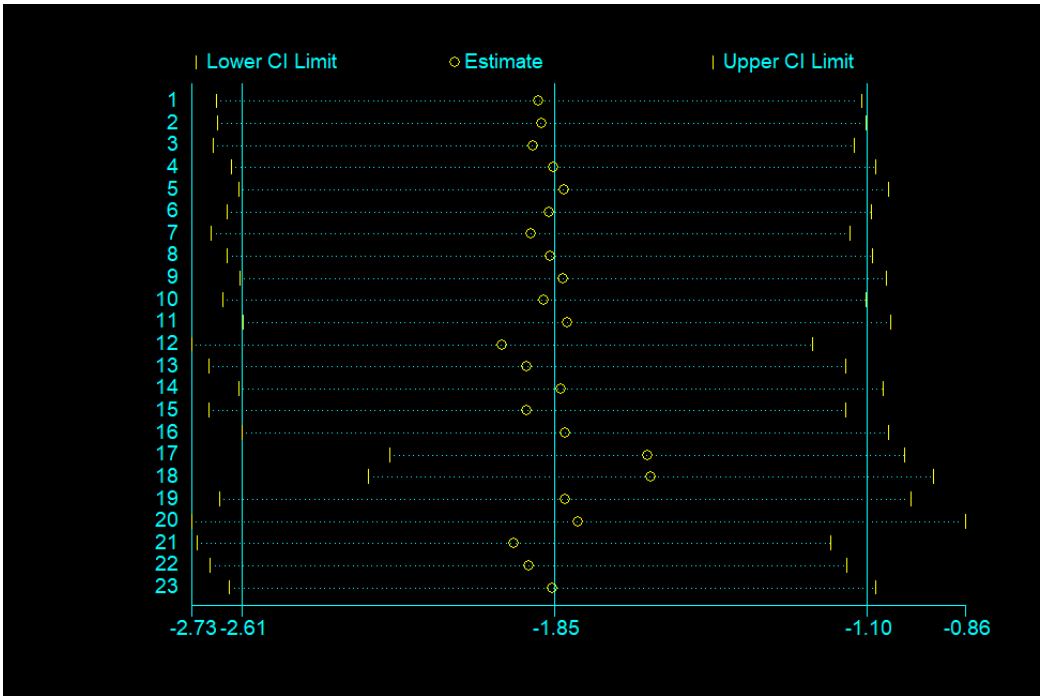


Figure 2.18: $FePO_4$ versus $FeSO_4$ sensitivity analysis plot

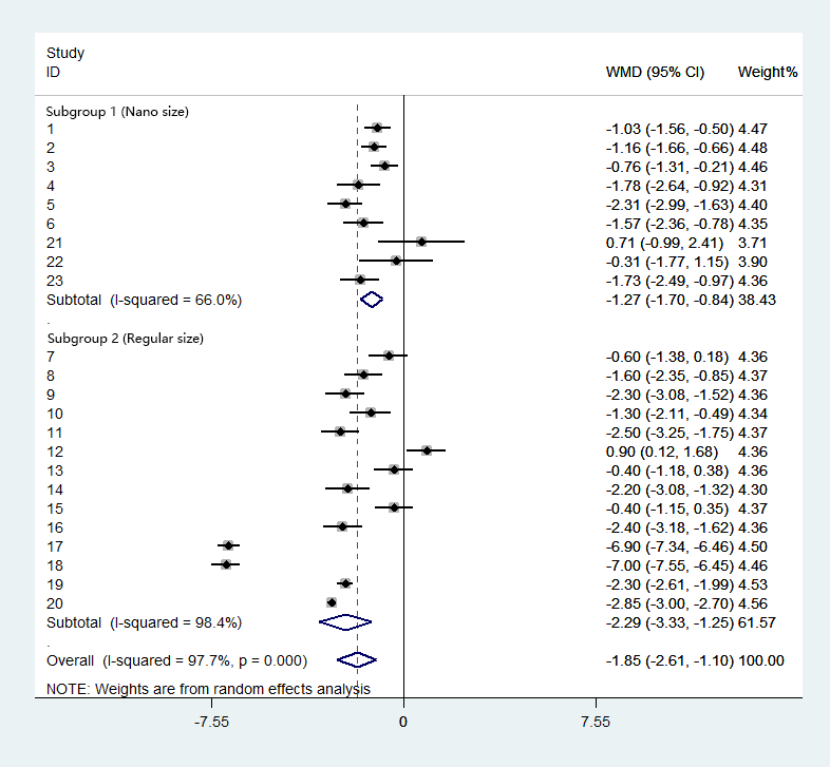


Figure 2.19: $FePO_4$ versus $FeSO_4$ subgroup (particle size) forest plot

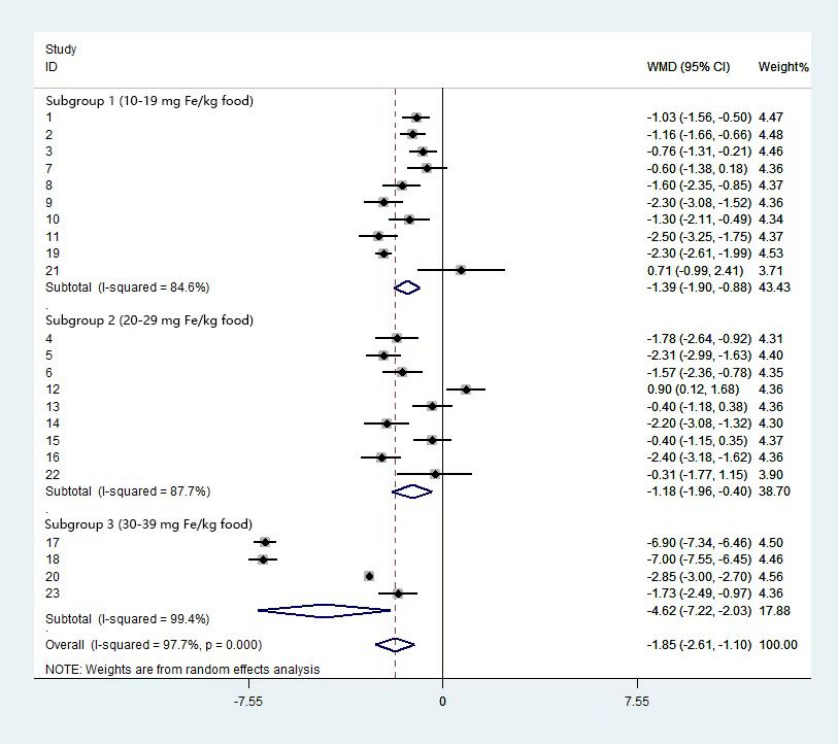


Figure 2.20: $FePO_4$ versus $FeSO_4$ subgroup (iron concentration) forest plot

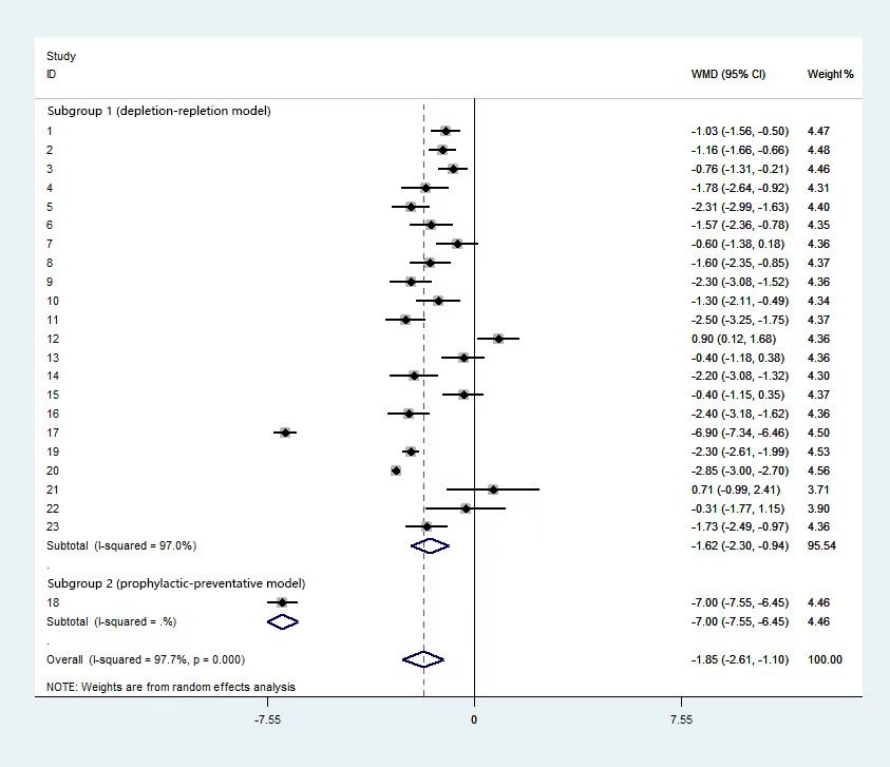


Figure 2.21: $FePO_4$ versus $FeSO_4$ subgroup (intervention model) forest plot

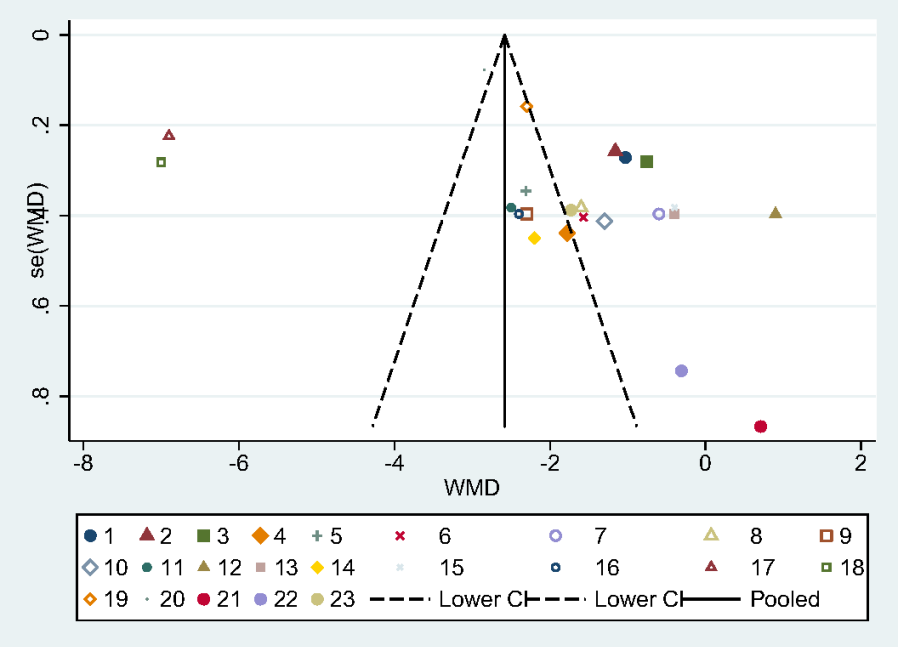
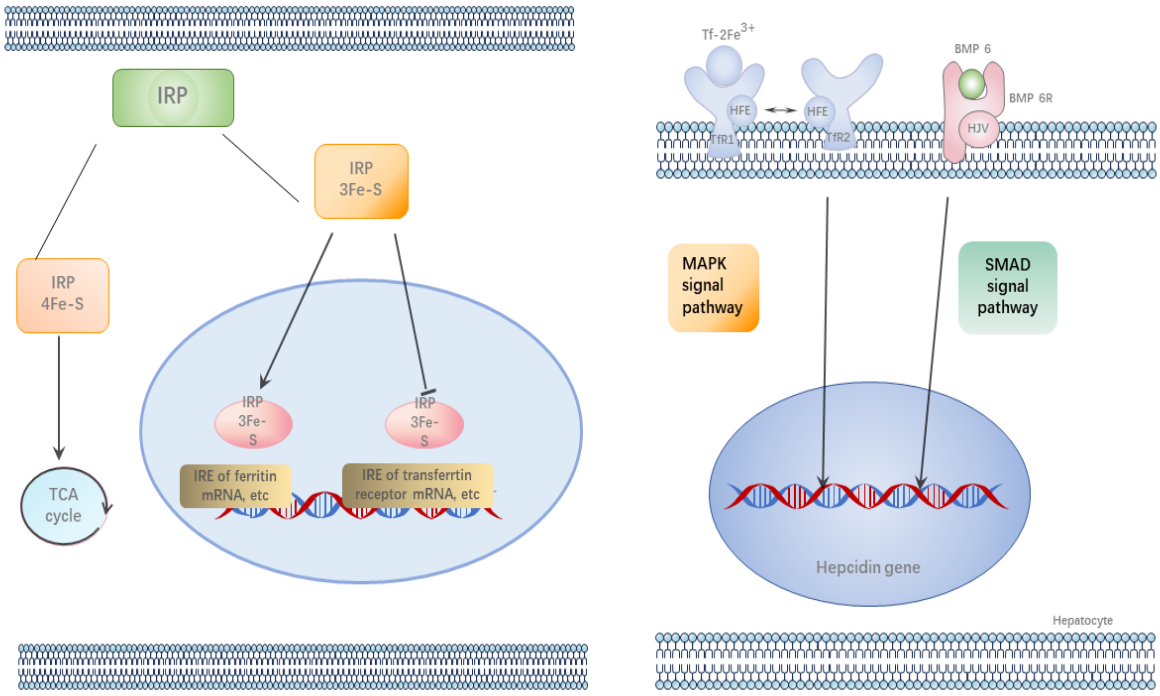


Figure 2.22: $FePO_4$ versus $FeSO_4$ funnel plot



a IRE-IRP system

b Hepcidin-Ferroportin interaction

Note: " ⟶ " represents "promote" or "activate". " ⊣ " represents "inibit" or "inactivate".

Figure 2.23: Regulation mechanisms of iron absorption

Table 2.1: *Characteristics of $FePO_4$ rat studies included in meta-analysis*

Study ID	**Author, publication year**	**Species**	**Sample size**	**Intervention Model**	**Intervention duration (day)**	**Food matrix**	**Iron type**	**Particle size**	**Iron concentration (Fe mg/kg food)**
1	Rohner 2006	Male SD rats	9	Depletion-repletion	Depletion: Unknown Repletion: 15	AIN-93G	$FePO_4$	Nanoparticle (64 nm)	10
							$FePO_4$	Nanoparticle (31 nm)	10
							$FePO_4$	Nanoparticle (11 nm)	10
							$FePO_4$	Nanoparticle (64 nm)	20
							$FePO_4$	Nanoparticle (31 nm)	20
							$FePO_4$	Nanoparticle (11 nm)	20
2	Dickmann 2016	Male SD rats	10	Depletion-repletion	Depletion: 15 Repletion: 21	AIN-76A	$FePO_4$ (Source No.1)	Regular	12
							$FePO_4$ (Source No.2)	Regular	12
							$FePO_4$ (Source No.3)	Regular	12
							$FePO_4$ (Source No.4)	Regular	12
							$FePO_4$ (Source No.5)	Regular	12

Table 2.1: *Characteristics of $FePO_4$ rat studies included in meta-analysis(continued)*

Study ID	**Author, publication year**	**Species**	**Sample size**	**Intervention Model**	**Intervention duration (day)**	**Food matrix**	**Iron type**	**Particle size**	**Iron concentration (Fe mg/kg food)**
							$FePO_4$ (Source No.1)	Regular	24
							$FePO_4$ (Source No.2)	Regular	24
							$FePO_4$ (Source No.3)	Regular	24
							$FePO_4$ (Source No.4)	Regular	24
							$FePO_4$ (Source No.5)	Regular	24
3	Whittaker 1989	Male SD rats	10	Depletion-repletion	Depletion: 7 Repletion: 10	AIN-76A	$FePO_4$	Regular	35
							$FePO_4$ +ascorbic acid	Regular	35
4	Lysinek 2001a	Female SD rats	10	Prophylactic-preventative	Intervention: 23	Petit-Suisse Cheese	$FePO_4$	Regular	15
5	Benito 1997	Male Wistar rats	10	Depletion-repletion	Depletion: 13 Repletion: 11	AIN-76A	$FePO_4$	Regular	35

Table 2.1: *Characteristics of $FePO_4$ rat studies included in meta-analysis(continued)*

Study ID	**Author, publication year**	**Species**	**Sample size**	**Intervention Model**	**Intervention duration (day)**	**Food matrix**	**Iron type**	**Particle size**	**Iron concentration (Fe mg/kg food)**
6	Srinivasu 2015	Rat	8	Depletion-repletion	Depletion: 30 Repletion: 15	AIN-93G	$FePO_4$	Nanoparticle	10
							$FePO_4$	Nanoparticle	20
							$FePO_4$	Nanoparticle	30

Table 2.2: *Characteristics of FePP rat studies included in meta-analysis*

Study ID	**Author, publication year**	**Species**	**Sample size**	**Intervention Model**	**Intervention duration (day)**	**Food matrix**	**Iron type**	**Particle size**	**Iron concentration (Fe mg/kg food)**
7	Vicente 2008	Male SD rats	6	Depletion-repletion	Depletion: 28 Repletion: 21	Fruit juice	FePP	Micronized	46
						Fruit juice	FePP+ ascorbic acid	Micronized	46
8	Sakaguchi 2003	Male SD rats	6	Depletion-repletion	Depletion: 28 Repletion: 14	Corn starch	FePP	Micronized	35
							FePP	Regular	35
9	Wegmuller 2004	Male SD rats	8	Depletion-repletion	Depletion: 24 Repletion: 14	AIN-93G	FePP	Regular	20

Table 2.2: *Characteristics of FePP rat studies included in meta-analysis(continued)*

Study ID	**Author, publication year**	**Species**	**Sample size**	**Intervention Model**	**Intervention duration (day)**	**Food matrix**	**Iron type**	**Particle size**	**Iron concentration (Fe mg/ kg food)**
						AIN-93G	FePP	Micronized	20
						AIN-93G	FePP (Encapsulated)	Micronized	20
						AIN-93G	FePP	Small micronized (0.5μm)	20
						AIN-93G	FePP	Regular	10
						AIN-94G	FePP	Micronized	10
						AIN-95G	FePP (Encapsulated)	Micronized	10
						AIN-96G	FePP	Small micronized (0.5μm)	10
10	Salgueiro 2008	Male SD rats	10	Prophylactic-preventative	Intervention:21	AIN-93G	FePP	Regular	16.25
						Yogurt	FePP	Regular	15.4
11	Lucia 2013	Male Wistar rats	8	Depletion-repletion	Depletion: 21 Repletion: 14	AIN-93G	FePP	Micronized	12
						Yancon flour	FePP	Micronized	12

Table 2.3: *Characteristics of NaFeEDTA rat studies included in meta-analysis*

Study ID	**Author, publication year**	**Species**	**Sample size**	**Intervention Model**	**Intervention duration (day)**	**Food matrix**	**Iron type**	**Particle size**	**Iron concentration (Fe mg/kg food)**
12	Zhu 2006	Male SD rats	6	Depletion-repletion	Depletion: 7 Repletion: 12	AIN-93G	NaFeEDTA	Regular	35
13	Malika 2022	SD rats	3	Prophylactic-preventative	Intervention: 60	Wheat flour	NaFeEDTA	Regular	15
							NaFeEDTA	Regular	25
							NaFeEDTA	Regular	35
							NaFeEDTA	Regular	40
14	Ologunde 1992	Male SD rats	10	Depletion-repletion	Depletion: 7 Repletion: 14	AIN-76A	NaFeEDTA	Regular	35
3	Whittaker 1989	Male SD rats	10	Depletion-repletion	Depletion: 7 Repletion: 10	AIN-93G	NaFeEDTA	Regular	30
						Bread	NaFeEDTA	Regular	30
15	Olivira 1995	Male Wistar rats	6	Prophylactic-preventative	Intervention: 35	Basal diet	NaFeEDTA	Regular	20

Table 2.4: *Characteristics of ferrous fumarate rat studies included in meta-analysis*

Study ID	**Author, publication year**	**Species**	**Sample size**	**Intervention Model**	**Intervention duration (day)**	**Food matrix**	**Iron type**	**Particle size**	**Iron concentration (Fe mg/ kg food)**
16	Hernandez 2003	Male and female SD rats	6	Depletion-repletion	Depletion: 10 Repletion: 14	Wheat flour	Ferrous fumarate	Regular	56
						Corn flour	Ferrous fumarate	Regular	59
14	Ologunde 1992	Male SD rats	10	Depletion-repletion	Depletion: 7 Repletion: 14	AIN-76A	Ferrous fumarate	Regular	35
17	Suva 2022	Wistar rats	6	Depletion-repletion	Depletion: 5 Repletion: 15	Commercialdiet	Ferrous fumarate	Regular	35

Table 2.5: *Characteristics of animal studies that were not included in meta-analysis*

Study ID	**Author, publication year**	**Species**	**Sample size**	**Intervention Model**	**Intervention duration (day)**	**Food matrix**	**Iron type**	**Particle size**	**Iron concentration (Fe mg/ kg food)**
18	Lysionek 2001b	Male SD rats	9	Prophylactic-preventative	Intervention: 28	Basal diet	NaFe-EDTA	Regular	20
19	Mazgaj 2020	Pregnancy piglets	5	Prophylactic-preventative	Intervention: 45	Standard fodder	FePP	Regular	80
20	Valcarcel 2018	Male newborn piglets	6	Prophylactic-preventative	Intervention: 28	Basal diet	FePP	Micronized	120
							Ferrous fumarate	Regular	120
21	Anderson 1972	Male Carworth rats	8	Prophylactic-preventative	Intervention: 28	Creal-milk	FePP	Regular	15
22	Carretero 2006	Half male and half female Wistar rats	8	Prophylactic-preventative	Intervention: 28	Cocoa powder	FePP	Regular	35
							Ferrous fumarate	Regular	35
23	Sachdeva 2015	Male Wistar rats	8	Prophylactic-preventative	Intervention: 28	Milk	FePP	Regular	25
							FePP +vitamin A +acetate	Regular	

Table 2.5: *Characteristics of animal studies that were not included in meta-analysis (continued)*

Study ID	Author, publication year	Species	Sample size	Intervention Model	Intervention duration (day)	Food matrix	Iron type	Particle size	Iron concentration (Fe mg/kg food)
			8	Depletion-repletion	Depletion: 7 Repletion: 21		FePP	Regular	
							FePP +vitamin A +acetate	Regular	
24	Ahmad 2021	Female SD rats	Unknown	Depletion-repletion	Depletion: 14 Repletion: 90	Basal diet +inulin (722 mg/kg)	NaFe-EDTA	Regular	10
							NaFe-EDTA	Regular	20
						Basal diet +inulin (963 mg/kg)	NaFe-EDTA	Regular	10
							NaFe-EDTA	Regular	20
25	Douglas 1981	Male SD rats	Unknown	Depletion-repletion	Depletion: 28 Repletion: 10	Milk +whey protein	$FePO_4$	Regular	40
			Unknown				$FePO_4$	Regular	20
			Unknown				$FePO_4$	Regular	10

Table 2.5: *Characteristics of animal studies that were not included in meta-analysis (continued)*

Study ID	**Author, publication year**	**Species**	**Sample size**	**Intervention Model**	**Intervention duration (day)**	**Food matrix**	**Iron type**	**Particle size**	**Iron concentration (Fe mg/ kg food)**
						Milk	FePP	Regular	40
							FePP	Regular	20
							FePP	Regular	10
26	Theuer 1971	Male rats	Unknown	Depletion-repletion	Depletion: 32 Repletion: 28	Soy Formula	FePP	Regular	7
27	Theuer 1973	Male rats	Unknown	Depletion-repletion	Depletion: 39 Repletion: 28	Milk Formula	FePP	Regular	7
28	Sudargo 2015	Female Wistar rats	9	Depletion-repletion	Depletion: 10 Repletion: 17	Soybean	NaFe-EDTA	Regular	6
								Regular	12
								Regular	24
29	Fritz 1975	Male albino rats	≥8	Depletion-repletion	Depletion: 28 Repletion: 14	Basal diet	$FePO_4$	Regular	24
30	Carretero 2014	Half male and half female Wistar rats	8	Prophylactic-preventative	Intervention: 28	AIN-93G diet	FePP	Regular	15
31	Li 2009	Rats	8	Depletion-repletion	Depletion: 14 Repletion: 14	Salt	FePP	Micronized	250

Table 2.5: *Characteristics of animal studies that were not included in meta-analysis (continued)*

Study ID	**Author, publication year**	**Species**	**Sample size**	**Intervention Model**	**Intervention duration (day)**	**Food matrix**	**Iron type**	**Particle size**	**Iron concentration (Fe mg/ kg food)**
							Ferrous fumarate	Microencapsulated	250
							FePP	Micronized	500
							Ferrous fumarate	Microencapsulated	500
32	Kosonen 1991	Male Wistar rats	6	Depletion-repletion	Depletion: 28 Repletion: 16	Basal diet (Flour)	$FePO_4$	Regular	6
							$FePO_4$	Regular	12
							$FePO_4$	Regular	24
33	Yeung 2005	Male SD rats	10	Prophylactic-preventative	Intervention: 39	AIN-93G diet	NaFeEDTA (isotope)	Regular	35
						AIN-93G diet +tea	NaFeEDTA (isotope)		35
34	Amine 1974	Female SD rats	6	Depletion-repletion	Depletion: 28 Repletion: 10	Farina	$FePO_4$	Regular	10
						Farina	$FePO_4$	Regular	20
						Farina	$FePO_4$	Regular	30
						Farina +baked apple	$FePO_4$	Regular	5

Table 2.5: *Characteristics of animal studies that were not included in meta-analysis (continued)*

Study ID	**Author, publication year**	**Species**	**Sample size**	**Intervention Model**	**Intervention duration (day)**	**Food matrix**	**Iron type**	**Particle size**	**Iron concentration (Fe mg/ kg food)**
						Farina +baked apple	$FePO_4$	Regular	10
						Farina +baked apple	$FePO_4$	Regular	15
						Farina +maple sugar	$FePO_4$	Regular	5
						Farina +maple sugar	$FePO_4$	Regular	10
						Farina +maple sugar	$FePO_4$	Regular	15

Table 2.6: *Characteristics of cell-culture studies*

Study ID	Author, publication year	Species	Food matrix	Iron type	Particle size	Iron concentration
35	Zhu 2009	Caco-2	Nonfat dry milk	Soluble FePP	Regular	100μmol/L
				NaFeEDTA	Regular	100μmol/L
			Rice powder	Soluble FePP	Regular	50μmol/L
				Soluble FePP +ascorbic acid	Regular	50μmol/L
36	Yeung 2002	Caco-2	Bread flour	NaFeEDTA	Regular	37 mg/kg
				Ferrous fumarate	Regular	37 mg/kg
			Nonfat dry milk	NaFeEDTA	Regular	50μmol/L
				Ferrous fumarate	Regular	50μmol/L
				Ferrous fumarate (encapsulated)	Regular	50μmol/L
37	Wortley 2005	Caco-2	Wheat-cereal	Ferrous fumarate (encapsulated 65%)	Regular	30 mg Fe/100g food
				Ferrous fumarate (encapsulated 85%)	Regular	30 mg Fe/100g food
				FePP (encapsulated)	Regular	30 mg Fe/100g food
				NaFeEDTA	Regular	30 mg Fe/100g food
38	Sabatier 2020	Caco-2	Milk	FePP	Regular	2.8-2.9 mg Fe/100g food
				FePP+ascorbic acid (1:2)	Regular	2.8-2.9 mg Fe/100g food
				FePP+ascorbic acid (1:4)	Regular	2.8-2.9 mg Fe/100g food

Table 2.6: *Characteristics of cell-culture studies(continued)*

Study ID	**Author, publication year**	**Species**	**Food matrix**	**Iron type**	**Particle size**	**Iron concentration**
39	Karn 2011	Caco-2	Curry powder	Ferrous fumarate	Regular	38 mg Fe/100g food
				NaFeEDTA	Regular	90 mg Fe/100g food
40	Cercamondi 2016	Caco-2	Bouillon cube	FePP	Regular	62.5 mg Fe/100g food
				FePP+tetra sodium pyrophosphate	Regular	62.5 mg Fe/100g food
41	Valcarcel 2022	Caco-2	Infant cereal	FePP	Micronized	7.5 mg Fe/100g food
				Ferrous fumarate	Regular	7.5 mg Fe/100g food
42	Micheletto 2022	Caco-2	Starch	FePP+ phospholipid	Regular	30 mg Fe/sample

Table 2.7: *Characteristics of outcome effects in $FePO_4$ rat studies*

Study ID	**Comparison No**	**Primary outcome (Hb)**	**Other primary and secondary outcomes**
1	1	$FePO_4$<$FeSO_4$	In vitro iron solubility (pH = 1, time = 15 mins, 0.1N HCL): $FePO_4$ (11 nm)>$FePO_4$ (31 nm)>$FePO_4$ (64 nm). In vitro iron solubility (pH = 1, time = 30 mins, 0.1N HCl): $FePO_4$ (11 nm) = $FePO_4$ (31 nm) (No significant difference), $FePO_4$ (31 nm)=$FePO_4$ (64 nm) (No significant difference), $FePO_4$ (11 nm)>$FePO_4$ (64 nm).
	2	$FePO_4$<$FeSO_4$	
	3	$FePO_4$<$FeSO_4$	
	4	$FePO_4$<$FeSO_4$	
	5	$FePO_4$<$FeSO_4$	
	6	$FePO_4$<$FeSO_4$	
2	7	No significant difference	In vitro iron solubility (pH = 1.2, time = 30 mins, 0.02N HCl, 0.05N HCl, 0.1N HCl, respectively): Source No.1>Source No.4>Source No.2>Source No.3=Source No.5 (No significant difference)
	8	$FePO_4$<$FeSO_4$	
	9	$FePO_4$<$FeSO_4$	
	10	$FePO_4$<$FeSO_4$	
	11	$FePO_4$<$FeSO_4$	
	12	No significant difference	
	13	No significant difference	
	14	$FePO_4$<$FeSO_4$	
	15	No significant difference	
	16	$FePO_4$<$FeSO_4$	
3	17	$FePO_4$<$FeSO_4$	Hepatic iron (μg): $FePO_4$<$FeSO_4$.
	18	$FePO_4$<$FeSO_4$	
4	19	$FePO_4$<$FeSO_4$	Hepatic iron (mg): No significant difference.
5	20	$FePO_4$<$FeSO_4$	Hepatic iron (μg/g): $FePO_4$<$FeSO_4$.
6	21	No significant difference	Not reported

Table 2.7: *Characteristics of outcome effects in $FePO_4$ rat studies(continued)*

Study ID	**Comparison No**	**Primary outcome (Hb)**	**Other primary and secondary outcomes**
	22	No significant difference	
	23	$FePO_4$<$FeSO_4$	

Table 2.8: *Characteristics of outcome effects in FePP rat studies*

Study ID	**Comparison No**	**Primary outcome (Hb)**	**Other primary and secondary outcomes**
7	24	No significant difference	Hepatic iron (mg/g): FePP<$FeSO_4$.
	25	No significant difference	Hepatic iron (mg/g): No significant difference.
8	26	No significant difference	Not reported
	27	FePP<$FeSO_4$	
9	28	No significant difference	Not reported
	29	No significant difference	
	30	FePP<$FeSO_4$	
	31	No significant difference	
	32	FePP<$FeSO_4$	
	33	FePP<$FeSO_4$	
	34	FePP<$FeSO_4$	
	35	FePP<$FeSO_4$	
10	36	No significant difference	Not reported
	37	FePP>$FeSO_4$	
11	38	No significant difference	
	39	No significant difference	

Table 2.9: *Characteristics of outcome effects in NaFeEDTA rat studies*

Study ID	**Comparison No**	**Primary outcome (Hb)**	**Other primary and secondary outcomes**
12	40	No significant difference	Hepatic iron (μg/g): NaFeEDTA<$FeSO_4$. Bone iron (μg/g): No significant difference.
13	41	No significant difference	Total iron binding capacity (μg/dL): No significant difference.
	42	No significant difference	Serum iron (μg/dL): NaFeEDTA=$FeSO_4$. Hepatic iron (μg): NaFeEDTA>$FeSO_4$.
	43	No significant difference	
	44	No significant difference	
14	45	No significant difference	
3	46	NaFeEDTA >$FeSO_4$	Hepatic iron (μg): No significant difference
	47	NaFeEDTA >$FeSO_4$	
15	48	No significant difference	Hematocrit (%): No significant difference. Transferrin saturation (%): No significant difference. Hepatic iron (μg/g): No significant difference

Table 2.10: *Characteristics of outcome effects in ferrous fumarate rat studies*

Study ID	**Comparison No**	**Primary outcome (Hb)**	**Other primary and secondary outcomes**
16	49	No significant difference	Not reported
	50	Ferrous fumarate<$FeSO_4$	
14	51	No significant difference	Total iron binding capacity (μg/dL): No significant difference. Serum iron (μg/dL): No significant difference. Hepatic iron (μg): Ferrous fumarate>$FeSO_4$
17	52	No significant difference	Hematocrit (%): No significant difference

Table 2.11: *Characteristics of outcome effects in animal studies that were not involved in meta-analysis*

Study ID	**Comparison No**	**Primary outcome (Hb)**	**Other primary and secondary outcomes**
18	53	Hb gain (g/dL): NaFeEDTA< $FeSO_4$	Not reported
19	54	Hb value (g/dL): No significant difference	Plasma/serum iron (μmol/L): No significant difference. Plasma/serum ferritin (ng/mL): No significant difference. Hepatic iron (nM): No significant difference.
20	55	Hb value (g/dL): FePP<$FeSO_4$	Plasma iron (μg/dL): Ferrous fumarate vs $FeSO_4$: No significant difference. FePP<$FeSO_4$. Total iron binding capacity (μg/dL): No significant difference. Transferrin saturation (%): Ferrous fumarate vs $FeSO_4$: No significant difference. FePP<$FeSO_4$.
	56	Hb value (g/dL): Ferrous fumarate>$FeSO_4$	
21	57	Hb value (g/dL): FePP<$FeSO_4$	Hematocrit gain (%): FePP<$FeSO_4$. Hepatic iron (μg/g): FePP<$FeSO_4$
	58	Hb value (g/dL): FePP< Blank control	Total iron binding capacity (μg/dL): FePP>Blank control, Ferrous fumarate vs Blank control: No significant difference. Hepatic iron (μg/g): FePP<Blank control, Ferrous fumarate vs Blank control: No significant difference.
	59	Hb value (g/dL): No significant difference	
23	60	Hb gain (%): FePP>Blank control; FePP+vitamin A+acetate> Blank control	Plasma ferritin gain (%): No significant difference. Plasma transferrin gain (%): No significant difference. Hepatic iron (ppm): No significant difference.
	61		

Table 2.11: *Characteristics of outcome effectsin animal studies that were not involved in meta-analysis(continued)*

Study ID	**Comparison No**	**Primary outcome (Hb)**	**Other primary and secondary outcomes**
	62	Hb gain (%): FePP> Blank control; FePP+vitamin A+acetate> Blank control	
24	64	No Hb outcome reported.	Serum ferritin gain (ng/mL): All NaFeEDTA< $FeSO_4$. Serum transferrin gain (mg/dL): NaFeEDTA (722mg inulin/kg)<$FeSO_4$, NaFeEDTA (963mg inulin/kg)>$FeSO_4$. Total iron binding capacity (μg/dL): NaFeEDTA (722mg inulin/kg)<$FeSO_4$, NaFeEDTA (963mg inulin/kg)>$FeSO_4$.
	65		
	66		
	67		
25	68	Hb value (g/dL): No significant difference	Not reported
	69		
	70		
	71	Hb value (g/dL): No significant difference	
26	74	Hb value (g/dL): No significant difference	Hematocrit gain (%): FePP<$FeSO_4$.
27	75	Hb value (g/dL): No significant difference	Not reported
28	76	No Hb outcome reported.	Serum iron gain (μg/L): No significant difference. Serum ferritin (mg/dL): NaFeEDTA>$FeSO_4$.
	77		Serum iron gain (μg/L): NaFeEDTA>$FeSO_4$. Serum ferritin (mg/dL): NaFeEDTA<$FeSO_4$.

Table 2.11: *Characteristics of outcome effectsin animal studies that were not involved in meta-analysis(continued)*

Study ID	**Comparison No**	**Primary outcome (Hb)**	**Other primary and secondary outcomes**
	78		Serum iron gain (μg/L) and serum ferritin (mg/dL): No significant difference.
29	79	Hb value (g/dL): No significant difference	Not reported
30	80	No Hb outcome reported.	Hepatic iron (mg): No significant difference.
31	81	Hb gain (g/dL): No significant difference	Not reported
	82		
	83		
	84		
32	85	Hb-Fe (mg, iron incorporated into hemoglobin): No significant difference	Not reported
	86		
	87		
33	88	No Hb outcome reported.	Hepatic iron (μmol/g): NaFeEDTA<$FeSO_4$. Hematocrit (%): No significant difference.
	89		
34	90	Hb value (g/dL): No significant difference	
	91		
	92		
	93		
	94		
	95		
	96		
	97		
	98		

Table 2.12: *Characteristics of outcome effects in cell-culture studies*

Study ID	Comparison No	Primary outcome (Hb)	Other primary and secondary outcomes
35	99	No significant difference	Not reported
	100	NaFeEDTA <$FeSO_4$	
	101	No significant difference	
	102	No significant difference	
36	103	No significant difference	Not reported
	104	No significant difference	
	105	No significant difference	
	106	No significant difference	
	107	No significant difference	
37	108	Ferrous fumarate>$FeSO_4$	Not reported
	109	Ferrous fumarate>$FeSO_4$	
	110	FePP>$FeSO_4$	
	111	NaFeEDTA> $FeSO_4$	
38	112	No significant difference	In vitro iron solubility (pH = 2, time=30,60,90 mins, 0.02mol/L HCL): all FePP<$FeSO_4$.
	113	FePP<$FeSO_4$	
	114	FePP<$FeSO_4$	
39	115	No significant difference	Not reported
	116	No significant difference	
40	117	No significant difference	Not reported

Table 2.12: *Characteristics of outcome effects in cell-culture studies(continued)*

Study ID	Comparison No	Primary outcome (Hb)	Other primary and secondary outcomes
	118	FePP<$FeSO_4$	
41	119	FePP<$FeSO_4$	In vitro iron solubility (pH = 2, time=30,60,90 mins, Diluted HCL): Ferrous fumarate<FePP< $FeSO_4$.
	120	Ferrous fumarate<$FeSO_4$	
42	121	FePP(phospholipid)=2.54-fold of FePP	Not reported

Table 2.13: *Summary of primary study effect*

The iron effect of comparison	Cell-culture studies (Ferritin-not involved in meta-analysis)	Rat studies (Hb-involved in meta-analysis)	Animal studies (Hb-not involved in meta-analysis)	Total number
NaFeEDTA < $FeSO_4$	1	0	1	2
NaFeEDTA > $FeSO_4$	1	2	0	3
NaFeEDTA = $FeSO_4$ (No significant difference)	3	7	3	13
Ferrous fumarate < $FeSO_4$	1	1	0	2
Ferrous fumarate > $FeSO_4$	2	0	1	3
Ferrous fumarate = $FeSO_4$ (No significant difference)	4	3	2	9
FePP < $FeSO_4$	4	6	2	12
FePP > $FeSO_4$	1	1	0	2
FePP = $FeSO_4$ (No significant difference)	4	9	8	21
$FePO_4$ < $FeSO_4$	0	17	0	17
$FePO_4$ > $FeSO_4$	0	0	0	0
$FePO_4$ = $FeSO_4$ (No significant difference)	0	6	19	25
Total number	21	52	36	109

Note: Number represented the number of comparison.

Table 2.14: *Quality assessments of included studies*

ID	Authors, year of publication	1	2	3	4	5	6	7	8	9
1	Vicente 2008	L	L	H	H	H	L	L	L	L
2	Sakaguchi 2003	L	L	L	H	L	U	L	L	L
3	Wegmuller 2004	L	L	L	H	L	L	L	L	L
4	Salgueiro 2008	L	L	L	H	L	L	L	L	L
5	Lucia 2013	L	L	H	H	H	U	L	L	L
6	Rohner 2006	L	L	L	H	L	H	L	L	L
7	Dickmann 2016	L	L	L	H	L	L	L	L	L
8	Whittaker 1989	L	L	L	H	H	L	L	L	L
9	Lysionek 2001a	L	L	H	H	H	L	L	L	L
10	Benito 1997	L	L	L	H	L	L	L	L	L
11	Srinivasu 2015	L	L	L	H	L	L	L	L	L
12	Zhu 2006	L	L	L	H	H	L	L	L	L
13	Malika 2022	L	L	H	H	L	L	L	L	L
14	Ologunde 1992	L	L	H	H	H	U	L	L	L
15	Olivira 1995	L	L	L	H	H	U	L	L	L
16	Hernandez 2003	L	L	H	H	H	L	L	L	L
17	Suva 2022	L	L	L	H	H	L	L	L	L
18	Lysionek 2001b	L	L	L	H	H	L	L	L	L
19	Mazgaj 2020	L	L	L	H	L	L	L	L	L
20	Valcarcel 2018	L	L	L	H	L	L	L	L	L
21	Anderson 1972	L	L	L	H	L	L	L	L	L
22	Carretero 2006	L	L	L	H	L	L	L	L	L
23	Sachdeva 2015	L	L	L	H	L	L	L	L	L
24	Ahmad 2022	L	L	H	H	L	L	L	L	L
25	Douglas 1981	L	L	H	H	H	L	L	L	L
26	Theuer 1971	L	L	H	H	L	L	L	L	L
27	Theuer 1973	L	L	H	H	L	L	L	L	L
28	Sudargo 2015	L	L	H	H	L	L	L	L	L
29	Fritz 1975	L	L	H	H	L	H	L	L	L
30	Carretero 2014	L	L	L	H	L	L	L	L	L
31	Li 2009	L	L	H	H	H	L	L	L	L
32	Kosonen 1991	L	L	H	H	L	H	L	L	L
33	Yeung 2005	L	L	H	H	H	L	L	L	L
34	Amine 1974	L	L	H	H	L	H	L	L	L
35	Zhu 2009	L	L	L	H	H	L	L	L	L

Table 2.14: *Quality assessments of included studies(continued)*

ID	Authors, year of publication	1	2	3	4	5	6	7	8	9
36	Yeung 2002	L	L	H	H	L	L	L	L	L
37	Wortley 2005	L	L	L	H	L	L	L	L	L
38	Sabatier 2020	L	L	H	H	L	L	L	L	L
39	Karn 2011	L	L	L	H	L	L	L	L	L
40	Cercamondi 2016	L	L	L	H	L	L	L	L	L
41	Valcarcel 2022	L	L	H	H	L	L	L	L	L
42	Micheletto 2022	L	L	H	H	H	H	L	L	L

Note: "1" represented "There were clear research purposes". "2" represented "Relevant background information was presented". "3" represented "Randomized allocation methods were employed". "4" represented "The investigators were blinded". "5" represented "Dosage-response relationships were investigated or the fortification levels were explained". "6" represented "The attritions or negative results were explained". "7" represented "The data was related to methodological design". "8" represented "The conclusions and discussions matched up with the findings". "9" represented "The findings contributed to the current theories or future research/practice". "L" represented low risk. "H" represented high risk. "U" represented unclear.

Table 2.15: *GRADE assessments on Hb of included studies involved in meta-analysis*

Iron type	Number of studies	Risk of bias	Inconsistency	Indirectness	Imprecision	Other considerations	Certainty
NaFeEDTA	5	Not serious	Serious	Not serious	Not serious	Dose response gradient	⊕⊕⊕◯ Moderate
Ferrous fumarate	3	Not serious	Serious	Not serious	Not serious	Dose response gradient	⊕⊕⊕◯ Moderate
FePP	5	Not serious	Serious	Not serious	Not serious	Dose response gradient	⊕⊕⊕◯ Moderate
$FePO_4$	6	Not serious	Serious	Not serious	Not serious	Dose response gradient; Publication bias strongly suspected	⊕⊕◯◯ Low

Chapter 3

Comparative Efficacy of Iron Fortificants in Populations: A Clinical Systematic Review and Network Meta-analysis

Abstract

Background: It is well known that the prevalence of iron deficiency anemia can be down-regulated by the applications of food aid. Many clinical questions on iron bioavailability have been raised based on the rational addition of iron frotificants to foods to correct a dietary iron insufficiency. However, the lack of sufficient and robust evidence on overall clinical effect limits the development of iron fortification. Clinical systematic review and network meta-analysis can partially answer these clinical questions by combining the results of all relevant clinical trials, computing the direct and indirect effects of the included studies, and ranking the treatments from the best to the worst within a network. At present, no clinical systemic reviews with network meta-analysis on iron bioavailability have been published yet. Thus, this review is the first study in this field. It can provide references about direct and indirect effects of iron fortifications for better clinical applications.

Objective: To identified, evaluated, and summarized the evidence regarding the efficacy of iron fortifications in clinical trials.

Methods: The search strategy was employed to obtain initial articles. After scanning and sorting, final included articles were collected. The characteristics of included studies was summarized for the systematic review. The data was extracted for network meta-analysis.

Main Results: 145 studies were included in this systemic review. 46 studies including 88 comparisons, 5 iron fortificants ($FeSO_4$, electrolytic iron, NaFeEDTA, ferrous fumarate, and FePP), and 24107 participants (under 18 years old) contributed to the network meta-analysis in Hb. $FeSO_4$, NaFeEDTA, ferrous fumarate, and FePP were more effective than placebo, and NaFeEDTA was more effective than $FeSO_4$. The rank order of Hb effect was NaFeEDTA>ferrous fumarate>FePP>$FeSO_4$>electrolytic iron>placebo.

Conclusions: The findings of this systematic review showed that NaFeEDTA, ferrous fumarate, FePP and $FeSO_4$ fortifiactions were effective treatments in Hb. NaFeEDTA was suggested to be the most effective iron fortificant, followed by ferrous fumarate, FePP and $FeSO_4$ in population under-18-year-old. The findings of network meta-analysis are supposed to be applied with caution as conditional recommendations.

Keywords: Iron bioavailability, Iron fortification, Iron deficiency, Clinical trial, Network meta-analysis

3.1 Background

3.1.1 Description of the condition

Anemia is recognized by the WHO as a major global concern of public health and a common comorbidity of many chronic diseases. In 2011, approximately 270 million children, 550 million women including 30 million pregnant women had anemia in the world. Although there is a downward trend in anemia incidence over the pass few decades, the prevention and treatment of anemia remains a huge socioeconomic burden worldwide every year, especially in low-income regions. The World Bank estimates that an investment of one US dollar in anemia yields an economic return of twelve US dollar. The largest proportion of anemia worldwide is iron deficiency anemia. Iron insufficiency uptake and low iron absorption rate are considered to be the main etiological factors of iron deficiency anemia.[55–57;121–124]

3.1.2 Description of intervention

Iron fortification has been recommended as one of the most cost-effective ways to eradicate iron deficiency anemia. It can improve the iron status of people with iron deficiency or at

the risk of iron deficiency. There are diverse strategies to fortify food products with iron. The common food vehicles are staple food (e.g., rice, wheat, corn, maize, etc.), drinks (e.g., water, milk, beverages, etc.), and condiments (e.g., curry powder, soy sauce, salt, etc.). Some food processing approaches such as precooking and extrusion are usually used to lessen the inhibitors of iron absorption such as polyphenols, tannic, and phytic acid. However, it is still challenging to achieve high iron bioavailability. Therefore, the WHO drafted a new-round resolution to accelerate efforts for the micronutrient deficiency prevention and treatment with effective food fortification in 2023. It can help upgrade the current food fortifications to the planning clinical care and the public health intervention for all people throughout the whole life cycle.[121–123]

3.1.3 Why it is important to do this review

Although the prevalence of iron deficiency anemia can be down-regulated by the applications of food aid, iron deficiency anemia is still a severe public health issue.[121–123] The lack of sufficient and robust evidence on overall clinical effect limits the development of iron fortification. Currently, there are no published clinical systematic reviews on the efficacy of iron fortifications across the life course using network meta-analysis. Thus, this systematic review can complement the direct comparison results of other systematic reviews in specify populations and fill the gap in the field of indirect comparison on clinical iron bioavailability for all populations. This clinical systematic review has been registered on the PROSPERO (CRD42023461043).

3.2 Objectives

In this systematic review, participants (P) were all populations including male or female infants, children, adult, and the aged. Interventions (I) were iron fortificants. Comparisons (C) were positive control or blank controls. The primary outcomes (O) were Hb. The PICO questions were as follows: (1) In high-risk people (P), does iron fortification (I) compared with blank or positive control (C) prevent iron-deficient anemia (O)? (2) In people with iron deficient anemia (P), does iron fortification (I) compared with blank or positive control (C) improve anemia status (O)? We identified, evaluated, and summarized the findings of all relevant clinical trials to provide evidence-based suggestions for the better human applications in food aid. The process was schematically shown in Figure 3.1.

3.3 Methods

3.3.1 Criteria for considering studies (inclusion and exclusion criteria)

They must be randomized control clinical trials and primary research. Iron treatments must be given in the food vehicle. Iron supplementation studies and special food studies (e.g., testing foods were naturally high in iron) were not included. At least one iron fortificant with identified iron type and quantity was used. The health condition of participants was healthy, compromised iron status, iron deficient status or iron deficient anemia. Participants with acute and sever conditions were not included. At least one desired outcome was reported. The minimum of intervention duration was 2 weeks. The manuscript must be peer-reviewed and in English. Theses, dissertations, book sections, meeting abstracts and proceedings were not included in this systemic review. The preclinical trials, observational studies, case reports, reviews, protocols, and secondary research were excluded. Non-randomized or non-control clinical trials were excluded.

3.3.2 Types of outcomes

Hb was the primary outcome in the assessment of iron deficient anemia status. Secondary outcomes included but were not limited to ferritin, transferrin saturation, transferrin, plasma iron, serum iron, total iron binding capacity, and hematocrit, which were the indicators in the assessment of early negative iron storage and iron depletion status.[73] They were continuous outcomes. No dichotomous outcomes were collected.

3.3.3 Search strategy

We carried out the search strategy in 4 scientific databases (Pubmed, Web of Sciences Core Collection, Scopus, and EBSCO Agricola) on January 18, 2023. Snowball and citation search were used to extend the search strategy for additional articles using other academic search engines (e.g., Kansas state university libraries, Google Scholar, etc.). In this search strategy, a special syntax was employed. The words in the quotation marks could not be separated. The asterisk indicated word variations. The search strategy followed the user guides of the databases and was classified into 5 categories. Each Category contained a series of search items.

(1) Category #1 contained 5 search items: ferric, ferrous, NaFeEDTA, "reduced iron" and "elemental iron", to ensure that the testing diet must be fortified with an iron foritificant. (2) Category #2 contained 14 search items, which enabled iron status must be investigated. They were "iron status", "iron deficiency", "iron deficient", "iron depleted", "iron depletion",

"iron stores", anaemia, anemia, anemic, anaemic, microcyt*, hypochrom*, "iron state", "iron balance". (3) Category #3 including 9 search items: hemoglobin, haemoglobin, hematocrit, haematocrit, "total iron binding capacity", "plasma iron", ferritin, "transferrin saturation", and "serum iron" showed that at least one desired outcome was reported. (4) 4 search items, forti*, enrich*, food, "micronutrient powder" in Category #4 indicated that iron additives must be presented in food. (5) Category #5 had 8 search items, clinical, trial, intervention, controlled, cross-over, randomized, randomized, human, so that the included study must be a clinical randomized control trial.

All relevant literatures were identified in "all fields or topic" using advanced search builder and Boolean operators (AND and OR). All search items under one category were typed into one query search box with "OR" to get a query search result. All query searches were combined with "AND" (#1 query AND #2 query AND #3 query AND #4 query AND #5 query) into the final search box to obtain the initial articles. The filters were applied to specify publication types in peer-reviewed article and language in English.

3.3.4 Data managements, data extractions and quality assessments

Initial articles from 4 databases were imported into an online data manager, Zotero. After automatic duplicate/triplicate/quadruplicate merging, title- and abstract-reviewed articles were manually scanned. The remaining articles were thoroughly reviewed and further sorted to obtain the final included articles. The data and characteristics of included studies were extracted using Excel: author, year of publication, age, setting, number of enrolled participants, number of completed participants, study type, iron type, iron concentration, food vehicle, control type, number of arms, intervention duration, calculation of sample size, blinding, randomization, allocation, ethic approval and outcome.

The quality assessments of the included studies were conducted in accordance with modified "Risk of bias" tools in the Cochrane Handbook. The quality assessments were evaluated with three grades (high risk, unclear, and low risk) and further expressed with the total number of grade items.[76] The checklist comprised of 6 items: (1) Were randomized methods employed? (2) Was the allocation concealed? (3) Were the participants and researchers blinded from group assignment? (4) Were there similarities in baseline characteristics? (5) Did researchers interpret the attritions (incomplete outcomes)? (6) Did researchers interpret the negative results?[76] In the network plot of risk of bias percentage and the bar diagram of risk of bias percentage, red represented the percentage of high risk of bias. Yellow represented the percentage of moderate risk of bias. Green represented the percentage of low risk

of bias.

3.3.5 Statistical analyses

Hb data for network meta-analysis was performed in R (version 4.3.2) and STATA (version 18.0, USA) with the significance at p-value<0.05. SD could be calculated using the following formulas: SD=SEM×sqrt (n) or SD=$\sqrt{n}$*(upper limit value-lower limit value)/3.92, where n was the number of participants, if only the SEM or 95% CI was reported.[79;125] The study effects were evaluated with Bayesian network meta-analysis model, which could be employed to get a posterior probability distribution of study effects. The weighted MD, CIs, normal likelihood, and random-effect model were used to assess the overall effect of the continuous outcome. In the network plot, the node represented the treatment type. The size of node represented the number of participants. Width of the lines represented the number of comparisons. The size of circle represented the sample size of trials. The heterogeneity was quantified by I^2 , Chi^2 and Tau^2 indexes to examine the intra-study and inter-study variability.[76;126;127] Treatment effects were ranked according to estimated effects on mean ranks using the surface under the cumulative ranking curve (SUCRA).[76;128] The transitivity was estimated by comparing the methodological variables.[76;129] The consistency was assessed by loop-specific approach, and side-splitting approach, which could separate direct evidence from indirect evidence.[76;127;130] Meta-regression was conducted to detect the source of high heterogeneity. Publication bias was displayed in the funnel plot. A symmetrical funnel plot implied the less possibility of publication bias, and vice versa.[76;80–82] The certainty of evidence was evaluated using the Confidence in Network Meta-Analysis (CINeMA) online tool (https://cinema.ispm.unibe.ch/) and the Grading of Recommendations Assessment, Development and Evaluation (GRADE) method.[76;131;132]

3.4 Results

3.4.1 Description of search results

Our search yielded 2749 initial articles. 4 additional articles were added. After merging 870 iterations and screening 1883 titles or abstracts, 1608 articles were excluded. The full texts of 275 remaining articles were reviewed. 130 studies were further excluded. Thus, the number of final included articles was 145. The included studies were listed in Appendix A (page 191). The sorting and screening process was shown in Figure 3.2. All included

studies contributed to the summary of data and characteristics. 46 grain-based studies including 88 comparisons, 5 iron fortificants ($FeSO_4$, ferrous fumarate, NaFeEDTA, FePP, and electrolytic iron), and 24107 participants (under 18 years old) could be analyzed with network meta-analysis.

3.4.2 Characteristics and quality assessments of included studies

The characteristics of 145 studies were summarized in Table 3.1 and Table 3.2. 14 types of iron fortificants were studied, which were $FeSO_4$, ferrous fumarate, NaFeEDTA, FePP, $FePO_4$, electrolytic iron (EI), ferric ammonium citratecitrate, ferrous glycine sulfate, ferrous gluconate, iron trisglycinate chelate, ferrous bisglycinated, ferrous glucie phosphate, hydrogen reduced iron, ferrous chloride. The predominant particle size of iron fortificants was regular size (20-30 μm, n=128, 88.3%). 17 micronized (0.3-3 μm, 11.7%) size of iron fortificants were investigated. Among these studies, the food matrix of 99 studies was grain-based diets (68.2%), and others were milk or milk formular (n=17, 11.7%), drinking water (n=5, 3.5%), beverage (n=5, 3.5%), condiment (n=17, 11.7%), and snack (n=2, 1.4%). There were 13 dose-response studies (9.0 %), and 50 studies (34.5 %) explained the rationale of iron fortification level such as using a licensed dosage, while 82 studies (56.6 %) did not state it.

The included studies were divided into 5 groups according to age and gender, under 18 years old male and female populations (n=111, 76.6%), families (n=7, 4.8%), the aged (n=1, 0.7%), women aged 12-55 years old (n=23, 15.8%), and pregnant women (n=3, 2.1%). The distribution of study setting was in six continents, Africa (n=36, 24.8%), Asia (n=59, 40.7%), Europe (n=12, 8.3%), North America (n=9, 6.2%), Oceania (n=3, 2.1%), and South America (n=26, 18%). Considering anemia condition, 35 studies (24.1%) recruited only healthy participants, and 5 studies (3.5%) recruited anemia participants. In the rest of 105 studies (72.4%), some of the participants were healthy and others had anemia. The drop rate of 98 studies (67.6%) was less than or equal to 20%, others were larger than 20% (n=47, 32.4%). The duration of intervention lasted 0.5 to 36 months (<4 months, n=29, 20%; 4-6 months, n=80, 55.2%; >6 months, n=36, 24.8%). 87 studies (60%) utilized the completely randomized control model and the rest chose the cluster randomized control model (n=58, 40%). 81 studies clearly explained the method of sample size calculation (55.9%), while others (n=64, 44.1%) did not mention it. 12 studies (8.3%) did not indicate that they had the ethical approval.

In the direct Hb effects of 145 iron-fortification studies (Table 3.3 and Table 3.4), 18

$FeSO_4$, 12 ferrous fumarate, 11 NaFeEDTA, 7 FePP, 2 ferrous gluconate, 1 EI, 1 ferrous glucie phosphate, 1 ferrous bisglycinate, 1 ferric ammonium citrate, 2 combinations of NaFeEDTA and ferrous fumarate, and 1 combination of $FeSO_4$ and $FePO_4$ were significantly higher than placebo, respectively.

As shown in the table of quality assessments (Table 3.5), 93 studies (64.1%) did not clearly elucidate the generation of randomized sequence. 126 (86.7%) studies did not use the allocation method. There were 85 double blinding studies (58.6%), and 14 single blinding studies (8 studies masked participants, 6 studies masked operators) [9.7%], while 46 studies (31.7%) did not utilize the blinding method. 3 studies (2.1%) had high risk of imbalance baseline. 12 studies (8.2%) did not explain the incomplete data. 1 study (0.7%) did not interpret the negative results. The overall risks of bias were suggested to be low in 16 trials (11%), moderate in 78 trials (53.8%), and high in the remaining trials (n=51, 35.2%).

3.4.3 Network meta-analysis results and certainty of evidence

Figure 3.3 showed the Hb network meta-analysis of eligible comparisons in Hb. Except that $FeSO_4$- fumarate, EI-FePP, and NaFeEDTA-FePP did not have direct comparison, the rest could be directly compared with another iron fortificants or placebo in the network. The forest plot (Figure 3.4) of pairwise meta-analysis in Hb showed that $FeSO_4$, NaFeEDTA, fumarate, and FePP were more effective than placebo, and NaFeEDTA was more effective than $FeSO_4$ and EI, while EI-placebo, EI-$FeSO_4$, NaFeEDTA-$FeSO_4$, FePP-$FeSO_4$, fumarate-EI, FePP-EI, fumarate-NaFeEDTA, FePP-NaFeEDTA, and FePP-fumarate were equivalent to each other, respectively. As illustrated in SUCRA plot (Figure 3.5), the order of Hb effect was NaFeEDTA>fumarate>FePP>$FeSO_4$>EI>placebo.

In the side-split analysis, except that $FeSO_4$-placebo, EI-placebo, and EI-NaFeEDTA had a significant inconsistency, there was no significant inconsistency in other comparisons. No significant loop-specific inconsistency was found. Furthermore, the direct heterogeneity (pairwise heterogeneity) and indirect heterogeneity (network heterogeneity) were suggested to be low in FePP-placebo, EI-$FeSO_4$, FePP-$FeSO_4$, fumarate-NaFeEDTA, and FePP-fumarate, moderate in NaFeEDTA-$FeSO_4$ and fumarate-EI, and high in the remaining comparisons (Figure 3.6). In terms of transitivity (Figure 3.7), most of the comparisons had similar durations, while FePP-fumarate and fumarate-NaFeEDTA had relatively low duration means. Meta regression of duration had no impact on the network estimation. No significant publication bias was found in the funnel plot (Figure 3.8).

As shown in Figure 3.9 and Figure 3.10, the percentage of high risk of bias for EI-

$FeSO_4$, EI-NaFeEDTA, EI-placebo, $FeSO_4$-NaFeEDTA, $FeSO_4$-placebo, and NaFeEDTA-placebo were large. The percentage of high risk of bias for EI-fumarate, FePP-$FeSO_4$, fumarate-NafeEDTA, EI-FePP, FePP-NaFeEDTA, and $FeSO_4$-fumarate were moderate. The percentage of high risk of bias for FePP-fumarate, FePP-placebo, and fumarate-placebo were small. The certainty of network meta-analysis evidence was evaluated to be at very low level in EI-placebo, low in EI-$FeSO_4$, EI-NaFeEDTA, $FeSO_4$-NaFeEDTA, and $FeSO_4$-Placebo, and moderate in the remaining comparisons (Table 3.6).

3.5 Discussion

3.5.1 Characteristics and quality assessments of included studies

Based on 2749 initial articles, this systematic review included 145 clinical trials. The WHO reported that children in Asian and African were most affected by anemia. In 2019, 186 million children in Asia and Africa suffered from anemia, so most of participants were Asian and African populations under 18 years old.[133]. Since the major food matrix was the grain-based diet, the data of 46 grain-based studies including 24107 under-18 years old participants treated with iron fortifications was extracted for the network meta-analysis. In general, the average life span of red blood cells is around 4 months. Macrophages can clear old red blood cells and perform iron recycle for new erythroblasts in the circulation. Thus, 4-6 months intervention duration was chosen by most researchers to meet the erythrocyte iron turnover.[134;135]

Iron fortification is one of the preferred approaches to combat iron deficiency. The current network meta-analysis focused on 5 common iron fortificants ($FeSO_4$, EI, NaFeEDTA, ferrous fumarate, and FePP). Based on bioavailability, they could be divided into highly bioavailable iron fortificants such as NaFeEDTA, ferrous fumarate, and $FeSO_4$, and those with poor bioavailability such as FePP and EI.[136;137] They had been strongly recommended with a licensed dosage by WHO, United States Department of Agriculture (USDA), and United States Agency for International Development Organization in multiple guidelines and Food Aid Quality Review.[138–140]

Sample size should be estimated in preparatory phase. It is based on previously reported studies. This step was not considered in nearly half of studies. If the participants are more than required, it might be a waste of research resources. If sample size is too small, the results

of study might not be accurate. High dropout rate can reduce the generalizability of result. Inadequate sample size or imbalance intergroup sample size might give rise to the weak statistical powder. Ethical approval was not stated in some studies published from 1970s to1980s. Probably, it was not a requirement of publication at that time. However, some ethical paradoxes need to be further justified. For example, it needs to be verified whether recruiting children from juvenile criminal school and orphanage violate ethical rules.[141] In most of studies, healthy participants and anemia participants were involved. Considering ethics, should some participants with anemia take iron supplementation rather than iron fortifications? Therefore, some studies take anemia status as a cluster factor. Others cluster factors such as age and gender were also employed, while many clinical trials chose a completely randomized design to examine the efficacy of one major factors without taking covariant factor into account.

Most clinical trials had a moderate or high-level risks of bias due to unknown randomized sequence generation, unclear allocation method, and unblinded design. Specific randomization and allocation methods such as "generating a random number table from Excel" and "employing the sealed envelope system to assign the treatments" should be illustrated. The unblinded method in clinical trials might taint the results due to the participant bias and observer bias.

Moreover, the table of direct effect indicated that NaFeEDTA, ferrous fumarate, $FeSO_4$, and FePP fortificants were significantly higher than placebo in Hb and serum ferritin. Based on these individual direct comparisons, two hypotheses were proposed, namely, "Do these iron fortificants have positive overall effects of outcomes?" and "What is the rank order of iron fortificants in the effects of outcomes?". Network meta-analysis was used to verify these hypotheses with both direct and indirect comparisons.[142;143]

3.5.2 Network meta-analysis results

46 grain-based clinical trials contributed the Hb data to this network meta-analysis. These studies were combined and extended in a network plot to provide intuitive and visual understandings on the direct and indirect associations of multiple iron-treatment effects. The overall Hb effects of pairwise meta-analysis in the forest plot aligned the individual effects in (Table 3.4). $FeSO_4$, NaFeEDTA, ferrous fumarate, and FePP were effective iron treatments. In a recent Cochrane systemic review with pairwise meta-analysis conducted by Deregil et al. 13 clinical trials and 5810 children were included. It showed that NaFeEDTA, $FeSO_4$, and ferrous fumarate working as point-of-use food fortifications could alleviate the

risk of anemia prevalence.[144] Similarly, da Silva et al. summarized 75 systematic reviews and reported that $FeSO_4$, NaFeEDTA, ferrous fumarate, and FePP could increase Hb levels and control anemia in infants, children, and women.[145] Controversially, in Garcia-Casa et al.'s pairwise meta-analysis, the effects of maize flour fortified with $FeSO_4$, NaFeEDTA, and ferrous fumarate were uncertain in over 2-year-old children and adults.[146]

In population under 18 years old consuming grain-based fortification, NaFeEDTA was suggested to be the most effective iron fortificant, followed by ferrous fumarate, FePP, $FeSO_4$, and EI based on the results of SUCRA analysis. NaFeEDTA had the superior iron bioavailability due to its iron-EDTA chelate structure. The binding of ferric iron with EDTA is favorable in human gastrointestinal tract. EDTA can protect the non-heme iron from the iron inhibitors. The absorption of NaFeEDTA is usually 2 times higher than that of $FeSO_4$.[62;92;97] Ferrous fumarate and FePP also showed good efficacy with the assistance of encapsulation, micronize-sized technique, and iron enhancer (e.g., ascorbic acid). Encapsulation could block the interactions between iron fortificants and food components, especially iron inhibitors. Micronize-sized iron fortificants had a larger surface area, thus making iron fortificants easier to be hydrolyzed in gastrointestinal tract.[90;91] Enhancers can work as a reducing agent or a chelate to improve iron absorption. $FeSO_4$ could be dissolved in water to supply highly bioavailable iron but it had poor sensory properties.

Except for the short intervention duration of the FePP-fumarate and fumarate-NaFeEDTA (<2.5 months), the intervention duration of the other comparison was similar duration (3-6 months), so the transitivity in duration was balanced. Meanwhile, except for $FeSO_4$-placebo, EI-placebo, and EI-NaFeEDTA comparisons, there is no direct and indirect conflict of similarity in the remaining comparisons. However, more than half of the comparisons had the moderate or high-level heterogeneities. The variation of intergroup and intragroup in effect might not be the same across all comparisons.

3.5.3 Certainty of evidence and limitations

In the current systemic review, the moderate and high-level overall risk of bias, the moderate and high heterogeneities, the concerns of imprecision and inconsistency, and other limitations downgrade the evidence level of network meta-analysis. The predominant limitation of this systemic review was the small number of included studies. Due to insufficient data, sensitivity analysis, transitivity analysis and meta regression of other impact facts, network meta-analyses of secondary outcomes, and the meta-analysis of subgroup data were not conducted. Additionally, the Hb concentration of residents in plateau was physiologically

higher than that of residents in flat areas. Thus, diagnosis criteria of anemia might need to be adjusted in some studies according to elevation. New clinical trials will be included to update this network meta-analysis every 3-5 years.

3.6 Conclusion

The results of this systematic review and network meta-analysis provided the comprehensive evidence on the efficacy of iron fortificants. NaFeEDTA, ferrous fumarate, FePP and $FeSO_4$ fortifiactions were effective treatments in Hb for people with iron deficiency or healthy people with high risk of iron deficiency. In grain-based studies, NaFeEDTA was suggested to be the most effective iron fortificant, followed by ferrous fumarate, FePP and $FeSO_4$ in population under 18 years old. The certainty of network meta-analysis evidence was downgraded due to significant inconsistency, unsolvable risk of biases, and moderate or high heterogeneities in part of comparisons. Moreover, limited information and insufficient data reduced the quality of network meta-analysis. Therefore, the results of network meta-analysis should be applied with caution as conditional recommendations. Nevertheless, this clinical systemic review with network meta-analysis is still a crucial footstone of the iron fortification clinical research. More high-quality clinical trials are needed to elevate the evidence level of the network meta-analysis results, explore innovative frameworks for the prevention, therapy, and management on iron deficiency, and support the global implementations of the comprehensive frameworks through multisectoral anemia action alliances.

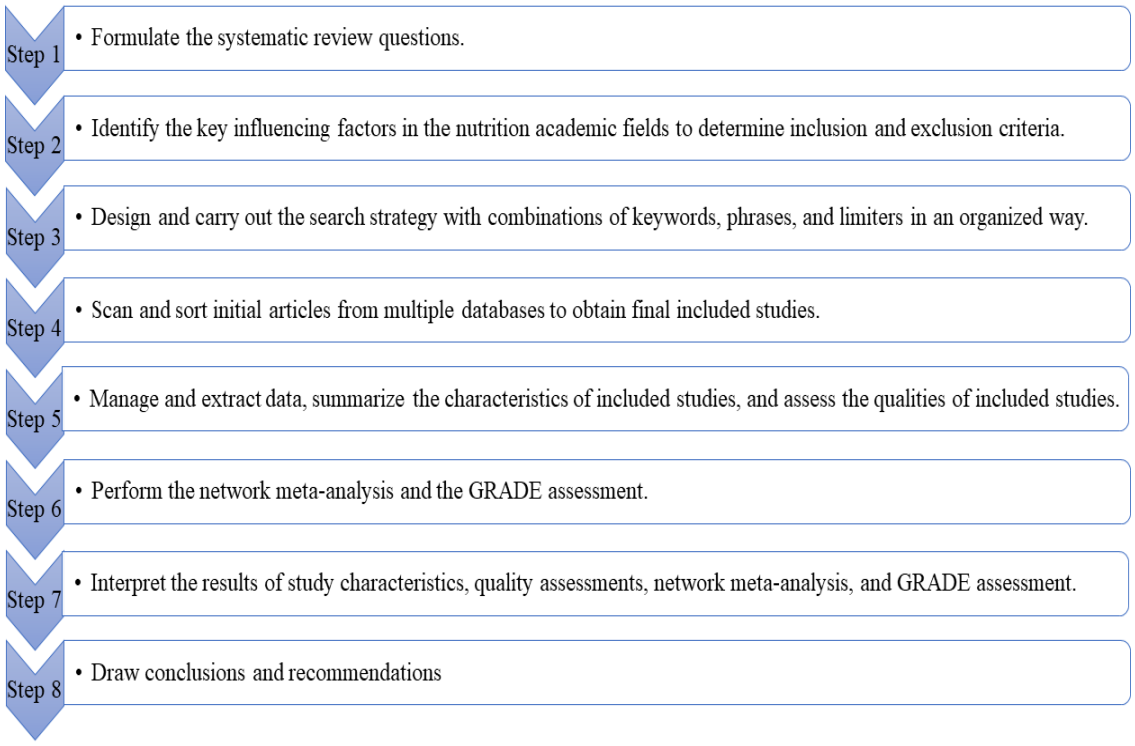


Figure 3.1: Flow diagram of steps in the clinical systematic review process

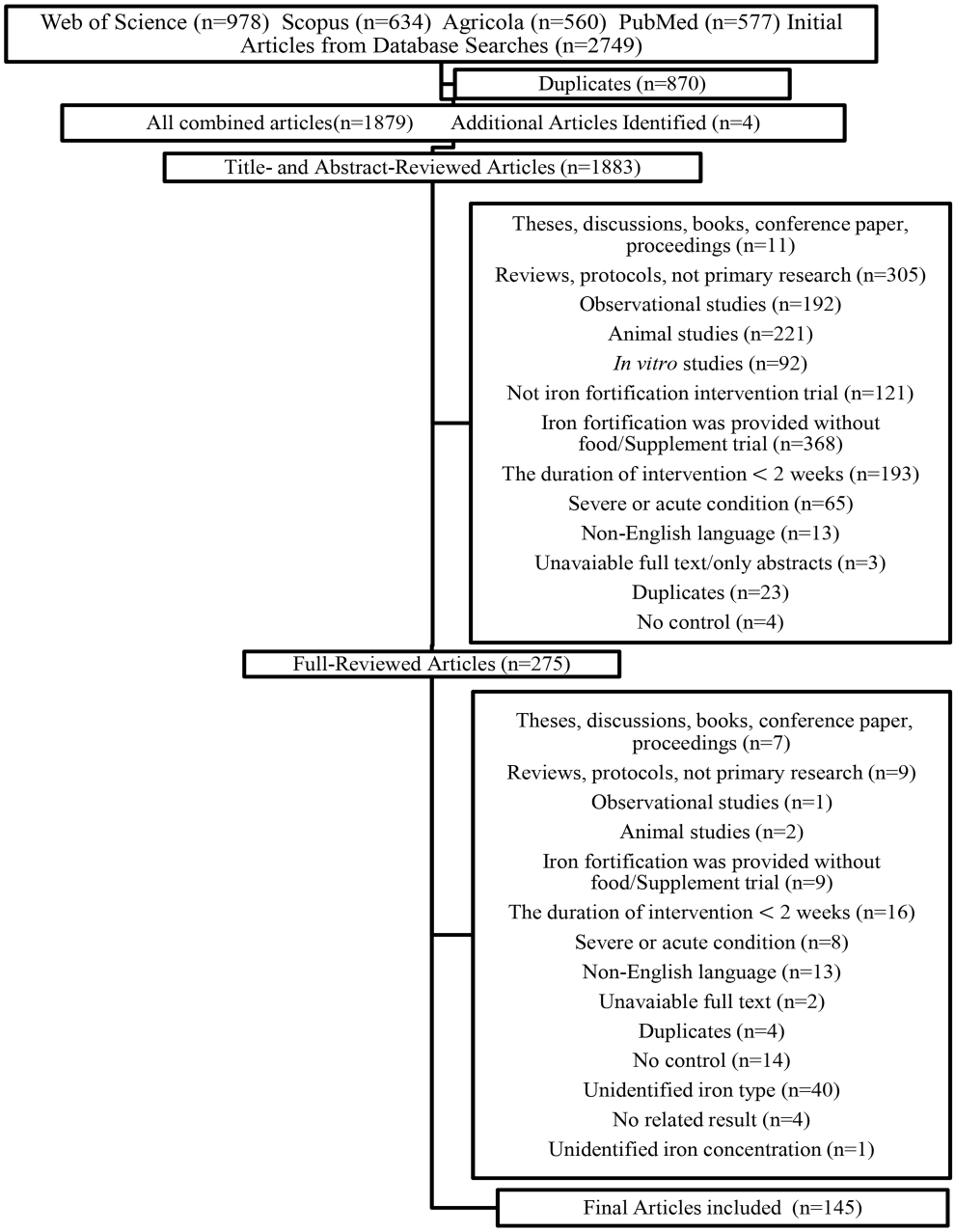


Figure 3.2: Flow diagram of scanning and sorting studies

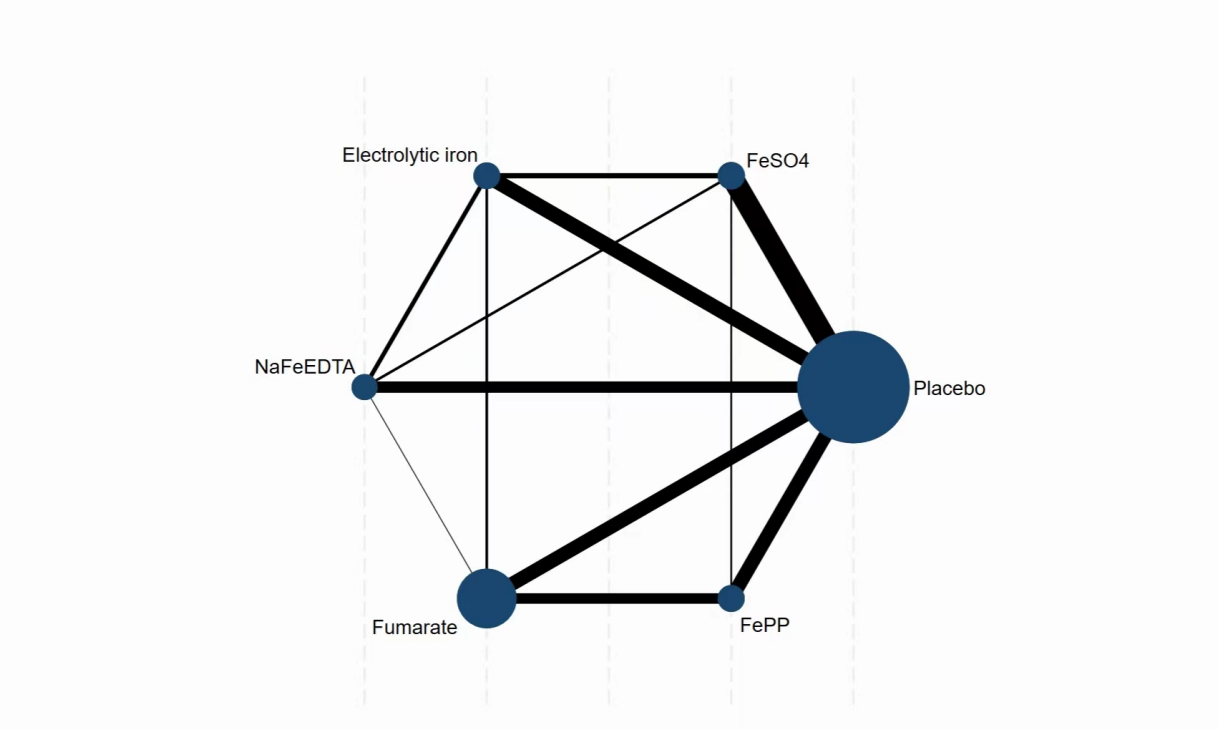
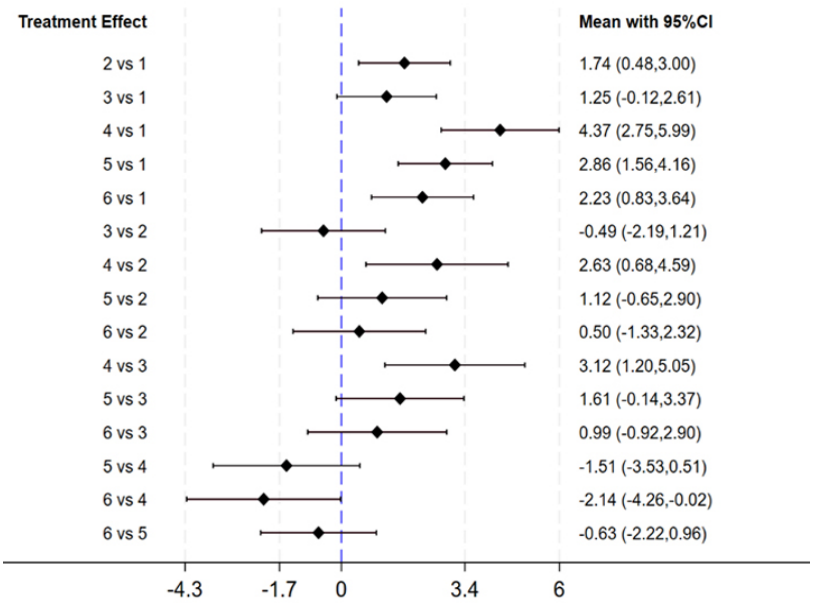


Figure 3.3: Network plot of comparisons in Hb



Note: "2 vs 1" represented FeSO4 vs placebo. "3 vs 1" represented electrolytic iron vs placebo. "4 vs 1" represented NaFeEDTA vs placebo."5 vs 1" represented Fumarate vs placebo. "6 vs 1" represented FePP vs placebo. "3 vs 2" represented electrolytic iron vs FeSO4. "4 vs 2" represented NaFeEDTA vs FeSO4. "6 vs 2" represented Fepp vs FeSO4. "4 vs 3" represented NaFeEDTA vs electrolytic iron. "5 vs 3" represented Fumarate vs electrolytic iron. "5 vs 4" represented Fumarate vs NaFeEDTA. "6 vs 5" represented FePP vs Fumarate.

Figure 3.4: Forest plot of comparisons in Hb

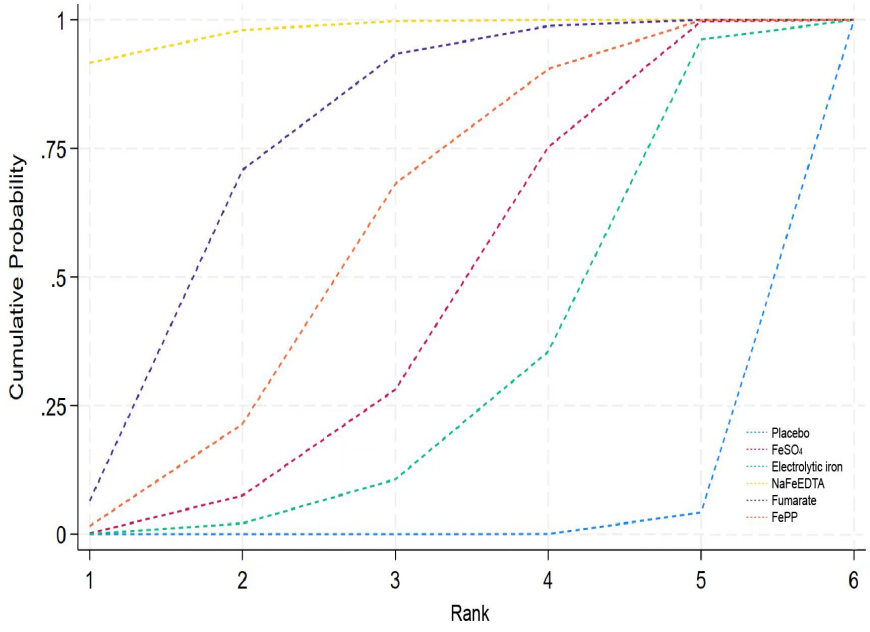
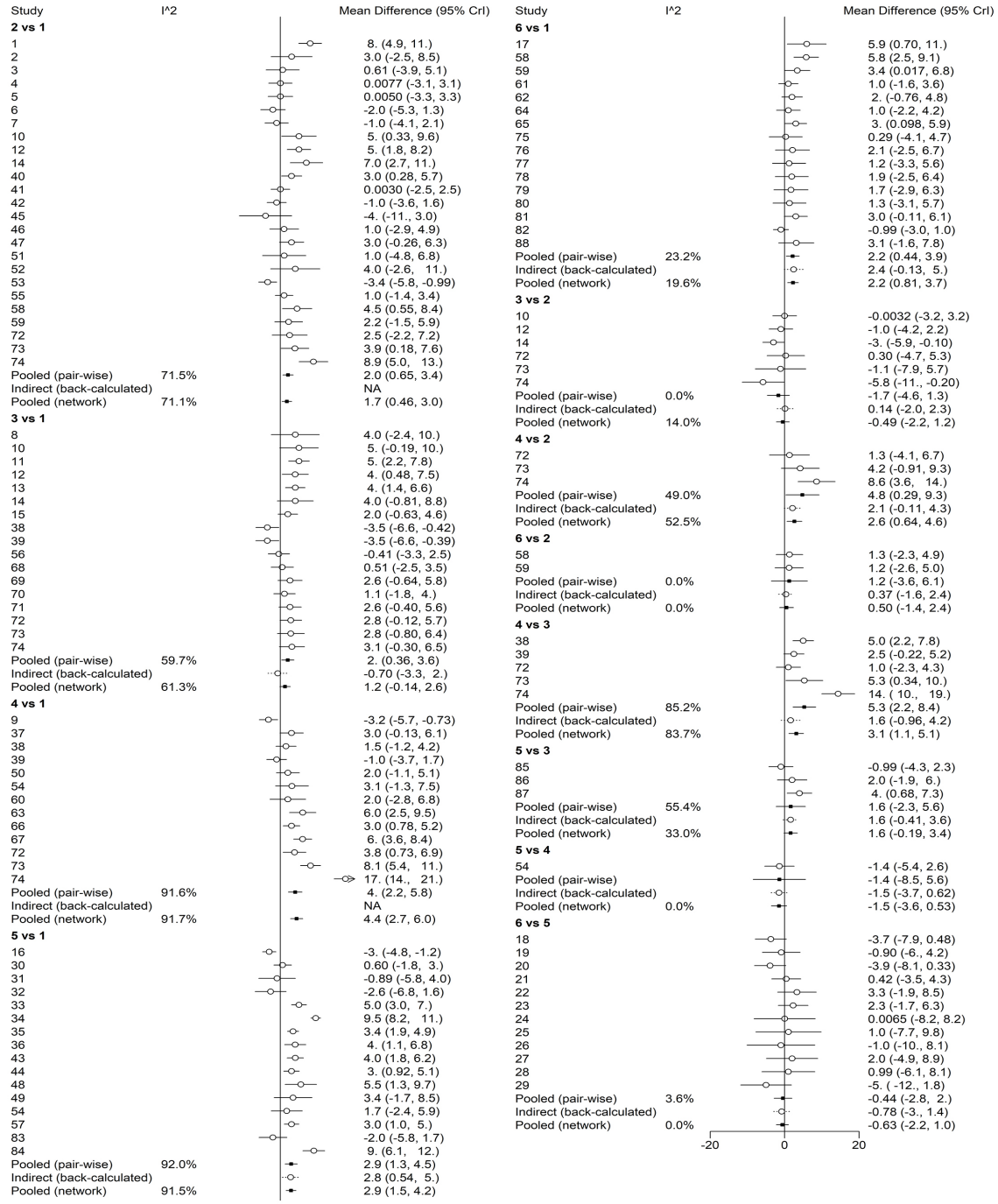


Figure 3.5: SUCRA rank analysis plot



Note: “2 vs 1” represented FeSO4 vs placebo. “3 vs 1” represented electrolytic iron vs placebo. “4 vs 1” represented NaFeEDTA vs placebo. “5 vs 1” represented Fumarate vs placebo. “6 vs 1” represented FePP vs placebo. “3 vs 2” represented electrolytic iron vs FeSO4. “4 vs 2” represented NaFeEDTA vs FeSO4. “6 vs 2” represented Fepp vs FeSO4. “4 vs 3” represented NaFeEDTA vs electrolytic iron. “5 vs 3” represented Fumarate vs electrolytic iron. “5 vs 4” represented Fumarate vs NaFeEDTA. “6 vs 5” represented FePP vs Fumarate.

Figure 3.6: Side-split heterogeneity analysis

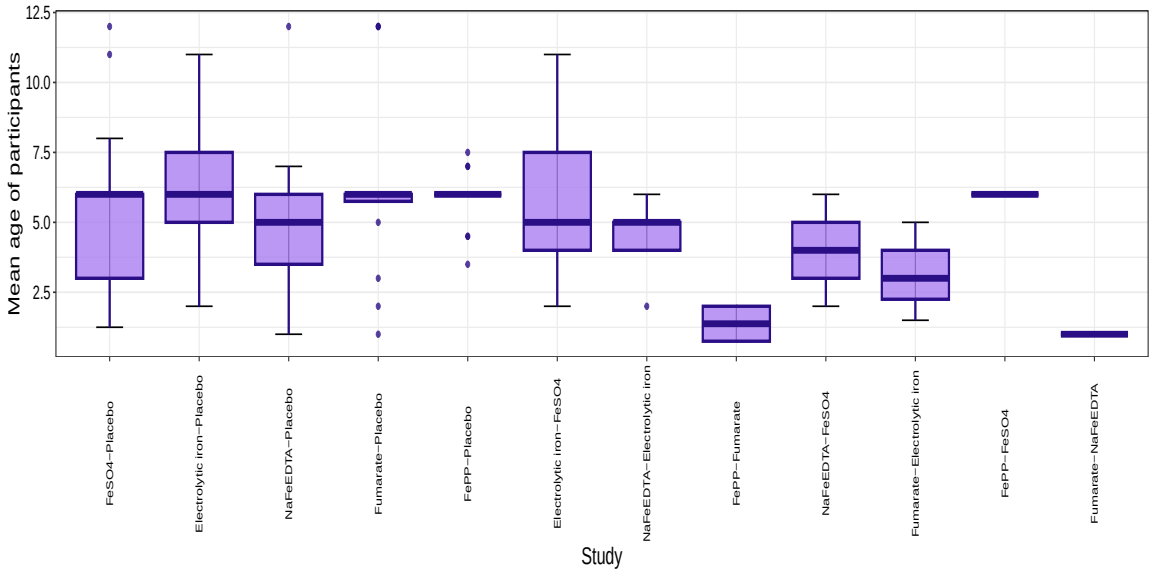


Figure 3.7: Transitivity analysis of durationt

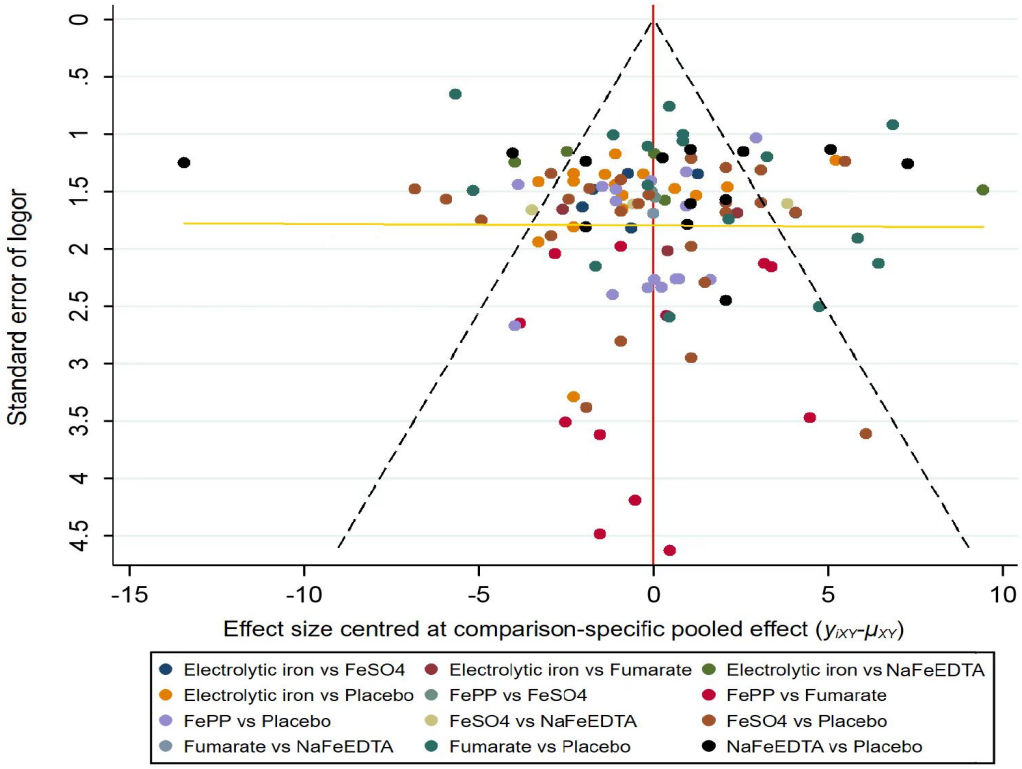


Figure 3.8: Funnel plot

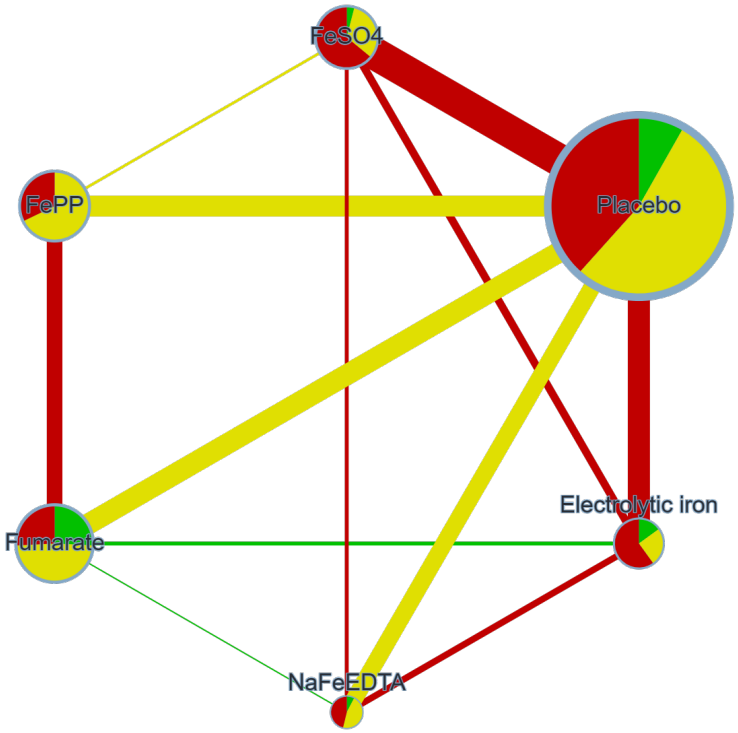


Figure 3.9: Network plot of risk of bias percentage

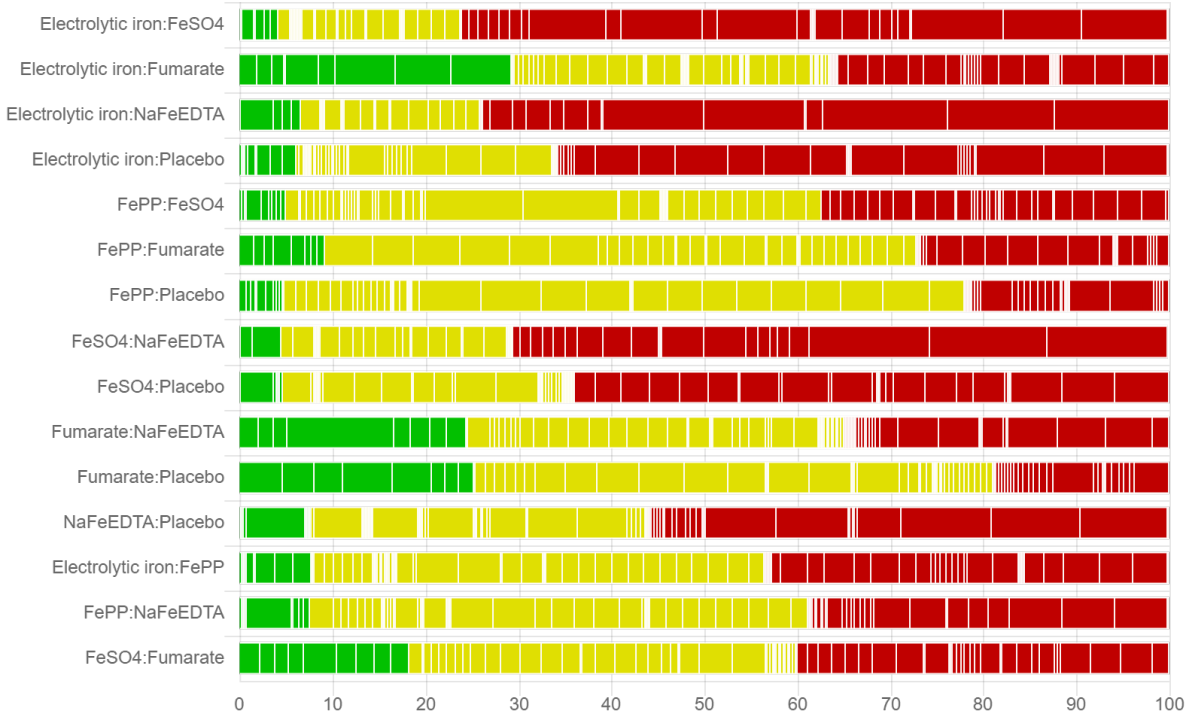


Figure 3.10: Bar diagram of risk of bias percentage

Table 3.1: *Basic characteristics of the included studies*

Study ID	Age (month, year)	Gender	Enrollment number	Intervention number	Drop rate (%)	Study setting	Study type	Arm	Sample size calculation	Anemia condition
1	Birth-2 months	M&F	347	347	0.0%	USA	Cluster	3	No	No
2	Birth-4 months	M&F	96	88	8.3%	Austria	Completely	2	No	No
3	6-9 months	M&F	335	306	8.7%	Nepal	Cluster	3	Yes	Yes (portion)
4	6.5-9.5 months	M&F	80	75	6.3%	Kenya	Completely	3	No	Yes (portion)
5	6 months	M&F	200	170	15.0%	Palestine	Completely	2	Yes	Yes (portion)
6	4 months	M&F	183	130	29.0%	Pakistan	Completely	3	No	Yes (portion)
7	6-12 months	M&F	361	289	19.9%	South Africa	Completely	2	Yes	Yes (portion)
8	4 months	M&F	95	65	31.6%	USA	Completely	2	Yes	Yes (portion)
9	6 months	M&F	287	170	40.8%	Kenya	Completely	2	Yes	Yes (portion)
10	6-12 months	M&F	216	117	45.8%	Ghana	Completely	4	No	Yes (portion)
11	6-7 months	M&F	225	115	48.9%	Guatemalan	Cluster	4	No	No
12	1-6 months	M&F	56	56	0.0%	Sweden	Completely	5	No	No
13	1 month	M&F	1239	394	68.2%	Chile	Completely	2	No	Yes (portion)
14	6 months	M&F	1179	835	29.2%	Chile	Completely	2	No	No
15	6 months	M&F	92	50	45.7%	UK	Completely	2	No	Yes (portion)
16	1-2 months	M&F	50	50	0.0%	Sweden	Completely	6	No	Yes (portion)

Table 3.1: *Basic characteristics of the included studies(continued)*

Study ID	Age (month, year)	Gender	Enrollment number	Intervention number	Drop rate (%)	Study setting	Study type	Arm	Sample size calculation	Anemia condition
17	6 months	M&F	164	139	15.2%	Honduras	Cluster	2	No	Yes (portion)
18	<3 months	M&F	554	400	27.8%	Chile	Completely	4	No	Yes (portion)
19	<3 months	M&F	554	270	51.3%	Chile	Completely	2	No	Yes (portion)
20	6-12 months	M&F	234	211	9.8%	North Korea	Completely	3	No	Yes (portion)
21	1 months	M&F	152	136	10.5%	USA	Completely	3	Yes	No
22	6-18 months	M&F	102	102	0.0%	Canada	Completely	2	Yes	No
23	6-18 months	M&F	213	208	2.3%	Ghana	Cluster	2	Yes	No
24	4 months	M&F	515	340	34.0%	Chile	Completely	5	No	No
25	6-18 months	M&F	4360	4292	1.6%	India	Cluster	2	Yes	Yes (portion)
26	6-18 months	M&F	304	239	21.4%	Ghana	Completely	2	Yes	Yes (all)
27	6-13 months	M&F	226	162	28.3%	China	Completely	2	No	No
28	10-23 months	M&F	171	142	17.0%	Brazil	Completely	2	Yes	No
29	6-24 months	M&F	196	175	10.7%	Brazil	Completely	2	Yes	Yes (portion)
30	6-18 months	M&F	432	381	11.8%	India	Cluster	5	Yes	Yes (portion)
31	10-23 months	M&F	216	188	13.0%	Brazil	Cluster	2	Yes	Yes (portion)
32	6-18 months	M&F	362	313	13.5%	Iran	Completely	3	Yes	Yes (portion)

Table 3.1: *Basic characteristics of the included studies(continued)*

Study ID	Age (month, year)	Gender	Enrollment number	Intervention number	Drop rate (%)	Study setting	Study type	Arm	Sample size calculation	Anemia condition
33	<24 months	M&F	133	118	11.3%	Ghana	Cluster	5	Yes	Yes (portion)
34	6-23 months	M&F	1704	1288	24.4%	Thailand	Completely	2	No	Yes (portion)
35	6-24 months	M&F	362	338	6.6%	Bangladesh	Cluster	3	Yes	Yes (portion)
36	6-23 months	M&F	407	345	15.2%	Gambia	Cluster	3	Yes	Yes (portion)
37	6-11 months	M&F	344	259	24.7%	Ethiopia	Completely	2	Yes	Yes (portion)
38	7-24 months	M&F	235	157	33.2%	Switzerland	Completely	3	Yes	No
39	6-18 months	M&F	557	493	11.5%	Ghana	Completely	2	Yes	Yes (portion)
40	8-20 months	M&F	437	324	25.9%	Ghana	Completely	4	Yes	No
41	6 months	M&F	743	576	22.5%	Zambian	Completely	2	Yes	Yes (portion)
42	12-23 months	M&F	70	70	0.0%	Kenya	Completely	3	Yes	Yes (portion)
43	6-8 months	M&F	193	112	42.0%	Brazil	Completely	2	Yes	Yes (portion)
44	12-36 months	M&F	133	128	3.8%	Guatemalan	Cluster	3	Yes	Yes (portion)
45	12-24 months	M&F	152	136	10.5%	Bangladesh	Cluster	2	Yes	Yes (portion)
46	12-20 months	M&F	225	205	8.9%	Newzealand	Cluster	3	Yes	No
47	18 months	M&F	752	750	0.3%	Bangladesh	Cluster	5	No	Yes (portion)
48	12-36 months	M&F	184	105	42.9%	Nigeria	Cluster	3	Yes	Yes (portion)

Table 3.1: *Basic characteristics of the included studies(continued)*

Study ID	Age (month, year)	Gender	Enrollment number	Intervention number	Drop rate (%)	Study setting	Study type	Arm	Sample size calculation	Anemia condition
49	12-30 months	M&F	486	415	14.6%	Mexico	Completely	3	Yes	No
50	12-36 months	M&F	338	315	6.8%	Kenya	Cluster	3	No	Yes (portion)
51	12-36 months	M&F	629	403	35.9%	Cote-dlvoire	Cluster	5	Yes	Yes (portion)
52	12-30 months	M&F	795	567	28.7%	Mexico	Cluster	2	No	Yes (portion)
53	6-35 months	M&F	2220	1958	11.8%	Ghana	Cluster	2	No	Yes (portion)
54	6-36 months	M&F	2283	1869	18.1%	Kyrgyz	Cluster	2	Yes	Yes (portion)
55	6-42 months	M&F	169	151	10.7%	Brazil	Cluster	2	Yes	Yes (portion)
56	6-35 months	M&F	1958	1806	7.8%	Ghana	Cluster	2	Yes	Yes (portion)
57	6-35 months	M&F	1420	862	39.3%	Kenya	Cluster	2	Yes	Yes (portion)
58	10-30 months	M&F	154	115	25.3%	Mexico	Completely	2	Yes	No
59	10-48 months	M&F	94	93	1.1%	Brazil	Completely	2	No	Yes (portion)
60	24-53 months	M&F	2186	1873	14.3%	Tanzania	Cluster	16	No	Yes (portion)
61	1.5-6 years	M&F	205	153	25.4%	Come-roon	Cluster	2	Yes	Yes (portion)
62	1-6 years	M&F	335	150	55.2%	Brazil	Completely	2	No	No
63	2-5 years	M&F	279	239	14.3%	Kenya	Cluster	3	Yes	No
64	2-5 years	M&F	327	314	4.0%	Bang-ladesh	Completely	2	Yes	Yes (portion)

Table 3.1: *Basic characteristics of the included studies(continued)*

Study ID	Age (month, year)	Gender	Enrollment number	Intervention number	Drop rate (%)	Study setting	Study type	Arm	Sample size calculation	Anemia condition
65	2-5 years	M&F	332	307	7.5%	Brazil	Cluster	4	Yes	Yes (portion)
66	2-5 years	M&F	270	259	4.1%	Brazil	Cluster	2	No	Yes (portion)
67	3-6 years	M&F	415	415	0.0%	China	Cluster	3	Yes	Yes (portion)
68	4 years	M&F	131	120	8.4%	Brazil	Cluster	2	No	Yes (portion)
69	36-66 months	M&F	684	513	25.0%	India	Cluster	2	Yes	Yes (portion)
70	3-6 years	M&F	47	40	14.9%	Mexico	Completely	3	No	No
71	2-6 years	M&F	268	173	35.4%	Brazil	Cluster	2	Yes	Yes (portion)
72	3-12 years	M&F	200	192	4.0%	South Africa	Completely	2	Yes	Yes (portion)
73	3-8 years	M&F	515	505	1.9%	Kenya	Completely	4	No	Yes (portion)
74	3-7 years	M&F	31	24	22.6%	USA	Completely	2	No	Yes (portion)
75	5-9 years	M&F	136	136	0.0%	India	Completely	2	Yes	Yes (portion)
76	5-15 years	M&F	207	123	40.6%	Cote-dlvoire	Completely	2	No	Yes (portion)
77	5-15 years	M&F	245	245	0.0%	India	Completely	2	Yes	Yes (portion)
78	5-11 years	M&F	140	128	8.6%	India	Cluster	2	Yes	Yes (portion)
79	5-13 years	M&F	566	534	5.7%	Nigeria	Cluster	2	Yes	Yes (portion)
80	5-13 years	M&F	186	130	30.1%	India	Completely	2	No	Yes (portion)

Table 3.1: *Basic characteristics of the included studies(continued)*

Study ID	**Age (month, year)**	**Gender**	**Enrollment number**	**Intervention number**	**Drop rate (%)**	**Study setting**	**Study type**	**Arm**	**Sample size calculation**	**Anemia condition**
81	5-12 years	M&F	241	224	7.1%	Ghana	Cluster	2	Yes	Yes (portion)
82	6-10 years	M&F	375	300	20.0%	Pakistan	Cluster	4	No	No
83	7-10 years	M&F	227	224	1.3%	India	Cluster	2	Yes	Yes (portion)
84	6-8 years	M&F	247	179	27.5%	South Africa	Completely	4	No	Yes (portion)
85	11-17 years	M&F	304	240	21.1%	China	Completely	3	No	Yes (portion)
86	6-9 years	M&F	403	384	4.7%	Vietnam	Completely	2	Yes	Yes (portion)
87	6-11 years	M&F	361	339	6.1%	South Africa	Cluster	4	Yes	Yes (portion)
88	6-9 years	M&F	180	171	5.0%	Phil-lippines	Completely	3	Yes	Yes (portion)
89	6-11 years	M&F	471	425	9.8%	Vietnam	Completely	5	Yes	Yes (portion)
90	6-11 years	M&F	252	228	9.5%	South Africa	Cluster	2	Yes	Yes (portion)
91	5-15 years	M&F	1645	546	66.8%	India	Completely	2	No	Yes (portion)
92	5-18 years	M&F	402	402	0.0%	India	Completely	2	Yes	Yes (portion)
93	7-11 years	M&F	123	123	0.0%	India	Completely	2	Yes	Yes (portion)
94	6-11 years	M&F	160	153	4.4%	South Africa	Completely	3	Yes	Yes (portion)
95	7-11 years	M&F	129	129	0.0%	India	Completely	2	No	Yes (portion)
96	6-15 years	M&F	382	353	7.6%	Mexico	Completely	2	Yes	Yes (portion)

Table 3.1: *Basic characteristics of the included studies(continued)*

Study ID	Age (month, year)	Gender	Enrollment number	Intervention number	Drop rate (%)	Study setting	Study type	Arm	Sample size calculation	Anemia condition
97	6-14 years	M&F	640	591	7.7%	Cote-dlvoire	Completely	8	Yes	Yes (portion)
98	11-18 years	M&F	418	418	0.0%	China	Completely	4	No	Yes (portion)
99	6-21 years	M&F	140	137	2.1%	Cambodia	Completely	3	Yes	Yes (portion)
100	6-16 years	M&F	2501	2380	4.8%	Cambodia	Cluster	5	Yes	Yes (portion)
101	6-12 years	M&F	258	232	10.1%	India	Cluster	3	Yes	Yes (portion)
102	6-12 years	M&F	250	238	4.8%	Phillippines	Cluster	4	Yes	Yes (portion)
103	6-13 years	M&F	179	179	0.0%	India	Completely	2	No	Yes (portion)
104	6-13 years	M&F	184	184	0.0%	India	Cluster	2	Yes	Yes (portion)
105	6-15 years	M&F	401	379	5.5%	India	Cluster	2	No	Yes (portion)
106	6-15 years	M&F	351	334	4.8%	Bangladesh	Cluster	2	Yes	Yes (portion)
107	6-14 years	M&F	159	157	1.3%	Morocoo	Completely	2	No	No
108	6-15 years	M&F	377	367	2.7%	Morocoo	Completely	2	No	No
109	6-15 years	M&F	163	163	0.0%	Morocoo	Completely	2	No	Yes (portion)
110	5-15 years	M&F	458	345	24.7%	India	Completely	3	Yes	No
111	11-17 years	M&F	1071	904	15.6%	Burundi	Cluster	2	No	No
112	Family	M&F	212	212	0.0%	India	Cluster	2	Yes	Yes (portion)
113	Family	M&F	3228	2005	37.9%	Srilanka	Cluster	3	Yes	Yes (portion)

Table 3.1: *Basic characteristics of the included studies(continued)*

Study ID	Age (month, year)	Gender	Enrollment number	Intervention number	Drop rate (%)	Study setting	Study type	Arm	Sample size calculation	Anemia condition
114	Family	M&F	88	88	0.0%	Brazil	Cluster	2	No	No
115	Family	M&F	14395	10714	25.6%	India	Cluster	2	No	Yes (portion)
116	Family	M&F	4000	3868	3.3%	China	Cluster	2	No	Yes (portion)
117	Family	M&F	984	598	39.2%	India	Cluster	2	Yes	Yes (portion)
118	Family	M&F	545	448	17.8%	China	Completely	2	Yes	Yes (portion)
119	>60 years	M&F	221	160	27.6%	USA	Cluster	2	No	Yes (all)
120	18-50 years	Female	304	237	22.0%	UK	Completely	2	No	Yes (portion)
121	18-55 years	Female	131	126	3.8%	India	Completely	2	No	Yes (portion)
122	18-35 years	Female	279	181	35.1%	Kuwaiti	Completely	3	No	Yes (portion)
123	18-49 years	Female	201	145	27.9%	Mexico	Completely	2	Yes	No
124	25-40 years	Female	417	273	34.5%	Norway	Completely	2	No	No
125	18-50 years	Female	330	330	0.0%	Thailand	Completely	3	No	Yes (portion)
126	18-25 years	Female	75	75	0.0%	Pakistan	Completely	5	No	Yes (portion)
127	18-35 years	Female	126	108	14.3%	Spain	Completely	2	Yes	No
128	18-45 years	Female	30	25	16.7%	Iran	Completely	2	No	No
129	18-45 years	Female	282	243	13.8%	Poland	Completely	2	No	No
130	18-35 years	Female	130	122	6.2%	Spain	Completely	2	Yes	Yes (portion)
131	18-35 years	Female	41	41	0.0%	Spain	Completely	2	No	Yes (portion)

Table 3.1: *Basic characteristics of the included studies(continued)*

Study ID	Age (month, year)	Gender	Enr-oll-ment num-ber	Inter-ven-tion num-ber	Drop rate (%)	Study setting	Study type	Arm	Sample size calcul-ation	Anemia condition
132	18-40 years	Female	142	105	26.1%	Swit-zerland	Completely	3	Yes	No
133	20-38 years	Female	44	43	2.3%	Denmark	Completely	2	No	No
134	18-44 years	Female	89	69	22.5%	Newz-ealand	Cluster	2	Yes	No
135	15-49 years	Female	51	51	0.0%	USA	Completely	5	No	No
136	12Y-P	Female	124	116	6.5%	China	Completely	2	Yes	Yes (all)
137	12-49 years	Female	152	136	10.5%	Vietnam	Completely	2	Yes	Yes (all)
138	16-49 years	Female	576	389	32.5%	Vietnam	Completely	2	Yes	No
139	18-50 years	Female	17	15	11.8%	USA	Completely	2	No	Yes (portion)
140	15-19 years	Female	140	123	12.1%	India	Completely	2	No	Yes (all)
141	18-55 years	Female	245	212	13.5%	India	Cluster	2	Yes	Yes (portion)
142	17-25 years	Female (soldier)	142	113	20.4%	USA	Cluster	2	No	Yes (portion)
143	Preg-anant	Female	106	96	9.4%	Nepal	Completely	2	No	Yes (portion)
144	Preg-anant	Female	70	70	0.0%	India	Completely	3	No	Yes (portion)
145	Preg-anant	Female	176	168	4.5%	Vietnam	Cluster	4	Yes	Yes (portion)

Note: "M&F" represented male and female. "12Y-P" represented 12 years old-premenopausal females.

Table 3.2: *Methodological characteristics of the included studies*

Study ID	**Duration (month)**	**Food matrix**	**Iron type**	**Control**	**Iron concentration**	
1	6, 12	Milk Formula	$FeSO_4$	Placebo	12.7, 7.4	mg/L
2	4, 6, 9, 12	Milk Formula	$FeSO_4$	Placebo	2	mg/100g food
3	6	Comple-mentary food	Encapsulated fumarate	Enhanced homestead food, Placebo	10	mg/sachet
4	4	Maize flour	Fumarate+ NaFeEDTA, fumarate+ NaFeEDTA +prebiotic	Placebo	5	mg/sachet
5	12	Semi solid food	Fumarate+ $FeSO_4$	$FeSO_4$	12,2	mg/sachet
6	8	Milk cereal	Fumarate, FePP	Placebo	7.5	mg/100 g food
7	6	Maize-porridge	Fumarate	Placebo	27.5	mg/100 g food
8	1.5,3,5	Cereal	Fumarate, EI	Each other	52.2, 54.5	mg/100 g food
9	12	Maize porridge	NaFeEDTA	Placebo	2.5	mg/sachet
10	6	Comple-mentary food	EI, fish powder, KOKO (fermented maize dough)	Placebo	18.3, 36.6	mg/100 g food
11	8	Maize	EI+whey protein, EI+bovine serum	Placebo	10	mg/day
12	3,5	Milk powder	$FeSO_4$, $FeSO_4$+ bovine lactoferrin, $FeSO_4$+ nucleotides	Placebo	2,4	mg/L
13	8, 14	Milk powder	$FeSO_4$	Placebo	15	mg/100g food

Table 3.2: *Methodological characteristics of the included studies(continued)*

Study ID	**Duration (month)**	**Food matrix**	**Iron type**	**Control**	**Iron concentration**	
14	6	Milk	$FeSO_4$ (12.7 mg/L)	$FeSO_4$ (2.3 mg/L)	12.7, 2.3	mg/L
15	1.25,2.5	Milk	$FeSO_4$	Placebo	12	mg/L
16	4.5	Milk powder	$FeSO_4$, bovine lactoferrin	Placebo	4, 7	mg/L
17	6	Comple-mentary food	$FeSO_4$ (exclusively breast-fed)	Placebo	5	mg/100g food
18	12	Milk powder	$FeSO_4$	Placebo	15	mg/100g food
19	12	Milk powder	$FeSO_4$	Placebo	15	mg/100g food
20	6	Rice	$FeSO_4$	Placebo	10	mg/day
21	3, 4.5, 6.5, 8	Fruit-Cereal	$FeSO_4$	$FeSO_4$ supplement, Placebo	7.5	mg/day
22	6	Comple-mentary food	Fumarate	Placebo	30	mg/100g food
23	6, 8	Cereal	Fumarate	Placebo	7.5	mg/day
24	4, 8, 11	Cereal and formula	Electrolytic Fe, $FeSO_4$	Placebo	55,12	mg/100g food
25	12	Comple-mentary food	Fumarate	Placebo	12.5	mg/day
26	2	Meal	Microenca-psulated fumarate	Fumarate+ zinc	8	mg/L
27	3	Rusk	Ferric ammonium citratecitrate	Placebo	0.29	mg/g
28	4.5	Rice	FePP	Placebo	56.4	mg/week
29	5	Rice	μFePP	Placebo	10	mg/day

Table 3.2: *Methodological characteristics of the included studies(continued)*

Study ID	Duration (month)	Food matrix	Iron type	Control	Iron concentration	
30	0.75, 2	Meal	Microencapsulated Fumarate, μFePP, ferrous glycine sulfate drops	Each other	12.5, 20, 30	mg
31	4.5	Rice	μFePP	Placebo	56.4	mg/week
32	4	Semi solid food	Microencapsulated Fumarate	$FeSO_4$ supplement	10,12.5	mg/day
33	0.75, 2	Semi solid food	Microencapsulated fumarate, FePP	$FeSO_4$ supplement	12.5, 20, 30	mg
34	9	Suitable food	Fumarate	Placebo	6	mg/sachet
35	2 (daily), 3 (flexibly), 4 (flexibly)	Rice	Fumarate (daily)	Fumarate (flexibly)	12.5	mg/sachet
36	2.5	Orange flavoured drink	Encapsulated fumarate	Placebo	6, 12	mg/day
37	6	Complementary food	Fumarate	Placebo	6	mg
38	9	Cereal	Fumarate, FePP, $FeSO_4$	Each other	9.3	mg/day
39	2	Complementary food	Microencapsulated fumarate	$FeSO_4$ supplement	40, 80	mg/day
40	6	Meal	Microencapsulated fumarate	Placebo, $FeSO_4$ supplement	40	mg/day
41	12	Porridge	Fumarate	Placebo	25	mg/100g food
42	4	Maize	NaFeEDTA	Placebo	4.2	mg/100g food

Table 3.2: *Methodological characteristics of the included studies(continued)*

Study ID	**Duration (month)**	**Food matrix**	**Iron type**	**Control**	**Iron concentration**	
43	2	Complementary food	Fumarate	Placebo	10	mg/sachet
44	1.25, 2.5	Black bean	$FeSO_4$, haem iron	Placebo	20	mg/100g food
45	2	Complementary food	Fumarate (high forfication level)	Fumarate (low forfication level)	4.3,12.5	mg/day
46	5	Milk	$FeSO_4$	Placebo	4.2-4.8	mg/day
47	12	Complementary food	NaFeEDTA+ famarate	Placebo	7.6	mg/L
48	6	Drinking water	$FeSO_4$ (High and moderate forfication levels)	$FeSO_4$ (Low forfication level)	2.24, 4.48, 6.72	mg/100ml
49	6	Complementary food	$FeSO_4$, ferrous gluconate	Placebo	10	mg/day
50	1	Semi solid food	NaFeEDTA, encapsulated fumarate	Placebo	3,12.5	mg/day
51	9	Complementary food	NaFeEDTA+ Fumarate, NaFeEDTA+ FePP	Placebo	2,3.8	mg
52	12	Milk	Ferrous gluconate	Placebo	5.28	mg
53	5	Semi liquid food	Fumarate	Placebo	12.5	mg/day
54	2	Complementary food	Fumarate	Placebo	12.5	mg/day
55	2	Rice	Fumarate	$FeSO_4$	1,4, 3.2, 4.2,12.5	mg/day

Table 3.2: *Methodological characteristics of the included studies(continued)*

Study ID	**Duration (month)**	**Food matrix**	**Iron type**	**Control**	**Iron concentration**	
56	5	Semi solid food	Fumarate	Placebo	12.5	mg/day
57	12	Staple food	Fumarate	Placebo	12.5	mg/day
58	6	Milk	Ferrous gluconate	Placebo	14.5	mg/L
59	6	Orange juice	Iron trisglycinate chelate+ level 1 sugar	Iron trisglycinate chelate+ level 2 sugar	100	mg/kg
60	5	Grain based food	Fumarate+ NaFeEDTA	Placebo	13	mg/100g food
61	3, 6	Cereal meal	Fumarate	Placebo	7.5	mg/day
62	6	Drinking water	$FeSO_4$	Placebo	50	mg/L
63	4	Maize	NaFeEDTA, amaranth	Placebo	6.9,23	mg
64	2	Meal	Fumarate (high forfication level)	Fumarate (low forfication level)	5,12.5	mg
65	4	Drinking water	$FeSO_4$ (high, moderate, low forfication level)	Placebo	5, 7.5, 10	mg
66	3	Meal	$FeSO_4$ (high forfication level)	$FeSO_4$ (low forfication level)	5,10	mg/day
67	3	Semi solid food	Fumarate (high, low forfication level)	Placebo	4.3,30	mg/day
68	3.5	Corn	$FeSO_4$	Placebo	10	mg
69	6	Rice	Fumarate	Placebo	7	mg/100g food

Table 3.2: *Methodological characteristics of the included studies(continued)*

Study ID	**Duration (month)**	**Food matrix**	**Iron type**	**Control**	**Iron concentration**	
70	2.5	Biscuit	$FeSO_4$, Heme Fe	Placebo	13.5, 6	mg
71	3, 6	Wheat	$FeSO_4$	Placebo	4	mg
72	6	Porridge	NaFeEDTA	Placebo	2.5	mg/day
73	5	Maize flour	NaFeEDTA, EI	Placebo	28, 56	mg/day
74	1.5, 3	Milk formula, soy formula	$FeSO_4$, ferrous bisglycinated (milk)	$FeSO_4$, ferrous bisglycinated (soy)	5,6	mg/day
75	6	Meal	Ferrous glucie phosphate	Placebo	28	mg/day
76	6	Salt	μFePP	Placebo	10.5	mg/day
77	12	Salt	$FeSO_4$ (chelated)	Placebo	1.1	mg/g
78	8	Rice	μFePP	Placebo	19	mg/day
79	6	Beverage	$FeSO_4$, ferrous bisglycinated	Placebo	14,17.5	mg/day
80	7.5	Rice	FePP	Placebo	15	mg/day
81	3.5	Cowpea meal	NaFeEDTA	Placebo	4.3	mg/day
82	1.5	Gum	$FeSO_4$, NaFeEDTA, $FeSO_4$+ NaFeEDTA	Placebo	30	mg/100g
83	5	Milk beverage	FePP	Placebo	18	mg/day
84	6,11	Soup powder	Fumarate	Placebo	20	mg/meal
85	3	Soy Sauce	NaFeEDTA	Placebo	5, 20	mg/day
86	6	Biscuit	Fumarate	Placebo, Fe supplement	30-40	mg/day
87	8.5	Bread	NaFeEDTA, fumarate, EI	Placebo	100, 200, 350	mg/100 g food
88	6	Rice	FePP, $FeSO_4$	Placebo	10	mg/day

Table 3.2: *Methodological characteristics of the included studies(continued)*

Study ID	**Duration (month)**	**Food matrix**	**Iron type**	**Control**	**Iron concentration**	
89	6	Noodle	NaFeEDTA	Placebo	20.6	mg/100g food
90	11	Biscuit	Fumarate	Placebo	13.1	mg/100g food
91	6, 12	Salt	$FePO_4$+ $FeSO_4$	Placebo	3500-5000	mg/kg
92	9	Salt	$FeSO_4$ (chelated)	Placebo	1	mg/g
93	12	Meal	$FeSO_4$	Placebo	10	mg/day
94	7.5	Bread	EI, ferrous bisglycinate	Placebo	3.66	mg/day
95	12	Salt	$FeSO_4$	Placebo	1	mg/g food
96	12	Milk	$Fe(II)Cl_2$	Placebo	14.3	mg/day
97	6	Biscuit	EI	Placebo, intermittent preventive and anthelmintic treatment	50	mg/day
98	2, 4, 6	Wheat flour	EI, NaFeEDTA, $FeSO_4$	Placebo	60, 20, 30	mg/kg
99	5	Fish sauce meal	NaFeEDTA, $FeSO_4$	Placebo	1	mg/ml
100	6	Rice	FePP	Placebo	10.7, 7.6, 7.5	mg/100g
101	6	Rice	FePP (high, low forfication level)	Placebo	6.25,12.5	mg/100g
102	8	Wheat flour	Hydrogen reduced Fe, EI, Fumarate	Placebo	80,80,40	mg/kg
103	7	Wheat meal	NaFeEDTA	Placebo	6	mg/100g
104	3.5, 7	Rice	μFePP	Placebo	5	mg/day
105	3.5, 7	Wheat meal	NaFeEDTA	Placebo	5	mg/day

Table 3.2: *Methodological characteristics of the included studies(continued)*

Study ID	**Duration (month)**	**Food matrix**	**Iron type**	**Control**	**Iron concentration**	
106	6	Wheat flour	Hydrogen reduced Fe	Placebo	66	mg/kg
107	5, 10	Salt	μFePP	Placebo	2	mg/g
108	5, 10	Salt	$FeSO_4$	Placebo	1	mg/g
109	5,10	Salt	μFePP	Placebo	2	mg/g
110	5, 10	Salt	μFePP, encapsulated fumarate	Placebo	5.7	mg/day
111	7	Rice	FePP	Placebo	17.8	mg/day
112	8	Salt	$FeSO_4$	Placebo	10	mg/10g
113	12, 24	Wheat flour	Hydrogen reduced Fe, EI	Placebo	66	mg/kg
114	4	Water	$FeSO_4$	Placebo	10	mg/L
115	12, 18	Salt	$FeSO_4$	Placebo	3.5	mg/g
116	6, 12, 18	Soy diet	NaFeEDTA	Placebo	29.6	mg/100g
117	12, 24	Curry powder	NaFeEDTA	Placebo	10	mg/g
118	6, 12, 18, 24, 30, 36	Wheat flour	EI	Placebo	20	mg/kg
119	6, 8	Wheat	$FeSO_4$	Placebo	15-22	mg/day
120	6, 12	White flour	Ferric ammonium citrate	Placebo, $FeSO_4$ supple-mentation	1	mg/day
121	10	Salt	Encapsulated fumarate	Placebo	3.3	mg/g
122	5.5	Biscuit	Encapsulated $FeSO_4$, hydrogen reduced Fe	Placebo	10, 20	mg/day
123	6	Rice	μFePP	Placebo	20	mg/day
124	5	Bread	$FeSO_4$	Placebo	6.2	mg/100g

Table 3.2: *Methodological characteristics of the included studies(continued)*

Study ID	**Duration (month)**	**Food matrix**	**Iron type**	**Control**	**Iron concentration**	
125	5, 9	Wheat flour	$FeSO_4$, hydrogen reduced Fe, electrolytic Fe	Placebo	12	mg/day
126	3	Wheat flour	NaFeEDTA, $FeSO_4$	Placebo	10, 20, 15, 30	mg/g
127	4	Milk	μFePP	μFePP+vitD	15	mg/day
128	2	Wheat flour	$FeSO_4$	Na_2EDTA	10	mg
129	6	Bread	$FeSO_4$	Placebo	15	mg/day
130	4	Fruit juice	Microencapsulated FePP	Placebo	18	mg/day
131	4	Fruit juice	Microencapsulated FePP	Placebo	18	mg/day
132	8	Margarine	μFePP, NaFeEDTA	Placebo	14	mg/day
133	2.5, 5	Bread	Fumarate	Placebo	6	mg/day
134	4	Cereal meal	$FeSO_4$+ kiwifruit (high level of ascorbic acid)	$FeSO_4$+ banana (high level of lutein and zeaxanthin)	16	mg/day
135	3	Wheat	Heme Fe, $FeSO_4$, hydrogen reduced Fe, EI	Placebo	5,50	
136	3	Drink	$FeSO_4$	Placebo	21	mg/day
137	3, 6	Fish sauce meal	NaFeEDTA	Placebo	10	mg
138	18	Fish sauce meal	NaFeEDTA	Placebo	9	mmol Fe/L
139	0.5	Rice	$FeSO_4$	Placebo	18	mg/100g
140	2	Meal	NaFeEDTA	Placebo	25	mg/day
141	7.5, 9	Salt	Fumarate	Placebo	3.3	mg/kg

Table 3.2: *Methodological characteristics of the included studies(continued)*

Study ID	**Duration (month)**	**Food matrix**	**Iron type**	**Control**	**Iron concentration**	
142	2.15	Food bar	$FeSO_4$	Placebo	56	mg/day
143	1.5	Rice	$FeSO_4$	Placebo	30	mg
144	3	Rice	$FeSO_4$	Placebo, $FeSO_4$ supplement	16	mg
145	4	Milk	Fumarate	Fuma tablet, $FeSO_4$ tablet, Placebo	15,60	mg/day

Table 3.3: *Characteristics of outcome effects*

Study ID	**Data contribution of network meta-analysis**	**Primary outcome (Hb g/L)**	**Secondary outcome**
1	No	No significant difference	Hematocrit (%), serum iron ($\mu g/L$), serum ferritin ($\mu g/dL$): no significant difference.
2	No	No significant difference	Hematocrit (%), serum ferritin ($\mu g/dL$): no significant difference.
3	Comparison No. 83: Femarate vs Placebo	No significant difference	Not reported
4	No	No significant difference	Plasma ferritin ($\mu g/L$): No significant difference
5	No	No significant difference	Plasma ferritin ($\mu g/L$): No significant difference
6	No	No significant difference	Serum ferritin ($\mu g/L$): No significant difference
7	Comparison No. 84: Femarate vs Placebo	Fumarate> placebo	Serum ferritin ($\mu g/L$): Fumarate> placebo
8	Comparison No. 85-87: Femarate vs Electrolytic iron	No significant difference	Plasma ferritin ($\mu g/L$), plasma iron ($\mu g/L$): No significant difference
9	Comparison No.9: Electrolytic iron vs Placebo	No significant difference	Serum iron ($\mu g/L$): No significant difference
10	Comparison No.8: NaFeEDTA vs Placebo	No significant difference	Plasma ferritin ($\mu g/L$), plasma transferrin saturation (%): No significant difference
11	No	No significant difference	Serum iron (μg/L): Electrolytic iron+whey protein<placebo, no significant difference between electrolytic iron+bovine serum and placebo.

Table 3.3: *Characteristics of outcome effects(continued)*

Study ID	**Data contribution of network meta-analysis**	**Primary outcome (Hb g/L)**	**Secondary outcome**
12	No	No significant difference	Serum iron (μg/L), total iron binding capacity (μmol/L) at 4 and 6 months: no significant difference. Serum ferritin (μg/L) at 4 months: $FeSO_4$ (4 mg/L)<placebo. Serum ferritin (μg/L) at 6 months: no significant difference
13	No	$FeSO_4$>placebo	Serum iron (μg/L): $FeSO_4$>placebo
14	No	$FeSO_4$ (12.7 mg/L) >>$FeSO_4$ (2.3 mg/L)	Serum ferritin (μg/L): $FeSO_4$ (12.7 mg/L)> $FeSO_4$ (2.3 mg/L)
15	Comparison No. 51-52	No significant difference	Serum iron (μg/L): no significant difference
16	No	No significant difference	Serum iron (μmol/L): no significant difference
17	Comparison No. 2-3	No significant difference	Hematocrit (%): no significant difference
18	No	$FeSO_4$>placebo	Serum iron (μg/L): $FeSO_4$>placebo
19	No	$FeSO_4$>placebo	Serum iron (μmol/L), plasma transferrin saturation (%), total iron binding capacity (μmol/L), serum ferritin (μg/L): $FeSO_4$>placebo
20	Comparison No.1	$FeSO_4$>placebo	Serum ferritin (mcg/L): $FeSO_4$ >placebo
21	Comparison No. 4-7	No significant difference	Serum ferritin (mcg/L): $FeSO_4$ >placebo
22	No	No significant difference	Serum ferritin (μg/L): no significant difference.
23	No	Change in Hb: fumarate> placebo	Not reported
24	Comparison No. 10-15	No significant difference	Tranferrin saturation (%) and Serum ferritin (μg/L): No significant difference.
25	Comparison No. 16	No significant difference	Not reported

Table 3.3: *Characteristics of outcome effects(continued)*

Study ID	**Data contribution of network meta-analysis**	**Primary outcome (Hb g/L)**	**Secondary outcome**
26	No	Fumarate> Fumarate+zinc	Not reported
27	No	No significant difference	Not reported
28	Comparison No. 17	FePP>placebo	Not reported
29	No	No significant difference	Not reported
30	Comparison No. 18-23	No significant difference	Serum ferritin (μg/mL): No significant difference.
31	Comparison No. 88	No significant difference	Not reported
32	No	No significant difference	Serum ferritin (μg/L): No significant difference.
33	Comparison No. 24-29	No significant difference	Serum ferritin (μg/L): No significant difference.
34	No	Fumarate> placebo	Fumarate>placebo
35	No	No significant difference	Not reported
36	No	Fumarate< placebo	Serum ferritin (μg/L), plasma iron (μmol/L), transferrin saturation (mg/L): fumarate<placebo.
37	Comparison No. 30	No significant difference	Serum iron (μg/L): fumarate<placebo
38	No	No significant difference	Plasma ferritin (μg/L): No significant difference
39	No	No significant difference	Serum ferritin (μg/L): fumarate<$FeSO_4$
40	Comparison No. 31-32	No significant difference	Serum ferritin (μg/L): No significant
41	Comparison No. 33: Femarate vs Placebo	Fumarate> placebo	Serum ferritin (μg/L): fumarate>placebo

Table 3.3: *Characteristics of outcome effects(continued)*

Study ID	**Data contribution of network meta-analysis**	**Primary outcome (Hb g/L)**	**Secondary outcome**
42	No	No significant difference	Serum ferritin (μg/L): NaFeEDTA>placebo
43	No	Not indicated	Plasma ferritin (μg/L): Fumarate>placebo
44	Comparison No. 50: Femarate vs Placebo	No significant difference	Serum ferritin (μg/L): No significant difference
45	No	No significant difference	Serum ferritin (μg/L): No significant difference
46	No	No significant difference	Serum ferritin (μg/L): $FeSO_4$>placebo
47	No	NaFeEDTA+ famarate> placebo	Not reported
48	No	$FeSO_4$ (High and moderate) >$FeSO_4$ (Low)	Serum ferritin (μg/L): No significant difference
49	Comparison No. 53: $FeSO_4$ vs Placebo	No significant difference	Serum ferritin (μg/L): No significant difference
50	Comparison No. 54: NaFeEDTA vs Fumarate vs Placebo	NaFeEDTA, fumarate> placebo	Plasma ferritin (μg/L): NaFeEDTA and fumarate>placebo
51	No	No significant difference	Plasma ferritin (μg/L): NaFeEDTA+fumarat and NaFeEDTA+FePP>placebo
52	No	Not stated	Serum ferritin (mg/L): no significant difference
53	No	No significant difference	Serum ferritin (μg/L): No significant difference
54	Comparison No. 34: Femarate vs Placebo	Fumarate> placebo	Not reported
55	No	No significant difference	Serum ferritin (μg/L): No significant difference

Table 3.3: *Characteristics of outcome effects(continued)*

Study ID	**Data contribution of network meta-analysis**	**Primary outcome (Hb g/L)**	**Secondary outcome**
56	Comparison No.35: Femarate vs Placebo	No significant difference	Serum ferritin (μg/L): fumarate>placebo
57	Comparison No. 36: Femarate vs Placebo	Fumarate> placebo	Not reported
58	No	Ferrous gluconate>placebo	Serum ferritin (μg/L): No significant difference
59	No	Ferrous gluconate>placebo	Not reported
60	No	Fumarate+ NaFeEDTA> placebo (except for corn-soy blend plus group)	Not reported
61	Comparison No. 48-49: Femarate vs Placebo	Fumarate> placebo	Serum ferritin (μg/L): No significant difference.Serum iron (μg/dL), transferrin saturation (%): fumarate>placebo
62	No	No significant difference	Not reported
63	Comparison No. 50: NaFeEDTA vs Placebo	No significant difference	Plasma ferritin (μg/L): no significant difference
64	No	No significant difference	Serum ferritin (ng/mL): no significant difference
65	No	$FeSO_4$ (High, moderate)>placebo. No significant difference between $FeSO_4$ (low) and placebo.	Hematocrit (%): $FeSO_4$<placebo.
66	No	No significant difference	Hematocrit (%): no significant difference

Table 3.3: *Characteristics of outcome effects(continued)*

Study ID	**Data contribution of network meta-analysis**	**Primary outcome (Hb g/L)**	**Secondary outcome**
67	Comparison No. 41-42: $FeSO_4$ vs Placebo	No significant difference	Serum ferritin (μg/L)): no significant difference
68	Comparison No. 40: $FeSO_4$ vs Placebo	No significant difference	Not reported
69	Comparison No. 43-44: Fumarate vs Placebo	No significant difference	Serum ferritin (μg/L): Fumarate>placebo
70	Comparison No. 45: $FeSO_4$ vs Placebo	No significant difference	Plasma ferritin (μg/L): no significant difference
71	Comparison No. 46-47: $FeSO_4$ vs Placebo	No significant difference	Not reported
72	No	No significant difference	Serum ferritin (μg/L): No significant difference
73	Comparison No. 38-39: NaFeEDTA vs Electrolytic iron vs Placebo	No significant difference	Plasma ferritin (μg/L): No significant difference
74	No	No significant difference	Serum ferritin (μg/L), transferrin saturation (%): No significant difference
75	No	Ferrous glucie phosphate> placebo	Not reported
76	No	No significant difference	Serum ferritin (μg/L): FePP>placebo
77	No	$FeSO_4$>placebo	Hematocrit (%): $FeSO_4$>placebo
78	No	No significant difference	Serum ferritin (μg/L): FePP>placebo

Table 3.3: *Characteristics of outcome effects(continued)*

Study ID	**Data contribution of network meta-analysis**	**Primary outcome (Hb g/L)**	**Secondary outcome**
79	No	No significant difference	Serum ferritin (μg/L): no significant difference
80	Comparison No. 81: FePP vs Placebo	No significant difference	Serum ferritin (μg/L): FePP>placebo
81	Comparison No. 37: NaFeEDTA vs Placebo	NaFeEDTA> placebo	Serum ferritin (μg/L): NaFeEDTA>placebo
82	No	No significant difference	Hematocrit (%), serum ferritin (μg/L), iron (μg/dL): no significant difference
83	No	No significant difference	Serum ferritin (μg/L): no significant difference
84	No	No significant difference	Serum ferritin (μg/L): Fumarate>placebo
85	No	NaFeEDTA> placebo	Not reported
86	Comparison No. 56: Fumarate vs Placebo	Fumarate> placebo	Not reported
87	No	No significant difference	Serum ferritin (μg/L), serum iron (μg/L), transferrin saturation (%): no significant difference
88	Comparison No. 58-59: $FeSO_4$ vs FePP vs Placebo	FePP, $FeSO_4$> placebo	Serum ferritin (μg/L): FePP, $FeSO_4$>placebo
89	Comparison No. 60: NaFeEDTA vs Placebo	NaFeEDTA> placebo	Serum ferritin (μg/L): NaFeEDTA>placebo
90	No	Fumarate> placebo	Serum ferritin (μg/L), serum iron (μmol/L) Transferr
91	No	$FePO_4$+$FeSO_4$> placebo	Not reported
92	No	$FeSO_4$>placebo	Serum ferritin (μg/L): $FeSO_4$>placebo

Table 3.3: *Characteristics of outcome effects(continued)*

Study ID	**Data contribution of network meta-analysis**	**Primary outcome (Hb g/L)**	**Secondary outcome**
93	Comparison No. 55: $FeSO_4$ vs Placebo	$FeSO_4$>placebo	Hematocrit (%): $FeSO_4$>placebo
94	Comparison No. 55: Electrolytic iron vs Placebo	No significant difference between electrolytic iron and placebo. Ferrous bisglycinate>placebo.	Serum ferritin (μg/L), transferrin saturation (%): no significant difference between electrolytic iron and placebo. Ferrous bisglycinate>placebo.
95	No	No significant difference	Hematocrit (%): $FeSO_4$>placebo
96	No	No significant difference	Hematocrit (%), serum ferritin (μg/L), transferrin saturation (%), serum iron (ng/mL): no significant difference
97	Comparison No. 68-71: Electrolytic iron vs Placebo	No significant difference	Plasma ferritin (μg/L): no significant difference.
98	Comparison No. 72-74: NaFeEDTA vs $FeSO_4$ vs Electrolytic iron vs Placebo	No significant difference between electrolytic iron and placebo. Electrolytic. NaFeEDTA, $FeSO_4$>placebo	Serum iron (μmol/L): no significant difference between electrolytic iron, NaFeEDTA and placebo, $FeSO_4$>placebo. Serum ferritin (μg/L), total iron binding capability: no significant difference between electrolytic iron and placebo, NaFeEDTA, $FeSO_4$>placebo.
99		No significant difference	Serum ferritin (μg/L): NaFeEDTA, $FeSO_4$ >placebo
100	Comparison No. 75-80: Fepp vs Placebo	FePP>placebo	Serum ferritin (μg/L): FePP>placebo
101	Comparison No. 61-62: Fepp vs Placebo	No significant difference	Serum ferritin (μg/L): FePP>placebo

Table 3.3: *Characteristics of outcome effects(continued)*

Study ID	**Data contribution of network meta-analysis**	**Primary outcome (Hb g/L)**	**Secondary outcome**
102	No	No significant difference	Not reported
103	Comparison No. 63: Fepp vs Placebo	NaFeEDTA> placebo	Serum ferritin (μg/L): NaFeEDTA>placebo
104	Comparison No. 64-65: Fepp vs Placebo	No significant difference	Serum ferritin (μg/L): FePP>placebo
105	Comparison No. 66-67: NaFeEDTA vs Placebo	NaFeEDTA> placebo	Serum ferritin (mg/L): NaFeEDTA> placebo
106	No	No significant difference	Serum ferritin (μg/L): no significant difference
107	No	FePP>placebo	Serum ferritin (μg/dL): FePP>placebo
108	No	$FeSO_4$>placebo	Serum ferritin (μg/dL): $FeSO_4$>placebo
109	No	FePP>placebo	Serum ferritin (μg/dL): FePP>placebo
110	No	No significant difference between μFePP and plac -ebo, fumarate >placebo	Serum ferritin (μmol/L): μFePP, fumarate>placebo
111	Comparison No. 82: FePP vs Placebo	No significant difference	Not reported
112	No	$FeSO_4$>placebo	Serum ferritin (μg/L): $FeSO_4$>placebo
113	No	No significant difference	Not reported
114	No	$FeSO_4$>placebo	Serum ferritin (ng/dL): $FeSO_4$>placebo
115	No	$FeSO_4$>placebo	Not reported
116	No	NaFeEDTA> placebo	Plasma ferritin (μg/L): NaFeEDTA> placebo

Table 3.3: *Characteristics of outcome effects(continued)*

Study ID	**Data contribution of network meta-analysis**	**Primary outcome (Hb g/L)**	**Secondary outcome**
117	No	Females: NaFeEDTA> placebo. Males: no significant difference	Serum ferritin (μg/dL): NaFePP>placebo. Transferrin saturation (%): no significant difference.
118	No	Electrolytic Fe>placebo	Serum iron (mg/L): electrolytic Fe>placebo.
119	No	No significant difference	Hematocrit (%): no significant difference
120	No	Ferric ammonium citrate>placebo after 6-month interventi on, but no significant difference after 12-month intervention	Hematocrit (%): Ferric ammonium citrate >placebo after 6-month intervention, but no significant difference after 12-month intervention
121	No	No significant difference	Serum ferritin (μg/dL): Fumarate>placebo
122	No	No significant difference	Serum ferritin (μg/dL): $FeSO_4$>placebo
123	No	No significant difference	Plasma ferritin (μg/dL): no significant difference
124	No	No significant difference	Hematocrit (%): no significant difference
125	No	No significant difference	Serum ferritin (μg/dL): $FeSO_4$ hydrogen reduced Fe, electrolytic Fe>placebo
126	No	NaFeEDTA (10, 20 mg/g), $FeSO_4$<placebo	Hematocrit (%), serum iron (μg/dL), serum ferritin (ng/mL): NaFeEDTA, $FeSO_4$>placebo.Total iron binding capacity (μg/dL): no significant difference
127	No	No significant difference	Hematocrit (%), serum iron (μg/dL), serum ferritin (ng/mL), transferrin saturation (%), total iron binding capacity (μmol/L): no significant difference

Table 3.3: *Characteristics of outcome effects(continued)*

Study ID	**Data contribution of network meta-analysis**	**Primary outcome (Hb g/L)**	**Secondary outcome**
128	No	Hb changes: $FeSO_4$> NaFeEDTA	Serum ferritin changes (μg/L): $FeSO_4$>NaFeEDTA
129	No	$FeSO_4$> NaFeEDTA	Hematocrit (%), serum iron (μg/dL): no significant difference. Transferrin saturation (%), total iron binding capacity (μmol/L): $FeSO_4$>NaFeEDTA
130	No	FePP>placebo	Hematocrit (%), serum ferritin (ng/mL), transferrin saturation (%): no significant difference. Serum iron (mmol/L): FePP>NaFeEDTA
131	No	FePP>placebo	Hematocrit (%), serum ferritin (ng/mL), transferrin saturation (%): FePP>placebo.
132	No	No significant difference	Serum ferritin (μg/dL): μFePP, NaFeEDTA>placebo
133	No	No significant difference	Serum ferritin (μg/dL): no significant difference
134	No	No significant difference	Serum ferritin (μg/L): no significant difference
135	No	No significant difference	Hematocrit (%), serum iron (μg/dL), transferrin saturation (%), total iron binding capacity (μmol/L): no significant difference
136	No	$FeSO_4$>placebo	Serum ferritin ratio: $FeSO_4$>placebo
137	No	NaFeEDTA> Placebo	Serum ferritin (μg/L): NaFeEDTA>Placebo
138	No	NaFeEDTA> Placebo	Serum ferritin (μg/L): NaFeEDTA>Placebo
139	No	$FeSO_4$>placebo	Hematocrit (%): $FeSO_4$>placebo.Serum iron (μg/dL), serum ferritin (ng/mL): no significant difference
140	No	NaFeEDTA> placebo	Serum ferritin (μmol/L): NaFeEDTA> placebo
141	No	No significant difference	Serum ferritin (μg/L): fumarate>Placebo

Table 3.3: *Characteristics of outcome effects(continued)*

Study ID	**Data contribution of network meta-analysis**	**Primary outcome (Hb g/L)**	**Secondary outcome**
142	No	$FeSO_4$>placebo	Serum ferritin (ng/mL): $FeSO_4$>placebo
143	No	No significant difference	Plasma ferritin (μg/L): $FeSO_4$>placebo.
144	No	No significant difference	Not reported
145	No	No significant difference	Transferrin saturation (%): no significant difference

Table 3.4: *Outcome effect summary of comparisons*

Effect			**Hb**	**Serumferritin**	**Plasma ferritin**	**Transferrin saturation**	**Total iron binding capability**	**Serum iron**	**Plasma iron**	**Hematocrit**
FeSO4	>	placebo	18	18	1	1	2	5	0	5
EI	>	placebo	1	1	0	0	0	1	0	0
Ferrous fumarate	>	placebo	12	12	2	2	1	1	0	1
NaFeEDTA	>	placebo	11	10	2	2	0	1	0	0
FePP	>	placebo	7	13	0	0	0	0	0	1
Ferrous gluconate	>	placebo	2	0	0	0	0	0	0	0
Ferrous glucie phosphate	>	placebo	1	0	0	0	0	0	0	0
Ferrous bisglycinate	>	placebo	1	1	0	1	0	0	0	0
Ferric ammonium citrate	>	placebo	1	0	1	0	0	0	0	1
Hydrogen reduced iron	>	placebo	0	1	0	0	0	0	0	0

Table 3.4: *Outcome effect summary of comparisons(continued)*

Effect			**Hb**	**Serumferritin**	**Plasma ferritin**	**Transferrin saturation**	**Total iron binding capability**	**Serum iron**	**Plasma iron**	**Hematocrit**
Combination of NaFeEDTA and fumarate	>	placebo	1	0	0	0	0	0	0	0
Combination of NaFeEDTA and FePP	>	placebo	0	0	2	0	0	0	0	0
Combination of $FeSO_4$ and $FePO_4$	>	placebo	1	0	0	0	0	0	0	0
$FeSO_4$	<	placebo	0	1	0	0	0	0	0	1
EI	<	placebo	0	0	0	0	0	1	0	0
Ferrous fumarate	<	placebo	0	1	0	0	0	1	1	0
NaFeEDTA	<	placebo	0	0	0	1	0	0	0	0
FePP	<	placebo	0	0	0	0	0	0	0	0
NaFeEDTA	<	FePP	0	0	0	0	0	1	0	0
NaFeEDTA	<	FeSO4	0	1	0	0	0	0	0	0
Ferrous fumarate	<	FeSO4	0	1	0	1	1	0	0	0

Note: Number represented the number of comparison

Table 3.5: *Quality assessments of included studies*

Study ID	**Random sequence generation**	**Allocation concealment**	**Blinding type**	**Blinding**	**Similarity of baseline characteristics**	**Incomplete Data**	**Selective reporting**	**Overall of bias level**
1	Unclear	High	Double	Low	Low	Low	Low	Moderate
2	Unclear	High	Double	Low	Low	Low	Low	Moderate
3	Unclear	High	None	High	Low	Low	Low	High
4	Low	High	Double	Low	Low	Low	Low	Low
5	Unclear	Low	Single-P	Low	Low	Low	Low	Low
6	Low	High	None	High	Low	Low	Low	High
7	Unclear	Low	Double	Low	Low	Low	Low	Low
8	Low	Low	Double	Low	Low	Low	Low	Low
9	Low	High	Double	Low	Low	Low	Low	Moderate
10	Low	High	None	High	Low	Low	Low	High
11	Low	Low	Double	Low	Low	Low	Low	Low
12	Unclear	High	Single-O	Low	High	Low	Low	High
13	Unclear	High	Double	Low	High	Low	Low	High
14	Low	Low	Double	Low	Low	Low	Low	Low
15	Unclear	High	Double	Low	Low	Low	Low	Moderate
16	Unclear	High	Double	Low	Low	Low	Low	Moderate
17	Unclear	High	None	High	Low	Low	Low	High
18	Unclear	High	None	High	High	Low	Low	High
19	Unclear	High	None	High	Low	Low	Low	High
20	Unclear	High	Double	Low	Low	Low	Low	Moderate
21	Unclear	High	None	High	Low	Low	Low	High
22	Low	High	Double	Low	Low	Low	Low	Moderate
23	Unclear	High	Double	Low	Low	Low	Low	Moderate
24	Unclear	High	None	High	Low	Low	Low	High
25	Unclear	Low	Double	Low	Low	Low	Low	Low
26	Low	Low	Double	Low	Low	Low	Low	Low
27	Unclear	High	None	High	Low	Low	Low	High
28	Unclear	High	None	High	Low	Low	Low	High
29	Low	High	Double	Low	Low	Low	Low	Moderate
30	Unclear	High	Double	Low	Low	Low	Low	Moderate
31	Unclear	High	Double	Low	Low	Low	Low	Moderate
32	Low	High	None	High	Low	Low	Low	High
33	Low	High	None	High	Low	Low	Low	High

Table 3.5: *Quality assessments of included studies(continued)*

Study ID	Random sequence generation	Allocation concealment	Blinding type	Blinding	Similarity of baseline characteristics	Incomplete Data	Selective reporting	Overall of bias level
34	Low	High	Double	Low	Low	Low	Low	Moderate
35	Unclear	High	None	High	Low	Low	Low	High
36	Low	Low	Double	Low	Low	Low	Low	Low
37	Unclear	High	None	High	Low	Low	Low	High
38	Unclear	High	Double	Low	Low	Low	Low	Moderate
39	Unclear	High	None	High	Low	Low	Low	High
40	Low	Low	None	High	Low	Low	Low	Moderate
41	Low	High	Double	Low	Low	Low	Low	Moderate
42	Low	Low	Double	Low	Low	Low	Low	Low
43	Low	Low	None	High	Low	Low	Low	Moderate
44	Low	Low	Single-O	Low	Low	Low	Low	Low
45	Low	High	Double	Low	Low	Low	Low	Moderate
46	Low	High	Single-O	Low	Low	Low	Low	Moderate
47	Unclear	High	Double	Low	Low	Low	Low	Moderate
48	Unclear	High	None	High	Low	Low	Low	High
49	Low	Low	None	High	Low	Low	Low	Moderate
50	Unclear	High	None	High	Low	Low	Low	High
51	Unclear	High	None	High	Low	Low	Low	High
52	Unclear	High	Double	Low	Low	Low	Low	Moderate
53	Low	High	Single-P	Low	Low	Low	Low	Moderate
54	Unclear	High	Double	Low	Low	Low	Low	Moderate
55	Unclear	Low	None	High	Low	Low	Low	Moderate
56	Unclear	High	Single-O	Low	Low	Low	Low	Moderate
57	Unclear	Low	Double	Low	Low	Low	Low	Low
58	Low	Low	Double	Low	Low	Low	Low	Low
59	Unclear	High	None	High	Low	Low	Low	High
60	Unclear	Low	Double	Low	Low	Low	Low	Low
61	Low	Low	Double	Low	Low	Low	Low	Low
62	Low	High	None	High	Low	Low	Low	High
63	Low	High	Double	Low	Low	Low	Low	Moderate
64	Low	Low	Double	Low	Low	Low	Low	Low
65	Low	High	Double	Low	Low	Low	Low	Moderate
66	Unclear	High	Double	Low	Low	Low	Low	Moderate

Table 3.5: *Quality assessments of included studies(continued)*

Study ID	Random sequence generation	Allocation concealment	Blinding type	Blinding	Similarity of baseline characteristics	Incomplete Data	Selective reporting	Overall of bias level
67	Unclear	High	None	High	Low	Low	Low	High
68	Low	High	Double	Low	Low	Low	Low	Moderate
69	Low	High	Double	Low	Low	Low	Low	Moderate
70	Low	High	None	High	Low	Low	Low	High
71	Unclear	High	Double	Low	Low	Low	Low	Moderate
72	Unclear	High	Double	Low	Low	Low	Low	Moderate
73	Low	High	None	High	Low	Low	Low	High
74	Low	High	None	High	Low	Low	Low	High
75	Unclear	High	None	High	Low	Low	Low	High
76	Unclear	High	Double	Low	Low	Low	Low	Moderate
77	Unclear	High	Double	Low	Low	High	Low	High
78	Low	High	Double	Low	Low	Low	Low	Moderate
79	Unclear	High	Double	Low	Low	High	Low	High
80	Unclear	High	Double	Low	Low	Low	Low	Moderate
81	Unclear	High	Double	Low	Low	Low	Low	Moderate
82	Low	High	None	High	Low	Low	Low	High
83	Low	High	Double	Low	Low	Low	Low	Moderate
84	Unclear	High	None	High	Low	Low	Low	High
85	Unclear	High	None	High	Low	Low	Low	High
86	Low	High	Double	Low	Low	Low	Low	Moderate
87	Low	High	Single-O	Low	Low	Low	Low	Moderate
88	Unclear	High	Double	Low	Low	Low	Low	Moderate
89	Low	High	Double	Low	Low	Low	Low	Moderate
90	Low	High	Single-P	Low	Low	Low	Low	Moderate
91	Unclear	High	None	High	Low	Low	Low	High
92	Unclear	High	None	High	Low	High	Low	High
93	Unclear	High	None	High	Low	Low	Low	High
94	Low	High	Single-O	Low	Low	Low	Low	Moderate
95	Unclear	High	None	High	Low	Low	Low	High
96	Unclear	High	None	High	Low	Low	Low	High
97	Low	High	Double	Low	Low	Low	Low	Moderate
98	Unclear	High	None	High	Low	High	Low	High
99	Low	High	Double	Low	Low	Low	Low	Moderate

Table 3.5: *Quality assessments of included studies(continued)*

Study ID	Random sequence generation	Allocation concealment	Blinding type	Blinding	Similarity of baseline characteristics	Incomplete Data	Selective reporting	Overall of bias level
100	Unclear	High	Double	Low	Low	Low	Low	Moderate
101	Unclear	High	Double	Low	Low	Low	Low	Moderate
102	Low	High	Double	Low	Low	Low	Low	Moderate
103	Unclear	High	None	High	Low	High	Low	High
104	Unclear	High	Double	Low	Low	High	Low	High
105	Low	High	Double	Low	Low	Low	Low	Moderate
106	Low	High	Double	Low	Low	Low	Low	Moderate
107	Unclear	High	Double	Low	Low	Low	Low	Moderate
108	Unclear	High	Double	Low	Low	Low	Low	Moderate
109	Unclear	High	Double	Low	Low	Low	Low	Moderate
110	Low	High	Double	Low	Low	Low	Low	Moderate
111	Unclear	High	Double	Low	Low	Low	Low	Moderate
112	Unclear	High	Single-P	Low	Low	Low	Low	Moderate
113	Unclear	High	None	High	Low	Low	Low	High
114	Unclear	High	Double	Low	Low	Low	Low	Moderate
115	Unclear	High	Double	Low	Low	Low	Low	Moderate
116	Unclear	High	Single-P	Low	Low	Low	Low	Moderate
117	Unclear	High	Double	Low	Low	Low	Low	Moderate
118	Unclear	High	Double	Low	Low	Low	Low	Moderate
119	Low	High	Double	Low	Low	Low	Low	Moderate
120	Unclear	High	Double	Low	Low	Low	Low	Moderate
121	Unclear	High	Double	Low	Low	Low	Low	Moderate
122	Unclear	High	None	High	Low	Low	Low	High
123	Unclear	High	Double	Low	Low	Low	Low	Moderate
124	Unclear	High	Double	Low	Low	High	Low	High
125	Low	High	Double	Low	Low	Low	Low	Moderate
126	Unclear	High	Single-P	Low	Low	Low	Low	Moderate
127	Unclear	High	None	High	Low	Low	Low	High
128	Unclear	High	Double	Low	Low	High	Low	High
129	Unclear	High	Double	Low	Low	Low	Low	Moderate
130	Unclear	High	Double	Low	Low	Low	Low	Moderate
131	Unclear	High	Double	Low	Low	Low	Low	Moderate
132	Unclear	High	Double	Low	Low	Low	Low	Moderate

Table 3.5: *Quality assessments of included studies(continued)*

Study ID	**Random sequence generation**	**Allocation concealment**	**Blinding type**	**Blinding**	**Similarity of baseline characteristics**	**Incomplete Data**	**Selective reporting**	**Overall of bias level**
133	Unclear	High	Double	Low	Low	Low	Low	Moderate
134	Unclear	High	Single-P	Low	Low	Low	Low	Moderate
135	Unclear	High	None	High	Low	High	High	High
136	Unclear	High	Single-P	Low	Low	Low	Low	Moderate
137	Unclear	High	Double	Low	Low	Low	Low	Moderate
138	Low	High	Double	Low	Low	High	Low	High
139	Unclear	High	Double	Low	Low	Low	Low	Moderate
140	Unclear	High	None	High	Low	High	Low	High
141	Unclear	High	Double	Low	Low	High	Low	High
142	Unclear	High	Double	Low	Low	Low	Low	Moderate
143	Low	High	Double	Low	Low	Low	Low	Moderate
144	Unclear	High	None	High	Low	Low	Low	High
145	Unclear	High	None	High	Low	Low	Low	High

Note: "Low" represented low risk. "Moderate" represented Moderate risk. "High"represented high risk. "Single-P" represented "Single blind-participants were masked". "Single-O"represented "Single blind-operators were masked".

Table 3.6: *Certainty of evidence evaluation*

Comparison	**# of studies**	**Within-study bias**	**Reporting bias**	**Indirectness**	**Imprecision**	**Heterogeneity**	**Incoherence**	**Certainty of evidence**
EI-$FeSO_4$	6	Major concerns	Low risk	No concerns	Major concerns	No concerns	No concerns	Low
EI-Fumarate	3	Some concerns	Low risk	No concerns	Major concerns	No concerns	No concerns	Moderate
EI-NaFeEDTA	5	Major concerns	Low risk	No concerns	No concerns	Major concerns	Major concerns	Low
EI-Placebo	17	Major concerns	Low risk	No concerns	Major concerns	No concerns	Major concerns	Very low
FePP-$FeSO_4$	2	Some concerns	Low risk	No concerns	Major concerns	No concerns	No concerns	Moderate
FePP-Fumarate	12	Some concerns	Low risk	No concerns	Major concerns	No concerns	No concerns	Moderate
FePP-Placebo	16	Some concerns	Low risk	No concerns	No concerns	Major concerns	No concerns	Moderate
FeSO4-NaFeEDTA	3	Major concerns	Low risk	No concerns	No concerns	Major concerns	No concerns	Low
FeSO4-Placebo	25	Major concerns	Low risk	No concerns	No concerns	Major concerns	Major concerns	Low
Fumarate-NaFeEDTA	1	Some concerns	Low risk	No concerns	Major concerns	No concerns	No concerns	Moderate
Fumarate-Placebo	16	Some concerns	Low risk	No concerns	No concerns	Major concerns	No concerns	Moderate
NaFeEDTA-Placebo	13	Some concerns	Low risk	No concerns	No concerns	Major concerns	No concerns	Moderate

Chapter 4

Bioavailability of NaFeEDTA, Ferrous Fumarate and Ferrous Sulfate in Different Methodological Caco-2 Cell Models with INFOGEST Digestion Method

Abstract

Background: The static digestion/Caco-2 cell model has been widely employed as a bioassay to assess iron bioavailability. However, methodological differences in this model reduce reproducibility of inter-experiments. Thus, a standard static digestion method named INFOGEST, a classic dual-chamber system, an alternative dual-chamber system, and a triple-chamber system were tested to identify an optimal digestion/Caco-2 cell model using NaFeEDTA, ferrous fumarate, and $FeSO_4$. Furthermore, the iron bioavailability of NaFeEDTA and ferrous fumarate/$FeSO_4$ combinations at high or low weight ratios was measured for better human applications of food aid.

Objective: To test whether INFOGEST digesta can clog the dialysis membrane. To examine whether micronized iron (0.4 µm) can be absorbed by Caco-2 cells. To assess

the feasibility of triple-chamber system of Caco-2 cell model. To identify the optimal iron treatment system for Caco-2 cell model. To measure the bioavailability of precooked corn flour fortified with NaFeEDTA, ferrous fumarate, $FeSO_4$ and their combinations.

Methods: Alternative dual-chamber system (insert membrane) and triple-chamber system (insert membrane+dialysis membrane) were designed based on the classic dual-chamber system (dialysis membrane). Precooked corn flour fortified with NaFeEDTA, ferrous fumarate, or $FeSO_4$ were digested with INFOGEST method. Caco-2 cells were treated with the iron digesta or supernatant in dual-chamber systems or triple-chamber system for 2 hours, respectively. The ferritin concentrations were examined to compare systems. Finally, the bioavailability of NaFeEDTA, ferrous fumarate, and $FeSO_4$ combinations was measured using the optimal system.

Main Results: Ferritin concentrations of NaFeEDTA, ferrous fumarate, and $FeSO_4$ samples in classic dual-chamber system were higher than those of NaFeEDTA, ferrous fumarate, and $FeSO_4$ samples in alternative dual-chamber system and triple-chamber system. In classic dual-chamber system, NaFeEDTA digesta and ferrous fumarate digesta had higher ferritin concentrations than NaFeEDTA supernatant and ferrous fumarate supernatant. There was no significant difference in ferritin response between $FeSO_4$ digesta and $FeSO_4$ supernatant. NaFeEDTA digesta was significantly higher than $FeSO_4$ digesta in ferritin response. The combinations of NaFeEDTA and $FeSO_4$ at low weight ratio (2:11) had the highest ferritin concentration among the combination samples.

Conclusions: The Caco-2 cell model with the classic dual-chamber system and INFOGEST digesta was a reliable iron bioassay. NaFeEDTA had higher bioavailability than $FeSO_4$. The combination of NaFeEDTA and $FeSO_4$ at low weight ratio had the highest bioavailability among the combination samples.

Keywords: INFOGEST, Caco-2 cell model, Dual-chamber system, Triple-chamber system, Iron digesta, Iron supernatant, Iron bioavailability, NaFeEDTA, Ferrous fumarate, $FeSO_4$.

4.1 Introduction

It is estimated that anemia affects 2 billion people worldwide. The most common cause is nutritional iron deficiency. It usually arises when iron is not absorbed well from diet for

physiological requirements. Iron fortification is one of the most sustainable, practical, low-cost, and long-term strategies to combat nutritional iron deficiency. However, improving iron bioavailability of fortificants is a challenge.[55–57] *In vitro* iron bioassays are constantly developed, refined, and advanced as scanning tools and prediction models for the *in vivo* studies and clinical trials.

Iron solubility is the water solubility of iron compound. In theory, *in vitro* iron solubility is a good predictor of *in vivo* iron absorption and bioavailability. The more solubilized iron, the more iron can be absorbed and utilized.[147] Therefore, as a standardized *in vitro* static digestion method, INFOGEST was employed to determine the iron solubility. It simulates oral cavity and gastrointestinal tract digestions with the use of electrolytes and digestive enzymes.[148] The INFOGEST method or other *in vitro* digestion method can be paired with the Caco-2 cell model to examine how the iron solubility of samples correlates with iron bioavailability. This model could mimic the physical process of iron absorption in intestinal epithelial cells with a piece of dialysis membrane and an insert frame. This model has been utilized by countless researchers for more than two decades.[149]

However, with the development of experimental materials, old version of insert (insert without insert membrane) was no longer sold. Additionally, in Glahn's study, 1.5ml digesta was added to the upper chamber to treat Caco-2 cells with low molecular weight (nanometer-level particle size) iron. Glahn's food concentration of digesta is 0.5-1 g food/15-17mL digestion solution (0.029-0.067 g/mL), while INFOGEST's food concentration of digesta is 5 g food/35mL digestion solution (0.143 g/mL). We wondered if the thicker INFOGEST digesta would work in this system. Therefore, we designed two alternative Caco-2 cell-culture systems based on Glahn's model to test whether they could be employed as iron bioassays.[139] This study was split to two experiments. In Experiment 1, the objectives were to test whether INFOGEST digesta can clog the dialysis membrane, examine whether micronized iron (0.4 µm) can be absorbed by Caco-2 cells, assess the feasibility of triple-chamber system of Caco-2 cell model, identify the optimal iron treatment system for Caco-2 cell model, and measure the bioavailability of NaFeEDTA, ferrous fumarate, $FeSO_4$. After that, the optimal iron treatment system was applied to the Experiment 2. The objective of Experiment 2 was to measure the iron bioavailability of combination fortifications (NaFeEDTA+fumarate/$FeSO_4$).

4.2 Materials and methods

4.2.1 Materials

All materials and supplies used in the present experiment were listed in Appendix B (page 227).

4.2.2 Sample preparation

Samples No.1 to 3 were NaFeEDTA, ferrous fumarate, $FeSO_4$ fortifications. They were designed for the Experiment 1. Samples No.4 to 7 were the combination fortifications of NaFeEDTA and fumarate/$FeSO_4$ at high/low weight ratio (4:9 or 2:11). They were designed for the Experiment 2 (Table 4.1). The target level of iron fortifications recommended by Food Aid Quality Review Report (United States Agency for International Development) was 130 mg/kg corn flour.[139] The food matrix was the commercial corn product (P.A.N. precooked corn flour). The product label showed that every 100 g of corn flour contained 6.67 g of dietary fiber, 6.67 g of protein 6.67 g, 4.67 mg of iron, 273.33 mg of potassium, 0.67 mg of thiamin, 3.67 mg of niacin, 0.33 mg of riboflavin, and 163.33 mcg of folic acid.

4.2.3 INFOGEST procedure

Initially, electrolyte solutions, simulated salivary fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF) were prepared and used for all digestion phases (Table 4.2).[148] In the oral phase, 5 g of food samples were mixed with 4 mL of SSF and 1 mL of amylase solution (83 mg amylase/ml water, 9 enzymatic activity unit/mg amylase) in the 50 ml centrifuge tube. The oral boluses were manually minced with metal spatulas and incubated at 37°C for 2 minutes. The oral boluses were added to 8 mL of SGF in the gastric phase. After the pH values were reduced to 3±0.1 with 6M hydrochloric acid, 1 mL of pepsin solution (66 mg pepsin/ml water, 605 enzymatic activity unit/mg pepsin) and 1 mL of lipase solution (80 mg lipase/ml water, 15 enzymatic activity unit/mg lipase) were added and then incubated on the shaking plate of incubator at 37°C and 150 rpm for 2 hours. In the intestinal phase, 8 mL of SIF was mixed with the gastric chyme and then pH values were adjusted to 7±0.1 with 5M sodium hydroxide. 3 mL of bile solution (54 mg bile/ml SIF, 10mM), 5 mL of pancreatin solution (4 mg pancreatin/ml SIF, 200 enzymatic activity unit/mg pancreatin), and 4 mL of water were added and incubated at 37°C and 150 rpm for 2 hours. Digesta was heated at 100°C for 5 minutes to stop intestinal digestion before being centrifuged at 5000g and 5°C for 45 minutes. The data of enzymatic activities was provided by Sigma Aldrich company. The iron contents of dry samples and supernatant

were analyzed with Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES) method by Soil Testing lab at Kansas State University. Solubility (%)= 100*soluble iron content (supernatent)/total iron content (dry sample).[148;150–154]

4.2.4. Preparation of three iron treatment systems.

4.2.4.1 Classic dual-chamber system

The insert membrane was cut out with scissor. A piece of dialysis membrane (15 KDa, 1-2 nm, regenerated cellulose) was held by the silicon "O" ring at the bottom of insert frame. The well was separated by the dialysis membrane-insert system to upper chamber and lower chamber. Iron digesta or iron supernatant was added to the upper chamber. After they were filtered by the dialysis membrane, the low molecule weight iron could be absorbed by Caco-2 cells in the lower chamber (Figure 4.1).[149]

4.2.4.2 Alternative dual-chamber system

Used the insert membrane (0.4 µm, polyethylene terephthalate) instead of the dialysis membrane. Iron digesta or iron supernatant was added to the upper chamber. Micronized iron entered the lower chamber across the insert membrane (Figure 4.2).

4.2.4.3 Triple-chamber system

Used the insert membrane and the dialysis membrane simultaneously. A piece of dialysis membrane was held by the silicon "O" ring at the bottom of insert membrane. Two membranes separated the well into three compartments: upper chamber, middle chamber, and lower chamber. The height of the middle chamber was approximately 1mm. Iron digesta or iron supernatant was added to the upper chamber. Micronized iron could pass through the insert membrane. The low molecule weight iron diffused into the lower chamber and could be absorbed by Caco-2 cells (Figure 4.3).

4.2.5 Caco-2 cell culture procedure

Caco-2 cells were purchased from ATCC at passage 18. The Caco-2 cells were cultured in T25, T75 and T225 flasks with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 1% Zellshield, and 25 mM HEPES in 37°C and 5% CO_2 air.[149]

Caco-2 cells were detached with trypsin (0.25%)-EDTA (0.53 mM) for 5-10 minutes at 80-100% confluency. Dissociated cells were centrifuged at 125g for 5-10 minutes. Cells were counted with hemocytometer. The sufficient Caco-2 cells (passage 23) from T225 flasks were transferred to collagen coated 6-well plates with 2mL of DMEM per well at a seeding density of 50,000 cells/cm^2 and allowed to grow for 12 days with media being refreshed every two days.[149]

On the 12^{th}day of the 6-well plates culture phase, DMEM was replaced by 2 mL of Minimum Essential Medium (MEM) supplemented with 4 mg/L hydrocortisone, 10 mM Piperazine-1,4-bis (2-ethanesulfonic acid) disodium salt, 20 μg/L epidermal growth factor, 5 μg/L selenium, 34 μg/L triiodothyronine, 5 mg/L insulin, and 1% Zellshield.[149] Iron digesta and supernatant were produced using INFOGEST method and stored overnight at -80°C.[153]

On the next day, iron treatment systems were prepared and sterilized with 0.5 M HCl solution for at least 1 hour, and rinsed with autoclaved deionized water. 2mL of MEM was replaced by 1mL of MEM into the lower chamber and 1.5 mL of iron digesta or supernatant into the upper chamber. Iron treatments were randomly assigned to the Caco-2 cells. The 6-well plates were placed on the rocking shaker (6 vibrations/min) and incubated for 2h. The dual- or triple- chamber system was removed and 1mL of MEM was added. The Caco-2 cells were returned to the incubator for another 22 hours. Blank control (only media) was used.

On the last day, MEM was removed and autoclaved deionized water was added to osmotically lyse the Caco-2 cells.[149] Placed a tube rack in the sonicator and then placed the cells on the tube rack for a 30-second sonication cell lysis.[155] Finally, cell lysate was collected for ferritin and protein analyses according to the instructions of ferritin and protein kits.[149]

4.2.6 Statistical analysis

Data were analyzed in SAS online Studio (https://welcome.oda.sas.com/) with significance at p-value<0.05. The Shapiro-Wilk's test was used for normality. Levene's test was used for homogeneity. Normally distributed data were assessed by one-way analysis of variance (ANOVA) with Least Significant Difference (LSD) test. The random number table was generated with Excel. ELISA analysis was performed using the Arigo online calculator at https://www.arigobio.com/elisa-analysis.[156]

4.3 Results

4.3.1 Iron solubility

In the Experiment 1, the iron solubility of NaFeEDTA was significantly higher than that of fumarate and $FeSO_4$ (Figure 4.4, Table 4.3). In the Experiment 2. The combinations of NaFeEDTA and fumarate/$FeSO_4$ at high weight ratio had significantly higher iron solubility than the combinations of NaFeEDTA and fumarate/$FeSO_4$ at low weight ratio (Figure 4.5,

Table 4.4).

4.3.2 Ferritin concentrations

In the Experiment 1, the ferritin concentrations of NaFeEDTA, ferrous fumarate, and $FeSO_4$ in classic dual-chamber system were significantly higher than those of NaFeEDTA, ferrous fumarate, and $FeSO_4$ in the alternative dual-chamber system and triple-chamber system, respectively. In classic dual-chamber system, NaFeEDTA digesta and ferrous fumarate digesta had higher ferritin concentrations than NaFeEDTA supernatant and ferrous fumarate supernatant. There was no significant difference between $FeSO_4$ digesta and $FeSO_4$ supernatant in ferritin response (Figure 4.6, Table 4.5).Thus, the classic dual-chamber system with digesta was the optimal bioassay. It was used in the Experiment 2. In classic dual-chamber system, NaFeEDTA digesta was significantly higher than $FeSO_4$ digesta in ferritin response, while $FeSO_4$ supernatant was significantly higher than NaFeEDTA supernatant (Figure 4.6, Table 4.5). In the Experiment 2, the combinations of NaFeEDTA and $FeSO_4$ at low weight ratio had the highest ferritin concentration among the combination samples (Figure 4.7, Table 4.6).

4.4 Discussion

In the 1930s, British scientists started using ococ'-dipyridyl to test ionisable iron in food.[157] In the 1960s, pepsin came into use to measure the iron content of food.[158;159] Until the 1970s, a relatively mature *in vitro* static method accompanied by both pepsin digestion and pancreatin digestion for the bioavailability prediction of iron in foods was established.[160;161] Recently, a better mechanistic understanding on *in vitro* static digestion method was reported by an international academic association named INFOGEST. In 2019, the protocol of INFOGEST digestion method was published on *Nature*. Compared with the conventional digestion method (pepsin-pancreatin digestion), INFOGEST had some stepwise refinements as follows: 1. Use amylase to simulate the oral digestion. 2. Use lipase to assist the overall digestion of triacylglycerides. 3 Use the digestive enzyme activities to determine their effects. 4. Optimize the electrolyte concentrations. 5. Provide the preservation method of digested samples. Thus, INFOGEST was selected as the static digestion strategy in our study. However, researchers should be aware of the weaknesses of INFOGST. 1. Enzymes on brush border membrane and microbiota had not been considered. 2. INFOGEST was

designed only for healthy adult digestion. If it is used for infants, children or patients, the parameters need to be adjusted. 3. The kinetic studies such as the gradual adjustments of gastrointestinal fluid volumes and pH values, gastrointestinal peristalsis and emptying, and the sieving activities of pylorus, were absent. Dynamic or Semi-dynamic digestion methods were being studied. Additionally, the bile solution was diluted to low bile concentration (54.4 mg bile/ml SIF) in this study, because the toxicity of high bile concentration (200 mg bile/mL SIF) might harm the Caco-2 cells, so approximate 4-flod dilution was adopted based on the INFOGEST suggestion.[148]

Next, the INFOGEST method was combined with Glanh's Caco-2 cell model. It included a classic dual-chamber system with dialysis membrane to evaluate the iron absorption of enterocyte. The dialysis membrane could mimic the mucin net so that the low molar weight iron could be absorbed.[162] The main component of intestinal mucus proteome is MUC2 mucin, which is synthesized in the nucleus of goblet cell (e.g., HT29 cell). After monomeric MUC2 mucin is glycosylated, it can be polymerized by N-terminal trimerizations and C-terminal dimerizations to form net-like structures parking on the top of goblet cells and enterocytes. This mucin net can be expanded to generate a mucus layer. The pore size of outer mucus layer is 440±50 nm when the ratio of Caco-2 cells and HT29 cells is 9:1. The permeability of the inner mucus layer (mucin net) is around 17kDa.[44;163–175] Thus, the 15kDa (1-2nm) dialysis membrane was used in the classic dual-chamber system to similar this mucin net. The insert membrane (0.4 μm) was chosen in the alternative dual-chamber system and triple-chamber system. As shown in Figure 4.6, the alternative dual-chamber system and triple-chamber system did not work well. The micronized iron (0.4 μm) was not be absorbed in Caco-2 cells. One possibility was that the micronized iron was not diffused into the lower chamber. Additionally, the permeability of dialysis membrane covering with insert membrane was significantly reduced. It might be because the distance between the two membranes could not be maintained. Briefly, they overlapped.

Similarly, Bering et al., also designed a triple-chamber system. Caco-2 cells were seeded in the 6-well insert and then placed it into 6-well plate for a 19-day cell monolayer culture. An additional 12-well insert was used for iron treatment. The insert membrane of the 12-well insert was removed. A piece of dialysis membrane was fixed at the bottom of the 12-well insert frame with the silicone O ring. Then, they were placed into the 6-well insert. 0.5mL of Fe^{55} digesta was added to the 12-well insert and incubated for 2 hours. The iron absorption was determined with the assessment of Fe^{55} activity. In their opinions, growing Caco-2 cells

in the 6-well insert could provide the space for the transportation of cellular iron. However, excessive cellular iron could be stored as ferritin, which might not significantly rise in 2 hours after iron treatment. This system held that the strength of growing cells in insert might lie in minimizing the bubbles or domes in the monolayer, but the limitation that insert could not be coated by collagen should be considered. In addition, radiolabeled iron might just bind to the surface of Caco-2 cell and was not absorbed by cells. Third, the continuity of Caco-2 monolayer seemed to be compromised in Bering's study.[162;176] Thus, the necessity and reliability of this triple-chamber system were questioned.

The significant iron bioavailability was also reported in other Caco-2 cell-culture studies using classic dual-chamber system. In Wiesinger et al.'s study, the bioavailability of endogenous iron in bean pastas was assessed. White and yellow beans had high ferritin level (approximate 20-30 ng ferritin/mg protein).[177] In Yeung et al.'s study, the iron bioavailability of bread and milk fortified with NaFeEDTA, ferrous fumarate, or $FeSO_4$ was assessed. In bread, the ferritin concentrations of NaFeEDTA, ferrous fumarate, or $FeSO_4$ were 40-60 ng ferritin/mg protein. In milk, the ferritin results were 120-140 ng ferritin/mg protein.[178]

To our surprise, even if the food content of INFOGEST digesta was much higher than that of Glahn's method digesta, the dialysis membranes were not clogged, since NaFeEDTA digesta, ferrous fumarate digesta and $FeSO_4$ digesta were superior or equivalent to their supernatant in ferritin response. It also implied that plentiful soluble iron could not be separated from chyme-mass to supernatant using centrifuging treatment. Supernatant might just contain part of soluble iron. The rest of soluble iron was in the precipitate at the bottom of centrifuge tube. Thus, adding iron digesta instead of iron supernatant in Caco-2 cells led to this discrepancy between iron solubility and iron bioavailability. This was a possible explanation for the occasional poor predictive ability of iron solubility.

NaFeEDTA is an iron-EDTA chelate. EDTA moiety can hold the iron in acid environment (stomach), allowing the iron dissociation in duodenum to be exchanged with other nutrients and absorbed by the intestinal epithelial cells.[62;96;97] Ferrous fumarate and $FeSO_4$ are ferrous complexes. Compared with ferric complexes, they were easily hydrolyzed in stomach and absorbed in duodenum due to the weak electromagnetic field ($2O^{-}$-Fe^{2+}). As shown in Figure 4.6, NaFeEDTA and ferrous fumarate had the highest iron bioavailability (119.4 and 110.2 ng ferritin/mg protein). Since the iron element concentration of NaFeEDTA was much lower than that of ferrous fumarate, the weight of NaFeEDTA (530 mg NaFeEDTA/kg food) added was around twice than that of the ferrous fumarate (240 mg ferrous fumarate/kg food)

added to reach the target iron fortification level (130 mg Fe/kg food). However, the amount of NaFeEDTA was restricted by the WHO for children under 5 years old. The tolerable intake level was 1.9 mg of NaFeEDTA for per kg of body weight per day. Therefore, combining NaFeEDTA with another iron fortificants, ferrous fumarate or $FeSO_4$ at a ratio of 4:9 or 2:11 was recommended by the Food Aid Quality Review Report.[139;179;180] Despite the lack of authoritative information, too much EDTA might also bring about unexpected side effects in adults. Thus, these combinations of two types of irons were tested in current study.

We found that all of the combinations were effective (48.1-96.1 ng ferritin/mg protein). Surprisingly, the combination of NaFeEDTA and $FeSO_4$ at low weight ratio (2:11) had high ferritin response. When $FeSO_4$ was dissolved in water, it could form an aquo complex $[Fe(H_2O)_6]^{2+}$.[181] One possible reason was that EDTA could react with $[Fe(H_2O)_6]^{2+}$ to maximize the iron absorption at a specific ratio. There seemed to be a curve-fitting relationship depending on various influencing factors. In the clinical trial conducted by Peruvian et al., the levels of $^{57/58}$Fe absorption were significantly improved from 2.4% to 3.7%, from 2.2% to 3.5%, and from 2.9% to 3.8% at EDTA-Fe ($FeSO_4$) molar ratios of 1:1, 0.7:1, and 0.3:1.[182] In another clinical trial on radio-labelled EDTA-$FeSO_4$ fortifications, the results of iron absorption were 3.8%, 11.3%, 13.5%, and 8.8% at EDTA-Fe molar ratios of 0:1, 0.25:1, 0.5:1, and 1:1.[183] Similarly, Hurrell et al. found that isotopic iron absorption could be elevated from 1.0% to 5.7% and from 0.7% to 2.9% at EDTA-Fe ($FeSO_4$) molar ratio of 0.67:1 and 1:1.[184] In a study of young Chinese children, $^{57}FeSO_4$-fortified meals with EDTA-Fe molar ratios of 0.4:1 or 0.7:1 were tested. Only the meal with a ratio of 0.4:1 could significantly increase the iron absorption.[185] However, the poor sensory properties of $FeSO_4$ should be taken into account.

4.5 Conclusion

The Caco-2 cell model with the classic dual-chamber system and INFOGEST digesta is a reliable iron bioassay. NaFeEDTA has higher bioavailability than $FeSO_4$. The combination of NaFeEDTA and $FeSO_4$ at low weight ratio (2:11) has the highest bioavailability among the combination samples.

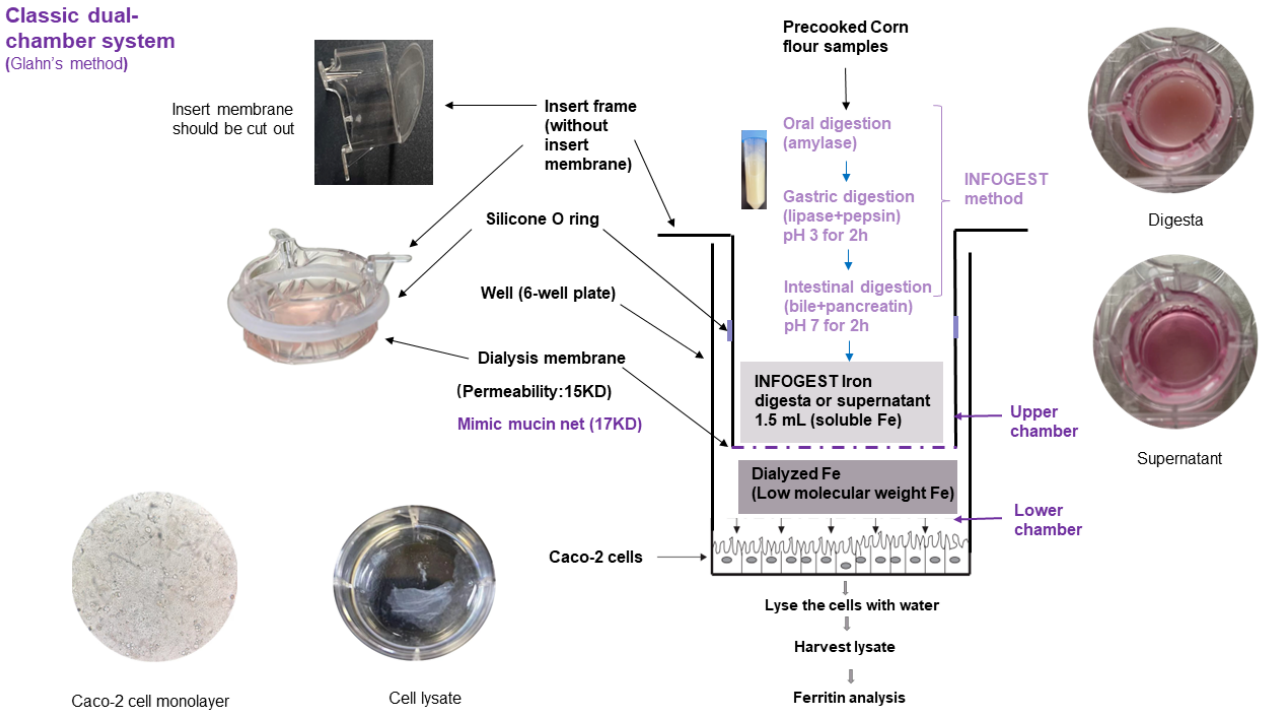


Figure 4.1: Classic dual-chamber system

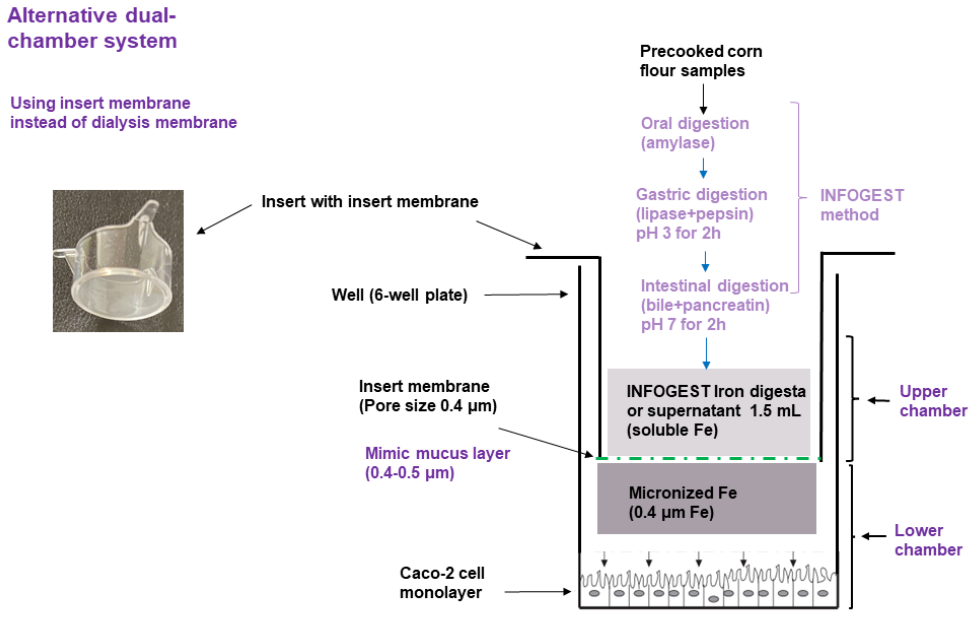


Figure 4.2: Alternative dual-chamber system

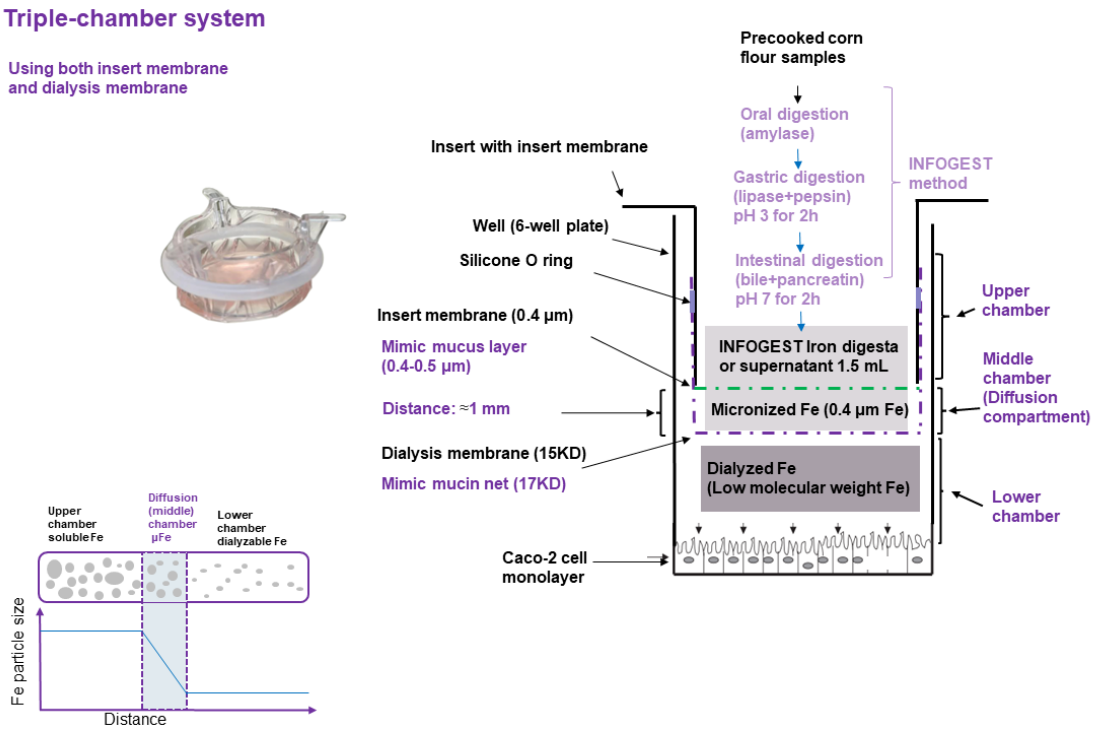


Figure 4.3: Triple-chamber system

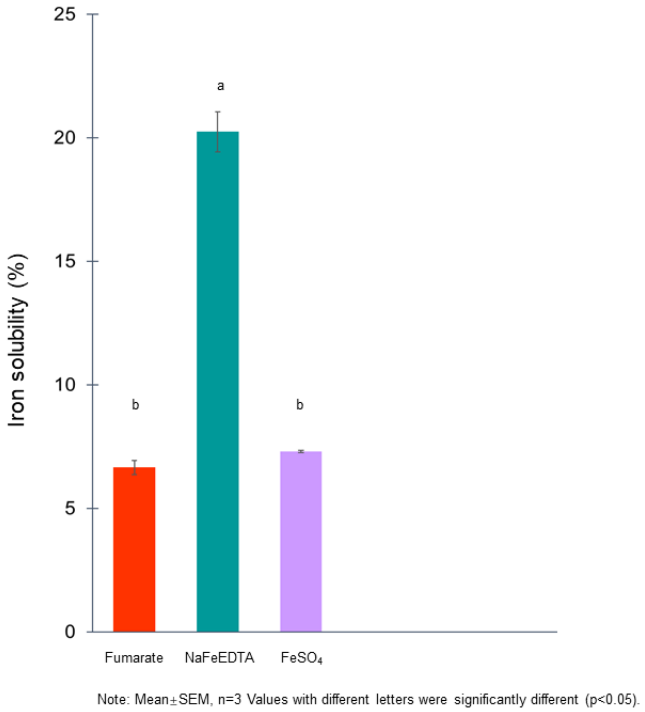


Figure 4.4: Iron solubility (Experiment 1)

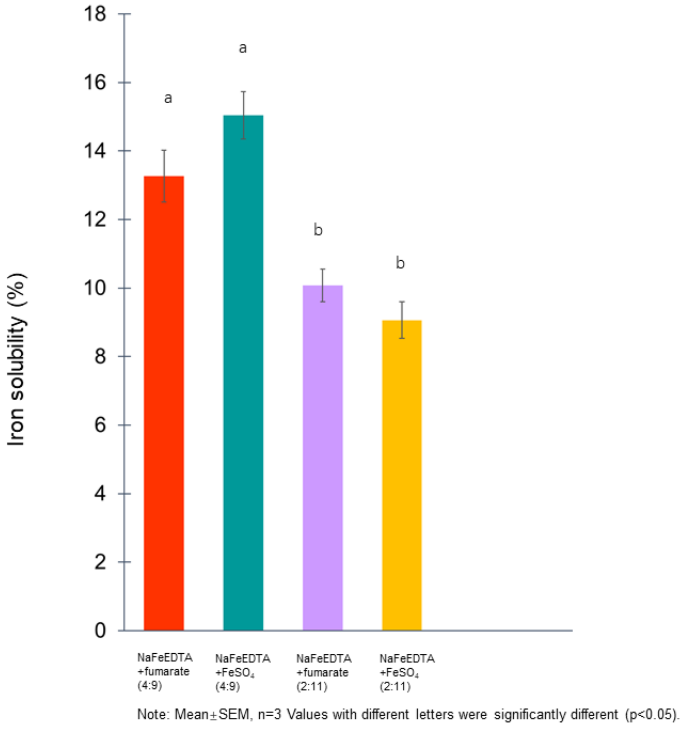


Figure 4.5: Iron solubility (Experiment 2)

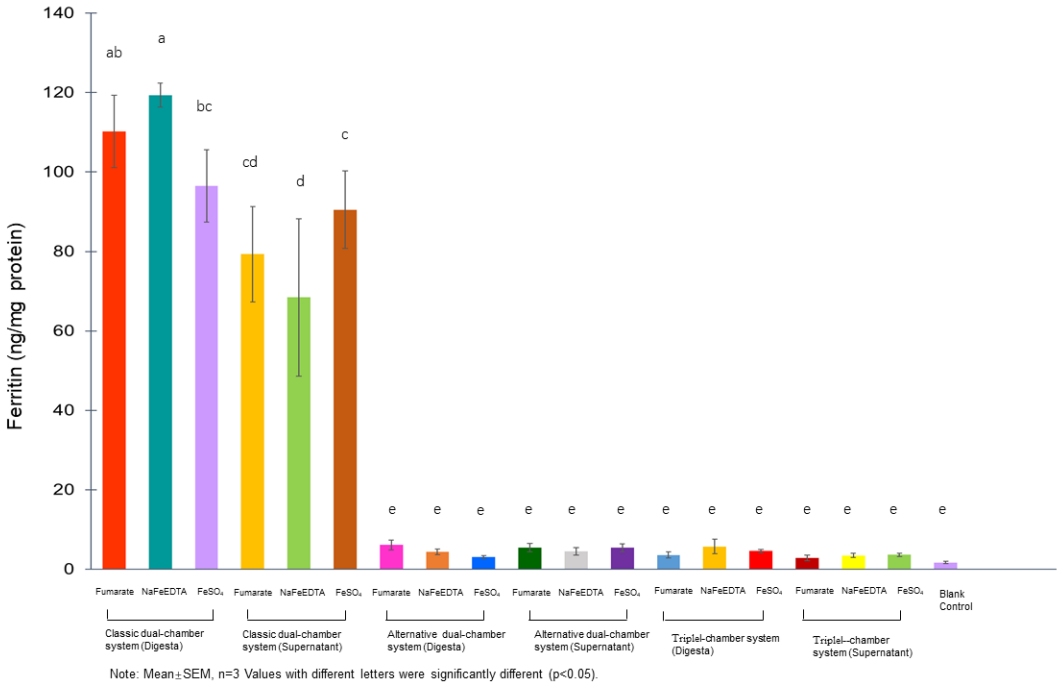


Figure 4.6: Ferritin concentrations (Experiment 1)

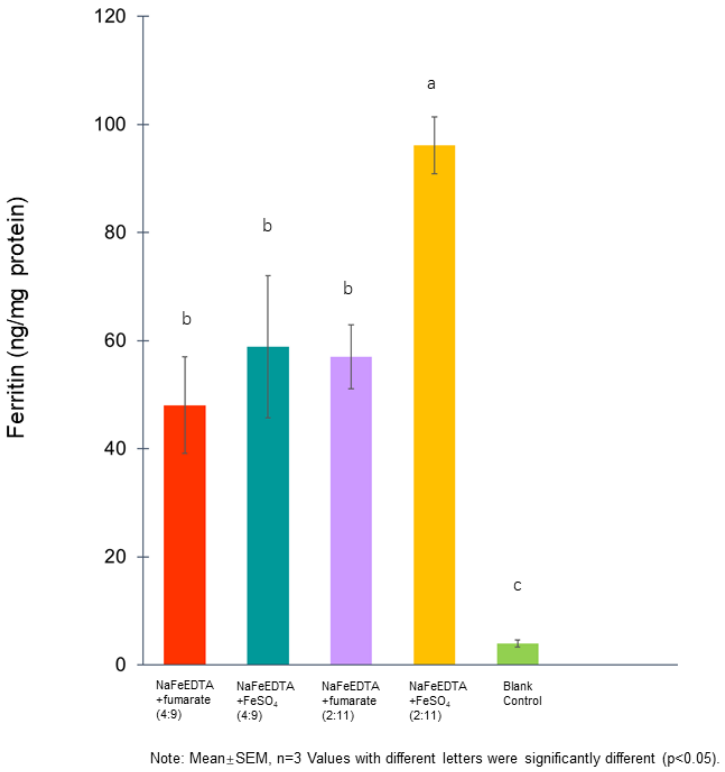


Figure 4.7: Ferritin concentrations (Experiment 2)

Table 4.1: *Iron fortification levels*

Sample ID	**Fe component**	**Target Fe fortification level (mg/kg food)**	**Fe compound concentration (mg/kg food)**	**Analytical Fe element concentration (mg/kg food)**
1	Fumarate	130	240.0	138.0
2	NaFeEDTA	130	530.0	121.4
3	$FeSO_4 \cdot 7H_2O$	130	390.0	129.6
4	NaFeEDTA	40	240.0	130.6
	Fumarate	90	190.0	
5	NaFeEDTA	40	198.6	131.3
	$FeSO_4 \cdot 7H_2O$	90	270.0	
6	NaFeEDTA	20	99.3	133.4
	Fumarate	110	191.3	
7	NaFeEDTA	20	99.3	118.6
	$FeSO_4 \cdot 7H_2O$	110	330.0	

Table 4.2: *Formulation of electrolyte solution and simulated digestion fluid*

Electrolyte Name	**Electrolyte Molecular Formula**	**Electrolyte Molecular Weight (g/mol)**	**Electrolyte solution concentration (M)**	**SSF (electrolyte solution mL/) water L)**	**SGF (electrolyte solution mL/ water L)**	**SIF (electrolyte solution mL/water L)**
Potassium chloride	KCl	74.6	0.5	37.75	17.25	17
Potassium dihydrogen phosphate	KH_2PO_4	136	0.5	9.25	2.25	2
Sodium hydrogen carbonate	$NaHCO_3$	84	1	17	31.25	106.25
Sodium chloride	NaCl	58.44	2	None	29.5	24
Magnesium chloride hexahydrate	$MgCl_2(H_2O)_6$	203.3	0.15	1.25	1	2.75
Ammonium carbonate	$(NH_4)_2CO_3$	96	0.5	0.15	1.25	None
Calcium chloride dihydrate	$CaCl_2(H_2O)_2$	147	0.3	0.0625	0.0125	0.1

Table 4.3: *Iron solubility (Experiment 1)*

Sample ID	**Fe component**	**Iron solubility (%)**
1	Fumarate	6.7 ± 0.28^{b}
2	NaFeEDTA	20.2 ± 0.82^{a}
3	$FeSO_4$	7.3 ± 0.04^{b}

Note: Mean±SEM, n=3. Values with different letters were significantly different ($p<0.05$).

Table 4.4: *Iron solubility (Experiment 2)*

Sample ID	**Fe component**	**Weight ratio**	**Iron solubility (%)**
4	NaFeEDTA+fumarate	4:9	13.3 ± 0.75^{a}
5	NaFeEDTA+$FeSO_4$	4:9	15.0 ± 0.69^{a}
6	NaFeEDTA+fumarate	2:11	10.1 ± 0.48^{b}
7	NaFeEDTA+$FeSO_4$	2:11	9.1 ± 0.54^{b}

Note: Mean±SEM, n=3. Values with different letters were significantly different ($p<0.05$).

Table 4.5: *Ferritin concentrations (Experiment 1)*

Sample ID	**Fe component**	**Iron additive form**	**Iron treatment system**	**Ferritin concentration (ng/mg protein)**
1	Fumarate	Digesta	Classic dual-chamber	110.21 ± 9.08^{ab}
2	NaFeEDTA	Digesta	Classic dual-chamber	119.37 ± 3.05^{a}
3	$FeSO_4$	Digesta	Classic dual-chamber	96.52 ± 9.08^{bc}
1	Fumarate	Supernatant	Classic dual-chamber	79.30 ± 12.03^{cd}
2	NaFeEDTA	Supernatant	Classic dual-chamber	68.43 ± 19.76^{d}
3	$FeSO_4$	Supernatant	Classic dual-chamber	90.51 ± 9.78^{c}
1	Fumarate	Digesta	Alternative dual-chamber	6.10 ± 1.29^{e}
2	NaFeEDTA	Digesta	Alternative dual-chamber	4.38 ± 0.70^{e}
3	$FeSO_4$	Digesta	Alternative dual-chamber	3.05 ± 0.38^{e}
1	Fumarate	Supernatant	Alternative dual-chamber	5.49 ± 1.07^{e}
2	NaFeEDTA	Supernatant	Alternative dual-chamber	4.50 ± 0.90^{e}
3	$FeSO_4$	Supernatant	Alternative dual-chamber	5.39 ± 1.01^{e}
1	Fumarate	Digesta	Triple-chamber	3.60 ± 0.80^{e}
2	NaFeEDTA	Digesta	Triple-chamber	5.73 ± 1.81^{e}
3	$FeSO_4$	Digesta	Triple-chamber	4.57 ± 0.43^{e}
1	Fumarate	Supernatant	Triple-chamber	2.82 ± 0.74^{e}
2	NaFeEDTA	Supernatant	Triple-chamber	3.49 ± 0.51^{e}
3	$FeSO_4$	Supernatant	Triple-chamber	3.63 ± 0.41^{e}
None	Control	None	None	1.67 ± 0.3^{e}

Note: Mean±SEM, n=3. Values with different letters were significantly different ($p<0.05$).

Table 4.6: *Ferritin concentrations (Experiment 2)*

Sample ID	**Fe component**	**Iron additive form**	**Iron treatment system**	**Ferritin concentration (ng/mg protein)**
4	NaFeEDTA +fumarate (4:9)	Digesta	Classic dual-chamber	48.05 ± 8.95^{b}
5	NaFeEDTA +$FeSO_4$ (4:9)	Digesta	Classic dual-chamber	58.87 ± 13.16^{b}
6	NaFeEDTA +fumarate (2:11)	Digesta	Classic dual-chamber	56.99 ± 5.92^{b}
7	NaFeEDTA +$FeSO_4$ (2:11)	Digesta	Classic dual-chamber	96.12 ± 5.24^{a}
None	Control	None	None	3.91 ± 0.67^{c}

Note: Mean±SEM, n=3. Values with different letters were significantly different ($p<0.05$).

Chapter 5

Bioavailability of Ferric Pyrophosphate and Ferric Orthophosphate with/without Extrusion and/or Citric Acid and/or Trisodium Citrate in the INFOGEST digestion and Caco-2 Cell Model

Abstract

Background: Iron bioavailability is an essential factor for the physiological maintenance of body and cellular iron levels. Currently, two screening methods for estimating iron absorption have been advocated. The first is the use of a single meal fortified isotopic iron in human participants. The second is the measurement of dialyzed iron uptake in Caco-2 cells after simulated gastrointestinal digestions. Some Swiss scientists reported that iron absorption of rice flour fortified and extruded with ^{57}FePP, citric acid (CA) and trisodium citrate (TSC) at low (1:0.1:2.1) and high (1:0.3:5.5) molar ratios could be significantly increased. Thus, we intended to carry out a Caco-2 cell experiment to examine the bioavailability of

regular FePP and $FePO_4$ using similar formulations and food processing strategies.

Objective: To determine the iron bioavailability of extruded rice flour fortified with the high/low molar ratio CA and TSC with FePP/$FePO_4$ using the *in vitro* INFOGEST digestion/Caco-2 cell model.

Methods: 13 extruded rice flour samples fortified with high/low molar ratio of FePP/$FePO_4$, CA, and TSC were digested with INFOGEST method. The iron solubility was measured by ICP-OES in Experiment 1. Subsequently, this method was repeated in Experiment 2 and then Caco-2 cells were treated with iron digesta in a classic dual-chamber system for 2 hours. The iron bioavailability of FePP and $FePO_4$ was determined by measuring ferritin concentrations.

Main Results: High ratio FePP, $FePO_4$, and μFePP were significantly higher than low/zero ratio FePP, $FePO_4$, μFePP, and control.

Conclusions: The iron bioavailability of FePP and $FePO_4$ was improved with high molar ratio (1:0.3:5.5) CA and TSC.

Keywords: Iron bioavailability, ferric pyrophosphate, ferric orthophosphate, extrusion, citric acid, trisodium citrate, INFOGEST, Caco-2 cell.

5.1 Introduction

Anemia is a serious public health problem. The largest proportion of anemia worldwide is iron deficiency anemia. Rice flour can be fortified with iron to combat deficiency anemia, but it is challenging to achieve high iron bioavailability and good organoleptic properties.[55–57] In general, common iron fortificants, ferric pyrophosphate and ferric orthophosphate, have relatively superior sensory properties but low iron bioavailability. Thus, iron absorption enhancers, food processing strategies and micronized size iron fortificants are used to improve the iron bioavailability.[186] Extrusion is a commercial food processing technology. Food is cooked, squeezed, and gelatinized by the mechanical and thermal energy of the extruder to improve mineral absorption.[187] CA and TSC can work as enhancers through redox reaction. Micronize-sized iron fortificants have a larger surface area so that iron fortificants can be more easily hydrolyzed in the gastrointestinal tract.[90;91]

Rice is a staple food for half of the world's population, but it is sensitive to color changes mixed with iron fortificants. Hackl et al., reported that the iron bioavailabil-

ity of rice flour fortified and extruded with ^{57}FePP, CA, and TSC at the molar ratio (Fe:CA:TSC=1:0.1:2.1) was significantly higher than that of ^{57}FePP rice extrudate without CA or TSC in iron-sufficient young women.[186] Scheuchzer et al., further found that a higher molar ratio (Fe:CA:TSC=1:0.3:5.5) could sharply increase the iron absorption of ^{57}FePP rice extrudate in iron-sufficient young women. It suggested that extrusion and enhancers could transform the insoluble FePP into soluble Fe citrate complexes without the color change of rice flour.[188]

Isotope iron study is based on a single meal after an overnight fasting. Isotopic iron is a good scanning tool, while it may overestimate the iron bioavailability.[189;190] Thus, compared with a daily diet fortified with regular iron compounds, isotopically labeled iron may not be directly applied in human food aid. Therefore, we examined the iron bioavailability of regular FePP and $FePO_4$ formulations using an *in vitro* INFOGEST digestion/Caco-2-cell model. This model could test the delivery of iron from diet to enterocytes as a proximately physiological bioassay. In this way, we aimed to determine whether CA, TSC, and extrusion could increase iron bioavailability and find out the most effective formulations in terms of the types of iron fortificants, the particle size of iron fortificants, the amount of iron absorption enhancers and the food processing strategies. The results can be further studied for the animal experiments and large-size clinical trials on the superiority and equivalence of iron fortifications.

5.2 Materials and methods

5.2.1 Materials

All materials and supplies used in the present experiment were listed in Appendix B (page 227)

5.2.2 Sample preparation

In this study, four impact factors (Table 5.1) were set up to analyze the iron bioavailability of samples. They were the type of iron (FePP or $FePO_4$), the particle size (regular or micronized), the molar ratio of enhancers (high or low ratio), and the food processing strategy (co-extruded or mixed). Two strategies of food processing were performed to identify which one could better improve the iron bioavailability.[187;191] This step was called minor mixing.

Strategy 1: Raw rice flour, iron, CA, and TSC were premixed at a high molar ratio

(1:0.3:5.5) or low molar ratio (1:0.1:2.1), and then extruded together by a lab-scale twin-screw extruder (Table 5.2). Spaghetti-shaped extrudates were produced, dried in the small-size oven at 70°C for 2 h, and manually cut to 1 cm. The extrudates were further ground by a coffer mill, sifted to reach the final particle size below 1 mm, and frozen until use. Since rice flour, iron, CA and TSC were extruded concurrently, this final product was called co-extruded rice flour sample (Figure 5.1).

Strategy 2: Raw rice flour was extruded by a pilot-scale twin-screw extruder (Table 5.2), dried by a gas-fired dryer at 115°C for 18 minutes with a 7-minute cooling, and ground by a hammer mill with a mesh size of 0.3 mm. Subsequently, iron, CA, and TSC were mixed with extruded rice flour at a high/low molar ratio, and frozen until use. Since iron, CA, and TSC were just mixed with the extruded rice flour, this final product was called mixed extruded-rice flour sample (Figure 5.1).

Thirteen rice flour samples were designed. The level of iron fortification in the minor mixing step was 700 mg/kg rice flour (Table 5.3). Since the final target level of iron fortifications recommended by the United States Department of Agriculture (USDA) was 40 mg/kg rice flour, the co-extruded rice flour samples and mixed extruded-rice flour samples were diluted with extruded rice flour that was produced using Strategy 2 (Table 5.4). This step was called major mixing. This study was split to two experiments. In the Experiment 1, the iron solubility of minor mixing products was measured. The objective of Experiment 2 was to assess the iron solubility and bioavailability of major mixing products.

5.2.3 INFOGEST procedure

Initially, electrolyte solutions, simulated salivary fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF) were prepared and used for all digestion phases (Table 5.5).[148] In the oral phase, 5 g of food samples were mixed with 4 mL of SSF and 1 mL of amylase solution (83 mg amylase/ml water, 9 enzymatic activity unit/mg amylase) in the 50 ml centrifuge tube. The oral boluses were manually minced with metal spatulas and incubated at 37°C for 2 minutes. The oral boluses were added to 8 mL of SGF in the gastric phase. After the pH values were reduced to 3±0.1 with 6M hydrochloric acid, 1 mL of pepsin solution (66 mg pepsin/ml water, 605 enzymatic activity unit/mg pepsin) and 1 mL of lipase solution (80 mg lipase/ml water, 15 enzymatic activity unit/mg lipase) were added and then incubated on the shaking plate of incubator at 37°C and 150 rpm for 2 hours. In the intestinal phase, 8 mL of SIF was mixed with the gastric chyme and then pH values were adjusted to 7±0.1 with 5M sodium hydroxide. 3 mL of bile solution (54 mg bile/ml

SIF, 10mM), 5 mL of pancreatin solution (4 mg pancreatin/ml SIF, 200 enzymatic activity unit/mg pancreatin), and 4 mL of water were added and incubated at 37°C and 150 rpm for 2 hours. Digesta was heated at 100°C for 5 minutes to stop intestinal digestion before being centrifuged at 5000g and 5°C for 45 minutes. The data of enzymatic activities was provided by Sigma Aldrich company. The iron contents of dry samples and supernatant were analyzed with ICP-OES method by Soil Testing lab at Kansas State University. Solubility (%)= 100*soluble iron content (supernatant)/total iron content (dry sample).[148;150–154]

5.2.4 Caco-2 cell culture procedure

Caco-2 cells were purchased from ATCC at passage 18. The Caco-2 cells were cultured in T25, T75 and T225 flasks with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 1% Zellshield, and 25 mM HEPES in 37 °C and 5% CO_2 air.[149]

Caco-2 cells were detached with trypsin (0.25%)-EDTA (0.53 mM) for 5-10 minutes at 80-100% confluency. Dissociated cells were centrifuged at 125g for 5-10 minutes. Cells were counted with hemocytometer. The sufficient Caco-2 cells (passage 23) from T225 flasks were transferred to collagen coated 6-well plates with 2mL of DMEM per well at a seeding density of 50,000 cells/cm^2 and allowed to grow for 12 days with media being refreshed every two days.[149]

On the 12^{th} day of the 6-well plates culture phase, DMEM was replaced by 2 mL of Minimum Essential Medium (MEM) supplemented with 4 mg/L hydrocortisone, 10 mM Piperazine-1,4-bis (2-ethanesulfonic acid) disodium salt, 20 μg/L epidermal growth factor, 5 μg/L selenium, 34 μg/L triiodothyronine, 5 mg/L insulin, and 1% Zellshield.[149] Iron digesta was produced using INFOGEST method and stored overnight at -80°C.[153]

On the next day, iron treatment systems were prepared and sterilized with 0.5 M HCl solution for at least 1 hour, and rinsed with autoclaved deionized water. 2mL of MEM was replaced by 1mL of MEM into the lower chamber and 1.5 mL of iron digesta into the upper chamber. Iron treatments were randomly assigned to the Caco-2 cells. The 6-well plates were placed on the rocking shaker (6 vibrations/min) and incubated for 2h. The dual- or triple- chamber system was removed and 1mL of MEM was added. The Caco-2 cells were returned to the incubator for another 22 hours. Blank control (only media) was used.

On the last day, MEM was removed and autoclaved deionized water was added to osmotically lyse the Caco-2 cells.[149] Placed a tube rack in the sonicator and then placed the cells on the tube rack for a 30-second sonication cell lysis.[155] Finally, cell lysate was collected for ferritin and protein analyses according to the instructions of ferritin and protein kits

(Figure 5.2).[149]

5.2.5 Statistical analysis

Data were analyzed in SAS online Studio (https://welcome.oda.sas.com/) with significance at p-value<0.05. The Shapiro-Wilk's test was used for normality. Levene's test was used for homogeneity. Normally distributed data were assessed by one-way analysis of variance (ANOVA) with Least Significant Difference (LSD) test. The random number table was generated with Excel. ELISA analysis was performed using the Arigo online calculator at https://www.arigobio.com/elisa-analysis.[156]

5.3 Results

5.3.1 Iron solubility

In the Experiment 1 (minor mixing products), co-extruded FePP samples were significantly higher than mixed FePP samples. Among co-extruded samples, high and low FePP samples were significantly higher than high and low $FePO_4$ samples. High FePP and $FePO_4$ samples were significantly higher than low FePP and $FePO_4$ samples. Among mixed samples, high FePP sample had higher iron solubility than low FePP, high $FePO_4$, high μFePP, and zero FePP samples. High and low $FePO_4$/μFePP samples were significantly higher than zero $FePO_4$/μFePP samples, respectively. (Figure 5.3, Table 5.6).

In the Experiment 2 (major mixing products), co-extruded high FePP sample was significantly higher than mixed high FePP sample. Among co-extruded samples, high FePP sample had higher iron solubility than low FePP and high $FePO_4$ samples. Among mixed samples, high FePP sample was significantly higher than low FePP, high $FePO_4$, high μFePP, and zero FePP samples, respectively. High and low $FePO_4$ samples had higher iron solubility than zero $FePO_4$ sample (Figure 5.4, Table 5.6).

5.3.2 Ferritin concentration

Ferritin concentrations of minor mixing products were not assessed with the Caco-2 cell model due to a potential risk of iron overload. In the Experiment 2, high ratio FePP, $FePO_4$, and μFePP were significantly higher than low/zero ratio FePP, $FePO_4$, μFePP, and control. Co-extruded low ratio FePP sample and mixed low ratio $FePO_4$ sample were significantly higher than control (Figure 5.5, Table 5.6).

5.4 Discussion

Extrusion can change the viscosity, hardness. crystallinity and other physicochemical properties of extrudate. In this study, the raw rice flour was put into the mixer and then went into the barrel that had several sections at different temperatures ranging from low to high, so the raw rice flour could be cooked and squeezed by the twin-screw movement (Figure 5.6). The physical-modified characteristics of extruded rice flour were verified by Scheuchzer et al. using the Small- and Wide-angle X-ray scatter test. The crystalline structure of starch was disrupted. Starch was degraded and gelatinized. In Hagenimana's experiment, water absorption and solubility of extruded rice flour could be increased. Besides, the inhibitors (e.g., phytic acid, phenolics, etc.) could be destroyed during extrusion. Tadesse et al. and Arun Kumar et al. found that phytate content was reduced in extruded sorghum flour.[192;193] Lubaaler et al. reported that the phenolic content of extruded sorghum porridge was lower than that of cooked sorghum porridge.[194] Thus, the extrude rice flour, instead of raw rice flour or cooked rice flour, was used to be mixed with iron and enhancers to improve iron hydrolytic capacity in the gastrointestinal tract.[188;195] In this study, we used the term "co-extrusion" to define the food processing that several micronutrients were extruded concurrently.[196] However, some scientists thought that "co-extrusion" usually referred to one staple food extruded with another staple food, fat-baser (e.g., cream), and/or water baser (e.g., jellies, jams.) simultaneously.[197]

Moreover, using infrared spectrum tests, Wu et al., investigated that iron could react with starch molecular to form a ferric starch complex through the coordinate bond C=O. In chemistry, ferric pyrophosphate was composed of multiple ferric orthophosphate groups with the P-O-P linkages (high-energy phosphate bond) and 3O-Fe^{3+} coordination bonds (Figure 8). If the physicochemical properties of FePP and its enhancers were changed, the environment of iron-ligand and the electromagnetic filed might be altered. Scheuchzer et al. indicated that the iron absorption of ^{57}FePP, CA, TSC and rice co-extrudate was higher than that of extruded rice flour mixed with FePP, CA and TSC. They employed electron paramagnetic resonance to find that the spin configuration of Fe^{3+}, the paramagnetic properties of FePP, the electromagnetic force of iron-ligand, the ligand fraction of Fe^{3+}, and the linear pyrophosphate structure of FePP were altered by the high temperatures, high pressure, and shear force during co-extrusion.[196;198;199]

Hence, the reasons mentioned above could interpret that FePP and enhancers concur-

rently extruded with rice flour at high molar ratio had the highest solubility. Whereas, co-extrusion did not improve the ferritin level. One possibility was the low concentration of co-extrudate. In addition, there was no significant difference between regular FePP, μFePP, and $FePO_4$ in ferritin response. One reason might be the amount of soluble iron in FePP digesta, μFePP digesta, and $FePO_4$ digesta was statistically equivalent. Another possibility might be the dose-response relationship. Since the iron solubility of FePP samples at 700mg Fe/kg level was much higher than that of FePP samples at 40mg Fe/kg, it was possible that the iron bioavailability of FePP could be greatly raised at higher fortification levels. Third, since the minimum concentration of iron detected by ICP-OES equipment is around 0.2 mg/L, the iron concentrations of samples' supernatant (0.13-0.19 mg/L) were lower than 0.2 mg/L except for the iron concentrations of high ratio FePP supernatant (0.27-0.29 mg/L). Thus, the sensitivity and accuracy of instrument might be reduced in this case.

Theoretically, micronized particle size of iron fortificants had higher iron bioavailability than the regular size iron, while μFePP (2.4 μm) was not superior in iron solubility or ferritin response to regular size FePP in this study. It might be because μFePP was not co-extruded with enhancers and rice flour. In line with our results, there was no significant difference in the relative bioavailability value between μFePP (2.5 μm) and regular FePP (21μm) in a rat study.[200] In an African clinical trial, the Hb concentrations of iron-deficient children were not improved by a 6-month μFePP (2.5μm) treatment.[201] Another possible explanation was that the particle size of μFePP was still too large to be absorbed. Sakaguchi et al. found that large μFePP (5.2 μm) had an inferior Hb gain to $FeSO_4$ in rats, while small μFePP (0.3μm) was as well bioavailable as $FeSO_4$ in Hb gain.[202] In another clinical trial, isotopic μFePP (0.3μm) also had the equivalent iron absorption to $FeSO_4$ in infant cereal and yoghurt.[203]

In the present study, the molar ratio of the enhancers was the principal determinant of iron bioavailability. The formulations of FePP and $FePO_4$ with a high molar ratio CA and TSC are promising food aid strategies for humans. CA and TSC are readily absorbed in the gastrointestinal tract as an energy resource for human body. Citrate is an intermediary in the tricarboxylic acid cycle to break down pyruvate through glycolysis. Most citrate can be reabsorbed in kidney. The rest is excreted through the urine.[204–209] CA and TSC may react with Fe^{3+} to generate an anti-caking iron complex, iron citrate ($FeC_6H_5O_6Na$). They can prevent the iron polymerization through their carboxylic bonds and hydroxyl bonds. Ferric citrate has good bioavailability and is generally recognized safe for use in food aid.[204–209] Sun et al. mixed the CA with the extractive of Tegillarca granosa, a kind

of seafood. It was suggested that citric acid could enhance the cellular iron absorption, because CA might reduce the attractive force of hydrogen bonds and disturb the secondary structures of Tegillarca proteins.[210] Salovaara et al. also investigated that CA could increase the Fe^{3+} uptake in the Caco-2 cell model. CA might chelate iron and lower pH value by carboxyl bonds and hydroxyl bonds.[211;212] Palika et al., conducted a Caco-2 cell experiment and reported that CA could improve iron absorption in milk at 1:1 molar ratio.[213] Except for CA and TSC, other organic acids can be effective as well. Taking Glahn's study as an example, a high ferritin level was observed in Caco-2 cells exposed to $FeSO_4$ and citric acid, while ascorbic acid had a better ferritin response.[214] More animal studies and clinical trials are needed to refine the iron formulations.

5.5 Conclusion

The iron bioavailability of FePP and $FePO_4$ was improved with high molar ratio (1:0.3:5.5) CA and TSC.

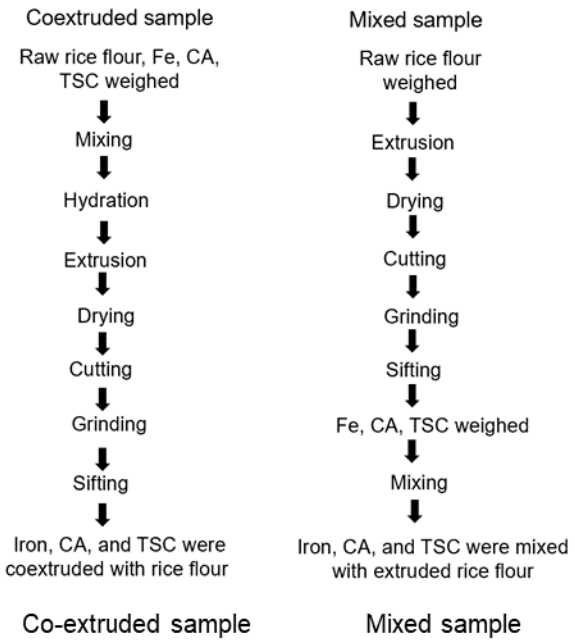


Figure 5.1: Flow chart of food processing strategies

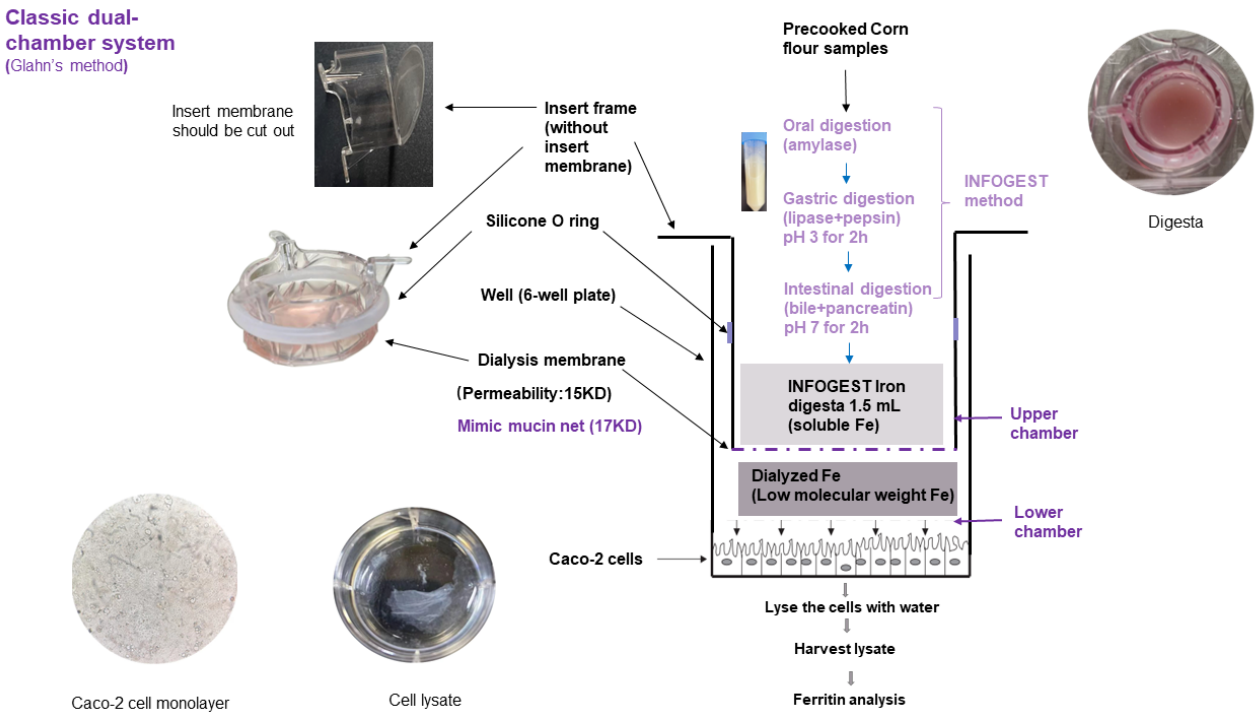


Figure 5.2: Classic dual-chamber system

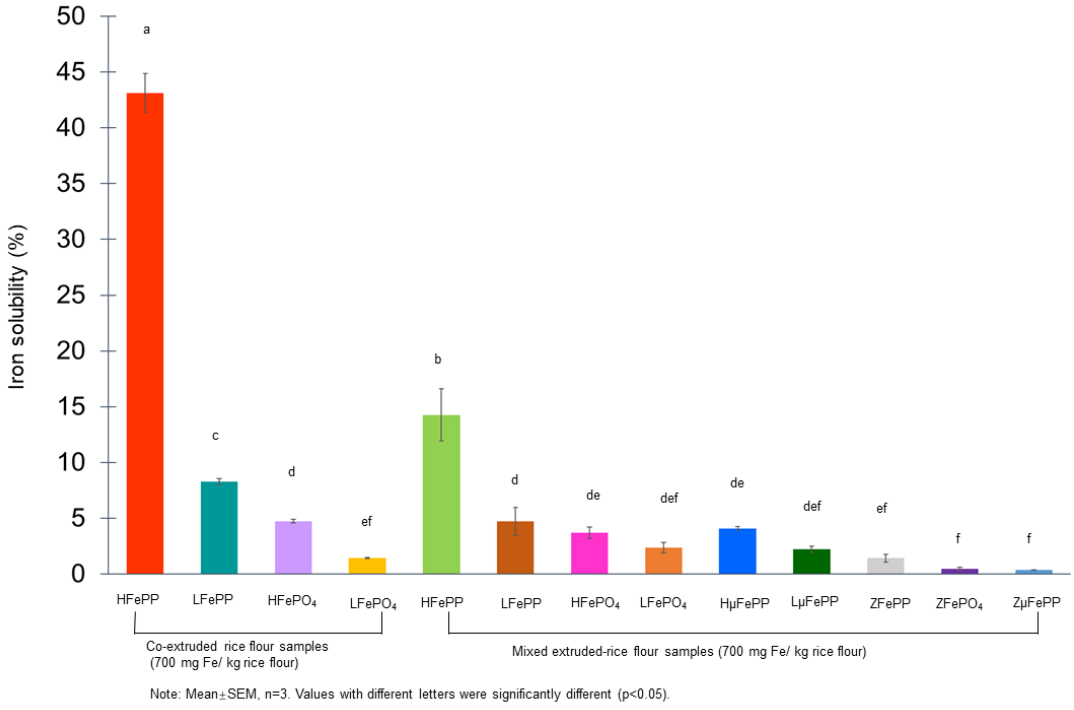


Figure 5.3: Iron solubility of Fe-fortified samples at 700 mg/kg level (Experiment 1)

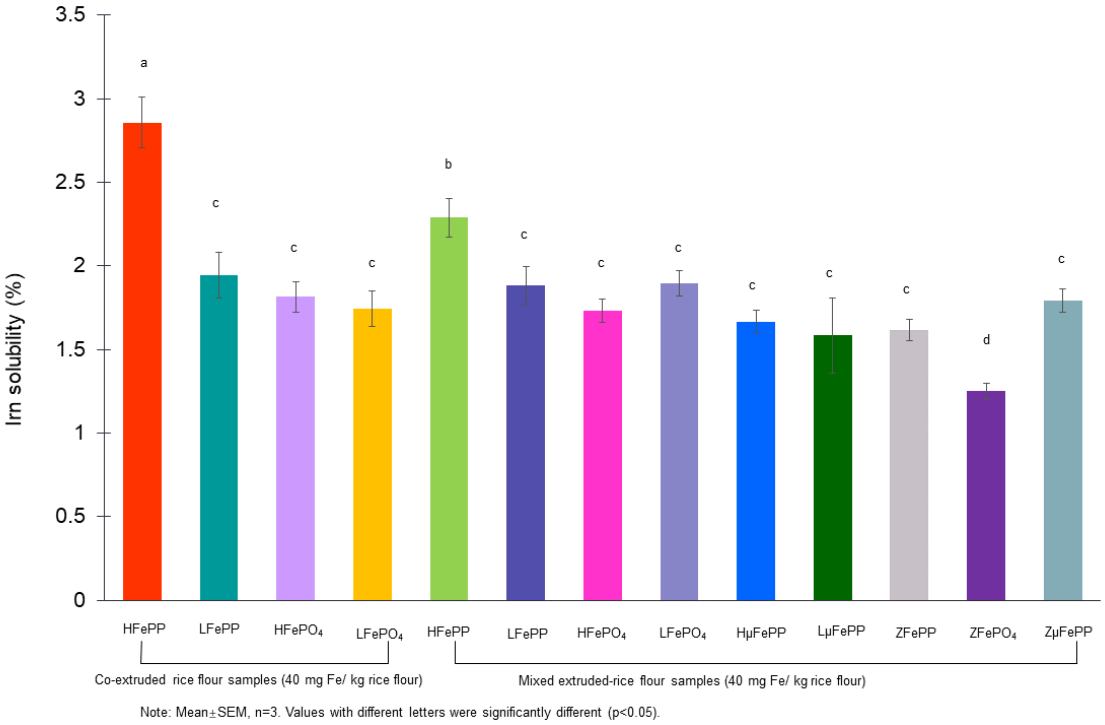


Figure 5.4: Iron solubility of Fe-fortified samples at 40 mg/kg level (Experiment 2)

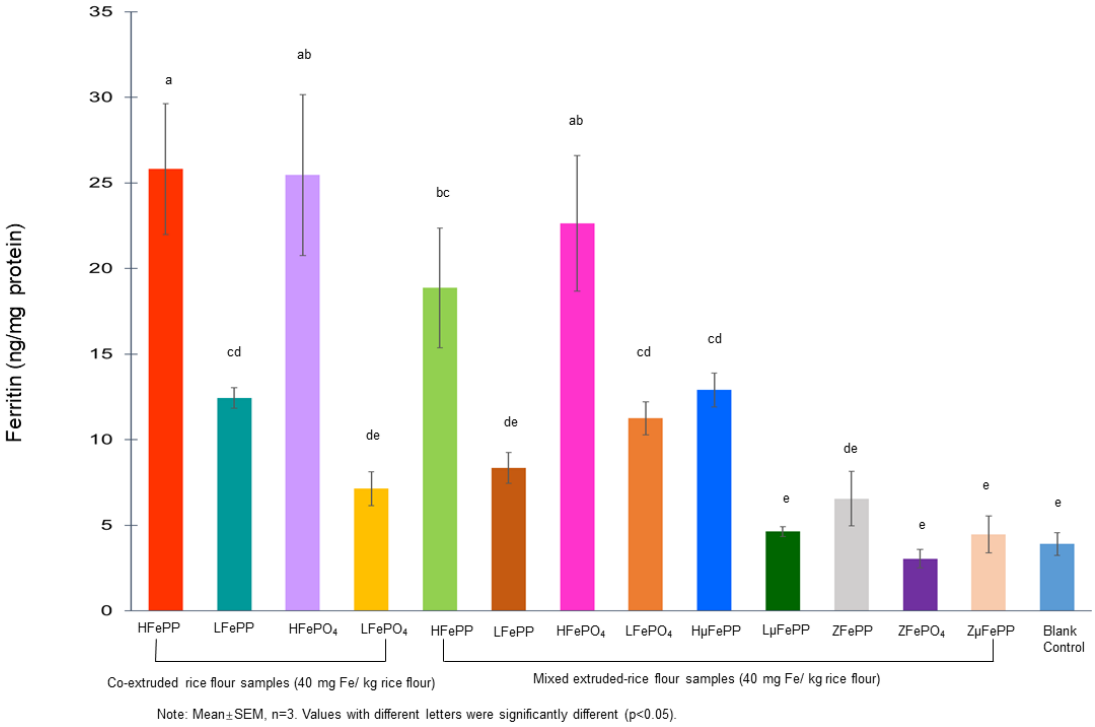


Figure 5.5: Ferritin concentrations

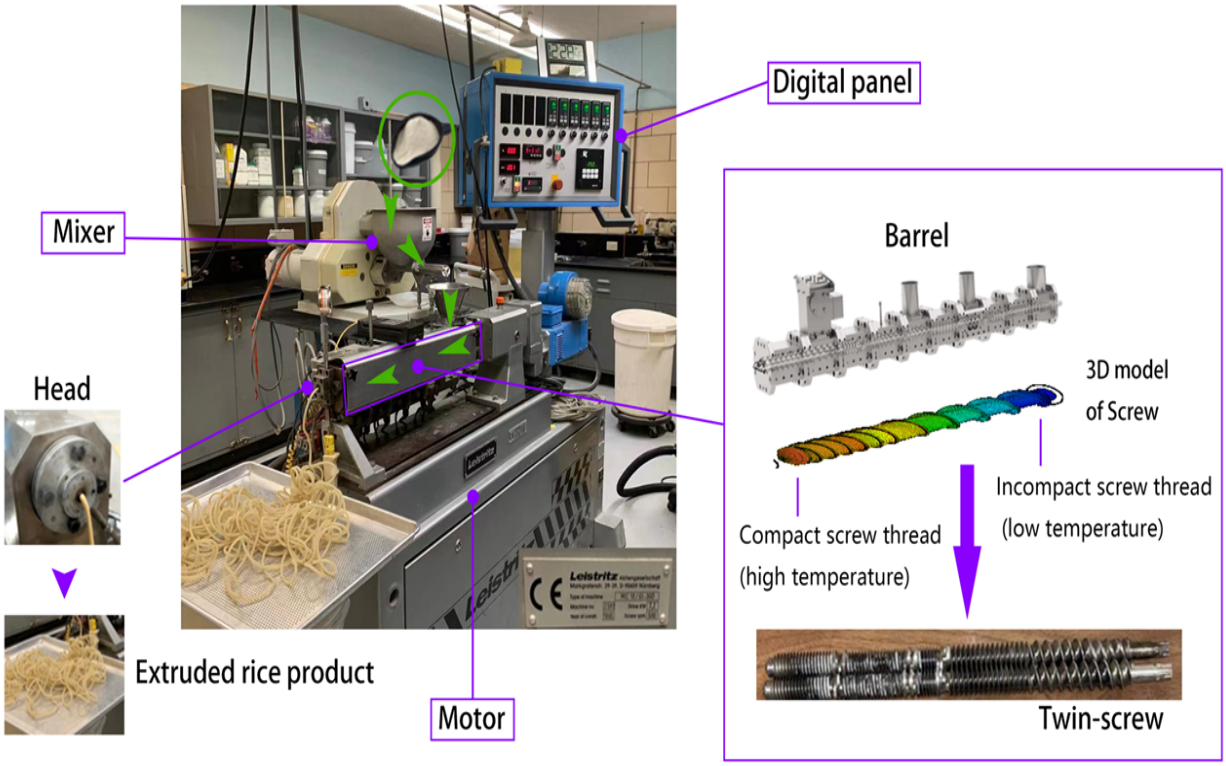


Figure 5.6: Extrusion procedure

Table 5.1: *Impact factors on iron bioavailability*

ID	Impact factors	Catalog 1	Catalog 2
1	Iron type	FePP	$FePO_4$
2	Molar ratio of Fe, CA, and TSC	1:0.3:5.5 (high molar ratio)	1:0.1:2.1 (low molar ratio)
3	Food Processing strategy	Co-extruded	Mixed
4	Particle size of iron additives	Regular FePP (20 μm)	Micronized FePP (µFePP) [2.4 μm]

Table 5.2: *Parameters of extruders*

Parameter	Pilot-scale	Lab-scale
Volumetric capacity (m^3)	0.056	None
Preconditioner shaft speed (rpm)	379	None
Average residence time (minute)	2.8	None
Barrel temperature (°C)	25-90	40-90
Screw diameter (mm)	52	18
L/D ratio	16	29
Feed speed (kg/h)	80	2.76
Screw speed fixed (rpm)	300	350
Circular die	3.7	3.1
Knife blades rotating speed (rpm)	530	Cut by hand

Table 5.3: *Sample design and iron fortification levels (700 mg/kg)*

Sample ID	**Fe component**	**Fe compound concentration (mg/kg)**	**Analytical Fe element concentration (mg/kg)**	**Fe enhancer compound**	**Enhancer concentration (mg/kg)**	**Molar ratio (Fe: enhancer)**	**Molar ratio level**	**Food processing method**
1	FePP	2820	713	CA	720	1:0.3	High	Co-extruded
				TSC	20280	1:5.5		
2	FePP	2820	725	CA	240	1:0.1	Low	Co-extruded
				TSC	7740	1:2.1		
3	$FePO_4$	2420	700	CA	720	1:0.3	High	Co-extruded
				TSC	20280	1:5.5		
4	$FePO_4$	2420	688	CA	240	1:0.1	Low	Co-extruded
				TSC	7740	1:2.1		
5	FePP	2820	823	CA	720	1:0.3	High	Mixed
				TSC	20280	1:5.5		
6	FePP	2820	767	CA	240	1:0.1	Low	Mixed
				TSC	7740	1:2.1		
7	$FePO_4$	2420	673	CA	720	1:0.3	High	Mixed
				TSC	20280	1:5.5		
8	$FePO_4$	2420	625	CA	240	1:0.1	Low	Mixed
				TSC	7740	1:2.1		
9	μFePP	2820	875	CA	720	1:0.3	High	Mixed
				TSC	20280	1:5.5		
10	μFePP	2820	910	CA	240	1:0.1	Low	Mixed
				TSC	7740	1:2.1		
11	FePP	2820	683	None	None	None	Zero	Mixed
12	$FePO_4$	2420	616	None	None	None	Zero	Mixed
13	μFePP	2820	927	None	None	None	Zero	Mixed

Table 5.4: *Sample design and iron fortification levels (40 mg/kg)*

Sample ID	**Fe component**	**Food processing method (minor mixing product)**	**Minor mixing product concentration (mg/100g)**	**Additional extruded rice flour (mg/100g)**	**Analytical Fe element concentration (mg/kg) (major mixing product)**	**Enhancer molar ratio level**
1	FePP	Co-extruded	4.92	95.08	56.9	High
2	FePP	Co-extruded	5.06	94.94	48.7	Low
3	$FePO_4$	Co-extruded	4.26	95.74	54.2	High
4	$FePO_4$	Co-extruded	5.3	94.7	51.1	Low
5	FePP	Mixed	4.86	95.14	41.9	High
6	FePP	Mixed	5.22	94.78	47.3	Low
7	$FePO_4$	Mixed	5.94	94.06	50.2	High
8	$FePO_4$	Mixed	6.4	93.6	45.9	Low
9	μFePP	Mixed	4.58	95.42	52.2	High
10	μFePP	Mixed	4.4	95.6	56.9	Low
11	FePP	Mixed	5.86	94.14	50.2	Zero
12	$FePO_4$	Mixed	6.5	93.5	60.3	Zero
13	μFePP	Mixed	4.32	95.68	42.1	Zero

Table 5.5: *Formulation of electrolyte solution and simulated digestion fluid*

Electrolyte Name	**Electrolyte Molecular Formula**	**Electrolyte Molecular Weight (g/mol)**	**Electrolyte solution concentration (M)**	**SSF (electrolyte solution mL/) water L)**	**SGF (electrolyte solution mL/ water L)**	**SIF (electrolyte solution mL/water L)**
Potassium chloride	KCl	74.6	0.5	37.75	17.25	17
Potassium dihydrogen phosphate	KH_2PO_4	136	0.5	9.25	2.25	2
Sodium hydrogen carbonate	$NaHCO_3$	84	1	17	31.25	106.25
Sodium chloride	NaCl	58.44	2	None	29.5	24
Magnesium chloride hexahydrate	$MgCl_2(H_2O)_6$	203.3	0.15	1.25	1	2.75
Ammonium carbonate	$(NH_4)_2CO_3$	96	0.5	0.15	1.25	None
Calcium chloride dihydrate	$CaCl_2(H_2O)_2$	147	0.3	0.0625	0.0125	0.1

Table 5.6: *Iron solubility and ferritin concentrations*

Sample ID	**Fe component**	**Molar ratio level**	**Iron solubility (%) (700mg/kg level)**	**Iron solubility (%) (40mg/kg level)**	**Ferritin concentration (ng/mg protein)**
1	FePP	High	43.1 ± 1.72^{a}	$2.9\pm0.15^{\alpha}$	25.82 ± 3.82^{A}
2	FePP	Low	8.3 ± 0.27^{c}	$1.9\pm0.14^{\gamma}$	12.44 ± 0.60^{CD}
3	$FePO_4$	High	4.7 ± 0.16^{d}	$1.8\pm0.09^{\gamma}$	25.46 ± 4.69^{AB}
4	$FePO_4$	Low	1.4 ± 0.04^{ef}	$1.7\pm0.10^{\gamma}$	7.14 ± 0.99^{DE}
5	FePP	High	14.3 ± 2.34^{b}	$2.3\pm0.12^{\beta}$	18.87 ± 3.49^{BC}
6	FePP	Low	4.7 ± 1.25^{d}	$1.9\pm0.11^{\gamma}$	8.34 ± 0.90^{DE}
7	$FePO_4$	High	3.7 ± 0.50^{de}	$1.7\pm0.07^{\gamma}$	22.64 ± 3.96^{AB}
8	$FePO_4$	Low	2.4 ± 0.46^{def}	$1.9\pm0.08^{\gamma}$	11.25 ± 0.96^{CD}
9	μFePP	High	4.1 ± 0.18^{de}	$1.7\pm0.07^{\gamma}$	12.90 ± 0.99^{CD}
10	μFePP	Low	2.2 ± 0.29^{def}	$1.6\pm0.22^{\gamma}$	4.63 ± 0.29^{E}
11	FePP	Zero	1.4 ± 0.34^{ef}	$1.6\pm0.06^{\gamma}$	6.56 ± 1.59^{DE}
12	$FePO_4$	Zero	0.5 ± 0.15^{f}	$1.3\pm0.05^{\delta}$	3.05 ± 0.54^{E}
13	μFePP	Zero	0.4 ± 0.01^{f}	$1.8\pm0.07^{\gamma}$	4.47 ± 1.08^{E}
Control	None	None	None	None	3.92 ± 0.67^{E}

Note: Mean±SE, n=3. Values with different letters were significantly different ($p<0.05$).

Chapter 6

Conclusions and Future Prospects

In the preclinical systemic review with pairwise meta-analysis, the overall Hb effect of NaFeEDTA was superior to that of $FeSO_4$. Ferrous fumarate was equivalent to $FeSO_4$.

In the clinical systemic review with network meta-analysis, NaFeEDTA, ferrous fumarate, and FePP were more effective than placebo, and NaFeEDTA was more effective than $FeSO_4$. The rank order of Hb effect was NaFeEDTA>ferrous fumarate>FePP>$FeSO_4$>electrolytic iron>placebo. Thus, NaFeEDTA, ferrous fumarate, FePP and $FeSO_4$ fortifiactions were effective treatments in Hb for people with iron deficiency anemia or healthy people with high risk of iron deficiency anemia. In grain-based trials, NaFeEDTA was suggested to be the most effective treatment in Hb, followed by ferrous fumarate, FePP and $FeSO_4$ in population aged under 18 years old.

However, the results of preclinical and clinical systemic reviews were supposed to be applied with caution as conditional recommendations due to unsolvable risk of biases, high heterogeneities, limited information, and insufficient data.

In the first cell-culture study, we observed that the micronized iron (0.4 μm) was not be absorbed by Caco-2 cellls. INFOGEST digesta did not clog the dialysis membrane. Thus, the dual-chamber system with dialysis membrane and INFOGEST digesta was the most reliable iron bioassay. In addition, NaFeEDTA had higher ferritin response than $FeSO_4$ in precooked corn flour. All of combination formulations were effective iron fortifications. The combination of NaFeEDTA and $FeSO_4$ at low weight ratio (2:11) had the highest ferritin concentration.

In the second cell-culture study, we investigated that ferritin concentrations of FePP and

$FePO_4$ could be increased with high molar ratio (1:0.3:5.5) CA and TSC in extruded rice flours.

In conclusion, the findings of systematic reviews and in vitro studies found that NaFeEDTA had the higher bioavailability than $FeSO_4$. The dual-chamber system with dialysis membrane and digesta was the most reliable iron bioassay. The combination of NaFeEDTA and $FeSO_4$ at low weight ratio had the highest bioavailability among the combination samples. The iron bioavailability of FePP and $FePO_4$ was improved with high molar ratio (1:0.3:5.5) CA and TSC. The results of our secondary studies and primary studies on iron bioavailability were consistent.

New primary studies will be included every 3-5 years to update the meta-analysis results. Meanwhile, more *in vitro* studies and *in vivo* animal studies will be conducted in our lab to upgrade the bioavailability of iron fortifications. Double-blind randomized control trials with large sample-size will be carried out to verify the efficacy of iron fortifications in humans. Moreover, observational research such as Cohort studies, Case-control studies and Cross-sectional studies will be performed to draw inferences from retrospective and prospective data.

Nowadays, a large amount of innovative technology has promoted the development of preclinical and clinical research on iron bioavailability. Recent insights show that iron absorption could be manipulated by prolyl hydroxylase inhibitors. They not only promote the production of endogenous erythropoietin, but also decrease the hepcidin level through the stimulation of dietary iron uptake.[215] Solid lipid nanoparticles can also overcome the barriers on iron bioavailability. Hong et al. assessed the solubility of solid Fe-lipid nanoparticle using the static digestion method. 80% of iron could be released in simulated intestinal condition.[216] Similarly, Hosny et al. conducted a pharmacokinetic experiment in rabbits. Solid $FeSO_4$-lipid nanoparticle had four-fold iron bioavailability compared with the marketed $FeSO_4$ tablet.[217] Regarding digestion model, dynamic digestion methods and semi-dynamic digestion methods have been recently developed due to the kinetic limitations of static digestion model. Kong and Singh built a dynamic digestion model that could mimic the stomach peristole, stomach emptying, and gastrointestinal secretions using a latex vessel and some rollers driven by a motor.[218] Compared with the dynamic digestion model, the semi-dynamic digestion method is simpler and more feasible. The simulated gastrointestinal solutions can be added at a constant rate by a microinjection syringe pump. Gastric chyme is taken from the bottom of container with a serological pipette to mimic the stomach emptying. Cabero et

al. assessed the nutrient level of milk and dairy-based food using the semi-dynamic digestion method. They observed that more nutrient could be released into the small intestine at an early stage of digestion.[219;220] Regarding Caco-2 cell monolayer, the conventional protocol required 2-3 weeks of Caco-2 cell monolayer culture. Corning company provided a 3- to 5-day of protocol for the Caco-2 cell monolayer culture using growth factors (epidermal growth factor and fibroblast growth factors), hormones (e.g. steroid hormones), and metabolites (e.g., insulin). The Caco-2 cell differentiation was assessed by brush border peptidase activity, alkaline phosphatase activity, and P-glycoprotein activity. Increased brush border peptidase activity, increased alkaline phosphatase activity, and functional P-glycoprotein activity proved that Corning's protocol was a feasible, productive, and convenient method of Caco-2 cell monolayer culture.[221;222] Likewise, the application of gene editing technology can build more and more advantageous cell models and animal models for iron bioavailability research. In Yu's experiment, CRISPR-Cas 9 was employed to knock out the divalent metal transporter 1 (DMT1) of Caco-2 cells, which were subsequently treated with ferrous bisglycinate (Fe-Gly) and $FeSO_4$. They reported that the cellular iron could be significantly increased with Fe-Gly treatment. Fe-Gly was absorbed by enterocytes through DMT1 pathway. The high expression of zinc-regulated transporter and iron-regulated transporter-like protein 14 might be the compensatory response to DMT1 deficiency.[223] Jackson et al., utilized the humanized mouse model and non-invasive Magnetic Resonance Imaging to assess the hepatic iron content of mouse. They developed a new animal model that was more similar to human pathophysiology, and then hepatic iron was suggested to be a promising biomarker for the early diagnosis of IDA.[224]

In 1980, the WHO declared that smallpox was eliminated. Hopefully, nutritional iron deficiency anemia will also be eradicated soon with the efforts of scientists.

Bibliography

[1] T. G. Janz, R. L. Johnson, and S. D. Rubenstein. Anemia in the emergency department: evaluation and treatment. *Emerg Med Pract*, 15:1–15, 2013. URL https://pubmed.ncbi.nlm.nih.gov/24716235/.

[2] F. Ferri. *Ferri's Clinical Advisor*. Elsevier, 2024. URL https://www.mea.elsevierhealth.com/ferris-clinical-advisor-2018-e-book-9780323529570.html.

[3] Rick D. Kellerman and David P. Rakel. *Conn's Current Therapy*. Elsevier, 2024. URL https://evolve.elsevier.com/cs/product/9780443121517?role=student.

[4] L. Goldman and A. Schafer. *Goldman-Cecil Medicine*, volume 25th, Ed. Elsevier, 2016. URL https://www.asia.elsevierhealth.com/goldman-cecil-medicine-2-volume-set-9780323930383.html.

[5] National Heart, Lung, and Blood Institute. How is iron-deficiency anemia diagnosed?, 2014. URL https://www.nhlbi.nih.gov/health/health-topics/topics/ida/diagnosis. (accessed March 7, 2024).

[6] World Health Organization. Micronutrient deficiencies, 2017. URL https://web.archive.org/web/20170713171156/http://www.who.int/nutrition/topics/ida/en/. (accessed March 7, 2024).

[7] National Heart, Lung, and Blood Institute. What are the signs and symptoms of iron-deficiency anemia?, March 2014. URL https://www.nhlbi.nih.gov/health/anemia/iron-deficiency-anemia. (accessed March 7, 2024).

[8] National Heart, Lung, and Blood Institute. What causes iron-deficiency anemia?, 2014. URL https://www.nhlbi.nih.gov/health/health-topics/topics/ida/causes. (accessed March 7, 2024).

[9] National Heart, Lung, and Blood Institute. What is iron-deficiency anemia? – nhlbi, nih, 2014. URL https://www.nhlbi.nih.gov/health/health-topics/topics/ida. (accessed March 7, 2024).

[10] Domenica Cappellini M and Motta I. Anemia in clinical practice—definition and classification: Does hemoglobin change with aging? *Semin Hematol*, 52:261–269, 2015. URL https://doi.org/10.1053/j.seminhematol.2015.07.006.

[11] D'Angelo G. Role of hepcidin in the pathophysiology and diagnosis of anemia. *Blood Res*, 48:10–15, 2013. URL https://doi.org/10.5045/br.2013.48.1.10.

[12] World Health Organization. The global prevalence of anaemia in 2011, 2011. URL https://iris.who.int/bitstream/handle/10665/177094/9789241564960_eng.pdf?sequence=1. (accessed March 12, 2024).

[13] Y.-J. Park, H.-S. Lim, and T.-H. Kim. Annual prevalence, health expenditures, and co-morbidities trend of iron deficiency anemia in korea: National health insurance service data from 2002 to 2013. *Int J Environ Res Public Health*, 17:4433, 2020. URL https://doi.org/10.3390/ijerph17124433.

[14] J. Tan, G. He, Y. Qi, H. Yang, Y. Xiong, and C. Liu. Prevalence of anemia and iron deficiency anemia in chinese pregnant women (iron women): a national cross-sectional survey. *BMC Pregnancy Childbirth*, 20:670, 2020. URL https://doi.org/10.1186/s12884-020-03359-z.

[15] N. I. Ugwu and C. J. Uneke. Iron deficiency anemia in pregnancy in nigeria-a systematic review. *Niger J Clin Pract*, 23:889–96, 2020. URL https://doi.org/10.4103/njcp.njcp_197_19.

[16] A. Habib, S. Kureishy, S. Soofi, I. Hussain, A. Rizvi, and I. Ahmed. Prevalence and risk factors for iron deficiency anemia among children under five and women of reproductive age in pakistan: Findings from the national nutrition survey 2018. *Nutrients*, 15:3361, 2023. URL https://doi.org/10.3390/nu15153361.

[17] A. C. Weyand, A. Chaitoff, G. L. Freed, M. Sholzberg, S. W. Choi, and P. T. McGann. Prevalence of iron deficiency and iron-deficiency anemia in us females aged 12-21 years,

2003-2020. *JAMA*, 329:2191–3, 2023. URL https://doi.org/10.1001/jama.2023.8020.

[18] S. Denic and M. M. Agarwal. Nutritional iron deficiency: an evolutionary perspective. *Nutrition*, 23:603–14, 2007. URL https://doi.org/10.1016/j.nut.2007.05.002.

[19] M. J. Cancelo-Hidalgo, C. Castelo-Branco, S. Palacios, J. Haya-Palazuelos, M. Ciria-Recasens, and J. Manasanch. Tolerability of different oral iron supplements: a systematic review. *Curr Med Res Opin*, 29:291–303, 2013. URL https://doi.org/10.1185/03007995.2012.761599.

[20] R. Olson, B. Gavin-Smith, C. Ferraboschi, and K. Kraemer. Food fortification: The advantages, disadvantages and lessons from sight and life programs. *Nutrients*, 13: 1118, 2021. URL https://doi.org/10.3390/nu13041118.

[21] R. D. Semba. The historical evolution of thought regarding multiple micronutrient nutrition. *The Journal of Nutrition*, 142:143S–156S, 2012. URL https://doi.org/10.3945/jn.110.137745.

[22] Arun Kumar T. V., Indra Mani, Pramod Aradwad, D.V.K. Samuel, Shivendra Jha, P.K. Sahoo, Jagnnath Prasad Sinha, and Abhijit Kar. Effect of extrusion technique on antinutritional factors of sorghum-soya blends. *Indian Journal of Agricultural Sciences*, 88, 2018. URL https://doi.org/10.56093/ijas.v88i3.78518.

[23] S. Saha, S. Jha, A. Tiwari, S. Jayapalan, and A. Roy. Considerations for improvising fortified extruded rice products. *J Food Sci*, 86:1180–200, 2021. URL https://doi.org/10.1111/1750-3841.15656.

[24] US Department of Agriculture. Fooddata central: Rice, white, medium-grain, cooked, unenriched, 2018. URL https://fdc.nal.usda.gov/fdc-app.html#/food-details/168930/nutrients. (accessed March 8, 2024).

[25] Trinidad P Trinidad, Aida C Mallillin, Rosario S Sagum, Dave P Briones, Rosario R Encabo, and Bienvenido O Juliano. Iron absorption from brown rice/brown rice-based meal and milled rice/milled rice-based meal. *International journal of food sciences and nutrition*, 60:688–693, 2009. URL https://doi.org/10.3109/09637480701830404.

[26] Micaela Albarracn, Rolando Jose Gonzalez, and Silvina Rosa Drago. Soaking and extrusion effects on physicochemical parameters, phytic acid, nutrient content and mineral bio-accessibility of whole rice grain. *International Journal of Food Sciences and Nutrition*, 66:210–215, 2015. URL https://doi.org/10.3109/09637486.2014.986070.

[27] Y. W. Jyrwa, R. Palika, S. Boddula, N. K. Boiroju, R. Madhari, and R. Pullakhandam. Retention, stability, iron bioavailability and sensory evaluation of extruded rice fortified with iron, folic acid and vitamin b12. *Matern Child Nutr*, 16:e12932, 2020. URL https://doi.org/10.1111/mcn.12932.

[28] D. Moretti, T.-C. Lee, M. B. Zimmermann, J. Nuessli, and R. F. Hurrell. Development and evaluation of iron-fortified extruded rice grains. *J Food Sci*, 70:S330–6, 2005. URL https://doi.org/10.1111/j.1365-2621.2005.tb09987.x.

[29] M. A. Beinner, A. D. N. Soares, A. L. A. Barros, and M. A. M. Monteiro. Sensory evaluation of rice fortified with iron. *Ciênc Tecnol Aliment*, 30:516–9, 2010. URL https://doi.org/10.1590/S0101-20612010000200034.

[30] B. Teucher, M. Olivares, and H. Cori. Enhancers of iron absorption: ascorbic acid and other organic acids. *Int J Vitam Nutr Res*, 74(6):403–19, Nov 2004. URL https://doi.org/10.1024/0300-9831.74.6.403.

[31] T. Suzuki, F.M. Clydesdale, and T. Pandolf. Solubility of iron in model systems containing organic acids and lignin. *J Food Prot*, 55(11):893–898, Nov 1992. URL https://doi.org/10.4315/0362-028X-55.11.893.

[32] Jack L. Smith Sareen S. Gropper. *Advanced Nutrition and Human Metabolism*, volume 6th Ed. Cengage Learning, 2012. URL https://books.google.com/books/about/Advanced_Nutrition_and_Human_Metabolism.html?id=9-C5DQAAQBAJ. (accessed March 1, 2024).

[33] M. Diaz, J. L. Rosado, L. H. Allen, S. Abrams, and O. P. García. The efficacy of a local ascorbic acid-rich food in improving iron absorption from mexican diets: a field study using stable isotopes. *Am J Clin Nutr*, 78:436–40, 2003. URL https://doi.org/10.1093/ajcn/78.3.436.

[34] M. Gillooly, T. H. Bothwell, J. D. Torrance, A. P. MacPhail, D. P. Derman, and W. R. Bezwoda. The effects of organic acids, phytates and polyphenols on the absorption of iron from vegetables. *Br J Nutr*, 49:331–42, 1983. URL https://doi.org/10.1079/BJN19830042.

[35] S. Salovaara, A.-S. Sandberg, and T. Andlid. Organic acids influence iron uptake in the human epithelial cell line caco-2. *J Agric Food Chem*, 50:6233–8, 2002. URL https://doi.org/10.1021/jf0203040.

[36] J. m. Porres, P. Etcheverry, D. d. Miller, and X. g. Lei. Phytase and citric acid supplementation in whole-wheat bread improves phytate-phosphorus release and iron dialyzability. *J Food Sci*, 66:614–9, 2001. URL https://doi.org/10.1111/j.1365-2621.2001.tb04610.x.

[37] J. D. Cook and E. R. Monsen. Vitamin c, the common cold, and iron absorption. *Am J Clin Nutr*, 30:235–41, 1977. URL https://doi.org/10.1093/ajcn/30.2.235.

[38] B. Holland, I. D. Dnwin, and D. H. Buss. Fruit and nuts, 1992. URL https://doi.org/10.1039/9781849735551-FP001. (accessed March 1, 2024).

[39] F. Boato, G. M. Wortley, R. H. Liu, and R. P. Glahn. Red grape juice inhibits iron availability: Application of an in vitro digestion/caco-2 cell model. *J Agric Food Chem*, 50:6935–8, 2002. URL https://doi.org/10.1021/jf025832q.

[40] L. Hallberg and L. Rossander. Improvement of iron nutrition in developing countries: comparison of adding meat, soy protein, ascorbic acid, citric acid, and ferrous sulphate on iron absorption from a simple latin american-type of meal. *Am J Clin Nutr*, 39(4): 577–583, Apr 1984. URL https://doi.org/10.1093/ajcn/39.4.577.

[41] M. Muleya, S. D. Young, and E. H. Bailey. A stable isotope approach to accurately determine iron and zinc bioaccessibility in cereals and legumes based on a modified infogest static in vitro digestion method. *Food Res Int*, 139:109948, 2021. URL https://doi.org/10.1016/j.foodres.2020.109948.

[42] A. P. Rebellato, J. G. Siqueira Silva, P. Probio de Moraes, B. Trajano, and J. Azevedo Lima Pallone. Static in vitro digestion methods for assessing essen-

tial minerals in processed meat products. *Food Res Int*, 155:111121, 2022. URL https://doi.org/10.1016/j.foodres.2022.111121.

[43] J. Martínez-Castro, A. de Haro-Bailón, S. Obregón-Cano, I. M. García Magdaleno, A. Moreno Ortega, and F. Cámara-Martos. Bioaccessibility of glucosinolates, isothiocyanates and inorganic micronutrients in cruciferous vegetables through infogest static in vitro digestion model. *Food Res Int*, 166:112598, 2023. URL https://doi.org/10.1016/j.foodres.2023.112598.

[44] R. Glahn. *Designing Functional Foods,the use of Caco-2 cells in defining nutrient bioavailability: application to iron bioavailability of foods*. Woodhead, 2009. URL https://doi.org/10.1533/9781845696603.2.340.

[45] H. Sun, E. Chow, S. Liu, Y. Du, and K.-C. Sandy. The caco-2 cell monolayer: Usefulness and limitations. *Expert Opinion on Drug Metabolism and Toxicology*, 4:395–411, 2008. URL https://doi.org/10.1517/17425255.4.4.395.

[46] G. Puyfoulhoux, J. M. Rouanet, P. Besançon, B. Baroux, J. C. Baccou, and B. Caporiccio. Iron availability from iron-fortified spirulina by an in vitro digestion/caco-2 cell culture model. *J Agric Food Chem*, 49:1625–9, 2001. URL https://doi.org/10.1021/jf001193c.

[47] G. C. Stefos, E. Dalaka, G. Papoutsi, I. Palamidi, V. Andreou, and G. Katsaros. In vitro evaluation of the effect of yogurt acid whey fractions on iron bioavailability. *J Dairy Sci*, 107:683–94, 2024. URL https://doi.org/10.3168/jds.2023-23643.

[48] A. L. Forbes, M. J. Arnaud, C. O. Chichester, J. D. Cook, B. N. Harrison, and R. F. Hurrell. Comparison of in vitro, animal, and clinical determinations of iron bioavailability: International nutritional anemia consultative group task force report on iron bioavailability. *Am J Clin Nutr*, 49:225–38, 1989. URL https://doi.org/10.1093/ajcn/49.2.225.

[49] J. C. Fritz, G. W. Pla, B. N. Harrison, G. A. Clark, and E. A. Smith. Measurement of the bioavailability of iron, using the rat hemoglobin repletion test. *J Assoc Off Anal Chem*, 61:709–14, 1978. URL https://pubmed.ncbi.nlm.nih.gov/649562/.

[50] S. Malika, A. Ullah, A. A. Anjum, M. M. K. Sattar, T. Ali, and R. Manzoor. Bio-efficacy of iron and zinc fortified wheat flour along with bio-assessment of its hepatic and renal toxic potential. *Braz J Biol*, 82:e261695, 2022. URL https://doi.org/10.1590/1519-6984.261695.

[51] M. Hernández, V. Sousa, A. Moreno, S. Villapando, and M. López-Alarcón. Iron bioavailability and utilization in rats are lower from lime-treated corn flour than from wheat flour when they are fortified with different sources of iron. *J Nutr*, 133:154–9, 2003. URL https://doi.org/10.1093/jn/133.1.154.

[52] S. K. Tchum, F. K. Arthur, B. Adu, S. A. Sakyi, L. A. Abubakar, and D. Atibilla. Impact of iron fortification on anaemia and iron deficiency among pre-school children living in rural ghana. *PLoS One*, 16:e0246362, 2021. URL https://doi.org/10.1371/journal.pone.0246362.

[53] M. B. Zimmermann, P. Winichagoon, S. Gowachirapant, S. Y. Hess, M. Harrington, and V. Chavasit. Comparison of the efficacy of wheat-based snacks fortified with ferrous sulfate, electrolytic iron, or hydrogen-reduced elemental iron: randomized, double-blind, controlled trial in thai women. *Am J Clin Nutr*, 82:1276–82, 45265. URL https://doi.org/10.1093/ajcn/82.6.1276.

[54] S. J. Henare, N. Nur Singh, A. M. Ellis, P. J. Moughan, A. K. Thompson, and T. Walczyk. Iron bioavailability of a casein-based iron fortificant compared with that of ferrous sulfate in whole milk: a randomized trial with a crossover design in adult women. *Am J Clin Nutr*, 110:1362–9, 2019. URL https://doi.org/10.1093/ajcn/nqz237.

[55] Jonathan R Carapetis, Abel Fekadu Dadi, et al. Global, regional, and national incidence, prevalence, and years lived with disability for 328 diseases and injuries for 195 countries, 1990-2016: a systematic analysis for the global burden of disease study 2016. *Lancet*, 390:1211–1259, 2017. URL https://doi.org/10.1016/S0140-6736(17)32154-2.

[56] A. Al-Naseem, A. Sallam, S. Choudhury, and J. Thachil. Iron deficiency without anaemia: a diagnosis that matters. *Clin Med*, 21:107–13, 2021. URL https://doi.org/10.7861/clinmed.2020-0582.

[57] S. Bathla and S. Arora. Prevalence and approaches to manage iron deficiency anemia. *Crit Rev Food Sci Nutr*, 62:8815–28, 2022. URL https://doi.org/10.1080/10408398.2021.1935442.

[58] D. D. Miller and L. A. Berner. Is solubility in vitro a reliable predictor of iron bioavailability? *Biol Trace Elem Res*, 19:11–24, 1989. URL https://doi.org/10.1007/BF02925446.

[59] R. Ghosh and J. Arcot. Fortification of foods with nano-iron: its uptake and potential toxicity: current evidence, controversies, and research gaps. *Nutr Rev*, 80:1974–84, 2022. URL https://doi.org/10.1093/nutrit/nuac011.

[60] M. C. Fidler, L. Davidsson, C. Zeder, T. Walczyk, and R. F. Hurrell. Iron absorption from ferrous fumarate in adult women is influenced by ascorbic acid but not by na 2 edta. *Br J Nutr*, 90:1081–5, 2003. URL https://doi.org/10.1079/BJN2003995.

[61] M. B. Zimmermann and F. M. Hilty. Nanocompounds of iron and zinc: their potential in nutrition. *Nanoscale*, 3:2390–8, 2011. URL https://doi.org/10.1039/C0NR00858C.

[62] L. Zhu, C. K. Yeung, R. P. Glahn, and D. D. Miller. Iron dissociates from the nafeedta complex prior to or during intestinal absorption in rats. *J Agric Food Chem*, 54: 7929–34, 2006. URL https://doi.org/10.1021/jf0616964.

[63] Q. Zuo, T. Wang, L. Zhu, X. Li, and Q. Luo. A systemic review and meta-analysis on the efficacy and safety of ferumoxytol for anemia in chronic kidney disease patients. *Ren Fail*, 44:94–102, 2021. URL https://doi.org/10.1080/0886022X.2021.2021237.

[64] M. H. Schwenk. Ferumoxytol: a new intravenous iron preparation for the treatment of iron deficiency anemia in patients with chronic kidney disease. *Pharmacotherapy*, 30: 70–9, 2010. URL https://doi.org/10.1592/phco.30.1.70.

[65] R. Hurrell. Use of ferrous fumarate to fortify foods for infants and young children. *Nutr Rev*, 68:522–30, 2010. URL https://doi.org/10.1111/j.1753-4887.2010.00312.x.

[66] K. F. Ofori, S. Antoniello, M. M. English, and A. N. A. Aryee. Improving nutrition through biofortification-a systematic review. *Front Nutr*, 9:1043655, 2022. URL https://doi.org/10.3389/fnut.2022.1043655.

[67] K. Mleczko-Sanecka and L. Silvestri. Cell-type-specific insights into iron regulatory processes. *Am J Hematol*, 96(1):110–127, Jan 2021. URL https://doi.org/10.1002/ajh.26001.

[68] G. Gao, J. Li, Y. Zhang, and Y.-Z. Chang. Cellular iron metabolism and regulation. *Adv Exp Med Biol*, 1173:21–32, 2019. URL https://doi.org/10.1007/978-981-13-9589-5_2.

[69] G. J. Anderson and D. M. Frazer. Current understanding of iron homeostasis. *Am J Clin Nutr*, 106:1559S–1566S, 2017. URL https://doi.org/10.3945/ajcn.117.155804.

[70] R. Coffey and T. Ganz. Iron homeostasis: An anthropocentric perspective. *J Biol Chem*, 292:12727–34, 2017. URL https://doi.org/10.1074/jbc.R117.781823.

[71] I. V. Milto, I. V. Suhodolo, V. D. Prokopieva, and T. K. Klimenteva. Molecular and cellular bases of iron metabolism in humans. *Biochemistry (Mosc)*, 81:549–64, 2016. URL https://doi.org/10.1134/S0006297916060018.

[72] N. Shokrgozar and H. A. Golafshan. Molecular perspective of iron uptake, related diseases, and treatments. *Blood Res*, 54:10–6, 2019. URL https://doi.org/10.5045/br.2019.54.1.10.

[73] C. M. Pfeiffer and A. C. Looker. Laboratory methodologies for indicators of iron status: strengths, limitations, and analytical challenges. *Am J Clin Nutr*, 106:1606S–1614S, 2017. URL https://doi.org/10.3945/ajcn.117.155887.

[74] Prisma 2020 flow diagram. Online, 2020. URL http://www.prisma-statement.org/PRISMAStatement/FlowDiagram?AspxAutoDetectCookieSupport=1. (accessed March 1, 2024).

[75] N. M. Delimont, S. K. Rosenkranz, M. D. Haub, and B. L. Lindshield. Salivary proline-rich protein may reduce tannin-iron chelation: a systematic narrative review. *Nutr Metab*, 14:47, 2017. URL https://doi.org/10.1186/s12986-017-0197-z.

[76] Julian Higgins and James Thomas. Cochrane handbook for systematic reviews of interventions, 2023. URL https://training.cochrane.org/handbook/current. (accessed March 7, 2024).

[77] The University of Edinburgh. Camarades tools and resources, 2022. URL https://www.ed.ac.uk/clinical-brain-sciences/research/camarades/tools-resources. (accessed September 6, 2023).

[78] Carlijn R. Hooijmans, Maroeska M. Rovers, Rob B. M. de Vries, Marlies Leenaars, Merel Ritskes-Hoitinga, and Miranda W. Langendam. Syrcle's risk of bias tool for animal studies. *BMC Med Res Methodol*, 14:43, Mar 2014. URL https://bmcmedresmethodol.biomedcentral.com/articles/10.1186/1471-2288-14-43.

[79] Graphpad prism 10 statistics guide - computing the sem, 2023. URL https://www.graphpad.com/guides/prism/latest/statistics/statcomputingsem.htm. (accessed August 29, 2023).

[80] Common mistakes in meta-analysis and how to avoid them: Fixed-effect vs. random-effects, 2023. URL https://www.meta-analysis-workshops.com/download/common-mistakes2.pdf. (accessed August 29, 2023).

[81] H. M. Vesterinen, E. S. Sena, K. J. Egan, T. C. Hirst, L. Churolov, G. L. Currie, and et al. Meta-analysis of data from animal studies: A practical guide. *Journal of Neuroscience Methods*, 221:92–102, 2014. URL https://doi.org/10.1016/j.jneumeth.2013.09.010.

[82] M. Leenaars, C. R. Hooijmans, N. van Veggel, G. ter Riet, M. Leeflang, L. Hooft, and et al. A step-by-step guide to systematically identify all relevant animal studies. *Lab Anim*, 46:24–31, 2012. URL https://doi.org/10.1258/la.2011.011087.

[83] M. J. Page, D. Moher, P. M. Bossuyt, I. Boutron, T. C. Hoffmann, C. D. Mulrow, and et al. Prisma 2020 explanation and elaboration: updated guidance and exemplars for reporting systematic reviews. *BMJ*, 2021. URL https://doi.org/10.1136/bmj.n160.

[84] Prisma 2020 checklist, 2020. URL http://prisma-statement.org/documents/PRISMA_2020_checklist.pdf. (accessed August 29, 2023).

[85] M. J. Page, J. E. McKenzie, P. M. Bossuyt, I. Boutron, T. C. Hoffmann, C. D. Mulrow, and et al. The prisma 2020 statement: an updated guideline for reporting systematic reviews. *BMJ*, 2021. URL https://doi.org/10.1136/bmj.n71.

[86] M. K. Swartz. The prisma statement: A guideline for systematic reviews and meta-analyses. *Journal of Pediatric Health Care*, 25:1–2, 2011. URL https://doi.org/10.1016/j.pedhc.2010.09.006.

[87] P. E. Johnson. Effect of food processing and preparation on mineral utilization. *Adv Exp Med Biol*, 289:483–98, 1991. URL https://doi.org/10.1007/978-1-4899-2626-5_32.

[88] E. Lemmens, N. De Brier, K. M. Spiers, C. Ryan, J. Garrevoet, G. Falkenberg, and et al. The impact of steeping, germination and hydrothermal processing of wheat (triticum aestivum l.) grains on phytate hydrolysis and the distribution, speciation and bio-accessibility of iron and zinc elements. *Food Chem*, 264:367–76, 2018. URL https://doi.org/10.1016/j.foodchem.2018.04.125.

[89] I. Paraman, M. E. Wagner, and S. S. H. Rizvi. Micronutrient and protein-fortified whole grain puffed rice made by supercritical fluid extrusion. *J Agric Food Chem*, 60: 11188–94, 2012. URL https://doi.org/10.1021/jf3034804.

[90] Q. Chaudhry, M. Scotter, J. Blackburn, B. Ross, A. Boxall, L. Castle, and et al. Applications and implications of nanotechnologies for the food sector. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess*, 25:241–58, 2008. URL https://doi.org/10.1080/02652030701744538.

[91] A. Perfecto, C. Elgy, E. Valsami-Jones, P. Sharp, F. Hilty, and S. Fairweather-Tait. Mechanisms of iron uptake from ferric phosphate nanoparticles in human intestinal caco-2 cells. *Nutrients*, 9:359, 2017. URL https://doi.org/10.3390/nu9040359.

[92] A. Nel, T. Xia, L. Mädler, and N. Li. Toxic potential of materials at the nanolevel. *Science*, 311:622–7, 2006. URL https://doi.org/10.1126/science.1114397.

[93] Carine Fillebeen, Konstantinos Gkouvatsos, Gonçalo Fragoso, Anne Calvé, Daniel Garcia-Santos, Manuel Buffler, Christian Becker, Klaus Schümann, Prem Ponka, Manuel M. Santos, and Kostas Pantopoulos. Mice are poor heme absorbers and do not require intestinal hmox1 for dietary heme iron assimilation. *Haematologica*, 100 (9):e334–e337, Sep 2015. URL https://pubmed.ncbi.nlm.nih.gov/25975840/.

[94] R. B. M. de Vries, C. R. Hooijmans, M. W. Langendam, J. van Luijk, M. Leenaars, M. Ritskes-Hoitinga, and et al. A protocol format for the preparation, registration and publication of systematic reviews of animal intervention studies. *Evidence-Based Preclinical Medicine*, 2, 2015. URL https://doi.org/10.1002/ebm2.7.

[95] Malcolm R. Macleod, Victoria O'Collins, David W. Howells, and Geoffrey A. Donnan. Pooling of animal experimental data reveals influence of study design and publication bias. *Stroke*, 35(5):1203–1208, May 2004. URL https://pubmed.ncbi.nlm.nih.gov/15060322/.

[96] G. Powis, B. A. Svingen, and C. Degraw. Iron-edta stimulated reduction of indicine n-oxide by the hepatic microsomal fraction, isolated hepatocytes, and the intact rat. *Biochem Pharmacol*, 31:293–9, 1982. URL https://doi.org/10.1016/0006-2952(82)90173-3.

[97] B. Th and M. Ap. The potential role of nafeedta as an iron fortificant. *International Journal for Vitamin and Nutrition Research Internationale Zeitschrift Fur Vitamin-Und Ernahrungsforschung Journal International de Vitaminologie et de Nutrition*, 74, 2004. URL https://doi.org/10.1024/0300-9831.74.6.421.

[98] P. Whittaker and J. E. Vanderveen. Effect of edta on the bioavailability to rats of fortification iron used in egyptian balady bread. *Br J Nutr*, 63:587–95, 1990. URL https://doi.org/10.1079/bjn19900145.

[99] O. Churio, E. Durán, S. A. Guzmán-Pino, and C. Valenzuela. Use of encapsulation technology to improve the efficiency of an iron oral supplement to prevent anemia in suckling pigs. *Animals (Basel)*, 9:1, 2018. URL https://doi.org/10.3390/ani9010001.

[100] C. Liyanage and S. Zlotkin. Bioavailability of iron from micro-encapsulated iron sprinkle supplement. *Food Nutr Bull*, 23:133–7, 2002.

[101] G. Wortley, S. Leusner, C. Good, E. Gugger, and R. Glahn. Iron availability of a fortified processed wheat cereal: a comparison of fourteen iron forms using an in vitro digestion/human colonic adenocarcinoma (caco-2) cell model. *Br J Nutr*, 93:65–71, 2005. URL https://doi.org/10.1079/bjn20041294.

[102] F. Rohner, F. O. Ernst, M. Arnold, M. Hilbe, R. Biebinger, F. Ehrensperger, and et al. Synthesis, characterization, and bioavailability in rats of ferric phosphate nanoparticles. *J Nutr*, 137:614–9, 2007. URL https://doi.org/10.1093/jn/137.3.614.

[103] A. Sabitova, R. McGranahan, F. Altamore, N. Jovanovic, E. Windle, and S. Priebe. Indicators associated with job morale among physicians and dentists in low-income and middle-income countries: A systematic review and meta-analysis. *JAMA Netw Open*, 3, 2020. URL https://doi.org/10.1001/jamanetworkopen.2019.13202.

[104] S. C. Morton, J. L. Adams, M. J. Suttorp, and P. G. Shekelle. Meta-regression approaches: What, why, when, and how? *Agency for Healthcare Research and Quality (US)*, pages 04–33, 2004. URL https://pubmed.ncbi.nlm.nih.gov/20734524/.

[105] S. G. Thompson and J. P. T. Higgins. How should meta-regression analyses be undertaken and interpreted? *Stat Med*, 21:1559–1573, 2002. URL https://pubmed.ncbi.nlm.nih.gov/12111919/.

[106] L. M. Spineli and N. Pandis. Problems and pitfalls in subgroup analysis and meta-regression. *American Journal of Orthodontics and Dentofacial Orthopedics*, 158:901–904, 2020. URL https://doi.org/10.1016/j.ajodo.2020.09.001.

[107] A. Granholm, W. Alhazzani, and M. H. Møller. Use of the grade approach in systematic reviews and guidelines. *British Journal of Anaesthesia*, 123:554–559, 2019. URL https://doi.org/10.1016/j.bja.2019.08.015.

[108] M. Micheletto, E. Gaio, E. Tedesco, G. Di Maira, E. Mantovan, M. Zanella, and et al. Intestinal absorption study of a granular form of ferric pyrophosphate. *METABOLITES*, 12, 2022. URL https://doi.org/10.3390/metabo12050463.

[109] A. Perfecto, C. Elgy, E. Valsami-Jones, P. Sharp, F. Hilty, and S. Fairweather-Tait. Mechanisms of iron uptake from ferric phosphate nanoparticles in human intestinal caco-2 cells. *Nutrients*, 9, 2017. URL https://doi.org/10.3390/nu9040359.

[110] J. M. Laparra, R. P. Glahn, and D. D. Miller. Different responses of fe transporters in caco-2/ht29-mtx cocultures than in independent caco-2 cell cultures. *Cell Biol Int*, 33:971–977, 2009. URL https://doi.org/10.1016/j.cellbi.2009.06.001.

[111] L. A. Martini, L. Tchack, and R. J. Wood. Iron treatment downregulates dmt1 and ireg1 mrna expression in caco-2 cells. *J Nutr*, 132:693–696, 2002. URL https://pubmed.ncbi.nlm.nih.gov/11880563/.

[112] B. Galy, D. Ferring-Appel, S. Kaden, H.-J. Gröne, and M. W. Hentze. Iron regulatory proteins are essential for intestinal function and control key iron absorption molecules in the duodenum. *Cell Metab*, 7:79–85, 2008. URL https://doi.org/10.1016/j.cmet.2007.10.006.

[113] H. Zoller, I. Theurl, R. Koch, A. Kaser, and G. Weiss. Mechanisms of iron mediated regulation of the duodenal iron transporters divalent metal transporter 1 and ferroportin 1. *Blood Cells Mol Dis*, 29:488–497, 2002. URL https://doi.org/10.1006/bcmd.2002.0587.

[114] W. Han, C. Wang, C. Su, and X. Xu. [effect of higher iron in diet on iron levels and hepcidin mrna expression levels in rats]. *Wei Sheng Yan Jiu*, 37:474–476, 2008. URL https://pubmed.ncbi.nlm.nih.gov/18540322/.

[115] K. Yeh, M. Yeh, and J. Glass. Hepcidin regulation of ferroportin 1 expression in the liver and intestine of the rat. *Am J Physiol Gastrointest Liver Physiol*, 286:G385–394, 2004. URL https://doi.org/10.1152/ajpgi.00246.2003.

[116] G. Nicolas, M. Bennoun, I. Devaux, C. Beaumont, B. Grandchamp, A. Kahn, and et al. Lack of hepcidin gene expression and severe tissue iron overload in upstream stimulatory factor 2 (usf2) knockout mice. *Proc Natl Acad Sci U S A*, 98:8780–8785, 2001. URL https://doi.org/10.1073/pnas.151179498.

[117] G. Nicolas, M. Bennoun, A. Porteu, S. Mativet, C. Beaumont, B. Grandchamp, and et al. Severe iron deficiency anemia in transgenic mice expressing liver hepcidin. *Proc Natl Acad Sci U S A*, 99:4596–4601, 2002. URL https://doi.org/10.1073/pnas.072632499.

[118] C. Pigeon, G. Ilyin, B. Courselaud, P. Leroyer, B. Turlin, P. Brissot, and et al. A new mouse liver-specific gene, encoding a protein homologous to human antimicrobial peptide hepcidin, is overexpressed during iron overload. *J Biol Chem*, 276:7811–7819, 2001. URL https://doi.org/10.1074/jbc.M008923200.

[119] D. M. Frazer, S. J. Wilkins, E. M. Becker, C. D. Vulpe, A. T. McKie, D. Trinder, and et al. Hepcidin expression inversely correlates with the expression of duodenal iron transporters and iron absorption in rats. *Gastroenterology*, 123:835–844, 2002. URL https://doi.org/10.1053/gast.2002.35353.

[120] G. J. Anderson, D. M. Frazer, S. J. Wilkins, E. M. Becker, K. N. Millard, T. L. Murphy, and et al. Relationship between intestinal iron-transporter expression, hepatic hepcidin levels and the control of iron absorption. *Biochem Soc Trans*, 30:724–726, 2002. URL https://doi.org/10.1042/bst0300724.

[121] Renata Micha. 2020 global nutrition report, 2020. URL https://globalnutritionreport.org/reports/2021-global-nutrition-report/foreword/. (accessed January 12, 2024).

[122] Global nutrition targets 2025: anaemia policy brief, 2014. URL https://www.who.int/publications-detail-redirect/WHO-NMH-NHD-14.4. (accessed January 12, 2024).

[123] Accelerating efforts for preventing micronutrient deficiencies and their consequences, including spina bifida and other neural tube defects, through safe and effective food fortification, 2023. URL https://apps.who.int/gb/ebwha/pdf_files/WHA76/A76_R19-en.pdf. (accessed January 12, 2024).

[124] Dylan Walters, Jakub Kakietek, Julia D. Eberwein, and Meera Shekar. An investment framework for meeting the global nutrition target for anemia, 2015. URL https://openknowledge.worldbank.org/server/api/core/bitstreams/91e969b7-f635-57b2-8fd0-8702d9b2357c/content. (accessed January 12, 2024).

[125] Confidence intervals, 2022. URL https://www.csus.edu/indiv/j/jgehrman/courses/stat1/misc/confintervals/7confi.htm. (accessed March 1, 2024).

[126] Kirsty M Rhodes, Rebecca M Turner, and Julian PT Higgins. Predictive distributions were developed for the extent of heterogeneity in meta-analyses of continuous outcome data. *Journal of clinical epidemiology*, 68:52–60, 2015. URL https://doi.org/10.1016/j.jclinepi.2014.08.012.

[127] Fengting Zhai, Dongmei Wang, Zhen Hua, Yuting Jiang, and Dandan Wang. A comparison of the efficacy and safety of complementary and alternative therapies for the primary dysmenorrhea: A network meta-analysis protocol. *Medicine*, 98:e15586, 2019. URL https://doi.org/10.1097/MD.0000000000015586.

[128] Georgia Salanti, AE Ades, and John PA Ioannidis. Graphical methods and numerical summaries for presenting results from multiple-treatment meta-analysis: an overview and tutorial. *Journal of clinical epidemiology*, 64:163–171, 2011. URL https://doi.org/10.1016/j.jclinepi.2010.03.016.

[129] Toshi A. Furukawa, Georgia Salanti, Lauren Z. Atkinson, Stefan Leucht, Henricus G. Ruhe, and Erick H. Turner. Comparative efficacy and acceptability of first-generation and second-generation antidepressants in the acute treatment of major depression: protocol for a network meta-analysis. *BMJ Open*, 6:e010919, 2016. URL https://bmjopen.bmj.com/content/6/7/e010919.

[130] Sofia Dias, Nicky J. Welton, Deborah M. Caldwell, and A. E. Ades. Checking consistency in mixed treatment comparison meta-analysis. *Stat Med*, 29:932–944, 2010. URL https://doi.org/10.1002/sim.3767.

[131] Adriani Nikolakopoulou, Julian P. T. Higgins, Theodoros Papakonstantinou, Anna Chaimani, Cinzia Del Giovane, Matthias Egger, and Georgia Salanti. Cinema: An approach for assessing confidence in the results of a network meta-analysis. *PLoS Med*, 17:e1003082, 2020. URL https://doi.org/10.1371/journal.pmed.1003082.

[132] Theodoros Papakonstantinou, Adriani Nikolakopoulou, Julian P. T. Higgins, Matthias Egger, and Georgia Salanti. Cinema: Software for semiautomated assessment of the confidence in the results of network meta-analysis. *Campbell Systematic Reviews*, 16: e1080, 2020. URL https://doi.org/10.1002/cl2.1080.

[133] World Health Organization. Anaemia, 2023. URL https://www.who.int/news-room/fact-sheets/detail/anaemia. (accessed January 14, 2024).

[134] Priyadharshini Thiagarajan, Cameron J. Parker, and Josef T. Prchal. How do red blood cells die? *Front Physiol*, 12:655393, 2021. URL https://doi.org/10.3389/fphys.2021.655393.

[135] M. D. Borges and R. Sesti-Costa. Macrophages: key players in erythrocyte turnover. *Rev Bras Hematol Hemoter*, 44:574–581, 2022. URL https://doi.org/10.1016/j.htct.2022.07.002.

[136] R. F. Hurrell. Preventing iron deficiency through food fortification. *Nutrition Reviews*, 55:210–222, 2009. URL https://doi.org/10.1111/j.1753-4887.1997.tb01608.x.

[137] R. F. Hurrell and J. D. Cook. Strategies for iron fortification of foods. *Trends in Food Science and Technology*, 1:56–61, 1990. URL https://doi.org/10.1016/0924-2244(90)90047-3.

[138] U.S. DEPARTMENT OF AGRICULTURE. Usda commodity requirements milled rice and fortified milled rice for use in international food assistance programs, 2018. URL https://www.ams.usda.gov/sites/default/files/media/mr26.pdf. (accessed March 7, 2024).

[139] U.S. Agency for International Development. Improving the nutritional quality of u.s. food aid: Recommendations for changes to products and programs, 2011. URL https://citeseerx.ist.psu.edu/document?repid=rep1&type=pdf&doi=51947bcdd7f09a0fe6fa4caa0f8680c77060912f. (accessed March 1, 2024).

[140] Lindsay Allen. Guidelines on food fortification with micronutrients, 2006. URL https://www.who.int/publications/i/item/9241594012. (accessed March 1, 2024).

[141] H. A. Nadiger, K. A. Krishnamachari, A. N. Naidu, B. S. Rao, and S. G. Srikantia. The use of common salt (sodium chloride) fortified with iron to control anaemia: results of a preliminary study. *Br J Nutr*, 43:45–51, 1980. URL https://doi.org/10.1079/bjn19800063.

[142] FS Tonin, I Rotta, AM Mendes, and R Pontarolo. Network meta-analysis: A technique to gather evidence from direct and indirect comparisons. *Pharmacy Practice (Granada)*, 15, 2017. URL https://doi.org/10.18549/pharmpract.2017.01.943.

[143] HC Bucher, GH Guyatt, LE Griffith, and SD Walter. The results of direct and indirect treatment comparisons in meta-analysis of randomized controlled trials. *J Clin Epidemiol*, 50:683–691, 1997. URL https://doi.org/10.1016/S0895-4356(97)00049-8.

[144] Luz Maria De-Regil, Maria Elena D Jefferds, and Juan Pablo Peña-Rosas. Point-of-use fortification of foods with micronutrient powders containing iron in children of preschool and school-age. *Cochrane Database of Systematic Reviews*, 11:CD009666, 2017. URL https://doi.org/10.1002/14651858.CD009666.pub2.

[145] Katharina da Silva Lopes, Noyuri Yamaji, Md Obaidur Rahman, Maiko Suto, Yo Takemoto, and Garcia-Casal. Nutrition-specific interventions for preventing and controlling anaemia throughout the life cycle: an overview of systematic reviews. *Cochrane Database of Systematic Reviews*, 9:CD013092, 2021. URL https://doi.org/10.1002/14651858.CD013092.pub2.

[146] Maria N Garcia-Casal, Juan Pablo Peña-Rosas, Luz Maria De-Regil, Jeffrey A Gwirtz, and Sant-Rayn Pasricha. Fortification of maize flour with iron for controlling anaemia and iron deficiency in populations. *Cochrane Database of Systematic Reviews*, 12: CD013092, 2018. URL https://doi.org/10.1002/14651858.CD010187.pub2.

[147] D. D. Miller and L. A. Berner. Is solubility in vitro a reliable predictor of iron bioavailability? *Biol Trace Elem Res*, 19:11–24, 1989. URL https://doi.org/10.1007/BF02925446.

[148] A. Brodkorb, L. Egger, M. Alminger, P. Alvito, R. Assunção, S. Ballance, et al. Infogest static in vitro simulation of gastrointestinal food digestion. *Nat Protoc*, 14:991–1014, 2019. URL https://doi.org/10.1038/s41596-018-0119-1.

[149] Raymond P. Glahn. The caco-2 cell bioassay for measurement of food iron bioavailability. *J Vis Exp*, 1(182), 2022. URL https://www.jove.com/v/63859/the-caco-2-cell-bioassay-for-measurement-of-food-iron-bioavailability.

[150] Mackie A, Rigby N., Verhoeckx Cotter, Lopez-Expósito I, Mackie A Kleiveland C, Lea T, and Requena T. *the Impact of Food Bioactives on Health: in vitro and ex vivo models, InfoGest Consensus Method*. Springer, 2015. URL https://doi.org/10.1007/978-3-319-16104-4_2.

[151] M Minekus, M Alminger, P Alvito, S Ballance, T Bohn, and C Bourlieu. A standardised static in vitro digestion method suitable for food – an international consensus. *Food Funct*, 5:1113–1124, 2014. URL https://doi.org/10.1039/C3FO60702J.

[152] K Penugonda, NM Fiorentino, S Alavi, and BL Lindshield. Bioavailable iron and vitamin a in newly formulated, extruded corn, soybean, sorghum, and cowpea fortified-blended foods in the in vitro digestion/caco-2 cell model. *Curr Dev Nutr*, 2:nzy021, 2018. URL https://doi.org/10.1093/cdn/nzy021.

[153] K Penugonda, S Alavi, and B Lindshield. Iron bioavailability of sorghum, cowpea, corn and soybean fortified blended foods. *The FASEB Journal*, 29:122.1, 2015. URL https://doi.org/10.1096/fasebj.29.1_supplement.122.1.

[154] Lluis M. Martínez. Sonication cell lysis: Step by step protocol, 2021. URL https://www.sepmag.eu/blog/sonication-cell-lysis-protocol-steps. (accessed March 6, 2024).

[155] Assay Genie. Sonication protocol for cell lysis, 2023. URL https://www.assaygenie.com/sonication-protocol-for-cell-lysis. (accessed March 1, 2024).

[156] T. Ma, X. Tian, B. Zhang, M. Li, Y. Wang, C. Yang, et al. Low-dose metformin targets the lysosomal ampk pathway through pen2. *Nature*, 603:159–165, 2022. URL https://doi.org/10.1038/s41586-022-04431-8.

[157] Leslie Shackleton and Robert Alexander McCance. The ionisable iron in foods. *Biochemical Journal*, 30:582, 1936. URL https://doi.org/10.1042/bj0300582.

[158] A Jacobs and DA Greenman. Availability of food iron. *The British Medical Journal*, 1: 673–676, 1969. URL https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1982684/.

[159] S. Lock and A. E. Bender. Measurement of chemically-available iron in foods by incubation with human gastric juice in vitro. *Br J Nutr*, 43:413–420, 1980. URL https://doi.org/10.1079/BJN19800109.

[160] Dennis D. Miller, Brian R. Schricker, Robert R. Rasmussen, and Darrell Van Campen. An in vitro method for estimation of iron availability from meals. *The American Journal of Clinical Nutrition*, 1981. URL https://pubmed.ncbi.nlm.nih.gov/6794346/.

[161] B. Narasinga Rao and T. Prabhavathi. An in vitro method for predicting the bioavailability of iron from foods. *The American Journal of Clinical Nutrition*, 31:169–175, 1978. URL https://doi.org/10.1093/ajcn/31.1.169.

[162] Susan Fairweather-Tait, Ian Phillips, Gary Wortley, Linda Harvey, and Raymond Glahn. The use of solubility, dialyzability, and caco-2 cell methods to predict iron bioavailability. *Int J Vitam Nutr Res*, 77(3):158–165, May 2007. URL https://www.ncbi.nlm.nih.gov/pubmed/18214016.

[163] D. Ambort, M. E. V. Johansson, J. K. Gustafsson, H. E. Nilsson, A. Ermund, B. R. Johansson, et al. Calcium and ph-dependent packing and release of the gel-forming muc2 mucin. *Proc Natl Acad Sci USA*, 109:5645–5650, 2012. URL https://doi.org/10.1073/pnas.1120269109.

[164] Y. Duangnumsawang, J. Zentek, and F. Goodarzi Boroojeni. Development and functional properties of intestinal mucus layer in poultry. *Front Immunol*, 12:745849, 2021. URL https://doi.org/10.3389/fimmu.2021.745849.

[165] T. L. Carlson, J. Y. Lock, and R. L. Carrier. Engineering the mucus barrier. *Annu Rev Biomed Eng*, 20:197–220, 2018. URL https://doi.org/10.1146/annurev-bioeng-062117-121156.

[166] J. McCright, A. Sinha, and K. Maisel. Generating an in vitro gut model with physiologically relevant biophysical mucus properties. *Cell Mol Bioeng*, 15:479–491, 2022. URL https://doi.org/10.1007/s12195-022-00740-0.

[167] H. E. Nilsson, D. Ambort, M. Bäckström, E. Thomsson, P. J. B. Koeck, G. C. Hansson, et al. Intestinal muc2 mucin supramolecular topology by packing and release resting on d3 domain assembly. *J Mol Biol*, 426:2567–2579, 2014. URL https://doi.org/10.1016/j.jmb.2014.04.027.

[168] P. Paone and P. D. Cani. Mucus barrier, mucins and gut microbiota: the expected slimy partners? *Gut*, 69:2232–2243, 2020. URL https://doi.org/10.1136/gutjnl-2020-322260.

[169] G. M. H. Birchenough, M. E. V. Johansson, J. K. Gustafsson, J. H. Bergström, and G. C. Hansson. New developments in goblet cell mucus secretion and function. *Mucosal Immunol*, 8:712–719, 2015. URL https://doi.org/10.1038/mi.2015.32.

[170] Y. Liu, Z. Yu, L. Zhu, S. Ma, Y. Luo, H. Liang, et al. Orchestration of muc2 — the key regulatory target of gut barrier and homeostasis: A review. *International Journal*

of Biological Macromolecules, 236:123862, 2023. URL https://doi.org/10.1016/j.ijbiomac.2023.123862.

[171] J. Leal, H. D. C. Smyth, and D. Ghosh. Physicochemical properties of mucus and their impact on transmucosal drug delivery. *International Journal of Pharmaceutics*, 532:555–572, 2017. URL https://doi.org/10.1016/j.ijpharm.2017.09.018.

[172] T. Pelaseyed, J. H. Bergström, J. K. Gustafsson, A. Ermund, G. M. H. Birchenough, A. Schütte, et al. The mucus and mucins of the goblet cells and enterocytes provide the first defense line of the gastrointestinal tract and interact with the immune system. *Immunological Reviews*, 260:8–20, 2014. URL https://doi.org/10.1111/imr.12182.

[173] J. P. Pearson, P. I. Chater, and M. D. Wilcox. The properties of the mucus barrier, a unique gel – how can nanoparticles cross it? *Therapeutic Delivery*, 7:229–244, 2016. URL https://doi.org/10.4155/tde-2015-0002.

[174] M. Herath, S. Hosie, J. C. Bornstein, A. E. Franks, and E. L. Hill-Yardin. The role of the gastrointestinal mucus system in intestinal homeostasis: Implications for neurological disorders. *Front Cell Infect Microbiol*, 10, 2020. URL https://doi.org/10.3389/fcimb.2020.00210.

[175] R. An, D. Robbins, F. E. Rey, and S. L. Thibeault. Vocal fold mucus layer: Comparison of histological protocols for visualization in mice. *Laryngoscope Investigative Otolaryngology*, 7:444–453, 2022. URL https://doi.org/10.1002/lio2.743.

[176] S. Bering, K. Bukhave, M. Henriksen, B. Sandström, S. J. Fairweather-Tait, et al. Development of a three-tier in vitro system, using caco-2 cells, to assess the effects of lactate on iron uptake and transport from rye bread following in vitro digestion. *J Sci Food Agric*, 86:2438–2444, 2006. URL https://doi.org/10.1002/jsfa.2637.

[177] Jason A. Wiesinger, Karen A. Cichy, Sharon D. Hooper, Jonathan J. Hart, and Raymond P. Glahn. Processing white or yellow dry beans (*phaseolus vulgaris* l.) into a heat treated flour enhances the iron bioavailability of bean-based pastas. *Journal of Functional Foods*, 71:104018, 2020. ISSN 1756-4646. doi: 10.1016/j.jff.2020.104018. URL https://www.sciencedirect.com/science/article/pii/S1756464620302425.

[178] A. Yeung, R. Glahn, and D. Miller. Comparison of the availability of various iron fortificants in bread and milk using an in vitro digestion/caco-2 cell culture method. *JOURNAL OF FOOD SCIENCE*, 67:2357–2361, 2002. URL https://doi.org/10.1111/j.1365-2621.2002.tb09554.x.

[179] N.M. Delimont, C.I. Vahl, R. Kayanda, W. Msuya, M. Mulford, P. Alberghine, and et al. Complementary feeding of sorghum-based and corn-based fortified blended foods results in similar iron, vitamin a, and anthropometric outcomes in the mffapp tanzania efficacy study. *Current Developments in Nutrition*, 3, 43466. URL https://doi.org/10.1093/cdn/nzz027.

[180] N. M. Delimont, S. Chanadang, M. V. Joseph, B. E. Rockler, Q. Guo, G. K. Regier, et al. The mffapp tanzania efficacy study protocol: Newly formulated, extruded, fortified blended foods for food aid. *Curr Dev Nutr*, 1:e000315, 2017. URL https://doi.org/10.3945/cdn.116.000315.

[181] Reactions of ions in aqueous solution, 2015. URL http://www.chemhume.co.uk/AS%20AQA%20CHEM/Inorganic/2.6%20Aqueous%20ions.pdf. (accessed March 1, 2024).

[182] L. Davidsson, T. Walczyk, N. Zavaleta, and R. Hurrell. Improving iron absorption from a peruvian school breakfast meal by adding ascorbic acid or na2edta. *Am J Clin Nutr*, 73:283–287, 2001. URL https://doi.org/10.1093/ajcn/73.2.283.

[183] A. P. MacPhail, R. C. Patel, T. H. Bothwell, and R. D. Lamparelli. Edta and the absorption of iron from food. *Am J Clin Nutr*, 59:644–648, 1994. URL https://doi.org/10.1093/ajcn/59.3.644.

[184] R. F. Hurrell, M. B. Reddy, J. Burri, and J. D. Cook. An evaluation of edta compounds for iron fortification of cereal-based foods. *Br J Nutr*, 84:903–910, 2000. URL https://pubmed.ncbi.nlm.nih.gov/11177208/.

[185] S. Chang, Z. Huang, Y. Ma, J. Piao, X. Yang, C. Zeder, et al. Mixture of ferric sodium ethylenediaminetetraacetate (nafeedta) and ferrous sulfate: an effective iron fortificant for complementary foods for young chinese children. *Food Nutr Bull*, 33:111–116, 2012. URL https://doi.org/10.1177/156482651203300204.

[186] L. Hackl, C. I. Cercamondi, C. Zeder, D. Wild, H. Adelmann, M. B. Zimmermann, and D. Moretti. Cofortification of ferric pyrophosphate and citric acid/trisodium citrate into extruded rice grains doubles iron bioavailability through in situ generation of soluble ferric pyrophosphate citrate complexes. *Am J Clin Nutr*, 103:1252–1259, May 2016. URL https://pubmed.ncbi.nlm.nih.gov/27053382/.

[187] K. Zhang, X. Jia, Z. Zhu, and W. Xue. Physicochemical properties of rice analogs based on multi-level: influence of the interaction of extrusion parameters. *Int J Food Prop*, 23:2033–2049, 2020. URL https://doi.org/10.1080/10942912.2020.1840389.

[188] P Scheuchzer, MB Zimmerman, C Zeder, A Sánchez-Ferrer, and D Moretti. Swiss paper no3 higt and low temperature. *The Journal of Nutrition*, 152:1220–1227, 2022. URL https://doi.org/10.1093/jn/nxab435.

[189] Tidehag P, Hallmans G, Wing K, Sjostrom R, Agren G, Lundin E, and Zhang JX. A comparison of iron absorption from single meals and daily diets using radiofe. *Br J Nutr*, 75:281–289, 1996. URL https://doi.org/10.1079/bjn19960130.

[190] J.D. Cook, S.A. Dassenko, and S.R. Lynch. Assessment of the role of nonheme-iron availability in iron balance. *Am J Clin Nutr*, 54:717–722, 1991. URL https://doi.org/10.1093/ajcn/54.4.717.

[191] Elisa L. Karkle, Sajid Alavi, and Hulya Dogan. Cellular architecture and its relationship with mechanical properties in expanded extrudates containing apple pomace. *Food Research International*, 46(1):10–21, 2012. URL https://www.sciencedirect.com/science/article/pii/S0963996911006120.

[192] S.A. Tadesse, G.B. Beri, and S. Abera. Chemical and sensory quality of sorghum-based extruded product supplemented with defatted soy meal flour. *Cogent Food and Agriculture*, 5:1653617, 2019. URL https://doi.org/10.1080/23311932.2019.1653617.

[193] T. V., Arun Kumar, Indra Mani, Pramod Aradwad, D.V.K. Samuel, Shivendra Jha, P.K. Sahoo, Jagnnath Prasad Sinha, and Abhijit Kar. Effect of extrusion technique on antinutritional factors of sorghum-soya blends. *Indian Journal of Agricultural Sciences*, 88, 2018. URL https://www.indianjournals.com/ijor.aspx?target=ijor:ijas&volume=88&issue=3&article=021.

[194] J. Lubaale, J.R.N. Taylor, M.N. Emmambux, and K.G. Duodu. Extrusion cooking of food-to-food fortified wholegrain sorghum-based porridges enhances caco-2 ferritin formation. *Cereal Chem*, 100:371–383, 2023. URL https://doi.org/10.1002/cche.10616.

[195] Tao Yue Xiqing Wu, Zhixi Wang. Ferric pyrophosphate forms soluble iron coordination complexes with zinc compounds and solubilizing agents in extruded rice and predicts increased iron solubility and bioavailability in young women. *Metal Mine*, 11:5, Mar 2017. URL https://www.doc88.com/p-9909660924724.htm.

[196] P. Scheuchzer, M. B. Zimmermann, C. Zeder, A. Sánchez-Ferrer, and D. Moretti. Ferric pyrophosphate forms soluble iron coordination complexes with zinc compounds and solubilizing agents in extruded rice and predicts increased iron solubility and bioavailability in young women. *J Nutr*, 153(3):636–644, Mar 2023. URL https://www.ncbi.nlm.nih.gov/pmc/articles/PMC10127525/.

[197] S. Apostol, V. Nentsislav, and L. Atanas. Coextrusion of food products – essence and technical characteristics. *Food Science, Engineering and Technologies*, 58: 617–623, 2010. URL https://www.researchgate.net/publication/273692346_Coextrusion_of_food_products_-_essence_and_technical_characteristics.

[198] Michael B. Zimmermann, Pattanee Winichagoon, Sueppong Gowachirapant, Sonja Y. Hess, Mark Harrington, Visith Chavasit, Sean R. Lynch, and Richard F. Hurrell. Comparison of the efficacy of wheat-based snacks fortified with ferrous sulfate, electrolytic iron, or hydrogen-reduced elemental iron: randomized, double-blind, controlled trial in thai women. *Am J Clin Nutr*, 82(6):1276–1282, Dec 2005. URL https://academic.oup.com/ajcn/article/82/6/1276/4863477.

[199] H. Mehansho. Chemical construction: Iron fortification technology development: New approaches 1. *The Journal of Nutrition*, 136:1059–1063, 2006. URL https://doi.org/10.1093/jn/136.4.1059.

[200] R. Wegmüller, M.B. Zimmermann, D. Moretti, M. Arnold, W. Langhans, and R.F. Hurrell. Particle size reduction and encapsulation affect the bioavailability of ferric pyrophosphate in rats. *J Nutr*, 134:3301–3304, 2004. URL https://doi.org/10.1093/jn/134.12.3301.

[201] R. Wegmüller, F. Camara, M.B. Zimmermann, P. Adou, and R.F. Hurrell. Salt dual-fortified with iodine and micronized ground ferric pyrophosphate affects iron status but not hemoglobin in children in côte d'ivoire. *Journal of Nutrition*, 136:1814–1820, 2006. URL https://doi.org/10.1093/jn/136.7.1814.

[202] N. Sakaguchi, T. Rao, K. Nakata, H. Nanbu, and L. Juneja. Iron absorption and bioavailability in rats of micronized dispersible ferric pyrophosphate. *International Journal for Vitamin and Nutrition Research*, 74:3–9, 2004. URL https://doi.org/10.1024/0300-9831.74.1.3.

[203] M.C. Fidler, T. Walczyk, L. Davidsson, C. Zeder, N. Sakaguchi, L.R. Juneja, and et al. A micronised, dispersible ferric pyrophosphate with high relative bioavailability in man. *Br J Nutr*, 91:107–112, 2004. URL https://doi.org/10.1079/BJN20041018.

[204] H.B. Abrahamson, A.B. Rezvani, and J.G. Brushmiller. Photochemical and spectroscopic studies of complexes, of iron with citric acid and other carboxylic acids. *Inorg Chim Acta*, 226:117–127, 1994. URL https://doi.org/10.1016/0020-1693(94)04077-X.

[205] W.L. Lau, N.D. Vaziri, A.C.F. Nunes, A.M. Comeau, M.G.I. Langille, W. England, and et al. The phosphate binder ferric citrate alters the gut microbiome in rats with chronic kidney disease. *J Pharmacol Exp Ther*, 367:452–460, 2018. URL https://doi.org/10.1124/jpet.118.251389.

[206] B.S. Rao, S. Kathoke, and S.V. Apte. Mono ferrous acid citrate as an iron fortificant. *Br J Nutr*, 39:663–665, 1978. URL https://doi.org/10.1079/bjn19780082.

[207] Association of Food and Drug Officials of the United States. *Appraisal of the safety of chemicals in foods, drugs, and cosmetics*. J Occup Environ Med, 1959. URL https://cir.nii.ac.jp/crid/1573105974419947520.

[208] T. Toyoda, Y.-M. Cho, Y. Mizuta, J. Akagi, and K. Ogawa. A 13-week subchronic toxicity study of ferric citrate in f344 rats. *Food Chem Toxicol*, 74:68–75, 2014. URL https://doi.org/10.1016/j.fct.2014.09.005.

[209] J.L. Pierre and I. Gautier-Luneau. Iron and citric acid: a fuzzy chemistry of ubiquitous

biological relevance. *Biometals*, 13:91–96, 2000. URL https://doi.org/10.1023/A:1009225701332.

[210] B. Sun, B. Tan, N. Sun, P. Huang, J. Hong, C. Li, and et al. Effect of ascorbic acid and citric acid on bioavailability of iron from tegillarca granosa via an in vitro digestion/caco-2 cell culture system. *Food Funct*, 12:11491–11502, 2021. URL https://doi.org/10.1039/d1fo01650d.

[211] S. Salovaara, A-S. Sandberg, and T. Andlid. Organic acids influence iron uptake in the human epithelial cell line caco-2. *J Agric Food Chem*, 50:6233–6238, 2002. URL https://doi.org/10.1021/jf0203040.

[212] S. Salovaara, A-S. Sandberg, and T. Andlid. Combined impact of ph and organic acids on iron uptake by caco-2 cells. *J Agric Food Chem*, 51:7820–7824, 2003. URL https://doi.org/10.1021/jf030177n.

[213] R. Palika, P.C. Mashurabad, S. Kilari, S. Kasula, K.M. Nair, and P. Raghu. Citric acid mediates the iron absorption from low molecular weight human milk fractions. *J Agric Food Chem*, 61:11151–11157, 2013. URL https://doi.org/10.1021/jf403973j.

[214] R. P. Glahn, O. A. Lee, A. Yeung, M. I. Goldman, and D. D. Miller. Caco-2 cell ferritin formation predicts nonradiolabeled food iron availability in an in vitro digestion/caco-2 cell culture model. *J Nutr*, 128:1555–1561, 1998. URL https://doi.org/10.1093/jn/128.9.1555.

[215] V. G. Sankaran and M. J. Weiss. Anemia: Progress in molecular mechanisms and therapy. *Nat Med*, 21:221–230, 2015. URL https://doi.org/10.1038/nm.3814.

[216] S-J. Hong, C. V. Garcia, G-H. Shin, and J-T. Kim. Enhanced bioaccessibility and stability of iron through w/o/w double emulsion-based solid lipid nanoparticles and coating with water-soluble chitosan. *Int J Biol Macromol*, 209:895–903, 2022. URL https://doi.org/10.1016/j.ijbiomac.2022.04.066.

[217] K. M. Hosny, Z. M. Banjar, A. H. Hariri, and A. H. Hassan. Solid lipid nanoparticles loaded with iron to overcome barriers for treatment of iron deficiency anemia. *Drug Des Devel Ther*, 9:313–320, 2015. URL https://doi.org/10.2147/DDDT.S77702.

[218] F. Kong and R.P. Singh. A human gastric simulator to study food digestion in human stomach. *J Food Sci*, 75:E627–E635, 2010. URL https://doi.org/10.1111/j.1750-3841.2010.01856.x.

[219] Ana I. Mulet-Cabero, Alan R. Mackie, André Brodkorb, and Peter J. Wilde. Dairy structures and physiological responses: a matter of gastric digestion. *Crit Rev Food Sci Nutr*, 60(22):3737–3752, 2020. URL https://www.tandfonline.com/doi/abs/10.1080/10408398.2019.1707159.

[220] A-I. Mulet-Cabero, A.R. Mackie, P.J. Wilde, M.A. Fenelon, and A. Brodkorb. Structural mechanism and kinetics of in vitro gastric digestion are affected by process-induced changes in bovine milk. *Food Hydrocolloids*, 86:172–183, 2019. URL https://doi.org/10.1016/j.foodhyd.2018.03.035.

[221] P. Flaherty. Corning® biocoattm: Comparison of 3-day system to 21-day protocol, 2013. URL https://www.corning.com/catalog/cls/documents/protocols/protocol_DL_090_Corning__BioCoat_Comparison_of_3-day_System_to_21-day.pdf.

[222] S. Yamashita, K. Konishi, Y. Yamazaki, Y. Taki, T. Sakane, H. Sezaki, and et al. New and better protocols for a short-term caco-2 cell culture system. *J Pharm Sci*, 91: 669–679, 2002. URL https://doi.org/10.1002/jps.10050.

[223] X. Yu, L. Chen, H. Ding, Y. Zhao, and J. Feng. Crispr iron transport from ferrous bisglycinate and ferrous sulfate in dmt1-knockout human intestinal caco-2 cells. *Nutrients*, 11:485, 2019. URL https://doi.org/10.3390/nu11030485.

[224] Laura H. Jackson, Evangelia Vlachodimitropoulou, Panicos Shangaris, Thomas A. Roberts, Thomas M. Ryan, Adrienne E. Campbell-Washburn, and et al. Non-invasive mri biomarkers for the early assessment of iron overload in a humanized mouse model of β-thalassemia. *Sci Rep*, 7:43439, 2017. URL https://doi.org/10.1038/srep43439.

Appendix A

Reference list of included studies

Preclinical systemic review (Chapter 2)

1.Vicente 2008

Author: Haro-Vicente JF, Pérez-Conesa D, Rincón F, Ros G, Martínez-Graciá C, Vidal ML.

Title: Does ascorbic acid supplementation affect iron bioavailability in rats fed micronized dispersible ferric pyrophosphate fortified fruit juice?

Link: https://doi.org/10.1007/s00394-008-0750-7.

2. Sakaguchi 2003

Author: Sakaguchi N, Rao TP, Nakata K, Nanbu H, Juneja LR.

Title: Iron absorption and bioavailability in rats of micronized dispersible ferric pyrophosphate.

Link: https://doi.org/10.1024/0300-9831.74.1.3.

3. Wegmuller 2004

Author: Wegmüller R, Zimmermann MB, Moretti D, Arnold M, Langhans W, Hurrell RF.

Title: Particle size reduction and encapsulation affect the bioavailability of ferric pyrophosphate

in rats.

Link: https://doi.org/10.1093/jn/134.12.3301.

4. Salgueiro 2008

Author: Salgueiro MJ, Arnoldi S, Kaliski MA, Torti H, Messeri E, Weill R, et al.

Title: Stabilized-solubilized ferric pyrophosphate as a new iron source for food fortification.

Bioavailability studies by means of the prophylactic-preventive method in rats.

Link https://doi.org/10.1007/s12011-008-8229-1.

5. Lucia 2013

Author: Della Lucia CM, Vaz Tostes Md, Silveira CM, Bordalo LA, Rodrigues FC, Pinheiro-

Sant'Ana HM, Martino HS, Costa NM.

Title: Iron bioavailability in Wistar rats fed with fortified rice by Ultra Rice technology with

or without addition of yacon flour (Smallanthus sonchifolius).

Link: https://pubmed.ncbi.nlm.nih.gov/24167960/

6. Rohner 2006

Author: Rohner F, Ernst FO, Arnold M, Hilbe M, Biebinger R, Ehrensperger F, et al.

Title: Synthesis, characterization, and bioavailability in rats of ferric phosphate nanoparticles.

Link: https://doi.org/10.1093/jn/137.3.614.

7. Dickmann 2016 Author: Dickmann RS, Strasburg GM, Romsos DR, Wilson LA, Lai GH,

Huang H.

Title: Particle Size, Surface Area, and Amorphous Content as Predictors of Solubility and

Bioavailability for Five Commercial Sources of Ferric Orthophosphate in Ready-To-Eat Cereal.

Link: https://doi.org/10.3390/nu8030129.

8. Whittaker 1989

Author: Whittaker P, Vanderveen JE. Title: Effect of EDTA on the bioavailability to rats of

fortification iron used in Egyptian balady bread.

Link: https://doi.org/10.1079/bjn19900145.

9. Lysionek 2002

Author: Lysionek AE, Zubillaga MB, Salgueiro MJ, Caro RA, Segal M, Shafran N, et al.

Title: Bioavailability of Petit-Suisse Cheese as Food Vehicle for Iron Fortification Estimated

by the Prophylactic Method.

Link: https://doi.org/10.3177/jnsv.48.315.

10. Benito1997

Author: Benito P, House W, Miller D. Title: Influence of meal frequency on iron absorption

as assessed by hemoglobin repletion in rats.

Link: https://doi.org/10.1016/S0271-5317(97)00066-3.

11. Srinivasu 2015

Author: Srinivasu BY, Mitra G, Muralidharan M, Srivastava D, Pinto J, Thankachan P, et al.

Title: Beneficiary effect of nanosizing ferric pyrophosphate as food fortificant in iron deficiency

anemia: evaluation of bioavailability, toxicity and plasma biomarker.

Link: https://doi.org/10.1039/c5ra07724a.

12. Zhu 2006

Author: Zhu L, Yeung CK, Glahn RP, Miller DD.

Title: Iron Dissociates from the NaFeEDTA Complex Prior to or during Intestinal Absorption

in Rats.

Link: https://doi.org/10.1021/jf0616964.

13. Malika 2022

Author: Malika S, Ullah A, Anjum AA, Sattar MMK, Ali T, Manzoor R.

Title: Bio-efficacy of iron and zinc fortified wheat flour along with bio-assessment of its hepatic

and renal toxic potential.

Link: https://doi.org/10.1590/1519-6984.261695.

14. Ologunde 1992

Author: Malika S, Ullah A, Anjum AA, Sattar MMK, Ali T, Manzoor R.

Title: Bio-efficacy of iron and zinc fortified wheat flour along with bio-assessment of its hepatic

and renal toxic potential.

Link: https://doi.org/10.1590/1519-6984.261695.

15. Olivira 1995

Author: Dutra-de-Oliveira JE, Freitas ML, Ferreira JF, Gonçalves AL, Marchini JS.

Title: Iron from complex salts and its bioavailability to rats.

Link: https://pubmed.ncbi.nlm.nih.gov/8789625/

16. Hernandez 2003

Author: Hernandez M, Sousa V, Moreno A, Villapando S, Lopez-Alarcon M.

Title: Iron bioavailability and utilization in rats are lower from lime-treated corn flour than

from wheat flour when they are fortified with different sources of iron.

Link: https://doi.org/10.1093/jn/133.1.154.

17. Suva2022

Author: Suva MA, Tirgar PR.

Title: Comparative assessment of anti-anemic effect of Sucrosomial iron in haloperidol-induced

iron deficiency anemia in Wistar rats. Journal of Integrated Science and Technology.

Link: https://www.pubs.iscience.in/journal/index.php/jist/article/view/1471.

18. Lysionek 2001b

Author: Lysionek A, Zubillaga M, Salgueiro J, Caro R, Ettlin E, Boccio J.

Title: Bioavailability studies of a new iron source by means of the prophylactic-preventive method in rats.

Link: https://doi.org/10.1385/BTER:84:1-3:123.

19. Mazgaj 2020

Author: Mazgaj R, Szudzik M, Lipiński P, Jończy A, Smuda E, Kamyczek M, et al.

Title: Effect of oral supplementation of healthy pregnant sows with sucrosomial ferric pyrophosphate on maternal iron status and hepatic iron stores in newborn piglets.

Link: https://doi.org/10.3390/ani10071113.

20. Valcarcel 2018

Author: Caballero Valcarcel AM, Martinez Gracia C, Martinez Miro S, Madrid Sanchez J,

Gonzalez Bermudez CA, Domenech Asensi G, et al.

Title: Iron bioavailability of four iron sources used to fortify infant cereals, using anemic weaning pigs as a model.

Link: https://doi.org/10.1007/s00394-018-1742-x.

21. Anderson 1972

Author: Anderson TA, Kim I, Fomon SJ.

Title: Iron status of anemic rats fed iron-fortified cereal-milk diets.

Link: https://doi.org/10.1159/000175401.

22. Carretero 2006 Author: Navas-Carretero S, Sarria B, Perez-Granados AM, Schoppen S,

Izquierdo-Pulido M, Vaquero MP.

Title: A comparative study of iron bioavailability from cocoa supplemented with ferric pyrophosphate or ferrous fumarate in rats.

Link: https://doi.org/10.1159/000104138.

23. Sachdeva 2015

Author: Sachdeva B, Kaushik R, Arora S, Kapila S.

Title: Bioavailability of iron in multiple fortified milk.

Link: https://doi.org/10.1007/s13197-015-1711-9.

24. Ahmad 2022

Author: Ahmad AMR, Farooq U, Anees M, Anis RA, Rashid S, Ahmed W.

Title: Co-administration of Inulin and Iron Fortificants improves Iron Deficiency Biomarkers

in Female Sprague Dawley Rats.

Link: https://doi.org/10.1002/fsn3.2337.

25. Douglas 1981

Author: ouglas FW, Rainey NH, Wong NP, Edmondson LF, LaCroix DE.

Title: Color, Flavor, and Iron Bioavailability in Iron-Fortified Chocolate Milk.

Link: https://doi.org/10.3168/jds.S0022-0302(81)82767-1.

26. Theuer 1971

Author: Theuer RC, Kemmerer KS, Martin WH, Zoumas BL, Sarett HP.

Title: Effect of Processing on Availability of Iron Salts in Liquid Infant Formula Products Experimental Soy Isolate Formulas.

Link: https://doi.org/10.1021/jf60175a017.

27. Theuer 1973

Author: Theuer RC, Martin WH, Wallander JF, Sarett HP.

Title: Effect of Processing on Availability of Iron Salts in Liquid Infant Formula Products.

Link: https://doi.org/10.1021/jf60187a002.

28. Sudargo 2015

Author: Sudargo T, Kusuma RJ, Arjuna T, Hasnawati RA, Rubi DS, Rohman A.

Title: Effect of sodium iron EDTA fortification in tempe in serum iron and ferritin level of

anemic female wistar rats.

Link: https://doi.org/10.3923/pjn.2015.88.93.

29. Fritz 1975

Author: Fritz JC, Pla GW, Harrison BN, Clark GA.

Title: Estimation of the bioavailability of iron.

Link: https://doi.org/10.1093/jaoac/58.5.902.

30. Carretero 2014

Author: Navas-Carretero S, Perez-Granados AM, Sarria B, Schoppen S, Vaquero MP.

Title: Iron Bioavailability from pate enriched with encapsulated ferric pyrophosphate or ferrous

gluconate in rats.

Link: https://doi.org/10.1177/1082013207077931.

31. Li 2009

Author: Li YO, Diosady LL, Wesley AS. Title: Iron in vitro bioavailability and iodine storage

stability in double-fortified salt.

Link: https://doi.org/10.1177/156482650903000403.

32. Kosonen 1991

Author: Kosonen T, Mutanen M Title: Relative bioavailability of iron in carbonyl iron and

complex ferric orthophosphate to rat.

Link: https://pubmed.ncbi.nlm.nih.gov/1587710/

33. Yeung 2005

Author: Yeung CK, Zhu L, Glahn RP, Miller DD.

Title: Tissue iron distribution and adaptation of iron absorption in rats exposed to a high

dietary level of NaFeEDTA.

Link: https://doi.org/10.1021/jf051242r.

34. Amine 1974

Author: Amine EK, Hegsted DM. Title: Biological Assessment of Available Iron in Food Products.

Link: https://doi.org/10.1021/jf60193a006.

35. Zhu 2009

Author: Zhu L, Glahn RP, Nelson D, Miller DD.

Title: Comparing Soluble Ferric Pyrophosphate to Common Iron Salts and Chelates as Sources

of Bioavailable Iron in a Caco-2 Cell Culture Model.

Link: https://doi.org/10.1021/jf900328t.

36. Yeung 2002

Author: Yeung A, Glahn R, Miller D.

Title: Comparison of the availability of various iron fortificants in bread and milk using an in

vitro digestion/Caco-2 cell culture method.

Link: https://doi.org/10.1111/j.1365-2621.2002.tb09554.x.

37. Wortley 2005

Author: Wortley G, Leusner S, Good C, Gugger E, Glahn R.

Title: 2 Iron availability of a fortified processed wheat cereal: a comparison of fourteen iron

forms using an in vitro digestion/human colonic adenocarcinoma (CaCo-2) cell model.

Link: https://doi.org/10.1079/bjn20041294.

38. Sabatier 2020

Author: Sabatier M, Rytz A, Husny J, Dubascoux S, Nicolas M, Dave A, et al. Title: 1 Impact

of Ascorbic Acid on the In Vitro Iron Bioavailability of a Casein-Based Iron Fortificant.

Link: https://doi.org/10.3390/nu12092776.

39. Karn 2011

Author: Karn SK, Chavasit V, Kongkachuichai R, Tangsuphoom N.

Title: 3 Shelf stability, sensory qualities, and bioavailability of iron-fortified Nepalese curry

powder.

Link: https://doi.org/10.1177/156482651103200102.

40. Cercamondi 2016

Author: Cercamondi CI, Duchateau GSMJE, Harika RK, van den Berg R, Murray P, Koppenol

WP, et al.

Title: 6 Sodium pyrophosphate enhances iron bioavailability from bouillon cubes fortified with

ferric pyrophosphate.

Link: https://doi.org/10.1017/S0007114516002191.

41. Valcarcel 2022

Author: Caballero Valcarcel AM, Lopez Nicolas R, Frontela Saseta C, Gonzalez Bermudez CA,

Martinez Gracia C, Santaella Pascual M.

Title: Comparison of bioavailability and transporters gene expression of four iron fortificants

added to infant cereals.

Link: https://doi.org/10.1016/j.fbio.2022.102023.

42. Micheletto 2022

Author: Micheletto M, Gaio E, Tedesco E, Di Maira G, Mantovan E, Zanella M, et al.

Title: Intestinal Absorption Study of a Granular Form of Ferric Pyrophosphate.

Link: https://doi.org/10.3390/metabo12050463.

Clinical systemic review (Chapter 3)

1. Bradley 1993

Author: Bradley CK, Hillman L, Sherman AR, Leedy D, Cordano A.

Title: Evaluation of two iron-fortified, milk-based formulas during infancy.

Link: https://doi.org/10.1093/ajcn/47.1.108.

2. Haschke 1988

Author: Haschke F, Pietschnig B, Vanura H, Heil M, Steffan I, Hobiger G, et al.

Title: Iron intake and iron nutritional status of infants fed iron-fortified beikost with meat.

Link: https://doi.org/10.1093/ajcn/47.1.108.

3. Osei 2015

Author: Osei AK, Pandey P, Spiro D, Adhikari D, Haselow N, De Morais C, et al.

Title: Adding multiple micronutrient powders to a homestead food production programme

yields marginally significant benefit on anaemia reduction among young children in Nepal.

Link: https://doi.org/10.1111/mcn.12173.

4. Paganini 2019

Author: Paganini D, Uyoga MA, Kortman GAM, Boekhorst J, Schneeberger S, Karanja S, et

al.

Title: Maternal human milk oligosaccharide profile modulates the impact of an intervention

with iron and galacto-oligosaccharides in Kenyan infants.

Link: https://doi.org/10.3390/nu11112596.

5. Albelbeisi 2020

Author: Albelbeisi A, Shariff ZM, Mun CY, Rahman HA, Abed Y.

Title: Multiple micronutrient supplementation improves growth and reduces the risk of anemia

among infants in Gaza Strip, Palestine: a prospective randomized community trial.

Link: https://doi.org/10.1186/s12937-020-00652-7.

6. Javaid 1991

Author: Javaid N, Haschke F, Pietschnig B, Schuster E, Huemer C, Shebaz A, et al.

Title: 158 Interactions between infections, malnutrition and iron nutritional status in Pakistani

infants. A longitudinal study.

Link: https://doi.org/10.1111/j.1651-2227.1991.tb12017.x.

7. Faber 2005

Author: Faber M, Kvalsvig JD, Lombard CJ, Benadé AJS.

Title: Effect of a fortified maize-meal porridge on anemia, micronutrient status, and motor

development of infants.

Link: https://doi.org/10.1093/ajcn/82.5.1032.

8. Ziegler 2011

Author: Ziegler EE, Fomon SJ, Nelson SE, Jeter JM, Theuer RC.

Title: Dry Cereals Fortified with Electrolytic Iron or Ferrous Fumarate Are Equally Effective

in Breast-fed Infants.

Link: https://doi.org/10.3945/jn.110.127266.

9. Jaeggi 2015

Author: Barth-Jaeggi T, Moretti D, Kvalsvig J, Holding PA, Njenga J, Mwangi A, et al.

Title: In-home fortification with 2.5mg iron as NaFeEDTA does not reduce anaemia but increases weight gain: a randomised controlled trial in Kenyan infants.

Link: https://doi.org/10.1111/mcn.12163.

10. Lartey 1999

Author:Lartey A, Manu A, Brown KH, Peerson JM, Dewey KG.

Title: A randomized, community-based trial of the effects of improved, centrally processed

complementary foods on growth and micronutrient status of Ghanaian infants from 6 to 12 mo

of age.

Link: https://doi.org/10.1093/ajcn/70.3.391.

11. Begin 2007

Author: Bégin F, Santizo MC, Peerson JM, Torún B, Brown KH.

Title:Effects of bovine serum concentrate, with or without supplemental micronutrients, on

the growth, morbidity, and micronutrient status of young children in a low-income, peri-urban

Guatemalan community.

Link: https://doi.org/10.1038/sj.ejcn.1602682.

12.Olle 2002

Author: Hernell O, Lönnerdal B.

Title: Iron status of infants fed low-iron formula: no effect of added bovine lactoferrin or nucleotides.

Link: https://doi.org/10.1093/ajcn/76.4.858.

13. Stekel 1988 Author: Stekel A, Pizarro F, Olivares M, Chadud P, Llaguno S, Cayazzo M,

et al.

Title: Prevention of iron deficiency by milk fortification III. Effectiveness under the usual operational conditions of a nation-wide food program.

Link:https://www.researchgate.net/publication/279617935_Prevention_of_iron_deficiency_by_milk_fortification_III_Effectiveness_under_the_usual_operational_conditions_of_a_nationwide_food_program

14. Walter 1998

Author:Walter T, Pino P, Pizarro F, Lozoff B.

Title:Prevention of iron-deficiency anemia: comparison of high- and low-iron formulas in term

healthy infants after six months of life.

Link: https://doi.org/10.1016/s0022-3476(98)70352-x.

15. David 1995

Author: Schümann K, Romero-Abal ME, Mäurer A, Luck T, Beard J, Murray-Kolb L, et al.

Title: Haematological response to haem iron or ferrous sulphate mixed with refried black beans

in moderately anaemic Guatemalan pre-school children.

Link: https://doi.org/10.1079/phn2004708.

16. Lonnerdal 1994

Author: Lönnerdal B, Hernell O.

Title: Iron, zinc, copper and selenium status of breast-fed infants and infants fed trace element

fortified milk-based infant formula.

Link: https://doi.org/10.1111/j.1651-2227.1994.tb18121.x.

17. Dewey 1998

Author: Dewey KG, Cohen RJ, Landa Rivera L, Brown KH.

Title: Effects of age of introduction of complementary foods on iron status of breast-fed infants

in Honduras.

Link: https://doi.org/10.1093/ajcn/67.5.878.

18. Olivares 1989

Author: Olivares M, Walter T, Hertrampf E, Pizarro F, Stekel A.

Title: Prevention of iron deficiency by milk fortification.

Link: https://doi.org/10.1111/apa.1989.78.s361.109.

19. Stekel 2019

Author: Stekel A, Olivares M, Cayazzo M, Chadud P, Llaguno S, Pizarro F.

Title: Prevention of iron deficiency by milk fortification.

Link: https://doi.org/10.1093/ajcn/47.2.265.

20. Rim 2008

Author: Rim HY, Kim SH, Sim BC, Gang HY, Kim HY, Kim YR, et al.

Title: Effect of iron fortification of nursery complementary food on iron status of infants in the

DPRKorea.

Link: https://pubmed.ncbi.nlm.nih.gov/18586646/

21. Zigler 2009

Author: 17Ziegler EE, Nelson SE, Jeter JM.

Title: Iron status of breastfed infants is improved equally by medicinal iron and iron-fortified

cereal.

Link: https://doi.org/10.3945/ajcn.2008.27350.

22. Christofides 2005

Author: Christofides A, Schauer C, Sharieff W, Zlotkin SH.

Title: Acceptability of micronutrient sprinkles: A new food-based approach for delivering iron

to First Nations and Inuit children in Northern Canada.

Link: https://pubmed.ncbi.nlm.nih.gov/16390629/.

23. Harrison 2021

Author: Harrison OA, Hays NP, Ansong RS, Datoghe D, Vuvor F, Steiner-Asiedu M.

Title: Effect of iron-fortified infant cereal on nutritional status of infants in Ghana.

Link: https://doi.org/10.1002/fsn3.2669.

24. Walter 1993

Author: Walter T, Dallman PR, Pizarro F, Velozo L, Pena G, Bartholmey SJ, et al.

Title: Effectiveness of iron-fortified infant cereal in prevention of iron deficiency anemia.

Link: https://pubmed.ncbi.nlm.nih.gov/8474819/.

25. Young 2021 Author:Young MF, Mehta RV, Gosdin L, Kekre P, Verma P, Larson LM, et al.

Title: Home fortification of complementary foods reduces anemia and diarrhea among children

aged 6-18 months in bihar, india: A large-scale effectiveness trial.

Link: https://doi.org/10.1093/jn/nxab065.

26. Zlotkin 2003 Author: Zlotkin S, Arthur P, Schauer C, Antwi KY, Yeung G, Piekarz A.

Title:Home-fortification with iron and zinc sprinkles or iron sprinkles alone successfully treats

anemia in infants and young children.

Link: https://doi.org/10.1093/jn/133.4.1075.

27. Liu 1993

Author: Liu D, Bates CJ, Yin T, Wang X, Lu C.

Title: Nutritional efficacy of a fortified weaning rusk in a rural area near Beijing.

Link: https://doi.org/10.1093/ajcn/57.4.506.

28. Arcanjo 2013

Author: Nogueira Arcanjo FP, Bastos Mota FS, Duarte Segall S, Madeiro Leite ÁJ, Roberto

Santos P.

Title: Rice fortified with iron given weekly increases hemoglobin levels and reduces anemia in

infants: a community intervention trial.

Link: https://doi.org/10.1024/0300-9831/a000145.

29. Beinner 2009

Author: Beinner MA, Velasquez-Meléndez G, Pessoa MC, Greiner T.

Title: Iron-Fortified Rice Is As Efficacious As Supplemental Iron Drops in Infants and Young

Children.

Link: https://doi.org/10.3945/jn.109.112623.

30. Hirve 2007

Author: Hirve S, Bhave S, Bavdekar A, Naik S, Pandit A, Schauer C, et al.

Title: Low dose 'Sprinkles'– an innovative approach to treat iron deficiency anemia in infants

and young children.

Link: https://pubmed.ncbi.nlm.nih.gov/17351300/

31. Arcanjo 2012

Author: Nogueira Arcanjo FP, Santos PR, Costa Arcanjo CP, Silverio Amancio OM, Pellegrini

Braga JA.

Title: Use of Iron-Fortified Rice Reduces Anemia in Infants.

Link: https://doi.org/10.1093/tropej/fms021.

32. Samadpour 2011

Author: Samadpour K, Long KZ, Hayatbakhsh R, Marks GC.

Title:Randomised comparison of the effects of Sprinkles and Foodlets with the currently

recommended supplement (Drops) on micronutrient status and growth in Iranian children.

Link: https://doi.org/10.1038/ejcn.2011.124.

33. Christofides 2006

Author: Christofides A, Asante KP, Schauer C, Sharieff W, Owusu-Agyei S, Zlotkin S.

Title:Multi-micronutrient Sprinkles including a low dose of iron provided as microencapsulated

ferrous fumarate improves haematologic indices in anaemic children: a randomized clinical

trial.

Link:https://doi.org/10.1111/j.1740-8709.2006.00060.x.

34. Hess 1992

Author: Hess SY, Wessells KR, Hinnouho G-M, Barffour MA, Sanchaisuriya K, Arnold CD,

et al.

Title:Iron status and inherited haemoglobin disorders modify the effects of micronutrient powders on linear growth and morbidity among young Lao children in a double-blind randomised trial.

Link: https://doi.org/10.1017/s0007114519001715.

35. Hyder 2007

Author: Ip H, Hyder SMZ, Haseen F, Rahman M, Zlotkin SH.

Title:Improved adherence and anaemia cure rates with flexible administration of micronutrient

Sprinkles: a new public health approach to anaemia control.

Link: https://doi.org/10.1038/sj.ejcn.1602917.

36. Wegmüller 2023

Author:Wegmüller R, Bah A, Kendall L, Goheen MM, Sanyang S, Danso E, et al.

Title: Hepcidin-guided screen-and-treat interventions for young children with iron-deficiency anaemia in The Gambia: an individually randomised, three-arm, double-blind, controlled, proof-of-concept, non-inferiority trial.

Link: https://doi.org/10.1016/S2214-109X(22)00449-1.

37. Samuel 2018

Author: Samuel A, Brouwer ID, Feskens EJM, Adish A, Kebede A, De-Regil LM, Osendarp

SJM.

Title: Effectiveness of a Program Intervention with Reduced-Iron Multiple Micronutrient Powders on Iron Status, Morbidity and Growth in Young Children in Ethiopia.

Link: https://doi.org/10.3390/nu10101508.

38. Davidsson 2009

Author: Davidsson L, Sarker SA, Jamil KA, Sultana S, Hurrell R.

Title: Regular consumption of a complementary food fortified with ascorbic acid and ferrous

fumarate or ferric pyrophosphate is as useful as ferrous sulfate in maintaining hemoglobin concentrations >105 g/L in young Bangladeshi children.

Link: https://doi.org/10.3945/ajcn.2008.27353.

39. Zlotkin 2001

Author: Zlotkin S, Arthur P, Antwi KY, Yeung G. Title: Treatment of anemia with

microencapsulated ferrous fumarate plus ascorbic acid supplied as sprinkles to complementary

(weaning) foods.

Link: https://doi.org/10.1093/ajcn/74.6.791.

40. Zlotkin 2003

Author:Zlotkin S, Antwi KY, Schauer C, Yeung G.

Title: Use of microencapsulated iron(II) fumarate sprinkles to prevent recurrence of anaemia

in infants and young children at high risk.

Link: https://pubmed.ncbi.nlm.nih.gov/12756979/

41. Cignis 2010

Author: Chilenje Infant Growth, Nutrition and Infection (CIGNIS) Study Team.

Title: Micronutrient Fortification to Improve Growth and Health of Maternally HIV-Unexposed

and Exposed Zambian Infants: A Randomised Controlled Trial.

Link: https://doi.org/10.1371/journal.pone.0011165.

42. Muite 2012

Author: Catherine W Macharia-Mutie 1, Agnes M Omusundi, John M Mwai, Alice M Mwangi,

Inge D Brouwer.

Title: Simulation of the effect of maize porridge fortified with grain amaranth or micronutrient

powder containing NaFeEDTA on iron intake and status in Kenyan children.

Link: https://10.1017/S1368980012005174

43. Oliveira 2017

Author: Oliveira CSM, Cardoso MA, Muniz PT, Sampaio P.

Title: Multiple micronutrients in powder delivered through primary health care reduce iron

and vitamin A deficiencies in young Amazonian children.

Link: https://doi.org/10.1017/s1368980016001294.

44. Schumann 2005

Author: Schumann K, Romero-Abal ME, Mäurer A, Luck T, Beard J, Murray-Kolb L, et al.

Title: Haematological response to haem iron or ferrous sulphate mixed with refried black beans

in moderately anaemic Guatemalan pre-school children.

Link: https://doi.org/10.1079/phn2004708.

45.Hyder 2007

Author: Hyder SMZ, Haseen F, Rahman M, Tondeur MC, Zlotkin SH.

Title: Effect of daily versus once-weekly home fortification with micronutrient Sprinkles on

hemoglobin and iron status among young children in rural Bangladesh.

Link: https://doi.org/10.1177/156482650702800204.

46. Gay 2009

Author: Szymlek-Gay EA, Ferguson EL, Heath A-LM, Gray AR, Gibson RS.

Title: Food-based strategies improve iron status in toddlers: a randomized controlled trial.

Link: https://doi.org/10.3945/ajcn.2009.27588

47.Campbell 2020

Author: Campbell RK, Shaikh S, Schulze K, Arguello M, Ali H, Wu L, et al.

Title: Micronutrient and Inflammation Status Following One Year of Complementary Food

Supplementation in 18-Month-Old Rural Bangladeshi Children: A Randomized Controlled

Trial.

Link: https://doi.org/10.3390/nu12051452.

48. Owolabi 2021

Author: Owolabi AJ, Senbanjo IO, Oshikoya KA, Boekhorst J, Eijlander RT, Kortman GAM,

et al.

Title: Multi-Nutrient Fortified Dairy-Based Drink Reduces Anaemia without Observed Adverse

Effects on Gut Microbiota in Anaemic Malnourished Nigerian Toddlers: A Randomised Dose-Response Study.

Link: https://doi.org/10.3390/nu13051566.

49. Levy 2008

Author: Shamah-Levy T, Villalpando S, Rivera-Dommarco JA, Mundo-Rosas V, Cuevas-Nasu

L, Jimenez-Aguilar A.

Title: Ferrous gluconate and ferrous sulfate added to a complementary food distributed by the

Mexican nutrition program Oportunidades have a comparable efficacy to reduce iron deficiency

in toddlers.

Link: https://doi.org/10.1097/MPG.0b013e318167b03b.

50. Teshome 2014

Author: Teshome EM, Andang'o PEA, Osoti V, Terwel SR, Otieno W, Demir AY, et al.

Title: Daily home fortification with iron as ferrous fumarate versus NaFeEDTA: a randomised,

placebo-controlled, non-inferiority trial in Kenyan children.

Link: https://doi.org/10.1186/s12916-017-0839-z.

51. Glinz 2015

Author: Glinz D, Hurrell RF, Ouattara M, Zimmermann MB, Brittenham GM, Adiossan LG,

et al.

Title: The effect of iron-fortified complementary food and intermittent preventive treatment of

malaria on anaemia in 12- to 36-month-old children: a cluster-randomised controlled trial.

Link: https://doi.org/10.1186/s12936-015-0872-3.

52.Rivera 2010

Author: Rivera JA, Shamah T, Villalpando S, Monterrubio E.

Title: Effectiveness of a large-scale iron-fortified milk distribution program on anemia and iron

deficiency in low-income young children in Mexico.

Link: https://doi.org/10.3945/ajcn.2009.28104.

53.Zlotkin 2013

Author: Zlotkin S, Newton S, Aimone AM, Azindow I, Amenga-Etego S, Tchum K, Mahama

E, Thorpe KE, Owusu-Agyei S.

Title: Effect of iron fortification on malaria incidence in infants and young children in Ghana:

a randomized trial.

Link: https://doi.org/10.1001/jama.2013.277129.

54. Lundeen 2010

Author: Lundeen E, Schueth T, Toktobaev N, Zlotkin S, Hyder SMZ, Houser R.

Title: Daily use of Sprinkles micronutrient powder for 2 months reduces anemia among children

6 to 36 months of age in the Kyrgyz Republic: a cluster-randomized trial.

Link: https://doi.org/10.1177/156482651003100307.

55. Machado 2021

Author: Machado MMA, Lopes M de P, Schincaglia RM, da Costa PSS, Coelho ASG, Hadler

MCCM.

Title: Effect of Fortification with Multiple Micronutrient Powder on the Prevention and

Treatment of Iron Deficiency and Anaemia in Brazilian Children: A Randomized Clinical Trial.

Link: https://doi.org/10.3390/nu13072160.

56. Tchum 2021

Author: Tchum SK, Arthur FK, Adu B, Sakyi SA, Abubakar LA, Atibilla D, et al.

Title: Impact of iron fortification on anaemia and iron deficiency among pre-school children

living in Rural Ghana.

Link: https://doi.org/10.1371/journal.pone.0246362.

57. Suchdew 2012

Author: Suchdev PS, Ruth LJ, Woodruff BA, Mbakaya C, Mandava U, Flores-Ayala R, et al.

Title: Selling Sprinkles micronutrient powder reduces anemia, iron deficiency, and vitamin A

deficiency in young children in Western Kenya: a cluster-randomized controlled trial.

Link: https://doi.org/10.3945/ajcn.111.030072.

58. Villalpando 2006

Author: Villalpando S, Shamah T, Rivera JA, Lara Y, Monterrubio E.

Title: Fortifying Milk with Ferrous Gluconate and Zinc Oxide in a Public Nutrition Program

Reduced the Prevalence of Anemia in Toddlers.

Link: https://doi.org/10.1093/jn/136.10.2633.

59. Paula 2001

Author: de Paula RA, Fisberg M.

Title: The use of sugar fortified with iron tris-glycinate chelate in the prevention of iron deficiency anemia in preschool children.

Link: https://pubmed.ncbi.nlm.nih.gov/11688083/

60. Delimont 2019

Author: Delimont NM, Vahl CI, Kayanda R, Msuya W, Mulford M, Alberghine P, et al.

Title: Complementary Feeding of Sorghum-Based and Corn-Based Fortified Blended Foods

Results in Similar Iron, Vitamin A, and Anthropometric Outcomes in the MFFAPP Tanzania

Efficacy Study.

Link: https://doi.org/10.1093/cdn/nzz027.

61. Ekoe 2020

Author: Ekoe T, Bianpambe O, Nguefack F, Pondi DM, Kana-Sop MM, Hays NP, et al.

Title: Efficacy of an iron-fortified infant cereal to reduce the risk of iron deficiency anemia in

young children in East Cameroon.

Link: https://doi.org/10.1002/fsn3.1639.

62. Almeida 2005

Author: de Almeida CAN, Dutra-De-Oliveira JE, Crott GC, Cantolini A, Ricco RG, Del

Ciampo LA, et al.

Title: Effect of fortification of drinking water with iron plus ascorbic acid or with ascorbic acid

alone on hemoglobin values and anthropometric indicators in preschool children in day-care

centers in Southeast Brazil.

Link: https://doi.org/10.1177/156482650502600302.

63. Mutie 2012

Author: Macharia-Mutie CW, Moretti D, Van den Brie N, Omusundi AM, Mwangi AM, Kok

FJ, et al.

Title: Maize Porridge Enriched with a Micronutrient Powder Containing Low-Dose Iron as

NaFeEDTA but Not Amaranth Grain Flour Reduces Anemia and Iron Deficiency in Kenyan

Preschool Children.

Link: https://doi.org/10.3945/jn.112.157578.

64. Rahman 2019

Author: Rahman S, Lee P, Raqib R, Roy AK, Khan MR, Ahmed F.

Title: Effect of Micronutrient Powder (MNP) with a Low-Dose of Iron on Hemoglobin and Iron

Biomarkers, and Its Effect on Morbidities in Rural Bangladeshi Children Drinking Ground-water

with a High-Level of Iron: A Randomized Controlled Trial.

Link: https://doi.org/10.3390/nu11112756.

65. Arcanjo 2016

Author: Arcanjo FPN, Amancio OMS, Braga JAP, de Paula Teixeira Pinto V.

Title: Randomized controlled trial of iron-fortified drinking water in preschool children.

Link: https://doi.org/10.1080/07315724.2010.10719825.

66. Arcanjo 2007

Author: Arcanjo FPN, Pinto VPT, Coelho MR, Amâncio OMS, Magalhães SMM.

Title: Anemia reduction in preschool children with the addition of low doses of iron to school

meals.

Link: https://doi.org/10.1093/tropej/fmm113.

67. Sharieff 2006

Author: Sharieff W, Yin S-A, Wu M, Yang Q, Schauer C, Tomlinson G, et al.

Title: Short-term daily or weekly administration of micronutrient Sprinkles has high compliance

and does not cause iron overload in Chinese schoolchildren: a cluster-randomised trial.

Link: https://doi.org/10.1079/phn2006841.

68. Arcanjo 2012

Author: Arcanjo FPN, Arcanjo CC, Arcanjo FCN, Campos L de A, Amancio OMS, Braga

JAP.

Title: Milk-based cornstarch porridge fortified with iron is effective in reducing anemia: a

randomized, double-blind, placebo-controlled trial.

Link: https://doi.org/10.1093/tropej/fms003.

69. Varma 2006

Author: Varma JL, Das S, Sankar R, Mannar MGV, Levinson FJ, Hamer DH.

Title: Community-level micronutrient fortification of a food supplement in India: a controlled

trial in preschool children aged 36-66 mo.

Link: https://doi.org/10.1093/ajcn/85.4.1127.

70.Msc 2016

Author: Quintero-Gutiérrez AG, González-Rosendo G, Pozo JP, Villanueva-Sánchez J.

Title: Heme Iron Concentrate and Iron Sulfate Added to Chocolate Biscuits: Effects on Hematological Indices of Mexican Schoolchildren.

Link: https://doi.org/10.1080/07315724.2015.1060875.

71. Barbosa 2011

Author: Navarro Barbosa TN, de Aguiar Carrazedo Taddei JA, Palma D, Ancona-Lopez F,

Pellegrini Braga JA.

Title: Double-blind randomized controlled trial of rolls fortified with microencapsulated iron.

Link: https://pubmed.ncbi.nlm.nih.gov/22392326/

72. Troesch 2011

Author: Troesch B, van Stujivenberg ME, Smuts CM, Kruger HS, Biebinger R, Hurrell RF, et

al.

Title: A micronutrient powder with low doses of highly absorbable iron

and zinc reduces iron and zinc deficiency and improves weight-for-age Z-scores in South African

children.

Link: https://doi.org/10.3945/jn.110.129247.

73. Andango 2007

Author: Andang'o PEA, Osendarp SJM, Ayah R, West CE, Mwaniki DL, De Wolf CA, et al.

Title: Efficacy of iron-fortified whole maize flour on iron status of schoolchildren in Kenya: a

randomised controlled trial.

Link: https://doi.org/10.1016/s0140-6736(07)60817-4.

74 James 2000

Author: Nickerson HJ, Silberman T, Park RW, DeVries EO, Broste SK.

Title: Treatment of iron deficiency anemia and associated protein-losing enteropathy in children.

Link: https://doi.org/10.1097/00043426-200001000-00010.

75 Vinodkumar 2009

Author: Vinodkumar M, Rajagopalan S.

Title: Efficacy of fortification of school meals with ferrous glycine phosphate and riboflavin

against anemia and angular stomatitis in schoolchildren.

Link: https://doi.org/10.1177/156482650903000307.

76. Wegmuller 2006

Author: Wegmuller R, Camara F, Zimmermann MB, Adou P, Hurrell RF.

Title: Salt Dual-Fortified with Iodine and Micronized Ground Ferric Pyrophosphate Affects

Iron Status but Not Hemoglobin in Children in Cote dIvoire.

Link: https://doi.org/10.1093/jn/136.7.1814.

77. Vinodkumar 2007

Author: Vinodkumar M, Rajagopalan S.

Title: Multiple micronutrient fortification of salt.

Link: https://doi.org/10.1038/sj.ejcn.1602955.

78. Radhika 2011

Author: Radhika MS, Nair KM, Kumar RH, Rao MV, Ravinder P, Reddy CG, et al.

Title: Micronized ferric pyrophosphate supplied through extruded rice kernels improves body

iron stores in children: a double-blind, randomized, placebo-controlled midday meal feeding

trial in Indian schoolchildren.

Link: https://doi.org/10.3945/ajcn.110.007179.

79. Aaron 2011

Author: Aaron GJ, Kariger P, Aliyu R, Flach M, Iya D, Obadiah M, et al.

Title: A Multi-Micronutrient Beverage Enhances the Vitamin A and Zinc Status of Nigerian

Primary Schoolchildren.

Link: https://doi.org/10.3945/jn.110.136770.

80. Zimmermann 2015

Author: Zimmermann MB, Muthayya S, Moretti D, Kurpad A, Hurrell RF.

Title: Iron fortification reduces blood lead levels in children in Bangalore, India.

Link: https://doi.org/10.1542/peds.2005-2440.

81. Abizari 2012

Author: Abizari A-R, Moretti D, Zimmermann MB, Armar-Klemesu M, Brouwer ID.

Title: Whole Cowpea Meal Fortified with NaFeEDTA Reduces Iron Deficiency among Ghanaian

School Children in a Malaria Endemic Area.

Link: https://doi.org/10.3945/jn.112.165753.

82 Azeem 2021

Author: Azeem M, Sharif MK, Shah FUH, Ali M, Nazir MA, Rizwan M, et al.

Title: Does iron-fortified chewing gum influence the biochemical profile of school- going children

(6-10 yrs.)?

Link: https://doi.org/10.15586/ijfs.v33i1.1804.

83 Kuriyan 2015

Author: Kuriyan R, Anura VK, Srinivasan K, Maria P, Prashanth T, Shilpa K-J, et al.

Title: The effects of regular consumption of a multiple micronutrient fortified milk beverage

on the micronutrient status of school children and on their mental and physical performance.

Link: https://doi.org/10.1016/j.clnu.2015.02.001.

84 Kruger 1996

Author: Kruger M, Benadé AJS, Chad JB, Erna PGM, Jacoba AL.

Title: Effects of Iron Fortification in a School Feeding Scheme and Anthelmintic Therapy on

the Iron Status and Growth of Six- to Eight-Year-Old Schoolchildren.

Link: https://doi.org/10.1177/156482659601700104.

85. Huo 2002

Author: Huo J, Sun J, Miao H, Yu B, Yang T, Liu Z, et al.

Title: Therapeutic effects of NaFeEDTA-fortified soy sauce in anaemic children in China.

Link: https://doi.org/10.1046/j.1440-6047.2002.00277.x.

86. Hieu 2012

Author: Hieu NT, Sandalinas F, de Sesmaisons A, Laillou A, Tam NP, Khan NC, et al.

Title: Multi-micronutrient-fortified biscuits decreased the prevalence of anaemia and improved

iron status, whereas weekly iron supplementation only improved iron status in Vietnamese school children.

Link: https://doi.org/10.1017/s0007114511006945.

87. Stuijvenberg 2008

Author: van Stuijvenberg ME, Smuts CM, Lombard CJ, Dhansay MA.

Title: Fortifying brown bread with sodium iron EDTA, ferrous fumarate, or electrolytic iron

does not affect iron status in South African schoolchildren.

Link: https://doi.org/10.1093/jn/138.4.782.

88. Agdeppa 2008

Author: Angeles-Agdeppa I, Capanzana MV, Barba CVC, Florentino RF, Takanashi K.

Title: Efficacy of iron-fortified rice in reducing anemia among schoolchildren in the Philippines.

Link: https://doi.org/10.1024/0300-9831.78.2.74.

89. Le 2006

Author: Le HT, Brouwer ID, Burema J, Nguyen KC, Kok FJ.

Title: Efficacy of iron fortification compared to iron supplementation among Vietnamese schoolchildren.

Link: https://doi.org/10.1186/1475-2891-5-32.

90. Stuijvenberg 1999

Author: Stuijvenberg ME, Kvalsvig JD, Faber M, Kruger M, Kenoyer DG, Benade AJS.

Title: Effect of iron-, iodine-, and beta-carotene-fortified biscuits on the micronutrient status

of primary school children: a randomized controlled trial.

Link: https://doi.org/10.1093/ajcn/69.3.497

91. Nadiger 1979

Author: Nadiger HA, Krishnamachari KA, Naidu AN, Rao BS, Srikantia SG.

Title: The use of common salt (sodium chloride) fortified with iron to control anaemia: results

of a preliminary study.

Link: https://doi.org/10.1079/bjn19800063.

92. Vinodkumar 2009

Author: Vinodkumar M, Erhardt JG, Rajagopalan S.

Title: Impact of a Multiple-micronutrient Fortified Salt on the Nutritional Status and Memory

of Schoolchildren.

Link: https://doi.org/10.1024/0300-9831.79.56.348

93. Kumar 2008

Author: Kumar MV, Rajagopalan S. Title: Trial using multiple micronutrient food supplement

and its effect on cognition.

Link: https://doi.org/10.1007/s12098-008-0127-1

94. Stuijvenberg 2006

Author: van Stuijvenberg ME, Smuts CM, Wolmarans P, Lombard CJ, Dhansay MA.

Title: The efficacy of ferrous bisglycinate and electrolytic iron as fortificants in bread in iron-

deficient school children.

Link: https://doi.org/10.1079/bjn20051669.

95. Kumar 2006

Author: Kumar MV, Rajagopalan S.

Title: Multiple micronutrient fortification of salt and its effect on cognition in Chennai school

children.

Link: https://pubmed.ncbi.nlm.nih.gov/17704033/

96. Rivera 1982

Author: Rivera R, Ruiz R, Hegenauer J, Saltman P, Green R.

Title: Bioavailability of iron- and copper-supplemented milk for Mexican school

children.

Link: https://doi.org/10.1093/ajcn/36.6.1162.

97. Rohner 2009

Author: Rohner F, Zimmermann MB, Amon RJ, Vounatsou P, Tschannen AB, N'Goran EK,

et al.

Title: In a Randomized Controlled Trial of Iron Fortification, Anthelmintic Treatment,

and Intermittent Preventive Treatment of Malaria for Anemia Control in Ivorian Children, only

Anthelmintic Treatment Shows Modest Benefit.

Link: https://doi.org/10.3945/jn.109.114256.

98. Sun 2006

Author: Sun J, Huang J, Li W, Wang L, Wang A, Huo J, et al.

Title: Effects of wheat flour fortified with different iron fortificants on iron status and anemia

prevalence in iron deficient anemic students in Northern China.

Link: https://pubmed.ncbi.nlm.nih.gov/17215188/.

99. longfils 2008

Author: Longfils P, Monchy D, Weinheimer H, Chavasit V, Nakanishi Y, Schuemann K.

Title: A comparative intervention trial on fish sauce fortified with NaFe-EDTA and FeSO4+citrate

in iron deficiency anemic school children in Kampot, Cambodia.

Link: https://pubmed.ncbi.nlm.nih.gov/18586644/.

100. Perignon 2016

Author: Perignon M, Fiorentino M, Kuong K, Dijkhuizen MA, Burja K, Parker M, et al.

Title: Impact of Multi-Micronutrient Fortified Rice on Hemoglobin, Iron and Vitamin A Status

of Cambodian Schoolchildren: a Double-Blind Cluster-Randomized Controlled Trial.

Link: https://doi.org/10.3390/nu8010029.

101. Thankachan 2012

Author: Thankachan P, Rah JH, Thomas T, Selvam S, Amalrajan V, Srinivasan K, et al.

Title: Multiple Micronutrient-Fortified Rice Affects Physical Performance and Plasma Vitamin

B-12 and Homocysteine Concentrations of Indian School Children.

Link: https://doi.org/10.3945/jn.111.149021.

102. Schumann 2007

Author: Cabalda AB, Tengco LW, Solon JAA, Sarol JN Jr, Rayco-Solon P, Solon FS.

Title: Efficacy of pandesal baked from wheat flour fortified with iron and vitamin a in improving

the iron and anthropometric status of anemic schoolchildren in the Philippines.

Link: https://doi.org/10.1080/07315724.2009.10719791.

103 Amalrajan 2012

Author: Amalrajan V, Anura K, Prashanth T, Sumithra S.

Title: Effect of wheat flour fortified with sodium iron EDTA on urinary zinc excretion in school-aged children.

Link: https://doi.org/10.1177/156482651203300301.

104. Moretti 2006

Author: Moretti D, Zimmermann MB, Muthayya S, Thankachan P, Lee T-C, Kurpad AV, et

al.

Title: Extruded rice fortified with micronized ground ferric pyrophosphate reduces iron

deficiency in Indian schoolchildren: a double-blind randomized controlled trial.

Link: https://doi.org/10.1093/ajcn/84.4.822.

105. Muthayya 2012

Author: Muthayya S, Thankachan P, Hirve S, Amalrajan V, Thomas T, Lubree H, et al.

Title: Iron fortification of whole wheat flour reduces iron deficiency and iron deficiency anemia

and increases body iron stores in indian school-aged children1-4.

Link: https://doi.org/10.3945/jn.111.155135.

106. Rahman 2015

Author: Benito P, House W, Miller D.

Title: Influence of meal frequency on iron absorption as assessed by hemoglobin repletion in rats.

Link: https://doi.org/10.1016/S0271-5317(97)00066-3.

107. Zimmermann 2018

Author: Zimmermann MB, Wegmueller R, Zeder C, Chaouki N, Biebinger R, Hurrell RF, et

al.

Title: Triple fortification of salt with microcapsules of iodine, iron, and vitamin A.

Link: https://doi.org/10.1093/ajcn/80.5.1283.

108. Zimmermann 2002

Author: Zimmermann MB, Zeder C, Chaouki N, Saad A, Torresani T, Hurrell RF.

Title:Dual fortification of salt with iodine and microencapsulated iron: a randomized, double-

blind, controlled trial in Moroccan schoolchildren.

Link: https://doi.org/10.1093/ajcn/77.2.425.

109. Zimmermann 2004

Author: Zimmermann MB, Wegmueller R, Zeder C, Chaouki N, Rohner F, Saïssi M, et al.

Title: Dual fortification of salt with iodine and micronized ferric pyrophosphate: a randomized,

double-blind, controlled trial.

Link: https://doi.org/10.1093/ajcn/80.4.952.

110. Andersson 2008

Author: Andersson M, Thankachan P, Muthayya S, Goud RB, Kurpad AV, Hurrell RF, et al.

Title: Dual fortification of salt with iodine and iron: a randomized, double-blind, controlled

trial of micronized ferric pyrophosphate and encapsulated ferrous fumarate in southern India.

Link: https://doi.org/10.3945/ajcn.2008.26149.

111. Parker 2015

Author: Parker ME, Dipika M, Dominique M, Emily M, Gloriose N, Kathryn R, et al.

Title: A Blinded, Cluster-Randomized, Placebo-Controlled School FeedingTrial in Burundi

Using Rice Fortified with Iron, Zinc, Thiamine, and Folic Acid.

Link: https://doi.org/10.1177/0379572115615234.

112. Kumar 2020

Author: Malavika VK, Juergen E.

Title: Improving micronutrient status of children and women in rural communities in India

using crystal salt enriched with multiple micronutrients.

Link: https://doi.org/10.3177/jnsv.67.111.

113. Nestel 2004

Author: Nestel P, Nalubola R, Sivakaneshan R, Wickramasinghe AR, Atukorala S,

Wickramanayake T.

Title: The use of iron-fortified wheat flour to reduce anemia among the estate population in

Sri Lanka.

Link: https://doi.org/10.1024/0300-9831.74.1.35.

114. Oliveira 2002

Author: Dutra-de-Oliveira JE, de Almeida CAN.

Title: Domestic drinking water–an effective way to prevent anemia among low socioeconomic

families in Brazil.

Link: https://pubmed.ncbi.nlm.nih.gov/12362799/.

115. Viteri 1982

Author: Working Group on Fortification of Salt with Iron.

Title: Use of common salt fortified with iron in the control and prevention of anemia–a collaborative study.

Link: https://doi.org/10.1093/ajcn/35.6.1442.

116. Chen 2005

Author: Chen J, Zhao X, Zhang X, Yin S, Piao J, Huo J, et al.

Title: Studies on the effectiveness of NaFeEDTA-fortified soy sauce in controlling iron deficiency: a population-based intervention trial.

Link: https://doi.org/10.1177/156482650502600201.

117. Ballot 1987

Author: Ballot DE, MacPhail AP, Bothwell TH, Gillooly M, Mayet FG.

Title: Fortification of curry powder with NaFe(111)EDTA in an iron-deficient population: report of a controlled iron-fortification trial.

Link: https://doi.org/10.1093/ajcn/49.1.162.

118. Huo 2012

Author: Huo J, Gary RG, Jian H, Jing S, Lijuan W, Lilian S, et al.

Title: Effectiveness of Fortified Flour for Enhancement of Vitamin and Mineral Intakes and

Nutrition Status in Northwest Chinese Villages.

Link: https://doi.org/10.1177/156482651203300210.

119. Gershoff 1977

Author: Gershoff SN, Brusis OA, Nino HV, Huber AM.

Title: Studies of the elderly in Boston, The effects of iron fortification on moderately anemic

people.

Link: https://doi.org/10.1093/ajcn/30.2.226

120. Elword 1970

Author: Elwood PC, Waters WE, Sweetnam P.

Title: The haematinic effect of iron in flour.

Link: https://doi.org/10.1042/cs0400031.

121.Wenger 2017

Author: Wenger MJ, Murray-Kolb LE, Nevins JEH, Venkatramanan S, Reinhart GA, Wesley

A, et al.

Title: Consumption of a Double-Fortified Salt Affects Perceptual, Attentional, and Mnemonic

Functioning in Women in a Randomized Controlled Trial in India.

Link: https://doi.org/10.3945/jn.117.251587.

122. Biebibinger 2009

Author: Biebinger R, Zimmermann MB, Al-Hooti SN, Al-Hamed N, Al-Salem E, Zafar T,

et al.

Title: Efficacy of wheat-based biscuits fortified with microcapsules containing ferrous sulfate

and potassium iodate or a new hydrogen-reduced elemental iron: a randomised, double-blind,

controlled trial in Kuwaiti women.

Link: https://doi.org/10.1017/s0007114509990353.

123. Hotz 2008

Author: Hotz C, Porcayo M, Onofre G, Garcia-Guerra A, Elliott T, Jankowski S, et al.

Title: Efficacy of iron-fortified Ultra Rice in improving the iron status of women in Mexico.

Link: https://doi.org/10.1177/156482650802900208.

124 Hygiene 1973

Author: H Natvig, O D Vellar.

Title: Iron-fortified Bread- a Long-term Controlled Therapeutic Community-based Experiment

with Ferrous Sulphate-enriched Flour.

Link: https://pubmed.ncbi.nlm.nih.gov/4585765/.

125. Zimmermann 2005

Author: Zimmermann MB, Winichagoon P, Gowachirapant S, Hess SY, Harrington M, Chavasit

V, et al.

Title: Comparison of the efficacy of wheat-based snacks fortified with ferrous sulfate,

electrolytic iron, or hydrogen-reduced elemental iron: randomized, double-blind, controlled

trial in Thai women.

Link: https://doi.org/10.1093/ajcn/82.6.1276.

126. Lqbal 2022

Author: Iqbal S, Ahmed W, Zafar S, Farooq U, Abid J, Shah HBU, et al.

Title: Effect of inulin, galacto oligosaccharides and iron fortification on iron deficiency anemia

among women of reproductive age; a randomized controlled trial.

Link: https://doi.org/10.3389/fnut.2022.1028956.

127.Msc 2013

Author: Toxqui L, Perez-Granados AM, Blanco-Rojo R, Wright I, Gonzalez-Vizcayno C, Pilar

Vaquero M.

Title: Effects of an Iron or Iron and Vitamin D-Fortified Flavored Skim Milk on Iron Metabolism:

A Randomized Controlled Double-Blind Trial in Iron- Deficient Women.

Link: https://doi.org/10.1080/07315724.2013.826116.

128. Fallahi 2003

Author: Fallahi E, Kimiagar M, Valaei N, Abbasi S.

Title: Effect of fortified flour with ferrous sulfate alone and with Na(2)EDTA on iron deficiency

anemia and serum zinc.

Link: https://www.researchgate.net/publication/299161133_Effect_of_fortified_flour_with_ferrous_sulfate_alone_and_with_Na2EDTA_on_iron_deficiency_anemi_and_serum_zinc.

129. Rafalski 1982

Author: Rafalski H, Ponomarenko W, Szyler I.

Title: Effect of iron-fortified bread roll on hematological indices and clinical symptoms in

menstruating women.

Link: https://pubmed.ncbi.nlm.nih.gov/7118577/

130.Rojo 2011

Author: Blanco-Rojo R, Pérez-Granados AM, Toxqui L, González-Vizcayno C, Delgado MA,

Vaquero MP.

Title: Efficacy of a microencapsulated iron pyrophosphate-fortified fruit juice: a randomised,

double-blind, placebo-controlled study in Spanish iron-deficient women.

Link: https://doi.org/10.1017/s0007114510005490.

131. Rojo 2012

Author: Blanco-Rojo R, Pérez-Granados AM, Toxqui L, Zazo P, de la Piedra C, Vaquero MP.

Title: Relationship between vitamin D deficiency, bone remodelling and iron status in iron-

deficient young women consuming an iron-fortified food.

Link: https://doi.org/10.1007/s00394-012-0375-8.

132. Andersson 2010

Author: Andersson M, Theis W, Zimmermann MB, Foman JT, Jäkel M, Duchateau GSMJE,

et al.

Title: Random serial sampling to evaluate efficacy of iron fortification: a randomized controlled

trial of margarine fortification with ferric pyrophosphate or sodium iron edetate.

Link: https://doi.org/10.3945/ajcn.2010.29523.

133. Hansen 2005

Author: Hansen M, Bæch SB, Thomsen AD, Tetens I, Sandström B.

Title: Long-term intake of iron fortified wholemeal rye bread appears to benefit iron status of

young women.

Link: https://doi.org/10.1016/j.jcs.2005.04.001.

134. Beck 2010

Author: Beck K, Conlon CA, Kruger R, Coad J, Stonehouse W.

Title: Gold kiwifruit consumed with an iron-fortified breakfast cereal meal improves iron status

in women with low iron stores: a 16-week randomised controlled trial.

Link: https://doi.org/10.1017/s0007114510003144.

135. Swain 2006

Author: Swain JH, Johnson LK, Hunt JR.

Title: Electrolytic iron or ferrous sulfate increase body iron in women with moderate to low

iron stores.

Link: https://doi.org/10.1093/jn/137.3.620.

136. Lee 2016

Author: Lee C-T, Jeng C-J, Yeh L-S, Yen M-S, Chen S-M, Lee C-L, et al.

Title: A double-blind, randomized, and active-controlled phase III study of Herbiron drink in

the treatment of iron-deficiency anemia in premenopausal females in Taiwan.

Link: https://doi.org/10.3402/fnr.v60.31047.

137. Thuy 2003

Author: Van Thuy P, Berger J, Davidsson L, Khan NC, Lam NT, Cook JD, et al.

Title: Regular consumption of NaFeEDTA-fortified fish sauce improves iron status and reduces

the prevalence of anemia in anemic Vietnamese women.

Link: https://doi.org/10.1093/ajcn/78.2.284.

138. Thuy 2005

Author: Van Thuy P, Berger J, Nakanishi Y, Khan NC, Lynch S, Dixon P.

Title: The use of NaFeEDTA-fortified fish sauce is an effective tool for controlling iron deficiency

in women of childbearing age in rural Vietnam.

Link: https://doi.org/10.1093/jn/135.11.2596.

139. Losso 2017

Author: Losso JN, Karki N, Muyonga J, Wu Y, Fusilier K, Jacob G, et al.

Title: Iron retention in iron-fortified rice and use of iron-fortified rice to treat women with iron

deficiency: A pilot study.

Link: https://doi.org/10.1016/j.bbacli.2017.09.001.

140. Kunnath 2021

Author: Kunnath AK, Mathew S, Mothadaka MP, Rao RCN.

Title: Iron-Enriched Fish Powder Improved Haemoglobin Levels in Adolescent Girls of West

Jaintia Hills District of Meghalaya, India.

Link: https://doi.org/10.1007/s12011-021-02820-0.

141. Haas 2014

Author: Haas JD, Rahn M, Venkatramanan S, Marquis GS, Wenger MJ, Murray-Kolb LE,

et al.

Title: Double-fortified salt is efficacious in improving indicators of iron deficiency in female

Indian tea pickers.

Link: https://doi.org/10.3945/jn.113.183228.

142. Karl 2010

Author: Karl JP, Lieberman HR, Cable SJ, Williams KW, Young AJ, McClung JP.

Title: Randomized, double-blind, placebo-controlled trial of an iron-fortified food product

in female soldiers during military training: relations between iron status, serum hepcidin, and

inflammation.

Link: https://doi.org/10.3945/ajcn.2010.29185

143. Graham 2007

Author: Graham JM, Haskell MJ, Pandey P, Shrestha RK, Brown KH, Allen LH.

Title: Supplementation with iron and riboflavin enhances dark adaptation response to vitamin

A-fortified rice in iron-deficient, pregnant, nightblind Nepali women.

Link: https://doi.org/10.3945/ajcn.2010.29185

144. Agarwal 2008

Author: Agarwal T, Kochar GK, Goel S.

Title: Impact of Iron Supplementation on Anemia During Pregnancy.

Link: https://citeseerx.ist.psu.edu/document?repid=rep1&type=pdf&doi=a149e6cadd0c9edd1

4fc8a2dfb63d83b0c7eab1b.

145. Hoa 2005

Author: Hoa PT, Christine van B, Ha Dui K, Nguyen Cong K, Rainer G, Wolney LC.

Title: Milk Fortif Ed with Iron Or Iron Supplementation to Improve Nutritional Status of

Pregnant Women: An Intervention Trial from Rural Vietnam.

Link: https://doi.org/10.1177/156482650502600104.

Appendix B

Material list and supply list

Material list

1. Human salivary α-amylase, Sigma Aldrich Co (A3176).
2. Lipase from porcine pancreas, Sigma Aldrich Co (L3126).
3. Porcine pepsin, Sigma Aldrich Co (P7000).
4. Porcine pancreatin, Sigma Aldrich Co (P7545).
5. Bovine bile, Sigma Aldrich Co (B3883).
6. 3,3',5-Triiodo-L-thyronine sodium salt, Sigma Aldrich Co (T6397).
7. Epidermal growth factor, Sigma Aldrich Co (E4127).
8. Hydrocortisone-Water Soluble, Sigma Aldrich Co (H0396).
9. Insulin, Sigma Aldrich Co (P3768).
10. PIPES disodium salt, Sigma Aldrich Co (P3768),

Piperazine-1,4-bis (2-ethanesulfonic acid) disodium salt. Liquid PIPES may be better.

11. Sodium selenite, Sigma Aldrich Co (S5261).
12. Citric acid, Sigma Aldrich Co (C0759).
13. Trisodium citrate, Sigma Aldrich Co (S1804).
14. NaFeEDTA, Sigma Aldrich Co (EDFS).
15. $FeSO_4 \cdot 7H_2O$, Sigma Aldrich Co (F8633).
16. Ferrous fumarate, Fisher Scientific (18-602-691).
17. 6 M HCl, Fisher Scientific (H16805).
18. NaOH, Fisher Scientific (A1839536).
19. Isopropyl Alcohol (C3H8O), Fisher Scientific (LC157502).

20. Cell culture flask T25, Fisher Scientific(10-126-28), Corning™ 430639.
21. Cell culture flask T75, Fisher Scientific(07-202-000), Corning™ 430641U.
22. Cell culture flask T225, Fisher Scientific(14-826-80),Falcon 353138.
23. Insert,Fisher Scientific (07-000-388), Greiner Bio-one 0.4 μm 657640.
24. 6-well plate, Fisher Scientific (720080), Costar 3506.
25. Cryovial, Fisher Scientific (22-010-1119), Globe scientific 3002.
26. HEPES, Fisher Scientific (15-630-080),

N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid, 1M, Gibco™ 15630080.

27. Potassium chloride, Fisher Scientific (AAA116620B).
28. Potassium dihydrogen phosphate, Fisher Scientific (AA1159430).
29. Sodium hydrogen carbonate, Fisher Scientific (AC447102500).
30. Sodium chloride, Fisher Scientific(S271-500).
31. Magnesium chloride hexahydrate, Fisher Scientific (AA1228836).
32. Ammonium carbonate, Fisher Scientific (AC401130250).
33. Calcium chloride dihydrate,Fisher Scientific (AC423520250).
34. Ethanol, Fisher Scientific(BP28184), Absolute (200 Proof).
35. Caco-2 cells, American Type Culture Collection (ATCC) (HTB-37).
36. Dulbecco's Phosphate Buffered Saline, ATCC (30-2200) No calcium or magnesium.
37. Dimethylsulfoxide (DMSO), ATCC (4-X).
38. Trypsin-EDTA Solution, ATCC, 30-2101,0.25% Trypsin/0.53 mM EDTA.
39. Trypsin-EDTA Solution, Gemini Bio (400151), 0.25% Trypsin/0.53 mM EDTA.
40. Minimum Essential Medium, Gibco (10370021), NEAA, no glutamine.
41. Dulbecco's Modified Eagle's Medium, Gibco (11965084),

High Glucose, L-glutamine, no sodium pyruvate.

42. L-Glutamine, Gibco (25030149),200 mM.
43. Collagen, Corning (354236).
44. Bottle top filter, Corning (430626),150mL.
45. Dialysis membrane, Spectrum Laboratories (Spectra/Por 7 Pretreated RC),

Catalog: 888-11026-EA. 45 mm Flat Width. Mfg. Part # 132123.

Dialysis Tubing 15,000 MWCO.

46. Ferritin ELISA Assay Kit, Eagle Biosciences (FRR31-K01).
47. Fetal bovine serum, R&D Systems (S12450).
48. Protein assay kit, Bio-Rad Laboratories Inc (5000116).

49. Protein stand II, Bio-Rad Laboratories Inc (5000007).

50. ZellShield, Minerva Biolabs (13-0050), Antibiotic/antimycotic.

51. Silicone O-Ring, Uxcell (a18111400ux0199).

This item is 100% silica gel and doesn't contain butyl rubber.

52. Deionized water, Kansas State University, Chemistry Storeroom.

53. Corn flour, International Grains and cereal Limited Liability Company, Texas, USA(P.A.N.).

Precooked white corn flour.

54. Rice flour, RIVLAND Partnership, Houston, Texas.

55. FePP, Wright Enrichment Inc., Lafayette, LA.

56. μFePP, Wright Enrichment Inc., Lafayette, LA.

57.$FePO_4$, Wright Enrichment Inc., Lafayette, LA.

Supply list

1. Pipetter 20, 50, 100, 200 μL, 0.5 1 mL, Fisher brand.

2. Pipet tips 20, 50, 100, 200 μL, 0.5 1 mL, Fisher brand.

3. Microtiter plate, Fisher brand.

4. Disposable reagent reservoir, Fisher brand.

5. Tube 2, 5, 15, 50 mL, Fisher brand.

6. Disposable serological pipet 1, 5, 10, 25mL, Fisher brand.

7. Transfer pipet (disposable polyothylene), Fisher brand.

8. Cell scraper, Fisher brand.

9. Tweezer, Fisher brand.

10. Vortex mixer, Fisher Scientific.

11. Heat Core Magnetic Stirring Hotplate, Fisher Scientific.

12. Hemocytometer, Fisher Scientific.

13. pH meter, Fisher Scientific.

14. Analytical Scale, Fisher Scientific.

15. Vortex mixer, Fisher Scientific.

16. Convection hot-air oven, Fisher Scientific.

17. Vacuum pump, Fisher Scientific.

18. -80 °C freezer, Fisher Scientific.

19. Tube rack, Thermo Scientific™ (Resmer Manufacturing Technology)

20. 100 place storage-box, Thermo Scientific™.
21. Mr. Forsty cryo 1 ℃ freezing container, Thermo Scientific™.
22. Liquid nitrogen dewar, Thermo Scientific™.
23. Autoclave, Tuttnauer Brinkmann 3850M.
24. Water bath and sonicator, Branson 2510.
25. Low speed centrifuge, Cgoldenwall, 80-2 table top low speed centrifuge.
26. High speed centrifuge, Eppendorf 5810R.
27. Electric Pipette Controller, EP-PRO B0BX9P7SXT.
28. Multichannel pipetter, Rainin.
29. Bleach, Clorox.
30. Trypan blue, Cytiva HyClone.
31. Microplate reader, BioTek Synergy HT.
32. Rocking Platform, Vavntor VWR.
33. Laboratory Incubator Shaker, 211DS, Labnet international, Inc.
34. Dry sterilizer, Germinator 500.
35. Microscope, Nikon.
36. Biological Safety Cabinets, NUAIR , Class II Type A2.
37. Co_2 incubator, Forma Scientific, Inc. Listed laboratory equipment 446U.
38. Co_2 tank, Matheson.
39. Dry sterilizer, Germinator 500.
40. Lab-scale twin-screw extruder, Micro-18, American Leistritz Extruder Corp., Somerville.
41. Pilot-scale twin-screw extruder,TX-52, Wenger Manufacturing, Sabetha, KS.
42. Coffer mill, Moongiantgo Grain Grinder.
43. Hammer mill, Fitzpatrick DKAS012.
44. Mixer, Hobart A200.
45. Gas-fired dryer, 4800, Wenger Manufacturing, Sabetha, KS.
46. Garden scissor, Amazon.
47. Stainless steel filter, Amazon.