Efficacy of iron fortification and protein efficiency in fortified blended foods and extruded rice in rats

by

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Abstract

Background & Objectives: Both fortified blended foods (FBFs) and fortified rice are important food aid products for addressing protein undernutrition and iron deficiencies globally and were evaluated in two rat studies. We previously found that extruded sorghum-soy blend (SSB) FBFs were equally nutritious compared to corn-soy blend (CSB) FBFs. In the first study, we assessed SSB and CSB FBFs with protein primarily provided by soy flour and compared outcomes to previously developed blends with whey protein concentrate (WPC) to evaluate reduced-cost options (FBF study). In the second study, we compared iron outcomes from four different iron fortificants in extruded rice (rice study). Ferric phosphate ($FePO_4$) and ferric pyrophosphate (FePP) were selected for their suitable organoleptic properties. Micronized FePP (μFePP) and the addition of trisodium citrate (TSC) and citric acid (CA) to FePP (FePP+TSC+CA) were suggested to increase FePP absorption.

Methods: In the FBF study, SSB and CSB FBFs were developed with soy flour and 0–15% sucrose in SSBs and 0–10% sucrose in CSBs. SSB and CSB FBFs with 9.5% WPC and 15% sucrose served as comparison diets.

In the rice study, extruded rice kernels were fortified with one of four iron fortificants: $FePO_4$, FePP, μFePP, or FePP+TSC+CA (ratio: 1:2.1:0.1). Each extruded rice was blended at 1% with natural white rice, soy protein isolate, and soybean oil and cooked.

In each study, weanling, male Sprague Dawley rats were individually housed and randomly assigned to a test or control diet (n=9-10). Food intake was measured every other day (FBF) or daily (rice) and body weights were taken weekly. At study conclusion (FBF: 28 days; rice: 21 days), blood and livers were collected to evaluate iron outcomes and body scans were performed to assess body composition and bone mineral density (BMD).

Results: In the FBF study, there were no differences in food intake, weight gain, lean mass, and iron outcomes among FBF groups. The CSB groups without WPC had significantly lower caloric efficiency and all groups without WPC had significantly lower protein efficiency compared to the groups with WPC. In combined analyses, groups consuming FBFs with 15% sucrose had significantly lower BMD compared to FBF groups with ≤10% sucrose.

In the rice study, all rice groups had significantly lower moisture-adjusted total food intake, weight gain, and BMD compared to the control group with no differences in these outcomes between the rice groups. Hemoglobin concentrations were significantly higher in FePP and μFePP groups compared to $FePO_4$ and control groups. Hepatic iron concentrations were significantly higher in FePP, μFePP, and FePP+TSC+CA groups compared to $FePO_4$ and control groups.

Conclusions: All factors considered, extruded SSB FBFs with soy protein and ≤10% sucrose are an efficacious alternative to WPC-containing FBFs in rats. While the rice study outcomes need to be interpreted with caution because of poor growth, these results suggest FePP leads to better iron outcomes than $FePO_4$. However, neither micronizing nor adding CA+TSC to FePP improved iron outcomes.

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Chapter 1 - Introduction

Global Status of Malnutrition

Protein-Energy Malnutrition (PEM)

Undernutrition Prevalence

821 million people, approximately 1-in-9, suffer from undernourishment – a global concern which only recently began increasing after decades of decline (1, 2). The majority of those who are suffering live in Asia and Africa at 514 million (11.3%) and 256 million people (19.9%), respectively (1). Children are most severely impacted by the negative effects of undernutrition, accounting for approximately one-quarter of the undernourished. Inadequate nutrition during the first 1,000 days of a child's life, starting at conception, contributes to nearly half of global deaths of children under the age of five, approximately three million children each year (3, 4). Primarily as a result of insufficient caloric intakes, 149 and 49 million children are stunted and wasted, respectively (4). While an estimated 16 million children suffer from the more deadly combination of stunting and wasting (5, 6). Globally, the numbers of stunted and wasted children are declining. However, similar to the trend of increased overall global hunger (chronic undernourishment), stunting and wasting are on the rise in certain areas of the world including West and Central Africa. (4).

Undernutrition Causes

Several factors contribute to global undernutrition. The recent increases in undernutrition are mostly attributed to economic slowdowns in middle-income countries (1). While climate change, which has brought unpredictable and varied weather patterns, has additionally contributed to recent increases in hunger (1, 7). Conflict also contributes to hunger, where in countries such as Yemen, more than half the population is food insecure (1, 5, 7). Poverty,

however, has been and continues to be the primary cause of global hunger, with the poorest 20% suffering from stunting at rates double those of the richest 20% (4).

Poverty and undernutrition exist in a cycle which is often challenging to break. Chronic undernutrition can result in permanent decreased cognitive function affecting competence, memory, motor skill development, and coordination (8, 9). These impairments negatively impact school and work performance, leading to reduced success rates and economic productivity (2, 4, 8, 10). Availability and quality of protein evaluated on a national-level was found to be associated with stunting prevalence. Regions with higher proportion of stunting were found to be negatively correlated with total energy, total protein consumption, and per capita gross domestic product (GDP, 11).

The cycle persists when children are permanently setback by poor nutrition and poor education due to lack of resources (8, 12). Undernutrition further decreases productivity due to weakened immune systems associated with increased risk of morbidity, longer recovery times, and mortality (4, 8, 10). Infections reduce the intestinal ability to absorb nutrients negatively impacting both linear and ponderal growth (11). The effects of poor nutrition can be permanent, multi-generational, and widespread, negatively impacting the entire community generation after generation.

Maternal undernutrition contributes to protein-energy malnutrition (PEM) in offspring by restricting intrauterine growth resulting in small-for-gestational-age infants, which is associated with increased risk of morbidity, mortality, stunting, and non-communicable diseases into adulthood (13). Adequate nutrition during pregnancy could prevent 32% of small-for-gestational-age infants in undernourished mothers and help break the cycle (13).

Manifestations

Protein-energy malnutrition is caused by chronic undernutrition and the resulting inadequate protein and caloric intakes leads to deficits in all macro- and micronutrients (14). PEM broadly describes several forms of undernutrition which include underweight, wasting, stunting, kwashiorkor, and marasmus. Wasting describes children who are too thin for their height while stunting refers to children who are too short for their age; these conditions may appear independently or simultaneously (4, 12). Generally, stunting is thought to be a reflection of past undernutrition while wasting is a reflection of present undernutrition (1). However, it may not be that simple. Which form presents may be a reflection of adaptation to specific conditions related to season of birth and specific nutritional deficits (6). Kwashiorkor, named for its typical appearance in children weaned from mother's breastmilk when a new child is born, is primarily a result of inadequate protein with adequate caloric intake. Clinical presentation of Kwashiorkor typically includes children with relatively normal weight and height and abdominal edema (15). Marasmus is primarily a result of chronic deficit in all macronutrients and is most often characterized by wasting with accompanied fat stores depletion (15).

Iron Deficiency

Prevalence

Anemia is the most prevalent micronutrient deficiency affecting approximately one-third of the world's population (16). An estimated 800 million of those with anemia are women and children (16-18). Anemia may be caused by infections or micronutrient deficiencies of iron, folate, riboflavin, and/or vitamin B_{12}. Iron deficiency, which in its most severe form is iron deficiency anemia (IDA), is estimated to be responsible for half of all anemia cases. With an

estimated 43% of children and 38% of pregnant women worldwide impacted by anemia, women of childbearing age and children are most at risk of iron deficiency (16, 17, 19).

Anemia disproportionately affects populations in Asia and Africa, where over half the children in many regions are affected (16). Iron deficiencies are more common in countries which consume primarily plant-based diets and overall caloric and iron intake is low. Iron levels may be further reduced by infections, parasites, and associated blood loss (20).

Hemoglobin concentrations are used to determine anemia status where 110 and 120 g/L are the established thresholds for classifying anemia in children and non-pregnant women, respectively (16, 21). Hemoglobin concentrations assess functional iron levels representing both red blood cell mass and plasma volume (21). The World Health Organization (WHO) estimates that iron deficiency is 2.5 times as common as anemia (20, 21). However estimation of true levels of iron deficiency is challenging because decreased hemoglobin concentrations may only be detected once a person is anemic and iron stores are depleted. Serum ferritin is an additional measure, reflective of body iron stores, and is recommended in addition to hemoglobin to properly assess iron deficiency status (17, 21). In regions where infectious disease and inflammation are endemic, serum ferritin may be artificially elevated and measurement of transferrin receptor should be measured to properly evaluate iron status in individuals (21).

Symptoms

Iron deficiency anemia negatively impacts oxygen transport and cellular oxidative capacity which result in fatigue and lethargy, negatively impacting productivity, cognitive function, and physical performance (19). Evidence suggests that cognition may be additionally impaired due to reduced function of dopamine neurotransmitters and receptors for serotonin and gamma-aminobutyric acid (22). Physical labor productivity is reduced by fatigue which may be

further reduced by increased heartrate during physical activity (22). When large portions of the country are affected, country productivity and economic development is reduced (14). Losses due to cognitive and labor productivity as a result of iron deficiency were calculated for ten developing countries to be approximately 0.81% GDP (22, 23). This results in an estimated $4.2 billion in annual South Asian losses from decreased physical activity capacity of the labor force (22).

Iron deficiency and/or anemia during pregnancy negatively impacts offspring. Children born to mothers with anemia are at increased risk of being born premature and small-for-gestational-age (20, 24). Smaller birth size is associated with inadequate iron stores. And because breastmilk is a poor source of iron, these small infants are at an increased risk of iron deficiency and therefore risk of infection. Iron deficiency during pregnancy is also associated with increased maternal and infant mortality (20).

Prevention

In many cases, IDA can be reversed and prevented. Nearly half of the cases of anemia in women and children could be corrected with iron supplementation (16). Three primary strategies may be employed: dietary modification and education, supplementation, and fortification (20). Food-based approaches, such as fortification of staples, is a recommended approach for improving general population iron intake (16, 19). Universal fortification of staple cereal crops, such as wheat flour, is recommended for countries with widespread iron deficiency risk in women and children (20). Economically, benefits from iron supplementation associated with improvements in both physical and cognitive capabilities were estimated to have a value 8.7 times greater than associated costs (22, 23).

Food Assistance

Food assistance is a broad approach which combines several long-term strategies to address undernutrition and micronutrient deficiencies. Two of the largest providers of food assistance are the World Food Programme (WFP), which serves to nearly 90 million recipients every year (25), and the United States Agency for International Development (USAID), which serves approximately 56 million recipients every year (26).

The Food for Peace Act of 1954 permitted the use of agricultural surpluses for emergency and non-emergency food relief (27) and was followed closely by President Kennedy signing the Foreign Assistance Act into law in 1961, which established USAID to oversee food assistance (28). A 1966 amendment permitted the purchase of non-surplus commodities, paving the way for fortified blended foods (FBFs). Known as the Food for Peace (Title II) provisions, these foods have grown to include minimally processed grains and pulses, fortified grains, FBFs, and ready-to-use specialty and therapeutic foods (29).

Of the $1.7 billion US dollars spent on Title II food in the 2017 fiscal year (FY17), the majority, 80%, was spent for emergency response in areas of conflict including Yemen, Nigeria, Somalia, and South Sudan (30). Nearly half of the foods supplied by USAID in FY17 were wheat products, 27% were other grain products including FBFs, and 3% were other foods including rice. Of the 27% grain products supplied, 81.5% was sorghum and the remaining 11.5% was corn-soy blend (CSB) FBFs. Of the 3% other foods, 64% was rice (30).

Fortified Blended Foods (FBFs)

Both the WFP and USAID provide specialized food products, which includes FBFs. Fortified blended foods are pre-cooked cereal grain and legume dry mixes fortified with

micronutrients. They may additionally include another protein source, vegetable oil, and sugar. FBFs are consumed as porridges, prepared by boiling the dry mix with water.

Corn-soy blends are the most common FBFs provided by both WFP and USAID. Target recipients are pregnant and lactating women and children aged 6-59 months. Fortified blended foods are formulated to prevent and treat undernutrition and micronutrient deficiencies in these populations, although they may be provided as emergency food aid (31). Fortified blended foods when used for complimentary, targeted feeding have been shown to improve micronutrient and undernutrition status of recipients (31, 32).

Both organizations currently provide two versions of CSB FBFs. The first, CSB Plus (CSB+, USAID)/Super Cereal (WFP), is formulated with 78% corn, 20% soybeans, and 2% vitamin/mineral premix (33, 34). CSB+ accounted for the vast majority, 94%, of FBFs provided by USAID in FY17 (30). The second formulation, Super Cereal *Plus* (USAID and WFP), includes an animal-source protein (skim milk powder), sugar, and oil in addition to the ingredients in CSB+. Super Cereal *Plus* formulation is 58% corn, 20% soybeans, 8% dried skim milk powder, 9% sugar, 3% soybean oil, and 2% vitamin/mineral premix (33, 34).

Recommendations

In 2011, a USAID Food Aid Quality Report (FAQR) provided a review of the evidence for reformulation of fortified foods and recommendations for improving existing food aid products. The report questioned whether the CSB, “workhouse of the FBF category,” was fit for purpose, promoting linear growth in children in the first 1,000 days (35). One recommendation within the report was to improve the macronutrient formulation of FBFs by increasing protein, fat, and total energy content.

Animal-source protein in the form of 3% whey protein concentrate (WPC, 80% protein, WPC80), was additionally recommended to provide high-quality protein. Dairy has been hypothesized to support growth by promoting insulin-like growth factor 1 production, increasing mineral absorption, and providing adequate quantities of sulfur-containing amino acids (AA, cysteine and methionine) which support growth plate development (36). Several have criticized this recommendation, questioning whether it is appropriate given a lack of evidence demonstrating that animal-source protein, specifically WPC, supports growth needs (37, 38). While WPC may be beneficial for increasing muscle mass, it may not support the desired improvements in linear growth needed to address stunting (38). The outcomes associated with whey protein in the FBF target populations requires additional research to support its use.

The report additionally recommended improvements to the micronutrient profile of FBFs. Specifically, recommendation was made to use a combination of both sodium iron ethylenediaminetetraacetic acid (NaFeEDTA) and ferrous fumarate iron fortificants to improve iron absorption (35).

One additional notable recommendation called for formulation of new cereal grain and legume-based FBFs. Sorghum was identified as a prospective cereal grain for inclusion in FBFs in part for its acceptability among many food assistance recipient nations in Africa and relatively low price (35). Per capita consumption of sorghum is higher in Africa than anywhere else in the world (39). In many regions in Africa, particularly Sub-Saharan Africa, sorghum remains a key staple food because it can thrive where there is insufficient rainfall and excess heat for other crops, such as corn (40). Sorghum's drought-tolerance and widespread familiarity across Africa makes it an appealing cereal grain for inclusion in FBFs.

Cost

The FAQR also identified the disproportionate cost of fortified products, such as CSB+, which in 2011 cost 44% but only represented 25% by volume of Title II foods (35). It is estimated that the recommendation of 3% WPC80 would increase costs of the FBFs by approximately 18% (37). However, this added cost may be advantageous if associated with reduced total time of treatment, resulting in reduced total cost per recipient, or increased consumption and acceptability (41). Other factors, including the overall cost-effectiveness of treatment and the mechanisms by which dairy products support growth, are needed to understand if the increased cost of FBFs with WPC are met with desired improvements (36, 41).

Based on the FAQR recommendations, novel FBFs were developed with 9.5% WPC80 or soy protein isolate (SPI), 9% vegetable oil, 3.2% micronutrients, and 15% sucrose with the remaining 63.3% the grain-legume blend (42). A 2019 publication on cost effectiveness of these novel FBFs determined the 9.5% addition of WPC80 accounted for 27-32% of the total costs (which includes ingredients, processing, production, and transportation) if the FBFs were produced in the United States, where the cost of WPC was lowest (compared to other countries analyzed). While the estimated cost of WPC80 increased to 43-44% of the total costs if produced in Tanzania, where cost of WPC was highest (43). In general, addition of 10-15% dairy-sourced protein (WPC or skim milk powder) nearly doubles the total cost of FBFs. Considering total nutrient cost effectiveness, the 2019 publication concluded that the novel FBF formulations with 9.5% WPC were not as cost effective per nutrient effectiveness as the existing CSB+ and Super Cereal *Plus* (43). At approximately half the cost with similar protein quality, SPI has been proposed as a cost-effective alternative to WPC in fortified food aid products (43).

Processing

Currently, USAID CSB+ and Super Cereal *Plus* may be processed by either roasting or extrusion (44). The novel FBFs described previously were processed with extrusion. Extrusion mixes and prepares dough through a single or twin screw extruder which exits through a die and is cut by a rotating blade producing cooked extrudates. Extrusion was selected in part for its ability to improve the nutritional profile of cereal grain products by decreasing naturally present antinutritional factors (45, 46). This processing technology is appealing over roasting which can result in lower-digestibility of final products (47).

Based on USDA viscosity requirements for CSB13, the ideal Bostwick consistency of prepared FBF porridges should be between 9.0-21.0 cm/minute with acceptable consistency ranging from 6.0-24.0 cm/minute (48). In the first iteration of the previously described novel FBFs, the initial viscosity was too thick to meet these consistency requirements. Sugars effectively decrease the viscosity of high-starch products, such as FBFs, by interfering with starch hydration (49). Therefore, sugar, as sucrose, was included in the formulation of these FBFs, which decreased viscosity of the cooked porridges and enhanced the sensory characteristics (42).

In a second iteration of these novel FBFs, adjustments were made to extrusion processing parameters. The resulting FBFs met viscosity requirements without addition of sucrose (50).

Rice

Rice a staple food for more than half the world and provides up to 70% of the daily calories for populations in many middle- to low-income South Asian countries (51-54). Regions of Asia with the highest rice consumption also tend to have a high prevalence of poverty, food insecurity, and political instability (52, 53). As a result, these regions experience high levels of

undernutrition and thus, a large proportion of the population suffers with PEM and anemia. Due to widespread consumption in particularly at-risk regions, rice fortification offers a promising opportunity to address a large proportion of the global micronutrient deficiencies.

In 2014, USAID approved fortified milled rice for use in government assistance programs (55). Fortified milled rice provided by USAID is a blend of milled (natural) rice with fortified rice, designed for use in both emergency and development settings (33). The fortified rice may either be coated rice kernels or rice-shaped extrudates (33, 56). Fortified rice kernels increases the cost of milled rice by 2-5% and represented more than 80% of the rice provided by USAID in FY17 (30, 55). Micronized ferric pyrophosphate (μFePP) is the recommended iron fortificant for fortified rice. Other fortificants may be used if determined suitable (56).

Fortification

The majority of micronutrients naturally present in rice are located in the outer layers of the rice kernel. Polished rice is first milled to remove these outermost layers, including husk, germ, and bran layers, and then polished to remove remaining bran and increase translucency (52). Approximately 75-90% of the micronutrients, including iron, zinc, and B vitamins, are lost during these processes. Resulting iron concentrations are 0.4-0.6 mg iron/100 g in polished white rice (18, 51, 53, 54). Antinutritional factors, such as phytate, which interfere with bioavailability of micronutrients, are also located in the outer layers and removed during processing which improves the phytate:iron ratio (51, 52).

Fortification of rice with iron is recommended by WHO as a means of addressing iron deficiencies in rice-consuming populations (54). Regular consumption of fortified rice has been shown to improve micronutrient status and anemia prevalence in at-risk populations (18). However, fortification of rice is more complex than with other cereal grains, such as wheat,

because rice is consumed largely as intact kernels and many iron fortificants can result in unsatisfactory olfactory characteristics in finished rice products. Certain preparation methods, such as rinsing before cooking and boiling in an excess of water (discarded after cooking), result in additional loss of micronutrients (18, 52).

Processing

Four technologies are currently utilized to fortify rice: dusting, coating, cold extrusion, and hot extrusion. Both dusting and coating apply micronutrients to the exterior of natural rice kernels. In dusting, powdered micronutrients adhere to the rice kernel surface with electrostatic forces (51, 57). This method is not recommended for communities where practices of rinsing and boiling in excess of water are common (18, 54). Coating improves micronutrient retention over dusting. In coating, micronutrients are sprayed onto the kernels in several layers with waxes and polymers, adhering the nutrients to the kernels (18, 51, 57, 58). If the kernels are washed, micronutrient losses may be as high as 60% with more significant losses of water-soluble micronutrients, as high as 90%, when cooked in an excess of water (51).

In both types of extrusion, a rice dough, made from rice flour and micronutrients, is forced through small openings for form rice kernel-shaped extrudates. Cold extrusion is similar to pasta making where only mechanical energy is applied to create extrudates and reaches typical maximum processing temperatures of 30-40°C. In contrast, hot extrusion applies thermal energy in the form of heated barrel jackets, water, and steam reaching temperatures of 70-110°C (18, 51, 57).

Generally, the process selected for fortifying rice should be based on resources available, preferences, and local practices. Dusting is the most affordable option. Coating is another less costly option, but resulting kernels can carry distinct physical properties which consumers may

find unappealing (57). Because micronutrients are applied to the outside of the kernels and then diluted at a ratio of 1:200 to 1:50 with natural rice, these kernels may be easily picked out and discarded (51). Both extrusion processes also fortify at higher levels and blend a small amount of fortified kernels with natural rice. However, unlike dusting and coating, the micronutrients are distributed throughout the extruded kernels making them less visually distinctive against the natural rice (51). Cold extrusion is more affordable than hot extrusion, however, it results in kernels which are slightly off-color and opaque. Hot extrusion is the most costly rice fortification option, however, it results in the highest-quality product which most closely resembles natural rice (51, 57).

Effectiveness and Acceptability

In a study which evaluated the simulated effect of rinsing extruded rice and measured iron losses in the water, mean iron loss from ferric pyrophosphate (FePP) and μFePP were 1.0-2.6%, which was not significantly from different ferrous sulfate ($FeSO_4$), but significantly higher than losses from elemental iron (0.01-0.03%, 59). In practice, iron retention during cooking and absorption from coating, cold extrusion, and hot extrusion technologies were compared in a stable isotope feeding study in women (58). In experiments where the rice was cooked in an ideal amount of water, 1:2 rice:water ratio, no significant differences were observed in iron retention when no precooking treatment (rinsing or soaking) was applied. However, when the rice was soaked, hot extrusion resulted in significantly higher iron retention compared to the coated rice. In experiments where the rice was cooked in excessive amounts of water, 1:6 rice:water ratio, iron retention from all types of pretreatment and extrusion technologies was lower than when cooked in an ideal amount of water. Hot extrusion resulted in significantly higher iron retention compared to both cold extrusion and coating when no pretreatment was

taken; while both extrusion methods, hot and cold, resulted in significantly higher iron retention for both rinsing and soaking pretreatments compared to coating (58).

In absorption studies, corrected fractional iron absorption was significantly higher for cold extrusion compared to hot extrusion. The higher solubility from the cold extruded rice may be attributed to its starch microstructure, which is more similar to parboiled rice. In a second series of absorption studies, the fractional iron absorption and relative bioavailability (RBV) from hot extruded rice was not found to be significantly different than that from $FeSO_4$, the reference fortificant. While absorption and RBV from the coated rice was significantly lower than that of $FeSO_4$ (58).

Micronized ferric pyrophosphate in fortified rice has been evaluated in several human clinical trials. A 5-month study observed that fortified rice was more effective than iron drops for increasing serum ferritin and hemoglobin concentrations in anemic children (60). Another similar study utilized school feedings over 8 months and observed decreased iron deficiency prevalence in the fortified rice group compared to a placebo group (61). And in an 18-month study in infants, anemia prevalence was reduced with fortified rice consumption (62).

In addition to less micronutrient losses and improved iron status, minimal negative sensory impacts have been observed in iron-fortified extruded rice. Texture of cooked extruded rice kernels were compared with natural Jasmine and long grain rice kernels using a texture analyzer to simulate a two-bite compression. The rice extrudates were found to have similar hardness and springiness, but significantly different cohesiveness and adhesiveness compared with the natural rice comparisons (59). Uncooked fortified rice diluted at ratios of 1:100 or 1:200 were not found to be visually different than natural rice in 3 out of 4 samples and trained subjects could not differentiate between 1.5:100 fortified rice samples and natural rice. Despite the study

being underpowered, the results suggest that extruded rice fortified at 1:100-2:100 ratio with natural rice result in minimal visual uncooked differences and cooked differences (59). Both children and adult recipients of fortified rice in field trials scored the multiple types of fortified rice as acceptable or undistinguishable from natural rice in sensory tests (60, 61). Fortified, extruded rice when diluted with natural rice at common dilution ratios appear to produce minimal perceptible differences and are likely to be accepted by food aid recipient populations.

Iron Fortificants

Iron fortification is the most challenging of all micronutrients because many iron compounds result in undesirable sensory properties and have low bioavailability (63). Iron compounds used in food fortification may be grouped into categories based on solubility: freely water-soluble, dilute acid-soluble, and water insoluble/poorly dilute acid-soluble. Freely water-soluble iron fortificants, such as $FeSO_4$, are commonly used in foods including cereal grain flours and infant formulas because of relatively low cost and high bioavailability (64). These iron fortificants dissolve freely in gastric juice and are readily available for uptake (64). However, freely water soluble iron fortificants are not recommended for use in all foods, such as white rice, due to unappealing organoleptic properties, including color changes and rancidity, which may occur during storage (57, 59, 63, 65).

Ferrous fumarate and ferrous succinate are two iron fortificants from the group described as poorly soluble in water, however, readily soluble in dilute acids, including gastric juice. These compounds result in fewer undesirable sensory changes than freely water-soluble iron compounds. Studies have suggested that ferrous fumarate absorption is at least as good as absorption from ferrous sulfate, making it a suitable alternative in for use in foods such as infant cereals and FBFs (64).

The least well-absorbed iron fortificants are water insoluble and poorly soluble in dilute acid. These include elemental iron and iron phosphate compounds, including ferric phosphate ($FePO_4$, also known as ferric orthophosphate) and ferric pyrophosphate (FePP, 63, 64). Despite poor solubility in gastric juice, these compounds are the only ones recommended for fortification of rice because they result in minimal organoleptic changes (64, 66). In iron-fortified simulated rice grains, FePP resulted in white-opaque and slightly yellow-tone kernels which more closely resembled natural Basmati and Jasmine rice than the $FeSO_4$ simulated rice grains (59). Animal studies indicate absorption from these compounds is approximately half that of $FeSO_4$ (64).

Ferric phosphate is reported to have a RBV compared with $FeSO_4$ of 6-46% and 25-32% in rats and humans, respectively. Ferric pyrophosphate is reported to have higher RBV than $FePO_4$ of 45-58% and 21-74% in rats and humans, respectively (63). A variety of factors including the biological system, physical properties (particle size), and food composition impact absorption of these compounds (63).

An additional group of novel iron fortificants includes NaFeEDTA and hemoglobin. Hemoglobin is very well absorbed, however, its red-brown color and low iron content are not suitable for many food fortification applications (64). NaFeEDTA causes few unsatisfactory organoleptic changes and boasts advantages over other iron fortificants. NaFeEDTA can prevent iron from binding to phytate, an antinutritional factor present in many cereal grains and legumes which interferes with iron absorption. The result is increased absorption 2-3 times greater than $FeSO_4$ in many foods (64). The combination of few organoleptic changes, increased absorption, and that NaFeEDTA does not promote lipid oxidation makes it a particularly appealing iron fortificant in food products including FBFs.

NaFeEDTA can be added to cereal grain-based foods to increase absorption from other iron forms including ferrous sulfate and ferrous fumarate (67). In the 2011 FAQR, NaFeEDTA was recommended in addition to ferrous fumarate to enhance iron absorption (35). This is not the first time NaFeEDTA was recommended for inclusion in FBFs. The switch to ferrous fumarate was recommended in 1994 and two years later, in 1996, NaFeEDTA was additionally recommended (68). The combination of ferrous fumarate and NaFeEDTA is required in current CSB+ and Super Cereal *Plus* formulations (44, 69, 70).

Improving Iron Bioavailability

Strategies to improve the absorption from the least-bioavailable iron fortificants, such as FePP, have been evaluated and include the modification of the iron fortificant, addition of complementary compounds, or reduction of particle size. Increasing the concentration of iron fortificant used compared to ferrous sulfate may be used, however it is associated with increased cost (66). A modification of the fortificant is chelation. When FePP is chelated, the iron becomes bonded to citrate and phosphate ligands resulting in a soluble compound. Soluble, chelated FePP was observed to increase the *in vitro* bioavailability of FePP higher than that of $FeSO_4$ (71).

In humans, the addition of compounds such as zinc sulfate ($ZnSO_4$), trisodium citrate and citric acid (TSC and CA), and ascorbic acid have been observed to increase FePP iron absorption in single meal feeding studies. Both FePP without zinc and co-fortified with $ZnSO_4$ resulted in greater iron absorption than FePP co-fortified with zinc oxide. Relative bioavailability of FePP co-fortified with $ZnSO_4$ was marginally higher when compared with the FePP without zinc and both were significantly lower than the reference meal fortified with $FeSO_4$ (72).

Iron absorption was observed from four test meals with different iron fortification strategies: FePP, FePP with TSC and CA (FePP+TSC+CA) added before extrusion,

FePP+TSC+CA added after cooking, and $FeSO_4$. The fractional iron absorption, total iron absorbed, and RBV from the meal fortified with FePP+TSC+CA before extrusion were not significantly different than that from the reference meal fortified with $FeSO_4$. While all iron outcomes were significantly lower for the other two meals compared to both FePP+TSC+CA before extrusion and $FeSO_4$ meals (73). Citric acid and trisodium citrate added before extrusion was effective for increasing iron absorption from FePP, comparable to absorption from $FeSO_4$.

Ascorbic acid was reported to increase absorption of FePP in women by 2.6 times in infant cereal (74). Another study observed that ascorbic acid increased the iron absorption from both µFePP, mean particle size (MPS) of 2.5µm, and $FeSO_4$ in rice meals. Although the RBV of iron from the µFePP meal was much lower than that of the $FeSO_4$ (75).

Reduction of the MPS of FePP increases the surface area which in turn increases the absorption in gastric juice (59). In iron-depleted rats, µFePP with a MPS of 0.5 µm resulted in RBV which was not significantly different compared to $FeSO_4$. However, FePP with larger MPSs, 2.5 µm and 21 µm, resulted in RBVs significantly lower than the $FeSO_4$ and not significantly different from each other (76). In humans, RBV was not significantly different in a study of FePP MPSs ranging from 6.7 to 12.5 µm (74).

Iron Measures

Several measures and methods are available to asses iron bioavailability. Absorption, as discussed previously, from iron fortificants can vary drastically. Absorption varies based on the ability of the iron fortificant to dissolve for uptake, nutritional/iron status of the subject, and inhibitors or enhancers of iron absorption present in a food/meal. Because of these differences, absorption measured as RBV is often compared to $FeSO_4$ (64). Absorption is commonly measured by feeding subjects a meal or food with stable or radioisotope labeled iron. Iron

incorporation in the erythrocytes is measured 14 days after meal/food consumption (77). The absorption values from test meals are compared to that of a control meal consumed by each subject to calculate RBV.

In longer-term human studies, measurements of iron status are typically performed a minimum at baseline and study endpoint. Common measures include hemoglobin, serum ferritin, plasma ferritin, and soluble transferrin receptor. In animal studies, additional invasive measures can be evaluated, such as concentration of iron in the liver, where approximately 20-30% of body iron is stored, and can more precisely indicate iron status (78).

In rats, two methods are commonly employed: depletion-repletion method and preventative-prophylactic method. In the depletion-repletion method, animals are put on a low-iron diet to deplete iron stores and then fed the test diets during the repletion period (79). In the preventative-prophylactic method, no repletion period is used and animals are placed on test diets immediately after weaning (80). Both methods have benefits, however the prophylactic method is more advantageous when also evaluating protein quality and growth because it requires fewer animals which may be evaluated during the linear growth phase.

Protein Quality

Rat Model

The Protein efficiency ratio (PER) has been used for evaluation of protein quality in human nutrition for a century (81). Protein efficiency ratio assesses protein quality by calculating the weight gain divided by protein consumed in rapidly growing rats.

$$PER = \frac{weight\ gain\ of\ test\ group\ (g)}{protein\ consumed\ by\ test\ group\ (g)}$$

Laboratory rats have been used as experimental animals since the 19th century and are often suitable models for nutrition experiments due to similarities of the digestive tracts of rats

and humans (82). However, there are key limitations which impact PER applicability for determining protein performance in humans. In part due to the rapid growth, rats require greater amounts of certain amino acids including histidine, isoleucine, threonine, and valine than humans (81). Rats additionally require higher levels of the sulfur-containing amino acids which support fur growth (83). Rapid growth is accompanied by use of some protein for body maintenance, which is not reflected in the PER calculation (81). The requirement of protein for maintenance is proportionally lower in rats than in humans. This additional difference contributes to discrepancies in PER for predicting protein performance in humans (83).

Generally, these discrepancies result in PER values which over-estimate the quality of animal-source proteins and under-estimate the quality of plant-source proteins for supporting human growth. This results in economic, rather than health, implications by potentially promoting a need for more expensive proteins than are necessary (81). PER is widely used and valuable for predicting protein performance in humans, however, the limitations must be considered when estimating protein quality.

Protein Measures

In addition to PER, several other methods are used to assess and report protein quality, although none are perfect measures of a protein's ability to support the needs of a target human population (84). Amino acid score (AAS) is a ratio of the amount of a certain limiting AA in a test protein compared to that of a reference protein, often egg (81). The first limiting amino acid, the essential/indispensable AA (EAA) available in the lowest quantity, is used for determining a protein's AAS.

$$AAS = \frac{AA\ in\ test\ protein\ (mg/g)}{same\ AA\ in\ reference\ protein\ (mg/g)}$$

Protein Digestibility Corrected Amino Acid Score (PDCAAS) was adopted by the Food and Agriculture Organization (FAO) and WHO in 1991. PDCAAS is the AAS for a protein adjusted for digestibility, the proportion of nitrogen absorbed from the protein source (81). The PDCAAS method is criticized for several reasons: scores are truncated at 100% (not accounting for increased nutritional value some proteins provide), it uses fecal digestibility (instead of ileal), does not account for antinutritional factors or bioavailability (over-estimating quality from some proteins), and the scoring pattern is not representative for all persons (84, 85).

$$PDCAAS\ (\%) = AAS \times true\ N\ digestibility\ (\%)$$

The newest measure was introduced in 2013 by the FAO, Digestible Indispensable Amino Acid Score (DIAAS), and is recommended as a replacement for PDCAAS. DIAAS improves upon the criticisms of PDCAAS by accounting for individual amino acid digestibility, not truncating scores, focusing on ileal digestibility, and utilizing three scoring patterns (84).

$$DIAAS\ (\%) = 100 \times \frac{digestible\ dietary\ indispensable\ AA\ in\ test\ protein(mg/g)}{same\ dietary\ indispensable\ AA\ in\ reference\ protein\ (mg/g)}$$

Protein Sources and Quality

The quality of protein provided by different sources varies on AA composition and content, speed of digestion, and the ability of AAs to be used for protein synthesis (86). The 9 EAAs which cannot be synthesized are: histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. Proteins from animal sources are considered to be complete because they contain adequate levels of all EAAs. Animal-source proteins are considered to be of high-quality because they are generally well digested and easily used for protein synthesis (86). Protein provided by most plant sources is considered incomplete due to providing an insufficient amount of at least one of the 9 EAAs and generally lower quality due to presence of antinutritional factors and faster rates of digestion (86, 87).

Lysine is most often the first limiting AA in plant proteins. However, not all plant proteins are limited by the same AAs – grains are limited by lysine while legumes are limited by methionine (86, 88). Despite limitations, a modest amount of food higher in the limiting AA, from plant or animal sources, can satisfactorily meet human needs (88). Combining complementary plant proteins, such as grains and legumes, is a common strategy used to overcome AA limitations. Some plant proteins, such as soy, contain all 9 EAAs and may be considered complete proteins. Although, the concentrations of some EAAs may be lower than found in animal-source proteins or availability of AAs may be reduced (86).

Plant sources of protein often have lower AA digestibility compared to AAs from animal sources. This, combined with faster digestion (increased urea synthesis), may explain the limited evidence which suggests there is lower muscle protein synthesis from plant sources compared to animal sources (89). While digestibility of protein from plant sources has been reported to be approximately 45-80% (compared to 90% from animal sources), processing can effectively and economically improve digestibility (66). For example, SPI has a reported equivalent protein digestibility compared with animal source proteins (84). In addition to processing, anabolic response from plant sources of protein can be increased with AA fortification, combining complimentary plant protein sources, and increasing total amount of plant-source proteins consumed (89).

Inhibitors are antinutritional factors present in plant sources of protein which reduce the bioavailability of AAs. Bioavailability is used describe the overall effects from a food on digestibility, usable proportion of AAs, and amount of metabolism interference. Digestibility accounts for the greatest amount of variation in reported bioavailability (84). Common inhibitors in plant sources of proteins include tannins, trypsin inhibitors, and phytate (phytic acid). Tannins

present in cereal grains and legumes can precipitate proteins (90). While trypsin inhibitors are present in legumes and phytate, present in grains, seeds, and nuts, chelates nutrients (90). Processing can remove or minimize negative effects of antinutritional factors. For example, wet methods of heating such as boiling can improve digestibility (86). Much of the phytate can be removed with processing (milling) which removes the outer layers, where phytate is most concentrated (90).

Protein Sources in Food Aid

Recent research has been conducted on efficacy of plant- versus animal-source proteins for supporting needs of the vulnerable recipients of food aid. Dairy products, primarily skim milk powder and WPC, are commonly used in food aid products and have shown to be effective for treating moderate acute malnutrition in children (41). WPC has been proposed to replace skim milk powder in products such as Super Cereal *Plus* because it has reduced cost as a co-product of cheese production compared to skim milk powder (41). However, some have criticized inclusion of animal-source proteins in food aid products. One criticism includes that rapid growth from consumption of dairy products may be associated with an increased non-communicable disease risk later in life (41). More information is needed for understanding how dairy product inclusion in food aid impacts long-term health in recipient populations.

Despite reported lower quality, in recent human trials, foods with only plant-source proteins have performed similarly to those which contain animal-source protein for managing PEM (91-95). Furthermore, exclusively plant-source protein food aid products have been associated with greater improvements in iron outcomes compared to foods which contain animal-source proteins (96, 97). A recent systematic review did not find a strong relationship of animal-source foods inclusion in food aid products for addressing PEM. However, the authors noted a

high amount of heterogeneity among studies, which included varied study designs and a wide variety of animal-source foods evaluated (98). Plant-source proteins are promising, cost-effective alternatives to animal-source proteins in food aid products. However, more research is needed to better understand the outcomes associated with plant- and animal-source proteins in food aid products.

Chapter 2 - Evaluation of protein quality and iron bioavailability from nine extruded corn-soy and sorghum-soy fortified blended foods with and without whey protein in rats

Abstract

Background: Previously we found that extruded corn-soy blend (CSB) and sorghum-soy blend (SSB) fortified blended foods (FBFs) containing whey protein concentrate (WPC) are equally nutritious food aid products. WPC is commonly added to FBFs as a source of high-quality protein, however, it is the most expensive ingredient in these FBFs.

Objectives: The primary objective of this study was to determine if protein from soy flour may serve as an alternative to WPC in FBFs. A secondary objective was to evaluate different sucrose concentrations in the FBFs.

Methods: Extruded CSB and SSB FBFs were developed with increased soy flour to meet protein requirements. Sucrose content ranged from 0–10% in CSBs (CSB-0, CSB-5, CSB-10) and 0–15% in SSBs (SSB-0, SSB-5, SSB-10, SSB-15). Previously developed FBFs with 9.5% WPC and 15% sucrose served as comparison diets (CSB-WPC, SSB-WPC). Male, weanling Sprague Dawley rats were individually housed and divided into 10 diet groups (n=9-10) which consumed assigned diet, either AIN-93G or one dry FBF, for 28 days. Food intake was measured every other day and body weights were recorded weekly. At study conclusion, blood and livers were collected to evaluate iron outcomes and body scans were performed to assess body composition and bone mineral density (BMD). Results were analyzed using one-way ANOVA with Tukey's test and significance at $p<0.05$.

Results: Outcomes were not significantly different among the SSB groups, with the exception of significantly higher protein efficiency for the SSB-WPC group. Among the CSB groups, both caloric and protein efficiencies were significantly higher for the CSB-WPC group compared to the non-WPC groups. There were no significant differences in hemoglobin or hepatic iron concentrations between FBF groups, but hepatic iron concentrations were significantly higher in all FBF groups compared to the AIN-93G group. In additional analyses grouped by sucrose content, the FBF groups consuming ≤10% sucrose diets had significantly higher BMD compared to groups with 15% sucrose.

Conclusion: With all findings considered, these results suggest that extruded SSB, but not necessarily CSB FBFs, with soy protein and up to 10% added sucrose are efficacious and cost-effective alternatives to WPC-containing FBFs in growing rats.

Background

Fortified blended foods (FBFs) are an important component of food aid for treatment and prevention of undernutrition and micronutrient deficiencies, including deficiencies of iron, the most prevalent micronutrient deficiency (16, 19, 35). Consumed primarily as complimentary foods, FBFs are distributed as partially cooked, energy-dense, dry grain-legume blends fortified with micronutrients. Sugar, oil, and an additional protein source may be included in FBF formulations. Recipients prepare FBF powders with water to form a porridge for consumption. Corn-soy blend (CSB) FBFs, including CSB+, represent a considerable portion of aid provided by the United States Agency for International Development (USAID) and partnering organizations (35, 99).

The 2011 Food Aid Quality Report (FAQR) recommended reformulation of FBFs to provide increased protein, fat, and total calories (35). Sorghum was recommended as an

alternative, non-genetically modified cereal crop for use in FBFs. Sorghum is appealing because it can be cultivated in hot, dry regions which experience low rainfall where crops like corn are less likely to thrive and because it is a familiar a food for many food aid recipient nations, such as those in Sub-Saharan Africa (35, 39, 40).

Extruded FBFs made with sorghum, corn, soy, and/or cowpea, formulated based on FAQR recommendations, improved iron bioavailability and protein digestibility compared to a non-extruded FBF, CSB+, in animal models (42, 50). Extrusion was selected for these FBFs because it utilizes heat, pressure, and mechanical stress to process and cook foods, reducing preparation time (46, 57, 100). These novel, extruded FBFs included 9.5% whey protein concentrate (WPC) or soy protein isolate (SPI), 9% vegetable oil, 3.2% micronutrients, and 15% sucrose, which was added to meet viscosity requirements (42). In Tanzanian children, significant improvements were observed from baseline in hemoglobin concentrations, anemia, and vitamin A statuses in the novel FBF-consuming groups, however, these findings were not significantly different for the CSB+ group (91).

Whey protein was included in the novel FBFs as a source of high-quality, animal-source protein, based on the FAQR recommendation (35). However, 3% addition of WPC increases the total cost of FBFs by approximately 18% (37). Available research led some to question whether the increased cost is justified to obtain the desired improvements (37, 38, 43). Soy protein, which is approximately half the cost of whey protein, may serve as an alternate plant-based, high-quality protein in FBFs (43, 101).

In support of this possibility, SPI was observed to be a viable, cheaper alternative to WPC in sorghum-cowpea FBFs in broiler chicks (50). In addition to the testing the efficacy of SPI in place of WPC in the chickens, changes in extrusion processing parameters allowed

sorghum-based FBFs to meet viscosity requirements without the addition of sugar. This additional reformulated, “overprocessed,” sorghum-cowpea blend resulted in similar anthropometric and iron outcomes to the blends with 15% added sucrose (50).

In the present study with male, weanling Sprague Dawley rats, 2 previously developed WPC-containing FBFs were prepared with corn-soy or sorghum-soy grain-legume blends and 15% sucrose. Based on an interest to test whether less expensive formulations may be equally efficacious and results from the study in chickens, 7 new corn-soy and sorghum-soy FBFs were formulated. These new blends align with FAQR nutrient recommendations and contain increased soy flour (compared to WPC-containing blends) and varying amounts to sucrose (35).

The primary objective of this study was to determine if protein provided by soy flour may serve as a suitable and cheaper alternative to 9.5% WPC in extruded corn-soy and sorghum-soy FBFs at several different sucrose concentrations. Secondary objectives were to evaluate feeding behaviors and outcomes as a result of different sucrose levels, from 0-15%, in the FBFs and further compare sorghum-soy and corn-soy blends.

Methods

Ethical Standards

Animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Kansas State University (protocol 4016). Animals were assessed for well-being prior-to and throughout the study for the duration of the experiment.

FBFs

Corn-soy blend and sorghum-soy blend (SSB) FBFs were developed based on recommendations in the FAQR (35) and previous studies (42, 50). Corn-soy and sorghum-soy flour blends were extruded, milled, and mixed with the additional ingredients as described

previously (42, 50, 57). Nine FBFs were formulated with different sucrose levels and either WPC or adjusted levels of soy flour to provide a similar amount of protein (Table 2.1). Four CSB FBFs were developed: 1 with 9.5% WPC and 15% sucrose (CSB-WPC, comparison FBF); 3 contained no WPC, increased soy flour, and varying amounts of sucrose: 0% (CSB-0), 5% (CSB-5), and 10% (CSB-10). A fourth non-WPC CSB FBF with 15% sucrose was developed, however, due to flow issues inside the extrusion barrel which locked the screw, it failed to extrude, and thus is not included in this study. Five SSB FBFs were developed: 1 with 9.5% WPC and 15% sucrose (SSB-WPC, comparison FBF); 4 contained no WPC, increased soy flour, and varying amounts of sucrose: 0% (SSB-0), 5% (SSB-5), 10% (SSB-10), and 15% (SSB-15). FBFs were evaluated for compliance with USDA FBF viscosity requirements (48). Vitamin and mineral contents of the FBFs are described previously and listed in Table 2.2 (42, 102).

Nutritional Analyses

Iron concentrations were assessed by atomic absorption spectrophotometry in duplicate (AACC Official Method 40-70.01) by AIB International (Manhattan, KS). Macronutrient proximate analyses, amino acid profiles, and available lysine were assessed by the University of Missouri–Columbia Agricultural Experiment Station Chemical Laboratories (Columbia, MO). Methods for macronutrients included: protein (combustion analysis, LECO; AOAC 990.03, 2006), fat (acid hydrolysis, 954.02, 2006), and carbohydrates by calculation. Total calories were determined by calculation where: protein = 4 kcal/g, carbohydrate = 4 kcal/g, and fat = 9 kcal/g. Amino acid profiles were determined by AOAC Official Methods 982.30 E(a,b,c), chp. 45.3.05, 2006, for tryptophan: alkaline hydrolysis 988.15, chp. 45.4.04, 2006 or enzymatic hydrolysis by colorimetric determination, and for available lysine: 975.44, chp. 45.4.03, 2006.

$$Carbohydrates = 100\% - \%(crude\ protein + ash + crude\ fat + moisture)$$

Study Design

Male, weanling Sprague Dawley rats (Charles River, Wilmington, MA) were randomized into 10 diet groups (n=10, 100 total). A control group was fed American Institute of Nutrition (AIN)-93G, standard diet for growing rats (Research Diets, Inc., New Brunswick, NJ) and the additional 9 groups were assigned to consume one of the dry FBFs. One rat (CSB-5 group) died and another (SSB-15 group) was euthanized, both attributed to preexisting health conditions. A rat in the CSB-10 group acutely lost weight at study midpoint and never recovered this loss; as a result, this animal was excluded from analyses. Two additional animals, 1 from the SSB-0 group and 1 from the SSB-10 group, were excluded for a concern that they may have consumed food beyond their assigned FBF. Animals were individually housed in wire-bottom cages and provided with a resting board, enrichment products, and *ad libitum* access to food and water for the 28-day study. The environment was temperature-controlled with 12-hour alternating light and dark cycles. Feedings occurred every other day where remaining food was weighed and fresh food was provided. The rats were weighed upon arrival and weekly thereafter. The study duration and size were based on the prophylactic (80) and protein efficiency ratio (PER, 103) methods that we have utilized previously (42).

Data and Sample Collection

At study conclusion, animals were euthanized with carbon dioxide (CO_2) inhalation followed by cardiac puncture. Blood was drawn and collected in K2 EDTA vacuette tubes for hemoglobin analysis. Tubes were stored on ice and later transferred to a 4°C refrigerator where they were stored for 36 hours prior to hemoglobin analysis. After blood collection, liver tissues were collected, weighed, and flash-frozen in liquid nitrogen. After flash-freezing, liver tissues were stored in a -80°C freezer until wet ashing. Following tissue removal, body scans were

performed on a dual energy x-ray absorptiometry (DEXA) PIXImus densitometer according to manufacturer's procedures (GE Lunar Corporation, Madison, WI) to determine body composition and bone mineral density (BMD).

Iron Analysis

Hemoglobin

QuantiChrom Whole Blood Hb Kit (DWHB-250, BioAssay Systems, Hayward, CA) was used to quantify hemoglobin concentrations. The kit used a triton/sodium hydroxide (NaOH) method to uniformly color the hemoglobin from whole blood samples. Color intensity was measured by spectrophotometry at 570 nm. Hemoglobin concentration was then determined via calculation.

$$Hemoglobin = \frac{Optical\ density\ (OD)_{Sample} - OD_{water}}{OD_{calibrator} - OD_{water}} \times Calibrator$$

Samples were analyzed in duplicate according to the manufacturer's procedure. An additional (triplicate) sample was assessed for duplicates with greater than 15% variance.

Hepatic Iron

Samples were prepared and analyzed at the Kansas State University Soil Testing Lab (Manhattan, KS) by inductively coupled plasma-optical emission spectrometry (ICP-OES, Varian 720-ES, Agilent Technologies, Santa Clara, CA). All glassware for the procedure was prepared in a 6% nitric acid solution prior to use. Samples were thawed from -80°C to 4°C in a refrigerator overnight prior to analysis and re-frozen to -80°C after 1 g samples were removed for analysis. Tissue samples were each covered with 10 mL of *TraceMetal* grade nitric acid solution (Fisher Chemical, Pittsburgh, PA) in a 50 mL beaker and allowed to degrade for 1 hour. Samples were then allowed to gently reflux on a hot plate until approximately 1 mL of solution remained in the beaker, approximately 1-3 hours. Once cool, samples were diluted to 10 mL with

distilled-deionized water and stored in 15 mL polypropylene tubes at room temperature prior to ICP-OES. Samples were prepared in duplicate. Duplicates with more than a 15% variance were assessed an additional time (triplicate).

Calculations

Calculations were performed to determine caloric and protein efficiencies and lean mass using individual animal data.

$$Caloric\ efficiency = \frac{weight\ gain\ (g)}{food\ intake\ (g)\ \times total\ calories\ per\ diet \div 100\ kal}$$

$$Protein\ efficiency = \frac{weight\ gain\ (g)}{food\ intake\ (g)\ \times\ total\ protein\ per\ diet\ (g)}$$

$$Lean\ mass\ (\%) = \frac{total\ weight\ (g) - fat\ mass\ (g)}{total\ weight\ (g)} \times 100$$

Statistical Analysis

Results were assessed for normality using Shapiro-Wilk's test and for homogeneity of variance with Levene's test. Natural log or square root transformations were used if assumptions for normality were not met. Group differences were assessed using one-way analysis of variance (ANOVA) with Tukey's test and significance at $p<0.05$. Differences in BMD between groups based on percent sucrose in formulation (≤10% and 15% sucrose) were assessed with a t-test at $p<0.05$. Data are reported as group means with standard deviation. Statistical analyses were performed in SAS Studio (Version 3.71, SAS Institute Inc., Cary, NC).

Results

FBF Composition

On average, the FBFs provided 5.1% more energy, 3.7% more protein, and 26.2% more fat than the AIN-93G diet (Table 2.3). The total energy and macronutrients were similar across all FBFs. The FBFs with increased soy protein contained on average 2.1% more protein, 53.2%

more fiber, 16.9% less available lysine, and 21.8% less cysteine and methionine than the FBFs with WPC. Iron content of the FBF diets was on average 75.1% greater than the AIN-93G diet. The FBFs without WPC contained on average 8.1% more iron than the FBFs with WPC. The SSB FBF diets contained on average 7.4% more iron than the CSB diets.

Food Intake and Efficiencies

No significant differences were observed among FBF groups for food intake with the exception of the SSB-0 group (Table 2.4, Figure 2.1). The SSB-0 group had significantly higher food intake compared to the CSB-WPC group.

Both WPC-containing FBF groups had significantly increased caloric efficiency compared to all non-WPC-containing CSB FBF groups and significantly increased protein efficiency compared to all other diet groups (AIN-93G, non-WPC SSBs, and non-WPC CSBs). The CSB-5 group's caloric efficiency was significantly decreased compared to AIN-93G, SSB-0, and SSB-5 groups and protein efficiency significantly decreased compared to the AIN-93G group.

Anthropomorphic Outcomes

No significant differences were observed among FBF groups for total weight gain and final weight with the exception of the SSB-0 group (Table 2.4, Figure 2.2). The SSB-0 group had significantly higher total weight gain and final weight compared to the CSB-5 group.

No significant differences were observed in lean body mass among groups (Table 2.5). No significant differences were observed between FBF groups for BMD. However, the three FBF groups which contained 15% sucrose (CSB-WPC, SSB-WPC, and SSB-15) had significantly lower BMD than the AIN-93G group.

Given that we found significantly reduced BMD in all 15% sucrose groups compared to the AIN-93G group, we were interested to further investigate a potential threshold effect among the FBF groups. FBF groups were organized into two new groups based on sucrose content: one with all ≤10% sucrose groups and another with all 15% sucrose groups. The ≤10% sucrose group mean BMD (83.2 ± 10.4 g/cm^2 x 1000) was significantly greater than the 15% sucrose group mean BMD (77.5 ± 10.1 g/cm^2 x 1000, p=0.0129).

Iron Outcomes

No significant differences were observed in hemoglobin concentrations among all groups (Table 2.5). Hepatic iron concentrations were significantly higher in all of the FBF groups compared to the AIN-93G group. No other differences were observed except the CSB-5 group had a significantly higher hepatic iron concentration than the SSB-15 group.

Discussion

CSB and SSB Group Comparisons

Previously we found that sorghum and cowpea are suitable alternative ingredients to corn and soy in extruded FBFs in animal studies and a human efficacy trial (42, 50, 91). We additionally found that a sorghum-cowpea blend with SPI performed similarly to the same blend with WPC and hypothesized that protein from soy flour may serve as a suitable alternative to WPC in FBFs (50). In the present study, the SSB FBFs all resulted in similar anthropometric and iron outcomes in weanling, male rats. The only significant difference among SSB groups was an increased protein efficiency for the SSB-WPC group compared to the non-WPC SSB FBFs. Among the CSB groups, there were similarly few significant differences in outcomes. While intake did not differ significantly, the CSB-WPC group had somewhat lower intake which corresponded with higher caloric and protein efficiencies compared to the non-WPC CSB FBFs.

Protein Outcomes

The increased protein efficiency for both SSB and CSB WPC groups compared to respective non-WPC groups may in part be explained by similar growth and food intake among all groups combined with overall less protein consumed by the WPC groups. The SSB-WPC FBF contained approximately 6.4% less protein compared to the SSB FBFs with increased soy flour. Because PER is calculated based on total protein consumed, slightly more or less protein magnifies differences in protein efficiency. PER is additionally not a proportional measure of protein intake and corresponding growth since it does not account protein used for maintenance (100, 104). The soy-based FBFs also contained limiting amounts of essential amino acids which were present at greater quantities in the WPC FBFs (lysine, cysteine and methionine). Another consideration for the observed significant differences in PER is that soy protein is generally less digestible than protein from animal sources (105).

Findings of reduced protein efficiency from soy protein-based diets with no differences in food intake has been observed previously. Significantly lower body weight gain and protein energy efficiency from a soy-based diet compared to a whey-based diet were observed in 5 week old male Sprague Dawley rats (106). In a study with Wistar rats, the soy group had significantly lower protein efficiency, body weight, total gain, and fat and lean mass gain compared to the whey group (107).

While PER in rat models is an important outcome for evaluating the quality of the FBFs, there are a few key differences between human and rat protein requirements which may further support the efficacy of soy flour-based SSB FBFs for humans. Protein used by weanling rats is predominantly for growth; in humans, even during phases of rapid growth, a higher proportion of protein is required for body maintenance (83, 104). Rats additionally require 50% more of the

sulfur-containing amino acids, cysteine and methionine, which support fur growth and were the most limiting amino acids in our soy-based FBFs (83). Differences in amino acid requirements result in lower PDCAAS values for rats than humans (SPI: 64 in rats, 100 in humans; skim milk powder: 74 in rats, 100 in humans, 103) and an over-estimation of quality of animal-source proteins compared to plant-source proteins for human growth (104).

In our 20-week trial with Tanzanian children, we observed that our novel extruded FBFs with WPC performed similarly to CSB+. However, this study was too short and underpowered to earnestly assess anthropometric outcomes (91). Despite limitations of being underpowered and early termination, in an unadjusted model, a 10-week study did not find a significant difference in the proportion of Sierra Leone children who recovered from moderate-acute malnutrition with a CSB FBF (similar to CSB+), which contained no animal-source foods, compared to a CSB FBF with WPC (94, 95). Additional trials have demonstrated that animal-source protein in ready-to-use food aid products do not necessarily result in better anthropometric outcomes in malnourished children (92, 93). Furthermore, a recent systematic literature review was unable to identify a relationship between animal-source foods and improved growth outcomes. This review was limited by large heterogeneity between studies, including study design and wide variety of animal-source foods evaluated (98).

Compared to CSB+, we believe our FBFs without WPC offer superior protein quality, in part due to extrusion processing improving bioavailability and because they offer more lysine and cysteine and methionine in comparison, and that they would perform at least as well as CSB+ in a human trial. Considering findings from these studies and the limitations of PER, we believe the SSB FBFs without WPC developed for this study are an efficacious alternative to the SSB-WPC FBF and may lead to similar anthropometric improvements in vulnerable children.

Iron Outcomes

The other main outcome of interest for this study was iron status. Hepatic iron concentrations were significantly higher in all of the FBFs compared to the AIN-93G group. This difference is most likely explained by the much higher iron content and more bioavailable iron fortificant used in the FBFs compared to AIN-93G. AIN-93G is fortified with ferric citrate, which is less bioavailable than ferrous fumarate and NaFeEDTA, iron fortificants in the FBFs (108). The hepatic iron concentration of the CSB-5 was marginally higher than all the other FBF groups, and statistically higher than only the SSB-15 group. The CSB-5 group was on average smaller than all the other groups at the study conclusion. Lower growth and a likely lower blood volume of the rats in the CSB-5 group may have resulted in decreased demand for circulating iron which allowed those animals to store more iron compared to the other groups (42).

We did not observe any differences in hepatic iron levels with increased sorghum content, unlike our previous FBF rat study (42). Soy has also been shown to inhibit iron absorption in humans (109), but no significant impacts on the iron outcomes with increased soy content of FBFs developed in this study were observed.

Sucrose and BMD Outcomes

In the previous extruded FBF rat study, CSB+, which contains 0% sucrose, was poorly consumed and it was hypothesized that the 15% sucrose content, which was added to meet viscosity requirements, may have contributed to animal feeding behaviors (42). Addition of sugar, either from sucrose or fruit, has been observed to reduce or mask potentially unappealing flavors such as soy, grain, and bitter notes in FBF cereals and porridges (110, 111). Rats have been observed to prefer the taste of sucrose (112), although there are differences in rat and human taste perceptions (113).

As a result of changes in extrusion processing parameters, we were able to test formulations in the present study with varying levels of sucrose. While we cannot be certain how the rats perceived the FBFs, it does not appear that sucrose content impacted feeding behaviors in this study because all the FBFs were equally well consumed.

Humans, particularly infants and children, tend to prefer the added sweetness that sugar or fruit provides to FBFs. Field observations in Tanzania found that caregivers added sugar and/or fruit to breakfast porridges made from corn and sorghum when feasible (110). And a 2018 study observed that children preferred SSB FBFs with 15% added sucrose over CSB+ and hypothesized it is due to their preference for sweeter foods and familiarity with sorghum (114).

The FBFs with added sucrose were thinner than the respective blends without added sucrose, and each 5% increase in sucrose resulted in a slight additional thinning of the porridge. Notably, the SSB FBFs, including the blend with 0% sucrose, were all thinner than all the CSB FBFs which may be a result of less starch accessibility and protein interference in sorghum compared to corn (115). The thinner prepared viscosities of the SSB FBFs is advantageous. Caregivers have been observed to thin porridges to their desired flow for infant feeding, which can result in insufficient caloric density of prepared FBFs (110, 116). The thinner nature of the SSB FBFs offers more nutrient density per volume of food consumed compared to CSB FBFs when thinned to similar viscosities which is advantageous in ensuring adequate nutrient intake by recipient populations.

Compared to our previous rat study, we observed similar BMDs for extruded FBFs, all of which contained 15% sucrose in the previous study. However, the BMD for the control group was higher in the present study than in the previous (95.4 vs. 87.4 g/cm^2). The higher control

group BMD compared to the previous study may explain why there were no significant differences between the extruded FBFs with 15% sucrose and AIN-93G previously (42).

The differences in BMD related to sucrose-content was an interesting and unexpected finding. AIN-93G is formulated with 10% sucrose (117) and possible that in weanling rats, BMD is negatively affected by a sucrose content of greater than 10% in the diet. One other rat study similarly did not find any significant differences in 2 month old Sprague Dawley rat BMD with diets which contained less than 10% sucrose (118).

In 5 week study of weanling Wistar rats, a 43% sucrose diet resulted in tibia/femur densities and breaking strengths that were significantly decreased compared to a control diet (43% potato starch, 119). In weanling male rats fed either a 68% corn starch or a 68% sucrose diet, there were no differences in bone composition or mechanical properties. However, BMD, total intake, and weight gain for animals was not reported and it is unclear if the diets were fortified with micronutrients (120).

Ad libitum access to AIN-93G and one solution: deionized distilled water or deionized distilled water with 13% sugar (glucose, sucrose, fructose, or high fructose corn syrup (HFCS)), was provided to 35 day old female Sprague Dawley rats for 8 weeks (121). Total sugar intake (sucrose from AIN-93G plus respective sugar solution) by groups with the sugar-sweetened solutions was approximately 3-7 times more than the control group. None of the sugar solution groups' whole-femur BMDs were significantly different compared to the control, but the glucose group had significantly reduced BMD compared to each sucrose, fructose, and HFCS groups. The glucose solution group consumed the most sugar of all groups; high sugar consumption, displacement of mineral-rich food, and increased mineral excretion are possible mechanisms

which contributed to the decreased BMD (121). Quality of data presented should be considered regarding these findings.

Additional studies have evaluated the potential effect of glucose, or more broadly sucrose/sugar, on bone mineralization, and mineral excretion. A review proposed that glucose may be primarily responsible for sucrose's adverse effects on bone. High glucose intake may inhibit osteoblast function, impeding bone mineralization and eventually leading to bone loss (122). Fewer minerals available for bone formation due to increased excretion may further contribute to decreased bone mineralization. Diets high in sucrose contribute to elevated insulin levels, which inhibit calcium reabsorption and lead to increased calcium excretion (123). These mechanisms are both biologically plausible as supported by animal models; decreased bone calcium concentrations have been observed in animals fed sucrose-containing diets compared to sucrose-free diets (119). While it is unclear how sucrose, or its constituents, glucose and fructose, may impact bone health, mechanisms have been proposed which may explain our findings. Further research is needed to elucidate the effects of high sucrose consumption on bone health and at which dietary concentrations negative clinical effects being to emerge.

Limitations

Several limitations for this study include the relatively short duration of 4 weeks, which took place during the rats' rapid, linear growth period, and the poor health of some of the animals, which resulted in the loss of two rats. In addition, the dry FBFs that the rats consumed is not typical of how humans consume the FBFs, as boiled porridges. We identified an interesting outcome of sucrose content related to BMD, however, due to not anticipating this difference, we did not gather additional data or samples that could have been used to better understand this outcome.

Conclusions

Similar growth, anthropometric, and iron outcomes were observed comparing the non-WPC containing sorghum-soy FBF groups to both WPC-containing FBF groups. Despite differences observed in protein efficiencies, the sorghum-soy FBFs with increased soy flour may be a suitable, less expensive alternative to FBFs with WPC considering the lack of significant differences in all outcomes evaluated. While protein quality is important and served as a main outcome of interest for this study, the differences in protein efficiency do not necessarily suggest that the FBFs without WPC will be inadequate to address the protein needs of food aid recipients. We believe the FBFs with increased soy flour evaluated in this study offer superior protein quality to CSB+ and are likely to result in similar outcomes in vulnerable children. The observed correlation between sucrose and BMD is of concern and this research supports the addition of ≤10% sucrose to the FBFs without negatively impacting BMD. The data does not provide enough evidence to suggest that sucrose contents from 10-15% should not be used in FBFs, however further research evaluating sucrose's role in bone health is warranted. These results suggest that SSB FBFs with 5-10% added sucrose, to increase appeal over 0% sucrose formulations, and protein from soy flour are efficacious and cost-effective alternatives to extruded FBFs with 15% sucrose and 9.5% WPC.

Tables

Table 2.1 FBF formulations (%)

	CSB-WPC	CSB-0	CSB-5	CSB-10	SSB-WPC	SSB-0	SSB-5	SSB-10	SSB-15
Low-fat Soy Flour	15.2	32.0	33.0	34.0	16.0	32.0	33.0	34.0	35.0
Degermed Coarse Corn Flour	48.1	55.8	49.8	43.8	—	—	—	—	—
Decorticated White Sorghum Flour	—	—	—	—	47.8	56.3	50.3	44.3	38.3
Whey Protein Concentrate	9.5	—	—	—	9.5	—	—	—	—
Sucrose	15.0	—	5.0	10.0	15.0	—	5.0	10.0	15.0
Vegetable Oil	9.0	9.0	9.0	9.0	8.5	8.5	8.5	8.5	8.5
Vitamin-Mineral Premix	3.2	3.2	3.2	3.2	3.2	3.2	3.2	3.2	3.2

AIN-93G formulation: cornstarch (39.7), casein (20.0), dextrinized cornstarch (13.2), sucrose (10.0), soybean oil (7.0), cellulose (5.0), mineral mix (3.5), vitamin mix (1.0), L-cysteine (0.3), choline bitartrate (0.25), tert-butylhydroquinone (TBHQ, 0.001).
CSB-WPC: corn-soy blend (CSB) with whey protein concentrate (WPC) and 15% sucrose; CSB-0: corn-soy blend with 0% sucrose; CSB-5: corn-soy blend with 5% sucrose; CSB-10: corn-soy blend with 10% sucrose; SSB-WPC: sorghum-soy blend (SSB) with WPC and 15% sucrose; SSB-0: sorghum-soy blend with 0% sucrose; SSB-5: sorghum-soy blend with 5% sucrose; SSB-10: sorghum-soy blend with 10% sucrose; SSB-15: sorghum-soy blend with 15% sucrose.

Table 2.2 Vitamins and minerals per 100g of FBF (mg)

Vitamin A Palmitate	0.488	Coated Ascorbic Acid	40.0
Thiamin Mononitrate (B_1)	0.652	Calcium (Tri-Calcium Phosphate)	279.08
Riboflavin (B_2)	0.933	Iron	13.0
Niacinamide (B_3)	9.07	Sodium Iron EDTA	1.47
Calcium D-Pantothenate (B_5)	3.646	Ferrous Fumarate	3.79
Pyridoxine Hydrochloride ($B_{6)}$	0.752	Iodine (Potassium Iodide)	0.23
Folic Acid (B_9)	0.087	Magnesium Oxide	9.47
Vitamin B_{12}	0.0015	Phosphorus (Tricalcium Phosphate)	290.97
Vitamin D3	0.0292	Potassium (Potassium Monophosphate)	163.19
Vitamin E	13.224	Sodium Chloride	225.67
Vitamin K	0.033	Zinc Sulfate	5.50

Table 2.3 FBF caloric, macronutrient, selected amino acids, and iron content

	CSB-WPC	CSB-0	CSB-5	CSB-10	SSB-WPC	SSB-0	SSB-5	SSB-10	SSB-15
Total Energy (kcal/100g)	417.1	414.0	414.8	415.5	410.6	398.3	407.1	408.3	408.7
Carbohydrate									
g/100g	62.5	60.8	61.0	60.8	62.7	59.3	60.1	60.4	60.0
% energy	60.0	58.8	58.8	58.6	61.1	59.6	59.0	59.1	58.8
Protein									
g/100g	20.4	21.0	21.0	20.6	19.7	20.6	21.2	21.0	21.2
% energy	19.5	20.3	20.2	19.8	19.2	20.7	20.8	20.6	20.8
Fat									
g/100g	9.5	9.6	9.7	10.0	9.0	8.7	9.1	9.2	9.3
% energy	20.5	20.9	21.0	21.6	19.7	19.7	20.1	20.3	20.5
Crude Fiber (g/100g)	0.4	0.9	0.9	0.7	0.4	0.6	0.8	0.7	0.7
Ash (g/100g)	3.6	4.2	4.2	4.3	3.6	4.3	4.3	4.3	4.4
Moisture (g/100g)	4.0	4.3	4.2	4.3	5.0	7.1	5.3	5.1	5.0
Lysine (mg/g)	13.8	11.0	11.1	11.3	13.3	10.9	11.6	12.0	12.2
Available Lysine (mg/g)	13.2	9.9	10	10.4	12.9	10.5	11	11.4	11.8
Cysteine + Methionine (mg/g)	7.6	5.9	5.8	6.0	7.4	6.0	6.2	6.2	6.1
Iron (mg/100g)	13.5	13.4	13.9	15.0	13.8	15.2	15.4	15.2	15.5

Macronutrients and amino acids analyzed by AOAC official methods at the University of Missouri-Columbia Agricultural Experiment Station Chemical Laboratories, Columbia, MO.
Iron content analyzed in duplicate by AOAC official methods at AIB International, Manhattan, KS.
AIN-93G provides 390.0 kcal/100g energy, 64.0 g/100g carbohydrate, 20.0 g/100g protein, 7.0 g/100g fat, and 6.6 mg/100g iron.

Table 2.4 Food intake and food efficiencies

	AIN-93G	CSB-WPC	CSB-0	CSB-5*	CSB-10*	SSB-WPC	SSB-0*	SSB-5	SSB-10*	SSB-15*
Total Food Intake (g)	502.4± 53.4^{a}	424.5± 38.8^{b}	439.2± 35.9^{ab}	436.0± 69.2^{ab}	472.8± 33.5^{ab}	447.0± 42.1^{ab}	501.6± 62.2^{a}	475.0± 59.6^{ab}	442.7± 34.4^{ab}	465.1± 46.4^{ab}
Final Body Weight (g)	297.1± 29.4^{ab}	284.1± 25.3^{ab}	261.5± 18.1^{ab}	255.4± 38.5^{b}	286.7± 23.8^{ab}	291.0± 30.7^{ab}	304.2± 33.8^{a}	294.3± 39.1^{ab}	272.7± 22.7^{ab}	283.8± 30.6^{ab}
Caloric Efficiency (g/100 kcal)	12.7± 0.4^{ab}	13.3± 0.5^{a}	11.8± 0.6^{bc}	11.5± 0.7^{c}	12.1± 0.6^{bc}	13.2± 0.7^{a}	12.7± 0.3^{ab}	12.7± 1.5^{ab}	12.4± 0.3^{abc}	12.4± 0.4^{abc}

Protein Efficiency (g/g)	2.5± 0.1^{b†}	2.7± 0.1^{a}	2.3± 0.1^{bc}	2.3± 0.2^{c}	2.5± 0.1^{bc}	2.8± 0.1^{a}	2.5± 0.1^{bc}	2.5± 0.3^{bc}	2.4± 0.1^{bc}	2.4± 0.1^{bc}

Data are mean ± standard deviation; values with different letters are statistically different ($p<0.05$) determined via one-way ANOVA with Tukey's test.
Food Intake: measured every other day by subtracting food remaining from food given.
Caloric efficiency: total weight gained (g) divided by total energy (kcal) consumed.
Protein efficiency: total weight gained (g) divided by total protein consumed (g).
n=10, *n = 9.
†Based on label protein value rather than analyzed protein content.

Table 2.5 Anthropometric and iron outcomes

	AIN-93G	CSB-WPC	CSB-0	CSB-5*	CSB-10*	SSB-WPC	SSB-0*	SSB-5	SSB-10*	SSB-15*
Lean Mass (%)	89.8± 1.4	89.7± 1.1	89.1± 1.4	88.5± 1.3	88.8± 1.6	89.6± 1.8	88.7± 1.6	89.5± 1.6	89.4± 1.2	89.4± 1.3
Bone Mineral Density (g/cm^2) x 1000	95.4± 12.0^{a}	78.0± 9.6^{b}	86.1± 16.4^{ab}	81.9± 9.9^{ab}	84.7± 10.3^{ab}	77.8± 7.7^{b}	83.1± 6.1^{ab}	81.4± 7.9^{ab}	82.2± 10.3^{ab}	76.8± 13.8^{b}
Hemoglobin (g/dl)	16.1± 1.3	15.8± 0.9	15.4± 1.1	15.3± 1.6	14.9± 0.6	14.8± 1.5	16.1± 2.5	15.0± 1.3	14.5± 1.1	14.7± 1.4
Hepatic Iron (μg/g)	9.5± 1.7^{c}	17.8± 2.5^{ab}	18.7± 3.1^{ab}	19.7± 4.4^{a}	17.2± 4.1^{ab}	15.9± 3.0^{ab}	15.9± 2.7^{ab}	16.4± 2.4^{ab}	15.5± 2.1^{ab}	15.0± 2.8^{b}

Data are mean ± standard deviation; values with different letters are statistically different ($p<0.05$) determined via one-way ANOVA with Tukey's test.
Lean mass: total weight minus fat mass divided by total weight x 100.
n=10, *n = 9.

Figures

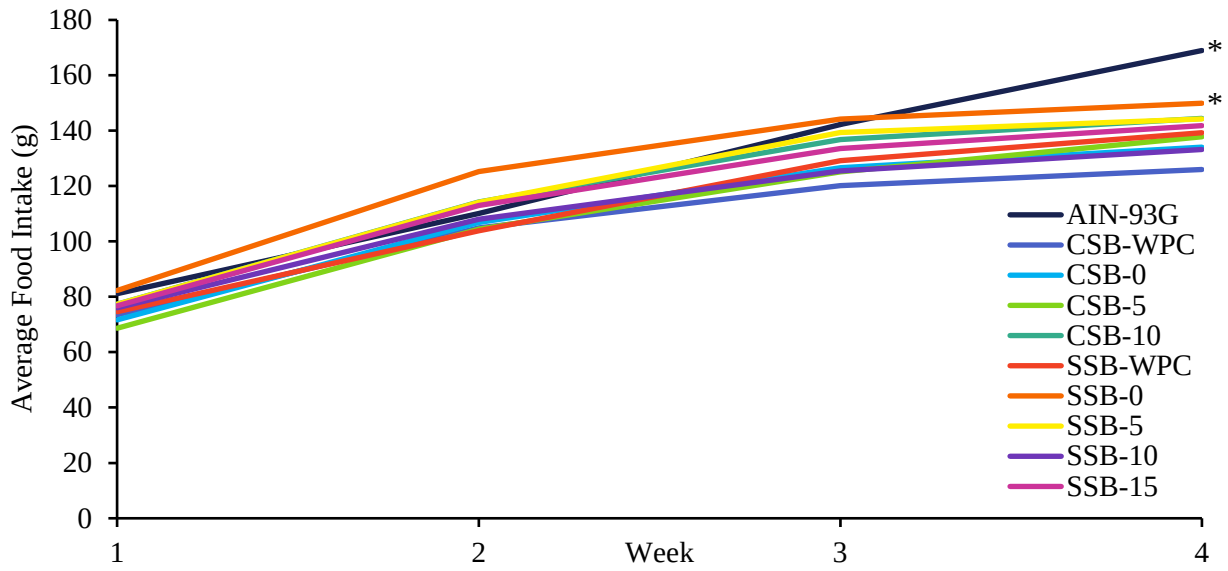

Figure 2.1 Mean weekly food intakes

*Total food intake for AIN-93G and SSB-0 were significantly higher compared to the CSB-WPC group with no other significant differences among groups. n=9-10.

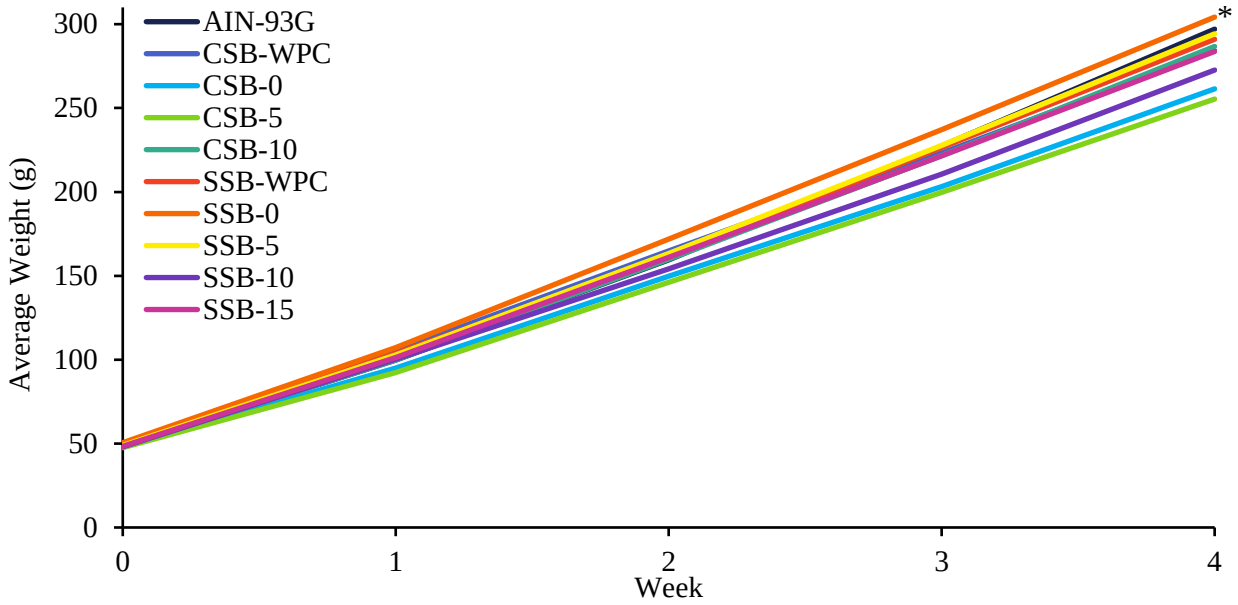

Figure 2.2 Mean weekly body weights

*Mean final body weight and total weight gain significantly higher for SSB-0 compared to CSB-5 with no other significant differences in total weight gain or final body weight among groups. n=9-10.

Chapter 3 - Evaluation of iron bioavailability from four iron fortificants in extruded rice in rats

Abstract

Background: Rice fortification is promising for reduction of micronutrient deficiencies primarily due to its high consumption in low-income countries. The most bioavailable forms of iron, such as ferrous sulfate ($FeSO_4$), contribute unpleasant sensory properties in neutral foods such as rice. Ferric phosphate ($FePO_4$) and ferric pyrophosphate (FePP) have been suggested suitable iron forms for use in rice considering their more acceptable organoleptic properties, however, they have lower bioavailability. Micronized FePP (µFePP) or the addition of trisodium citrate (TSC) and citric acid (CA) to FePP have been suggested techniques to increase FePP bioavailability.

Objective: Our primary objective was to evaluate hemoglobin and hepatic iron outcomes from extruded rice diets fortified with four types of iron.

Methods: Rice flour was fortified with a USDA MR24 vitamin/mineral blend and one of four iron fortificants: $FePO_4$, FePP, µFePP, or FePP with TSC and CA (FePP+TSC+CA, ratio 1:2.1:0.1). Each extruded rice was blended at a 1:99 ratio with unenriched white rice, soy protein isolate, and soybean oil, which were added to support the nutritional requirements of growing rats. Rice diets were cooked to approximately 40% added moisture. Weanling, male Sprague Dawley rats were randomly divided into 5 groups (n=10, 50 total). Daily food intake and weekly body weights were measured. Each group consumed assigned diet (AIN-93G or one fortified rice diet) for 21 days, when the study was terminated because of poor growth in the rice groups. Blood and livers were collected to evaluate iron outcomes and bone mineral density (BMD) and body composition were assessed with dual energy x-ray absorptiometry (DEXA) PIXImus scans.

Results: All rice groups had significantly lower moisture-adjusted total food intake, weight gain, final weight, and BMD compared to the AIN-93G group with no differences in these outcomes between the rice groups. There were no differences in either iron outcome between the different FePP fortificants. Hemoglobin concentrations were significantly higher in the FePP and µFePP groups compared to the $FePO_4$ and AIN-93G groups. Hepatic iron concentrations were significantly higher in the FePP, µFePP, and FePP+TSC+CA groups compared to the $FePO_4$ and AIN-93G groups.

Conclusions: While they need to be interpreted with some caution because of poor growth, our results suggest that FePP leads to better iron outcomes than $FePO_4$. However, neither micronizing nor adding TSC+CA to FePP improved hemoglobin or hepatic iron outcomes.

Background

Rice is a staple food for nearly half the global population providing as many as 50-70% of the calories consumed in many low-income Asian countries (51, 53). A concern with high rice consumption is the majority of the nutrients are stripped during the milling and polishing processes, including about 90% of the iron (53). Populations which consume large amounts of rice are at-risk for micronutrient deficiencies including iron deficiency anemia (IDA). Anemia is estimated to affect approximately 800 million children and women with half of these cases due to iron deficiency. Iron fortification and/or supplementation are promising approaches for addressing many cases of IDA, specifically those in high-risk groups including women and children (16).

Because rice is commonly consumed as intact kernels, fortification with essential micronutrients, including iron, presents a unique challenge. Current rice fortification strategies include dusting, coating, and extrusion. In these strategies, kernels are diluted into natural rice at

a ratio of 1:200–1:50 (51, 59). In extruded rice, added micronutrients are embedded into the kernels and maintain much of their functional properties (51). This is beneficial compared to coating and dusting strategies where some of the micronutrients may be lost during rice preparation with common practices of washing before cooking and boiling rice in an excess of water that is discarded after cooking (52, 54).

Ferrous sulfate ($FeSO_4$) is a commonly used food fortificant because it is a highly bioavailable form of iron, however, in products such as rice, it produces undesirable sensory changes (65). Ferric phosphate (also referred to as ferric orthophosphate, $FePO_4$) is used in some countries for technical reasons in rice fortification, but the bioavailability is low (57, 65). Ferric pyrophosphate (FePP) is commonly used in rice because it does not negatively impact color or organoleptic properties (51, 59, 73, 124). However, the bioavailability and absorption of FePP is low compared to other iron fortificants, with a reported 20-50% relative bioavailability (RBV) compared with $FeSO_4$, and even lower bioavailability, approximately 15-24%, in rice-based meals (72, 75).

Regular FePP has a mean particle size (MPS) of about 20 µm (51). Micronizing, reducing the particle size, is reported to improve bioavailability and absorption. In adult women, FePP with a MPS of 0.5 µm was shown to have comparable absorption to $FeSO_4$ from labeled test meals (74). With a particle size of 2.5 µm, the RBV is approximately 70% of $FeSO_4$ in rats (59, 75). Dual fortification of micronized FePP (µFePP) and iodine in salt resulted in decreased prevalence of IDA in a randomized controlled trial in iodine-deficient and anemic children (124).

Another approach to increase FePP bioavailability is to chelate ferric pyrophosphate to citrate and phosphate ligands making it soluble in aqueous solutions (soluble ferric pyrophosphate, SFP, 71). The addition of trisodium citrate (TSC) and citric acid (CA) to rice

flour before extrusion results in the formation of SFP because of the pressure, heat, and subsequent boiling. Addition of TSC and CA to rice flour before extrusion was found to double the absorption of FePP from extruded rice in a human stable iron isotope study in women (73). An increase in iron solubility and dialyzability in the rice extruded with TSC and CA compared to the other fortification approaches (no TSC+CA or TSC+CA added pre- or post-cooking) was additionally observed.

Our primary objective was to evaluate iron outcomes, hemoglobin and hepatic iron, in rats which consumed diets containing extruded rice fortified with one of four iron fortificants: $FePO_4$, FePP, µFePP, and FePP with TSC and CA added before extrusion (FePP+TSC+CA).

Methods

Ethical Standards

Procedures were approved by the IACUC at Kansas State University (protocol 4017). Animals were assessed for well-being prior-to and throughout the study.

Rice Diets Preparation

Iron fortificants were obtained from Wright Enrichment, Inc. (Lafayette, LA). Mean particle size for the micronized FePP was 2.4 µm; MPS for the regular FePP was considerably larger. Four extruded rice blends were developed according to USDA MR-24 Milled Rice Commodity Requirements (MR-24 Reference). Rice flour (Riviana Foods, Inc., Houston, TX) was blended with iron fortificant, vitamin and mineral premix (REPCO, Salina, KS), salt (Cargill, Inc., Wayzata, MN), and monoglycerides (DuPont Danisco Food Ingredients, Copenhagen, Denmark) before extrusion (Tables 3.1 & 3.2). For the FePP+TSC+CA blend, trisodium citrate (Sigma-Aldrich, St. Louis, MO) and citric acid (Sigma-Aldrich, St. Louis, MO) were added at a ratio of 1:2.1:0.1 (Fe:TSC:CA, 73).

Each blend was extruded on a double-screw extruder (TX-52, Wenger Manufacturing, Inc., Sabetha, KS) with a dry ingredient feed rate of 1 kg/minute. The downspout temperature was maintained above 80°C and steam and water were added during preconditioning. The preconditioner cylinder speed was 400 rpm and extruder speed was 200 rpm. The product was forced through rice-shaped openings and extrudates were cut with a knife cutting speed of 2750 rpm. After cutting, the rice-shaped extrudates were dried for 18 minutes and cooled for 10 minutes in a double-pass dryer/cooler (series 4800, Wenger Manufacturing, Inc., Sabetha, KS). The rice kernels were dried a second time with the same conditions to achieve a final moisture content between 12-13%.

The extruded rice blends were included in the final diet at 1%, a 1:99 ratio, with natural white rice (84.5%, JFC International, Inc., Commerce, CA), soy protein isolate (SPI, 10.5%, Know-How Foods, Faribault, MN), and soybean oil (4%, Wal-Mart Stores, Inc., Bentonville, AR) to meet protein and lipids macronutrient requirements of growing rats (82). The diets were cooked with equal part water to an added moisture content of approximately 40% which reduced further during cooling. A goal of 35% added moisture content was determined based on studies evaluating water addition levels on protein efficiency in rats (125) and the maximum observed water addition before intake was decreased in female weanling rats (82). The average moisture of the prepared diets was calculated to be approximately 42%. Moisture content was calculated from initial moisture of dry ingredients, moisture of freshly prepared cooked diets, and moisture loss after 24 hours at ambient conditions. Rice diets were prepared twice weekly and stored in a 4°C refrigerator until use.

Nutritional Analyses

Rice extrudates, natural white rice, and SPI iron concentrations, macronutrient proximate analyses, amino acid profiles, and available lysine were determined as previously described in Chapter 2 Methods: Nutritional Analyses.

Study Design

Male, weanling Sprague Dawley rats (Charles River, Wilmington, MA) were randomized into 5 diet groups (n=10, 50 total). Groups were assigned to consume either one of the extruded rice diets or a standard growing rat diet, AIN-93G (control, Research Diets, Inc., New Brunswick, NJ). Animals were individually housed in wire-bottom cages and provided with a resting board, enrichment products, and *ad libitum* access to food and water for the duration of the study. Temperature and 12-hour alternating light and dark cycles were maintained. Food intake, by food remaining, was measured and fresh food was provided daily. The animals were weighed upon arrival and weekly thereafter. The intended study duration, 28 days, and size were based on the prophylactic (80) and protein efficiency ratio (PER, 103) methods. The study was terminated after 21 days due to poor growth in the rice groups.

Data and Sample Collection

At study conclusion, animals were euthanized and samples were gathered and stored as described in Chapter 2 Methods: Data and Sample Collection. Moisture adjustments were calculated for food intake, caloric efficiency, and protein efficiency outcomes based on a 6.6% moisture basis, the moisture content of AIN-93G (117).

Iron Analysis

Hemoglobin

Samples were analyzed in duplicate as described in Chapter 2 Methods: Iron Analysis. No triplicate analyses were performed due to variances between all sample duplicates being less than 15%.

Hepatic Iron

Samples were analyzed in duplicate as described in Chapter 2 Methods: Iron Analysis. Triplicate analyses were performed when variance between duplicates was greater than 15%.

Calculations

Calculations were performed to determine caloric and protein efficiencies as described in Chapter 2 Methods: Calculations. Food intake, caloric efficiency, and protein efficiency outcomes are reported with as-is values in addition to adjusted 6.6% moisture basis values, the moisture content of AIN-93G (117).

$$6.6\%\ Moisture\ basis\ value = \frac{100\% - as\text{-}is\ moisture\ \%}{100\% - 6.6\%} x\ as\text{-}is\ value$$

Statistical Analysis

Shapiro-Wilk's test and for homogeneity of variance with Levene's test was used to evaluate data for normality. Square root transformations were performed if assumptions for normality were not met. Group differences were determined for data which met normality assumptions using one-way analysis of variance (ANOVA) with Least Significant Difference (LSD) test. Data which did not meet normality assumptions after transformation were assessed with Wilcoxon scores ranked sums with the Kruskal-Wallis Test and Dwass, Steel, Critchlow-Fligner pairwise multiple comparison analysis. Statistical analyses were performed in SAS Studio with significance at $p<0.05$ (version 3.71, SAS Institute Inc., Cary, NC).

Results

Diet Composition

The energy provided by the rice diets was 74% carbohydrates, 16.3% protein, and 9.7% fat (Table 3.3). Each rice diet provided 1.41 mg/100g iron, no fiber, 0.6 g/100g ash, 7.76 mg/g available lysine, and 5.15 mg/g cysteine and methionine. The average calculated total moisture of the cooked diets was 42%, which is 7% higher than our intended target of 35%.

Food Intake and Efficiencies

No significant differences in total food intake, caloric, and protein efficiency outcomes were observed among the rice groups (Table 3.4). Before moisture adjustment, only the μFePP and FePP+TSC+CA groups' total food intake was significantly lower than the AIN-93G group. Both unadjusted caloric and protein efficiencies for all rice groups were significantly lower than the AIN-93G group.

All rice groups were all significantly lower than the AIN-93G group for moisture-adjusted total food intake, caloric, and protein efficiency outcomes. Moisture-adjusted food intake for week 1 of the study was similar among groups; intake decreased in all rice groups during the second and third weeks (Figure 3.1).

Anthropomorphic Outcomes

No significant differences in weight gain or final body weights were observed between the rice groups and all rice groups weight gain and final weights were significantly lower than the AIN-93G group (Table 3.4). The similar week 1 moisture-adjusted food intakes did not correspond with similar week 1 weight gain among rice groups compared to the AIN-93G group (Figure 3.2).

There were no significant differences in lean mass among all groups (Table 3.5). All rice groups had significantly lower bone mineral density (BMD) compared to the AIN-93G group with no differences between rice groups.

Iron Outcomes

Hemoglobin concentrations were significantly higher in the FePP and µFePP groups compared to the $FePO_4$ and AIN-93G groups (Table 3.5). Hemoglobin concentrations of the FePP+TSC+CA group was significantly higher than only the AIN-93G group. Hepatic iron concentrations were significantly higher in all the FePP groups (FePP, µFePP, FePP+TSC+CA) compared to both $FePO_4$ and AIN-93G groups.

Discussion

Iron Outcomes

Despite low food intake and growth in the rice groups, bioavailability of iron from FePP was not improved by micronizing or adding trisodium citrate and citric acid. In alignment with our results, another rat study observed no significant differences in the RBV of µFePP (MPS 2.5 µm) and regular FePP (MPS 21 µm) in iron-depleted rats after 14 days (76).

Micronized ferric pyrophosphate with a mean particle size (MPS) of 2.5 µm in fortified salt was shown to reduce prevalence of iron deficiency anemia in children. Regular FePP was not tested as a comparison to the µFePP (124). The researchers selected µFePP with a 2.5 µm MPS due to reported increases in RBV compared to regular FePP (approximately 70% compared to ≤50%, respectively, 124).

In a stable isotope single meal feeding study, iron absorption from rice meals was increased when TSC+CA was added to FePP (73). While absorption was found to be increased in the stable isotope single-feeding study, we did not observe that TSC+CA added during

extrusion increased iron outcomes from FePP in rats in our study. This is the first study we are aware of which assessed longer-term hemoglobin and hepatic iron outcomes from FePP+TSC+CA.

Iron absorption is an important metric for understanding differences in iron fortificants, however, it is not necessarily reflective of longer-term uptake and use of iron. Variation within a single subject from the same iron fortified meal can vary between 20-30% from one day to the next (79). Recent research suggests that iron outcomes as related to inhibitory factors from long-term trials do not align with findings from absorption studies (126, 127). Antinutritional factors, such as tannins and phytate, have been observed to negatively impact iron outcomes in absorption studies. However, humans may be able to adapt to dietary tannins and phytates, overcoming negative impacts on iron bioavailability. Two studies in non-anemic women found that tannin supplementation and phytate intake over 4 weeks did not significantly affect iron status (128, 129). While research of the long-term impacts of iron inhibitory factors is limited, these results may be helpful in explaining differences observed in single meal absorption studies compared with longer-term iron status studies.

While we found no significant differences in iron outcomes among FePP groups, FePP lead to greater hemoglobin and hepatic iron concentrations than $FePO_4$. In anemic Wistar rats, day 7 hemoglobin and hematocrit concentrations were not significantly different for the $FePO_4$ group compared to the $FeSO_4$ group, while the FePP group concentrations were significantly lower compared to $FeSO_4$. However, on day 14 of the study, there were no significant differences in iron outcomes between the three groups (130). Other research suggests that $FePO_4$ bioavailability is lower (6-46%) compared with FePP bioavailability (45-58%) in rats (65).

Diet Composition

Protein Quality and Amino Acids

Poor intake among rice groups in the second and third weeks of the study was unexpected. Reduced food intakes may be attributed to inadequate nutrients provided by the rice diets to support the needs of rapidly growing rats. Protein quality may have been a contributing factor to significantly reduced growth and BMD in the rice groups. Evidence in growing rats suggests that low protein intake (10%) is significantly associated with reduced bone mass and strength compared to a moderate protein diet (20%, 131).

The rice diets met the total protein requirement of National Research Council recommendations for growing rats, however, the lysine and cysteine and methionine concentrations of the rice diets were lower than recommended – per 100g of diet, 0.92 g of lysine recommended vs. 0.80 g provided and 0.98 g of cysteine and methionine recommended vs. 0.52 g provided (82). A study found that a reduced methionine level (0.17%) was significantly associated with reduced BMD, bone volume, bone mineralization, and bone mineral content compared to a 0.52% methionine diet in 7 week old rats (132).

In previous animals studies of fortified blended foods, the amount of lysine and cysteine and methionine in the poorest performing diet (corn-soy blend plus, CSB+) were similar to the concentrations in the rice diets provided in this study (42, 50). CSB+ resulted in similar food intake and growth patterns to the rice diets in this study. In both our pre-cooked rice diets and CSB+, the amount of lysine was approximately 0.8% and cysteine and methionine is approximately 0.5%. After cooking and adjusting for total moisture content, the rice diets provided approximately 0.5% lysine and 0.3% cysteine and methionine.

Substantial improvements in growth of weanling rats was observed when diets of 90% rice was supplemented with 0.05-0.10% lysine compared to diets without lysine supplementation and diets with higher levels of supplemented lysine (0.2-0.8%, 133). However, the weanling male hooded rats only gained 100-121 g over 5 weeks, much lower than the total gain of our control group (171.3 ± 16.1 g gain over 3 weeks). At least some of this difference in gain is likely related to strain differences (82). In a follow-up study of amino acid supplementation to precooked rice diets, the best combined weight gain and feed efficiency in weanling rats was calculated to be with the addition of 0.34% lysine and 0.18% threonine (134). Our rice diets provided approximately 0.54% threonine (0.31% post-cooking). In the follow-up amino acid study with lysine and threonine supplementation over 5 weeks, the male weanling rats grew substantially more (nearly 200 g gain at various levels of supplementation) than in the initial study.

Increased amounts of additional essential amino acids beyond lysine, cysteine and methionine, and threonine, may be necessary to support rat growth. A study of low-protein diets with amino acid supplementation demonstrated that growing rats may need higher amounts of phenylalanine, valine and arginine (135). In another study, lysine, methionine, threonine, and tryptophan increased growth rates of weanling rats when added to wheat gluten (136).

Micronutrients

The micronutrients provided by the vitamin and mineral premix were added at 1% to the extruded rice which was added at 1% to the final rice diets. Due to dilution, these micronutrients (vitamins A, B6, B12, niacin, zinc, thiamin, and folic acid) were low in the final prepared rice diets. Rats consuming a diet similar to our rice diets (rice, oil, corn starch, and salt; approximately 7.8% protein) gained only 16 g in 28 days (consumed an average of 126 g of food

total) and addition of vitamins and minerals resulted in significantly increased growth (137). The low levels of micronutrients present in the rice diets in our study is another possible explanation for the poor intake and growth of the animals.

Moisture Content

The National Research Council supports diets with up to 40% moisture for adequately supporting caloric needs of weanling female rats (82) and research in 30 day old Sprague Dawley rats found 66% moisture to be acceptable (138). However, other research suggests that 40-66% moisture may be too high to support energy requirements for growing rats. Adult male rats were unable to maintain body weights when fed diets diluted with more than 25% water for longer than 3 days (139). At 50% and 80% added moisture to 12% protein diets, PER was significantly decreased compared to 0% and 20% added moisture diets in 21 day old male, Sprague Dawley rats (140). The group fed the 20% added moisture diet consumed the most protein and had the highest protein efficiency (140). In a follow-up study, water was added at concentrations from 5-35% to a basal diet with 8% moisture. Diets with 20-35% added moisture resulted in the highest protein efficiencies (125). Considering findings from all studies presented on moisture content of diets for rats, the 42% moisture content was not likely the primary concern with the rice diets, although it may be a contributing factor.

Limitations

The most notable limitation is the poor intake and growth observed in the groups consuming the rice diets and due to this, a follow-up animal study is required. The rice diets quality, including nutritional quality, cooked nature, and to a lesser extent, total moisture content, likely contributed to the poor intakes and growth. While our preparation was designed to mimic cooking and feeding practices in humans, it should be re-evaluated and optimal preparation to

benefit growing rats should be utilized instead. Extruded foods ground into dry powders have been well-received by the rats in our previous fortified blended foods studies and may be an appropriate format for delivering extruded rice diets to the rats. The study duration was also shortened to 3 weeks from the intended 4 weeks due to the poor growth which limits the quality of our iron outcome findings.

Conclusions

There were no significant differences in intake, growth, and anthropometric outcomes among rice groups, however, these outcomes were all significantly decreased compared to the control group. Despite poor growth which resulted in the early termination of the study, hemoglobin and hepatic iron concentrations were higher in the ferric pyrophosphate groups than the ferric phosphate and control groups. Neither strategy suggested to improve ferric pyrophosphate bioavailability, micronizing or adding trisodium citrate and citric acid, significantly improved iron outcomes compared to the regular ferric pyrophosphate group. Due to poor growth in the rice groups, an additional animal study is warranted to confirm the iron outcome findings from this study.

Tables

Table 3.1 Extruded rice formulations (%)

	Rice Flour	Iron	Vitamin-Mineral Premix	Monoglycerides	Salt
$FePO_4$	96.5	1.3	1.0	0.75	0.5
FePP	96.2	1.6	1.0	0.75	0.5
Micronized FePP	96.2	1.6	1.0	0.75	0.5
FePP+TSC+CA*	96.1	1.6	1.0	0.75	0.5

*Trisodium citrate, 0.013%; citric acid, 0.0004%.

Table 3.2 Vitamin and mineral fortification levels per 100 g extruded rice

Vitamin A (IU)	0.11
Niacinamide (mg)	5.6
Zinc (mg)	3.5
Pyridoxine HCL (mg)	0.6
Thiamine mononitrate (mg)	0.47
Folic acid (mg)	0.15
Vitamin B12 (mcg)	1.1

Table 3.3 Caloric, macronutrient, select amino acid, and iron content of rice and AIN-93G diets

	Rice Diets*	AIN-93G
Total Energy (kcal/100g)	373.9	390.0
Carbohydrate		
g/100g	69.2	64.0
% energy	74.0	
Protein		
g/100g	15.2	20.0
% energy	16.3	
Fat		
g/100g	4.0	7.0
% energy	9.7	
Crude Fiber (g/100g)	0.0	–
Ash (g/100g)	0.6	–
Moisture (g/100g)	11.0	6.6
Lysine (mg/g)	7.98	–

Available Lysine (mg/g)	7.76	–
Cysteine + Methionine (mg/g)	5.15	–
Iron (mg/100g)	1.41	6.6

*Nutrient profile for pre-cooked diets

Table 3.4 Food intake and food efficiencies

	AIN-93G	$FePO_4$	FePP	Micro FePP	FePP+TSC+CA
Total Food Intake (g)	338.4±30.5[a]	295.5±30.1[ab]	307.9±33.4[ab]	282.5±30.8[b]	292.5±36.5[b]
Adj. Total Food Intake (g)[†‡]	338.4±30.5[a]	191.1±19.5[b]	199.1±21.6[b]	182.7±19.9[b]	189.1±23.6[b]
Total Weight Gain (g)[†]	171.3±16.1[a]	23.2±4.7[b]	25.7±6.3[b]	21.2±4.3[b]	22.9±8.1[b]
Final Body Weight (g)[†]	229.7±23.0[a]	83.2±9.7[b]	83.1±9.8[b]	81.0±7.1[b]	82.1±12.7[b]
Caloric Efficiency (g/100 kcal)[†]	127.8±3.3[a]	20.9±3.3[b]	22.3±4.5[b]	19.9±3.1[b]	20.5±4.7[b]
Adj. Caloric Efficiency (g/100 kcal)[†‡]	127.8±3.3[a]	32.3±5.1[b]	34.4±6.9[b]	30.8±4.8[b]	31.7±7.2[b]
Protein Efficiency (g/10 g)[†]	25.3±0.6*[a]	5.1±0.8[b]	5.5±1.1[b]	4.9±0.8[b]	5.0±1.1[b]
Adj. Protein Efficiency (g/10 g)[†‡]	25.3±0.6*[a]	7.9±1.3[b]	8.5±1.7[b]	7.6±1.2[b]	7.8±1.8[b]

Data are mean ± standard deviation; values with different letters are statistically different ($p<0.05$).
Food Intake: measured every other day by subtracting food remaining from food given.
Caloric efficiency: total weight gained (g) divided by total energy (kcal) consumed.
Protein efficiency: total weight gained (g) divided by total protein consumed (g).
n=10.
*Based on label protein value rather than analyzed protein content.
[†]Data analyzed with nonparametric ANOVA.
[‡]Adjusted values: 6.6% moisture basis.

Table 3.5 Anthropometric and iron outcomes

	AIN-93G	$FePO_4$	FePP	Micro FePP	FePP+TSC+CA
Lean Mass (%)	89.2±1.1	90.1±1.0	90.5±0.9	89.9±0.8	89.9±1.0
Bone Mineral Density (g/cm^2) x 1000[†]	84.3±6.5[a]	44.3±1.8[b]	44.3±3.6[b]	44.2±4.1[b]	45.2±2.1[b]
Hemoglobin (g/dl)	12.5±1.0[c]	13.3±2.3[bc]	14.9±0.8[a]	15.0±1.0[a]	14.5±0.8[ab]
Hepatic Iron (µg/g)	8.7±2.0[b]	10.0±1.5[b]	16.0±4.8[a]	14.6±4.0[a]	15.8±4.1[a]

Data are mean ± standard deviation; values with different letters are statistically different ($p<0.05$).
Lean mass: total weight minus fat mass divided by total weight x 100.
n=10.
[†]Data analyzed with nonparametric ANOVA.

Figures

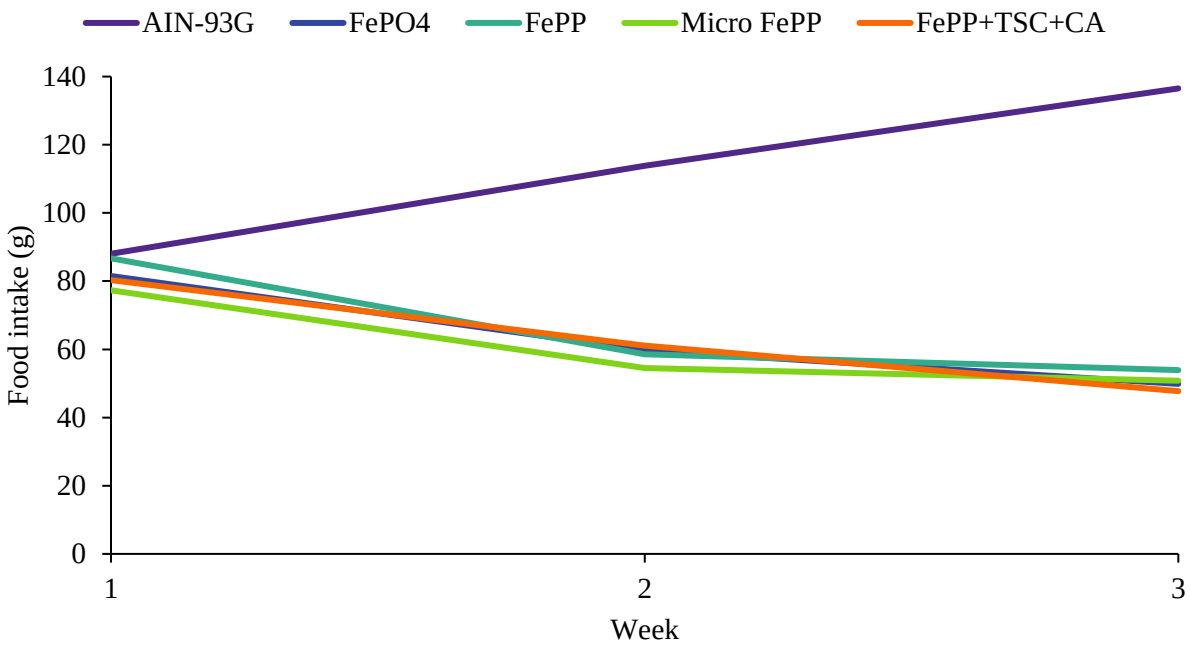

Figure 3.1 Moisture-adjusted* mean weekly food intakes

*All diets reported at 6.6% moisture basis
Total food intake for AIN-93G significantly higher compared to all rice groups. n=10.

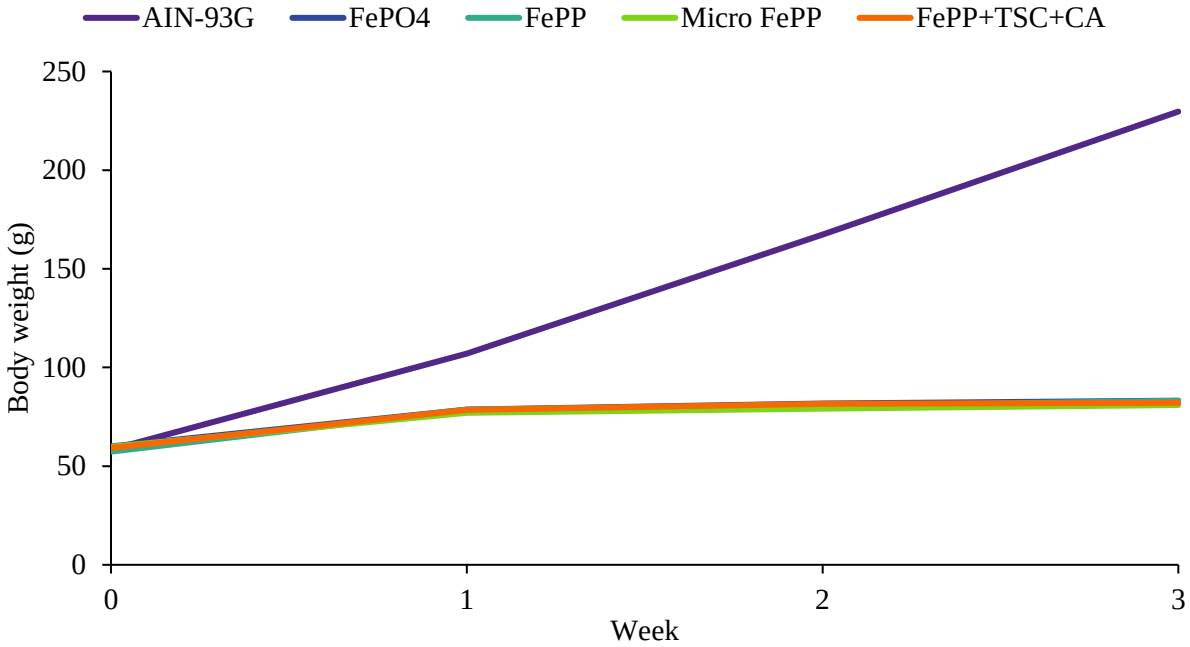

Figure 3.2 Mean weekly body weights

Total final body weight and total weight gain for AIN-93G significantly higher compared to all rice groups. n=10.

Chapter 4 - Conclusions

In the first study, we observed that protein provided from soy flour is an efficacious alternative to protein from WPC in FBFs made from sorghum and soy flours in rats. We additionally observed that 15% added sucrose in the FBFs was associated with decreased BMD in growing rats compared to 10% or less added sucrose.

No significant differences were observed among SSB-0, SSB-5, and SSB-10 groups. In future studies, it would be beneficial to evaluate how these formulations are perceived by children. Considering that children tend to prefer sweeter foods, they may prefer the FBFs with 5-10% added sucrose over the 0% sucrose FBF. Information about children's FBF perceptions and preferences would be beneficial for selecting blends for an efficacy trial.

The only significant difference observed in this study among the SSB groups was for protein efficiency. While protein efficiency was a primary outcome of interest, its significance for interpreting the quality of the SSB FBFs becomes less important when considering the limitations of PER and similarities in all other outcomes.

Considering the overall findings and outcomes in clinical trials, I believe that the novel, extruded SSB FBFs without WPC have at least similar, if not better, protein quality compared with CSB+. Additionally, these FBFs are more affordable and equally efficacious alternatives to extruded FBFs with WPC. A human efficacy trial which evaluates the SSB FBFs without WPC compared with current USAID FBFs, CSB+ and Super Cereal *Plus*, is a reasonable future research goal.

In the second study, we observed that neither of the proposed strategies of micronizing or adding trisodium citrate and citric acid to FePP, which have been observed to increase iron absorption in humans, resulted in improved hemoglobin or hepatic iron concentrations in

weanling Sprague Dawley rats. Additionally, we observed that $FePO_4$ resulted in significantly decreased iron outcomes compared to FePP despite study limitations including poor animal growth and short duration of the study. The quality of this study was diminished due to inadequate micronutrient fortification levels (excluding iron) and the presumed poor nutritional quality of diets (resulting in poor growth and early study termination). Due to these limitations, another animal study is needed which improves intake and growth of the animals consuming the rice diets.

While I feel that we gained important information from both of these studies, there are aspects which I would improve upon if given the opportunity. For the FBF study, we used "weanling" rats while for the rice study, we used a specified age of rats which I would recommend with any future rat studies. Additionally with the FBF study, concern for FBF consumption beyond which was assigned resulted in the omission of results for two animals from the SSB groups. To mitigate this concern in the rice study, all the animals were identified with unique tail markings which was effective for confirming animal identities. For the rice study, the biggest concern was nutritional quality of the rice diets. I would prefer to use only extruded rice (no natural kernels) and provide the rice in a ground powder mixed with the other ingredients, similar to the FBF studies. While I do not feel that the added moisture was a primary concern for reduced diet quality, the cooking process may have introduced unintended variables and should be avoided for a future study.

These studies are unified by a need to address effectiveness and cost of food aid products to nourish future generations of our global community. I believe that FBFs and rice play an important role for overcoming the currently increasing number of people suffering from undernutrition and micronutrient deficiencies. My hope is that findings from these and additional

studies may be used to inform the decisions of food aid organizations including USAID and WFP. Extruded sorghum-soy FBFs without animal-source protein are a promising alternative to CSB+/Super Cereal and Super Cereal *Plus* and I hope to see SSB blends available for food aid distribution in the future. A better understanding of iron fortificants in longer-term research studies can help guide the treatment and prevention of IDA. I look forward to seeing how the results from these and future studies can help decrease hunger and micronutrient deficiencies, improving local and global economies, and ultimately, our global community.

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Appendix A - List of Abbreviations

AACC	American Association of Cereal Chemists
AIN	American Institute of Nutrition
AA	Amino Acid
AAS	Amino Acid Score
ANOVA	Analysis of Variance
AOAC	Association of Official Analytical Chemists
BMD	Bone Mineral Density
CO_2	Carbon Dioxide
CDC	Centers for Disease Control and Prevention
CA	Citric Acid
CSB	Corn-Soy Blend
CSB+	Corn-Soy Blend Plus
DIAAS	Digestible Indispensable Amino Acid Score
DEXA	Dual Energy X-ray Absorptiometry
EAA	Essential Amino Acid
EDTA	Ethylenediaminetetraacetic Acid
$FePO_4$	Ferric Phosphate/Orthophosphate
FePP	Ferric Pyrophosphate
$FeSO_4$	Ferrous Sulfate
FY17	Fiscal Year 2017
FAQR	Food Aid Quality Report
FAO	Food and Agriculture Organization of the United Nations
FBF	Fortified Blended Food
GDP	Gross Domestic Product
ICP-OES	Inductively Coupled Plasma-Optical Emission Spectrometry
IACUC	Institutional Animal Care and Use Committee
IDA	Iron Deficiency Anemia
LSD	Least Significant Difference

MPS	Mean Particle Size
µFePP	Micronized Ferric Pyrophosphate
MFFAPP	Micronutrient Fortified Food Aid Pilot Project
PDCAAS	Protein Digestibility Corrected Amino Acid Score
PER	Protein Efficiency Ratio
PEM	Protein-Energy Malnutrition
RBV	Relative Bioavailability
NaOH	Sodium hydroxide
NaFeEDTA	Sodium Iron EDTA
SFP	Soluble Ferric Pyrophosphate
SSB	Sorghum-Soy Blend
SPI	Soy Protein Isolate
TSC	Trisodium Citrate
UNICEF	United Nations Children's Fund
USAID	United States Agency for International Development
USDA	United States Department of Agriculture
WPC	Whey Protein Concentrate
WPC80	Whey Protein Concentrate – 80% Protein
WFP	World Food Programme
WHO	World Health Organization