

2017

Iron absorption from a novel iron-enriched fungal fortificant in young women

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Iron absorption from a novel iron-enriched fungal fortificant in young women

by

Amanda Elizabeth Bries

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE

Major: Nutritional Sciences

Program of Study Committee:
Matthew J. Rowling, Major Professor
Manju Reddy
Rachel J. Derscheid

The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this thesis. The Graduate College will ensure this thesis is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University

Ames, Iowa

2017

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NOMENCLATURE

AA: ascorbic acid

AOAC: Association of Official Analytical Chemists

Ao: *Aspergillus oryzae*

ASP: Aspiron™

AUC: area under the curve

CRP: C-reactive protein

DCYTB: duodenal cytochrome b

DMT1: divalent metal transporter 1

Fe: iron

FePP: ferric pyrophosphate

Ferric: Fe³⁺

Ferrous: Fe²⁺

Fe-S: iron-sulfur

FeSO₄: ferrous sulfate

FPN1: ferroportin 1

Hb: hemoglobin

HCP1: heme carrier protein 1

HO-1: heme oxygenase 1

IBA: iron bioavailability

ICP: inductively coupled plasma spectrometry

ID: iron deficiency

IDA: iron deficiency anemia

IRE: iron response element

IRP: iron response element-binding protein

MCV: mean corpuscular volume

MDFP: micronized dispersable ferric pyrophosphate

MFP: meat, fish, poultry

MNP: micronutrient powders

NTBI: non-transferrin bound iron

PCFT: proton-coupled folate transporter

PP: polyphenol

RBV: relative biological value

ROS: reactive oxygen species

SF: serum ferritin

SF: serum ferritin

TIBC: total iron binding capacity

Tf: transferrin

Tfr: transferrin receptor

WHO: World Health Organization

ACKNOWLEDGMENTS

I would like to express my sincere gratitude for those who contributed to the success and completion of this project for my Master's Degree in Nutritional Sciences. First and foremost, I would like to greatly acknowledge my major professor, Dr. Manju Reddy who has seen me through my advancements in research both in my Undergraduate and Graduate career. Thank you for your unwavering support and mentorship over the course of one and a half years; I am grateful for the knowledge you have shared and the boundaries you have helped me surpass.

Sincere thanks to Dr. Matthew Rowling for your guidance and mentorship throughout my degree. I admire your confidence and strong listening ear. Thank you for being the lens that helps me see with clarity and my confidant during enduring times. To Dr. Derscheid, thank you for providing insight to my research and helping me see my project from a different angle.

To the current and previous members of Dr. Reddy's lab: Isaac Agbamalfe, Casey Johnson, Nicole Hanson, Samantha Matt, Qi Xu and Dan Chen, thank you for your selfless guidance and assistance in my projects and through the tribulations of graduate school. Thank you to Jeanne Stewart for your prompt responses and assistance on the human study. Truly without your help, none of this work would have exited with such precision. An extended thank you goes to Cura Global Health, Inc. Thank you for your generosity in supporting my scientific endeavors. Thank you to Yilin Bian, Bruce Wicking, Zoraida Defrietus and Anbien for opening up your lab and Cura family to me. Without you all, this growth would not have been possible. Lastly, I would like to give my thanks to my loved ones, graduate cohort, family, friends and departmental professors. Thank you for your patience, sacrifice and understanding throughout this time. Your support is what sought me through every day of this journey.

ABSTRACT

Iron deficiency is the most prevalent micronutrient deficiency, attributing to approximately 800 million individuals with anemia, worldwide. This nutrient deficiency is greatly attributed to consumption of high plant-based diets and foods that have low bioavailable iron. Challenges arise in iron fortification, as the most affordable and conventional iron compounds have low bioavailability, therefore efforts have been made towards finding an iron fortificant with high bioavailability. The objective of this research was to determine the iron absorption of an iron-enriched fungus, *Aspergillus oryzae* (ASP-p) and determine its effectiveness compared to current fortificants ferric pyrophosphate (FePP) and ferrous sulfate (FeSO₄). Two, single-blinded cross-over studies were conducted using a dual-labelled isotope method in young female subjects. Results indicated a 2.3 fold higher absorption in ASP-p compare to FePP ($P = 0.0001$), whereas ASP-p reported significantly lower absorption ($P = 0.0001$) than FeSO₄.

CHAPTER 1. GENERAL INTRODUCTION

Background

Iron deficiency anemia (IDA) is the most prevalent micronutrient deficiency, affecting approximately 30% of the world's population – primarily children and women of childbearing age (1). Despite astronomical advancements in agricultural practices, food technology and global distribution of resources, iron deficiency (ID) remains the greatest source of anemia (2). As a result from depleted iron stores, consequences of IDA include: cognitive impairment, decreased productivity, morbidity and mortality (3–5). Iron deficiency is often a result of low intake of total dietary iron or consumption of foods primarily containing low iron bioavailability (IBA) – characterized by the amount of iron absorbed and accessible to the body to support physiological functions (6). Iron absorption includes both heme and non-heme iron, whereby several dietary components such as ascorbic acid, meat, phytic acid, and polyphenols which influence the overall non-heme iron absorption in a given food (7,8).

Several strategies exist in addressing the global burden of IDA, such as supplementation, dietary modification and fortification programs. Of these, fortification is suggested the most cost effective strategy, however challenges remain in finding an acceptable iron fortificant (9,10). Iron compounds which employ the highest bioavailable iron, result in negative organoleptic properties when added to food. Whereas, foods fortified with iron compounds which don't exert adverse sensory changes, are those with the lowest bioavailability (11,12).

Establishing a widely consumed food is exceedingly important as IDA can only be addressed with consistent adequate iron intake. One of the most widely consumed condiments in Africa is chicken bouillon cubes (13). Ferric pyrophosphate (FePP) is the iron compound used in

fortification, however daily consumption only meets ~1% of daily requirements (14). Therefore this research was carried out to assess the iron absorption of a new fortificant – iron-enriched *Aspergillus oryzae* (ASP-p) – when fortified in chicken bouillon.

Objectives

1. To determine if iron-enriched *Aspergillus oryzae* (ASP-p) achieved higher iron absorption than currently used ferric pyrophosphate in chicken bouillon
2. To assess the relative biological value (RBV) of the ASP-p compared to the standard, ferrous sulfate in chicken bouillon.

Thesis Organization

This thesis contains four chapters including a general introduction, literature review, one manuscript and an overall summary. The manuscript is titled, “Iron Absorption From a Novel Iron-Enriched Fortificant in Young Female Subjects,” and was written in preparation for submission to the *Journal of Nutrition*. The research described in this manuscript examined the iron absorption from three iron fortificants when fortified in chicken bouillon. References throughout the thesis are formatted based on the *Journal of Nutrition*. References placed at the end of each chapter represent only that chapter’s content. This thesis concludes with a discussion and recommendations for future research stemming from our findings.

Author’s Roles

In the course of my master’s degree, I worked primarily on the clinical human iron absorption study. I did all of the initial paperwork alongside my major professor, Manju Reddy in filing Institutional Review Board applications, modifications and updating all certifications of myself and those who would be helping with the research project. I also functioned in

maintaining appropriate documentation and security of our study data and ClinicalTrial.gov updates.

The majority of my work is presented in Chapter 3 of this thesis, which includes the design and execution that I led as a graduate student under Dr. Reddy's oversight. I recruited, interviewed and performed all ELISA analyses for the context of this study. In regards to the manuscript, I wrote the draft and Dr. Manju Reddy edited the manuscript as lead investigator on the project.

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CHAPTER 2. LITERATURE REVIEW

Role of Iron in Human Health

Iron is an essential nutrient that is vitally important for countless biological processes. Its primary role is in the structure and function of hemoglobin and myoglobin, which are iron-containing oxygen transporters (1). Hemoglobin is present in red blood cells, facilitating oxygen transport throughout the body, whereas myoglobin is located in the muscle tissue to support oxygen diffusion. Iron is also necessary for hematopoiesis, synthesis of new red blood cells, occurring every 120 days (2,3)

Iron has a prominent role in the function of redox enzymes, which are non-heme iron-sulfur (Fe-S) proteins characterized by a ubiquitous Fe-S or cofactor (4). Proteins containing these Fe-S clusters include metalloproteins, a class of enzymes whose primary function is to conduct oxidation-reduction reactions, participate in electron transfer in the mitochondria, regulate gene expression and bind active sites to enhance enzyme activity (4). Iron-sulfur cluster biogenesis results in production of numerous enzymes, including cytochrome c reductase, hydrogenases, nitrogenases, succinate-coenzyme Q reductase, and others (5,6). One Fe-S dependent example is the enzyme aconitase; its function is the catalytic conversion of citrate to its isomer, isocitrate, which is an essential intermediate for the tricarboxylic acid cycle and the formation of energy products, NADH^+ and FADH_2 (7). Notably, various diseases are related to disruption of Fe-S clusters, such as Friedreich's ataxia and sideroblastic anemia (7,8) which result in the accumulation of reactive oxygen species (ROS) that target iron chaperone proteins (9).

Iron Metabolism

The functions of iron are dependent upon the total body iron stores since the utilization of iron by various tissues is a tightly organized and regulated process. The average adult contains a total of 3-5g of iron in the body (1).

Hemoglobin requires approximately 75% total body iron for its synthesis, whereas, 10-20% or 1 g represents the iron in the storage protein, ferritin, found in enterocytes, liver and heart (Figure 2.1) (1). The remaining 5-15% is used for the aforementioned processes such as gene expression, oxidation-reduction, etc. Unlike most nutrients, there is no inherent excretory mechanism for iron, other than the iron that is lost from epithelial turnover (10,11).

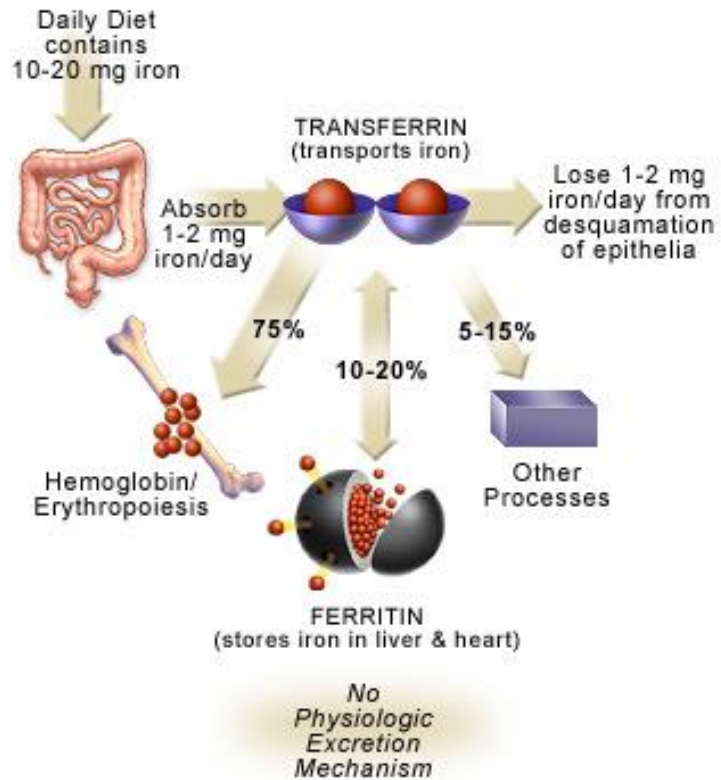


Figure 2.1. Iron utilization among body tissues. Storage form of iron is in ferritin and most iron is used to support erythropoiesis. No primary physiological excretion method apart from epithelial turnover. Abbaspour et al (1)

The 1-2 mg/d of absorbed iron is typically sufficient for the average adult, however it is estimated that approximately 50% of basal iron losses (0.5-1.5 mg Fe/d) is from menstruation in women (12,13). Additionally, an estimated permanent iron loss of during pregnancy is ~840 mg Fe, therefore the iron requirement during pregnancy is ~1,000 mg to support both mother and fetus (14,15). Likewise, there is an increased absolute iron requirement of 0.7-0.9 mg Fe/day (17) to support vital growth and development of a child in his or hers first 1000 days of life as shown

in **Table 2.1**. Iron requirements not only depend on the metabolism of the individual, but the absorption of dietary iron.

Iron Absorption

Iron absorption is dependent upon the type of iron consumed from the diet. There are three physiological states of iron, listed from the highest to lowest level of absorption: heme iron, ferrous iron (Fe^{2+}), and ferric iron (Fe^{3+}). All three are predominantly absorbed at the site of the duodenum (trace amounts in the jejunum) of the small intestine (17). The level of absorption or overall iron bioavailability (IBA) is highly variable. From a dietary standpoint, an estimated 5 - 35% of the iron we consume can be absorbed by the enterocytes (17). This range of absorption is in part due to the influence other nutrients (when concurrently consumed with iron) and the status of total body iron stores (18). Iron absorption is an intricately dynamic and controlled process as we conserve the iron we absorb through recycling mechanisms for iron reutilization by other tissues (19).

Heme iron absorption

Heme is a porphyrin compound consisting of a central Fe ion (**Figure 2.2**). Heme is found in animal products such as meat, fish and poultry (20) where Western diets contribute approximately 10-15% of the total dietary iron sources (21). Heme iron absorption contains higher bioavailability compared to non-heme, since heme iron is influenced less by additional dietary components (22). Heme is taken up by an independent mechanism. The formerly proposed transporter for heme was named

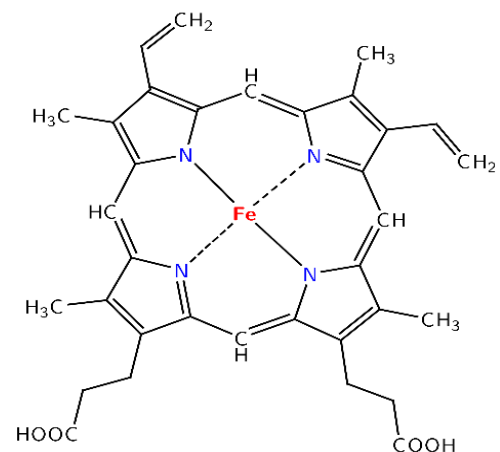


Figure 2.2 The structure of heme iron which is found in animal products. Iron is found within the porphyrin ring (Wikipedia image).

Table 2.1. Iron intake requirements for growth under the age of 18 years, median basal iron losses, menstrual losses in women, and total absolute iron requirements Joint FAO/WHO (16).

Group	Age (years)	Mean body weight (kg)	Required iron intakes for growth (mg/day)	Median basal iron losses (mg/day)	Menstrual losses		Total absolute requirements ^a	
					Median (mg/day)	95th percentile (mg/day)	Median (mg/day)	95th percentile (mg/day)
Infants and children	0.5–1	9	0.55	0.17			0.72	0.93
	1–3	13	0.27	0.19			0.46	0.58
	4–6	19	0.23	0.27			0.50	0.63
	7–10	28	0.32	0.39			0.71	0.89
Males	11–14	45	0.55	0.62			1.17	1.46
	15–17	64	0.60	0.90			1.50	1.88
	18+	75		1.05			1.05	1.37
Females	11–14 ^b	46	0.55	0.65			1.20	1.40
	11–14	46	0.55	0.65	0.48 ^c	1.90 ^c	1.68	3.27
	15–17	56	0.35	0.79	0.48 ^c	1.90 ^c	1.62	3.10
	18+	62		0.87	0.48 ^c	1.90 ^c	1.46	2.94
Postmenopausal		62		0.87			0.87	1.13
Lactating		62		1.15			1.15	1.50

^a Total absolute requirements = Requirement for growth + basal losses + menstrual losses.

^b Pre-menarche.

^c Effect of the normal variation in haemoglobin concentration not included in this figure.

Source: adapted, in part, from reference (8) and in part on new calculations of the distribution of iron requirements in menstruating women.

heme carrier protein 1 (HCP1), while more recently, it has been postulated that the heme is taken up by a proton-coupled folate transporter (PCFT); however both mechanisms are unclear (23–25). After the heme is absorbed into the mucosal cell, it is broken down by heme-oxygenase 1 (HO-1) enzyme thereby liberating iron from the porphyrin ring, where it enters the nonheme iron pool in the enterocytes (26).

Non-heme iron absorption

Dietary non-heme iron must be in the Fe^{2+} form prior to transportation from the lumen into the enterocyte (**Figure 2.3**) (27). This process reducing ferric iron to ferrous iron is achieved by the action of a reducing-like agent, ascorbic acid or more commonly by the brush border ferric reductase, duodenal cytochrome b (DCYTB) (27,28). The concentration of Fe^{2+} transported into the enterocyte is modulated by an enterocyte blocking effect, whereby the intracellular iron stores (ferritin) dictate the amount of iron that will be transported by the apical membrane-bound, proton-pump dependent, divalent metal ion transporter 1 (DMT1) (27,29). In addition to iron, the DMT1 transports other metals: cadmium, copper, zinc and manganese.

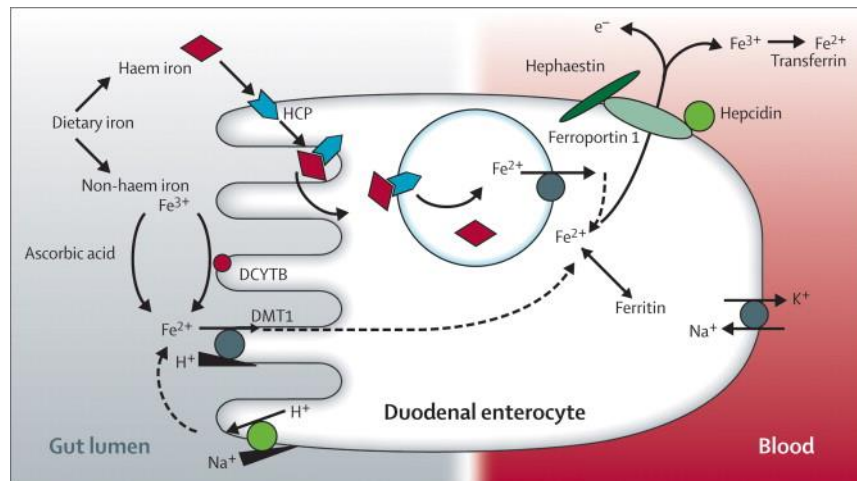


Figure 2.3. Duodenal enterocytic uptake of dietary iron sources: nonheme and heme. DMT1 facilitates the uptake of reduced Fe^{3+} to Fe^{2+} by DCYTB from Hurrell et al (28).

Arredondo et al (30) demonstrated antagonistic effects Fe, Cu, and Zn have on each other, suggesting there is competitive uptake among these minerals. Results indicated that disproportionate levels of the respective minerals was driven by saturation, whereby when more Cu was presented to the cell, there was a dampening effect on Fe and Zn uptake (29,30).

Iron Regulation and Homeostasis

Storage and Release

Following intestinal uptake, liberated heme-bound-Fe and non-heme Fe transported through the apical membrane enter the labile iron pool (31). This common pool serves as a crossroads between the cytosolic signaling of iron regulatory proteins and the shunting of iron into storage within a ferritin molecule, which can hold ~4500 Fe³⁺ iron atoms (32,33). Ferritin is an acute phase protein which is directly influenced by hepcidin and closely associated with c-reactive protein (CRP), two inflammatory markers affecting iron homeostasis (34,35). Under normal conditions the iron which is not used intracellularly or stored will enter into circulation. This efflux of the iron from the enterocyte into circulation is mediated by the basolateral transporter, ferroportin 1 (FPN1) along with the ferroxidase hephaestin, to oxidize Fe²⁺ to Fe³⁺ (36). After iron is released from the intestine as Fe³⁺, it is sequestered by transferrin (Tf), a circulatory glycoprotein chaperone to be carried in the blood. Various tissues will then take up iron through a receptor-mediated endocytosis pathway by the transferrin receptor (Tfr) (37). The Tfr has a high affinity for Tf, allowing for the clathrin-mediated endocytosis of holo-Tf-Tfr into the endosomes (37). An endosome's acidic pH results in the dissociation of the complex, iron is released via DMT1 for functional use. Tfr then relocates to the membrane after its dissociation with Tf (38).

Regulation of iron mobilization

There are distinct differences in iron absorption when comparing insufficient versus sufficient conditions as the body has the capacity to sense and regulate these factors. During an iron-deplete condition, iron absorption into the mucosal cells is upregulated (39). Conversely, when plasma iron concentrations are low, levels can be restored through the release of iron from ferritin storage protein in reticuloendothelial macrophages, enterocytes and hepatocytes (10). This release of iron into circulation is through FPN1, after which it is oxidized by the hephaestin homologue ceruloplasmin (40). This process of Fe^{2+} efflux is crucial in maintaining iron homeostasis in response to low serum Fe. Transferrin receptor expression is regulated at the transcriptional level by an iron-responsive element-binding protein (IRP) (41). Under circumstances of low circulating serum Fe, in the 3' untranslated region of mRNA, IRP binds to a stem loop structure, known as the iron response element (IRE) (42). When IRP is bound to the IRE, the mRNA is stabilized, allowing for increased translation to upregulate Tfr synthesis (43). In contrast, the IRP when bound to the IRE on the 5' end of the mRNA inhibits the translation of ferritin, thereby reducing iron storage. In iron excess condition, IRP is released from IRE and Tfr mRNA is degraded and the IRP is removed, allowing the translation of ferritin (42).

This homeostatic process can be disrupted when a large bolus of Fe^{2+} iron is absorbed, or when the FPN1 function is compromised. When a bolus of highly soluble iron (Fe^{2+}) is absorbed into the enterocyte, this floods the system, overwhelming the Tf, resulting in unbound Fe^{2+} in circulation, otherwise known as non-transferrin bound iron (NTBI) (44). This NTBI can induce free radical generation through the Fenton reaction, generating hydroxyl radicals (45).

Under conditions of systemic inflammation, upregulation of hepcidin, a key regulator in iron homeostasis, acts on the basal lateral transporter, FPN1, resulting in the internalization of

FPN1, causing decreased iron absorption (**Figure 2.4**) (46). Hepcidin regulates iron release into circulation at three locations: 1) duodenal iron absorption 2) macrophages' recycled iron from senescent and damaged red blood cells 3) hepatocytes' storage (47). Recent studies have examined the increased hepcidin levels due to NTBI (48,49). Moretti et al. conducted a dose-dependent study investigating the absorptive outcomes when subjects were administered FeSO_4 doses of 40, 60, 80, 160 and 240 mg daily. In their study, they observed a significant increase in circulating hepcidin and when subjects were given doses ≥ 60 mg FeSO_4 , there was a 35-45% observed decrease in iron absorption (50).

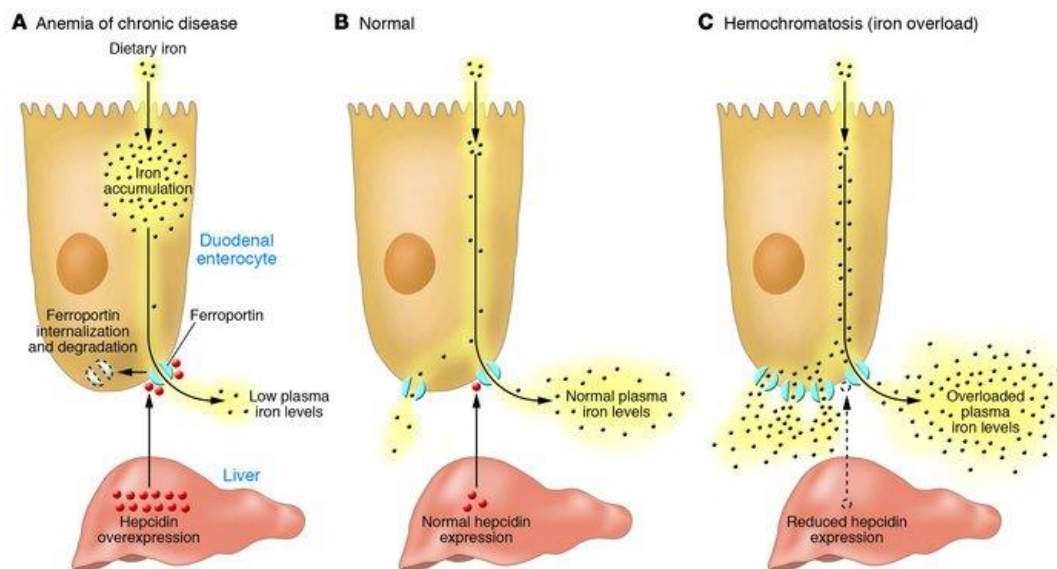


Figure 2.4. (A) Increased hepcidin expression by the liver, leading to internalization of FPN1, results in low plasma Fe levels due to iron accumulation inside enterocyte. (B) Normal hepcidin levels, regulating the iron exported into the plasma. (C) Hemochromatosis, or iron overload from insufficient hepcidin levels. No FPN1 internalization, therefore plasma Fe levels continue to increase. Domenico et al. (46).

Iron Deficiency Anemia

There are many factors that govern the utilization of iron in the body. When there is not enough iron ingested in the diet or excess iron losses, this leads to iron deficient conditions.

Inflammation is also known to cause anemia, apart from iron deficiency, due to the trapping of

iron in storage in the presence of hepcidin (34). During inflammation, the body's inability to mobilize the iron in storage, which may lead to the inadequate incorporation into hemoglobin, otherwise defined as iron deficiency anemia (IDA) (51).

Prevalence

According to the 2011 WHO report, over 1.6 billion individuals globally are afflicted with anemia and of these, it is estimated that 50% is attributed to iron deficiency (ID) (52). Iron deficiency anemia is the only micronutrient disorder that is commonly seen in both industrialized and developing countries. Reports indicate a 38% prevalence of IDA in pregnant women aged 15-49 years. Additionally, IDA is common among children, whereby 18.1% of those <5 years of age are classified anemic (53). These staggering numbers sparked the introduction of the "WHO 2025 Global Targets for maternal, infant and child nutrition". One specific aim was "targeted to support the Global Health Assembly initiative towards a 50% reduction of anemia in women of reproductive age by 2025" (50).

Consequences

Iron deficiency (ID) results in the reduced capacity to generate new red blood cells, and ultimately hemoglobin, causing IDA. Several ramifications stem from ID, especially during fetal and infant growth and development. A lack of iron attributes to slow neurodevelopment, causing behavioral and cognitive impairment (54,55). Other adverse outcomes, such as fatigue, decreased motor maturation, increased risk for preeclampsia and morbidity and mortality manifest from IDA (56,57). These implications further impose economic consequences with an estimated median total loss of \$16.78 per *capita* or 4.05% of gross domestic product due to both cognitive impairment and decreased productivity (58,59).

Stages of iron deficiency

Iron deficiency anemia (IDA) is a gradual and progressive nutritional condition as a result of multiple factors including inadequate iron intake, gastrointestinal related blood loss, inflammation, as well as infections (51,60). This progression of iron depletion to IDA is subdivided into three clinical stages – pre-latent, latent and IDA – with each stage containing various consequential outcomes (61). All three stages are defined by overlapping iron status indicators and diagnostic criteria outlined in **Table 2.2**. The first, or pre-latent stage of iron deficiency, reflects suboptimal/depleted iron stores. Clinical assessments often use serum ferritin (SF) as the primary indicator of iron deficiency (62), however SF is an acute phase protein, whereby low concentrations may be masked by inflammation-induced hepcidin (63).

The second stage is measured by total iron binding capacity (TIBC), whereby long-term inadequate iron intake leads to a state of negative iron balance, referred to as iron deficient erythropoiesis (62). This stage is characterized by increasingly exhausted SF levels ($<15 \mu\text{g/L}$), elevated TIBC ($>400 \mu\text{g/dL}$) and serum transferrin receptor of $>8.5 \text{ mg/L}$. Collectively, TIBC and serum iron is used for calculating percent saturation ($\text{TIBC/serum iron} \times 100$). Transferrin saturation and red blood cell protoporphyrin ($<70 \mu\text{g/dL}$) decrease in stage 2, with normal Hb ($>12 \text{ g/dL}$) (62).

The third stage is defined as IDA, whereby iron stores are diminished to the extent there is insufficient iron available for hematopoiesis and Hb maintenance. The clinical diagnosis for IDA is Hb $<12 \text{ g/dL}$ in females and $<13 \text{ g/dL}$ in males. Mean corpuscular volume (MCV) may be an indicator for IDA, but it is also affected by other nutrients causing microcytic or megaloblastic anemia (62).

These clinical parameters are good for distinguishing one stage from another, but there should be careful considerations when assessing these markers. For instance, more recently, the sTfR/log(ferritin) diagnostic ratio has shown to be more specific when classifying true IDA (64). Generally, clinicians often rely on multiple iron assessments to distinguish true ID from inflammatory or acutely-induced deficiency (63).

Table 2.2. Clinical iron status indicators for the three stages of iron deficiency.

<u>Stages of Iron Deficiency</u>	<u>Clinical Parameters</u>	<u>Diagnostic Range</u>
Storage Iron Depletion (prelatent)	Marrow iron stores	Absent
	Serum ferritin (ug/L)	<20
Iron Deficient Erythropoiesis (latent)	Serum iron (ug/dL)	<10
	TIBC (ug/dL)	>400
	Transferrin saturation (%)	<16
	sTfR (mg/L)	>8.5
	RBC protoporphyrin (ug/dL)	<70
Iron Deficiency Anemia	Hemoglobin (g/dL)	
	Females	<12
	Males	<13
	MCV	<80

*Adapted from WHO, Worwood 1997 (61).

Iron Bioavailability

Iron bioavailability (IBA) is described as the amount of absorbable iron from dietary components that is available for the physiological processes which require iron (65). Several dietary components, such as ascorbic acid (AA) and animal products like meat, fish and poultry (MFP), play a significant role in determining the fate of the iron bioavailability in a given food (66). On the other hand, dietary inhibitors, or antinutritive factors, are those which tightly bind iron or cause the inhibition of its absorption; these most often include phytic acid from bran and

seeds (67), and competitive inhibitors like polyphenols found in coffee, tea and beans (68).

While individual effect of each factor is well studied, understanding their effects in a mixed meal is complex (69).

Dietary enhancers

Ascorbic acid is a reducing agent of non-heme iron, which is a potent enhancer of iron absorption (70). It has the capacity to augment the iron absorption within complex food matrices, overcoming the inhibitory effects of chelating compounds such as phytic acid, polyphenols and calcium (71). In a single meal study, AA did notably increase iron absorption, but its effect is modest in a whole meal (70); this revealed AA shows greater effect on nonheme iron absorption when examined in a single meal rather than whole diet.

Meat, when concomitantly consumed iron containing foods, has demonstrated to increase non-heme iron absorption in plant-based foods, otherwise known as the, “meat factor” (72). The enhancing effect has been suggested to be due to the ability of maintaining iron in its soluble form during digestion (73). Several studies have examined the meat effect on non-heme iron absorption and concluded there are enhancing properties of meat when added to meals with low IBA (74). Engelmann et al (75) added meat to an infant meal and observed a 15% RBV compared to 9.9% with no added meat. Reddy et al found an increase from 4.8 to 6.5% in nonheme iron absorption when comparing individuals who consume no meat versus a high-meat diet (76).

Dietary inhibitors

Phytic acid is predominantly found in nuts, legumes and seeds. Its cyclic acid structure has a high affinity for minerals, especially iron, making iron almost completely unabsorbable.

When phytates were removed from cereal porridge, iron absorption of native iron showed to be

increased significantly (77), demonstrating the strong inhibitory effects PA has in cereal grains. A report by Joy et al (78) determined in African, the mean supply of phytate was 2770 mg per capita each day, suggesting African (where ID is common) diets contain high amounts of these iron inhibitors. Although phytate is known to have an impact on iron absorption, Armah et al observed a decreased inhibition of phytate on nonheme iron absorption when persons regularly consumed high phytate diets (79).

Polyphenols are commonly found in pulses and cereals with highest concentrations generally reported in coffee and tea (80). PP have antinutritive properties since they can bind iron in the gut, dampening intestinal Fe absorption (81). Many different types and amounts (grams) of PP are found in various foods. Black tea PP are known to have more of an inhibitory effect than PP in coffee (82). Herbal teas and cocoa are also known to inhibit absorption but less than black tea; an inverse relationship has been reported with PP content and Fe absorption (83,84).

Several studies have investigated how IBA of native or elemental iron can be dampened (85) or increased (86) depending on the matrix with which it's consumed. Moreover, when iron is administered in a liquid rather than a semi-solid or solid meal, there is a disproportionately higher iron absorption when consumed with liquid (69). Therefore, careful consideration must be taken when measuring the relative biological value (RBV) of a given iron compound.

Methods of Assessment for Iron Absorption/Bioavailability

Due to the nature of iron's absorption and the complex mechanisms by which it can be absorbed, there are several methods used in order to measure the bioavailability of various

dietary iron sources. As mentioned, iron absorption is manipulated by many factors, both positive and negative, so it is important to consider which methods are utilized.

In vitro solubility/dialyzability

In vitro methods are often used to determine the iron solubility of foods, because they are convenient and inexpensive. This methodology uses a controlled environment to mimic the human gastrointestinal digestion, whereby the food is subjected to brush border enzyme digest. The non-heme iron released during *in vitro* digestion is then measured using a colorimetric assay for total soluble iron content (87). A more traditional iron solubility method is the assessment of dialyzable iron (88,89). *In vitro* dialyzability is a two-step digestion whereby contents are fractionized in a dialysis bag with low molecular weight coefficient and measured dialyzable Fe as an index of IBA (90,91). Limitations of the solubility/dialyzability are that they are not a strong indicator of IBA, rather they are useful as a secondary measure to assess concentration of iron chelators prior to mucosal uptake (89).

Cell culture model

To mimic the human physiological conditions, *in vitro* solubility/dialyzability tests are commonly carried out further using a Caco-2 cell model, as the rat model is shown to be less sensitive to dietary factors (92). Caco-2 are human epithelial colorectal adenocarcinoma cells which spontaneously differentiate into epithelial enterocytes forming a brush border and corresponding digestive enzymes (93). These cells are grown under conditions mimicking human body temperature of 37C and 5% CO₂ for suitable growth. Bioavailability is then determined following treatment and % RBV is measured by using ferritin as a surrogate for IBA or via inductively coupled plasma spectrometry (ICP) (94). IBA of Caco-2 cells has been strongly correlated with human absorption (95). It should be noted there are limitations in using the Caco-

2 cell culture model, because the regulation of hepcidin is absent. New models attempt to integrate hepcidin's role by combining Caco-2 with the HepG2 cell model (90).

Animal models

To date, the hemoglobin depletion-repletion model in rats is the most standard method to assess iron absorption and was established by Association of Official Analytical Chemists (AOAC) (96,97). Predominantly rat species have been examined to determine responsiveness to iron repletion following iron depletion. Wistar rats were determined to be the best models when three rat species were compared (98,99). Pigs can be used for the same method but they are expensive (100).

The hemoglobin depletion-repletion assay (101) utilizing the Wistar animal model includes rats fed an iron-deficient (2.5 mg/kg or 5 mg/kg elemental Fe) diet for approximately 25-35 days to deplete hemoglobin levels prior to iron repletion. Iron repletion includes any experimental dietary iron being tested – both supplemental and dietary (96). Following the intervention, hemoglobin concentrations are determined and assessed among treatment and control (FeSO₄) groups to estimate two values: 1) relative biological values (RBV %) against FeSO₄ is 100% 2) Hb regeneration efficiency (HRE%) ratio to determine the IBA based upon the improvement in Hb concentration relative to the amount consumed.

Human studies

Human studies give direct measure of absorption, though they are expensive to conduct. Three common techniques are used to assess iron absorption: area under the curve (AUC), radioisotopes and stable isotopes. Serum iron curves can be used to measure the absorption from iron doses as low as 5-20 mg Fe (79,102). In this method, subjects consume an iron supplement or meal containing the iron treatment of interest. Following ingestion, blood samples are taken

every 30 minutes and plotted on a curve starting with baseline measures. The area under the curve (AUC) is then used to calculate the total iron absorbed in that timeframe. Benefit of the AUC method is it is inexpensive relative to isotope studies, as well as utilization to assess kinetics of Fe release. The limitation of AUC method is it takes extended time to get serum Fe levels to reach back to baseline, which can be hard on the subjects.

Tracer methodology in human research through radioisotopes is a powerful tool to assess how the body acquires the micronutrients following digestion and absorption. Radioisotopes, ^{55}Fe and ^{59}Fe are used both through intrinsic and extrinsic labelling. Due to the unconventional, cumbersome nature of intrinsic labelling in iron absorption studies, Cook et al (103) compared iron absorption with intrinsic and extrinsic methods, showing no significant differences between absorption of the two labelled iron methods, proposing use of extrinsic-labelling in the later years. Limitations are exposing humans to radio isotopes, though it is never more than a single chest x-ray dose.

Stable isotopes are the preferred methodology as they are safer than radioisotopes, however they are exceedingly more expensive. In nature, our human body contains four main iron isotopes with differing abundance (^{54}Fe , ^{56}Fe , ^{57}Fe and ^{58}Fe). Stable iron isotopes for use in human metabolic studies, are often ^{57}Fe and ^{58}Fe since the natural abundance is the lowest among the naturally occurring isotopes, 2.1% and 0.28% iron, respectively. Since these isotopes are already prevalent in our bodies, analysis requires a sensitive instrument to detect the enrichment. Similar to radio isotope studies, two meals with two isotopes are fed to the same subject to reduce inter-subject variability (103,104).

Iron incorporation into red blood cells or iron content of Hb is used to assess stable isotope enrichment in the blood. Following consumption of the total isotope dose, 14 days is

allowed for the incorporation of the isotope into the hemoglobin. Iron is then separated by use of anion exchange chromatography (105). Following, iron ratios (baseline enrichment to 14 d post-dosing) can be measured through mass spectrometry (104). Fractional iron absorptions are calculated based on the assumption that 80% of the iron is incorporated into the hemoglobin after estimating total blood volume by height and weight of subjects (**Figure 2.5**) (107,108,109).

$${}^{57}\text{Fe}_{(\text{inc})} = \frac{R^t{}^{57}:56 - R^0{}^{57}:56}{R^0{}^{57}:56} \times \text{Fe}_{(\text{circ})} \times A_{57},$$

$${}^{58}\text{Fe}_{(\text{inc})} = \frac{R^t{}^{58}:56 - R^0{}^{58}:56}{R^0{}^{58}:56} \times \text{Fe}_{(\text{circ})} \times A_{58},$$

Figure 2.5. Isotope enrichment calculations where $R^t{}^{57}:56$ and $R^t{}^{58}:56$ are the enriched isotope ratios at time t (days after final dose). $\text{Fe}_{(\text{circ})}$ is the total circulating Fe on t . A_{57} and A_{58} are the natural abundances (2.1) and (0.28). These are measured against baseline indicated by R^0 . From Kastenmayer et al. (108).

Although there are several methods to accurately determine iron absorption in humans, long-term efficacy studies are needed to observe changes in iron status as measured by many status indicators accounting for confounding factors. Trials determining the efficacy of an iron intervention are conducted in a large, controlled environment typically over the course of a several months to assess its effectiveness on treating an ailment. Efficacy studies are expensive to conduct, take a lot of coordination among individuals as well as demand a large number of subjects, in contrast to the small number required for isotope studies (109). They are necessary however, to assess the impact on a larger population. Several efficacy studies have helped advance the field of finding a suitable, effective iron treatment for various populations on improving the iron status (110–112).

Interventions to Treat Iron Deficiency Anemia

To address ID and IDA, there are challenges in finding a suitable, sustainable and effective intervention. There are barriers to finding an appropriate treatment since cost, accessibility and distribution all play a vital role in iron outcomes. It is well established that not all forms of iron treatment are suitable for every population. The economics, comorbidities, disease and cultural aspects are driving forces for designing and establishing effective interventions for IDA. Currently, there are four main intervention strategies used to help ameliorate the incidence of IDA including: supplementation, home fortification, biofortification, dietary modifications and food fortification. All come with their own benefits and drawbacks which are assessed and summarized below.

Supplementation

Iron supplementation is the most frequently used strategy because of its targeted approach in providing high doses of iron in a convenient and direct form (113). Frequently associated consequences of IDA have been suggested to be adequately addressed through supplementation due to its cost-effectiveness. Studies have demonstrated improvements in mental performance, fine motor movements, as well as decreased fatigue and anxiety outcomes in iron deficient individuals given daily iron supplements (114,115). Among pregnant women in China with low iron stores taking Fe supplements, Zhao et al (116) found significantly higher maternal hemoglobin levels, reduced iron deficiency anemia, and greater concentrations of umbilical cord serum iron than those women not taking iron supplements.

Several iron supplements, such as ferrous sulfate heptahydrate, ferrous gluconate, and ferrous fumarate have been identified as primary supplemental treatments for iron deficiency anemia (117). Recently, the WHO set recommendations for daily doses of these compounds

ranging from 30-60 mg elemental iron from FeSO₄, ferrous gluconate and fumarate, in pregnant women (118). The WHO switched from daily to intermittent administration of iron supplements particularly in malaria endemic areas (50). This was based upon the understanding that there is a mucosal blocking effect of iron, dampening the ability for the release of iron from duodenum into the plasma (99,119). Differences between daily and intermittent iron administration were analyzed and such findings showed both weekly and daily supplementation were efficacious (69,120). A recent study by (50) suggests 48 hours between iron supplements, rather than 5 to 6 days, provides adequate time for recovery of the enterocytes from down regulating hepcidin and other hormonal factors. Although daily administration improves iron status, tri-weekly intake of iron supplements may improve compliance by reducing associated side effects, systemic inflammation and ultimately provide better long term outcomes in iron status indicators (50,121,122).

Effectiveness of iron supplementation has confounding factors such as limited distribution to developing nations and non-compliance due to adverse effects from high iron doses to the gastrointestinal tract (123). Aside from oral iron causing gastrointestinal distress, bloating and constipation, a high bolus of an iron supplement has been shown to cause NTBI, further exacerbating certain disease conditions (124). For instance, iron is an essential nutrient for the malaria pathogen, *Plasmodium falciparum* (125). It is well established that the non-transferrin bound iron in the plasma is a primary nutrient for the pathogen's integrity, contributing to the parasitic growth of the *Plasmodium*, causing increased exposure of the pathogen in circulation of the brain and intestines (126). Therefore, the WHO stated iron supplements should be provided intermittently alongside other strategies to control malaria to prevent those with malaria from dying of excess iron supplements (127,128).

Home Fortification of Micronutrient Powders

Micronutrient powders (MNPs) are otherwise recognized as at-home or point-of-use fortification programs, first established after the creation of MNP Sprinkles by Schauer and his colleagues in 2003 (129). Each MNP sachet provides a single-serving containing 15 vitamin and minerals and was designed to be “sprinkled” on semi-solid food to serve as an efficacious strategy to address concomitant nutritional deficiencies (130,131). The novelty of these Sprinkles pertains to the microencapsulation of the iron in the form of ferrous (III) fumarate to provide protection from processing, storage and competitive absorption with other minerals such as zinc (132–134).

Since 2003 when MNP programs were implemented, many countries have shown improvements in stunted growth and overall health. In Nepal, refugee children were given daily *Vita-Mix-It* containing the recommended nutrient intake of 16 vitamins and minerals from age 1-3 y (135). Reports from the 18 month intervention indicated no significant reduction in anemia, however parents did indicate a significant reduction in diarrhea, and vomiting as well as increased energy levels in their children (135). Additional long-term MNP programs have attributed their non-significant improvements in iron status to the unacceptability and non-compliance of the Sprinkles due to reported fear of artificial and synthetic ingredients and a preference to nurture their children with culturally familiar foods (136,137).

Micronutrient powder programs have also been explored in pregnant women. Lower doses of iron (2.5mg) combined with ascorbic acid and exogenous phytase consumption reduced iron deficiency from 75% to 18.9% following 6 months (138). Low iron dose in presence of exogenous phytase demonstrated to be efficacious in reducing IDA, however residual concerns regarding the bitter taste of the MNP when added to rice is a foreseeable problem (131). Overall,

MNP programs have been successful, however they are expensive to implement and only reach specific countries who are participating.

Biofortification

Iron biofortification is an agricultural practice in which the nutrients of staple crops are improved by modes of conventional plant breeding technology. Foods most commonly biofortified with iron are cassava, rice and grains, as well as beans and legumes (139). These are often native staple crops in areas with low accessibility to iron-rich foods and are commonly multi-bred for various nutrients such as vitamin A carotenoids, zinc, and iodine (140).

Biofortification of iron can be achieved through several strategies: 1) decreasing antinutritive factors like polyphenols and phytates, 2) increasing the concentration of iron within the respective crop, 3) increasing nutrient iron enhancers, such as ascorbic acid (141). Following a nine month feeding trail of biofortified (1.79 mg/d) and unfortified (0.37 mg/d) rice in Filipino women, there were observed increases in serum ferritin stores, but not hemoglobin in only the nonanemic group, suggesting this isn't a sustainable/effective strategy for addressing IDA (142). Recent reviews highlight the efficacy of iron biofortification, showing positive improvement for the overall nutrition and health outcomes of a given population (143,144). However, the upfront investment and time in cross-breeding crops for high quality nutrients becomes costly to ensure the iron is deposited in the seed of the crop rather than trapped in the inedible portions like roots and leaves.

Dietary Modifications

There exist inherent limitations when attempting to modify the foods people regularly consume as ingredient variety is typically not an option in most developing countries making dietary modifications difficult to implement. A recent review discussed ways to improve the iron

status of children and women of child-bearing age in affluent countries (145). Results indicated most women were meeting their daily requirements for iron, however this was achieved by high consumption of heme-iron containing foods like meat. Since meat is rarely consumed in most developing countries, modifying the cultural diets and behaviors of people is not appropriate where adequate high iron foods are not accessible nor affordable (145).

Fortification

Iron fortification has been described as the most cost-effective, sustainable strategy of the primary approaches (146). Utilizing micronutrient fortification of foods allows diverse subsets of populations to be reached. By employing local, commercialized products as the vehicle for iron fortification, there is a greater dispersion of iron fortificants to all populations irrespective of location, culture, economic class, and social dependencies (58).

Iron fortification of regularly consumed condiments can aid in the amelioration of this widespread deficiency. However, established barriers exist in the formulation of these vehicles (147). Iron demands considerable attention when being added to food, as it can react with the food matrix, resulting in organoleptic problems such as rancidity, alterations in color, shelf-life stability, and consumer acceptability (148,149). The highest bioavailable iron sources are the most soluble and thus cause sensory problems in the food, whereas the iron compounds which do not cause adverse sensory outcomes are typically of the lowest bioavailable iron sources (110).

Staple foods such as wheat flour, rice, milk and infant formulas have been long used for iron fortification (150–152). Many countries have implemented programs focused around fortifying staple foods with iron, however there are drawbacks, such that certain members of the population may not regularly consume these staple foods or have access to them. Unless these staple foods are fortified with multiple nutrients, regular consumption may also impose

deficiencies of other micronutrients. There also exist technological problems in fortifying foods with iron, as there are barriers in the amount of iron a given food may be able to tolerate in relation to its sensory characteristics. Therefore, there is no silver bullet when it comes to finding an iron intervention strategy, thus by combining strategies, there may be added benefits.

The implementation of iron fortification in condiments and commonly consumed food vehicles has shown to be effective as this strategy focuses on fortifying regularly consumed condiments, eliminating concern of adherence and affordability (153). A recent meta-analysis analyzed the comparison of iron status outcomes from iron fortified condiments compared to noodles (154). The review found a significant increase in hemoglobin of adults and children consuming iron-enriched/fortified condiments in comparison to noodles, 0.74g/dL Hb and 0.3g/dL Hb, respectively (154).

Condiments and Bouillon Spices

Despite the advanced research in various iron interventions and multiple governmental programs established, there are still technical challenges when attempting to fortify foods with iron. Since iron is highly reactive and readily undergoes oxidation and reduction reactions, industries have introduced more conventional approaches such as adding iron to condiments and spices to provide a matrix to support the iron and provide soluble iron carriers (155,156). For instance, insoluble iron compounds are best suited for dry spices, whereas soluble iron compounds which notably turn food green, have been integrated into soy sauce to appear more inert (155).

Bouillon cubes are most commonly consumed in the addition to soups, stews, curries and sauces in place of common spices such as salt and pepper. They contain dehydrated stock in the forms of either vegetable, chicken, seafood, or beef as well as salt, vegetable fat, flavor

enhancers (monosodium glutamate) and spices to be sprinkled on top of any dish (mainly rice) and boiled in stews. Due to their wide distribution and frequent consumption, alongside their affordable cost and accessibility, bouillon cubes are an appropriate vehicle for iron fortification, especially in areas of highly prevalent iron deficiency, such as West and Central regions of Africa (157).

Several well-established transnational companies, like Unilever, Nestle' and GB foods have designed multiple-fortified and commercialized chicken bouillon cubes with vitamin A, iodine and iron (158,159). Though not all chicken bouillon cubes are micronutrient fortified, the ones currently distributed to regions of Africa are brand named including Maggi®, Knorr®/ Royco® and Jumbo® (156,160). In 2015, Nestle reported an estimated 44 billion iron fortified chicken bouillon cubes were dispersed. In places such as Nigeria, chicken bouillon cube sales reaches a staggering 80 million cubes sold each day.

In addition to the convenience and acceptability of fortifying chicken bouillon cubes, their vast, frequent consumption is what makes them a promising IDA intervention strategy. A summarized study by Hess and colleagues, examined survey outcomes from 12 sub-Saharan African countries' using the Fortification Rapid Assessment Tool to estimate the consumption of fortified foods (157). The survey focused on the consumption patterns of four commonly consumed foods (wheat flour, sugar, vegetable oil and chicken bouillon cubes) in hopes to determine the best national food fortification programs to expand. Findings from the study reported a range from 79-99% of women in those surveyed countries consumed bouillon cubes within that week. Women in Cameroon had the highest chicken bouillon cube consumption with reporting mean consumption of 13.8 times per week (161). On average among the four surveyed nation, consumption ranged from approximately 1.5-4 g/day (161).

Iron Fortificants

Many iron fortificants are available, all with varying levels of iron bioavailability, cost and solubility. Finding a single iron compound that is cost-effective and easy to fortify foods with has been the challenge in this realm of research. Food vehicles themselves have functional groups which interact differently depending on the iron compound with which they are fortified, therefore finding the appropriate iron compound to fortify a vehicle of interest is essential.

Currently, there are a wide array of iron compounds being used for fortification, and can be categorized into: 1) water soluble; 2) acid-soluble; 3) insoluble; and 4) encapsulated. Most commonly used fortificants have been described and adequately classified based on their cost, bioavailability and solubility in solutions (**Table 2.3**).

Water soluble iron compounds

Water soluble iron compounds are highly bioavailable, owing to their high solubility. However, being soluble results in interactions with the food matrix, resulting in negative organoleptic outcomes (162).

Ferrous sulfate

Ferrous sulfate is used for comparison in both supplementation and fortification as it contains 100% RBV. FeSO_4 has two forms in fortification, both heptahydrate (20% Fe) and dried (33% Fe). Because of its high solubility, FeSO_4 is functional in fortifying dry foods with little moisture, such as pastas, low extraction wheat flour, milk powder and dried milk-based infant formulas (163). A 35-week study examined ferrous sulfate's efficacy on improving iron status in Thai women given fortified wheat-based snacks containing 12 mg Fe/d 6 out of the 7d/wk (164). The study resulted in significant improvement of iron status, indicated by total body iron stores increasing from 1.5 to 5.3 mg/kg body weight and an 80% reduction of women with iron

deficiency. Studies have shown ferrous sulfate fortified in milk based infant formula is effective in reducing ID in infants (165).

Principally, FeSO_4 is the most frequently used iron compound due to its inexpensive cost. Though it is cheap, there are limitations to its use in fortification. For instance, it has been shown to cause unacceptable organoleptic problems including: lipid oxidation, rancidity, and vitamin oxidation ultimately provoking off flavors and colors in the foods like rice, soups and milk (21,166).

Ferrous bisglycinate

Ferrous bisglycinate has shown high RBV but is rapidly oxidized to Fe^{3+} in the presence of water. Addition of citric acid (redox stabilizing iron) prevents iron oxidation, eliminating off-color effects of iron. Moreover, this redox-stabilized FBG when administered to women and children, has shown to improve iron levels (167,168).

Acid Soluble Iron Compounds

Ferrous fumarate

Ferrous fumarate without capsulation is soluble only in dilute acid. It is a dark red, fine powder which contains comparable RBV to ferrous sulfate due to its solubility in the gastric juice (163). Several efficacy studies have demonstrated the effectiveness of ferrous fumarate in enhancing iron stores. Using a hemoglobin repletion study, Mehansho et al (169) examined the hemoglobin differences and RBV among FeSO_4 , encapsulated ferrous fumarate and ferrous fumarate without the encapsulation and concluded there were significant hemoglobin gain in the rats, however the two forms of ferrous fumarate were not significantly different from one another. Additionally, the RBV for the encapsulated and unencapsulated ferrous fumarate was 98 and 107, respectively (169). Since the iron in the form of ferrous fumarate is insoluble in water,

it is used in reconstituted dry infant cereals as source of high bioavailable iron, as reported in comparison to FeSO_4 (170). Ziegler and his colleagues assessed the efficacy of both ferrous fumarate (52.5 mg Fe/100g dw) and electrolytic iron (54.5 mg Fe/100g dw) fortified dry infant cereal on the ability to maintain iron status and prevent ID onset with daily consumption in 4-9 month old infants (170). Daily ferrous fumarate showed when consumed daily, they were effective in maintaining iron status during exponential growth period (170).

Insoluble iron compounds

Elemental iron

Elemental iron is the most prevalently utilized source of iron in fortifying commercial food products because of its significantly low cost and inertness in the food (171). While the former is true, the bioavailability of elemental iron compounds is so low that despite large quantities fortified in foods, the absorbable dietary amount of elemental iron is negligible. Therefore, companies have manipulated the particle size (<45 microns or 325 mesh) and surface area in an aim to increase its bioavailability, which has been modestly successful, however the IBA remains unresolved because of the vast differences in manufacturing (172) The different forms of elemental iron include: H-reduced, atomized, CO-reduced, electrolytic and carbonyl – all containing vastly different RBVs as reported in **Table 2.3**. Human bioavailability studies have evaluated the differences among these commercial iron sources. Walter et al. (2014) reported bioavailability of H₂-reduced elemental iron of 62% RBV when fortified in white bread (173); electrolytic Glidden A-131 iron showed a RBV of 50% compared to FeSO_4 when fortified in bread rolls (174); following a feeding study of doubly-labelled wheat rolls containing carbonyl iron (^{55}Fe) and native iron ($^{59}\text{FeCl}_3$), reported RBV percentages ranged from 5-20% depending on the food matrix it was consumed with (14).

Ferric pyrophosphate

Ferric pyrophosphate (FePP) has an off-white color, which is favorable in commercial fortification, because unlike the soluble iron compounds, it remains inert in the food and may contain more absorbable iron than an elemental iron source (175). The versatility of FePP and its acceptable sensory characteristics, owes to its popularity in fortifying sensitive food vehicles such as bouillon cubes, chocolate drinks and powders as well as infant cereals (176). The RBV of FePP has been reported to be in the range of 25-75% of FeSO₄ (177,178), however strategies exist to improve the bioavailability of this compound.

Particle size reduction is one employed strategy in increasing the bioavailability of insoluble iron compounds that are poorly absorbed (179,180). Wegmuller et al. used a hemoglobin-repletion animal model to assess the RBV's of various sized micronized FePP fortificants (21µm, 2.5µm and 5µm) (181). They determined by reducing FePP from 21 µm to 0.5 µm, micronized dispersible ferric pyrophosphate (MDFP) improved its bioavailability by almost 2-fold with a reported Hb comparison of 21.5 g/L to 43.7, respectively (181). Long-term efficacy studies have also demonstrated the effectiveness of MDFP consumption (182,183). In a 32 week feeding trial with fortified margarine containing 14 mg micronized, ground FePP, Andersson et al. reported MGF₂FePP improved SF and total body iron in young women (184). Although shown effective, the RBV of MDFP is primarily driven by the food matrix in which it is administered. A study investigated the influence two different food matrices (wheat-milk infant cereal and a rice meal) had on the overall RBV of MDFP with particle size 0.77 µm (185). Results indicated MDFP had an RBV of 62% when fortified in the infant cereal compared to only 15-25% RBV when consumed with the rice meal. This study demonstrated the importance food processing and food matrix has on the bioavailability of an iron fortificant. Currently,

chicken bouillon cubes are fortified with FePP, but a recent study reported its bioavailability to be only 4.4% when consumed as a broth, not as a complex meal (186). Although iron absorption almost doubled (6%) with the addition of sodium pyrophosphate, the RBV was only 19%.

Table 2.3. Key characteristics including Fe content, bioavailability and properties of iron compounds commonly used for food fortification.

Iron Compounds	Iron Content (%)	RBV(%)	Contribution to Changes in Sensory Properties
Water soluble			
Ferrous sulfate, 7H ₂ O	20	100	Highly oxidative, causing rancidity
Ferrous sulfate, dried	33	100	Highly oxidative, causing rancidity
Ferrous bisglycinate	20	>100	Low induction of taste and color changes
Acid soluble			
Ferrous fumarate	33	100	Low alterations to food matrix
Insoluble			
Elemental iron	96-99	12-148	Variable characteristics of altering food matrix
Ferric pyrophosphate	25	21-74	Low to no changes in organoleptic properties

*Modified from Prentice et al (110).

Natural iron compounds

Industries are looking toward providing a “clean label” for their consumers. Although there is no clear definition of “clean label” by the FDA, more natural ways of providing iron as a fortificant are newly being explored (187). To date, research is scant in the realm of natural iron fortificants, with very few studied as potential candidates.

A recent study by Sabatier et al (188) explored the IBA from a mutant yeast of *Saccharomyces cerevisiae*. This particular biomass in the mutant yeast was able to take up ~0.9% iron (9 mg/g Fe). When the iron-enriched mutant yeast was fortified in a fresh cheese and fed to 16 female subjects, an RBV of 72% of FeSO₄ was achieved (189). To our knowledge, this

is the only natural iron fortificant for which there is published data. Due to its low iron acquisition, there remains a concern of the overall efficacy this particular iron-enriched yeast has on long-term iron status improvements due to its low daily contribution.

Aspergillus oryzae, otherwise known as koji, is a filamentous fungus that has been used for centuries to ferment soybeans and rice to make miso paste, sake, soy sauce, and other food products. It is structurally and fundamentally different than *Aspergillus fumigatus* otherwise known to cause detrimental health outcomes, especially those in which are immune-deprived. *A. oryzae*, has the capability of acquiring high amounts of iron, ~6-8%, and when grown in iron-rich FeSO₄ media, iron was found to be stored within the mycelia (190). Due to its strong capacity to sequester iron in media, *A. oryzae* was studied in humans as a supplement. Reddy et al saw a comparable iron absorption of the iron-enriched *A. oryzae* in FeSO₄ rich media (ASP-s) compared to gold standard, FeSO₄. Results from this study proposed further investigation into the capabilities of *A. oryzae* to be enriched in other iron-rich media, such as ferric pyrophosphate (ASP-p) due to its promising results in humans (191).

The biological processes of iron acquisition in *Aspergillus* genus has been investigated. In *A. fumigatus* Haas et al. identified iron regulation processes whereby there is siderophore-mediated iron storage as well as storage within the iron vacuole (192). If iron is stored within the vacuole, it may provide a protective barrier against when added to various foods (193). Because *A. oryzae* has the versatility to be enriched with different iron sources, this warrants further investigation on iron absorption of ASP-p as a fortificant in food vehicles compared to more commonly used iron fortificants, such as FePP.

Conclusion

This literature review examined the resounding impact IDA has on our global population. Iron deficiency plays a significant role on the growth and development of women of child-bearing age, infants and children and the consequences have been explained in this review. In addition, this review has explored the interface between dietary iron sources and the food matrices' influence on iron absorption. This imposes a clear need to determine the most effective strategy towards providing regular consumption of sufficient, highly bioavailable iron sources to support iron maintenance. Food fortification appears to be the most cost-effective strategy but challenges remain in finding the most efficacious iron compound and vehicle for fortification. Due to this obstacle in iron fortification and the evolving public interest for more natural iron fortificants, this research was pursued to find a more iron bioavailable source for vehicle fortification to address global iron deficiency anemia.

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CHAPTER 3: IRON ABSORPTION FROM A NOVEL IRON-ENRICHED FUNGAL FORTIFICANT IN YOUNG FEMALE SUBJECTS

A manuscript prepared for the submission to the *Journal of Nutrition*

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Abstract

Background: Fortification is a sustainable, cost-effective strategy in addressing global iron deficiency anemia. Iron compounds, such as ferrous sulfate (FeSO₄) are highly absorbed, but interact with food matrices, whereas ferric pyrophosphate (FePP) does not react with food, but it has low absorption. The objective of this research was to compare iron absorption from *Aspergillus oryzae* enriched with FePP (ASP-p), FePP and FeSO₄ by humans using a dual labelled staple isotope methodology.

Methods: In two single blinded cross-over studies, healthy adult females with serum ferritin levels <40 µg/L were randomized to consume a meal with rice, vegetable sauce and chicken bouillon fortified with iron. Subjects in study I (n=17, 18-35y) consumed a total of 4.2 mg of elemental iron from both iron sources as either ⁵⁷FePP or ⁵⁸ASP-p with natural abundance iron in each meal fed on 3 consecutive days. Similarly, in study II (n=18, 18-35y) subjects consumed ⁵⁷FeSO₄ and ⁵⁸ASP-p. Iron absorption was measured using isotope enrichment in the whole blood 14 days following the final iron dosing. Hemoglobin, hematocrit, serum ferritin (SF) and serum C-reactive protein (CRP) were analyzed at baseline and at 14d.

Results: Our results indicated the iron absorption from FePP:0.94% ± 0.43-2.03%; (geometric mean ± SD) was significantly ($P<0.0001$) lower than ASP-p:2.20% ± 1.00-4.83%, whereas in

Study II, ASP-p absorption of $2.98\% \pm 1.37$ - 6.46% was significantly ($P < 0.0001$) lower than FeSO₄: $9.89\% \pm 4.52$ - 21.62% . After correcting the absorption values to serum ferritin of 15 µg/L, ASP-p absorption was 2.6-fold higher than FePP and 72% lower than FeSO₄.

Conclusions: The higher iron absorption of ASP-p compared to currently used FePP, suggests its potential to be used in fortifying chicken bouillon.

Keywords: *iron absorption; stable iron isotope; iron fortification; ferrous sulfate; ferric pyrophosphate; Aspergillus oryzae*

Introduction

Iron deficiency anemia (IDA) is the most prevalent nutrient deficiency, affecting 1.62 billion people, globally (1). Iron deficiency is common among populations with high consumption of plant-based diets and higher rates of infections such as malaria (2,3). For instance, West and Central regions of Africa which are affected by the above factors, are also afflicted with the greatest incidence of anemia, with roughly 70% of the children having IDA (4,5). Associated consequences of IDA include decreased cognitive function, productivity, and an increase in morbidity and mortality (6–9).

Several strategies are implemented to address IDA including the use of supplementation with FeSO₄; dietary modifications aimed at increasing daily iron intake; iron biofortification of staple foods (such as rice, flour and beans) (10); and iron fortification of foods. Despite improvements in iron status, supplements like FeSO₄ are known to release a large bolus of iron into the gastrointestinal tract, leading to systemic inflammation and potential worsening of infections, like malaria. Dietary modifications are often unconventional due to the barriers in altering culturally adherent foods, especially in impoverished regions where there is a low accessibility to iron rich foods (11). Although staple food biofortification is the most sustainable

approach, fortifying affordable and widely-consumed food vehicles, such as condiments can also make a significant contribution in meeting daily iron requirements (12,13).

There are numerous iron compounds available for food fortification, but not all are acceptable for many reasons. The challenges in fortification include finding a suitable iron compound with high iron bioavailability (IBA) while remaining inert in the food matrix. Soluble iron compounds such as FeSO_4 and ferrous gluconate are highly absorbed, however they are also highly reactive in food, causing lipid oxidation and rancidity, among other adverse sensory outcomes (14). Elemental iron sources are ideal for fortification as they do not alter the organoleptic properties of the food; unfortunately, these are the least absorbed iron compounds (15). FePP is widely used in foods sensitive to color changes and generally known to be absorbed at 21-74% compared to the standard of FeSO_4 (14,16). Attempts have been made to improve iron absorption by reducing the particle size of FePP or through the addition of trisodium citrate during the rice extrusion process (17).

Chicken bouillon cubes were selected as a candidate for fortification because of their regular consumption by many African countries. As already mentioned, FeSO_4 is not suitable for bouillon fortification while FePP is commonly used. A recent study showed only 4.4% of the iron was absorbed from FePP when chicken bouillon cubes were dissolved in water and consumed as a drink, compared to 27% absorption from FeSO_4 fortified cubes (18). The relative bioavailability of FePP is known to be influenced by the food matrix (19,16), therefore a lower iron absorption can be expected from FePP when consumed with a meal. This study's results suggest the need to identify new iron sources for bouillon fortification.

Recently, some studies focused on using yeast as natural iron fortificants (20,21) Although cheese fortified with yeast shows promising results, the amount of yeast needed to

achieve the required iron fortification would be higher because it uptakes low amounts of iron (20,21). *A. oryzae* is a filamentous fungus, otherwise known as koji, with a US FDA Generally Recognized as Safe (GRAS) status (22). It has been used for thousands of years to make fermented soy and rice products like sake, soy sauce, amazake, and miso paste (23,24). *A. oryzae* has the potential to be used as an iron fortificant due its unique ability to take up high amounts of iron (25). In a previous human study conducted by our team, *A. oryzae* grown in FeSO₄ containing media (ASP-s) showed similar absorption as FeSO₄ when given with a semipurified meal., suggesting its potential as a new highly bioavailable source of iron for food fortification (manuscript in review). However, studies conducted with ASP-s showed slight color reaction in bouillon cubes. To solve this problem Cura Global Health, Inc. developed another iron-enriched *A. oryzae* product, grown in FePP (ASP-p) (26). Hence, the objective of this study was to investigate the effectiveness of the ASP-p iron absorption and compare it with commonly used fortificants FePP and FeSO₄.

Subject and Methods

Subjects: Healthy, nonsmoking, females of 18-35 y, with ferritin < 40μg/L, but not anemic, were recruited for both iron isotope studies by sending a mass email to faculty and students at Iowa State University (ISU). These criteria were used because individuals in this category have a high risk for developing iron deficiency anemia (IDA) and an increased response to iron fortified foods due to low iron stores (27). Exclusion criteria included history of chronic or gastrointestinal conditions, in addition to having known food allergies to the ingredients in our administered test meal. Participants were excluded from the study if they were vegetarian, pregnant or lactating, or clinically classified as underweight (BMI <18.5 kg/m²) or overweight (BMI ≥ 25kg/m²). Participants were ineligible if they were taking any medications other than

oral contraceptives. Those consuming vitamin and/or mineral supplements were asked to discontinue their use at least two weeks prior and throughout the study period. Participants were not allowed to donate blood or plasma one month prior or while in the study. All subjects formally agreed to participate and signed consent forms. The study was approved by the Institutional Review Board of Iowa State University.

In study 1 comparing the absorption of FePP to ASP-p, a total of 58 subjects were screened, of which only 18 were eligible based on inclusion criteria. One subject dropped out of the study halfway through the feeding trial after becoming ill with the flu, therefore 17 subjects completed the study (*Figure 1*). In study II evaluating FeSO₄ and ASP-p, 77 individuals were screened and 19 were eligible. Similarly, one subject dropped out midway through the study unable to finish eating the meal containing the iron treatment. Eighteen subjects participated in the final analyses for study II (*Figure 1*). Of the total 35 participants, 77% were White, 11.4 % Hispanic/Latino, 5.7% Asian, 2.9% African American and American Indian. Both iron absorption studies required at least 16 participants to detect a 30% difference in iron absorption with 80% power at $P < 0.05$ using previously reported standard deviation of 0.2 (within-subject) for absorption following \log_{10} transformation (20).

Stable Isotope Analysis: Stable isotopes (⁵⁷Fe and ⁵⁸Fe) were purchased from Chemgas (Boulogne, France) and shipped directly to Dr. Paul Lohmann® (Emmerthal, Germany) for labelling ⁵⁷FePP, ⁵⁷FeSO₄, and ⁵⁸FePP using their commercial preparation for iron salts. Iron salts with natural abundance iron were made by the same company using similar methodology. Iron content and enrichment of compounds were as follows: [⁵⁷Fe]-FePP (26.4% Fe with 95.8% enrichment), [⁵⁷Fe]-FeSO₄ (35.95% Fe with 95.3% enrichment) and unlabeled, naturally abundant FePP containing 22.3% Fe and FeSO₄ with 37.0% Fe. ⁵⁸FePP was used to intrinsically

label *A. oryzae* to make $^{58}\text{ASP-p}$. Methodology for intrinsically labelled ^{58}ASP was based on our previous study (21)^C Briefly, *A. oryzae* was grown in the presence of $^{58}\text{FePP}$, the intrinsically labelled ^{58}ASP compound was then harvested, oven dried, and ground to 100 mesh-size powder, under food grade conditions. The ^{58}ASP powder contained 5.0% iron with 99.5% ^{58}Fe enrichment, as measured by Magnetic Sector Thermal Ionization Mass Spectrometry at Cornell University. Inductively Coupled Mass Spectrometry was used to measure iron content in all iron sources. Unenriched *A. oryzae* (Ao) and enriched ASP-p with natural abundance iron contained 0.5 mg/g and 87.9 mg/g of iron, respectively. The Ao was used to match the fungi content of ASP-p in all other test meals.

Test Meal: The test meal composition used to feed the stable isotopes was modified based on a previous study (19). The meal was formulated to contain low elemental iron and low amounts of iron absorption inhibitors and enhancers. Each meal consisted of 42g of each zucchini, carrots, green cabbage, and 24g yellow onion, 6.3g corn oil (Mazola[®], ACH Food Companies, Inc., Cordova, TN), 6.6g Maggi's unenriched granulated chicken flavor bouillon (Nestlé USA, Inc., Glendale, CA), and 75g dry weight unenriched, medium-grain 80% jasmine, 20% white rice blend (Three Elephant Brand, AAA Grade, Thailand). Total iron content was measured in both chicken bouillon and jasmine rice using Inductively Coupled Mass Spectrometry and shown to contain 0.56 mg Fe together per test meal. Thirteen servings of each vegetable (546 g of each zucchini, cabbage, carrot, and 312 g onion) were diced, added to 234 g nanopure water in an instant 6-quart pressure cooker (Power Pressure Cooker XL, Inc., Tristar Products), cooked for 14 minutes at 109°C and allowed to cool for one hour. Following cooling, vegetables were pureed and corn oil was added to make a sauce that was stored at -20°C until feedings. The day

preceding our served meal, jasmine rice was prepared at 2:1 water to rice ratio and 200g cooked rice was pre-portioned for the following day. Chicken bouillon was weighed in individual one-ounce cups and all iron treatments (tracers and natural abundance) were meticulously added on top of the bouillon to prevent any isotopic loss. On the day of the feeding, the rice and vegetable sauce were individually heated thoroughly in a microwave, combined, and mixed with the chicken bouillon containing the respective iron tracers. The cups were rinsed three times with purified water to ensure all residual isotope was added to the meal. All the ingredients were mixed very carefully prior to serving.

Fortification and Labelling of Test Meals: Two test meals were fed in both studies, referred to as A and B in study1; C and D in study 2. All test meals were designed to contain equal amounts of added iron (4.2 mg Fe). Rationale for the addition of iron is based on current iron fortified chicken bouillon; 3.3 g bouillon with 2.1 mg Fe/ meal or 6.6 g bouillon with 4.2 mg Fe/d. Test meals contained a total of 4.76 mg Fe, including the iron from the bouillon and rice. Meal A contained 3.34 mg ^{57}Fe from $^{57}\text{FePP}$, 0.87 mg natural abundance iron from FePP. Meal B contained 0.68 mg ^{58}Fe from $^{58}\text{ASP-p}$ and 3.52 mg as FePPAo (*A. oryzae* in FePP with natural abundance iron). Meal C contained 4.2 mg of ^{57}Fe from $^{57}\text{FeSO}_4$ plus the matching 3.52 mg Ao that was in the labelled ASP. Study I provided a total of 10 mg of ^{57}Fe and 2 mg of ^{58}Fe . Because of the low absorption values in the first study, we fed 12.5 mg ^{57}Fe and 3 mg ^{58}Fe total in the second study to ensure enough enrichment.

Study Designs: In two controlled, single-blinded, cross-over study designs, participants were randomized separately in each study (using the “RAND” function in Excel). In study I, people

consume both meal A ($^{57}\text{FePP}$) and meal B ($^{58}\text{ASP-p}$). The entire stable iron isotope dose (10.02 mg of ^{57}Fe or 2.04 mg ^{58}Fe) was fed to each subject in 3 consecutive mornings meals. In Study II, people were randomized similarly to consume meal C ($^{57}\text{FeSO}_4$) and meal D (^{58}ASP) and followed the same feeding protocol except with a few modifications as described below.

On day one of the study, participants were required to complete a 10 hour overnight fast prior to consuming the first test meal between the hours of 6:00 and 8:30 am at our Nutrition and Wellness Research Center (NWRC) at ISU. Baseline blood was drawn by a phlebotomist and followed by administration of the isotopes in either meal A, B, C or D. Participants were instructed to consume the entire meal within 15 minutes and bowls and spoons were rinsed with filtered bottled water for minimum three times or until all food residue was gone. Water was ingested after each rinse and bowls and spoons were checked by research personnel to confirm all isotope was consumed. Subjects were not allowed to eat or drink for an additional 3 h, except water. Fourteen days after the last test meal was eaten, participants' 10 h fasted blood was drawn for final hemoglobin and iron enrichment analyses.

The difference between study I and II were the alterations in feeding days to accommodate the students' 3 hour availability during the weekday with classes. Isotopes were still fed in three morning meals but over two consecutive weekends (Friday, Saturday and Sunday).

Blood analysis: Serum and whole blood were collected at screening and stored (-20°C) until time of measurement. Serum ferritin concentrations (S-22 Spectro Ferritin kit Ramco Laboratories, Inc., Stafford, TX) were assessed to determine participant eligibility ($<40\text{ ug/L}$) as well as at baseline and endpoint whole blood was sent to a Certified Diagnostic Laboratory

(Quest Diagnostics, Lenexa, KS) for blood chemistry analysis, including hemoglobin and iron status markers. Baseline and final serum were collected from participants and stored at -20°C until further analysis were performed for serum ferritin and serum C-reactive protein (CRP ELISA, American Laboratory Products Company, Salem, NH). Final whole blood samples (frozen) were sent to ETH Zürich (Zürich, Switzerland) for fractional stable isotope enrichment analysis.

Enrichment Calculations: Iron from the whole blood samples was extracted by the anion exchange chromatography method (28). The amounts of ^{57}Fe , ^{58}Fe isotopic labels in blood 14d after the second meal feeding was assessed on the basis of the shift in iron-isotope ratios and the estimated amount of iron circulating in the body. Circulating iron was calculated on the basis of blood volume estimated height, weight (29) and Hb concentrations. Fractional iron absorption was calculated on the assumption that 80% is incorporated into hemoglobin (30). Isotope measurements were performed using a negative thermal ionization –mass spectrometry.

Statistical analysis: All statistical analyses were performed by GraphPad Prism 6.07 software (GraphPad Software, Inc., San Diego, CA). Normally distributed data were presented as means \pm SDs. Non-normally distributed data were log transformed prior to statistical analysis; values were presented as geometric means \pm SDs for iron absorption, ferritin, and CRP values. Student t-test was used to compare the absorption of ASP-p with FePP to ferrous sulfate. There was an outlier was identified with absorption with Asp-p in the study and statistical analysis was performed with and without using the subject. Pearson correlation analyses were performed between absorption. To be able to combine two studies, we corrected absorption to a serum

ferritin of 15 μ g/L as described by Cook et al (31) and analyzed mean differences among groups using a one-way ANOVA followed by Tukey's multiple comparison test. All differences were considered significant at $P \leq 0.05$.

Results

Subject Characteristics: General baseline anthropometric and biochemical characteristics of subjects in both study I and II are presented in **Table 1**. Mean age of participants in Study I and Study II were 20 y and 21 y, respectively. Average BMI of 22.1 kg/m² and 22.2 kg/m² for study I and II, respectively, were within normal range (18.5-24.9 kg/m²). At screening, all participants in both studies had hemoglobin levels within normal levels (>12 g/dL) according to the reference values provided by the diagnostic lab (Quest DiagnosticsTM). The mean \pm 1SD hemoglobin in study 1 (12.8 \pm 1.4 g/dL) was similar to study II (12.9 \pm 0.7 g/dL). One subject in study I had elevated baseline (at the beginning of the study) serum ferritin (74.7 μ g/L), but met the inclusion criteria with a screening SF (33.1 μ g/L). This subject's baseline CRP concentration was not elevated (<5 mg/L) but did show trends in increasing ferritin throughout the study indicated by a final SF of 97.5 μ g/L. In study II, one reported to have high baseline SF (56.2 μ g/L), however ferritin at screening was 4.9 μ g/L and final value was 11.4 μ g/L with no elevated CRP, another study II subject did have an elevated baseline CRP concentration (8.073 mg/L), but all SF concentrations remained within range. Geometric means of serum ferritin were comparable to study I with 15.4 \pm 13.0 μ g/L and 18.2 \pm 18.0 μ g/L and not significantly different between two studies. None of the other characteristics were significantly different between two studies.

Iron absorption: Fractional iron absorption values are presented as geometric means \pm SD in **Figure 2**. Ferric pyrophosphate absorption was low (0.94% \pm 0.10-3.52%) and ASP was

significantly ($P < 0.0001$) higher by 2.3-fold ($2.20\% \pm 0.26-7.34\%$; **Figure 2A**) than FePP. The mean FeSO₄ and ASP percentages of absorptions were 9.89% (SD \pm 1.95-28.94%) and 2.98% (SD \pm 0.94-19.29%), respectively (**Figure 2B**). The 70% lower absorption of ASP compared to FeSO₄ was statistically ($P < 0.0001$) different (**Figure 2B**). One outlier was identified in the ASP group with similar absorption as FeSO₄, but the study outcome did not change leaving the subject in the analysis and the difference remained significant ($P < 0.0002$). Highly significant correlations were found between FePP and ASP-p ($r=0.92$, $P < 0.0001$) in study I and FeSO₄ and ASP-p ($r=0.537$, $P < 0.03$) in study II. When we combined the two studies by correcting individual absorption values to a serum ferritin of 15 $\mu\text{g/L}$ (**Figure 2C**), the RBV compared to FeSO₄, of ASP-p was 28% and FePP was 11% which were statistically different from each other ($P < 0.001$) as well with FeSO₄ ($P < 0.0001$)

Discussion

Iron fortification is a cost-effective strategy towards reducing the incidence of iron deficiency anemia. It is technically difficult to fortify with highly bioavailable iron salts, such as ferrous sulfate, due to unacceptable flavors and colors with the food matrix. Insoluble iron compounds are less reactive with food, however they have low bioavailability. Several iron compounds have been identified to provide higher bioavailable iron without altering the organoleptic properties (16). Because of higher bioavailability (greater than FeSO₄) of NaFeEDTA, it's been long used to fortify soy and fish sauce, but the high cost to produce NaFeEDTA prohibits its use (32). Additionally, the iron bound EDTA moiety has shown to slowdown the fermentation process of yeast, hindering its use in grain products such as bread (33) as well as showing signs of rancidity following sensory reports from industries (34). Another water-soluble iron compound, bis-glycinate, used in fortification reported to

immediately precipitate upon incorporation into soy sauce potentially causing lipid peroxidation (35).

Because of gaining interest in clean label of food products (36), use of natural iron sources may be promising in food fortification, but to date, there is limited research in this area. A recent study (20) that examined the iron absorption of iron-enriched brewer's yeast, *Saccromyces cerevisea* reported a 72% of RBV in humans when fed with cheese. Although the results of the above study look promising, the mutant yeast strain was shown to acquire only ~0.9% iron, necessitating a substantial amount of the yeast to be added in food products to meet daily iron needs. *A. oryzae* acquires high levels of iron when grown in iron-rich media at a level of 8-10% (Wicking patent). Although not much data exist on the iron metabolism in *A. oryzae*, other *Aspergillus* species and yeast known to acquire iron and store in the vacuoles, may be as polyphosphate or iron oxide forms (37). We can speculate a similar type of iron acquisition and storage in *A. oryzae*.

The aim of the current study was to determine whether iron absorption from ASP-p is greater than FePP, which is currently used for fortifying chicken bouillon. Chicken bouillon cubes are widely distributed (38) in Africa by multinational corporations and since it is a frequently consumed condiment by all age groups in Africa, with reports indicating weekly consumption as 96% across Cameroon households (39). Similarly, 97% of the respondents consumed bouillon cubes with a mean intake 3.7 g/d in women of childbearing age (40) and may be around 4.3-8.6 g/d in Senegal rural and urban areas, respectively.

Most of the chicken bouillon cubes are fortified with FePP because it causes minimal organoleptic problems and known to have RBV of 50% of ferrous sulfate (41). Since bouillon cubes are generally fortified with 0.6 mg Fe/g of bouillon, we used the same iron to bouillon

ratio (4.2 mg Fe/6.6g bouillon). Our study showed that FePP absorption is very low (around 1%) when consumed with a rice meal, and only 11% of FeSO₄ absorption when absorption values were corrected to ferritin of 15 µg/L. The iron absorption value in our study is four times lower than a previous study showing a 4.4% FePP absorption from bouillon cubes. The latter might be due to lower iron status of the subjects (9.4 µg/L) and/o lack of meal inhibition, since the bouillon cubes were given in a water-based drink rather than with food (18). Despite the differences in absorption values between two studies, the RBV of 11% in our study is comparable to the 13% reported by Cercamondi, *et al.*, . These researchers attempted to increase the absorption of FePP by adding sodium pyrophosphate which improved it by 46% (6.4%), but the RBV was still 19% (19). With the daily consumption of 4 g bouillon, the authors pointed out that women of child bearing age can meet 11% of their requirement (0.9 mg). However, meeting the daily iron requirements will be much lower, since the absorption will be dampened with the meal because of the iron interaction with the food components that has been shown previously (42).

Thus, finding an alternative iron fortificant with higher bioavailability but less reactivity than FeSO₄, will be advantageous for the individuals who consume lower amounts of bouillon in meeting meaningful daily iron requirements to improve iron status. To our knowledge, no current research has examined iron absorption from an iron-enriched fungal fortificant. Since iron may be stored in the vacuole, it may create a barrier between the food matrix and iron, thereby reducing rancidity, discoloration and taste change. Absorption of ASP-p was 2.3-fold higher than currently used FePP, suggesting it might be an effective fortificant in bouillon cubes. The RBV of ASP-p in our study was 28%, which is higher than the 19% reported with combined NaPP and FePP treatment. (19).

In our previous study a RBV of 78% (21) was shown with ASP-s, but a comparison cannot be made with the current study because in that study we provided the treatment with a semipurified liquid meal. As it was explained before, meal effect on bioavailability of iron is an important factor. Absorption of FeSO₄ is generally used to assess RBV of iron compounds but caution should be taken as RBV can vary based on the meal type and the iron status. The RBV of micronized dispersible FePP was reported to vary from 15-62% depending on the food matrix (19).

Since women of child-bearing age require 1.46 mg Fe/day based on 10% absorption (43) we estimated that the current iron fortification levels in bouillon cubes are not sufficient. If women consume 6.6 g of bouillon fortified with 4.2 mg Fe, they can only meet 3.4% of daily requirement with FePP. Fortification with ASP-p provides 8.2% of iron requirement as, almost 3 times iron than FePP (2.9% vs 1.1 % for ASP-p and FePP, respectively). However, since the 6.6 g we used in our study exceeds the average reported consumption of 1-4g bouillon/day, with 4 g consumption of bouillon will provide only 5% of the requirement. Therefore, considerations should be made in increasing the amount of iron to 5 mg instead of 2.5 mg per 4 g of bouillon in order to meet 10.3% daily requirement for women of child-bearing age instead of promoting higher amounts of bouillon cube consumption with high sodium content. In conclusion, meeting 10% of daily iron requirement through condiment fortification is a significant additional source of iron. The results of our study suggest that ASP-p is a promising, natural iron fortificant that may be used various food vehicles to improve iron status of the population who are vulnerable for iron deficiency anemia.

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Table 3.1. General baseline characteristics and iron status indicators of all subjects¹

	Study I (n=17)	Study II (n=18)
Age, y	20.1 ± 2.4 (18-26)	21.3 ± 2.7 (18-29)
Weight, kg	61.5 ± 6.3 (50.2-70.8)	62.2 ± 5.3 (49.6-71.3)
BMI, kg/m ²	22.1 ± 1.7 (18.8-24.6)	22.2 ± 1.2 (19.6-24.1)
Hemoglobin, g/dL	12.8 ± 1.4 (10.4-15.4)	12.9 ± 0.7 (11.7-14.3)
Hematocrit, %	38.3 ± 3.6 (32.2-45.1)	38.0 ± 2.0 (35.5-42.5)
Serum CRP, ² mg/L	0.41 ± 1.7 (0.002-5.16)	0.63 ± 2.2 (0.004-8.07)
Serum ferritin, ² µg/L	18.2 ± 18.0 (4.9-79.5)	15.4 ± 13.0 (6.5-56.2)

¹Values are means ± SD; (range). BMI, Body mass index; CRP, C-reactive protein

²Geometric means ± SD; (range)

No significant differences in the listed variables were found between two studies

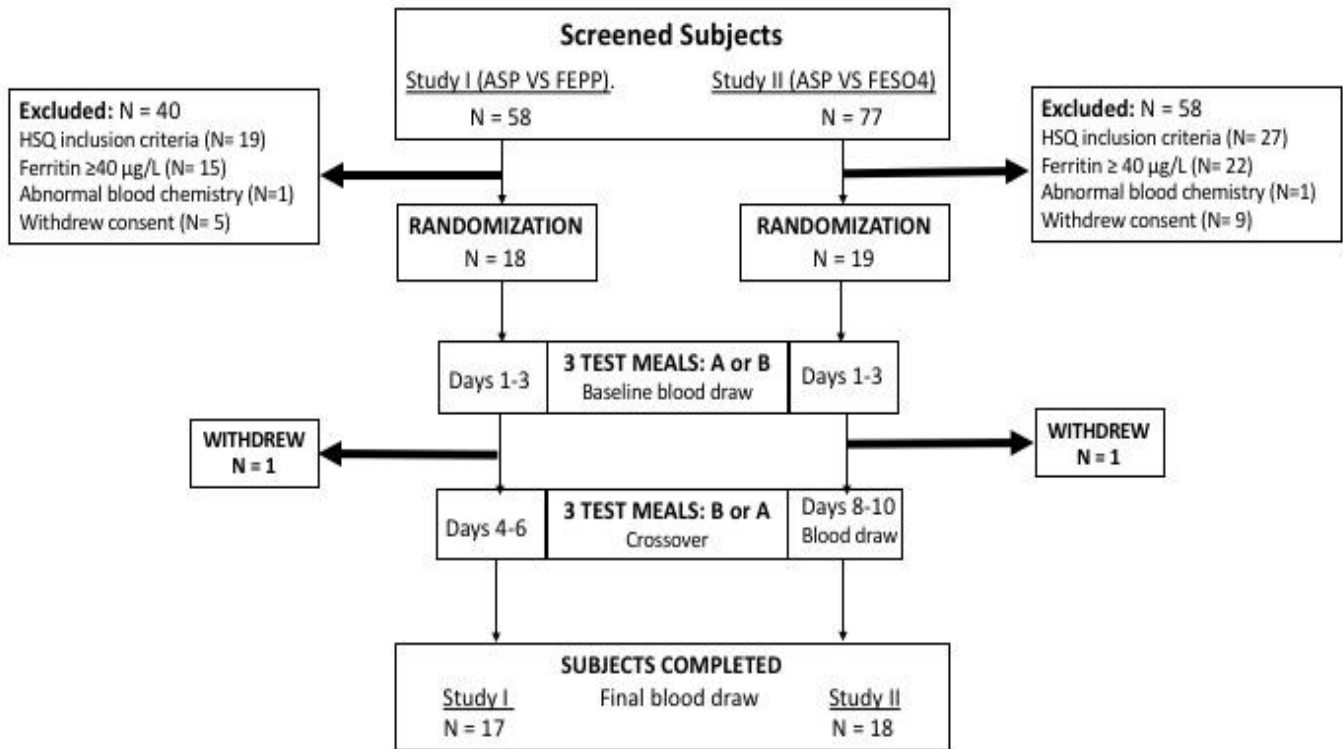


Figure 3.1. Study Design and eligibility; Study I and II presented. Participants were recruited based on eligibility from HSQ, health screen questionnaire including BMI 18.5 to 25.0 kg/m². Subjects had follow-up assessments for serum ferritin (<40 µg/L) and hemoglobin concentrations (age-dependency from Quest Diagnostics). Flowchart indicates test meal AB, BA in a crossover design. Isotopes were given to each subject over 3 test meals. Study I subject withdrew due to flu; study II subject withdrew from unfinished test meal.

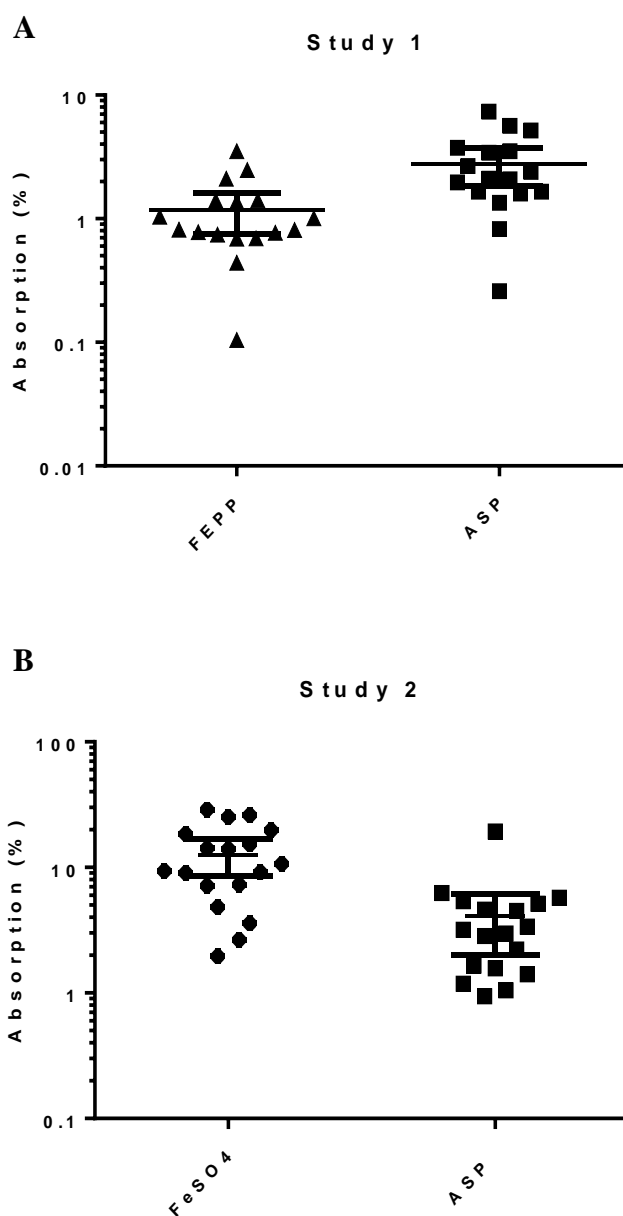


Figure 3.2. Iron absorption plot of $^{57}\text{FePP}$ (triangle), ^{58}ASP (square), and $^{57}\text{FeSO}_4$ (circle). for study I (n=17) and study II (n=18) in Figures 2A and 2B. ^{58}ASP were significantly different ($P<0.01$); ^{58}ASP was statistically significant from $^{57}\text{FeSO}_4$. ASP was significantly higher than FePP ($P<0.01$) and lower than FeSO₄. Values were significant at $P<0.05$ (paired t-test).

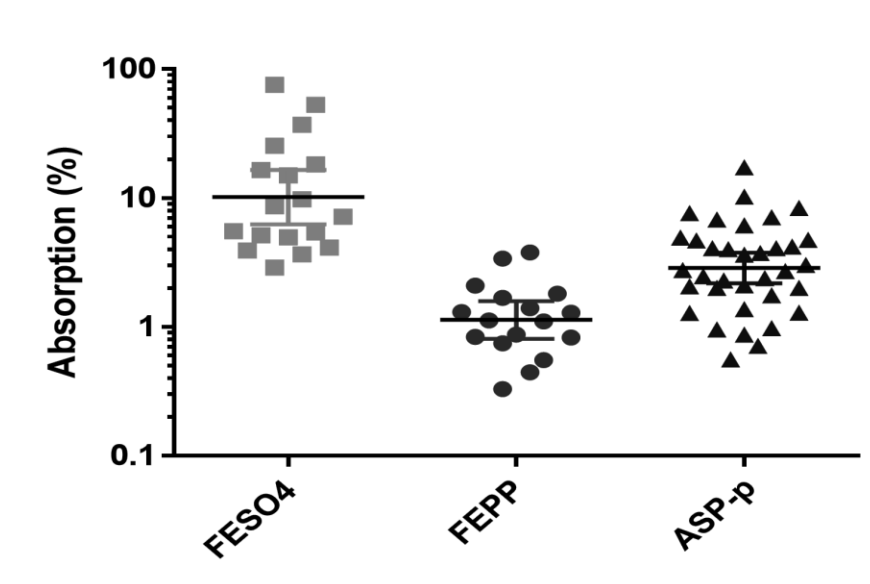


Figure 3.3. Mean iron absorption plots of FeSO₄, FePP and ASP-p combined absorptions after corrected to a SF of 15 µg/L. RBV of ASP-p compared to FeSO₄, was statistically different from each other ($P < 0.001$) as well with FeSO₄ ($P < 0.0001$). Values were significant at $P < 0.05$ (one-way ANOVA followed by Tukey's multiple comparisons test).

CHAPTER 4: GENERAL CONCLUSIONS

The research presented in this thesis investigated the iron absorption from three iron fortificants, of which one was a new, novel iron-enriched *A. oryzae* (ASP-p). Findings from this study demonstrated the highest absorption from FeSO₄, however we know from the literature that FeSO₄ is unsuitable for fortification as it alters the taste, color and shelf-life properties when added to food. Results from this study illustrated a higher iron absorption from ASP-p (28% RBV) than currently used, FePP (11% RBV). Although this finding of 28% RBV is the highest among those investigated in chicken bouillon, it does not provide sufficient amount of iron to meet daily requirements in women and children.

Based on our findings, we acknowledge chicken bouillon may not be the most effective vehicle for iron fortification due to the inhibiting dietary components in the condiment. Therefore, future research is warranted in finding a more widely consumed vehicle or through new technologies towards enhancing the iron bioavailability from cost-efficient iron compounds.

Future ideas are based on our team's previous work assessing iron-enriched *Aspergillus oryzae* as a supplement (ASP-s). In a human study, ASP-s demonstrated to be as bioavailable as FeSO₄. Because ASP-s cannot be added directly to the food, (as it results in food discoloration) future research aims would include investigation of the addition of sodium pyrophosphate (NaPP) to the ASP-s. NaPP would act as a protective compound by decreasing the ability of ASP-s to oxidize the food, ultimately reducing the organoleptic properties it exerts. If this is plausible, sensory studies and bioavailability through *in vitro* models are the first step towards assessing the outcomes of ASP-s plus NaPP in fortification.

APPENDIX: IRB APPROVAL

IOWA STATE UNIVERSITY
OF SCIENCE AND TECHNOLOGY

Institutional Review Board
Office for Responsible Research
Vice President for Research
2420 Lincoln Way, Suite 202
Ames, Iowa 50014
515 294-4566

Date: 1/20/2017

To: Dr. Manju Reddy
220 MacKay Hall

From: Office for Responsible Research

Title: Iron absorption from a novel iron enriched fungal supplement, Aspiron, in humans using stable isotope methodology

IRB ID: 16-378

Approval Date: 1/20/2017 **Date for Continuing Review:** 9/5/2017

Submission Type: Modification **Review Type:** Expedited

The project referenced above has received approval from the Institutional Review Board (IRB) at Iowa State University according to the dates shown above. Please refer to the IRB ID number shown above in all correspondence regarding this study.

To ensure compliance with federal regulations (45 CFR 46 & 21 CFR 56), please be sure to:

- Use only the approved study materials in your research, including the recruitment materials and informed consent documents that have the IRB approval stamp.
- Retain signed informed consent documents for 3 years after the close of the study, when documented consent is required.
- Obtain IRB approval prior to implementing any changes to the study by submitting a Modification Form for Non-Exempt Research or Amendment for Personnel Changes form, as necessary.
- Immediately inform the IRB of (1) all serious and/or unexpected adverse experiences involving risks to subjects or others; and (2) any other unanticipated problems involving risks to subjects or others.
- Stop all research activity if IRB approval lapses, unless continuation is necessary to prevent harm to research participants. Research activity can resume once IRB approval is reestablished.
- Complete a new continuing review form at least three to four weeks prior to the date for continuing review as noted above to provide sufficient time for the IRB to review and approve continuation of the study. We will send a courtesy reminder as this date approaches.

Please be aware that IRB approval means that you have met the requirements of federal regulations and ISU policies governing human subjects research. Approval from other entities may also be needed. For example, access to data from private records (e.g. student, medical, or employment records, etc.) that are protected by FERPA, HIPAA, or other confidentiality policies requires permission from the holders of those records. Similarly, for research conducted in institutions other than ISU (e.g., schools, other colleges or universities, medical facilities, companies, etc.), investigators must obtain permission from the institution(s) as required by their policies. IRB approval in no way implies or guarantees that permission from these other entities will be granted.

Upon completion of the project, please submit a Project Closure Form to the Office for Responsible Research, 202 Kingland, to officially close the project.

Please don't hesitate to contact us if you have questions or concerns at 515-294-4566 or IRB@iastate.edu.