Factors affecting food aid: evaluating new fortified-blended foods and the clinical impact of tannin and phytic acid consumption on iron bioavailability

by

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AN ABSTRACT OF A DISSERTATION

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DOCTOR OF PHILOSOPHY

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Abstract

Iron, vitamin A, and protein inadequacies are common in food-aid receiving countries, and maximizing nutrient intake and bioavailability are essential treatments. Fortified-blended foods (FBFs), are food-aid micronutrient-fortified legume-grain porridges distributed worldwide. FBFs have not consistently, effectively treated undernutrition, and it has been suggested that formulation and processing changes could improve their nutritional quality. Sorghum is a well suited FBF commodity, but high concentrations of 'antinutritional' tannin and phytic acid have limited its adoption. Iron bioavailability adaptation may be possible after long-term antinutritional factor consumption, but adaptive mechanisms are not well understood. In rats, salivary proline-rich proteins (PRPs) have been found to chelate tannins to improve iron bioavailability, this could be true for people as well. Several research design methods were employed to summarize FBF quality outcomes and the effect of tannin and phytic acid consumption on iron bioavailability. Extruded sorghum and corn FBFs were developed; protein quality, iron, and vitamin A outcomes were compared with a non-extruded corn-soy blend (CSB+) in rats. A narrative literature review and meta-analysis were conducted to determine tannin's antinutritional effects on iron bioavailability, and the potential for adaptation through salivary PRPs. Two clinical trials examined the effect of long-term tannin or phytic acid consumption on iron bioavailability, salivary protein production, and correlations between PRPs and iron bioavailability. There were no differences between iron (hepatic iron 207-300 µmol/g *100), vitamin A (hepatic retinol 423-585.5 ng/mg), or protein quality (caloric efficiency: 101.3-113.3 g/kcal*100) between extruded FBFs regardless of commodity in rats. Compared to extruded FBFs, CSB+ caloric efficiency (49.0 ± 2.2 g/kcal*100) and growth (96.3 ± 3.4g vs. 208.6-236.6) were significantly reduced. A literature review suggested that there were

differences in acute meal and long-term iron bioavailability with tannin consumption; tannic acid inhibited iron availability, while food-tannins did not. Meta-analysis suggested that tannin-PRP binding could protect iron bioavailability, that long-term tannin consumption did not significantly affect hepatic iron or non-heme iron absorption respectively in rats (d = -0.64-1.84; -2.7-0.13), and that PRP expression in rats during tannin consumption was correlated with improved iron bioavailability. There were no reductions in iron bioavailability or status based on long-term tannin (ps > 0.126) or phytic acid (ps > 0.08) consumption clinically, but basic PRP and cystatin subtypes were significantly correlated with improved iron bioavailability during tannin (ps < 0.03) and phytic acid (ps < 0.02) consumption. *In vitro*, it phytic acid-PRP binding did not occur, but phytic acid did specifically bind with cystatin SN, a non-enzymatic salivary protein. In conclusion, FBF formulation changes may improve protein quality, and provide needed macronutrients to food-aid receiving areas. Despite this, this research did not suggest that antinutritional factors affected iron bioavailability. In support of this finding, literature, and clinical studies presented here suggest that salivary proteins, including PRPs and cystatin, may serve as adaptive protective mechanisms against phytic acid and tannin consumption, and that further research may be warranted before further recommendations for their removal from food-aid are made.

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Dedication

This work is dedicated to the poor and malnourished, and to a vision for a world where nutritional and health equality exist for all people.

Chapter 1 - UNDERSTANDING AND OVERCOMING BARRIERS TO GLOBAL UNDERNUTRITION

Undernutrition

Global Prevalence of undernutrition

The United Nations Children's Fund (UNICEF) estimates that nearly half of under five deaths are attributable to undernutrition, including micronutrient deficiencies (MNDs), stunting, or wasting; this equates to almost 3 million children each year [1]. Micronutrient deficiencies are often referred to as 'hidden hunger' because of their significant impact on morbidity and mortality despite lack of overt signs and symptoms of deficiency [2]. Micronutrient deficiencies are commonly found with other nutritional deficiencies, although they are often found alone [2].

In the first 1000 days of life, poor nutrition is associated with stunting, an irreversible deficit in stature associated with cognitive impairment, and wasting, defined as rapid weight loss or failure to gain weight [1]. An estimated one in four children are stunted (156 million), which is a ten percent decrease in the past 15 years. Wasting remains more prevalent than obesity (50 versus 42 million children in 2015) [3], and the majority (93% and 92%) of the world's stunted or wasted children respectively live in Asia and Africa [3]. Most undernutrition occurs in low income countries, which are the only areas in the world to experience increases in stunting prevalence in the past ten years [3].

Reasons for undernutrition

Most malnutrition occurs due to inadequate intake or absorption of nutrients, although the complexity of this phenomena cannot be overstated [2]. For infants and children under five, undernutrition may occur due to maternal undernutrition or secondary to rapid growth [4], but the primary etiological processes that drive undernutrition are most likely food insecurity, inadequate feeding practices, lack of safe drinking water, climate change, and lack of access to health services [5]. Despite its complexity, undernutrition's superseding driver is ultimately poverty [2], which leads to high rates of stunting, wasting, and micronutrient deficiencies in resource poor countries [3].

Micronutrient deficiencies and protein-energy malnutrition

Micronutrient deficiencies

Children who have MNDs are at increased morbidity and mortality risk due to fatal infections, resultant cognitive, physical and economic losses, and deficits in reproductive capability; these factors ultimately lead to multigenerational consequences and the continued cycle of undernutrition [1,2]. Pregnant women and children under five are commonly micronutrient deficient, and the most widespread deficiencies are of iron, iodine, vitamin A, folate, and zinc [2] (Table 1-1). While iodine [6], and even folate [7] supplementation initiatives have been effective in combatting MNDs, vitamin A deficiency remains the leading cause of childhood blindness [8], and iron deficiency is the most common nutritional deficiency worldwide [9].

Table 1-1 Common micronutrient deficiencies and pathology (adapted from [10])

Deficiency	Prevalence	Pathology due to deficiency
Iron	2 billion	Iron deficiency anemia, malaise, low birth weight, increased infant mortality, stunting
Iodine	1.88 billion [11]	Goiter, cognitive deficit, hypothyroidism, infant mortality, stunting, inhibited sexual maturation
Vitamin A	240-250 million children and pregnant women [8]	Xeropthalmia, night blindness, increased maternal and infant mortality
Folate	Variable, but estimates as high as 30-40% of Western and non-Western cohorts [7]	Neural tube defects, megaloblastic anemia, heart disease, depression, stroke
Zinc	Global estimates of ~31% of poor children and women [12]	Stunting, decreased resistance to infectious disease, genetic disorders

Iron deficiency

Prevalence, pathology and etiology

Iron deficiency is linked to developmental [13], economic, and health [14] related complications. Economic losses due to iron deficiency exceed billions of dollars each year globally [15], and an estimated one billion people are anemic due to iron deficiency [9]. Iron deficiency anemia (IDA) rates are more than 30, 42, and 47% in non-pregnant women, pregnant women, and children worldwide [16], an improvement of only 4% in total iron deficiency prevalence in the past 20 years [17]. More anemic children are concurrently diagnosed with severe acute malnutrition (SAM) than in the general population, exacerbating anemia-mortality risk [18]. Simply, iron deficiency occurs with imbalances in iron intake, absorption, and demand [16]. Children and women are particularly vulnerable to iron deficiency due to physical factors such as rapid growth and blood loss [16],

respectively, but also because of economic, political, and institutional disadvantages that lead to infectious, health care related, and nutritional iron losses compared to their adult male counterparts (Figure 1-1) [16].

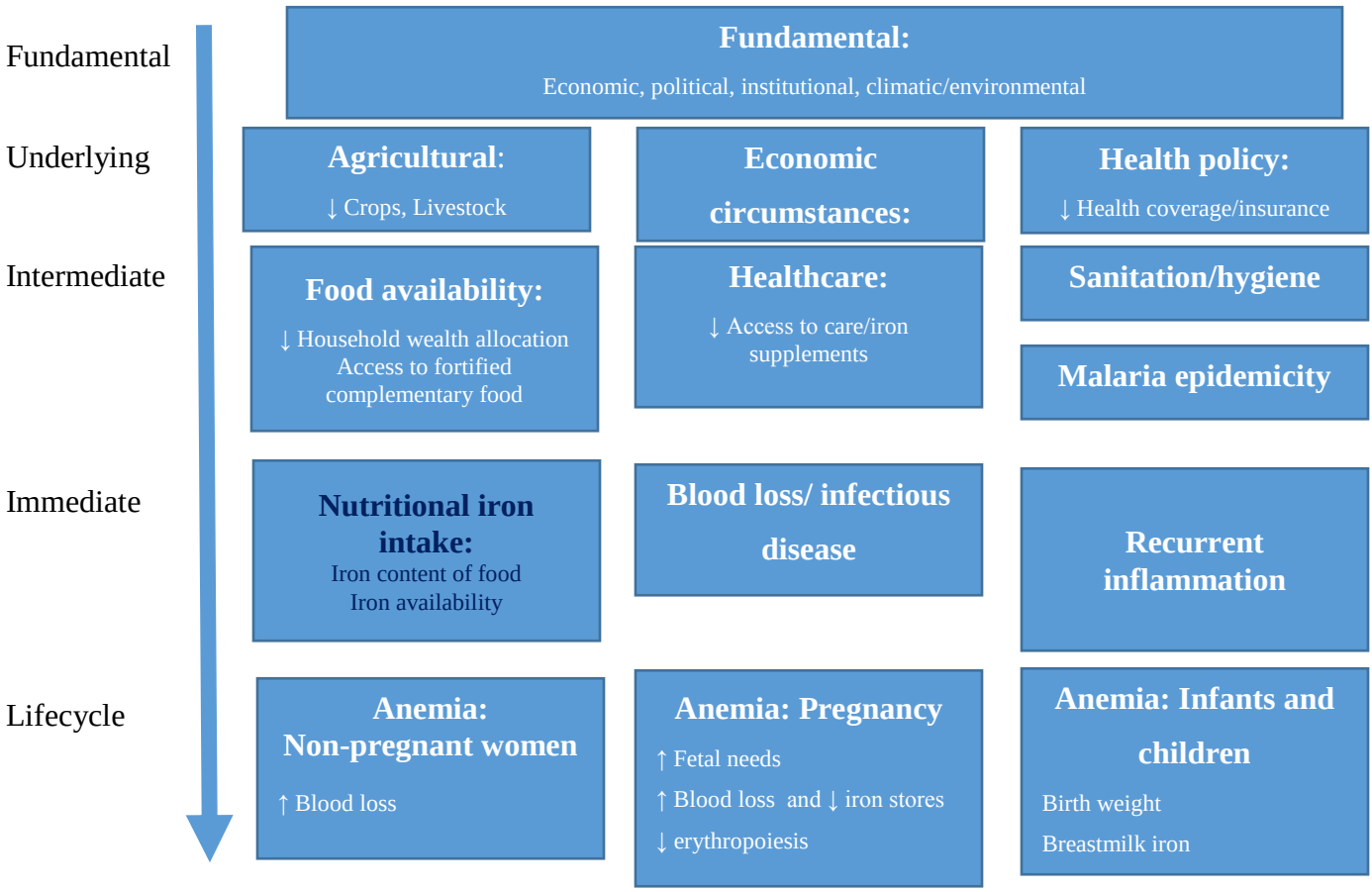

Figure 1-1: Iron deficiency determinants in women and children.

Adapted [16]. Women and children are particularly vulnerable to economic and political disadvantages. Lack of land ownership or agricultural outputs, lower literacy rates, decreased equal education and pay, and thus reduced health care coverage lead to less wealth and access to iron containing foods, access to healthcare to prevent and treat iron deficiency, and increased infectious burden and sanitation related illness. The anemia cycle continues as women experience increased fetal need for iron, coupled with blood loss during pregnancy and increased nutrient needs during breastfeeding.

Non-heme iron absorption and metabolism

Ten percent of iron stores are obtained through absorption, while the remainder comes from senescent red blood cell recycling [19,20]. Total body iron stores, hypoxia, and erythropoiesis regulate iron absorption, and these factors are balanced with multifactorial cell loss, menstruation, and intestinal iron losses through enterocyte sloughing [21]. Duodenal non-heme iron absorption occurs at the enterocyte apical cell membrane, where it is first reduced to its ferrous form by cytochrome Dcytb [22] and is transported into the cell by divalent metal transporter 1 [23]. Ferrous iron is then transported across the basal membrane of the enterocyte, exported by ferroportin [24], oxidized by hephaestin and bound to transferrin for transport and organ use [25].

Measurements of iron status and deficiency

Iron status and deficiency are often measured in context with hemoglobin, which is the iron-rich, oxygen-carrier molecule in red blood cells [26]. In iron deficiency, heme and red blood cell production are decreased [26], leading to an increased number of senescent cells, and later, a low proportion of reticulocytes, or immature red blood cells, which contain decreased heme content [27]. Decreased heme-cell availability then leads to decreased cell size and color (denoted microcytosis with hypochromia) [26], along with decreased total red blood cell counts, which collectively indicate iron deficiency [27]. Although anemia is a result of iron deficiency, heme production is tightly regulated, red blood cell production is conserved well into marginal iron status, and thus anemia itself is not indicative of iron stores [26] (Table 1-2). Serum ferritin, an iron storage molecule, coupled with total iron binding capacity, serum free iron, and transferritin saturation more accurately reflect iron balance [26,27] than hemoglobin itself. Serum ferritin is correlated with iron storage, although production is increased in inflammation, which may mask

iron insufficiency. Co-analysis of hemoglobin, which is decreased, along with mean corpuscular volume and TIBC, which are normal, in anemia of chronic disease, respectively [26,27], or an acute inflammatory biomarker such as C-reactive protein (CRP) can provide context to the inflammatory increase in ferritin levels.

Table 1-2: Trends in iron status markers with iron balance (Adapted [26]).

Measurement	Normal	Negative balance	Depletion	Deficiency	Anemia
Bone Marrow	↔	↓↓	↓↓	↓↓	↓↓
Plasma Ferritin	↔	↓	↓↓	↓↓↓	↓↓↓↓
TIBC[a]	↔	↔/↑	↑	↑↑	↑↑
sTr[b]	↔	↔/↓	↓	↓↓	↓↓
Plasma iron	↔	↔	↔	↓↓	↓↓
Hemoglobin	↔	↔	↔	↓ /↔	↓↓
MCV[c]	↔	↔	↔	↓/ ↔	↓

No change (↔), negative change (↓), positive change (↑). [a] Total iron binding capacity; [b]Serum transferritin saturation, [c]Mean corpuscular volume

Interventions targeting iron status

Iron homeostasis is mostly non-modifiable, so much of the nutritional focus around iron deficiency treatment has been on a few modifiable factors such as food inhibitors and iron absorption enhancers [19]. Strategies aimed at improving global iron status have centered around iron supplementation, and food fortification [16], although risks of supplementation in low income countries include increased malarial burden, iron overload (and related growth suppression), and supplement side effects [16]. Antinutritional factors, such as tannins and phytates, directly chelate

iron to form insoluble complexes [28], and thereby impair absorption. Due to the possibility for dietary restriction, and propensity for iron chelation, antinutritional factors have been criticized for their contribution to iron deficiency in low income countries [29,30] and are thus an area of focus for researchers and health workers.

Models assessing iron bioavailability

Among others, some commonly used iron bioavailability assessment methods include radioiron tagging or stable iron isotope ingestion [31], postabsorptive plasma iron measurement [32], *in vitro Caco-2* cell model[33], and the animal model based hemoglobin depletion-repletion method [34]. Radioiron tagging is often used as an endpoint measurement, meaning that ultimately, tagged iron is measured from red blood cells, and absorption is based on expected iron loss versus measured red blood cell iron-incorporation [31,34]. Postabsorptive plasma iron measures changes in plasma iron after oral iron administration [32]. Both radioiron tagging and postabsorptive iron measurement allow for direct measurement of human iron bioavailability, and can be modified to assess for acute or long-term nutrient analysis [31,32,34]. Despite their advantages, radioiron tagging and post-absorptive plasma iron measurement are costly, time-consuming, and often, populations of interest (particularly infants and children) are difficult to study.

Non-human iron bioavailability methods include *in vitro Caco-2* cell and the animal model based hemoglobin depletion-repletion method. Colorectal *Caco-2* cells have the ability differentiate to enterocytes with normal epithelial cell characteristics, including secretion of brush border enzymes [33]. After differentiation, *Caco-2* cells take up iron like typical enterocytes

[33]. Simulated digestion, and then *Caco-2* iron uptake have thus been used to measure acute iron bioavailability from meals [33,34].

Pig, rat, and chickens are all models used to simulate iron bioavailability *in vivo* [35]. Most animal studies use variants of the hemoglobin depletion-repletion model, which essentially creates increased iron demand to measure iron bioavailability differences in test meals [34]. Rats are commonly used experimental model for iron bioavailability, are simple and cost effective, and have been shown to accurately simulate human digestion [36]. Pigs are omnivorous, have similar nutrient requirements as humans [37,38], and studies in pigs have demonstrated similar pharmacokinetic and bioavailability results as humans [39]. Broiler chickens are cost effective, have similar intestinal anatomy to humans, mirrors human iron responses during anemia [35], and matches iron availability studies in *Caco-2* cells [40]. While rats, pigs, and chickens present cost-effective, feasible models for iron bioavailability simulation, there are dissimilarities between each model and human digestion that limit their broad application. Rats have been noted to have greater iron bioavailability than humans, potentially due to differences in human and rat gastrointestinal tracts, energy expenditure, and nutrient metabolism [38]. While pigs accurately simulate human iron digestion, they are cost prohibitive [39]. It is not well understood whether avian iron metabolism accurately reflects human bioavailability outside of *Caco-2* cell digestion.

Vitamin A deficiency

Prevalence, pathology, and treatment

Vitamin A deficiency is the third most common nutritional deficiency in the world [8], and its estimated prevalence in South Asia and Sub Saharan Africa nears 40% [41]. Morbidity and mortality rates due to infectious disease are doubled in deficient children [41], and supplementation has been shown to reduce all-cause mortality by as much as 24% in deficient children 6 months – 5 years [42]. Vitamin A deficiency most commonly occurs due to inadequate preformed vitamin A intake, as well as diets low in provitamin A-rich carotenoid fruits and vegetables [42]. The most common interventions to combat vitamin A deficiency include supplementation and food fortification with preformed vitamin A, carotenoid rich food consumption, and crop bio-fortification [43]. In 2014, fortification initiatives reached targeted estimates of 80% of East Asian, Pacific, and West and Central African nations [44], while Eastern, Southern African, and South Asian countries remained nearly 20% below targets [44].

Vitamin A absorption, metabolism, and measurement

Vitamin A is a fat-soluble vitamin commonly consumed as either preformed vitamin A (retinol or retinyl esters) or from provitamin A carotenoids [45]. Preformed vitamin A is found in animal products (dairy, fish, meat), while provitamin A carotenoids are generally plant-based [45]. Most dietary vitamin A, whether carotenoid or preformed, are transported by chylomicrons and converted to retinyl esters for storage in hepatic stellate cells, or are converted to retinol, bound to retinol binding protein, and directed to various organs for use [46]. Vitamin A (cis-retinal) is needed to form rhodopsin, needed for dim light accommodation in rod formation, and vitamin A deficiency leads to keratinization of corneal tissue by xeropthalmia [26]. Vitamin A deficiency

therefore leads to night blindness, and eventually, corneal scarring, and complete blindness [26]. Serum vitamin A stores are tightly homeostatically regulated by retinol binding protein (RBP), and thus, reductions in serum retinol (<0.7 μmol/L) reflect depleted vitamin A stores, and deficiency [43]. Due to tightly regulated serum vitamin A levels, blood measurement is not directly reflective of status, similar to iron metabolism, but is useful in identifying deficiency. Aside from overt vitamin A deficiency, hepatic RBP production is decreased during inflammation or illness, and thus the clinical picture of vitamin A deficiency based on serum retinol depends on the inflammatory state of the individual [47]. Hepatic retinol isotope dilution is a technique that allows for more accurate estimation of deficiency and moderate or marginal vitamin A status by using a small dose of tracer vitamin A followed by measurements of unlabeled vitamin A to tracer vitamin A ratios [46], however this technique is not field-friendly due to the need for blood storage, and sophisticated lab assessment. Retinol-binding protein has been found to be reflective of serum retinol [48], can be adjusted for inflammation [49], and can be performed in a simple ELISA from a dried blood spot [50].

Protein-energy undernutrition

Prevalence, pathology, and etiology

Protein-energy malnutrition (PEM) is an umbrella term for a constellation of conditions including underweight (<80% weight for age), marasmus (<60% weight for age), kwashiorkor (weight for age <80% + edema), stunting, wasting, mild, moderate, and severe acute malnutrition (SAM; weight for height, edema, MUAC <80%) [51,52]. Edema's presence in undernutrition indicates electrolyte abnormalities encompassed in protein deficiency with, or without, caloric deficit [52]. Increased vulnerability to gram negative septicemia is common with SAM, and treatment is complicated by commonplace and life-threatening refeeding syndrome [52]. In refeeding syndrome, isocaloric or hypercaloric diets cause functional electrolyte deficits that occur secondary to rapid changes in metabolic rate [2].

Infectious disease burden, impaired dietary absorption, rapid growth, and ultimately inadequate protein intake all contribute to PEM prevalence rates among children in low income countries [53]. Even with adequate PEM recovery, children have been found to be at increased risk for relapse or death more than a year after returning to normal anthropometric-for-age scores [54]. It has been suggested that extended feeding programs may help to prevent relapse [55], but above all, energy deficits and subsequent macronutrient imbalances compound the problem of PEM. Total, and utilizable, protein may differ among nourished and undernourished individuals; positive energy balance uses a significantly higher proportion of total protein for growth than negative energy balance [53]. Findings from one study estimated that 5% energy deficits in underweight men increased protein needs for growth by 10% [56]. These findings underscore the nuances in energy balance and macronutrient-diet composition that must be considered in

childhood PEM, where growth patterns lead to continual and rapid changes in macronutrient needs. Unfortunately, it is not well understood how to best treat PEM once a child is undernourished. Protein intake in infancy correlates with later total weight gain [53], and animal protein intake has been shown to be associated with linear growth with adequate weight for age [57], but it is not well understood whether protein intake results in improved linear growth or obesity later in life [58,59]. Amino acid composition and protein quality regulate growth rates among a variety of protein sources [60-62], although debate remains about plant versus animal source proteins' adequacy for PEM treatment [63]. In food secure areas, PEM may be combated effectively through community nutritional education [64] outlining treatment complexity that is beyond metabolic nutrition alone.

Measurements of protein quality

Common measurements for protein quality include the protein efficiency ratio (PER), amino acid scoring (AAS), the protein digestibility-corrected amino acid score (PDCASS), and the digestible indispensable amino acid score (DIAAS). While the PER has been commonly used for protein quality assessment, recent recommendations have favored models that more accurately simulate human digestion [65]. Despite this, it is important to consider models independently for their strengths to measure protein quality accurately.

The PER is a standardized method that measures weight gain for four weeks in weanling rats consuming a test diet, and compares efficiency of weight gain to a control, high quality (casein) protein diet [65]. Final PER values are calculated by dividing weight gain by the amount of protein consumed during the study [65]. The PER 'can be used when there is doubt about the protein quality of a food or diet,' but limitations include that animal feeding patterns do not

simulate post-prandial and post-absorptive changes in protein and amino acid balances in humans, and that the method does not account for caloric maintenance [65,66]. Despite questions regarding similarity of the method to simulating human digestion, strengths include 1) that the PER may be more accurate when considering antinutritional factors [67], and 2) that the PER allows for nutritive comparison of foods, outside of human digestion [65,67].

While the PER perhaps gives context to protein quality of foods independent of human digestion, there has been consensus that a method based on human amino acid requirements might be a more suitable approach [65]. The AAS was developed to score protein quality based on the first limiting amino acids in foods [65,68]. Despite its attempt to rate quantity needed for human amino acid needs, the AAS lacks context for amino acid bioavailability, protein digestion, and methodology for amino acid quantification [65,69], limiting its generalizability for human digestion. Currently, the FAO/WHO recommend the PDCAAS method for determination of protein quality, although the digestible indispensable amino acid score (DIAAS) has been proposed as a better measurement [66]. While the PDCAAS assesses the quality of protein based on total digestibility and amino acid composition, its limitations include measuring effects of antinutritional factors, as well as lack of distinct scoring differences among protein sources due to score truncation [53,66]. By contrast, the DIAAS allows for individual amino acids measurement in foods, from ileal digesta, without a truncated score, in age-defined populations [66]. These factors may be particularly important in low protein diet consumers, like children with PEM.

Strategies to reduce undernutrition: Food aid

Global food aid use and distribution

To combat undernutrition, food aid programs exist for pregnant women, malnourished individuals with tuberculosis or HIV/AIDs, school-aged children and children under five [70]. In-kind food aid, local and regional purchase, cash transfers for food, and food vouchers all constitute methods for food aid delivery [70]. The United States provides most food aid as Title II (in-kind) food aid, where U.S. commodities are purchased, processed, and transported to food aid beneficiaries [70]. In 2012, the World Food Program (WFP) estimated that 2,085,721 metric tons of food aid was sent from the United States (more than 50% of total food aid donations); and 2,976,688 tons of world food aid was received by Sub-Saharan Africa alone [70] (Table 1-3).

Table 1-3: Food aid received (2012) by region [71]

Country (2012)	Food aid received (metric tons)
Sub-Saharan Africa	2,976,688
North Africa and Middle East	472,995
Asia	1,090,719
Latin America and Caribbean	194,811
Europe	4,793
Total	4,740,011

Commonly distributed food aid products

Ready-to-use therapeutic food (RUTF) and fortified-blended food (FBF) aid products are approved for pediatric food aid, and have proven effectiveness in preventing stunting and wasting [72]. RUTFs are lipid-based or protein-rich, micronutrient-fortified supplements, while

FBFs are micronutrient fortified cereal-legume porridges that are partially precooked [73]. High energy biscuits, compressed food bars, and micronutrient sprinkle powders provide micronutrient-and-protein or micronutrient only fortification to improve nutrition where cooking facilities are scarce, and are less commonly distributed [71]. The most commonly distributed food aid are FBFs, which have been approved for treatment of acute malnutrition and stunting during complementary feeding, malnourished individuals on antiretroviral or tuberculosis treatments, pregnant and lactating women [71]. FBFs' nutrient-cost benefit has contributed to their widespread use [74]. It is recommended that FBFs be consumed as <50% of total caloric needs [71], although they are often consumed as a higher proportion than recommended. The most commonly produced FBF by the United States is CSB, or corn-soy blend [75], but wheat and rice are also combined with soy in FBFs [73] (Table 1-4).

Table 1-4: Commodities purchased for food aid FY 2014, WFP adapted [76]

Food Type	Percentage	Amount x1000 metric tons
Maize	16	350.1
Sorghum	7	169.5
Rice	16	360.5
Wheat	7	161.6
Cereal flours	7	168.9
Dried Pulses	11	247.9
Vegetable oil	6	141.1
Sugar	1	38.8
RUTF/HEB	10	227.8
MNP/compressed food bars	1	25.9
Canned foods	1	33
Cereal based processed foods	5	114.3
Family parcels	6	145.9
Total	100	2185.9

RUTF: ready to use therapeutic food; HEB: high-energy-biscuit; MNP: micronutrient powders/sprinkles

History of food aid development and use

The first FBF, 'corn-soy milk' consisted of vitamin and micronutrient powder, dried milk, corn, and soy flour, and was used in the 1960's for its ease and simplicity of delivery that was consistent with post-World War II pragmatism for 'efficient diets' that were nutrient dense and easily digestible [77]. FBFs' benefits, including ease of humanitarian programming around use, transport ease, and a 'one size fits all' approach [78] have been essential components to their continued use. FBFs can be culturally modified in their preparation, and their partially precooked

nature allows for recipient control over feeding practices while supplying consistent nutrition [77]. Consumption of FBFs has been shown to treat moderate acute malnutrition as effectively as RUTF [79], at a fraction of the price, which enhances recipient availability. Despite many benefits, in-country commodity sourcing, palatability barriers, and product misuse (improper preparation, selling or stealing product) complicate FBF use [77].

After their inception, few modifications were made to FBFs formulations for nearly forty years [74]. In 2004, WFP called for food aid programs that included development of FBFs conceived with participatory research for feasible, affordable, and culturally acceptable recipes [80]. The report noted that while FBFs could provide nutritious, micronutrient dense foods, they inconsistently met iron or zinc requirements in a Hatian cohort [80]. Five years later, a systematic review purported that despite positive effects on recovery from moderate acute malnutrition and weight gain due to FBF consumption, there was 'limited and weak' evidence for FBF nutritional outcomes improvement, including micronutrient status, in children [81]. Other reports have claimed that FBFs lack macronutrient and micronutrient composition necessary to prevent stunting or wasting, and that their composition inherently inhibits micronutrient absorption due to food matrix issues (i.e. antinutritional factors) [82]. In 2010, a review suggested that both development of new products, and improving FBF manufacturing and formulation to enhance delivery efficiency would better meet recipient needs [74].

Strategies to improve food aid

A 2011 review commissioned by the United States Agency for International Development (USAID) [82] called for formulation of new FBFs that prioritized nutritional improvement at the heart of programming. They emphasized in this document that FBFs needed to be

"1) energy-dense and rich in micronutrients, 2) easily digestible and palatable, and 3) able to be prepared relatively quickly, i.e. with minimal cooking" [82].

Suggested FBF changes included improvements in nutritional quality to enhance macronutrient and micronutrient blend composition, formulation of 'smart' food aid that was culturally acceptable and palatable to recipients, and use of processing methods and commodities that would improve nutritional quality, as well as delivery and availability efficiency for food aid beneficiaries [82]. Specifically, the report suggested that sorghum could be used for new FBFs, noting that it would meet beneficiaries' needs given its "acceptability in Africa, its relatively low price, and its acceptability among host governments [82]." To improve FBFs' nutritional quality, it was suggested that antinutritional factor reductions through processing "including extrusion," could enhance nutrient and energy density, and improve product quality through longer shelf life [82]. In this report, authors suggested that a prototype formula "CSB14" could be compared to CSB13, which at the time was the most commonly distributed USAID food aid product (Table 1-5) [82].

Table 1-5: Comparing CSB+, envisioned CSB14, and infant DRI/RNI values

	CSB13 (per 100 g)	CSB14 (envisioned, per 100g)	DRI[a]/RNI[b] for 6-11 month infants
Energy (kcal)	386.1	387	675
Protein (g)	15.9	17.7	16
PDCASS	0.85	0.87	-
Utilizable protein (g)	13.5	15.4	-
Animal source protein (g)	0	2.4	-
Fat (g)	8.7	8.8	31
Iron (mg)	10.6	15.5	7.7
Vitamin A (mg)	0.819	0.532	0.46

PDCAAS: Protein digestibility-corrected amino acid score. Modifications in envisioned CSB14 compared to CSB13. Recommendations included addition of animal source protein, increased blend fat content, decreased preformed vitamin A, and increased iron (as NaFeEDTA) [82].
[a]Dietary reference intake, [b]Reference nutrient intake

Commodities

Sorghum

Sorghum is the fifth most commonly produced cereal crop globally, and the second most produced cereal in Africa [83]. Due to its propensity for drought-tolerance, sorghum production is generally highest in semi-arid parts of India, Sub Saharan Africa, and the Mid-Western United States (Figure 1-2) [84]. In drought years, sorghum has documented greater yields, and less year to year variability, than its grass counterpart, corn [85,86]. Sorghum is not a genetically modified organism (GMO), and can thus be exported to food aid receiving counties that may not accept modified foods, and is often obtained at a lower price than corn [86]. One challenge to sorghum

production is the lack of post-emergent herbicides (largely due to its non-GMO status), which reduces overall crop yield [86]. Although most sorghum varieties grown in the U.S. have almost no tannin content through breeding [87], higher tannin content in sorghum largely dictates bird, mold, and insect resistance [88], and thus may improve crop yields in lieu of herbicide use.

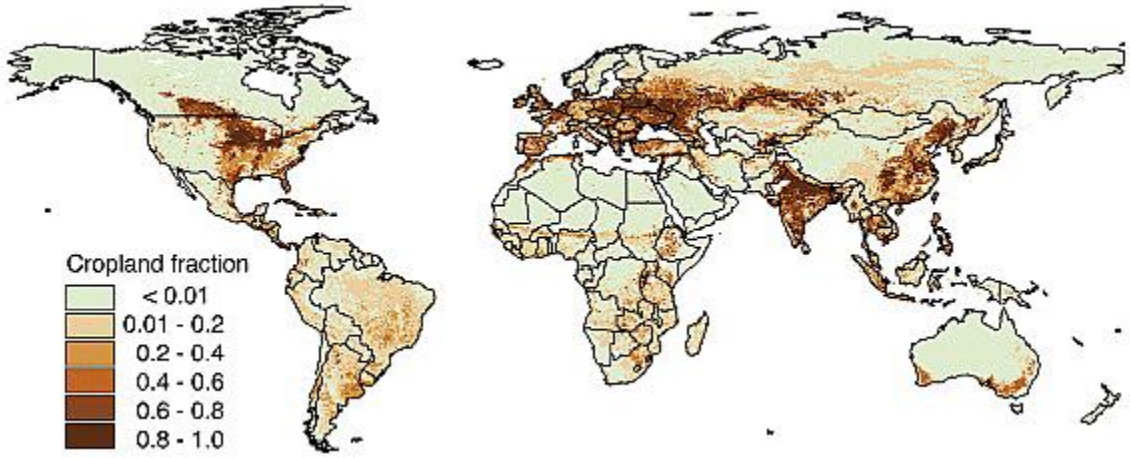

Figure 1-2: Cropland fraction of sorghum by geographical location with permission [84]

In many parts of Africa, sorghum is consumed as porridge, bread, or whole grain [89], but it is most commonly used as animal feed in wealthier countries [83]. Compared to corn, sorghum contains similar starch, protein, and fiber content [83,90-92], potentially making it a viable alternative to corn in complementary foods and FBFs (Table 1-6). Like corn, while sorghum is rich in cysteine and methionine, it lacks lysine necessary for complementary feeding (Table 1-6) [90-92]. Another challenge to its nutritional use is that its starch and protein digestibility when cooked are reduced, often up to 25 and 50% less than corn, respectively [91]. Strategies to improve sorghum digestibility include soaking, fermentation, and heat processing [91].

Table 1-6: Comparing sorghum and corn nutritional composition adapted [83,87,91-94]

Composition	Sorghum (whole, dry)	Corn (whole, dry)
Kcal (per 100g)	358	329
Starch (%)	63-68	60-64
Protein (%)	9-11	9-11
Fat (%)	1-1.5	3-5
Fiber (%)	1-2	1-2
Iron (mg/100g)	5.4	2.7
Cysteine/Methionine (mg/100g)	26.3	28.6
Lysine (mg/g)[a]	14.6	26.1
Polyphenols (mg/100g)	10-68	69-71

[a]DIAAS 6-30 month: 57 mg/g [90] [b]DIAAS 6-30 month: 27 mg/g [90]. DIAAS: Digestible indispensable amino acid score.

Cowpea

Cowpeas, also known as black-eyed peas, are drought-tolerant and nitrogen-fixing legumes often used for intercropping in arid and sub-tropical regions [95]. Sub-Saharan Africa produces 96% of global cowpea hectacres [96], where the legume is consumed as an important source of zinc and iron [97]. In addition to legume consumption as either fresh or dried seeds, cowpea leafy greens are commonly consumed [98]. Cowpea's relative lysine abundance to sorghum, coupled with cysteine and methionine deficiency [99], make it a complementary protein source to both sorghum and corn. Compared to soy, cowpea has similar lysine content, however, total protein, and fat content are lower, while starch content is higher [97,100,101] (Table 1-7).

Table 1-7: Comparing cowpea[97,101] and soy [100] nutrient composition

Composition	Cowpea (whole)	Soy (whole)
Kcal (per 100g)	336	382
Starch (%)	35-55	30
Protein (%)	18-30	42.6
Fat (%)	1-1.5	20
Fiber (%)	6.3	5.3
Iron (mg/100g)	6-7.6	15.7
Cysteine/Methionine (mg/100g)	9.6-11.1	25.9
Lysine (mg/g)[a]	47.7-67.8	63.8
Proanthocyanidins (mg/100g)	2.2-6.3	20-65

[a]DIAAS 6-30 month: 57 mg/g [90] [b]DIAAS 6-30 month: 27 mg/g [90]. DIAAS: Digestible indispensable amino acid score.

Food inhibitors common in cereal and legume products: antinutritional factors

Antinutritional factors are food factors that inhibit macronutrient and micronutrient absorption, and can be found within foods, or are sometimes the result of protein processing [102]. Trypsin inhibitors, hemmaglutinins, glucosinolates, gossypol, tannins and phytates occur naturally in legumes, grains, and oils; manufactured antinutritional factors often come from Maillard reactions [102]. Antinutritional factors are often discussed because they reduce food protein quality and inhibit mineral absorption [102].

Tannins

Tannins are water-soluble polyphenolic compounds, either hydrolyzable, or condensed (also known as proanthocyanidins) [103] (Figure 1-3). Due to their hydrophilic nature, tannins tend to form complexes with proteins and minerals most predominately in solutions [102]. Hydrolyzable

tannins are generally gallic acid esters of polyols, are easily hydrolyzed by acidic or basic conditions [104], and are rarely consumed [103] (Figure 1-3). Condensed tannins are comprised of oligomers and polymers of flavan-3-ols linked by carbon bonds [105], are difficult to hydrolyze [104] (Figure 1-3), and are commonly consumed, often found in sorghum, wine, tea, dark chocolate and berries [103]. Both hydrolyzable and condensed tannins have a multidendate nature that allows for precipitation of multiple cationic compounds to polyphenol hydroxyl groups, and complexes are generally insoluble during digestion [106,107]. Larger tannin molecules tend to bind to cationic metals with greater affinity, and acidic environments favor precipitation and binding, thereby reducing mineral bioavailability [106-108]. In general, tannins are heat-resistant, and processing treatments aimed at their removal from foods are often expensive and ineffective [102]. Despite cited antinutritional effects, condensed tannins may confer antioxidant [109], cancer fighting [110], and cardiovascular [111] health benefits.

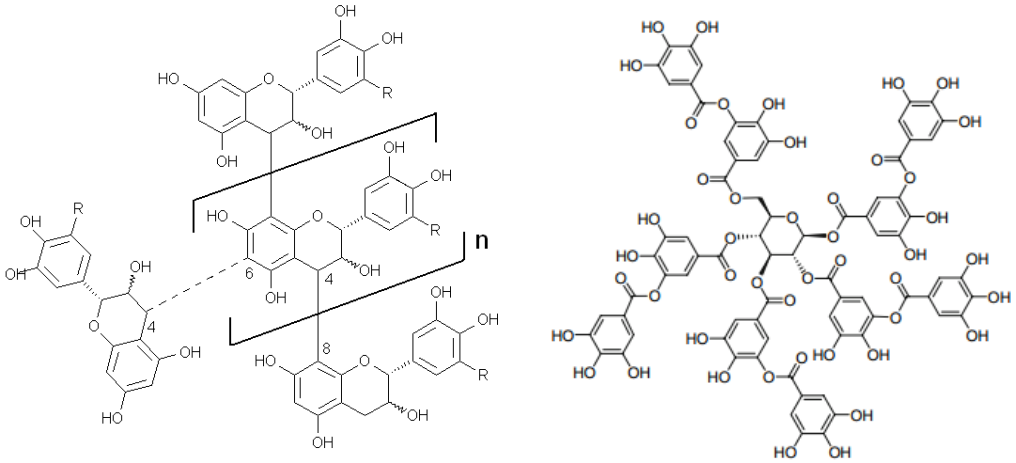

Figure 1-3: A: Condensed tannin Yikrazuul/ Wikimedia Commons / Public Domain and B: Tannic Acid Ronhjones/ Wikimedia Commons / Public Domain

Phytic acid

Phytic acid (*myo*-inositol hexakisphosphate) is a phosphorous storage compound for most plants, including grains and legumes, that often accounts for the majority of a plant's phosphorous content [112] (Figure 1-4). The *myo* denotation refers to the hydroxyl conformation around the inositol ring, and is the most commonly consumed phytate form [112]. Like tannins, phytic acid compounds are multidendate, meaning that they can bind to multiple sites on a given compound [113]. Increased inositol size leads to increased metal cation affinity and chelation [112], however, unlike tannins, both increased and decreased pH leads to increased precipitation depending on the bound agent pKa [114]. Negatively charged phosphates around the inositol ring chelate metal cations, exerting an 'antinutritional effect [112].' When metal ions are bound to phytic acid, the resultant insoluble compound is called a *phytate* [112]. Wheat and sorghum phytic acid content is generally highest in the grain bran, and thus the food matrix in which phytic acid is consumed plays a direct role in its antinutritional effect [112]. The phytic acid to iron molar ratio plays a role in iron bioavailability specifically, and amounts less than or equal to 10:1 phytic acid: iron tend to have less effect on absorption [115]. Phytic acid-iron chelation is less prone to stay precipitated during digestion than tannic acid, and can be reversed by ascorbic acid or certain iron salts [112,116]. Increased metal ion: phytic acid ratio (often other than iron: Ca^{2+}, Mg^{2+}, Cu^{2+}, Zn^{2+}) increases the propensity of phytate formation [117], meaning that increased food-mineral content increases the likelihood of iron precipitation regardless of food-iron content. Strategies aimed at phytic acid reduction in foods include milling, soaking, phytase treatment, and recently, genomic reduction [112]. Despite its general denotation as an antinutritional factor, phytic acid has potential cancer fighting antioxidant mechanisms [112], is antiviral [118], may lower glucose [119], and cholesterol [120].

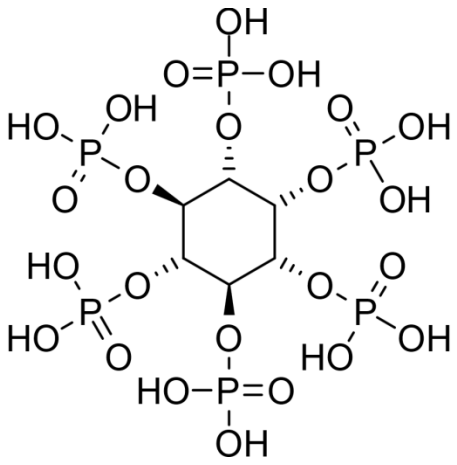

Figure 1-4: Chemical structure of phytic acid (*myo*-inositol hexakisphosphate) Harbinary/Wikimedia Commons/ Public Domain

Strategies aimed at reducing antinutritional content of foods

Bio-fortification, soaking, fermentation, milling, extrusion, enzymatic food treatment, and cooking are all used to overcome, destroy or denature antinutritional factors. Bio-fortification uses crop-gene modification or conventional plant breeding in commonly consumed plants to reduce negative food components (tannin or phytate plant content, for example) or improve positive attributes (increase iron or zinc content) [121]. Processing, including heat or plant protein hydrolysis has also been shown to reduce soybeans' antinutritional effect [122]. Phytates, which are not completely destroyed with cooking, are often reduced by application of phytase enzyme treatment [123,124], which is often only partially effective, and is a costly measure. Household strategies aimed at improving nutrient availability include grain and legume germination, fermentation, cooking, and soaking, as well as increased ascorbic acid consumption [125]. Despite multiple approaches aimed at antinutrient reductions, socio-cultural and economic factors may render household use unlikely [125].

Extrusion processing

Extrusion processing uses a combination of milling, steam and pressure to process foods with enhanced nutrient retention, and antinutritional factor reduction at a high rate of productivity [126]. An added benefit to extruded foods for undernourished children is that foods can be produced with a high caloric density [82]. From an ecological standpoint, extruded products are 'pre-cooked', meaning that they take less time and energy to prepare foods that must be boiled or cooked over a stove. Mechanical shearing, high temperature, and short times for processing allow for tannin or phytic acid polymer covalent bond destruction [126]. It is the processing and food-protein denaturation, through decreased temperature [127], screw speed [128], and pressure [129], that may allow for increased digestive enzyme binding to proteins, and thus better protein bioavailability [126]. Iron content in extrudates is actually enhanced, potentially due to metallic screws wear during processing [130].

Effects of extrusion processing on protein quality and iron bioavailability

Many studies have cited improvements in protein and iron-related outcomes with extruded compared to conventionally prepared foods. In weanling pigs, soy-meal extrusion significantly improved average daily gains and gain to feed ratios compared to roasted soybeans by nearly 31% over a 14-day feeding period [131]. Mineral bioavailability and protein digestibility significantly increased compared with roasting and malting in extruded soybeans in the *in vitro* Caco-2 digestion model (75-79 and 79-82% vs. 47-53 and 70-72% iron and protein in extruded vs. malted/roasted common beans, respectively) [132]. *In vitro* digestion of extruded vs. raw lentils was significantly improved from 60 to 90%, and polyphenol and phytic acid content were significantly reduced by more than half [133]. Extrusion of pearl millet significantly enhanced *in*

vitro iron bioavailability by 3.5 times, and protein digestibility by 10% compared to roasted millet [134]. Extruded, iron-fortified rice reduced anemia prevalence similar to ferrous sulfate fortification in Philipino children aged 6-9 [135]. Despite these results, findings citing improvement in iron incorporation into hemoglobin with extruded vs. non-extruded products have been mixed. In a study of 30 normal men and women consuming extruded vs. non-extruded wheat bran and flour, there were no differences in iron bioavailability measured by iron absorption after consuming meals [136]. Similarly, rats consuming extruded chickpeas had similar hemoglobin and hepatic iron levels at end line compared to their non-extruded meal consuming counterparts despite similar food intake [137].

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Chapter 2 - AN EVALUATION OF IRON, VITAMIN A, AND GROWTH OUTCOMES IN RATS CONSUMING EXTRUDED FORTIFIED-BLENDED FOODS COMPARED TO CORN-SOY BLEND PLUS

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Abstract

Corn and soybean micronutrient fortified-blended foods (FBFs) are commonly used for food aid. Sorghum and cowpeas have been suggested as alternative commodities because they are drought-tolerant, can be grown in many localities, and are not genetically modified. Change in formulation of blends may improve protein quality, vitamin A and iron availability of FBFs. The primary objective of this study was to compare protein quality, iron, and vitamin A availability of newly formulated, extruded, sorghum, cowpea, soy, and corn based FBFs, along with a current, USAID corn and soy blend FBF (CSB+). A second objective was to compare protein quality of whey protein concentrate (WPC) and soy protein isolate (SPI) containing FBFs to determine whether WPC inclusion improved outcomes. Eight groups of growing rats (n = 10)

consumed two white and one red sorghum-cowpea (WSC1, WSC2, RSC), white sorghum-soy (WSS), corn-soy (CSB14), white sorghum-cowpea with SPI (WSC1+SPI) extruded FBFs, CSB+, and AIN-93G, a weanling rat diet, for 4 weeks. There were no significant differences in protein quality, iron or vitamin A outcomes between WPC FBFs groups. The CSB+ group consumed significantly less food, gained significantly less weight, and had significantly lower caloric efficiency, protein efficiency, and length, compared to all other groups. Compared with WSC1, the WSC1 + SPI FBF group had significantly lower caloric efficiency, protein efficiency, and weight gain. These results suggest that a variety of commodities can be used in formulation of FBFs, and that newly formulated extruded FBFs are of better nutritional quality than CSB+.

Background

Protein-energy malnutrition, iron, and vitamin A remain some of the most common nutritional deficiencies worldwide [1], and food aid targeted at improving both food security and foreign agricultural development is necessary to create sustainable and effective programs to treat undernutrition. Fortified blended foods (FBFs), have traditionally consisted of micronutrient fortified, partially precooked blends of milled cereals and pulses, and the most commonly distributed micronutrient-fortified food aid by the United States Department of Agriculture (USDA) is the FBF corn soy blend (CSB). Hundreds of thousands of metric tons of CSB are distributed annually [2], and the most widely distributed is CSB+, a roasted CSB blend [3]. A recent report cited the importance of formulating new food aid products to improve treatment of malnutrition, which included suggestions to utilize crops that are adapted to climate change, locally-available, and utilizing processing methods that may destroy anti-nutritional factors

thereby improving the nutritional quality of FBFs [4]. Despite recommendations calling for new formulations, there is little research assessing nutritional outcomes from these changes compared with previous FBF formulations.

Corn, soy, sorghum, and cowpea are all crops suitable for food aid due to their availability and acceptability worldwide. In 2012, The World Food Program (WFP) invested 62% of its food aid efforts to support Sub-Saharan African nations[5], and sorghum and cowpea are appealing crops for use in FBFs here due to common consumption, drought-tolerance, and regional and local availability [6-9]. Sorghum porridge is an already acceptable and widely consumed staple in many areas throughout Africa, and from 1993-2013, 36% of global sorghum production came from African nations [9,10]. Cowpea is a nitrogen-fixing, drought-tolerant legume that can be utilized in intercropping because it is tolerant to shade [8,11], and Africa produces 96% of global cowpea hectares [6]. The combination of sorghum with cowpea in FBFs has potential to enhance low levels of cysteine and methionine found in cowpeas, and cowpeas' amino acid composition complements traditionally low lysine levels in sorghum [6,11]. Formulating new blends with sorghum and cowpea may allow for local and regional procurement aimed at improving local agricultural markets and nutritional outcomes in food aid receiving countries [4].

When consumed in large quantities, antinutritional factors such as trypsin and hemagglutinins in legumes, and phytates and tannins in cereals, may negatively affect the bioavailability of amino acids and minerals such as zinc and iron that may contribute to stunting, wasting and micronutrient deficiencies in low income countries [12,13]. Extrusion is a processing technique that has been shown to decrease antinutritional factors and improve protein and iron bioavailability [14-16] by an operation that first grinds, then partially cooks, and finally applies

pressure to products to promote expansion to a desired density [17]. Additionally, because density of extruded products is controlled, a unique benefit of this processing is its ability to create solids compositions that enhances caloric and micronutrient density of FBFs [4]. Extrusion may further benefit food aid consumers because it can create pre-cooked porridges, which take less energy to prepare and consume. In addition to suggestions to use alternative commodities and processing methods, it has been proposed that lack of animal source protein may be a reason that FBFs have not traditionally adequately prevented stunting and wasting [4], although this has not been supported in a recent review [18]. Limitations to utilizing animal proteins isolates like whey protein concentrate (WPC) include: they are costly, may not provide the protein quantity to support linear growth in suggested amounts, and may lack beneficial bioactive components reported as important components of supporting growth [19]. A recent field trial comparing CSB+ against an animal protein complementary food product found no difference in linear growth, iron status, or lean mass between protein sources in children consuming them as complementary food at 6 months of age for 9 months [20]. Utilization of soy based proteins may be a safe, cost-effective, and efficacious alternative to WPC [21], and therefore, whey and soy protein may similarly enhance protein quality of FBFs by providing amino acids that are highly bioavailable.

The primary objective in this study was to assess protein, iron, and vitamin A outcomes of newly formulated, (according to United States Agency for International Development (USAID) guidelines [4] extruded, sorghum, cowpea, corn and soy based FBFs, along with a current, non-extruded USAID corn and soy blend FBF, CSB+. Combinations of corn, soy, sorghum, and cowpea were chosen as commodities to assess whether sorghum or cowpea, recommended as alternative to corn or soy in the Food Aid Quality Report (FAQR), could be utilized with similar

or better protein, vitamin A, or iron outcomes. A second aim was to compare the protein quality of a whey protein concentrate (WPC) containing FBF to a soy protein isolate (SPI) containing FBF [18,19].

Methods

Ethical Standards

We chose weanling rats, which are a well-known nutritional model, to allow for assessment of FBF protein quality, vitamin A, and iron bioavailability during a linear growth period. The Institutional Animal Care and Use Committee (IACUC) at Kansas State University approved all animal procedures (protocol 3399). Welfare assessments were carried out prior to and during the experiment.

Diets

In order to compare nutritional outcomes related to recommended formulation, six FBFs were developed according to USAID food aid recommendations [4], and were later reformulated to meet viscosity requirements. In reformulation, sugar replaced 15% grain and legume flours, and additional WPC or SPI and oil were added to meet protein and fat requirements (Table 2-1). Vitamin and mineral premixes were formulated according to recommendations by the FAQR [4], as 3.2% of FBFs (Research Products Company, Salina, KS). Blends were created by extruding grain and legume flours, milling to powder, then adding sugar, vitamin and mineral premix, oil and whey protein concentrate 80% (WPC80) (Davisco Foods, Eden Prairie, MN) or SPI 80% (Organic Puris 1060, World Food Processing, Turtle Lake, WI). For comparison of commodity types within FBF formulation, two white (Fontanelle 4575, 738Y), one red (217X Burgundy) sorghum with cowpea

(WSC1, WSC2, RSC, respectively), a white sorghum (Fontanelle 4575) soy (WSS), and corn soy blend (CSB14), with whey protein concentrate, along with white sorghum (Fontanelle 4575) cowpea with soy protein isolate (WSC1+SPI) extruded blends, were developed. WSC1, WSC2, RSC, WSS, and CSB14 were formulated to compare outcomes related to consumption of different commodity types (sorghum-cowpea blends, sorghum-soy, and corn-soy). Further, CSB14 was developed to compare new formulation and extrusion of blends to a current USAID FBF (CSB+). WSC1+SPI was formulated to compare soy to whey protein in WSC1. CSB+ was purchased from a USDA producer (Bunge Milling, St. Louis, MO), with standard preparation, which includes utilization of heat treated corn and soybeans which are mixed, and micronutrient fortified. AIN-93G, which is a diet formulated to meet the National Research Council (NRC) requirements for growing rats, was included as a control diet group to facilitate assessment of adequacy of the FBFs. Of note, iron forms and concentrations, as well as vitamin A concentrations were different between the CSB+, extruded FBFs, and AIN-93G. AIN-93G contained ferric citrate (6.6g/100g), while extruded FBFs and CSB+ contained sodium iron EDTA iron/ferrous fumarate, although at different concentrations, vitamin A concentrations in CSB+ were nearly twice those in newly formulated FBFs, and more than 40 times the levels in AIN-93G (Table 2-2). Sodium-ETDA iron was chosen to reduce mineral-antinutrient interactions found in ionized iron forms, to improve bioavailability [4]. Therefore, iron availability of FBFs was expected to surpass AIN-93G (ferric citrate alone).

Fortified Blended Food Production

Sorghum-cowpea, sorghum-soy and corn-soy flours were extruded on a single screw extruder X-20 (Wenger Manufacturing Co., Sabetha, KS, USA). The dry feed rate was 200 kg/h for formulations made from commercially sourced flours and 166 kg/h for formulations that were obtained from flours produced from pilot milling (cowpea flour containing FBFs).
Steam and water were added in the preconditioner, where discharge temperature was maintained above 85°C, and screw speed ranged from 500-550 rpm. In-barrel moisture content ranged between 18-20%, the die had a single circular opening of 4.1 mm. After cutting, extrudates were dried using a double pass dryer/cooler (Series 4800, Wenger Manufacturing Co., Sabetha, KS, USA) operating at 104°C, where they were retained for 10 minutes, before being cooled for 5 minutes at room temperature. Vitamins and minerals were mixed in with other dry ingredients in steps to ensure mixing uniformity. Once dry ingredients were mixed combined through this process, oil was added and mixed.

Diet, Macronutrient, and Antinutrient Analysis

FBFs were analyzed by AOAC official methods by the University of Missouri Agricultural Chemical Laboratories. Methods included measurement for total calories (by calculation: protein = 4kcal/g, carbohydrate = 4kcal/g, fat= 9kcal/g), protein (LECO; AOAC 990.03, 2006), fat (acid hydrolysis, 954.02, 2006), carbohydrates (by calculation: 100% - % crude protein + ash + crude fat + moisture), and amino acids including available lysine (cEIC- HPLC; spectrophotometry AOAC 982.30E; 975.44). Phytate and tannin content of blends were analyzed as described in reference [22]. Briefly, phyates and tannins were assessed using a Megazyme kit (Megazyme International, Ireland) and methods described previously [23], respectively.

Study Design

Weanling, 21-23 day old male Sprague-Dawley rats (Charles River, Wilmington, MA) were randomized into 8 diet groups (n=10, 80 total). Animals were housed individually in wire-bottomed cages (to prevent corporophagy) with a resting board beneath food and water feeders, in a temperature controlled facility with 12-hour light and dark cycles. Rats were provided food and water *ad libitum*, fed every other day when food intake was measured, and weighed weekly for four weeks. Study length and size were based on the preventative prophylactic [24], and protein efficiency ratio (PER) [25] methods, respectively.

Data and Sample Collection

At study end, rats were anesthetized by CO_2 inhalation, weights and lengths were recorded, and they were euthanized by exsanguination. Length from nose to base of tail was measured as a comparison of overall linear growth. Blood collected from cardiac puncture was divided into 2 ml EDTA-K2 vacuum tubes (Fisher) and 2 ml microcentrifuge tubes for hemoglobin and serum, respectively. EDTA tubes were immediately placed on ice and subsequently stored at 4°C for 48 hours before analysis. Blood samples in microcentrifuge tubes collected for serum analysis were allowed to rest at room temperature under aluminum foil to protect them from light. They were then centrifuged at 3000 x G for 15 minutes, supernatant was pipetted into microcentrifuge tubes, flash frozen in liquid nitrogen, and stored at -80°C. Following blood collection, liver tissue was collected, weighed, flash frozen in liquid nitrogen, and stored at -80°C. After hepatic samples were collected, bone density and total body fat mass were measured via a PIXIMUS densitometer (Lunar, Madison WI) following manufacturer instructions. Prior to the study, it was

verified that hepatic removal had a consistent, and minimal effect, on fat mass and bone density measured.

Iron Quantification

Hepatic and Diet Iron

Hepatic iron analysis was determined by wet ashing before quantification by flamed atomic absorption spectrometry (AAS) (Perkin Elmer AAnalyst 100, Waltham, MA). Briefly, 1g of hepatic tissue was placed into a 50 ml acid washed beaker, 10 ml of full strength nitric acid was slowly added and left for one hour for chemical decomposition. Samples were then brought to boil, reduced to 1 ml over 2-3 hours, titrated to 10 ml with dd H2O, and quantified in duplicate (n=10) by atomic absorption spectrophotometry (AAS). Iron content of blends were analyzed in duplicate (n=1) by AAS (Great Plains Analytical Laboratory AACC method 40-70.01, Manhattan, KS).

Hemoglobin

Hemoglobin samples were prepared in triplicate (n=10) using Drabkin's reagent for cyanmethemoglobin measurement (Sigma Aldrich, St. Louis). Samples were compared with a standard hemoglobin curve prepared with lyophilized bovine hemoglobin and measured by spectrophotometer at an absorbance of 540 nm according to manufacturer instructions.

Retinol Quantification

Hepatic Retinol

Hepatic retinol concentrations were analyzed in duplicate (n=10) using an adapted protocol [26,27]. In initial samples analyzed, BHT was not found to protect retinol from oxidation, and was not included in the protocol. 0.1 g of liver sample was weighed and homogenized by vortexing well with 0.25 g ascorbic acid in 5ml ethanol [27,28]. Samples were placed on ice, and 1 ml of supersaturated KOH was added. After vortexing, samples were heated for 30 minutes in a water bath (70°C), vortexing every 10 minutes for 30 seconds. After ensuring that tissue was totally dissolved, samples were cooled on ice for 10 minutes. Six ml of hexane was added, the sample was vortexed, supernatant was removed, and samples were dried down in a Vacufuge (Eppendorf Vacufuge, Hamburg Germany) at 20°C. This process was repeated twice more. When approximately 1 ml of sample remained, it was vortexed for 30 seconds, pipetted into Eppendorf tubes, dried under nitrogen, and stored at -20C overnight (<24 hours). Samples were reconstituted into 400 µL of mobile phase, vortexed well, and 20 µl was injected into the HPLC.

Serum Retinol

Serum for all rats was pooled and prepared in duplicate (n=1), because of low volumes of CSB+ serum due to small body size. Pooling has been shown to be highly representative of individual serum samples *in vivo* [29]. Serum was extracted using a modified protocol [27,30]. Serum samples (150 µl) were added to an equal volume of ethanol with ascorbic acid (0.25 g per 5 ml), vortexed, and extracted three times with 1 ml of hexane, with the supernatant removed after each extraction. Supernatant samples were dried down under nitrogen, and stored at -20°C overnight

(<24 hours). Samples were reconstituted into 40 µL of mobile phase, vortexed well, and 30µl was injected into the HPLC.

Diet Retinol

Vitamin A content of blends was analyzed as described previously in duplicate [31]. 0.25 g of blend was weighed, transferred into a 50 ml glass centrifuge tube, then 3.5 ml of ethanol and 1.5 ml dd H_2O were added to the sample with 0.25g ascorbic acid, followed by 1 ml of supersaturated KOH. Samples were vortexed, then placed in a 60°C water bath for 30 minutes, vortexing every 10 minutes, then 2 ml of dd H_2O was added, and samples were cooled on ice. Seven ml of hexane was added, the sample was vortexed, supernatant was removed, and samples were dried down in a Vacufuge (Eppendorf Vacufuge, Hamburg Germany) at 20°C. This process was repeated twice more. When approximately 0.5 ml of sample remained, it was vortexed for 30 seconds, pipetted into Eppendorf tubes, dried under nitrogen, and stored at -32°C overnight (<24 hours). AIN93-G, extruded FBFs, and CSB+ were reconstituted in 40 µl, 80µl, and 160 µl, respectively, with 20µl injected into the HPLC. Different reconstitution volumes were utilized to obtain similar retinol values, across blends with a wide range of vitamin A content.

Sample Analysis

Samples were run on an Agilent Eclipse XDB 5µm C_{18} (250mm x 4.6mm, Santa Clara, CA,USA) analytical column at a flow rate of 1ml/min for 20 minutes at 23.4°C with an autosampler (Shimadzu SIL) on a HPLC system containing a LC20AB pump (Shimadzu), and a Shimadzu SPD-M20A PDA system. Mobile phase consisted of 47/47/6 methanol, acetonitrile, and chloroform. Samples were analyzed against an external standard curve prepared using retinyl acetate (U.S. Pharmacopeia, Rockville, MD); standards were prepared in duplicate daily from

stock solutions after analysis on spectrophotometer at 325 nm to quantify absorbance. Concentration was calculated using an absorbance coefficient of 0.155 for retinyl acetate in ethanol [32].

Calculations

Due to differences in protein, fat, carbohydrate and total caloric content between blends, as well as evidence suggesting that protein intake may not directly relate to linear growth as protein reaches a certain concentration in the diet [33], caloric efficiency was calculated along with protein efficiency as an indicator of protein quality.

$$Caloric\ efficiency\ (weight\ gain\ per\ kcal\ consumed) = \frac{weight\ gain\ (g)}{Food\ intake\ (g) x (100g \times (number\ of\ calories\ per\ FBF\ in\ 100g))}$$

$$\textit{Protein efficiency (weight gain per gram of protein consumed)} = \frac{Weight\ Gain\ (g)}{total\ protein\ intake\ (g)}$$

Lean mass was calculated to monitor for weight gain related to adiposity rather than linear or lean mass.

$$Lean\ mass\ (proportion\ of\ non-fat\ mass\ measured) = \frac{Weight - Fat\ Mass}{Weight}$$

Blends were compared with Digestible Indispensable Amino Acid Score (DIAAS) recommendations for protein quality assessment. DIAAS was utilized to analyze protein quality because of limitations of the Protein digestibility-corrected amino acid score (PDCAAS) as an estimate of crude protein digestibility, and the recent recommendation of the Food and Agricultural Organization (FAO) that DIAAS replace PDCAAS [21].

Statistical Analysis

Group differences were assessed using one-way ANOVA with Tukey's test after satisfying Levene's test for homogeneity. Significance was set at $p < 0.05$; statistics were performed using SAS version 9.3 (Cary, NC).

Results

FBF composition

CSB+ contained 8.3% fewer total kcals, 6.9% more carbohydrate, 23.9% less protein, and 41.5% less fat compared to newly formulated, extruded FBFs (Table 2-3). Lysine and sulfur-containing amino acids did not meet DIAAS requirements for children aged 6 months to 4 years in CSB+ and WSC1+SPI diets, respectively [21]. CSB+ and AIN-93G (6.6 mg/100g) contained 48% and 58% less iron than the newly formulated, extruded FBFs, respectively. Vitamin A content of blends was higher and lower in CSB+ and AIN-93G, respectively, compared with newly formulated, extruded FBFs. WPC containing FBF groups had comparable macronutrient and micronutrient compositions (Table 2-3). CSB+ mean phytate content was more than three times greater than newly formulated, extruded FBFs (Table 2-3) [22]. Phytate content of WSC2, WSC3, and WSS were similar, and more than 1.5 times greater than CSB14; WSC1 mean phytate content was 1.2-2.5 times greater than other newly formulated blends [22].

Food intake, anthropomorphic and micronutrient outcomes

Food intake, weight gain, final body weights, caloric efficiency, protein efficiency, and linear growth changes were not significantly different between WPC-containing FBF groups (Table 2-4, Figures 2-1, 2-2). The CSB+ group's total intake was significantly reduced by 30%, final body weight, protein efficiency were significantly decreased by greater than 50%, caloric efficiency was significantly decreased by 20%, and length was significantly reduced by greater than 20% compared to all groups (Table 2-4). During week 1, all groups consumed the same amount of FBF, while weight gain was significantly decreased (>50%) in the CSB+ consuming group. In subsequent weeks, CSB+ consumption and growth were significantly decreased (Figures 2-1, 2-2). Compared with the AIN-93G group, the WSC1+SPI group gained significantly less total weight (Figure 2-2). Compared with the WSC1 and AIN-93G groups, the WSC1+SPI group had significantly lower caloric and protein efficiency (Table 2-4).

There were no differences in lean mass, bone mineral density, hemoglobin, hepatic iron, serum retinol, or hepatic iron in newly formulated, extruded FBFs groups. Bone mineral density was significantly lower in the CSB+ group compared to the AIN-93G group. Liver weight as a percentage of body mass was significantly lower in the CSB+ group compared with all groups (Table 2-5). The AIN-93G group had significantly, and CSB14 non-significantly, lower hepatic iron levels compared to remaining groups (Table 4). WSC2, RSC, and WSC1+ SPI groups had significantly lower hemoglobin levels than the CSB+ group (Table 2-6). Serum retinol levels were not significantly different between groups, while CSB+ and AIN-93G had significantly higher and lower hepatic retinol concentrations than all groups, respectively (Table 2-6).

Comparing FBFs to National Research Council Recommendations

Due to significantly different anthropometric outcomes, WSC1+SPI and CSB+ macronutrient and micronutrient content were compared with NRC recommendations for growing rodents [34]. WSC1 content is also included as a representative WPC-containing FBF given its similarity in formulation to WSC1+SPI. Comparing WSC1 composition to the NRC recommendations assisted in identifying composition differences that may not have contributed to significant outcomes observed in the CSB+ and WSC1+SPI groups. Micronutrient and macronutrient formulation of CSB+ and WSC1+SPI met or exceeded recommended requirements for weaning rodents with the exception of vitamin B_{12} (Table 2-7). CSB+ and WSC1+SPI levels were below requirements for sulfur-containing amino acids (53% and 48% of recommendation, respectively), and CSB+ lysine concentration was 15.2% less than requirement. WSC1 met all NRC recommendations [34].

Discussion

In this study, consumption of newly formulated blends resulted in improved protein quality, vitamin A, and iron availability outcomes compared to a current FBF (CSB+) and a control diet formulated for growing rats regardless of cereal or legume combination. Further, there were no differences in protein quality, vitamin A, and iron outcomes among newly formulated, extruded FBFs. This suggests that cowpea and sorghum based FBFs support protein, vitamin A, and iron outcomes as effectively as the newly formulated corn and soy FBF blends.

CSB+ consumption resulted in poor protein quality outcomes. The CSB+ group consumed less FBF, had weight and length suppression, lower caloric and protein efficiency compared to all groups. The SPI containing FBF consuming group also had significantly lower caloric efficiency, protein efficiency, and weight gain compared to a similar FBF group with WPC. Adiposity did not differ between FBF groups, micronutrient outcomes were similar among extruded FBFs, however, the CSB+ group's vitamin A and iron hepatic levels were significantly greater than other groups.

Several factors likely led to changes in growth observed in the CSB+ group, and to a lesser extent, the SPI consuming group. Blends met requirements of total protein and fat intake when compared with NRC recommendations for rodents [34], however, selected amino acids were lower than recommendations, including methionine + cysteine (WSC1+SPI and CSB+) and lysine (CSB+, Table 2-7). While severe limitations in lysine may reduce rodent growth, it was unlikely the only cause of growth restriction in the CSB+ consuming group. For example, up to 50% of lysine recommendations in feed did not reduce growth in 6-week-old Sprague-Dawley rats [35]. Relative deficiency of methionine is a well-known growth inhibitor in weanling rats [36], but given that the methionine content was lower in WSC1+SPI than CSB+, it is unlikely that lack of methionine was the cause of observed growth suppression. These findings may however, explain the small but significant decreases in weight gain and caloric efficiency in the WSC1+SPI group compared to the WSC1 group. Some of growth impairment in the CSB+ group may have been due to several limiting amino acids (methionine, cysteine, lysine, leucine, and tryptophan), or more likely, reduction in protein and starch digestibility. The NRC recommends that protein sources be "high quality" [34], and while individual protein sources and amino acid content were

adequate, protein digestibility may have been poor in the CSB+ group compared with the newly formulated, extruded FBFs. One noteworthy consideration is that CSB+ is partially cooked, but its preparation requires boiling to complete cooking, while extruded blends are considered completely cooked. Complete cooking improves starch and protein digestibility, supported by multiple observations that extrusion improves cereal and legume amino acid digestibility [37-39]. Perhaps more importantly, antinutritional content of blends was greatly reduced in newly formulated, extruded blends. CSB+ phytate content were more than three times the levels found in newly formulated, extruded blends [22], and inhibition of growth may be attributed in part to reductions in amino acid bioavailability and enzyme activity of dietary and mucosal proteins, found in vivo with consumption of tannins [40].

In the SPI consuming group, it is possible that reduced protein digestibility when compared with WPC may have accounted for the small decrease in growth. It is unlikely that when consuming a varied diet, changes in growth would be observed with SPI inclusion in FBFs for complementary foods for children. For example, no differences in growth were found in 6-12-month-old infants consuming soy, casein, or rice formula along with complementary feeding [41]. In field trials, CSB+ has been as efficacious as other ready-to-use foods for growth [20,42,43], despite possibly poor protein quality identified in our study. Dietary variety beyond food aid may contribute to these findings as well. A 2014 review found that FBFs containing isocaloric, isonitrogenous sources of animal source proteins did not enhance linear growth compared to plant proteins, suggesting that animal protein itself may not be needed to be included in FBFs [18]. Our study supports that a WPC containing FBF did not significantly enhance linear growth

compared to a SPI containing FBF. Given the higher cost of WPC when compared with SPI, it may be prudent to further explore the use of SPI, or other plant protein sources within FBFs.

It is possible that animal feeding behaviors had effect on growth in the CSB+ group. Certainly, significant reduction in CSB+ consumption contributed to growth suppression, but growth was inhibited with similar food intake to other groups from week 1. Although reductions in growth were seen in the first week of feeding, when intake was consistent with other groups, CSB+ intake in subsequent weeks was significantly less than all other groups. One possibility is that the CSB+ group did not consume CSB+ as well as other FBFs groups because they contained sugar. Given that lean mass and food intake were unchanged in newly formulated, extruded FBFs groups compared to the AIN-93G group, which also did not contain sugar, it is unlikely that sugar led to overeating of blends. It is possible that sugar enhanced taste, or masked unappealing flavors of the extruded FBFs. For example, some studies have cited improved taste of corn and soy blended foods with enhanced sweetness [44].

Similarities in the micronutrient outcomes make it unlikely that micronutrient differences were responsible for the observed growth suppression. While extrusion has been demonstrated to enhance micronutrient bioavailability [45,46], the combination of higher levels of vitamin A in CSB+ and subsequently less demand for micronutrients due to slower growth rates likely resulted in elevated hepatic iron and retinol levels observed. Additionally, animals in the CSB+ group did not show overt signs of other micronutrient deficiencies, and their livers were not enlarged compared with other groups. Given that circulating retinol and hemoglobin differences were not observed among groups, vitamin A or iron toxicity were also unlikely causes for growth suppression. Despite these findings, given the non-significant relationship between hepatic iron

and grain types, it may be most important to consider availability, cost, and preference of consumers of these products rather than small changes in biochemical markers when selecting commodities for FBFs.

Interestingly, our findings do not support further differences in protein quality or in biochemical markers with consumption of newly formulated blends containing varying levels of antinutritional factors regardless of grain type. For example, WSC1 contained more tannins and phytates than its sorghum cowpea counterparts, but had similar caloric, protein efficiency, and micronutrient outcomes. Our results may suggest that differences in digestibility and bioavailability of nutrients in sorghum and cowpea may be negated by reductions in antinutritional factors. Further, lack of differences in outcomes between groups consuming newly formulated, extruded blends regardless of antinutritional factor level may suggest possible threshold, or dose-mediated adaptation to antinutritional factors such as tannins and phytates, cited previously [47-49].

Limitations

Given consumption level and composition differences, it is not possible to specifically identify factors that contributed to inhibition of CSB+ growth outcomes compared with other groups. FBFs were consumed as dry powders rather than cooked porridges. Sorghum protein digestibility has been reported to decrease after cooking in water [50], although our blends are cooked during extrusion, and it is possible that protein digestibility would not be decreased with addition of hot water during their preparation. Lack of cooking may have contributed to poor protein and starch digestibility of CSB+. We did not obtain antinutritional information for WSC1+SPI, so our interpretation of findings in this group is limited. Newly formulated,

extruded FBF prepared porridges contain increased solids when compared with CSB+ (20% and 13.79%, respectively), which is not a difference we were able to assess for in this study. This study was limited to a rapid growth period, but did not follow animals through transitions into later life. This limits the ability to ascertain whether newly formulated, extruded FBFs support long term growth. Additionally, the study was limited to FBF consumption only, rather than "complementary" consumption along with other food items.

Conclusions

These results suggest that a variety of commodities can be used in newly formulated, extruded FBFs, which are of better nutritional quality than CSB+. Further studies that compare prepared FBF porridges to gain a better understanding of poor growth outcomes in the CSB+ group are warranted. Given the potential cost savings of using plant protein sources, further research comparing soy, or other plant proteins, versus whey protein in FBFs is warranted. A field trial is currently assessing the efficacy of these newly formulated, extruded porridges in combating micronutrient deficiencies and supporting linear growth in children. Lastly, long-term studies exploring protein and micronutrient adaptation may enhance understanding of FBF quality and efficacy over the lifespan.

Tables

Table 2-1: Newly Formulated Extruded FBFs, CSB+, and AIN-93G Formulations (%)

	Sorghum flour	Cowpea flour	Soy flour	Corn Flour	Sugar	Whey protein	Soy protein	Vegetable oil	Micronutrient Premix
WSC1+WPC, WSC2+WPC, RSC+WPC	24.7	38.6	0	0	15	9.5	0	9.0	3.2
WSS+WPC	47.6	0	15.7	0	15	9.5	0	9.0	3.2
WSC1 + SPI	24.7	38.6	0	0	15	0	9.5	9.0	3.2
CSB14+WPC	0	0	15.2	48.1	15	9.5	0	9.0	3.2

CSB+: Whole corn (78.4), whole roasted soy (20), vitamin mineral (0.2), tricalcium phosphate (1.16), potassium chloride (0.17)

AIN-93G: corn starch (39.7), casein (20), maltodextrin (13.2), sucrose (10), soybean oil (7), powdered cellulose (5), AIN-93 vitamin and mineral mix (4.5), L-cystine (0.3), choline bitartrate (.25), t-buylhydroquinone (0.001)

White sorghum-cowpea 1 with whey protein concentrate (WSC1+WPC), White sorghum-cowpea 2 + WPC (WSC2+WPC), Red sorghum-cowpea + WPC (RSC+WPC), WSC1+ + soy protein isolate (SPI), White sorghum-soy + WPC (WSS+WPC), Corn-soy blend 14 + WPC (CSB14+WPC), Corn soy blend Plus (CSB+).

Table 2-2: Newly formulated, extruded FBFs and CSB+ vitamin and mineral forticant levels (mg per 100g)

adapted [51].

Newly formulated, extruded FBF		CSB+	
Vitamin A Palmitate	0.488	Vitamin A Retinyl Ester	1.04
Thiamin Mononitrate (B_1)	0.652	Thiamin Mononitrate (B_1)	0.2
Riboflavin (B_2)	0.933	Riboflavin (B_2)	1.4
Niacinamide (B_3)	9.07	Niacinamide (B_3)	8
Calcium D-Pantothenate (B_5)	3.646	Calcium D-Pantothenate (B_5)	1.6
Pyridoxine Hydrochloride ($B_{6)}$	0.752	Pyridoxine Hydrochloride	1
Folic Acid (B_9)	0.087	Folic Acid (B_9)	0.11
Vitamin B_{12}	0.0015	Vitamin B_{12}	0.002
Vitamin D3	0.0292	Vitamin D3	0.011
Vitamin E	13.224	Vitamin E	8.3
Vitamin K	0.033	Vitamin K	0.03
Coated Ascorbic Acid	40.0	Coated Ascorbic Acid	90
Calcium (Tri-Calcium Phosphate)	279.08	Calcium (Tri-Calcium Phosphate)	452
Iron	13.0	Iron	6.5
Sodium Iron EDTA	2.0	Sodium Iron EDTA	2.5
Ferrous Fumarate	11.0	Ferrous Fumarate	4.0
Iodine (Potassium Iodide)	0.23	Iodine (Potassium Iodide)	0.04
Magnesium Oxide	9.47	Phosphorus (Tri-Calcium Phosphate)	290
Phosphorus (Tricalcium Phosphate)	290.97	Potassium (Potassium Chloride)	140
Potassium (Potassium Monophosphate)	163.19	Zinc Sulphate Monohydrate	5
Sodium Chloride	225.67	Sodium Chloride	326
Zinc Sulfate	5.50		

*Amount of iron the forticant is providing

Table 2-3: Analyzed FBF macronutrient, micronutrient, and antinutrient content

	White Sorghum Cowpea Variety 1 (WSC1+WPC)	White Sorghum Cowpea Variety 2 (WSC2+WPC)	Red Sorghum Cowpea Variety (RSC+WPC)	WSC1 + Soy Protein Isolate (SPI)	Sorghum Soybean Blend (WSS+WPC)	Extruded Corn-Soybean Blend (CSB14+WPC)	Corn-Soybean Blend + (CSB+)
Total Calories (kcal/100g)	394.6	396.5	397.1	395.1	392.19	392.4	361.64
Carbohydrate (g/100g)	60.8 (61.6%)	59.6 (60.1%)	60.7 (61.1%)	59.9 (60.6%)	60.7 (61.9%)	61.1 (62.3%)	64.7 (71.6%)
Protein (g/100g)	19.0 (19.2%)	19.7 (19.9%)	19.5 (19.6%)	19.2 (19.4%)	19.4 (19.8%)	19.3 (19.7%)	14.7 (16.3%)
Fat (g/100g)	8.4 (19.2%)	8.8 (20%)	8.5 (19.2%)	8.7 (20%)	8.0 (18.3%)	7.7 (18%)	4.9 (12.1%)
Lysine (mg/g)	74.1	70.9	72.2	60.5	69.5	68.3	52.9[a]
Cysteine + methionine (mg/g)	33.1	30.9	32.2	24.5[a]	35.0	35.7	35.3
Available lysine (mg/g)[+]	72.0	67.9	68.6	58.9	67.4	66.2	52.2
Iron (mg/100g)	15.2	15.9	15.2	16.3	15.6	15.6	8.2
Vitamin A (µg/100g)	598.9	496.9	527.7	488.4	553.7	462.6	846.0
Phytates (mg/100g)*	832.0	561.0	689.0	ND	557.0	318.0	1885.0
Tannins (mg/100g)*	0.00	0.00	0.00	ND	0.00	0.00	0.00

a. Does not meet recommended mg/g amino acid content for children aged 6 months-3 years [20]
+ by HPLC

* From Reference[21], ND Not Determined

Note: AIN-93G is formulated to contain 6.6 mg/100g iron; 23.1 µg/100g vitamin A; Macronutrient and micronutrient content analyzed in duplicate.

Table 2-4: Food intake, food efficiencies, and length (n=10)

	AIN-93G	WSC1+ WPC	WSC2+ WPC	RSC+WPC	WSC1 + SPI	WSS+ WPC	CSB14+ WPC	CSB+
Total Food intake (g)	432.6 ± 15.7[a]	414.3 ± 15.3[a]	414.4 ± 11.4[a]	429.7 ± 12.5[a]	415.6 ± 14.8[a]	414.3 ± 8.9[a]	412.7 ± 9.2[a]	293.0 ± 10.0[b]
Total weight gained (g)	195.9 ± 6.7[a]	188.7 ± 6.9[a]	184.3 ± 6.7[ab]	188.5 ± 6.9[ab]	168.9 ± 7.5[b]	194.4 ± 3.2[ab]	189.0 ± 4.2[ab]	54.1 ± 2.6[c]
Final Body Weight (g)	238.1 ± 8.0[a]	228.5 ± 8.4[a]	225.8 ± 7.3[a]	230.6 ± 8.0[a]	208.6 ± 9.1[a]	236.3 ± 4.2[a]	228.9 ± 4.7[a]	96.3 ± 3.4[b]
Caloric Efficiency (g/kcal* 100)	113.2 ± 4.0[a]	111.8 ± 1.9[a]	109.1 ± 2.9[ab]	107.6 ± 2.5[ab]	101.3 ± 1.8[b]	114.7 ± 1.4[a]	113.3 ± 1.6[a]	49.0 ± 2.2[c]
Protein Efficiency (g/g)	2.40 ± 0.08[a]	2.40 ± 0.04[a]	2.22 ± 0.06[ab]	2.19 ± 0.05[ab]	2.14 ± 0.04[b]	2.35 ± 0.28[ab]	2.29 ± 0.03[ab]	1.16± 0.17[c]
Length (cm)	21.8 ± 2.3[a]	21.6 ± 0.3[a]	21.4 ± 0.3[a]	21.5 ± 3.2[a]	20.7 ± 3.1[a]	21.8 ± 0.2[a]	21.9 ± 2.3[a]	16.8 ± 0.4[b]

White sorghum-cowpea 1 (WSC1+WPC), White sorghum-cowpea 2 (WSC2+WPC), Red sorghum-cowpea (RSC+WPC), WSC1+ + soy protein isolate (SPI), White sorghum-soy (WSS+WPC), Corn-soy blend 14 (CSB14+WPC). Food intake: measured every other day by subtracting food remaining from food given (g). Total weight gained: cumulative weight gain from weeks 1-4 (g). Caloric efficiency: total weight gain (g) divided by total caloric content of food intake per gram consumed (calculated). Protein efficiency: total weight gained (g) divided by total protein consumed (g).
Data are mean ± SEM; values with different letters are statistically different ($p<0.05$).

Table 2-5: Anthropometric outcomes

	AIN-93G	WSC1+WPC	WSC2+WPC	RSC+WPC	WSC1 +SPI	WSS+WPC	CSB14+WPC	CSB+
Lean mass (%)	89.9 ± 0.5[a]	90.0 ± 0.4[a]	89.6 ± 0.3[a]	89.7 ± 0.4[a]	88.1 ± 0.6[a]	90.3 ± 0.3[a]	89.5 ± 0.5[a]	90.4 ± 0.5[a]
Bone Mineral Density (g/cm^2) x1000	87.4 ± 3.1[a]	80.4 ± 2.2[ab]	78.3 ± 1.7[ab]	82.0 ± 2.5[ab]	77.3 ± 1.0[ab]	79.0 ± 1.3[ab]	80.7 ± 4.6[ab]	73.7 ± 1.6[b]
Liver weight/body weight (%)	5.63 ± 0.2[a]	5.46 ± 0.1[a]	5.50 ± 0.2[a]	5.47 ± 0.2[a]	4.90 ± 0.2[a]	5.58 ± 0.2[a]	5.73 ± 0.2[a]	4.00 ± 0.1[b]

White sorghum-cowpea 1 (WSC1+WPC), White sorghum-cowpea 2 (WSC2+WPC), Red sorghum-cowpea (RSC+WPC), WSC1 + soy protein isolate (SPI), White sorghum-soy (WSS+WPC), Corn-soy blend 14 (CSB14+WPC). Lean mass: total weight minus fat mass and divided by total weight x100. Liver weight/body weight%: liver weight divided by body weight x100. Data are mean ± SEM; values with different letters are statistically different ($p<0.05$).

Table 2-6: Circulating and hepatic iron and vitamin A levels

	AIN-93G	WSC1+ WPC	WSC2+ WPC	RSC+ WPC	WSC1 + SPI	WSS+ WPC	CSB14+ WPC	CSB+
Hemoglobin (g/dl)	18.6 ± 0.6^{ab}	17.9 ± 0.5^{ab}	17.7 ± 0.9^{a}	17.9 ± 0.6^{a}	17.3 ± 0.5^{a}	18.3 ± 0.4^{ab}	18.7 ± 0.9^{ab}	20.2 ± 0.4^{b}
Hepatic Iron (µmol/g *1000)	142.4 ± 16.8 ^{a}	293 ± 29.3^{b}	272 ± 30.2^{b}	300 ± 26.5 ^{b}	271.8 ± 32.1^{b}	207 ± 29.1^{ab}	277 ± 32.1^{b}	310.5 ± 25.2^{b}
Serum Retinol (ng/µl)	89.6 ± 3.5^{a}	73.9 ± 11.2^{a}	74.5 ± 2.2^{a}	77.9 ± 2.7^{a}	82.6 ± 1.8^{a}	71.3 ± 1.2^{a}	67.4 ± 10.1^{a}	55.7 ± 5.3^{a}
Hepatic Retinol (ng/mg)	78.1 ± 5.0^{a}	479.4 ± 17.8^{b}	473.8 ± 27.8^{b}	482.5 ± 20.4^{b}	585.5 ± 28.6^{b}	423.8 ± 19.4^{b}	460.9 ± 28.9^{b}	1478.8 ± 95.2^{c}

White sorghum-cowpea 1 (WSC1+WPC), White sorghum-cowpea 2 (WSC2+WPC), Red sorghum-cowpea (RSC+WPC), WSC1 + soy protein isolate (SPI), White sorghum-soy (WSS+WPC), Corn-soy blend 14 (CSB14+WPC). Data are mean ± SEM; values with different letters are statistically different ($p<0.05$).

Table 2-7: **Comparison of NRC growing rodent dietary needs versus formulation per 100g of corn soy blend plus (CSB+), white sorghum cowpea-1 with soy protein isolate (WSC1+SPI), and white sorghum cowpea 1 (WSC1+WPC).**

	NRC	CSB+	WSC1 +SPI	WSC1+WPC		NRC	CSB+	WSC1+ SPI	WSC1+WPC
Vitamin A (µg)	70	1038	488	488	Total Fat (g)	5	4.88	8.74	8.37
Vitamin D_3 (µg)	2.5	11.04	29.2	29.2	Protein (g)	15	14.74	18.53	19.02
E (mg)	1.8	8.3	13.2	13.2	Arginine (g)	0.43	0.93	1.33	0.99
K (µg)	100	30	33	33	Aromatic amino acids[a] (g)	1.02	1.17	1.21	1.42
Thiamin(mg)	0.4	0,2	0.652	0.652	Histidine (g)	0.28	0.41	0.53	0.49
Riboflavin(mg)	0.3	1.4	0.933	0.933	Isoleucine (g)	0.62	0.61	0.87	0.98
B_6 (mg)	0.6	1	0.752	0.752	Leucine (g)	1.07	1.34	1.59	1.82
Pantothenic acid (mg)	1	1.6	3.646	3.646	Lysine (g)	0.92	0.78	1.16	1.41
Folic acid (µg)	100	110	87	87	Methionine + Cysteine (g)	0.98	0.52	0.47	0.63
Niacin (mg)	1.5	8	9.07	9.07	Threonine (g)	0.62	0.54	0.68	0.94
B_{12} (µg)	5	2	1.5	1.5	Tryptophan (g)	0.2	0.18	0.25	0.28
Iodine (µg)	15	40	23	23	Valine (g)	0.74	0.74	0.98	1.04

Total Iron (mg)	3.5	6.5	13	13	
Zinc (mg)	1.2	5	5.5	5.5	

[a]Aromatic amino acids: phenylalanine, tyrosine, tryptophan

Figures

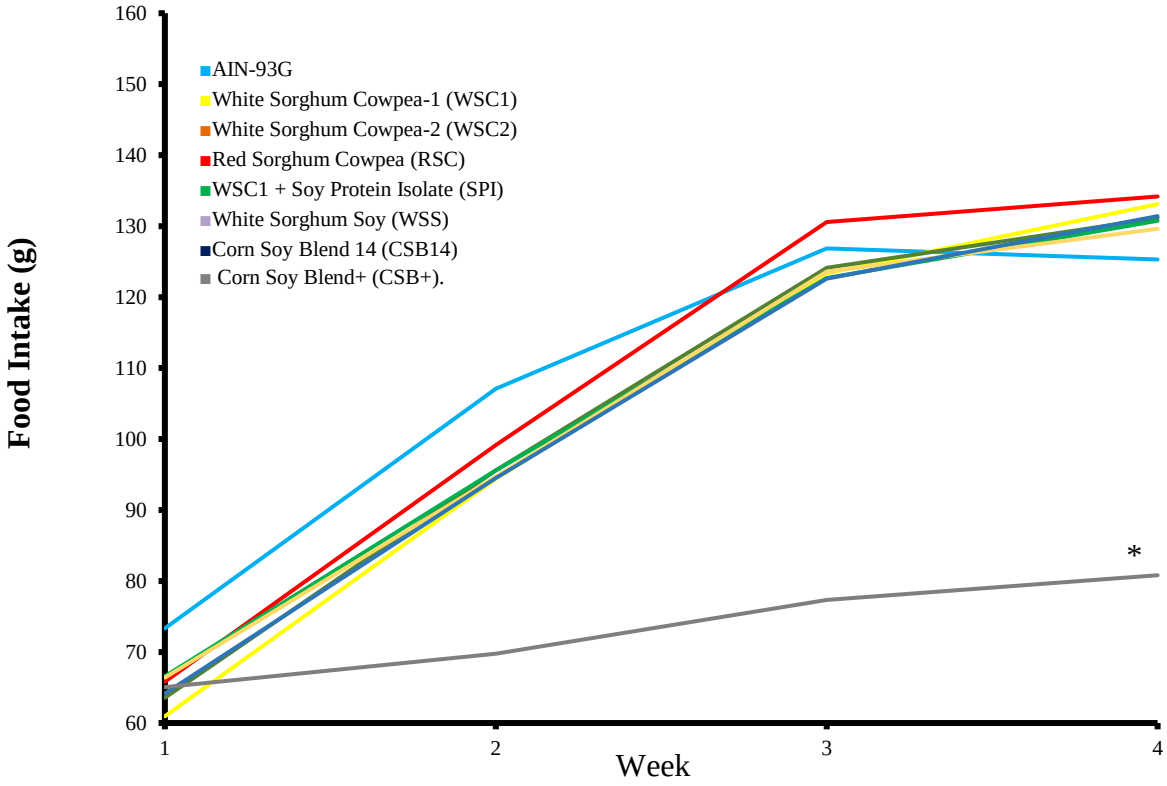

Figure 2-1: Average weekly food intake. The CSB+ group's average weekly food intake was significantly decreased during weeks 2-4 compared to extruded fortified blended food (FBF) groups and AIN-93G (n=10; *p<0.05).

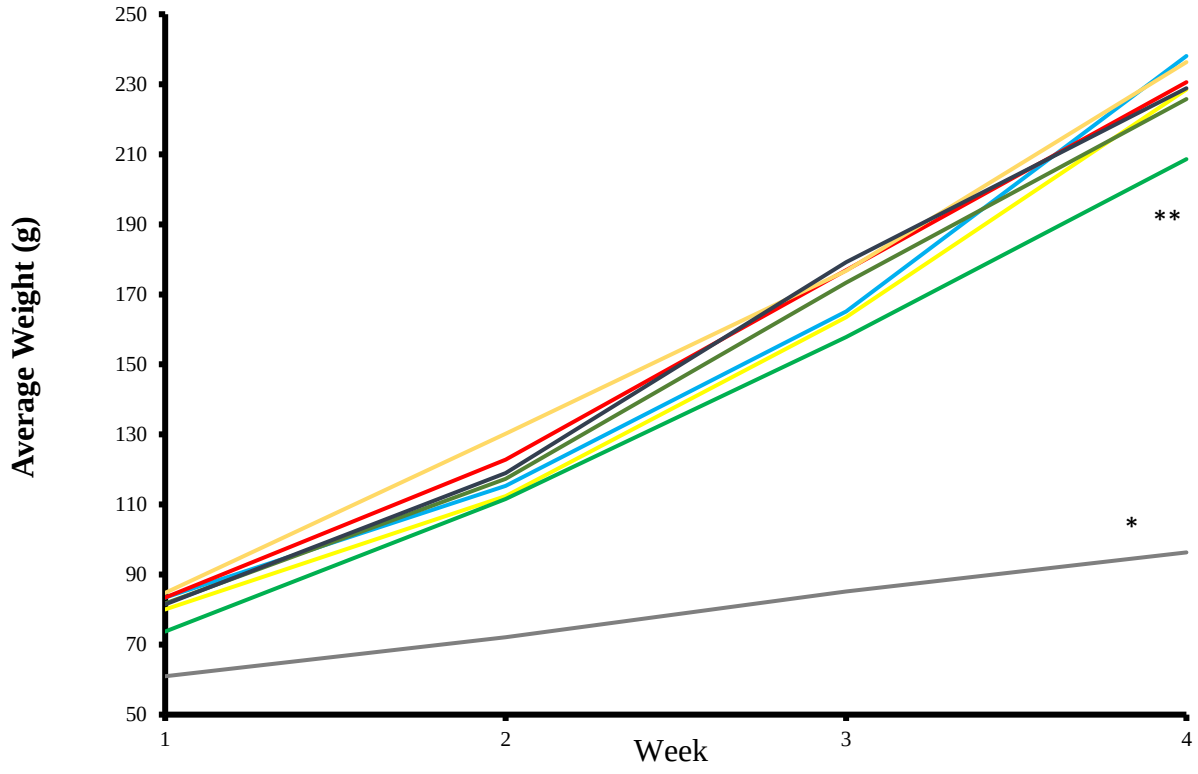

Figure 2-2: Weekly average body weights. The CSB+ group's average body weight was significantly reduced compared with extruded fortified blended food groups; the WSC1 +SPI group's body weight was reduced compared with AIN-93G and WSC1+WPC (n=10; *p<0.05 CSB+ versus comparison to all groups, ** p<0.05 WSC1 +SPI versus WSC1 +WPC and AIN-93G).

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Chapter 3 - THE IMPACT OF TANNIN CONSUMPTION ON IRON BIOAVAILABILITY AND STATUS: A NARRATIVE REVIEW

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Abbreviations:

IDA: Iron deficiency anemia

Hb: Hemoglobin

TIBC: Total iron binding capacity

WHO: World Health Organization

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Abstract

Iron deficiency remains a global health issue, and antinutritional factors, such as tannins, are often cited as contributors to the high prevalence of deficiency. Despite this, tannin-rich diets may have potential beneficial cardiovascular and cancer fighting properties due to tannins' antioxidant activity. Further, epidemiological studies and long-term trials involving participants who consumed diets rich in antinutritional factors, particularly tannins, conflict with single-meal bioavailability studies. The purpose of this narrative review is to determine tannins' effects on iron bioavailability and status, and establish whether adaptation to tannins reduces tannins' antinutritional effects over time. We also aimed to compare tannins used in iron studies. Common themes related to iron bioavailability and iron status with tannin consumption were collected and collated for summary and synthesis based on models and subjects used. Overall, there was dissonance among iron bioavailability and status in studies. Single meal studies with hydrolyzable and oligomeric catechin/epicatechin tannins (tea and tannic acid) generally support reductions in bioavailability with tannin consumption compared to condensed tannin's consumption, although condensed tannins are more commonly found in food. Long term animal models, epidemiological data, and multi-meal studies generally do not support changes in iron status related to tannin intake. Studies suggest that long-term tannin consumption may reflect iron status differently than single meal studies or bioavailability iron models predict. Further, iron bioavailability studies employing condensed tannins, which are more commonly consumed, may better predict mealtime iron bioavailability. More research is needed to develop

representative antinutritional-iron studies, as well as the possibility of, and mechanisms underlying the adaptation to tannins and other antinutritional factors that occurs over time.

Key Words: iron bioavailability, antinutritional factors, tannins, proanthocyanidins, iron deficiency anemia

Introduction

Iron deficiency is common worldwide, and nearly 1 billion people suffer from iron deficiency anemia (IDA) [1]. Adequate iron stores are required for normal growth and development, and IDA has been associated with loss of productivity, reduced cognitive functioning [2], increased prematurity, and perinatal, childhood, and maternal mortality [3]. While the World Health Organization (WHO) approximates that IDA contributes to 3% of all disability life years lost, more recent estimates suggest that 2013 economic losses related to IDA in Indian children 6-59 months alone were nearly 24 billion U.S. dollars [4]. Populations with higher prevalence of IDA include women, children, people consuming a vegetarian or meatless diet, and those consuming insufficient iron in developing countries [1], and an estimated 30-40% of women and children younger than 5 years develop IDA without iron fortification [1]. Despite prevalence rates and multiple initiatives aimed at improvement of IDA, an estimated 29% of non-pregnant women were anemic in 2011, a reduction of only 4% from 1995 [5].

The absorption, incorporation, and use of iron in the body is a strictly regulated process in which the homeostatic regulation of iron is primarily mediated through absorption and recycling [6]. Nearly 90% of iron stores are retained through senescent red blood cell recycling; nutritional intake accounts for the remaining 10% [7]. While a multitude of genetic and disease related factors influence the pathophysiology and prevalence of IDA (6), interest in its treatment has been

largely focused on readily modifiable factors, such as nutritional enhancement and iron absorption inhibitors. Inhibitors found in diets rich in legumes and grains (termed 'antinutritional factors') are particularly criticized as contributors to the high prevalence of deficiency in developing and low income countries [8,9], despite cited health benefits of diets rich in these staples [10,11]. Antinutritional factors, such as tannins and phytates, in cereals have been found to negatively impact the bioavailability of minerals, such as iron, when consumed in large quantities [12,13]. It is accepted that tannins reduce iron availability before absorption through the formation of insoluble antinutritional-mineral complexes [14], and reported exacerbation of IDA by foods high in phytates or tannins is common [15-17]. Single meal studies have confirmed iron bioavailability inhibition with phytate [12,18,19], and tannin [20-22] consumption.

The term 'tannin' denotes a broad class of compounds, which can be further classified into hydrolyzable, or the more commonly consumed, condensed tannins (also known as proanthocyanidins) [23] **(Figure 1)**. Tannins' ability to precipitate proteins has been linked to the sensation of astringency [24], and plant tannin content is linked to insect, animal, and mold resistance [25]. It may be these defense mechanisms that lead to antioxidant [26], cancer fighting [27], and cardiovascular benefits [28] derived from antioxidant properties tannin-rich foods, such as wine and tea. Considering both the potential detrimental and beneficial properties of tannins, a dichotomy exists between limiting the tannin consumption of those at risk of IDA and the potential health benefits derived from tannin-rich diets.

Due to tannins' iron absorption inhibition, prudent food system and agricultural efforts have been made to reduce tannin content of grains, legumes, and foods in an effort to enhance the iron status of those consuming them [29]. Despite this, there is a wealth of information that refutes

conventional ideology that tannins contribute to chronic changes in iron deficiency. In studies that have supported reduction in bioavailability through tannin consumption, individual iron absorption has been highly variable [30,31], and the majority of individuals consuming diets with high concentrations of tannins, as well as antinutritional factors in general, have reported normal iron status [32,33]. Further, removal of antinutritional factors has not been shown to improve iron bioavailability [34], nor have diets rich in tannin content [35,36].

Evidence suggests that individuals can adapt to antinutritional factors over time. Repeated consumption of antinutritional factors has been shown to blunt reductions in iron bioavailability in animal [37-39] and clinical [40,41] models alike. Interestingly, tannins administered *per rectum* [42] or topically [43] have been linked to hepatotoxicity, while oral consumption of condensed tannins has not, suggesting that inherent defense mechanisms may exist that respond to tannins consumption over time. While adaptation to antinutritional factors may be plausible, it is not currently well understood whether 1) tannins at commonly consumed levels are linked to changes in iron status, 2) short term tannin-mediated reductions in iron bioavailability continue over time, 3) tannins alone (rather than consumed with phytates) reduce iron status, and 4) single meal study inhibition levels result in meaningful reductions in iron status. The focus of this review is to explore tannins' effects on iron bioavailability and status.

Methods

Interventional and epidemiological studies that examined the relationship between tannin consumption on iron bioavailability, or iron status was identified using PubMed, Web of Science, and Google scholar databases using the search terms "iron availability AND/OR iron bioavailability" AND "tannin AND/OR polyphenol AND/OR antinutritional factors", "iron"

[Mesh] AND “bioavailability” [Mesh] AND “tannin” [Mesh], “polyphenol” AND iron bioavailability. Terms also included were “sorghum AND/OR tea” due to the common citation of these tannin containing factors on iron bioavailability. In addition, the method of snowball article collection (citations from relevant journal articles) was also utilized.

In vivo articles were included that gave some indication of bioavailability/iron status at the end of the study. Studies excluded were ecological, rather than human nutrition application, *in vitro*, or focused on special populations (genetically defined illness), or did not attempt to quantify tannins’ effects alone when consumed with other antinutritional factors.

Results

The original search terms generated 497 articles, which were narrowed first by inclusion of at least 2 search terms (217). Exclusions for *in vitro studies* (133), review articles (38), for specialized populations (9), were conducted during abstract reviews. A second abstract review excluded studies for manual combing of duplicates (34). Full article review of the 64 remaining studies was conducted, and further exclusion for non-iron outcomes (13), as well as lack of direct assessment of tannins’ effects on iron status (16), were applied. In total, 37 studies were reviewed **(Figure 2)**.

Iron bioavailability, iron status in tannin consumers

Animal Studies

Animal studies using tea to measure inhibition of iron status or bioavailability often exceeded or met levels that could be expected in common consumption. For example, one cup of tea may contain about 25-80 mg tannins per 150 ml [21,22], and three cups of tea daily would mean consumption of anywhere from 75 – 240 mg tannins per day. Most studies exceeded this, especially when accounting for weight/weight dosing in animals. The majority studies isolating the influence of tannin consumption over time without confounding antinutritional factors have been in animals. This enabled comparison of tissue level iron depletion/repletion, and the direct comparison of bioavailability versus iron status with tannin consumption.

Studies supporting reduced iron bioavailability and/or iron status in animals consuming tannins over time

Studies supporting reductions in iron bioavailability and/or iron status in animal models have typically employed use of tannic acid or tea as study interventions **(Table 1)**. The consumption of 100g/L green tea polyphenols compared to water consumption in rats over eight weeks [44], resulted in a significant reduction in hepatic iron and hemoglobin (25% and 10%, respectively), although food intake was also significantly reduced. A 28-day pig study, consumption of 125, 250, 500, or 1000 mg/kg tannic acid in feed resulted in significant and linear depletion of serum iron levels, as well as hemoglobin. In this study, there was a significant decline in erythrocyte count, hemoglobin, and hematocrit seen in the control group similar to the 125, 250, and 500 mg/kg diet consuming groups, and mean corpuscular volume was unaffected

by tannic acid consumption [45]. Interestingly, there were significant reductions in gain to feed ratios seen in days 0-14 that were normalized during days 15-28, suggesting adaptation to the diets over time. Serum and hepatic iron levels were significantly reduced in rats consuming diets containing a 5, 10, 15, or 20% tannic acid/kg diet for 3 weeks [46], but there were no significant differences in other tissue iron levels, body weight gain, or food intake with increasing tannin dose. Despite reductions in iron stores, rats consuming tannic acid in weeks two and three had non-significant improvement in hematocrit levels, possibly suggesting adaptation or demand facilitated increases in uptake to blunt iron losses. Another depletion-repletion rat study utilizing a bean ragout meal with green or black tea compared to water for 14 days found that iron bioavailability and change in hemoglobin decreased significantly with tea intake when iron and food intake were similar, but hepatic iron and total hemoglobin levels were not significantly different at study end [47]. In a study examining effects of habituated versus black tea naïve diet on iron bioavailability in rats (n = 6), iron bioavailability, along with final body weight and food consumption of rats consuming a powdered black tea diet was significantly reduced compared to control, although hepatic iron stores were normal [48]. In the long-term tea consuming group, food intake and iron bioavailability from baseline to end line significantly improved over time, suggesting adaptation.

Studies supporting no differences in iron bioavailability or status with tannin consumption over time

Many animal studies have not reported differences in iron status after consuming tannin-rich diets **(Table 2).** In iron replete and depleted rats consuming a diet containing 20 mg of condensed tannin/kg body weight + phytoferritin for 4 weeks in a hemoglobin depletion-

repletion model, a significant reduction in hemoglobin, weight gain, and serum iron in the condensed tannins + phytoferritin consuming, compared to phytoferritin alone-consuming rats was observed [49]. Despite reductions in overall iron absorption, ferritin was not reduced, rats were not iron deficient, and achieved similar iron repletion to control while consuming condensed tannins with adequate iron intake. Food intake was not measured in this study. It is important to note that anemic rats consuming condensed tannins died by study end, pointing to toxicity at the daily dose of 20 mg/kg. In both 16 and 30-day rat studies comparing 0.35, 1.17, or 3.50% weight per weight black tea consumption, or green tea oral and powdered diet (daily dose of 20 mg/kg) to control diets, there were no differences in tissue iron levels or hemoglobin at study end [38,50]. In the study employing a green tea challenge, iron absorption was similarly unchanged among tea consumers compared to control without changes in body weight or feed intake [38]. A study in weanling rats looked at differences in iron availability with condensed tannin habituated vs. naïve animals. Both habituated and naïve rats did not have significant differences in iron status at study end [51]. A 4-week hemoglobin depletion-repletion study in piglets consuming meals with significantly different tannin levels from red or white bean feed found no differences hemoglobin, hemoglobin repletion-efficiency (hemoglobin replaced per iron intake), or weight gain at endpoint [36]. Interestingly, this study did find initial downward trends in hemoglobin repletion efficiency at day 7 (49.9% versus 55.6% in red versus white bean consumers, respectively) that were compensated for by endpoint, potentially indicating adaption to tannin consumption over the study period. In pigs consuming grape meal or hops polyphenol-rich diets compared to control diet for 4 weeks, there were no significant differences in plasma iron, total iron binding capacity, transferrin saturation, tissue iron, or fecal iron compared to control [52].

Human studies

While many studies have linked tannin consumption to iron bioavailability, there are a paucity of human studies directly examining the relationship between tannin intake and iron status. Studies are presented by their methodology to accurately portray the information available from the design. Single meal studies using tea often used a standard dose of 150 – 300 ml of tea, while the majority of studies using condensed polyphenols exceeded the 75 – 240 mg polyphenol per day that could be expected with tea consumption three times daily [21,22].

Epidemiological studies

The majority of epidemiological studies that included isolated tannin-iron interactions focused on tea consumption, and have not found an influence of tea consumption on iron status **(Table 3)**. One study collected two 24-hour recalls from 173 Indian, premenopausal, parous women, and found that in multiple regression analysis, tannin intake was not a significant regression factor correlated with anemia [53]. Similarly, tea consumption in 2573 French men (954) and women (1639) had no influence on iron status [54]. Another cross-sectional study with 157 Indian participants did not find differences in anemia prevalence between men and women consuming "high" and "low" tannin-containing diets [55]. Notably, condensed tannin/polyphenol consumption varied widely in these studies (from 36 mg tannin intake daily to greater than 5000 ml of tea per week); however, none of them found an impact of tannin consumption levels on iron status.

Single meal studies

Since the 1970's, researchers have found statistically significant reductions in iron absorption measurements with tannin consumption in single meal studies. The majority of these studies have been in iron-replete individuals, both male and female, who consume a meal with tannin compared to the meal alone. Almost all single meal bioavailability studies use radioactive iron, most use hemoglobin incorporation, and fewer use direct measurement of iron absorption through area under the curve serum iron levels to understand iron uptake. Additionally, most studies have used iron absorption ratios to compare tannin-containing meals to control.

In premenopausal anemic and non-anemic Indian women (n =10) consuming either 200 ml of black tea or warm tap water with a control meal on two consecutive days reduced iron absorption by 21%, although this inhibition was reduced when tea was consumed with milk [20]. These findings have been since supported in a variety of foods, but generally, reductions in bioavailability of iron with tannin consumption are linked to consuming black tea **(Table 4)** [22,56-63]. These studies all reported notable iron absorption variability between participants. Further, iron absorption between studies varied, from as little as 1% [63] to as much as 50% [31], pointing to the wide variability in maintenance iron homeostasis through iron absorption.

Contrary to the previous studies mentioned, other single meal studies in men and women have suggested that tannin consumption alone, particularly of condensed tannins, may not contribute to reductions in iron bioavailability **(Table 5)**. One study in anemic and non-anemic Indian men found that while there was a two-fold reduction in iron absorption after consumption of a phytate and tannin-rich sorghum meal (20 versus 136 mg/100g and 160 vs. 273 mg/100g tannin and phytates in low and high tannin meals, respectively), there was no significant

difference in iron absorption when meals were normalized for phytic acid (n = 12, 4.0 vs. 3.1% iron absorption in low and high tannin meals) [35]. A comparison of red to white cowpea meals found no differences in iron bioavailability with a two-fold increases in tannin levels in premenopausal Dutch women [64], and another study found that despite similar tannic acid equivalents, spinach consumption resulted in a two-fold increase in iron bioavailability compared to black tea (n = 9, 5, respectively) in 19-51-year-old Swedish men and women [56]. Other studies have supported minimal [16,61,65], no reductions [66], or enhanced uptake in iron absorption [67] with tannin consumption.

Phytate content may conflate findings in these studies. Women consuming low polyphenol beans (29 versus 180 mg/100g in low versus high polyphenol beans) did not significantly increase iron absorption, while high phytate beans significantly reduced iron bioavailability (n = 20) [68]. Similarly, adding polyphenol oxidase to high tannin sorghum meals did not improve iron bioavailability in iron replete women consuming them, suggesting that tannins were not the cause of reduced iron bioavailability (n = 16,18) [62].

Long-term/multi-meal clinical studies

Few multi-meal antinutritional factor iron bioavailability studies have been conducted **(Table 6)**. In 19- to 32-year-old German vegetarian (n = 6, 8 in black and green tea groups, respectively) and omnivorous (n = 10, both groups) men and women consuming green or black tea for 4 weeks, ferritin was significantly decreased in omnivorous women consuming black tea, or in a subgroup analysis of anemic women consuming black or green tea, respectively. There were no changes in ferritin in men or non-anemic vegetarian women consuming tea, nor did total iron binding capacity, hemoglobin, serum iron, or hematocrit change in groups from baseline to

end line [69]. In a study comparing iron status of Indian men and women aged 20-25 years old (n= 46), polyphenol-rich leafy vegetable supplement diets did not significantly impact individuals' hemoglobin levels after three weeks of supplementation compared to a control meal with no green leafy vegetables [70].

Confounding antinutritional diet factors make intervention increases in tannin consumption alone difficult. Two studies that followed 31 healthy men aged 32-56 [40] and later [71] 36 premenopausal women consuming a "high" or "low" bioavailability diet for more than 12 weeks. High bioavailability diets consisted of meat and poultry, refined cereal and grain products, no coffee or tea, and foods with > 75 mg ascorbic acid in each meal, while low bioavailability diets consisted of whole grains, with limited meat, tea, and 60 mg/d ascorbic acid equivalent. Iron and calcium content of diets were similar and researchers conducted two, 24-hour iron absorption studies comparing iron bioavailability between the two groups before and after the study period was complete. While tannin burden of diets was not measured, it was noted that absorption efficiency of the low bioavailability diets significantly increased over time, while absorption efficiency of the high bioavailability diets decreased [40,71]. This was supported in 2015, when marginally iron deficient women consuming a high phytate (and tannin, although this was not measured) diet for 8 weeks (n = 16) significantly increased iron absorption from a high phytate test meal, while iron absorption in low phytate diet consumers was non-significantly decreased. In this study, consumers' iron status was not changed by high or low phytate diets [41].

Discussion

Tannins' effect on iron bioavailability

Studies reviewed that noted reductions in iron bioavailability with tannin consumption commonly used hydrolyzable tannins (tannic acid) or epicatechin and catechins monomers, dimers and oligomers found in tea. Animal or single meal studies that resulted in significant reductions in iron bioavailability or iron status almost exclusively used tannic acid [31,45,46,60,63], a mixture of hydrolyzable gallo-tannins that are "virtually absent from our diet" [23], or tea [20,22,44,50,56,58,61,72], which contains thearubigins and a low density of condensed tannins found in most foods [73]. Of studies reviewed, only four that utilized condensed tannins supported reductions in iron bioavailability during single meals (out of 16 total). No studies reviewed using multi-meal animal or clinical models, or epidemiological analysis found reductions in iron status or bioavailability with condensed tannin consumption, which may point to a limitation of single meal studies to assess iron bioavailability and status for long-term outcomes. This could highlight the importance of using condensed, rather than hydrolyzable tannins or tea to assess bioavailability of iron in tannin containing meals. It is also interesting to note that some significant findings have used polyphenol beverages, rather than food, which may increase tannin-iron interaction in the food matrix [24]. Plants such as sorghum contain proteins rich in proline, similar to salivary proteins that may protect consumers from antinutritional properties of tannins [24]. It may be that consumption of such plants may result in an inherent mediation of tannin-nutrient binding with whole plant or food consumption, not found with tannin extracts or beverages. Phytic acid may be another factor that affects outcomes describing tannin induced

reductions in iron availability, considering negation of antinutritional effects in the setting of normalized phytate levels found in two studies reviewed [35,68].

It may be important to also consider outcome measures from single meal studies. While several single meal studies reviewed noted reductions in iron bioavailability, these findings were often non-significant until data was adjusted into iron absorption ratios. Often, total iron absorption differences were less than 10% between tannin consumers and non-consumers, which may not impact iron status long-term. This is an important normalizing factor given the wide variability of iron absorption, but may point to significant outcomes that make little meaningful impact on iron status when consuming tannin-rich diets over time. It is similarly important to take into consideration population, iron status, and study design when determining whether this research will result in meaningful outcomes in the context of global nutrition. Studies considering iron bioavailability from iron replete and deplete populations, who fully randomize their interventions order may carry more significant weight in this context.

Tannins' effect on iron status

Although tannin consumption impairs iron bioavailability, the majority of epidemiological and long-term human studies reviewed have not supported reductions in iron *status* with consumption of tannin-rich diets over time. In epidemiological studies, tannin compounds that inhibit iron bioavailability in single meal studies have not been correlated with iron status changes [54], or iron deficiency [53,74]. Further, four week or more studies focused on tannin-rich foods have not found alterations in iron status [69,70]. Some animal [75,76], and human [40,41] studies reviewed point to the idea that long-term adaptation to antinutritional factors, including tannins, instead. It is important to notice that these findings are confused by several factors, including

lack of control for concurrent antinutritional, or iron enhancing factors in diets, as well as assessment of iron deficiency, rather than adequate iron stores. More importantly, there are few studies that have looked into long-term antinutritional effects on either iron bioavailability or iron status, and no studies that we found have isolated the effects of condensed tannin consumption over time.

Conclusion

The focus of this review was to determine tannins' effects on iron bioavailability and status, and attempt to answer to whether possible adaptation to tannins could reduce the antinutritional effects of tannin consumption over time. Evidence from animal and single-meal studies suggests that tannic acid and tea consumption more consistently impair iron bioavailability than condensed tannins, although the connection between these studies' findings and individual iron status findings are not established. Certain tannins may inhibit iron bioavailability, but a lack of long-term studies and confounding factors in most studies reviewed assessing tannins' effects limit this generalizability. Additionally, epidemiological studies and long-term trials reviewed suggest that individuals' iron status is often not affected by tannin consumption, although there is a dearth of this type of research conducted compared to single meal studies. Future studies focusing on the long-term effects of condensed tannins (proanthocyanidins) on iron status are needed to determine their impact on iron bioavailability and status, and whether adaptation is the missing piece to explain the inconsistency between single meal and longer-term studies. In addition, further studies are needed to characterize the mechanism that results in adaptation.

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Author Contributions

ND conceived, researched, wrote and reviewed the manuscript. MH conceived and reviewed the manuscript. BL reviewed and edited the manuscript.

Tables

Table 3-1: Studies reporting reductions in iron bioavailability in animal models

Reference	n	Model	Intervention	Tannin type	Intervention length	Outcome
[46]	6	Rat	5, 10, 15, 20 g tannin/kg diet vs. control	Tannic acid	3 weeks	Significant linear reduction in hemoglobin (up to 27%) and hepatic (up to 61%) iron levels with tannic acid consumption
[45]	9	Pig	125, 250,500, 1000 mg tannin/kg diet vs. control	Tannic acid	4 weeks	Significant linear reduction in hemoglobin (up to 21%; $p = 0.028$) and serum iron levels (29%, $p = 0.12$) with tannic acid consumption
[44],	7	Rat	100 g tea consumption vs. 100 g tea (beverage) with various levels of aluminum and control	Green tea	8 weeks	Significant reduction in hemoglobin (11.0 vs. 10.0 g/L; 9% reduction) and hepatic (750 vs. 250 µg/liver; 71% reduction) iron with tea consumption compared to control
[47].	8	Rat	Green or black tea decoction with bean ragout meal	Green or black tea	14 days	Significant depletion of hemoglobin (-1.1 and -0.95 g/L black and green tea) and iron bioavailability (19.6% and 14.9% in black and green tea, respectively) compared to control during the study. Normal hepatic iron in tea groups compared to control (65, 89.4 and 66.3 µg/g in control, black, and green tea)
[76]	6	Rat	Black tea powdered diet as 25 g/kg vs. control	Black tea	12 days	Significant reduction (26%) in iron absorption compared to control, although there was significant increase over time (24 vs. 42% at baseline and end line among tea consumers). No reduction in hepatic iron levels.

Table 3-2: Studies reporting no or inconsistent reductions in iron bioavailability or iron status in animal models

Reference	n	Model	Intervention	Tannin type	Intervention length	Outcome
[50]	6	Rat	Control vs. various types of tea in food	Black tea	16 days	No significant differences in iron absorption or hepatic iron compared to control
[75]	6	Rat	Green tea diet, or gavage vs. control	Green tea	30 days	No significant differences in iron absorption (3.7 vs. 5.6 over time and 43 vs. 63% in control vs. tea, $p = 0.292$) or hepatic iron (60.9 vs. 54.2 control vs. tea, $p = 0.521$) compared to control
[49]	10	Rat	Meal with phytoferritin vs. condensed tannins (PA) and phytoferritin	Condensed tannins	4 weeks	Significant reduction in hemoglobin (11.9 vs. 10.0 g/L); serum iron (10.33 vs. 21.43) control vs. proanthocyanidins). Iron repletion and ferritin (23.4 vs. 20.98) not significantly different from no proanthocyanidin group.
[51]	7	Rat	Casein, soy, chickpea, red kidney bean flour based meals	Condensed tannins	1 week	No significant differences in iron retained, total hemoglobin from meals containing various levels of polyphenols and control, no difference between iron retention in high and low tannin kidney bean meals
[52]	16	Pig	Grape meal, hops diets vs. control	Condensed tannins	4 weeks	No significant differences in iron, TIBC*, transferrin, hepatic iron, fecal iron between groups
[36]	8	Pig	Red vs. white bean meal	Condensed tannins	4 weeks	No significant difference in hemoglobin/hemoglobin repletion efficiency in white and red bean (26 vs. 30%)

*TIBC: Total iron binding capacity

Table 3-3: Epidemiological study outcomes related to iron status

Reference	n	Intervention	Analysis	Tannin type	Conflation of phytates	Iron status affected?
[53]	173	2- 24 hour recalls	Multiple regression	"Dietary tannin" including tea and other polyphenol	yes	No IDA correlated with "tannin" intake. No significant effect in regression model
[74]	143	24 hour diet recall	Multiple regression	"Dietary tannin" including tea and other polyphenol	yes	No significant IDA correlation with "tannin" intake
[54]	1639	3- 24 hour diet recall, venous blood draw	Multiple regression	Black, green, and herbal tea	yes	IDA, or marginal iron status not correlated with "tannin" intake (ferritin 48, 50, 49 compared to 50, 47, 46 µg/l in control, regular black, green, and herbal tea drinkers, respectively; p = 0.71, 0.34, 0.36 in premenopausal women with green, black, and herbal tea)

Table 3-4: Single meal bioavailability studies showing reductions in iron bioavailability with tannin consumption

Reference	N	Iron status	Population	Intervention	Tannin type	Outcome
[20]	10	Replete	Women	Control meal with water vs. with meal with tea	Black tea	Significant reduction in iron bioavailability by 20%
[56]	10	Replete and deplete	Men and Women	Oregano, spinach, coffee, tea, or tannic acid vs. control	Black tea, tannic acid, polyphenol/ condensed tannin	Tannic acid significantly reduced iron bioavailability; oregano, tea, and coffee % inhibited bioavailability by greater than >60, which was less than their respective equivalent tannic acid doses. Spinach reduced bioavailability by 30% despite similar tannic acid equivalents to its tannic acid, tea, coffee, oregano counterparts.
[57]	6 (C), 13 (I)	Replete	Men and Women	High vs. low availability meal in vegetarian vs. non vegetarians	Polyphenol/ condensed tannin	Significant impairment of iron absorption from low bioavailability meals in vegetarian and non-vegetarian consumers. Similar iron bioavailability between vegetarian (1.4% vs. 14.9 in bran vs. whole wheat rolls) and non-vegetarian (22.3 vs. 2.2%) despite higher average phytate intake in vegetarian group
[22]	10	Replete and deplete	Women	Meal with black tea, ascorbic acid, vs. control meal	Black tea	Significant reduction in iron bioavailability with tea consumption (18.2 vs. 7.1% in control vs. 150 ml, and 19.7 vs. 5.6% in control vs. 300 ml tea drinkers), not dependent on polyphenol burden (1 vs. 2 cups tea)
[58]	8	Replete and deplete	Men and Women	10 different beverages	Black tea, herbal tea, cocoa, or coffee	Significant reduction in iron bioavailability with tannin consumption (tea); ranges in reductions tea compared to water: 3-27% dependent on whether food consumed.
[59]	13	Replete and deplete	Men and Women	Control breakfast vs. coffee, tea	Black tea, polyphenol	Significant reduction in bioavailability with tea or coffee consumption compared to control (60-90% reduction compared to control, average 10% less iron absorbed)
[61]	22	Replete	Women	Control meal vs. meal with tea	Black tea	Non-significant differences in iron absorption with tea consumption (reduction 1.7%), although effects of tea noted with ascorbic acid consumption (20% reduction vs. ascorbic acid alone)
[60]	8	Replete	Women	Broccoli with tannic acid (500mg) or	Tannic acid	Significant reduction (10% vs. 0.3%) iron absorption in broccoli vs. broccoli + tannic acid meal

				broccoli alone vs. control		
[31]	14 (C), 15 (I)	Replete	Women	Control meal with 5mg FeSO4 vs. tannic acid, phytic acid, or pectin	Tannic acid	16-25% significant reduction in iron absorption with tannic acid vs. no tannin consumption
[63]	10 (C), 16 (I)	Replete	Women	Bread baked with tannic acid (12-884 mg) vs. control	Tannic acid	Significant reduction in iron absorption ratio, serum iron with tannic acid consumption in bread (average reduction of iron absorbed 3-10%)

Table 3-5: Single meal bioavailability studies showing no or minimal reductions in iron bioavailability with tannin consumption

Reference	n	Iron status	Population	Intervention	Tannin type	Outcome
[65]	14	Replete	Women	Meal with green tea, meal with rosemary extract, control meal	Green tea or polyphenol	No significant differences in iron absorption 12.1 vs 8.9% (control vs. green tea) and 7.5 vs. 6.4% (control vs. rosemary oil)
[66],	8	Replete and deplete	Men and Women	Meal with orange juice or tea compared with control (water)	Black tea	No significant differences in iron bioavailability between tea and control, ascorbic acid and control
[35]	7 (D), 12 (R)	Replete and deplete	Men	Vegetable and low or high tannin sorghum roti	Polyphenol/ condensed tannins	Significant 5.2% reduction in absorption of iron in anemic men, normalized and non-significant when adjusted for phytates (0.83%). No significant difference in iron replete men (5.05 vs. 3.81% in low and high tannin sorghum)
[62]	16 (C), 18 (I)	Replete	Women	Meal with white vs. polyphenol rich sorghum	Condensed tannins	5.2 and 5.8% significant reduction ($p < 0.001$) in iron bioavailability from high/medium tannin sorghum compared with low tannin sorghum. No differences noted with polyphenol oxidase addition to meal
[64]	16	Replete	Women	Meal containing red or white cowpea	Condensed tannins	No significant differences in iron bioavailability between red and white cowpea (11%, both groups, $p = 0.69$)
[67]	16	Replete	Men and Women	Maize meal compared to algae containing maize meal	Polyphenol/ condensed tannins	Dose-dependent, significant enhancement of iron bioavailability with algae polyphenols (6.8-17.8% more iron absorbed with algae compared to maize meal)
[68]	20	Replete and deplete	Women	2x2 factorial structure with low phytate, low polyphenol, high phytate, or high polyphenol bean meals	Polyphenol/ condensed tannins	Polyphenol-rich, low-phytate beans non-significantly increased iron bioavailability (6.14, 95% CI: 2.57-14.65 vs. 3.99; 95% CI 1.83-8.71), compared to low-polyphenol, low-phytate beans. High-phytate beans significantly reduced iron bioavailability (6.14, CI 2.57-14.65% vs. 3.84, 1.76-8.38).

Table 3-6: Long-term studies investigating the impact of tannin consumption on iron bioavailability

Reference	n	Iron status	Intervention	Tannin type	Intervention length	Outcome
[69]	9 (D), 25 (R)	Deplete and Replete	Green or black tea supplementation in either vegetarian or omnivorous participants	Green or Black Tea	4 weeks	Significant decrease in ferritin in anemic and omnivorous women consuming black tea without change in TIBC*, Hb+, serum iron
[70]	11 (C), 12 (I)	Replete	Green leafy vegetable supplementation vs. standard meal	Polyphenol/ condensed tannins	3 weeks	No significant inhibition of iron bioavailability after supplementation of tannin containing meal compared to control; significant increase in Hb+ by 11% compared to baseline with leafy vegetable intake
[40]	14 (C), 17 (I)	Replete	"High" or "Low" bioavailability diet	Polyphenol/ condensed tannins	12 weeks	Trends toward increases in bioavailability of low bioavailable diet over time with reduction in bioavailability of high bioavailable diet. No significant differences in non-heme iron absorption between groups at study end
[71]	9	Replete	"High" or "Low" bioavailability diet	Polyphenol/ condensed tannins	12 weeks	Significant 8.8% greater iron absorption efficiency in high bioavailability group ($p < 0.0001$) compared with low bioavailability group.
[41]	16	Deplete	"High" or "Low" phytate diet with high phytate diet challenge at baseline and endpoint	Polyphenol/ condensed tannins	8 weeks	Significantly increased uptake of iron in high phytate diet (29.3% increase) despite no changes in iron markers (ferritin, TIBC*, hepcidin) compared to low phytate group. Decrease in absorption of iron to high phytate meal with low phytate consuming group

+ Hb: Hemoglobin; *TIBC: Total iron binding capacity;

(C) Control; (D) Deplete participants; (I) Intervention; (R) Replete Participants

Figures

Figure 3-1: A: Condensed tannin Yikrazuul/ Wikimedia Commons / Public Domain and B: Tannic Acid Ronhjones/ Wikimedia Commons / Public Domain

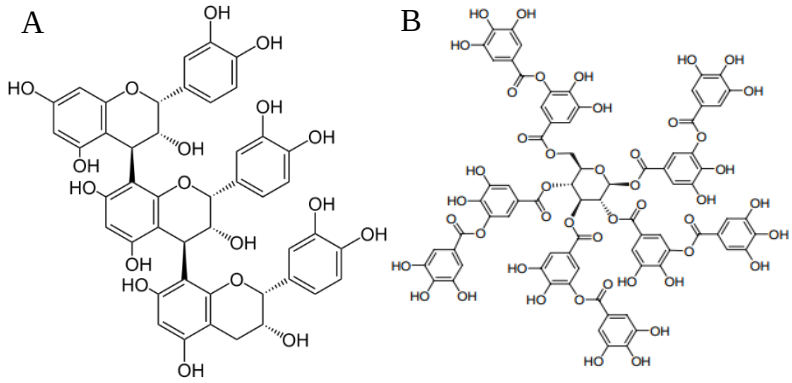
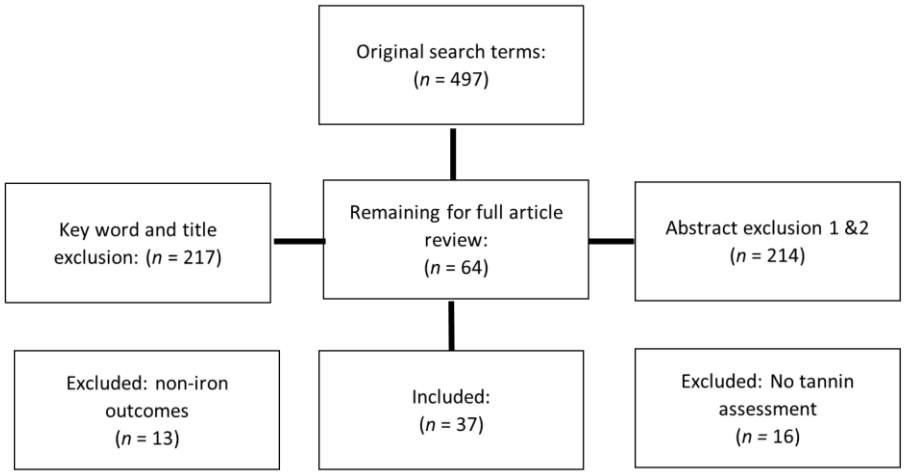


Figure 3-2: Inclusion and exclusion criteria for review. Articles were excluded related to key terms, *in vitro analysis*, lack of tannin assessment in dietary analysis, and analysis of non-iron outcomes iron bioavailability, iron status in tannin consumers



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Chapter 4 - SALIVARY PROLINE-RICH PROTEIN MAY REDUCE TANNIN-IRON CHELATION: A SYSTEMATIC NARRATIVE REVIEW

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Abstract

Background: Tannins are often cited for antinutritional effects, including chelation of non-heme iron. Despite this, studies exploring non-heme iron bioavailability inhibition with long-term consumption have reported mixed results. Salivary proline-rich proteins (PRPs) may mediate tannin-antinutritional effects on non-heme iron bioavailability.

Aim: To review evidence regarding biochemical binding mechanisms and affinity states between PRPs and tannins, as well as effects of PRPs on non-heme iron bioavailability with tannin consumption *in vivo.*

Method: Narrative systematic review and meta-analysis. Common themes in biochemical modeling and affinity studies were collated for summary and synthesis; data were extracted from *in vivo* experiments for meta-analysis.

Results: Thirty-two studies were included in analysis. Common themes that positively influenced tannin-PRP binding included specificity of tannin-PRP binding, PRP and tannin stereochemistry. Hydrolyzable tannins have different affinities than condensed tannins when binding to PRPs. *In vivo,* hepatic iron stores and non-heme iron absorption are not significantly affected by tannin consumption (d = -0.64-1.84; -2.7-0.13 respectively), and PRP expression may increase non-heme iron bioavailability with tannin consumption.

Conclusions: *In vitro* modeling suggests that tannins favor PRP binding over iron chelation throughout digestion. Hydrolyzable tannins are not representative of tannin impact on non-heme iron bioavailability in food tannins because of their unique structural properties and PRP affinities. With tannin consumption, PRP production is increased, and may be an initial line of defense against tannin-non-heme iron chelation *in vivo.* More research is needed to compare competitive binding of tannin-PRP to tannin-non-heme iron complexes, and elucidate PRPs' role in adaption to non-heme iron bioavailability *in vivo.*

Key words: iron bioavailability, antinutritional factors, tannin, proanthocyanidins, salivary proline-rich proteins

Introduction

Non-heme iron-tannin binding

Tannins are defensive metabolites classified as either hydrolyzable or condensed [1], that protect plants from insect, animal predators, and mold [2]. Hydrolyzable tannins are esters of polyols with phenolic acids (generally gallic acid) that are readily hydrolyzed by acidic or basic conditions [1], and are "virtually absent from the diet" [3] **(Figure 1a**). Condensed tannins, also known as proanthocyanidins, are more commonly consumed in sorghum, wine, tea, dark

chocolate and berries [3], and are comprised of oligomers and polymers of flavan-3-ols linked by carbon bonds [4] that are difficult to hydrolyze [1] **(Figure 1b)**.

While tannin-rich foods may confer potential antioxidant [5], cancer fighting [6], and cardiovascular [7] health benefits, a potential drawback to their consumption is that they inhibit non-heme iron bioavailability [8-20]. However, the studies supporting this antinutrient function have mostly been single meal studies utilizing hydrolyzable tannic acid or oligomeric epigallocatechin gallate found in tea. Studies that have explored long-term consumption of tannins [21,22] and other antinutritional factors [23,24], as well as epidemiological studies [25-27] have questioned whether potent tannin-non-heme iron inhibition is sustained over time.

Traditionally, protein-tannin binding has been cited as a major contributor to the antinutritional effect of tannins outside of mineral metabolism. It is tannins' predilection toward protein interaction that may mediate non-heme iron-chelation by salivary proline-rich proteins (PRPs), which bind to tannins in lieu of non-heme iron to make the mineral more bioavailable. Tannins' ability to interact with proteins varies widely, and it has been noted that protein characteristics that increase binding include large protein size, amino acid sequences that are rich in proline, and a lack of protein structure [28]. Due to their specific preference for tannin binding, PRP's have been studied in disciplines that have aimed to understand adaption to seasonally tannin-rich diets in animals [29-32]. Salivary PRPs have been of interest in sensory studies due to their ability to irreversibly precipitate tannins, contributing to the sensation of astringency [28]. Tannin-PRP complexes have been found to be insoluble throughout the gastrointestinal tract [33,34], thus complexes are maintained during digestion. Moreover, like non-heme iron absorption, PRP profiles tend to show high intrapersonal, rather than interpersonal variability [35], that is

genetically linked [36,37], which may explain why some individuals have enhanced capacity for 'antinutritional' consumption without impaired iron status. Increases in PRP secretion with tannin consumption have been shown to improve protein [38], and non-heme iron availability [39,40], in rats, and hamsters without capacity to enhance PRP synthesis tannin consumption resulted in poorer protein availability [31,41].

This systematic review aims to explore whether tannin-PRP complexes may reduce tannin-non-heme iron chelation during digestion, and to determine whether biochemical mechanisms behind tannin-PRP binding could reduce tannin-non-heme iron chelation during digestion. This review will identify *in vitro* and *in vivo* research exploring biochemical mechanisms and outcomes related to tannin-PRP binding and non-heme iron. Secondary aims are to compare potential differences in non-heme iron bioavailability attributed to hydrolyzable versus condensed tannins PRP-binding mechanisms in order to explore whether food, or condensed tannins, may affect non-heme iron chelation differently than hydrolyzable tannins commonly used in non-heme iron absorption studies.

Methods

Primary outcomes and search strategy

Primary search subjects included non-heme iron and/or salivary proline-rich proteins exposed to tannins, both condensed and hydrolyzable. Search outcomes were targeted toward research exploring biochemical modeling for binding methods, binding strength, mechanism of binding comparatively, effects of binding of one compound on another, and long-term effects of tannin consumption on PRP and non-heme iron availability. Studies were not excluded for sample or effect size. To capture as many relevant citations as possible, medical and scientific

databases were used (Pubmed, Web of Sciences Core Collection, Cochrane Database, Medline, Proquest Nursing, and CABI), as well as snowball article collection (citations from relevant journal articles) and internet search engines (Google Scholar) to look for other references. All publication dates were included and searches were completed by July 2016. Articles reviewed were in English.

Search terms and inclusion

Article searches in PubMed included ("Salivary Proline-Rich Proteins"[Mesh] AND "Non-heme iron"[Mesh]) AND "Tannins"[Mesh];("Salivary Proline-Rich Proteins"[Mesh] AND "Non-heme iron"[Mesh]) 2012; ("Salivary Proline-Rich Proteins"[Mesh] AND "Tannins"[Mesh] 2009-2014; ("Non-heme iron"[Mesh]) AND "Tannins"[Mesh] 1945-2015; ("Salivary Proteins and Peptides"[Mesh]) AND "Non-heme iron"[Mesh] 1983-2015; (("Salivary Proteins and Peptides"[Mesh]) AND "Non-heme iron"[Mesh]) AND "Tannins"[Mesh];("Proline-Rich Protein Domains"[Mesh]) AND "Tannins"[Mesh] 2009-2016;"Proline-Rich Protein Domains"[Mesh] AND "Non-heme iron"[Mesh] ("Tannins"[Mesh]) AND "Salivary Proteins and Peptides"[Mesh] 1983-2015; (salivary protein[All Fields] OR salivary proteinase[All Fields] OR salivary proteins[All Fields]) AND ("non-heme iron"[MeSH Terms] OR "non-heme iron"[All Fields]) 1966-2016; salivary[All Fields] AND ("proline"[MeSH Terms] OR "proline"[All Fields]) AND rich[All Fields] AND ("proteins"[MeSH Terms] OR "proteins"[All Fields] OR "protein"[All Fields]) 1973-2016. Filters included searches for *in vivo, in vitro,* human and animal models. Web of Science Core Collection and CABI search terms included “salivary proline-rich proteins AND tannins (condensed OR proanthocyanidins AND/OR hydrolyzable) AND/OR non-heme iron”; “tannins AND non-heme iron” (All years). Proquest nursing and MEDLINE (all years;

terms included: "salivary proline-rich protein*" AND proanthocyanidins; "salivary proline-rich protein*" AND non-heme iron; ("salivary protein*" AND non-heme iron) AND tannin; ("salivary protein*" AND tannin); ("salivary protein*" AND non-heme iron) (All years). Cochrane search terms were: #1 MeSH descriptor: [Non-heme iron] explode all trees 1785 #2 MeSH descriptor: [Proanthocyanidins] explode all trees; #3 MeSH descriptor: [Salivary Proline-Rich Proteins] explode all trees (all years).

Selection, quality assessment, data extraction and analysis

Articles retrieved were collected into an online database, and duplicates were removed. Remaining articles were included with the presence of at least two key terms, and then by abstract reviews for relevance to study outcomes. After initial inclusion criteria were met, articles were collected for full review. Quality assessment of data was completed using the Cochrane Quality Guide [42] adapted for in vitro studies (Appendix A). In brief, articles were reviewed for risk of bias based on methodology, results presented and discussion. They were classified as having high, unclear, or low risk of bias based on their overall study characteristics. Studies with overall high or unclear risk of bias were excluded from the review. Data from remaining articles were collated into summary tables (biochemical study characteristics, Appendix B). For biochemical binding analysis, a narrative synthesis approach was chosen due to the heterogeneity of studies explored and lack of studies exploring tannin-non-heme iron: tannin-PRP binding comparisons. This process included developing a theory of how study interventions worked, developing a preliminary synthesis of findings in included studies, exploring relationships within and between studies, and assessing the robustness of the synthesis

formulated **(Figure 2)** [43]. For analysis, studies were grouped primarily by study question, then by subgroup topics.

For *in vivo* analysis, study outcomes were collected, mean outcome measurements and standard deviations were used to calculate Hedges' g estimates, and results were used to calculate effect size [(40)]. Upper and lower confidence limits of effect size were calculated using a 95% confidence interval, and total effect size was calculated from normalized values [44]. PRP synthesis relative to control gave reference to PRP production with non-heme iron-tannin outcomes using the equation:

$$Relative\ PRP\ expression = \frac{Average\ increase\ in\ PRP\ production\ (intervention)}{Average\ increase\ in\ PRP\ production\ (control)}$$

Results

Inclusion Criteria

Original search terms generated 1220 articles that were added into an electronic database **(Figure 3)**. After duplicates were automatically removed, 752 articles were reviewed for title, abstract, and key term relevance. Articles were removed without presence of at least two key terms in abstracts (one of two key subject terms; non-heme iron and/or salivary proline-rich proteins) AND an intervention term (tannin exposure). Excluded studies at this stage included validity studies for measurement tools, assessment studies (including tannin content of foods, food non-heme iron content), studies in ruminant animals, research exploring topic subjects in specialized conditions (burning mouth syndrome, Sjogren's syndrome, Appendix C). The 107 articles remaining were manually combed for duplicates, and a second review of abstracts for

specific population intervention comparison and outcomes (PICO) specified further narrowed articles. Articles were obtained for full article review from online journal archives, printed journals, and interlibrary loan. Because of the heterogeneity of remaining studies available to answer the research question, full text review of the remaining 81 articles was divided into two groups: biochemical mechanisms for tannin binding, and *in vivo* comparisons of non-heme iron status with tannin consumption and PRP production. For biochemical mechanisms, articles were excluded that did not address PRP interactions with tannins specifically. This exclusion criterion was applied due to suggestion that PRPs have different binding capacity and mechanisms than other proteins, limiting efficacy of substitute models [45]. Other reasons for exclusion at this stage were that articles did not include biochemical mechanisms explaining tannin-PRP binding. Remaining *in vivo* articles were excluded that did not include all three outcomes related to the study question: non-heme iron, tannins, and salivary PRPs. Full text review of articles was completed including analysis for internal and external bias. Final analysis included 32 studies (for biochemical modeling: 30*in vitro* and 2 *in vivo,* both animal studies).

Part I: Mechanisms behind tannin-PRP binding, effects of tannin and PRP characteristics on binding affinity

Of studies reviewed, three themes potentially important to considering binding and affinity of PRPs to tannins were identified. The first was that tannin-PRP binding mechanisms are specific, and that binding affinity and efficiency are affected by tannin and PRP concentration. Next was that ionic and digestive influences favor tannin-PRP complex formation throughout digestion. Finally, the stereo-chemical makeup of tannins and PRPs themselves is important to preferential binding and affinity of tannin-PRP connections.

Tannin-PRP binding specificity

PRPs are randomly structured, unfolded proteins [46] where open and flexible conformation of helical and extended coils allows for binding of tannins to proline-rich residues **(Figure 4)** [47-49]. Interactions between PRPs and tannins first occur through hydrogen bonds between compounds enhanced by glycine, arginine, and alanine [46,48,50]. It is important to note that PRPs bind specifically to tannins [46,50-55] **(Table 1)**, and with an increased affinity compared to digestive enzymes and proteins [4,56], which supports the role of PRPs serving as biological protectors against tannins. Selectivity is regulated by both tannin and PRP properties enhancing affinity of binding; high-affinity tannin molecules are bound first, then lower affinity molecules are bound at higher concentrations [57]. Enzyme activity inhibited by tannins is enhanced in the presence of PRPs, pointing to tannin-PRP preferential binding [55], and similarly structured compounds to PRPs have not been found to bind to tannins with the robust affinity found in tannin-PRP binding [53].

As tannins bind to and saturate proline-rich residues, their multidendate, or self-associating nature [54], leads to cross-linked binding between tannin molecules, formation of hydrophobic tannin-PRP bonds [46], and more efficient and stable precipitation of tannin-PRP complexes [52,54]. The concept of affinity and eventual tannin-PRP precipitation may explain the effectiveness of tannin-PRP complexes in preventing tannin-iron chelation. In a non-precipitated state, tannins are highly bound to PRPs, however, lack of saturation of proline-rich residues increases the likelihood that tannin-PRP complexes will dissociate [46,54]. This may lead to tannin-iron chelation later during digestion. Tannin-PRP binding at low tannin concentrations may follow a ‘poisoned growth model,’ wherein aggregation of tannins through crosslinking is

limited by PRPs that do not have sufficient saturated proline residues to continue the process [54]. In this model, a lack of saturated proline-rich residues on PRPs favors redistribution of tannin molecules among all PRPs, reducing the crosslinks formed by tannins and leading to dissociation and 'freeing' of tannins from PRPs [54].

Concentration effects

High tannin concentrations support almost immediate formation of hydrophobic bonds and tannin crosslinks with PRPs, along with subsequent precipitation [46]. In contrast, lower tannin concentrations resist precipitation due to lack of tannin crosslinking and hydrophobic bonds in favor of weaker hydrogen bonds that may later dissociate **(Table 2)** [50,54,56,58-62]. Thus, as concentration of tannin increases, PRPs have the ability to bind tannin molecules beyond the number of proline residues that they contain [50,54], leading to more efficient tannin 'capture' by PRPs. After concentration-dependent hydrophobic interactions and tannin-crosslinking is established, conformational changes in the PRP molecule provides additional stability of tannin-PRP complexes, further reducing likelihood of digestive dissociation [51,63].

Thus, the process of tannin-PRP binding is proposed in three stages: first, proline residues are saturated by tannins that bind selectively, and hydrogen bonds strengthen the tannin-PRP complex. Next, tannins self-associate and hydrophobic stacking of multiple tannins promotes crosslinking of tannin-PRP complexes via tannin-tannin bonds, and conformational PRP changes stabilize the bond. Last, tannin-PRP complexes aggregate and separate from solution (precipitation, **Figure 5**) [46,52].

Ionic Effects

Ionic concentration and solvent effects also affect tannin-PRP binding affinity. Hydrophilic [56] and basic [55,62,64-66] environments tend to reduce tannin-PRP affinity, partly due to reductions in hydrogen and hydrophobic binding [56,66] (**Table 3**). Potential of hydrogen (pH) in gastric compared to enteric environments significantly increases precipitation of PRPs [65], and lower ionic concentrations may inhibit secondary structural changes needed for PRP-tannin association [54,67]. Tannic acid is more soluble in acidic environments [62], suggesting that precipitation may be necessary for PRP-induced protective mechanisms against tannins. Food matrix components, carbohydrates and ethanol particularly [56,66], disrupt precipitation of tannin-PRP aggregation, necessitating increased concentrations of tannins to precipitate PRPs. It is important to note that matrix disrupted interactions may limit tannin-iron exposure as well.

Digestive Effects

In vitro digestion experiments have shown that tannin-PRP bonds are highly resistant to digestion [55,58,59,65,68]. Tannin-PRP bonds are resistant to trypsin cleavage [58], and in the presence of enteric digestive protease enzymes, tannin-PRP complexes are more likely to stay insoluble than in their absence [65]. Gastric digestion and pH favor precipitation of tannin-PRP aggregates [55,58,59,65,68], although smaller tannin size and lower concentration reduce stability in gastric digestion [59]. PRPs are also highly bound and recovered in enteric digestion [59], and may block enteric absorption and digestion of tannins themselves [55,65]. Binding affinity of tannin-PRP complexes seems to remain specific outside of the mouth; addition of PRP to hydrolyzable tannin has been found to reduce tannin absorption 2-3 fold in Caco-2 cells [68].

PRP characteristics and tannin interactions

Many classifications of PRPs have been identified in saliva, and are grouped according to their biochemical properties. When referencing their ability to precipitate tannins in whole saliva, acidic PRPs (aPRP) tend to bind to tannins with the highest affinity, followed by basic (bPRP), and glycosylated PRPs (gPRPs) [58,59]. While tannin-PRP affinity may explain the predilection for complex formation, this does not always correlate to the ability to *efficiently* precipitate tannins. Greater proline content in bPRP [65,69], and gPRP [69] is associated with increased ability to precipitate higher concentrations of tannins compared to their acidic counterparts. Longer PRP sequences found in bPRP and gPRP with more proline-rich residues result in more tannin-proline-rich residue interactions [70], and allow for secondary conformational changes around tannin molecules [50], likely reducing complex dissociation compared with shorter PRP sequences [48]. Glycosylation may increase the threshold for precipitation of tannins without impeding tannin-PRP affinity [61], although this finding has been inconsistent [71].

In vivo, PRP profiles differ among regular tannin consumers and non-consumers. For example, gPRP have been found more prevalently in tannin consumers' salivary profiles, and tannins are precipitated at a lower rate (30%) in consumers than in non-consumers (85%), possibly due to increased capacity of PRP types produced [4]. Types of PRPs that were precipitated also varied between tannin and non-tannin consumers. Tannin consumer PRP profiles consisted of higher prevalence of bPRP and gPRP, which were most commonly precipitated [4].

Tannin Polymerization, galloylation, and hydroxylation

Tannin polymerization, galloylation, and hydroxylation play a role in tannin-PRP affinity and precipitation **(Table 4)**. Increased polymerization, or increases in the relative size of tannin molecules, may favor tannin-PRP affinity through hydrophobic interactions and subsequent increases in self-association of tannin molecules to form precipitating complexes [51]. Increased galloylation [69,72], as well as hydroxyl group positioning [53,70], may increase affinity, as well as binding strength of tannins to PRPs through promotion of hydrogen bonds. Perhaps less importantly, the location of carbon-carbon bonds within polymers may improve stability of precipitates formed by tannin-PRP complexes [72].

Tannin polymerization affects tannin-PRP affinity and dissociation more than either tannin galloylation or hydroxylation [52,53]; larger tannin molecules precipitate PRPs more efficiently [51-53,59,61,61,70,72], and selectively [4], than smaller molecules. Smaller molecules do bind PRPs, but do not crosslink [72], and the lack of tannin self-association increases the likelihood that tannin-PRP complexes dissociate [50,54,65,69], found *in vitro* [59]. *In vivo*, tannin polymerization has been shown to be positively correlated with precipitation; however, tannin consumers' saliva is able to precipitate smaller tannins, and given that greater polymer size is bound preferentially, does not leave tannins unbound. Non-consumers, on the other hand, may bind only large tannins[4].

Binding of hydrolyzable vs. condensed tannins

PRP binding to hydrolyzable versus condensed tannins is influenced both by differences in binding affinity [50,64,65,69] and subsequent likelihood of bond dissociation during digestion [50,51,65,66] (**Table 5**). PRP affinity toward hydrolysable tannins is greater than condensed tannins [50,64,65,69]. Affinity of hydrolyzable tannins toward PRPs may reduce the number of available proline residues for tannin-binding compared with condensed tannins [50,65,69]. It has been noted that there is a greater stability of PRP with quebracho (a condensed tannin), measured by precipitation, in both gastric and enteric conditions compared to tannic acid [65]. This may be due to weaker hydrogen bonds that hold hydrolyzable tannin-PRP complexes together [50,51] compared with more prominent hydrophobic bonds found in condensed tannin-PRP precipitates [66]. The ring structure in condensed tannins may favor a more stable hydrophobic bond compared with hydrolyzable tannins [72]. It has been suggested that hydrolyzable tannins may not crosslink compared to their condensed counterparts [51], reducing bond stability and increasing likelihood of complex dissociation. Differences in binding mechanisms described above may also reduce the physiological stability of hydrolyzable compared to condensed tannin-PRP complexes throughout the digestive process [50,51,65,66].

Part II: Effect of tannin consumption on PRP expression and non-heme iron bioavailability

In vivo comparisons

Two studies have explored the interaction of chronic tannin ingestion, PRPs and non-heme iron bioavailability [39,40] **(Table 6).** Due to the similarities of the two studies, it is possible to compare effects of several treatments to control conditions in order to isolate findings for synthesis. Thus, PRP production and the effects of tannin consumption on non-heme iron absorption versus hepatic iron status are highlighted. Further, isolation of experiments employing chronic ingestion of tannins is included to give context to effect of tannin ingestion over time.

Study characteristics:

Both of the studies reviewed included a diet challenge after either acute or chronic ingestion of a tannin-rich diet in weanling Sprague-Dawley rats, however they employed different diet mechanisms to attempt to explore the effect of PRP expression on non-heme iron absorption and hepatic iron stores. The first (Study 1) study provided tannin-containing green tea powder or a control diet, fed for 7 or 30 days, compared with a tannin-containing gavage (green tea) diet. In the gavage diet, exposure of tannin to the oral cavity was bypassed with the goal to reduce expression of PRPs [39]. This study included a diet challenge at either day 1 (acute) or day 24 (chronic) to measure non-heme iron absorption, after collection of baseline bloodwork. Green tea powder and control diets were given as powdered diets, or without green tea powder; animals in these groups were given a twice daily phosphate buffered saline gavage. Green tea gavage groups were given a control powdered diet with green tea gavages twice daily throughout the

study [39]. Tannin concentration in gavage diets was based on oral tannin consumption of green tea diets by rats.

The second (Study 2) study included a control or tannin-containing diet (as black tea, half the amount of tannin given at challenge) with tannin challenge at midpoint (day 11) for all groups. Substitution of protein with gelatin (proline-rich) in separate chronic and acute ingestion groups aimed to assess the effects of PRPs on non-heme iron absorption with proline addition [40]. In study 2, intervention groups were given a diet with brewed black tea that was made into a slurry with powdered diets. Slurry diet components were then freeze dried and milled into powdered diets [40]. Diets consumed in both studies contained similar tannin and iron content (Table 6). Study 2 diet intake was not significantly different between groups with chronic consumption, but was ~3x less days 1-3 of the study. Both studies used a 2 g test meal for iron absorption, which was totally consumed by rats.

Comparison I: Effect of tannin on non-heme iron absorption and stores with acute and chronic tannin ingestion

Data are presented in means ± SEM. Study 1 hepatic iron stores were not significantly different among treatment groups ($p = 0.521$) [39]. Acute treatment outcomes were not different from chronic outcomes in control (53.5 ± 11.1 vs. 61.0 ± 4.0 µg Fe/g liver), gavage (58.4 ± 2.0 vs. 57.6 ± 7.6 µg Fe/g liver), or green tea powder (47.2 ± 2.7 vs. 54.2 ± 7.5 µg Fe/g liver) groups [39] (**Table 7, Figure 3**). Study 2 hepatic iron stores were not significantly different among treatment groups (p-value not presented). Acute and chronic groups' hepatic iron levels were not significantly different (58.5 ± 5.2 and 58.8 ± 6.9 vs. 43.5 ± 3.4 and 46.6 ± 3.6 µg Fe/g liver) in challenge and long-term tea and gelatin diets, respectively, (Figure 1) [40]. Calculated confidence

intervals supported that overall, there was a non-significant, but positive, effect of tannin consumption on hepatic iron stores (calculated effect size d= 0.57, 95% CI -0.64 to 1.84 in favor of treatment; Table 7). This trend was more positive in chronic tannin consumers (calculated effect size d= 1.16, 95% CI -0.18 to 2.51 in favor of treatment).

Data for non-heme iron absorption are also presented in means ± SEM. Non-heme iron absorption percentage was not significantly different among treatment groups in study 1 (p = 0.104, p= 0.292 acute and chronic, respectively). Acute non-heme iron absorption was much higher than chronic non-heme iron absorption in all groups, possibly due to sufficient iron stores in the rats at study end, but absorption among treatment groups were not significantly different in control (43.1% ± 4.9 vs. 5.6% ± 1.0), gavage (56.5% ± 7.5 vs. 6.0% ± 1.0), or oral (63.8% ± 6.4 vs. 3.7% ± 1.2) groups (**Table 8, Figure 4**) [39]. Non-heme iron absorption was significantly reduced in Study 2 tannin-consuming groups, although there was significantly greater absorption of non-heme iron from groups consuming tannins over time (7.5% ± 2.3 and 25.1% ± 7.4 vs. 6.8% ± 1.6 and 20.8% ± 4.5) in challenge and long-term tea diet and gelatin diets, respectively, (Figure 4) [40]. Calculated confidence intervals supported that overall, there was a non-significant but negative effect of tannin consumption on non-heme iron absorption (calculated effect size d= -1.30, 95% CI -2.73 to 0.13 in favor of treatment; Table 8). This trend was slightly more positive with chronic tannin consumption (calculated effect size d= -1.11, 95% CI -2.37 to 0.16 in favor of treatment).

Comparison II: Effects of PRP production on non-heme iron bioavailability

While study 1 presented a more detailed analysis of PRP production relative to control, study 2 presented total PRP production for each chronically consuming tannin group and control. Because total PRP production was assessed differently by studies, and is presented in relative amounts compared to control analysis (**Table 9**). In study 1, total PRP production in oral-tannin ingestion groups was greater than either control or gavage groups [39]. Chronic oral tannin ingestion resulted in reduction of PRP production relative to acute ingestion. In study 2, there were no significant differences in PRP production between control and intervention groups [40].

Given that direct tannin precipitation of PRPs would most likely affect non-heme iron absorption with proposed biochemical binding mechanisms, relative PRP production to control was calculated and compared to experimental non-heme iron absorption. Given the somewhat qualitative nature of the findings in both studies, statistical analysis has not been applied. Overall, relative PRP expression is greater in groups that found greater non-heme iron absorption, although this is not true in gavage groups, where PRP induction was low and non-heme iron absorption was generally improved compared with control (Table 8, **Figure 6**). Groups where PRP induction was low (chronic tea and chronic gelatin ingestion) trended toward lower non-heme iron absorption.

Discussion

Comparison of tannin non-heme iron and tannin PRP binding

PRP tannin binding and affinity

Comparing the nature of tannin-PRP binding elucidates mechanisms that may deter tannin-non-heme iron chelation throughout digestion. Tannin exposure to PRPs in the mouth allows for selective complex formation in lieu of tannin-iron chelation on first contact with the food bolus, and conformational changes, along with tannin-crosslinking, effectively allows PRPs to efficiently bind tannins. The strength of hydrophobic bonds formed with tannin-crosslinking, along with secondary PRP conformational changes with residue saturation, may prevent tannin dissociation during digestion and limit the number of unbound hydroxyl groups with the potential to chelate iron throughout the alimentary tract [55,63].

Similarly to tannin-PRP binding, non-heme iron species are bound to hydroxyl groups of polyphenols [73], and tannins with greater affinity toward non-heme iron (such as tannins with galloyl groups, or larger tannin-polymers) [74] may bind to tannins more efficiently (and thus fewer tannins chelate more non-heme iron molecules). Because PRPs favor binding to higher order (higher polymerization) polyphenol complexes, it may be that PRPs specifically precipitate tannins most likely to bind to iron during digestion. Beyond the nature of tannin-PRP binding, production of PRPs that better bind tannins (such as bPRP or gPRP) [58,59] with common tannin consumption [4] may facilitate adaptation to tannin-iron chelation over time.

Digestion and ionization

At physiological conditions, tannins tend to be deprotonated [75], forming Lewis bases that are stabilized with Lewis acids, such as Fe^{3+}, and to a lesser extent, Fe^{2+} [73,76]). In acidic conditions, protonated tannins, and can be stabilized after bound ferric iron is reduced to its ferrous state, and reduce another ferric iron molecule [73]. Acidic conditions where iron is easily oxidized therefore favor tannin-iron affinity. Interestingly, gallate complexes oxidize at a much higher rate than catecholate complexes [77], which may be why tannic acid strongly chelates non-heme iron compared with condensed tannin-containing foods. Reductions in iron bioavailability are found at higher pH environments, where polyphenol-non-heme iron complexes prevent Fe^{3+} reduction to Fe^{2+} [78], possibly reducing the amount of non-heme iron that can be taken up by the enterocyte.

Similar tannin-iron and tannin-PRP properties regarding pH may explain why pH strongly influences the nature and strength of tannin-PRP bonds. Condensed tannins and bPRP tend to precipitate within similar, more basic ionic conditions [79]; aPRP tend to precipitate closer to the pH of saliva [58], suggesting that PRPs may have different functions at different stages of digestion [80]. Tendencies of aPRP to precipitate at salivary pH may also favor immediate tannin-PRP binding before non-heme iron can be chelated in the mouth. Importantly, the affinity of tannin-PRP binding is resistant to digestion, thereby reducing exposure of tannins to non-heme iron throughout the digestive tract [55,58,59,65,68]. Compared with the more basic isoelectric point of condensed polyphenols, tannic acid is deprotonated at a pH closer to 5 [81], where tannin-non-heme iron precipitation is highest [74]. This may explain why tannic acid has more potent affinity and efficient binding than condensed tannins for both PRP and non-heme iron complexes.

Hydrolyzable versus condensed tannins

Effective PRP protection against hydrolyzable and condensed tannins may be different due to multiple factors. Tannic acid binds more efficiently to non-heme iron as compared to catechins or food polyphenols [74,82,83], increasing efficiency of tannin acid-iron chelation comparatively. As mentioned previously, larger tannin polymers increase non-heme iron-tannin binding [74]. Condensed tannin polyphenols tend to be higher order polymers [59] than tannic acid [68], are more likely to cross-link [51], and less likely to dissociate in digestion. By comparison, tannic acid is not as efficiently bound by PRPs during digestion, increasing the antinutritional potency of these tannins. The overall smaller molecular size of hydrolyzable tannins (especially in tannic acid) decreases non-heme iron binding relative to higher order condensed tannin polymers, but an increased number of galloyl groups on tannins increase affinity compared to condensed tannins [74]. Subsequently, a higher concentration of small monomeric compounds may allow hydrolyzable tannins to more efficiently bind PRPs and non-heme iron, leading to an increased burden on salivary glands to produce PRPs, as well as more effective chelation of non-heme iron when PRPs cannot meet tannin demand. In digestion, the hydrolyzable tannins may more likely dissociate from PRPs [65], further increasing potential non-heme iron chelation compared with their condensed counterparts. *In vivo,* PRP production is more highly upregulated with exposure to a mix of condensed and hydrolyzable tannins compared to tannic acid alone [84], suggesting that PRPs may not be an effective defense mechanism against this compound. It may be important to consider these differences when comparing accommodation of non-heme iron status and availability between food tannins (condensed tannins) and hydrolyzable tannins (tannic acid).

Limitations in protective Tannin-PRP binding

Despite attributes that potentially favor tannin-PRP binding as a mechanism to prevent tannin-non-heme iron chelation, inefficiencies seem to exist. Similar to tannin-PRP interactions, low concentrations of polyphenols associate with non-heme iron more efficiently than higher concentrations [82]. This phenomenon may importantly explain the inefficiency of tannin-PRP complexes in preventing tannin-non-heme iron chelation, especially in adaption. Following the 'poisoned growth model,' tannins must be highly bound, and possibly precipitated in order to prevent tannin-non-heme iron chelation [50,54]. As PRP profiles change due to tannin consumption, favoring bPRPs and gPRPs [4] that may bind a greater number of tannins before crosslinking leads to complex formation, molecules that randomly dissociate may chelate non-heme iron in digestion. This may be more commonly found in tannins of lower affinity (lower polymerization, galloylation, or hydroxylation).

In vivo findings

In vivo, it is interesting to note that overall PRP production closely follows non-heme iron absorption (Figure 5). Protective benefits of PRP production against tannins have been particularly highlighted in hamsters, which do not produce PRPs [85]. Other studies have shown that PRPs play a role in acclimation to tannins [86-89], although this is not always without reductions in growth overall compared to control [30]. Non-heme iron absorption is generally impacted by tannin intake, although major limitations, including interaction of tannins and non-heme iron in solution before exposure to PRPs [40], reductions in overall chronic non-heme iron absorption [39], and differences in measurement of PRPs between both studies limits the generalizability of these findings. It is particularly important to point out that non-heme iron

absorption was highly variable in all experiments presented, highlighting the importance of individual variability in non-heme iron absorption and the need to understand mechanisms behind non-heme iron inhibitors and enhancers from a physiological point of view. Interestingly, non-heme iron absorption and non-heme iron status (measured by hepatic non-heme iron) were not correlated in these studies, possibly indicating that either non-heme iron supplied was greater than the burden of tannin supplied, or that numerical non-heme iron absorption may be a poor indicator of non-heme iron status (in this limited model). Adequate hepatic non-heme iron stores likely impacted non-heme iron absorption percentage changes when comparing acute to chronic tannin challenges [39], although there was an increase in non-heme iron absorption suggesting that there was accommodation to poor non-heme iron absorption in acute challenges [40]. Perhaps more interestingly, rats gavaged with tannins had similar non-heme iron absorption and hepatic status compared to those with oral tannin exposure, suggesting that mucosal or endocrinological protection mechanisms are additionally important to non-heme iron bioavailability with tannin consumption. It may be that immediate tannin accommodation starts with PRPs, but is more efficiently dealt with elsewhere.

Conclusion

Similarities in binding mechanisms to tannins may support the hypothesis that PRPs play a role in protecting against tannin-non-heme iron chelation during digestion. Additionally, PRP production is linked to non-heme iron absorption, although absorption is poorly correlated with non-heme iron status in studies reviewed. More research is needed to explore changes in PRP production in humans related to tannin-non-heme iron chelation, and *in vitro* studies specifically modeling tannin-non-heme iron chelation in the presence of food tannins. Further *in vivo*

research should explore the differences between condensed and hydrolyzable tannins on non-heme iron status, and explore potentiating effects of antinutritional factors when consumed together.

List of abbreviations:

Salivary Proline-Rich Proteins: PRPs

Population intervention comparison and outcomes: PICO

Acidic proline-rich proteins: aPRP

Basic proline-rich proteins: bPRP

Glycosylated proline-rich proteins: gPRP

Declarations:

Availability of data and materials: All data generated or analysed during this study are included in this published article [and its supplementary information files]

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Author Contributions

Nicole Delimont, Mak Haub, Sara Rosenkranz, and Brian Lindshield conceived, and edited the manuscript. Nicole Delimont researched, reviewed and wrote the manuscript.

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Tables

Table 4-1 Tannin-PRP specificity

Reference	Method	Tannin type	PAC-TA comparison?	Conditions of assay	Outcome	Mechanism agreement
[51]	NMR	B2, PGG, TGG, PAC monomer, epicatechin	yes	40 mM B2, other assays 50 mM; either 0.5 ml of 2mM or 4mM PRP from mouse; pH 3.8	N-terminal proline residues linked to amide and amino structures bind tannin, then secondary interactions with galloyl groups changes structure of open conformation around PRP (specific binding)	Yes
[52]	NMR	PAC as B1, B3, C2	No	0.5-20mM PRP (IB9) with 15.7 mM tannin; pH 3.5	Tannin-PRP binding is specific to a certain concentration of tannin; then becomes random	Yes
[53]	ESI-MS	EgCG, ECG, B2, B2 3-O gallate, reserpine	No	1:10 ratio protein: polyphenol; pH 3.2	Tannin-PRP binding is specific; PRP-reserpine did not bind (similar structure to studied tannins)	Yes
[54]	ESI-MS; DLS, SAXS	EgCG	No	0.336 mM (1-3.5 mg/ml) PRP (IB5); 2:1 protein: polyphenol; pH 5.5	Tannin-PRP interaction is specific and dependent on tannin interactions; PRP sites for tannin binding are independent and have free energy; at a threshold, multidendate tannin crosslinks strengthen Tannin-PRP bonds	Yes
[46]	DLS, ITC	EgCG	No	6.4 or 12.8 EgCG with 0.25-2 mg IB5; pH 3.5	Tannin-PRP interaction is concentration dependent; there is slow and specific binding of tannins followed by rapid and non-specific aggregation as Tannin-PRP binding sites are saturated.	Yes
[55]	*In vitro* digestion, SDS-PAGE, HPLC	EgCG	No	0.05-0.5 mM EgCG, Human salivary PRPs; protein: tannin ratio 3:1; pH gastric 2.07; duodenal pH 7.8	Preferential tannin-PRP binding compared to lipase, alpha amylase, chymotrypsin, trypsin, lactase	Yes
[90]	DLS, ITC	EgCG	No	1:5 ratio saliva: wine in 1% TFA compared to physiological conditions	Salivary PRP 'moderately' bound tannins	Yes
[50]	NMR, DLS	EgCG, EGC, PGG	Yes	20 mM polyphenol with 2 mM mouse PRP; pH 3.8	There is preferential binding of tannin to proline residues of PRPs vs. alternative amino acids	Yes

NMR: Nuclear magnetic resonance imaging; ESI-MS: electrospray ionization mass spectrometry; DLS: dynamic light scattering; SAXS: small angle X-ray scattering; ITC: isothermal titration calorimetry; SDS-PAGE: sodium dodecyl polyacrylamide gel electrophoresis; HPLC: high performance liquid chromatography. B1, B2, B3: proanthocyanidin B1, B2, B3; PGG: pentagalloylglucose; TGG: tetragalloylglucose; PAC- proanthocyanidin; EgCG: epigallocatechin gallate; ECG: epigallocatechin; PRP: proline-rich protein

Table 4-2: Effects of tannin, PRP concentration on binding

Reference	Method	Tannin type	PAC-TA comparison	Conditions of assay	Outcome	Concentration effect
56	DLS	PAC as tetramers, pentamers, gallates	No	31.2 mg/L GSE: 0.5-5 mg/L IB-8c or 3.12 mg/L IB-8c: 19.5-46.8 mg/L GSE; pH 5.0, 12% ethanol	Increase in PRP concentration increases aggregation and precipitation of tannins to a maxima, then increased protein concentrations favors dissociation due to reductions in tannin-cross linking	Tannin stacking and crosslinking at higher concentrations
58	SDS-PAGE, HPLC, tryptic digestion	PAC as dimers, trimers, tetramers	No	0.00-1.5 mM GSE in saliva; pH 5.0, 12% ETOH	At higher tannin concentrations, less PRP are required for similar binding at lower tannin concentrations.	Tannin stacking and crosslinking at higher concentrations
59	ESI-MS	EgCG, ECG, B2, B2 3-O gallate, reserpine	No	1:10 ratio protein: polyphenol; pH 3.2	Higher tannin concentration of tannins favor Tannin-PRP stability in gastric digestion	Stability of tannin-PRP binding
54	ESI-MS; DLS,SAXS	EgCG	No	0.336 mM (1-3.5 mg/ml) PRP (IB5); 2:1 protein: polyphenol; pH 5.5	At lower concentrations, PRP are bound to tannins, but soluble. At higher concentrations, more tannin is needed to effectively bind the same amount of PRP; this happens as binding occurs regardless of proline terminal residue numbers.	Tannin stacking and crosslinking at higher concentrations
60	HPLC-DAD	PAC as monomers, dimers, trimers	No	1-8 ml saliva mixed with 40 ml GSE or 20 or 40 ml sipped	Increased tannin concentration increases precipitation.	Tannin stacking and crosslinking at higher concentrations
61	ITC	GSE as catechin, epicatechin, epicatechin 3-O gallate	No	5-25 µg PAC and 40 µl saliva with 10% ethanol	Increased tannin concentration increases precipitation.	Tannin stacking and crosslinking at higher concentrations
50	NMR, DLS	EgCG, EGC, PGG	Yes	20 mM polyphenol with 2 mM mouse PRP; pH 3.8	The number of PRP binding sites does not correlate with the corresponding decrease in tannin concentration after Tannin-PRP binding at higher concentrations	Tannin stacking and crosslinking at higher concentrations
62	NMR	Tannic acid	No	1:0-1:5.6 ratio of PRP to tannic acid	More Tannin-PRP complexes that are bound, the less that the complexes dissociate	Stability of tannin-PRP binding

NMR: Nuclear magnetic resonance imaging; ESI-MS: electrospray ionization mass spectrometry; DLS: dynamic light scattering; SAXS: small angle X-ray scattering; ITC: isothermal titration calorimetry; SDS-PAGE: sodium dodecyl polyacrylamide gel electrophoresis; HPLC: high performance liquid chromatography. B1, B2, B3: proanthocyanidin B1, B2, B3; PGG: pentagalloylglucose; TGG: tetragalloylglucose; PAC- proanthocyanidin; EgCG: epigallocatechin gallate; ECG: epigallocatechin; PRP: proline-rich protein

Table 4-3: Effects of pH on tannin-PRP solubility

Reference	Method	Tannin type	pH	Effect
[64]	Diffusion precipitation interaction assay	Hydrolyzable and condensed wine extracts, catechin, tartaric acid, gallic acid	3.5 vs. 7.0	↓pH ↑precipitation
[65]	Competitive binding assay	Quebracho and tannic acid	2.0 vs. 7.4	↓pH ↑precipitation
[55]	*In vitro* digestion precipitation interaction assay, HPLC, SDS-PAGE	EgCG	2.07, 7.8, 5.0-9.0	↓pH + ↑ tannin = enzyme inhibition, blunted by PRP
[62]	NMR	Galloyl rings from tannic acid	3.5 vs. 1.7	↓pH ↑precipitation
[66]	SDS PAGE	Wine or tannic acid	2.9, 3.0, 3.6	↓pH ↓solubility of tannin-PRP

NMR: Nuclear magnetic resonance imaging; SDS-PAGE: sodium dodecyl polyacrylamide gel electrophoresis; HPLC: high performance liquid chromatography. EgCG: epigallocatechin gallate; PRP: proline-rich protein

Table 4-4: Effect of tannin polymerization, galloylation, and hydroxylation on PRP affinity and precipitation

Reference	Method	Tannin type	Polymerization (1)	Galloylation (2)	Hydroxylation of the B ring (3)
[53]	ESI-MS	EgCG, ECG, B2, B2 3-O gallate	↑↑↑	↑↑	↑
[51]	NMR	B2, PGG, TGG, PA monomer	↑↑↑	↑↑	*nd*
[72]	nephelometry	B1-9, C1, B2 3-O gallate, E	↑↑↑	↑↑	*nd*
[52]	NMR	PAC as B1, B3, C2	↑↑↑	↑↑	↑
[70]	ESI-MS	B1, B2, B3, B4, C2, C, E, quercetin derivatives	↑↑↑	*nd*	↑↑
[59]	ESI-MS	EgCG, ECG, B2, B2 3-O gallate, reserpine	↑↑↑	*nd*	*nd*
[61]	ITC	GSE as catechin, epicatechin, epicatechin 3-O gallate	↑↑↑	*nd*	*nd*
[69]	Competitive binding assay	5GG, gallic acid, EGC	*nd*	↑↑↑	*nd*

NMR: Nuclear magnetic resonance imaging; ESI-MS: electrospray ionization mass spectrometry; ITC: isothermal titration calorimetry; B1, B2, B3, B4, C1: proanthocyanidin B1, B2, B3, B4, C1; PGG: pentagalloylglucose; TGG: tetragalloylglucose; PAC- proanthocyanidin; EgCG: epigallocatechin gallate; ECG: epigallocatechin; PRP: proline-rich protein

Table 4-5: PRP binding to hydrolyzable vs. condensed tannins

Reference	Method	Tannin type	Bond stability	Binding affinity
[69]	Competitive binding assay	5GG, gallic acid, EGC	*nd*	Hydrolyzable tannin > condensed tannin
[65]	Competitive binding assay	Quebracho and tannic acid	Hydrolyzable: 20% greater dissolution of bonds in gastric and enteric digestion	Hydrolyzable tannin > condensed tannin
[51]	NMR	B2, PGG, TGG, PAC monomer	Hydrogen bonds associated with hydrolyzable tannins	B2>PGG>TGG>
[50]	NMR, DLS	EgCG, EGC, PGG	Hydrogen bonds associated with hydrolyzable tannins	Hydrolyzable tannin > condensed tannin
[66]	SDS PAGE	Wine or tannic acid	Condensed tannins associated with hydrophobic bonds	*nd*
[64]	HPLC	Hydrolyzable vs. condensed wine extracts	*nd*	Increased precipitation of condensed tannin at pH 7.5 c/t hydrolyzable tannin

NMR: Nuclear magnetic resonance imaging; DLS: dynamic light scattering; SDS-PAGE: sodium dodecyl polyacrylamide gel electrophoresis; HPLC: high performance liquid chromatography. B2: proanthocyanidin B2; PGG: pentagalloylglucose; TGG: tetragalloylglucose; PAC-proanthocyanidin; EgCG: epigallocatechin gallate; ECG: epigallocatechin; PRP: proline-rich protein. *nd*= not done

Table 4-6: *In vivo* studies comparing PRP expression and non-heme iron availability with tannin ingestion

Study	Model	Intervention	Study conditions	Tannin concentration in challenge diet (g/kg)	Fe(diet)	n	Study duration	Measurement of bioavailability	PRP measurement
39	Sprague Dawley rats	Green tea	Control (acute and chronic)	28.6 g/kg green tea (nd)	20 mg/kg	6	7, 30 days	% absorption (^{59}Fe test meals), Hepatic non-heme iron	SDS-PAGE, MALDI-MS/MS,DIGE
			Acute gastric gavage			6	7 days		
			Chronic gastric gavage			6	30 days		
			Acute tea powder			6	7 days		
			Chronic tea powder			6	30 days		
40	Sprague Dawley rats	Black tea	Control	0.3 ± 0.0	35.9 ± 1.3	6	24 days	% absorption (^{59}Fe test meals), Hepatic non-heme iron	SDS-PAGE
			5% tea solids challenge tannin free diet	8.9 ± 0.5	35.9 ± 1.3	6	24 days		
			5% tea solids + 6% gelatin challenge tannin free diet	9.9 ± 0.7	35.9 ± 1.3	6	24 days		
			5% tea solids chronic diet	8.9 ± 0.5[a]	34.3 ± 1.5	6	24 days		
			5% tea solids + 6% gelatin chronic	9.9 ± 0.7[a]	34.6 ± 1.0	6	24 days		

a. Supplied as 25 g/kg in diet.

SDS-PAGE: sodium dodecyl polyacrylamide gel electrophoresis; MALDI-MS/MS: Matrix-assisted laser desorption/ionization/mass spectrometry; DIGE:difference gel electrophoresis

Table 4-7: Effect size of acute and chronic hepatic iron levels

Study	Treatment	Effect size (*d*)	Lower CI (95%)	Upper CI (95%)
[39]	Acute gavage	0.17	-0.96	1.31
	Chronic Gavage	-0.20	-1.33	0.94
	Acute Powder	-0.22	-1.36	0.91
	Chronic Powder	-0.40	-1.55	0.74
[40]	5% tea challenge	-0.34	-1.48	0.80
	5% tea chronic	2.87[a]	1.26	4.48
	5% tea + gelatin challenge	0.50	-0.64	1.65
	5% tea + gelatin chronic	2.39[a]	0.91	3.87
Total (CI % hepatic iron)		0.57	-0.64	1.84
Total (CI % chronic hepatic iron)		1.16	-0.18	2.51
Total (CI % acute hepatic iron)		0.03	-1.11	1.17

[a] $p < 0.05$ (95% CI)

Table 4-8: Effect size of acute and chronic % non-heme iron absorption

Study	Treatment	Effect size (*d*)	Lower CI (95%)	Upper CI (95%)
[39]	Acute gavage	0.79	-0.38	1.97
	Chronic Gavage	0.14	-0.99	1.27
	Acute Oral	1.37[a]	0.11	2.62
	Chronic Oral	-0.66	-1.81	0.51
[40]	5% tea challenge	-3.99[a]	-5.94	-2.03
	5% tea chronic	-1.62[a]	-2.92	-0.31
	5% tea + gelatin challenge	-4.11[a]	-6.1	-2.11
	5% tea + gelatin chronic	-2.29[a]	-3.74	-0.83
Total (CI % absorption)		-1.30	-2.73	0.13
Total (CI % absorption chronic consumers)		-1.11	-2.37	0.16
Total (CI % absorption acute consumers)		-1.61	-3.08	0.11

[a] $p < 0.05$ (95% CI)

Table 4-9: PRP production in groups relative to control

Study	Treatment	PRP production/control
[39]	Acute gavage	0.73
	Chronic Gavage	2.4
	Acute Oral	9.9
	Chronic Oral	4.1
[40]	5% tea chronic	1.22
	5% tea + gelatin challenge	1.10

PRP: salivary proline-rich protein

Figures

Figure 4-1: Condensed tannin Yikrazuul/ Wikimedia Commons / Public Domain and B: Tannic Acid Ronhjones/ Wikimedia Commons / Public Domain

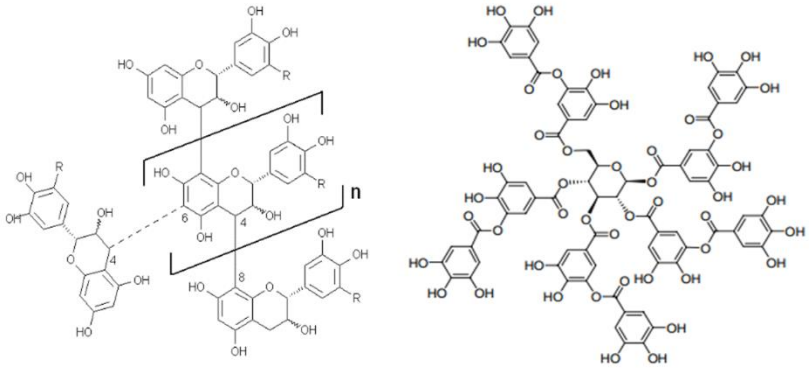


Figure 4-2**: Data analysis process for narrative review of tannin-non-heme iron and tannin-PRP interactions adapted from the Center for reviews and dissemination** [43]**.**

Figure 4-3: Inclusion and exclusion criteria for review. Articles were excluded related to key terms, non-comparative studies, and biological modeling dissimilarities to saliva. PRP: proline-rich proteins.

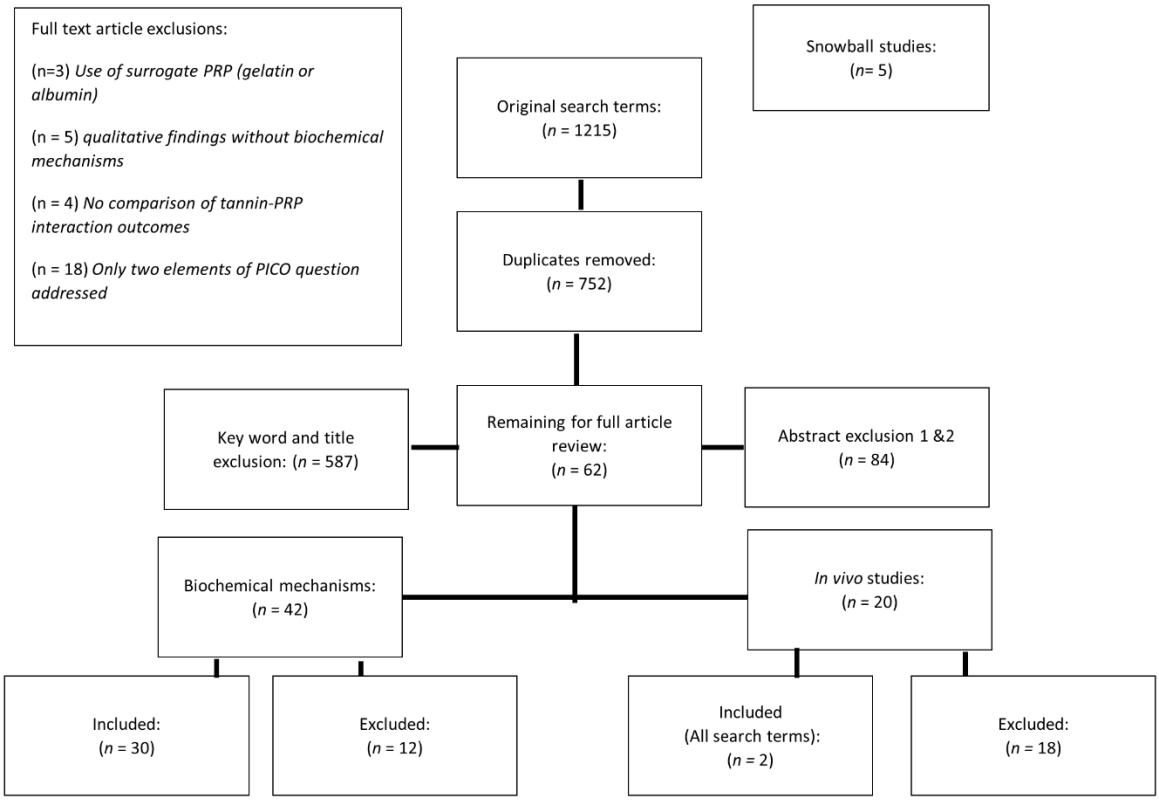
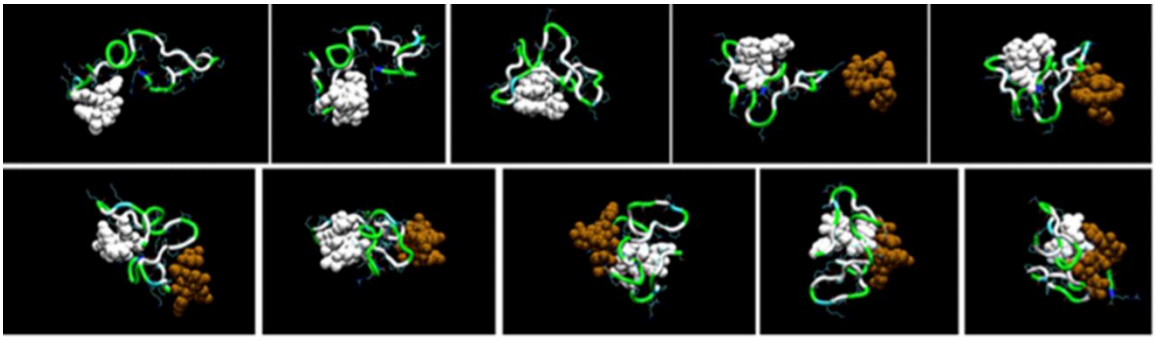


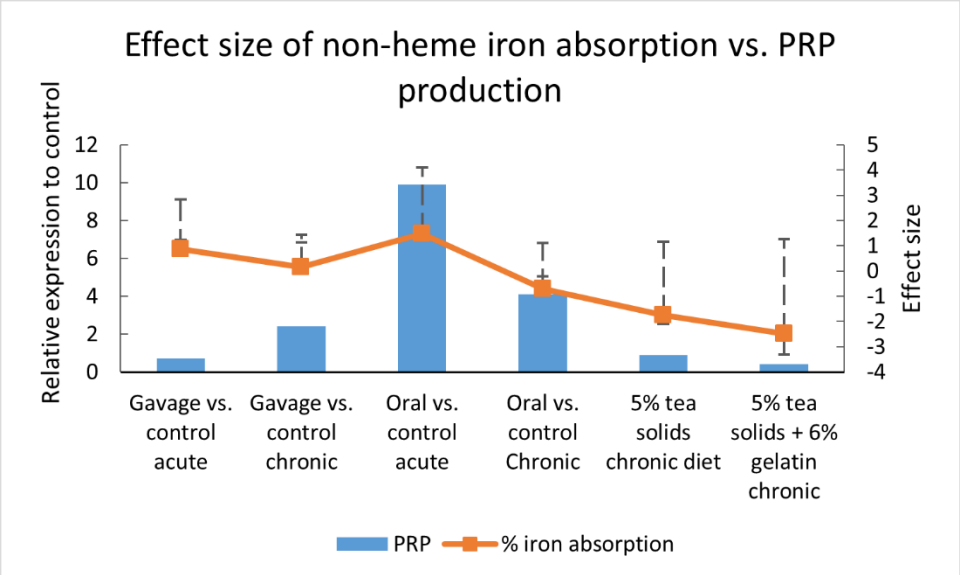
Figure 4-4: Open and flexible conformations of a basic PRP molecule with proline residues [49], with permission

Figure 4-5**: condensed tannin (white and brown) interaction with PRP peptide (green ribbon) during molecular dynamics run [52], with permission**



Tannins associate with PRP molecules and attach to proline-rich residues through hydrophobic bonds. On binding to PRPs, the multidendate nature of tannins allows for hydrophobic bond formation and conformational changes in the PRP molecule to stabilize the complex.

Figure 4-6: Relative PRP induction compared to non-heme iron absorption effect size.



PRP expression follows iron absorption with the exception of oral bypass of tannins in gastric gavage groups.

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Chapter 5 - LONG-TERM DOSE-RESPONSE CONDENSED TANNIN SUPPLEMENTATION DOES NOT AFFECT IRON STATUS OR BIOAVAILABILITY

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Abbreviations:

iAUC: incremental area under the curve

ANOVA: analysis of variance

aPRP: acidic salivary proline-rich protein

ASA24: automated system for 24-hour dietary recalls

BMI: body mass index

bPRP: basic proline-rich proteins

CI: Confidence interval

CRP: C-reactive protein

EDTA: ethylene diaminetetraacetic acid

gPRP: glycosylated salivary proline-rich protein

Hb: Hemoglobin

HPLC: High performance liquid chromatography

IDA: Iron deficiency anemia

KSU: Kanas State University

MANOVA: Multiple factor analysis of variance

PIV: peripheral indwelling IV catheter

PRP: salivary proline-rich proteins

RDA: Recommended Dietary Allowance

SST: serum separator tube

TFA: Trifluoroacetic acid

USDA: United States Department of Agriculture

Conflict of interest: The authors declare no conflicts of interest.

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Abstract

Background: Repeated consumption of phytic acid leads to iron absorption adaptation, but the influence of repeated condensed tannin consumption has not been well established. Salivary proline-rich proteins (PRPs) have been shown to protect iron absorption by precipitating tannins and reducing tannin-iron binding with repeated consumption in animal models. Upregulation of PRP synthesis may partially mediate the effects of tannin consumption on iron bioavailability.

Objective: The primary objectives of the current tannin dose-response trial were to determine the effect of long term dose-response condensed-tannin supplementation on iron bioavailability, and status, and to understand the effect of salivary proteins on iron bioavailability during prolonged condensed tannin consumption. A secondary objective was to assess astringency as a potential marker for adaptation to tannins and iron bioavailability.

Methods: Premenopausal women aged 18-35 yrs were enrolled in a double-blind three dose crossover trial (n = 11). During the 22-week study, one of three (1.5, 0.25, or 0.03 g) condensed proanthocyanidin tannin supplement doses was taken three times daily for four weeks in random order, with a two-week washout in-between. Dietary recalls were recorded for each supplementation period to assess dietary covariates for outcomes assessments. Before and after supplementation periods, meal challenges were employed to assess iron bioavailability, iron status, salivary PRP changes, and astringency perception.

Results: Tannin supplementation did not change iron bioavailability at any dose (incremental serum iron AUC: $\beta = 0.022$, $p = 0.83$; and % max iron absorption: $\beta = -0.023$, $p = 0.82$) from Week 0 to 4 in any dose. Iron status (hemoglobin: $\beta = 0.14$, $p = 0.126$ and serum ferritin: $\beta = -0.11$, $p = 0.83$) was unchanged by tannin dose from Week 0 to Week 4. There were significant positive correlations between iron bioavailability (by % max iron absorption), basic PRP ($r = 0.366$, $p = 0.003$), and cystatin production ($r = 0.27$, $p = 0.03$) with tannin supplementation. Astringency ratings did not change significantly within or between tannin doses (ps > 0.126), but there were significant reductions in astringency ratings with 1.5 g tannin supplementation at the lowest astringency testing concentration ($p < 0.047$), and there were negative relationships between bPRP (rs < -0.32, ps < 0.21), cystatin (rs < -0.2, ps < 0.28) salivary protein production and astringency ratings.

Conclusions: Condensed-tannin consumption did not affect iron bioavailability or status regardless of supplementation period in premenopausal women. Correlation analyses suggest that basic PRPs, and cystatins are associated with improved iron bioavailability, and that lower ratings of astringency may predict improved iron absorption with repeated tannin consumption.

Keywords: *tannins, iron bioavailability, salivary proline-rich proteins, adaptation, antinutritional factors, proanthocyanidins, iron deficiency anemia*

Background

An estimated one billion people have iron deficiency anemia (IDA) worldwide [1]. Most commonly, iron deficiency is found in women, children, vegetarians, and in people who have insufficient iron intake [1]. Despite multiple initiatives aimed at reducing IDA in the past 20 years, an estimated 29% of non-pregnant women were anemic in 2011, constituting a 4% reduction since 1995 [2].

Tannins have been found to negatively impact iron bioavailability [3-8] by formation of insoluble antinutritional-mineral complexes [9], and this has deterred tannin-rich foods, such as sorghum, from being used for food-aid in regions that are largely undernourished[10]. Previous research suggests that long-term tannin consumption may not inhibit iron bioavailability as much as acute studies would estimate [11,12]. For example, long term antinutritional factor consumption in animals [13-15] and humans [11,16] has resulted in improved non-heme iron bioavailability compared to single-meal studies. In these studies, the negative effects (reduced iron status or bioavailability) of antinutritional factor intake over time has not been sustained, these findings have been proposed to be due to adaptation. In studies that have found reductions in iron bioavailability with tannin consumption, individual iron absorption has been highly variable [17,18], and many individuals consuming diets with large concentrations of tannins maintain normal iron stores [19].

Many studies finding reduced iron bioavailability with tannin consumption have used hydrolyzable tannic acid, which is not commonly consumed, or tea tannins, which may be metabolized differently than condensed tannins, which are commonly found in food [20]. To the best of our knowledge, no studies have determined the long-term effects of tannin consumption

apart from other antinutritional factors such as fiber or phytates on iron bioavailability or status. In addition, it has not been determined whether long-term condensed tannin results in adaptation, or what mechanisms underlie adaptation if it does occur.

Mechanistically, adaptation to tannins may start in the mouth [21]. Saliva contains six main classes of salivary proteins: histatins, cystatins, statherins, acidic proline-rich proteins (aPRP), basic proline-rich proteins (bPRP), and glycosylated proline-rich proteins (gPRP) [22] that may exert independent effects on tannins. Proline-rich proteins (PRPs) in particular have been noted in sensory studies for their ability to precipitate condensed tannins, which results in an astringent oral sensation [21,22]. Tannin-PRP complexes are insoluble within the GI tract [23,24], preventing tannin-iron chelation throughout digestion. There are a wide variety of PRP subtypes that make up different salivary profiles, which may be largely genetically determined [25,26]. Genetic determination of salivary profiles favoring effective tannin precipitation may explain why some individuals have greater capacity to consume tannins than others without negative impacts on iron status. Upregulation of PRP secretion when consuming tannins has been shown to improve protein [27] and iron bioavailability in animal studies [14,28], and animals that do not upregulate PRP synthesis in response to tannin consumption have poor growth outcomes [29].

Previously, PRP-tannin binding has been identified in sensory studies for creation of astringent taste sensation during interaction [22]. Theoretically, identification of PRP "adapters" may then be possible through simple, inexpensive astringency testing [22]. Further, changes in astringency sensation may indicate an upregulation of PRP production over time with repeated tannin consumption [30].

The primary objectives of the current tannin dose-response trial were to determine the effect of long term dose-response condensed-tannin supplementation on iron bioavailability, and status, and to understand the effect of salivary proteins on iron bioavailability during prolonged condensed tannin consumption. A secondary objective was to assess astringency as a potential marker for adaptation to tannins and iron bioavailability.

In this study, we hypothesized that 1) Condensed-tannin supplementation would not change iron bioavailability (determined by iron absorption) or status (determined by hemoglobin and ferritin) regardless of dose over four weeks, 2) Salivary proline-rich protein production would not be induced by tannin consumption over time and by higher versus lower tannin doses, and 3) Proline-rich protein production would be positively associated with improved iron bioavailability after tannin consumption. Secondary hypotheses were that 1) Astringency perception would be changed with tannin consumption over time, 2) Salivary proline-rich protein production would predict astringency with tannin consumption, and 3) Astringency could be used as a surrogate marker for iron bioavailability with tannin consumption.

Methods

Inclusion/exclusion

The study protocol was approved by the Institutional Review Board at Kansas State University (KSU; IRB #8121). Due to the length of the study, enrollment was rolling, and is outlined (**Figure 5-1**). An announcement requesting participants was sent to faculty and students through a University email digest, as well as disseminated though departmental social media channels. Total, 48 women responded, and potential participants were screened in person or via phone (Figure 5-1). Before screening, participants were required to read and sign an informed

consent document, and all procedures, risks and benefits of the study were reviewed verbally. During screening, participants were asked to complete a medical history questionnaire. Premenopausal women, aged 18-35, who were non-obese (body mass index, BMI ≤ 30.0 kg/m^2), had no history of oral or gastrointestinal disease, were moderate (≤ 1 drink per day) or non-alcohol consumers, and non-tobacco users we eligible for participation. Further exclusion criteria included blood disorders affecting iron status or absorption, current supplementation or medication that would impair iron status, food allergies to supplements, pregnancy or breastfeeding (see Appendix D for screening questionnaire and exclusion criteria). Participants were compensated for completing study activities.

Study Design

Blinding and randomization

Participants were assigned participant ID numbers, and a researcher not involved in data collection randomized each participant number to a tannin supplement order (SAS, Carey, NC). Participants, the principle investigator, and the project coordinator were blinded to dose order.

Supplementation periods

Supplementation periods consisted of Week 0 and Week 4 meal challenges, with four weeks of tannin supplementation in between (**Figure 5-2**). Each participant consumed a condensed-tannin (Nusci grape seed extract, 95% condensed proanthocyanidins) supplement, provided in an opaque bottle with a non-caloric flavor enhancer and sweetener (Mio Original) to improve its palatability, three times daily for four weeks. High (1.5 g), medium (0.25 g), or low (0.03 g) condensed-tannin doses were provided for consumption with three daily meals. These doses represented 100g of high tannin red sorghum, 1 cups of tea, or a meal with one serving of

fruit or vegetable, respectively, three times daily. Each Week 4 meal challenge was followed with a two-week washout period to allow normalization of iron absorption and ferritin values to the participant's usual diet [31]. Two participants had a single washout period of three, instead of two weeks due to availability for meal challenges.

Tannin meal challenges

At Week 0 and Week 4 of each supplementation period, participants completed meal challenges at the KSU Physical Activity and Nutrition Clinical Research Consortium (PAN-CRC). Participants were asked to come in fasted (at least 8 hours) at 7:00 am, having abstained from teeth cleaning (2 hours) and exercise (24 hours) to minimize diurnal variations or other confounding factors in salivary production [32] and iron uptake [33] (**Figure 5-2b)**. Pre-meal saliva was collected by passive drool (2 ml total) in cryovials, and samples were immediately stored in a freezer set at -71^0 C. A 20-gauge indwelling peripheral IV (PIV) catheter was placed either in the median cubital, cephalic, or basilic vein for multiple blood samples, which was flushed and saline locked with 10 ml of 0.9% isotonic saline between blood collections. From the fasting blood draw, two separate samples were collected in 5 mL serum separator (SST) and 3 mL ethylenediaminetetraacetic acid (EDTA) vacutainer tubes to measure serum iron (by spectrophotometry), CRP (by nephelometry), ferritin (by immunoassay), and whole blood hemoglobin concentrations (by electronic cell cytometry). After fasted blood collection, a challenge meal of a 95 g bagel with 12 g sugar-free strawberry jam, sprinkled with 15 mg ferrous sulfate and 75 mg ascorbic acid [11,34] supplements separately, and a 90 g banana, was consumed with the participant's assigned dose (1.5 g, 0.25 g, or 0.03 g). Salivary samples were collected 15 minutes following the final bite of the meal to determine salivary protein stimulation after tannin

consumption [35]. Subsequent blood samples were collected in SST tubes and analyzed for serum iron at 180 and 240 minutes. After collection, serum samples were centrifuged at 2500 RPM for 15 minutes after clotting for 20 minutes, and kept at room temperature for analysis. All blood samples were analyzed by a certified laboratory (Quest Diagnostics, Lenexa, KS) within 24 hours.

Regression analysis of three-point time draw for % maximum iron absorption

To minimize blood loss, validity of a three-time blood draws to determine iron bioavailability by serum iron was determined. In the first supplementation period of the study, four participants had blood drawn every thirty minutes for four hours to establish correlation with a three-time point draw (0, 180, and 210 minutes) system previously proposed [34]. Regression of polynomial lines from data points were calculated on a computer data system (Microsoft Excel, 2013), and R^2 values were calculated for goodness-of-fit. From these data, it was verified that time points proposed were representative of the full models previously used [11,34]. Correlations of 0.99+ were seen, thus blood samples were also drawn for serum iron at these three time points to determine % max iron absorption and incremental serum iron area under the curve (iAUC) [34].

Incremental AUC and maximum percentage of iron absorption calculation

Serum iron data were used to calculate percentage of maximum iron recovery and iAUC for iron bioavailability analysis.

Percentage Iron Recovery: Percentage iron recovery was calculated as below [34].

$$\%\ iron\ recovery\ \max = (\frac{serum\ iron\ \max \times plasma\ volume)}{total\ iron\ ingested} \times 100$$

Where: $iron\ \max = \frac{\mu mol}{L}, plasma\ volume = L, and\ amount\ of\ iron\ is\ in\ \mu mol$

$$plasma\ volume\ (L) = \frac{Blood\ volume\ (ml) \times \left(1 - Packed\ cell\ volume\ (decimal)\right)}{1000}$$

And $blood\ volume = 69.6 \frac{ml}{kg} body\ weight$

iAUC by trapezoidal integration

$$\int AUC = \frac{\Delta\ time}{2} \times [\Delta Serum\ iron_{\ time\ 0-180} + (2 \times \Delta Serum\ iron_{time\ 0-210})]$$

Astringency testing

After the PIV was removed following each meal challenge, participants were asked to complete an astringency test [36,37]. Each participant was given four different concentrations of alum powder in 10 ml distilled water (0.20, 0.15, 0.07, and 0.03%) in random order to sip. They were first given a verbal description of the sensation of astringency and were asked to rate each solution based on their perception of astringency on a 5 point Likert scale (1 = not astringent, 5 = extremely astringent). Participants waited for 30 seconds before testing the next sample.

Dietary analysis in supplementation periods

Within each supplementation period, 24-hour dietary recalls were collected on three different days (two weekdays and one weekend day) [38]. At the beginning of week two of each four-week supplementation period, participants were emailed a unique username and password to complete 24-hour dietary recalls for 2 weekdays and 1 weekend day on the Automated self-administered 24-hour recall (ASA24®). After all recalls were collected in each supplementation period, dietary data were extracted, and total caloric intake (kcal), protein (g), fat (g), carbohydrate (g), iron (mg), ascorbic acid (mg), meat protein (oz.), sugar (g), fiber (g), Zn (mg), and Cu (mg) content were averaged from system calculated amounts for each participant. Food intake logs were downloaded from the ASA24® for manual calculation of proanthocyanidins and polyphenols. During this process, a research assistant reviewed all dietary data for each participant using an electronic spreadsheet (Microsoft Excel). Food items were referenced from United States Department of Agriculture (USDA) tables pulled into an electronic spreadsheet, and total proanthocyanidin (condensed tannin) [39] and polyphenol [40] amounts were calculated and summated for each recall. From these summations, averages were calculated. All assessments and calculations were reviewed by the project coordinator before analyses were completed.

Salivary PRP measurement:

Acidified saliva sample preparation

Frozen salivary samples were thawed overnight in a refrigerator. Before sample analysis, consistency in chromatogram output with duplicate samples was verified, and samples were analyzed in a single run. For PRP extraction, 900 µl of saliva was mixed with 10 µl of 10% trifluoroacetic acid (TFA) in water, centrifuged for 5 min at 8000 RPM, and the supernatant was filtered through a 0.2 µm PVDF syringe filter as described previously [41]. Before samples were analyzed, it was verified that there was no PRP peak loss with use of syringe filters by testing in trial HPLC runs. The supernatant was then analyzed by high performance liquid chromatography (HPLC).

HPLC parameters and equipment

All reagents were analytical grade. Acetonitrile, TFA, and HPLC grade water were purchased from Fisher Scientific. Ninety µl of salivary supernatant was injected into a Fisher 2.1x150 mm, 5µm BioBasic C8 analytical column at a flow rate of 0.3 ml/min for 49 minutes at 40˚C with an autosampler (Shimadzu SIL) on a HPLC system containing a LC20AB pump (Shimadzu), and a Shimadzu SPD-M20A PDA system. Detection of PRPs was carried out at 214 nm [41,42]. Mobile phase consisted of 0.2% TFA in HPLC grade water (A) and 0.2% TFA in 80/20 acetonitrile and HPLC grade water (B) [32,41-43]. A linear gradient was applied from 0-39 minutes from 0-54% (B), then from 39-49 minutes at 54-100% B to elute late proteins [32,41-43]. After each run, the column was washed and stabilized with initial conditions by increasing linear gradient back to 100% A over 10 minutes.

Statistical analyses

Data were analyzed using SAS statistical software (SAS Studio version 3.6, Cary, North Carolina), statistical significance was set at $p < 0.05$. All data are presented as mean ± standard deviation (SD). Before analysis, all data were analyzed for normality and homogeneity of data in Q-Q plots and with Levene's tests. Variables that were non-normal (proanthocyanidin monomers, dimers, total proanthocyanidin, ascorbic acid, sugar, and iron intake) were log transformed, and determined to be normal before further analysis. Log-transformed variables were included in stepwise variable selection in adjusted model building (below). All log-transformed data were back-transformed for results presentation.

Sample size

A sample size calculation determined that four participants would be needed to detect a change in iAUC of 25% [11,34] (SAS studio version 3.6, Cary, NC) as statistically significant with 80% power and at an α-level of 0.05.

Demographic data, washout, and randomization order analysis

Week 0 demographic and nutritional intake data were analyzed by analysis of variance (ANOVA) by supplementation period. Randomization order and previous dose effect were analyzed by Chi-square testing to assess for bias in supplementation period order, or previous effect of supplementation period. Changes between previous and next supplementation period during washout were analyzed for hemoglobin, ferritin, iAUC for serum iron, and % max iron absorption in ANOVA analysis.

Hematological outcomes analysis

Regression analysis of hematological outcomes

Linear regression of raw outcomes data was used to determine whether four weeks of multiple daily tannin supplementation would change iron absorption or status within nor between supplementation periods. In regression analysis, differences between supplementation periods were analyzed for % max iron absorption, ferritin, and hemoglobin at Weeks 0 and 4 (to analyze for within dose responses). Multiple regression was used to adjust models for repeated (participant) and random (ferritin, CRP, dietary intake, weight, and age) covariates after stepwise selection for variables. To maximize analysis of individual iron bioavailability and status within different supplementation periods, individual movements (increase, decrease, or maintain) in dose-responses (hemoglobin, ferritin, % max iron absorption, and serum iron iAUC) were analyzed with Chi-square testing and Fisher's exact tests.

PRP and astringency outcomes analysis

PRP changes with tannin supplementation and correlations with iron bioavailability

To determine whether salivary PRP production would be inducible by tannin consumption both over time and in a dose-dependent manner, salivary proteins were divided into type by retention times [32,41-43], peak mAu were recorded for each, and protein subtypes were aggregated to quantify total salivary proteins and PRPs. Salivary protein subtypes were further analyzed by proportion to total mAu from the equation:

$$PRP\ subtype\ proportion = PRP\ type\ (area\ sum) \div Total\ PRP\ area$$

Or

$$PRP\text{: } Acidic\ salivary\ proteins = Total\ PRP\ area\ \div Total\ salivary\ protein\ area$$

Differences in salivary protein production from Week 0 to 4 within doses were analyzed by MANOVA. To determine whether PRP production would impact iron bioavailability with tannin consumption, Pearson's product-moment correlations were used to determine correlations between % max iron absorption, iAUC for serum iron, randomization order, and PRP types.

Astringency perception, connections to salivary protein production, and iron bioavailability

We determined whether astringency perception was changed within or between tannin doses using Chi-square testing and Fisher's exact tests by allocated, and previous dose. Connections between salivary protein production, iron bioavailability and astringency were analyzed by Pearson's product-moment correlations.

Results

Week 0 demographics

Mean participant age was 26 ± 1.2 yrs, and ranged from 20-35. All participants were occasional (2-3 drinks/month) or moderate (2-3 drinks/week) alcohol consumers. Aside from one participant, who consumed a vegan diet, and took vitamin B12 supplements, no participants took vitamin or mineral supplements during the study period. The average BMI of participants was 24 ± 2.4 kg/m^2 (range 18.2-28.9). Participant weights (kg) did not significantly change between tannin doses nor from Week 0 to Week 4 of each supplementation period.

Supplementation order and outcomes measures

With our randomization procedure, six of the eleven participants were randomized to 1.5 g tannin doses during the first supplementation period, and low doses during the second supplementation period. Incremental AUC for serum iron (p = 0.118), hemoglobin (p = 0.87), and ferritin (p = 0.15) were not different by order of tannin dose in any supplementation period. Supplementation order did significantly positively impact % max iron absorption following the 1.5 g tannin dose when taken in the third versus the first supplementation order (p = 0.046), meaning that lower doses taken before the 1.5 g dose led to significantly improved iron bioavailability. There were no significant differences in Week 0 to Week 4 dose-responses for hemoglobin, ferritin, serum iron iAUC, or % max iron absorption when accounting for previous dose by Chi-square testing (results shown in Appendix E).

There were no significant changes in hemoglobin (p = 0.993), ferritin (p = 0.982), iAUC for serum iron (p = 0.984), or % max iron absorption (p = 0.998) at each Week 0-time point, and

previous tannin dose did not affect outcomes changes during washout for hemoglobin (p = 0.68), ferritin (p = 0.511), % max iron absorption (p = 0.735), or iAUC for serum iron (p = 0.137). No salivary protein measurements were significantly correlated with tannin dose order (ps > 0.62).

Study dietary intake

There was wide variability in nutrient consumption during supplementation periods, but no significant differences in total calorie, macronutrient, meat, fiber, or micronutrient consumption between tannin doses (**Table 5-1**). Despite wide variability in nutrient consumption, individual macronutrient and micronutrient intake were not different between tannin doses. Iron intake was 7-18% less than the recommended dietary allowance (RDA) of 18 mg in all supplementation periods, ascorbic acid exceeded the RDA by 15-80%. While not significant, proanthocyanidin intake trended towards lower amounts in the 0.03 g (69.1 ± 78.9 mg) and 0.25 g (82.3 ± 85.1 mg) doses compared to the 1.5 g dose (123.2 ± 136.6 mg; ps > 0.09). On average, 0.03 g, 0.25 g, and 1.5 g tannin supplements constituted 2, 8, and 35-fold the typical proanthocyanidin intake for their respective supplementation period.

Supplementation period iron absorption, hematological indices of iron status

Unadjusted regression outcomes

Individual level data are included in Appendix F. There were no changes in unadjusted iron bioavailability (by iAUC and % max iron absorption) within or between tannin supplementation periods (**Table 5-2**). In addition, there were no differences at Week 0 (p = 0.82) or Week 4 (p = 0.92) for unadjusted serum iron iAUC or % max iron absorption at Week 0 (p = 0.82) or Week 4 (p = 0.62, Table 5-2) between tannin doses.

Hemoglobin and ferritin values were not different at Week 0 or Week 4 for any tannin dose (Hb: p = 0.838, Week 0; 0.68, Week 4 and ferritin: p = 0.855, Week 0; 0.575, Week 4, Table 2). There were no significant differences in hemoglobin (p = 0.90), ferritin (p = 0.81), % max iron absorption (p = 0.39), or serum iron iAUC (p = 1.0) for improvement, deterioration, or maintenance by any tannin dose through Chi-square testing (**Table 5-3**).

Stepwise linear regression analysis and adjusted regression models

To test the impact of dietary and individual physiological differences (iron status, anthropometric, salivary protein) on iron bioavailability and status, we employed stepwise regression analysis to establish significant covariates to build an adjusted model for hematological outcomes. Covariates that were significantly positively associated with serum iron iAUC and % max iron absorption included bPRP and cystatin production (**Table 5-4**). Significant covariates that were negatively associated with serum iron iAUC and % max iron absorption included aPRP and total salivary protein production, higher rating of 0.2 mg/dL astringency testing, and total meat consumption. Significant covariates associated with ferritin levels included bPRP production, and zinc consumption.

Significant covariates for each outcome measure were added to the linear regression for adjusted outcomes analysis. Following the full adjustment for significant covariates, serum iron iAUC nor % max iron absorption were statistically different between or within each tannin dose (Table 5-4, **Figures 5-3, 5-4**). There were not significant differences in adjusted hemoglobin or ferritin values within or between tannin supplementation periods (Table 5-4).

Correlations between salivary protein production and iron absorption with tannin supplementation

There were no significant correlations between total salivary protein production and iron absorption (by % max iron absorption and iAUC for serum iron) during the study. In all tannin-doses, and when combining all data from 4-week supplementation periods, bPRP production was significantly and positively correlated with % max iron absorption at Week 0 and Week 4 (**Table 5-5**). There were more positive correlations with Week 4 0.03 and 0.25 g dose-max iron absorption % and bPRP production than 1.5 g challenge (Table 5-5), suggesting that bPRP production was potentially important for lower, but not higher doses of tannins. Week 0 and Week 4 aPRP production was significantly negatively correlated with iron absorption in each supplementation period (Table 5-5). Total gPRP production was significantly negatively correlated with iron bioavailability at Week 4 in the 1.5 g supplementation period (Table 5-5). Statherin production was non-correlated with iron absorption, while cystatin was overall positively significantly correlated with iron absorption (Table 5-5).

Astringency testing

Astringency ratings with tannin consumption

Astringency ratings did not change among participants with changes in tannin doses, and were not statistically affected by order of tannin dose in Chi-square testing (ps > 0.09), except for the lowest astringency doses, which were rated significantly lower after 1.5 g tannin doses (p = 0.047; **Table 5-6**). There were no significant effects from tannin dose on changes in ratings of astringency (ps > 0.126), however, overall ratings of astringency were lower due to 1.5 g tannin dose compared to 0.03 g tannin dose for 0.07 and 0.15 mg/dL astringency doses (p = 0.0655 and 0.013, respectively).

Astringency ratings with PRP production

Cystatin and bPRP production were correlated with lower sensation of astringency in all astringency doses. In correlations from individual participants with astringency ratings at the highest alum concentration (0.2 mg/dL) there were significant and positive correlations between total salivary proteins (7 of 11 participants, r > 0.49, p < 0.05) and astringency, and negative relationships between bPRPs (9 of11 participants, r = -0.32 to -0.81, p = 0.001 – 0.21), cystatins (9 of 11 participants, r = -0.2 to-0.76, p = 0.03 – 0.28) and astringency.

Discussion

The primary objectives of this trial were to determine the effect of long term dose-response condensed-tannin supplementation on iron bioavailability, and status, and to understand the effect of salivary proteins on iron bioavailability during prolonged condensed tannin consumption. Secondarily, the study assessed astringency as a potential marker for adaptation to tannins and iron bioavailability.

Hematological outcomes and tannin supplementation periods

Overall, our results support the hypotheses of no significant reductions in iron bioavailability or status with three supplementation periods of long-term, multiple-daily tannin supplements over four weeks. Despite non-significant negative trends in Week 0 iron absorption with 1.5 g (highest) compared to 0.25 and 0.03 mg (lowest) doses, hemoglobin and ferritin were maintained in all groups throughout supplementation periods (Table 5-2). There were no differences in ferritin or CRP measurements within individuals, or among tannin doses throughout the study (Table 5-2). To our knowledge, this is the first study that has quantified effects of long-term, dose-response condensed tannin effects on iron bioavailability and status.

Our findings of no significant changes in iron bioavailability or status within or among tannin doses are contrary to previous single meal studies using black tea [6,44,45], which contain theaflavins and thearubigins [46] or tannic acid [3,4,45]. Tannic acid and tea may bind to salivary proteins and chelate iron differently than condensed tannins (proanthycyanidins), which are typically larger in size and consumed within a complex food matrix [47,48]. Condensed tannin models in humans and rats [49-52], also found no changes in iron bioavailability or status with tannin consumption over time are consistent. In contrast, dose-dependent inhibition of grape seed extract on iron bioavailability has been cited in *Caco-2* cells [53]. There have been similar discrepancies in *in vivo* and *in vitro* models cited previously. Iron status in pigs consuming red and white beans with different tannin levels resulted in no difference in iron status outcomes, while the *Caco-2* cell model found higher iron bioavailability from white than red beans [54]. Inconsistencies between long-term *in vivo* and *in vitro studies* noted may be partially a result of the complexity of factors contributing to human and animal digestion, including salivary

proteins, which may not be accounted for in simulated digestion. In addition, the acute nature of *Caco-2* cells might have the same limitations as short-term bioavailability studies. The discrepancies between human, and C*aco-2* findings may suggest that caution needs to be exercised when using the *Caco-2* model to predict chronic consumption *in vivo* iron outcomes.

The current study is the first that we know of that has quantified the effects of multiple daily, multi-dose condensed proanthocyanidins on iron bioavailability or status. While supplementation levels at 1.5 g were 50 x greater than 0.03 g and 10 x greater than 0.25 g tannin doses, iron absorption was only modestly reduced in the 1.5 g supplementation period (equivalent to consuming 100g of high tannin sorghum three times daily), and there were no significant reductions in iron status over time. There were no changes in iron absorption, ferritin, or hemoglobin over time in any adjusted or unadjusted models, suggesting that condensed tannin intake at any dose did not affect iron absorption. While other studies have noted reduction in iron bioavailability with condensed tannin intake [4,55], the current study is the first that we know of to isolate supplementation of proanthocyanidins outside of other antinutritional factors commonly consumed concurrently *in vivo*.

PRP production and iron bioavailability

The current study is the first study, to the best of our knowledge, that has assessed correlations between salivary protein production and iron bioavailability, or measured long-term tannin supplementation effects on salivary profiles in humans. Overall, our hypotheses that salivary PRP production would be inducible in higher compared to lower tannin doses, and that PRP production would impact iron bioavailability with tannin consumption were partially supported. There were not significant changes in PRP or salivary protein production within or

among tannin doses, however, there were significant correlations between PRPs, non-PRP salivary proteins, and iron bioavailability suggesting that participants producing higher quantities of total salivary proteins, bPRPs, and cystatins had improved iron absorption with condensed tannin intake. Correlations between bPRPs, cystatins, and iron absorption tended to be stronger at Week 4 in lower doses, suggesting that salivary protein subtypes may change with regular tannin consumption to improve iron bioavailability, but are not likely the only physiological adaptation when consuming higher tannin doses. In *caco-2* cells, bPRPs have been found to inhibit uptake of small tannin molecules through formation of insoluble complexes, but this process was mediated in part by sodium-glucose transporter-1 (SGLT-1) and multidrug resistance protein (MRP2) [56]. It may be that bPRPs signal changes in these receptors that mediate tannin absorption and iron related sequelae.

Binding of bPRP to polyphenols may be preferential versus other PRP subtypes [57], and production of larger bPRPs that would efficiently bind to tannins tends to be genetically determined [57-59]. This idea may help to explain the wide variability in iron absorption among participants, and could help to explain age-related changes in iron absorption with tannin consumption. In pre-term infants, salivary protein profiles vary widely from adults [43], and bPRPs are almost non-existent.

Contrary to findings that bPRP supported iron bioavailability with tannin consumption, gPRP and aPRP production, especially at Week 0 for each tannin dose, were significantly negatively correlated with iron bioavailability, possibly meaning that individuals producing higher levels of these proteins less efficiently absorb iron until other homeostatic protective mechanisms are employed. This is the first time, to the best of our knowledge, that aPRP and

gPRP interactions with tannins over time have been determined *in vivo.* Reasons for poorer iron bioavailability with increased aPRPs may be due to their predilection to bind calcium [60], which is also a divalent cation. It may be that these aPRP and gPRP are upregulated with tannin consumption, but do not bind to condensed tannins effectively, thereby increasing protein-iron chelation. Further, aPRP and gPRP may be effectively inhibited by carbohydrate consumption [61] compared to bPRP, meaning that individuals producing higher quantities of these proteins may less effectively prevent tannin-iron chelation.

Astringency as a predictor of iron bioavailability with tannin consumption

Our secondary hypotheses that: 1) Astringency perception would be changed with tannin consumption over time, 2) Salivary proline-rich protein production would predict astringency, and 3) Astringency could be used as a surrogate marker for iron bioavailability, based on PRP expression with consumption were also partially supported by our findings. Astringency ratings did not change within or among tannin doses throughout the study, except for the highest (1.5g) tannin dose. Astringency ratings were lower with higher tannin concentrations, and were also significantly negatively correlated with bPRP and cystatin production, suggesting that reductions in ratings of very astringent or bitter foods may help predict iron bioavailability with tannin exposure. Despite this, we did not find consistent associations between iron bioavailability and astringency ratings within or between supplementation periods.

Limitations

There are several important limitations that must be considered when interpreting results from the current study. It must be acknowledged that tannin-supplementation limits the generalizability of these findings to food tannins, which commonly co-exist with other antinutritional factors, such as phytic acid. This limitation constrains our findings to tannin-rich food commodities, which may confer different effects with antinutritional-food matrix interactions. In addition, the population assessed in this study consisted of non-anemic, pre-menopausal adult women with a sufficient and varied diet. It may be problematic to generalize these findings to a clinical population, for example, anemic women and children, who may have a different response to tannin exposure.

Participants noted that they experienced increased salivary flow rates during 1.5 g compared to 0.3 tannin doses, although flow rates were not measured quantitatively. It is also important to realize that while concentrations of PRPs themselves did not change through the study, subjective experiences of salivary flow rates among participants was greater at Week 4 in higher, 0.25 and 1.5 g, supplementation periods than at Week 0. Previous research findings have indicated that salivary flow along with PRP concentration have given more accurate estimates of total production than concentration alone [62]. Salivary flow rate has been found to be an independent factor in reducing ratings of astringency along with salivary protein concentration [63] suggesting that in our study, total PRP production may have been increased with increasing salivary flow rates. Lack of measurement of salivary flow rate is a limitation in PRP-iron outcomes analysis because we were not able to assimilate total PRP quantification from a pre-determined 2 ml salivary sample (which was obtained over varying time spans). Lastly, we

grouped salivary types based on elution times. This has been previously employed [41], but is not an accurate representation of salivary protein quantification.

Future directions

Foremost, better characterization of proanthocyanidin-phytic acid interactions on iron bioavailability and salivary protein production needs to be explored, including the effects of mixed antinutritional factor outcomes regarding iron bioavailability over time. Mixed diets have conferred different findings in the past [64] than those presented in the current research study, and understanding nutrient interactions may be key to understanding these discrepancies. In addition, effects of tannins in anemic populations, who may have disease burden or dietary deficiencies, need to be explored.

Due to the complexity of PRP subtypes, determining which specific bPRP and cystatins improve iron bioavailability with tannin challenge may enable diet-specification in both children and adults [57]. Determination of PRP genetic makeup in anemic and non-anemic tannin consumers may help to determine those with tannin-binding subtypes, and protein production could later be determined based on findings. More studies are needed to determine the effects of tannin supplementation on iron bioavailability in infants, and the effects of different tannin types (tannic acid, theaflavins and thearubigins) on salivary proteins. Further comparison of oral and enteric tannin exposure may help to determine non-salivary determinants of physiological tannin mediation.

Conclusions

Condensed tannins did not impair iron bioavailability, ferritin, or hemoglobin levels in non-anemic, premenopausal women after long-term supplementation. Iron absorption following tannin supplementation was positively correlated with bPRP and cystatin production, and tannin supplementation was associated with significantly reduced ratings of astringency over time. These findings suggest that individual physiology may need to be accounted for when considering nutritional impact on iron bioavailability and status. Given the lack of impact of tannins on iron status over time, these results suggest that further consideration may be justified to efforts to remove tannins from the diet, considering their potential health benefits.

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Author Contributions

ND conceived and conducted experiments, analyzed data, and wrote the manuscript. NF randomized participants to supplementation periods, prepared supplements throughout the study, and edited the manuscript. KK analyzed salivary proteins on HPLC and edited the manuscript. SR conceived the experiment and edited the manuscript, MH conceived and edited the manuscript. BL conceived and oversaw the experiment, analyzed data, and edited the manuscript.

Tables

Table 5-1: Dietary intake of calories, macronutrients, micronutrients, and proanthocyanidins by supplementation period

Supplementation period	0.03 g	0.25 g	1.5 g
	Mean ± SD	Mean ± SD	Mean ± SD
N	11	11	11
Kcal/day	2186.2 ± 570.9	2230.5 ± 640.6	1957.8 ± 348
Protein (g/day)	80.8 ± 27.2	79.7 ± 21.6	71.6 ± 16.6
Fat (g/day)	90.3 ± 30	93.5 ± 27.9	71.1 ± 19.5
Carbohydrates (g/day)	259.3 ± 96.2	268.4 ± 109.9	252.1 ± 126.2
Meat (oz/day)	3.61 ± 2.5	3.81 ± 2.6	3.6 ± 1.9
Sugar (g/day)	122.8 ± 57.9	127.7 ± 69.3	120.0 ± 99.8
Fiber (g/day)	21.2 ± 12	19.2 ± 10	21.6 ± 14.3
Iron (mg/day)	15.1 ± 6.6	15.7 ± 7.2	14.7 ± 6.2
Ascorbic acid (mg/day)	109.7 ± 87.9	80.9 ± 66.9	110.4 ± 142.8
Zinc (mg/day)	12.6 ± 5	12.8 ± 5.6	10.2 ± 2.5
Copper (mg/day)	1.5 ± 0.69	1.5 ± 0.91	1.4 ± 0.61
Monomers (mg/day)	8.0 ± 7.5	18.2 ± 23.4	16.1 ± 21.8
Dimers (mg/day)	8.6 ± 6.6	13.5 ± 15.7	14.8 ± 17.3
Trimers (mg/day)	5.3 ± 4.5	6.7 ± 8.0	8.9 ± 12.5
4-6 mers (mg/day)	15 ± 16.3	16.6 ± 19.4	27.5 ± 36.3
7-10 mers (mg/day)	9.6 ± 12.7	9.0 ± 9.8	15.7 ± 18.7
Polymers (mg/day)	22.7 ± 39.5	18.4 ± 25.7	40.3 ± 51.3
Total proanthocyanidin intake (mg/day)	69.1 ± 78.9	82.3 ± 85.1	123.2 ± 136.6
Total polyphenol intake (mg/day)	1106.6 ± 531.1	1139.6 ± 647.3	1108.9 ± 590

No significant differences ($p > 0.05$)

Table 5-2: Unadjusted iron bioavailability, status, and inflammatory markers at Week 0 and Week 4 of each supplementation period

	0.03 g Week 0	0.03 g Week 4	0.25 g Week 0	0.25 g Week 4	1.5 g Week 0	1.5 g Week 4
	Mean 95% CI	Mean 95% CI	Mean 95% CI	Mean 95% CI	Mean 95% CI	Mean 95% CI
% Max iron absorption	12.7 (7.5, 17.9)	10.7 (5.4, 15.9)	12.1 (6.9, 17.3)	12.4 (7.2, 17.6)	11.2 (6.0, 16.5)	10.3 (5.0, 15.5)
iAUC for serum iron (µg/dL*hr)	2155 (612, 3696)	2269 (727, 3810)	2461 (919, 4003)	2769 (1228, 4311)	2237 (696, 3779)	2277 (735, 3819)
Hemoglobin (g/dL)	13.2 (13.0, 13.4)	13.3 (13.1, 13.5)	13.3 (13.1, 13.5)	13.4 (13.2, 13.6)	13.4 (13.1, 13.5)	13.3 (13.2, 13.6)
Ferritin (ng/ml)	35.4 (28.4, 42.4)	42.3 (35.3, 49.3)	35.8 (28.8, 42.8)	37.3 (30.3, 44.3)	40.0 (33.0, 47.0)	44.5 (37.5, 51.5)
CRP (mg/dL)	0.2 (0.0, 0.5)	0.3 (0.0, 0.5)	0.3 (0.1, 0.5)	0.2 (0.0, 0.4)	0.2 (0, 0.4)	0.3 (0.1, 0.6)

No significant differences ($p > 0.05$).

iAUC: incremental area under the curve; CRP: C-reactive protein.

Table 5-3: Comparison of improvement, maintenance, or deterioration of iron bioavailability and status within each supplementation period

	Supplementation period	Improvement	Maintenance	Deterioration	Fisher's exact (*p)*
% max iron absorption	0.03 g	5	0	6	0.394
	0.25 g	8	0	3	
	1.5 g	5	0	6	
	Total	18	0	15	
iAUC serum iron µg/dL*hr	0.03 g	6	0	5	1.0
	0.25 g	6	0	5	
	1.5 g	6	0	5	
	Total	18	0	15	
Hemoglobin (g/dL)	0.03 g	6	2	3	0.896
	0.25 g	5	2	4	
	1.5 g	4	4	3	
	Total	15	8	10	
Ferritin (ng/dL)	0.03 g	7	1	3	0.816
	0.25 g	5	1	5	
	1.5 g	6	0	5	
	Total	18	2	13	

Significance: $p < 0.05$

iAUC: incremental area under the curve; CRP: C-reactive protein.

Table 5-4: Estimation of iron bioavailability and status due to supplementation period, time, and significant covariates

		B	SE *B*	β	t	*p*
% max iron absorption	Model				6.92	<0.0001
	Constant	48.9	22.7	0	2.16	0.004
	Supplementation period	-0.36	1.5	-0.023	0.81	0.82
	Week 0	1.3	1.9	0.07	0.70	0.49
	Hemoglobin	-4.2	1.7	-0.20	-1.54	0.034
	Ferritin	-0.06	0.03	-0.23	-2.00	0.023
	CRP	-26.1	3.7	-0.74	-5.91	<0.0001
	bPRP	10.22	3.02	0.25	2.00	0.023
	aPRP	-16.9	6.5	-0.21	1.86	0.012
	Cystatin	0.0016	0.0004	0.06	2.51	0.0008
iAUC serum iron µg/dL*hr	Model				9.81	<0.0001
	Constant	10281	1869.2	0	5.5	
	Supplementation period	65.94	299.6	0.022	0.22	0.83
	Week 0	-210.6	379.4	-0.05	-0.56	0.58
	CRP	-2091.3	514.5	-0.40	-4.06	0.0002
	bPRP	0.0042	0.001	0.31	3.12	0.003
	Total salivary protein	-6606	1731	-0.38	-3.82	0.0004
	meat	-185.7	78.3	-0.23	-2.37	0.022
	0.2 astringency	-237.3	238.4	-0.31	-2.83	0.0066
Hemoglobin (g/dL)	Model				5.54	<0.0001
	Constant	12.4	0.27	0	41.55	<0.0001
	Supplementation period	0.17	0.11	0.14	1.55	0.126
	Week 0	-0.05	0.13	-0.03	-5.12	<0.0001
	CRP	-0.97	0.19	-0.47	4.14	0.0005
	Ferritin	0.007	0.001	0.36	3.32	0.0001
	Fat	0.008	0.002	0.32	-3.82	0.002
Ferritin (ng/dL)	Model				6.47	<0.0001
	Constant	-213.7	69.5	0	-3.33	0.0016
	Supplementation period	-5.44	5.64	-0.11	-0.97	0.83
	Week 0	-0.54	7.06	-0.01	-0.08	0.58
	Hemoglobin	22.95	4.49	0.50	4.60	0.0002
	bPRP	38.76	14.4	0.29	2.69	0.003
	%max iron	-0.70	0.40	-0.19	-1.72	0.0004
	Zinc	-2.24	0.85	-0.32	-2.63	0.022

Significance: $p < 0.05$ SE: standard error; CRP: C-reactive protein; bPRP: basic proline-rich protein; aPRP: acidic proline-rich protein; iAUC: area under the curve; Zn: zinc

Table 5-5: Correlations between % max iron absorption and salivary proteins at Week 0 and Week 4 of each supplementation period

Dose	Week 0/ Week 4	N	bPRP	aPRP	gPRP	Statherin	Cystatin	Total
			R (*p*)	*R* (*p*)	*R* (*p*)	*R* (*p*)	*R* (*p*)	*R* (*p*)
0.03 g	Week 0	11	0.218 (0.518)	-0.18 (0.596)	-0.131 (0.70)	-0.241 (0.475)	0.290 (0.387)	-0.078 (0.82)
	Week 4		0.605* (0.049)	-0.198 (0.56)	0.184 (0.587)	-0.055 (0.872)	-0.007 (0.985)	0.09 (0.793)
0.25 g	Week 0	11	0.25 (0.46)	-0.645* (0.03)	-0.204 (0.547)	-0.245 (0.469)	0.326 (0.328)	0.047 (0.892)
	Week 4		0.489 (0.07)	0.057 (0.876)	0.111 (0.76)	0.112 (0.757)	0.138 (0.704)	0.158 (0.66)
1.5 g	Week 0	11	0.297 (0.438)	0.075 (0.861)	0.391 (0.298)	0.01 (0.80)	0.201 (0.60)	0.46 (0.182)
	Week 4		0.173 (0.611)	-0.483 (0.133)	-0.595* (0.05)	0.10 (0.767)	0.114 (0.739)	-0.076 (0.825)
Total			0.366* (0.003)	-0.20* (0.028)	-0.23 (0.06)	0.07 (0.57)	0.27* (0.03)	0.20 (0.11)

*$p < 0.05$ basic proline-rich protein: bPRP, acidic proline-rich protein: aPRP, glycosylated proline-rich protein (gPRP)

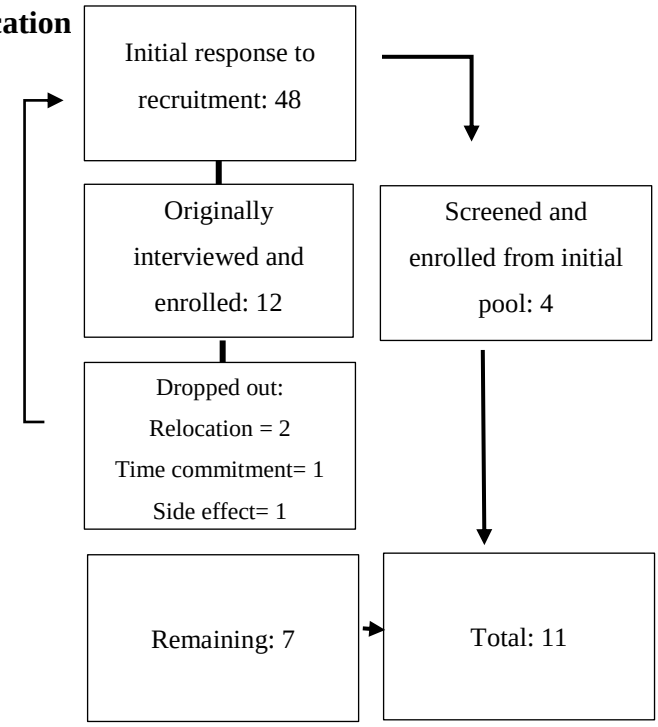
Table 5-6: Mean astringency ratings, and changes from Week 0 to Week 4 of supplementation periods

Dose*	0.03 g Week 0	0.03 g Week 4	0.25 g Week 0	0.25 g Week 4	1.5 g Week 0	1.5 g Week 4	Fisher's exact Week 0 to 4
	Mean (95% CI)	Mean (95% CI)	Mean (95% CI)	Mean (95% CI)	Mean (95% CI)	Mean (95% CI)	*p*
0.3 mg/dL	1.4 (1.1, 1.6)	1.1 (0.9, 1.3)	1.2 (0.9, 1.4)	1.3 (1.0, 1.5)	1.2 (0.9, 1.4)	1* (0.8, 1.2)	0.31
0.7 mg/dL	2.2 (1.8, 2.5)	2.3 (1.9, 2.6)	2 (1.7, 2.4)	1.9 (1.6, 2.3)	2.1 (1.7, 2.4)	1.8* (1.5, 2.2)	0.50
1.5 mg/dL	3.3 (2.8, 3.7)	3.4 (2.9, 3.8)	3.2 (2.7, 3.6)	3.5 (3, 3.9)	2.4 (1.9, 2.8)	3.3+ (2.8, 3.7)	0.126
2.5 mg/dL	4.2 (3.7, 4.7)	4.4 (3.9, 4.8)	4.3 (3.8, 4.7)	4.5 (4, 5)	3.3 (2.9, 3.8)	4.4 (3.9, 4.8)	0.55

*$p < 0.05$. CI: confidence interval. *$p < 0.05$ Week 4 vs. Week 0. +$p < 0.05$ 0.03 g vs. 1.5 g supplementation period. Tannin doses approximated consuming one fruit or vegetable, 3 cups of tea, or 100 g of sorghum, three times daily for four weeks. Scale 1 = not astringent, 5 = extremely astringent

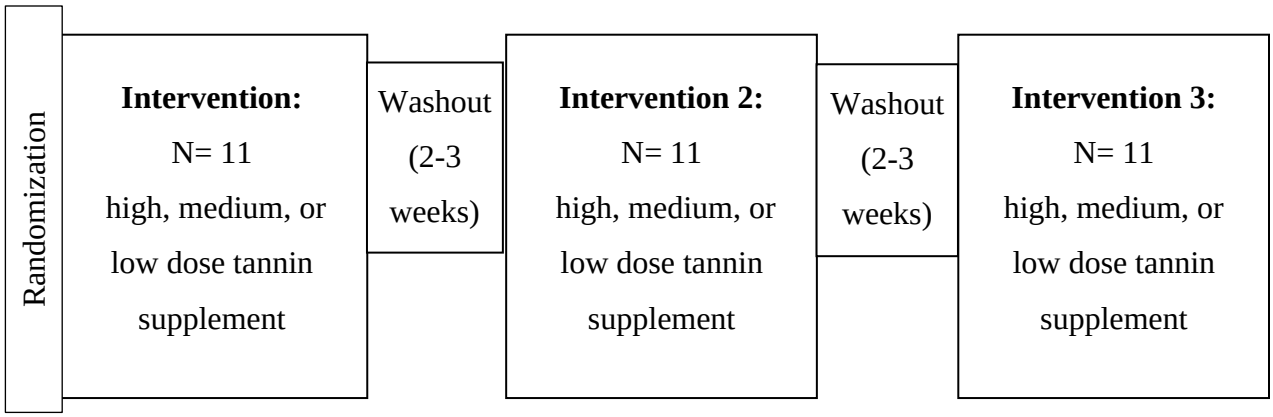
Figures

Figure 5-1: Enrollment allocation

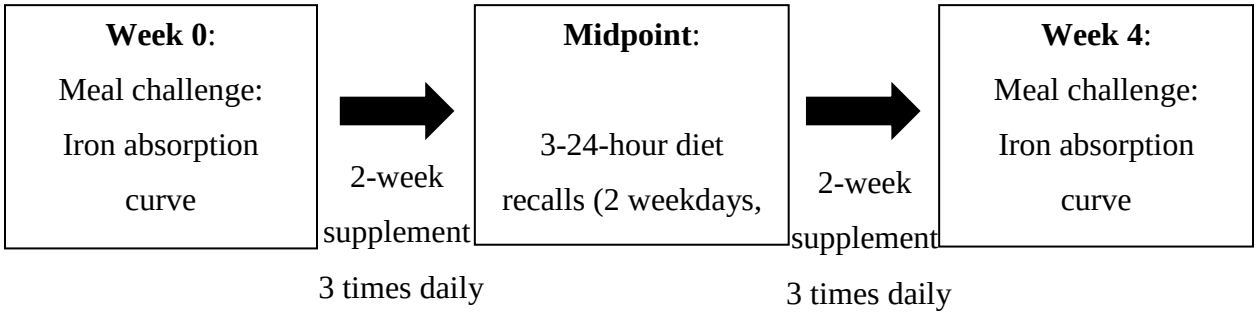


Enrollment allocation. Forty-eight potential participants responded to the study call and were screened; the study was conducted on a rolling basis. Twelve participants were initially enrolled, and during study duration, two participants dropped out due to relocation, one due to time commitment in the study, another due to intolerance to blood draws, and one due to supplement intolerance. These participants all dropped out after supplementation period I of the study was completed, and another four participants were recruited on a rolling basis from the initially screened pool of 48.

Figure 5-2: Supplementation periods (A) and supplementation period activities (B).

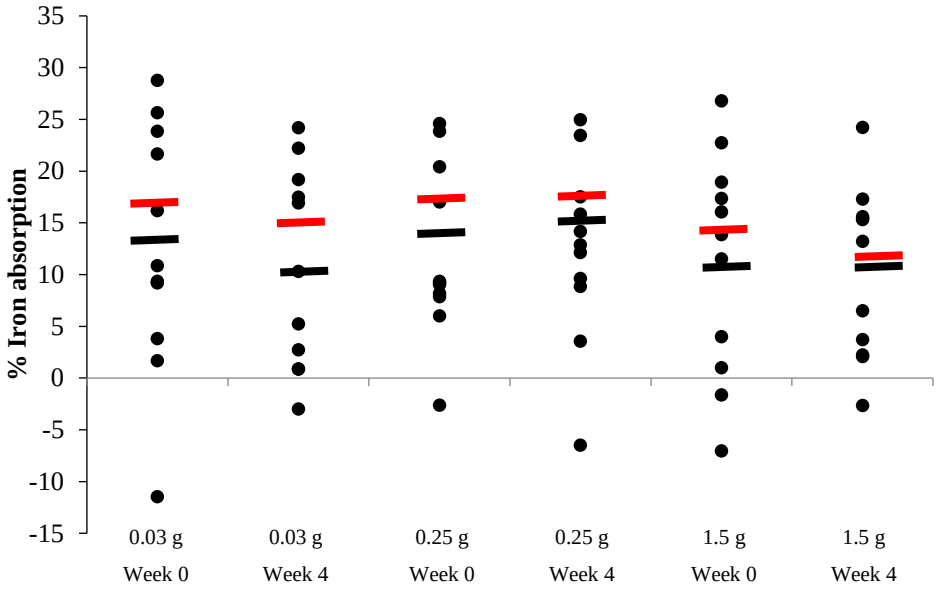


2b:



The study consisted of three supplementation periods for each participant, a high (1.5 g), medium (0.25 g), or low (0.03 g) condensed tannin supplement was provided for four weeks. Two to three-week washout periods between supplementation periods aimed to stabilize salivary protein and iron biomarkers. Supplementation periods consisted of Week 0 and Week 4 meal challenges, salivary collection, and astringency testing. At midpoint of each supplementation period, there were 3 24- hour dietary recalls (2 weekday, and 1 weekend day) collected from each participant.

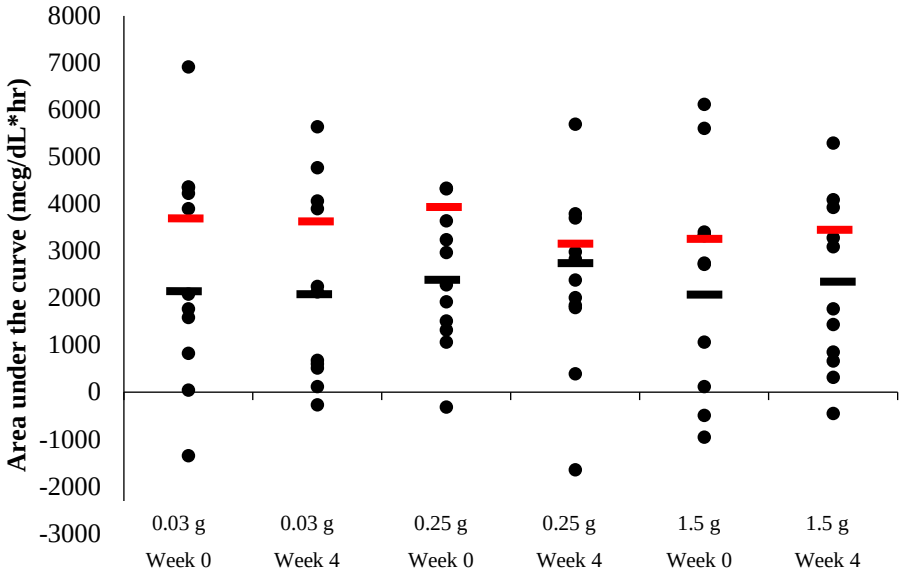
Figure 5-3: Mean adjusted and unadjusted individual level iron absorption at Week 0 and Week 4 of each supplementation period.



Adjusted for hemoglobin, ferritin, C-reactive protein, basic proline-rich proteins, and acidic proline-rich proteins, (red) and unadjusted (black)

There were no significant differences (*p*s > 0.05) in iron absorption at any dose of condensed tannin before or after supplementation periods.

Figure 5-4: Individual level incremental area under the curve for serum iron at Week 0 and Week 4 of each supplementation period



Mean regression-adjusted for C-reactive protein, basic proline-rich protein, total salivary protein, meat consumption, and rating of highest level of astringency: red; and unadjusted: black.There were no significant differences (*p*s > 0.05) in iron absorption within tannin supplementation periods.

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Chapter 6 - SALIVARY CYSTATIN SN BINDS TO PHYTIC ACID AND PREDICTS NON-HEME IRON BIOAVAILABILITY WITH PHYTIC ACID SUPPLEMENTATION

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Abstract

Background: Repeated consumption of phytic acid has been shown to lead to iron absorption adaptation. Mechanistically, adaption is not well understood, but salivary proline-rich proteins have been shown to block iron chelation by antinutritional factors other than phytic acid in animal models. It is therefore possible that upregulation of salivary protein synthesis partly diminishes the antinutritional effects of phytic acid.

Objective: The primary aims of this study were to determine whether salivary proteins bind phytic acid *in vitro*, and to understand the impact of daily phytic acid supplementation on individuals' iron status, bioavailability, and salivary proline-rich protein (PRP) production. A secondary aim was to investigate the correlations between astringency ratings and iron status. A

p*ost hoc* objective was to determine whether cystatin SN levels were correlated with iron bioavailability after phytic acid supplementation.

Method: In phase I, high performance liquid chromatography (HPLC) and matrix-assisted laser desorption/ionization: time of flight (MALDI-TOF) were used to characterize *in vitro* salivary protein-phytic acid interactions. Phase II was a clinical arm where women (n = 7) aged 18-35 consumed 350 mg phytic acid supplements three times daily for four weeks. Post-meal challenges were employed to determine iron bioavailability and status before and after supplementation periods. Three 24-hour dietary recalls were collected to assess for dietary covariates. At Week 0 and Week 4, phytic acid post-meal challenges were employed to assess iron bioavailability, ferritin, hemoglobin, salivary PRP changes, and astringency perception. In phase III, enzyme-linked immunosorbent assay (ELISA) analysis of purified protein fractions, and participant saliva from phase II were used to identify protein bound to phytic acid.

Results: *In vitro* salivary protein-phytic acid interaction showed dose-dependent reductions in a protein peak which correlated with MALDI-TOF and phytase treated phytic acid-salivary pellets. This peak was identified as cystatin SN through ELISA analysis. Neither iron bioavailability ($p = 0.32$), hemoglobin ($p = 0.72$), nor serum ferritin ($p = 0.08$) concentration were reduced from Week 0 to Week 4 after regular phytic acid supplementation. Basic proline-rich proteins were associated with improved iron bioavailability by study end ($r = 0.82$, $p = 0.02$). In *post hoc* analysis, cystatin SN concentrations were not significantly changed from Week 0 to Week 4 (R^2: -0.55, 2.01, $p = 0.23$). There was a significant positive correlation between cystatin SN concentration at Week 4 and improved iron bioavailability from Week 0 to

Week 4 (r = 0.996, $p < 0.001$), suggesting that upregulation may improve iron bioavailability with phytic acid consumption.

Conclusions: Proline-rich proteins did not bind to phytic acid *in vitro.* There were no changes in iron bioavailability, hemoglobin, or ferritin levels in participants consuming phytic acid supplements for four weeks. Basic proline-rich proteins were associated with improved iron bioavailability, while cystatin SN was found to be an independent predictor of poor iron bioavailability with phytic acid supplementation. Overall, these findings suggest that salivary proteins may play a role in protecting against phytic acid-iron chelation during digestion.
Keywords: *phytic acid, cystatin SN, iron bioavailability, salivary proline-rich proteins, adaptation, antinutritional factors, iron deficiency anemia*

Background

Phytic acid is the major phosphorous storage compound found in plants, including grains and legumes [1], which are commonly consumed in countries with high rates of iron deficiency. Phytic acid's propensity to bind to metal cations and proteins reduces their nutritional bioavailability [1], and phytic acid is thus denoted as an 'antinutritional' factor when it forms a phytic acid-nutrient complex (denoted phytate). The amount of phytic acid consumed [2], the food matrix in which it is consumed [1], and food preparation [1] all determine its ultimate 'antinutritional effect,' but human single-meal studies have repeatedly found that phytic acid reduces iron bioavailability [3-5].

Despite conventional views that support phytic acid's contribution to marginal iron status, there are also studies that dispute this idea. The individual effect of phytic acid consumption on iron bioavailability has been found to be highly variable [6], and many individuals consuming diets rich in grains and legumes, and thus phytic acid content maintain normal iron stores [7,8], pointing

to possible gaps in the understanding of phytates' antinutritional effects. Adaptation, or homeostatic maintenance of iron storage despite such antinutritional factor consumption, is one possible explanation. An experimental study showed that increased dietary phytic acid consumption over eight weeks improved iron absorption of a high phytate meal compared to a Week 0 meal and a low phytate control in marginally iron deficient women [9]. Another study examining long-term consumption (10 weeks) of high and low bioavailability diets in iron replete men also suggested adaptation to low bioavailability diets from Week 0 to Week 4, and authors suggested that single meal bioavailability may have exaggerated long-term effects of antinutritional factors [10]. In a 12-week crossover study that divided women into "high" (rich in heme protein and ascorbic acid) and "low" (rich in grains, legumes, and fiber) bioavailability diets, only women consuming a low bioavailability diet over time could absorb more non-heme iron from Week 0 to Week 4 with either a high or low bioavailable meal challenge, although iron storage was better correlated with a high bioavailability diet [11].

Saliva is the first defense mechanism of the alimentary tract to toxins and pathogens [12], and salivary proline-rich proteins (PRPs) in particular have been noted in sensory studies because of their ability to precipitate tannins [12,13], thereby reducing tannin-iron chelation, and creating an astringent sensation. In saliva, there are six main classes of salivary proteins: histatins, cystatins, statherins, acidic proline-rich proteins (aPRP), basic proline-rich proteins (bPRP), and glycosylated proline-rich proteins (gPRP) [13] that may exert independent effects on tannins. Statherin and aPRPs are commonly known for their ability to regulate oral calcium [14,15]. Cystatins have been linked to bitter sensation acceptance [16,17], and function to inhibit cysteine proteases [18]. Basic PRPs (bPRP) are thought to protect against the

negative effects of polyphenols [19], while the function of gPRP [12] is not well characterized. Production of PRPs when consuming tannins has been shown to improve protein [20] and iron availability [21,22] in animal studies, while hamsters, that do not upregulate PRP synthesis in response to tannin consumption, have poor growth outcomes [23] when consuming tannins. While PRP binding to tannins has been characterized previously [24-28], PRP-phytate interaction has not. Phytates may not directly interact with salivary PRPs, but it is important to establish whether PRP synthesis can be upregulated by phytates, which often accompany tannin consumption. Therefore, the primary objectives of this study were to determine: 1) whether salivary proteins would interact with phytic acid *in vitro* 2) whether iron bioavailability, or markers or iron status would be affected with repeated phytic acid consumption, 3) whether there would be changes in salivary protein production with phytic acid consumption over time, and 4.) whether salivary proteins could be associated with improved iron bioavailability during supplementation periods. *Post hoc* study objectives included determining whether cystatin SN could be correlated with improvements in iron bioavailability during the clinical study duration.

Methods

Study activities were divided into three phases to explore answer study objectives. In phase I, *in vitro* analysis of phytic acid-salivary proteins was designed to determine whether these interactions occurred. In phase II, a clinical arm was designed to measure iron bioavailability with repeated phytic acid consumption over time, as well as salivary protein production with phytic acid consumption. In phase III, *in vitro* and clinical arm salivary protein samples were analyzed via ELISA for confirmation of phytic acid-protein interactions.

Phase I: In vitro phytic acid-salivary protein interactions

Salivary PRP measurement

High performance liquid chromatography (HPLC) parameters and equipment

All reagents were analytical grade. Acetonitrile, trifluoroacetic acid (TFA), and HPLC grade water were purchased from Fisher Scientific.

Acidified saliva sample preparation

Frozen salivary samples from an individual donor, and later, clinical trial participants, were thawed in a refrigerator overnight. Before sample analysis, we verified consistency in chromatogram output with duplicate samples by HPLC, and because samples were analyzed relative concentrations, rather than for quantitative protein amounts, peak consistency allowed for samples to be analyzed in a single run. For PRP extraction, 900 µl of saliva was mixed with 10 µl of 10% TFA in water, centrifuged for 5 min at 8000 RPM, and the supernatant was filtered through a 0.2 µm polyvinylidene fluoride (PVDF) syringe filter described previously [29]. Before samples were analyzed, it was verified by investigators with HPLC analysis that there was no PRP peak loss with use of syringe filters. The supernatant was then analyzed by HPLC. Ninety µl of salivary supernatant was injected into a Fisher 2.1x150 mm, 5µm BioBasic C8 analytical column at a flow rate of 0.3 mL/min for 49 minutes at 40˚C with an auto sampler (Shimadzu SIL) on a HPLC system containing a LC20AB pump (Shimadzu), and a Shimadzu SPD-M20A PDA system. Detection of PRPs was carried out at 214 nm [29,30]. Mobile phase consisted 0.2% TFA in HPLC grade water (A) and 0.2% TFA in 80/20 acetonitrile and HPLC grade water (B) [29-32]. A linear gradient was applied from 0-39 min from 0-54% (B), then from

39-49 min at 54-100% B to elute late proteins [29-32]. After each wash, the column was stabilized with initial conditions by returning to 100% A over 10 min.

In Vitro Phytate-PRP testing

Matrix-assisted laser desorption/ionization: time of flight (MALDI-TOF) and HPLC determination of protein-phytate binding

Acidic saliva PRP-fractions were prepared, and phytic acid (inositol hexaphosphate, ACOS Organics) was added to samples at concentrations of 0.000512 (physiological, equivalent to 2:1 phytic acid supplement drink: saliva concentration, pH 4.5), 0.00512 (10:1, pH 2.3), or 0.0512 (100:1X, pH 1) mg/100 µl saliva and compared with an acidic saliva sample (buffered to 100 X saliva-phytic acid sample pH of 1 with TFA), or a saliva-only control. All samples were prepared to equivalent sample dilutions by adding aliquots from a standard saliva sample. After preparation, samples were shaken for 30 seconds and allowed to sit at room temperature (20°C) for five minutes. Samples were spotted on MALDI-TOF/TOF in several fractions: 1) whole extracted saliva 2) washed supernatant from samples centrifuged at 8000 RPM for 5 min 3) pellet digestion. In pellet digestion, 2,2,2-trifluoroethanol was used to disassociate aggregated hydrophobic proteins before spotting samples to MALDI-TOF/TOF. Pellets were then digested with trypsin (Promega, Trypsin Gold) to verify that protein was bound in phytic acid salts.
To reduce MALDI signal loss from phytic acid interference and quantify protein losses with phytic acid interaction, acidic saliva PRP-fractions were prepared at lower concentrations with phytic acid concentrations of 0.000512 (physiological), 0.000256 (0.5 X), or 0.000064 (0.125 X) mg/100 µl saliva, and a control saliva sample, to equivalent sample dilutions and buffered to pH of the physiological phytic acid supplement (pH 5.5). Samples were vortexed for 30 seconds,

then allowed to sit at room temperature (20°C) for five minutes, and finally centrifuged for 5 min at 8000 RPM. The supernatant was collected, filtered with a 0.2 µg PVDF syringe filter, and immediately run on HPLC. The remaining precipitate pellet was digested with addition of 10 µl wheat phytase (Sigma Aldrich) in hydrochloric acid buffered distilled water (62 mg/ mL phytase in water; pH 5.5) at 20^0C for five minutes, filtered with a 0.2 µg PVDF syringe filter, and run on HPLC. Chromatograms were analyzed for peak reductions at 214 nm, HPLC purified peak loss fractions from phytic acid and control samples were collected from control saliva for tryptic digestion and MALDI-TOF/TOF analysis. A phytase standard was run on HPLC to assess peak changes in the phytase sample created by the enzyme or enzyme impurities. Protein peaks recovered from phytase digestion were also collected and run on MALDI-TOF/TOF for identification.

Tryptic digestion

Pellets and HPLC fractions of interest were subjected to in-solution tryptic digestion. Trypsin (Promega, Trypsin Gold) was added to HPLC purified peaks and phytate pellets at a 1:200 trypsin:protein ratio. Samples were digested in 50 mM ammonium bicarbonate overnight, and subsequently spotted to MALDI after acidification with 1% TFA.

MALDI TOF analysis

Intact mass and in-solution trypsin digestion were analyzed using a MALDI-TOF mass spectrometer (Bruker Daltonics Ultraflex III) in linear mode for intact proteins, and reflectron mode for peptides in the Biotechnology/Proteomics Core Facility at Kansas State University. Intact proteins were spotted with 20 mg/mL sinapinic acid in 1:1 0.1% TFA/acetonitrile. Peptide digest samples were spotted with 2,5 dihydroxybenzoic acid (50 mg/mL) in 1:1 0.1%

TFA/acetonitrile. Digested mass spectra were matched against a SwissProt database for proteins within the intact mass range using mMass software (http://www.mmass.org).

Phase II: Clinical Arm

Inclusion/exclusion

The study protocol was approved by the Institutional Review Board at Kansas State University (KSU; IRB #8121). An announcement requesting participants was sent to faculty and students through a university email digest, as well as disseminated though departmental social media channels. Women who participated in a similar clinical trial were recruited for the current study, enrollment was rolling, potential participants were screened in person. Before screening, participants were required to read and sign an informed consent document, and all procedures, risk and benefits of the study were reviewed verbally. Premenopausal women, aged 18-35, who were non-obese (body mass index, BMI $\leq$ 30.0 kg/m^2), had no history of oral or gastrointestinal disease, were moderate ($\leq$ 1 drink per day) or non-alcohol consumers, and non-tobacco users we eligible for participation. Further exclusion criteria included blood disorders affecting iron status or absorption, current supplementation or medication that would impair iron status, food allergies to supplements, pregnancy or breastfeeding (see Appendix D for screening questionnaire and exclusion criteria). Participants were compensated for completing study activities. In total, seven participants were enrolled in the current study. Participants were compensated monetarily for completing study activities.

Study conditions

The study consisted of Week 0 and Week 4 meal challenges, with four weeks of daily phytic acid supplementation in between. Each participant consumed 350 mg phytic acid supplements

(inositol hexaphosphate, ACOS organics), to mirror a dose from a previous trial [9]. Supplements were provided in an opaque bottle with a non-caloric sweetener/flavoring (Mio Original) to improve palatability, three times daily for four weeks (**Figure 6-1**).

Phytic acid meal challenges

At Week 0 and at the end of Week 4, participants completed meal challenges at the KSU Physical Activity and Nutrition Clinical Research Consortium (PAN-CRC). Participants were asked to come in fasted (at least 8 hours) at 7:00 am, having abstained from teeth cleaning (2 hours) and exercise (24 hours) to minimize diurnal variations or other factors in salivary production [32], and iron uptake [33]. Pre-meal saliva was collected by passive drool (2 mL total) into cryovials, and samples were immediately placed into a freezer set to -71^0 C for storage. A 20-gauge indwelling peripheral intravenous (PIV) catheter was placed either in the median cubital, cephalic, or basilica vein for multiple blood samples, which was flushed and saline locked with 10 mL of 0.9% isotonic saline between blood collections. After fasted blood collection for serum ferritin, C-reactive protein (CRP), hemoglobin, and serum iron, a phytic acid challenge meal consisting of supplemented 15 mg ferrous sulfate and 75 mg ascorbic acid [9,34] (each sprinkled on a 95 g bagel with 12 g sugar free strawberry jam), and a 90 g banana, which were consumed with the phytic acid supplement. Salivary samples were collected 15 minutes following the final bite of the meal to determine salivary protein stimulation after phytic acid consumption [35]. After a waste blood sample was drawn [36], blood samples were drawn from the PIV for serum iron at 180 and 210 minutes to determine percentage of max iron absorption and incremental AUC for serum iron [34].

Astringency testing

After the PIV was removed at Week 0 and Week 4, participants were asked to complete an astringency test [37,38]. Each participant was given four different concentrations of 10 mL alum powder in distilled water (0.20, 0.15, 0.07, and 0.03%) to sip in randomized order. They were given a verbal description of the sensation of astringency asked to rate each solution based on their perception of astringency on a 5 point Likert scale (1 = not astringent, 5 = extremely astringent). Participants waited 30 seconds between astringency tests.

Dietary Analysis

At the beginning of week two of the study, participants were emailed a unique username and password to complete 2 weekdays and 1 weekend day, 24-hour dietary recalls on the Automated self-administered 24-hour recall (ASA24®). After all recalls were collected, dietary data were extracted, and total caloric intake (kcal), protein (g), fat (g), carbohydrate (g), iron (mg), ascorbic acid (mg), meat protein (oz.), sugar (g), fiber (g), Zn (mg), and Cu (mg) content were averaged from system calculated amounts for all participants. Food intake logs were downloaded from the ASA24® for manual calculation of proanthocyanidins, polyphenols, and phytic acid in the diet. During this process, a research assistant reviewed all dietary data for each participant kept in an electronic spreadsheet (Microsoft Excel). Food items were referenced from USDA tables transferred to an electronic spreadsheet, and total proanthyocyanidin [39], amounts were calculated and summated for each recall. Individual meal recalls were added into the Nutrition Data System for Research (University of Minnesota, Minneapolis, MN) *post hoc* to quantify phytic acid meal content. From these summations, group averages were calculated.

Hematological Parameters

From the fasting blood draw, two separate samples were collected 5 mL serum separator (SST) and 3 mL ethylene diaminetetra acetic acid (EDTA) vacutainer tubes to measure serum iron (by spectrophotometry), CRP (by nephelometry), ferritin (by immunoassay), and whole blood hemoglobin concentrations (by electronic cell cytometry). Subsequent blood samples were samples were collected in SST tubes and analyzed for serum iron. After collection, serum samples were centrifuged at 2500 RPM for 15 min after clotting for 20 minutes, and kept at room temperature for analysis. All blood samples were analyzed by a certified laboratory (Quest Diagnostics, Lenexa, KS) within 24 hours.

Incremental AUC and maximum percentage of iron absorption calculation

Serum iron data were used to calculate percentage of maximum iron recovery and incremental AUC for iron bioavailability analysis.

Percentage Iron Recovery: Percentage iron recovery was calculated as below [34].

$$\%\ iron\ recovery\ \max = (\frac{serum\ iron\ \max \times plasma\ volume)}{total\ iron\ ingested} \times 100$$

Where: $iron\ \max = \frac{\mu mol}{L}, plasma\ volume = L, and\ amount\ of\ iron\ is\ in\ \mu mol$

$$plasma\ volume\ (L) = \frac{Blood\ volume\ (ml) \times \left(1 - Packed\ cell\ volume\ (decimal)\right)}{1000}$$

And $blood\ volume = 69.6 \frac{ml}{kg} body\ weight$

Incremental serum iron AUC by trapezoidal integration

$$\int AUC = \frac{\Delta\ time}{2} \times [\Delta Serum\ iron_{\ time\ 0-180} + (2 \times \Delta Serum\ iron_{time\ 0-210})]$$

Phase III: ELISA confirmation of protein and protein-phytate binding

To confirm MALDI-HPLC findings, peak loss fractions from *in vitro* phytic acid and control samples were tested for presence and absence of cystatin SN in samples, respectively, using an enzyme-linked immunosorbent assay (ELISA) kit (Raybiotech, Norcross GA). Samples were prepared according to manufacturer instructions by adding the instructed sample amount, and read on a 96-well plate reader (Biotek Synergy HT, Winooski, VT) at 450 nm immediately after adding stop solution. Participant saliva from the clinical arm of this study was analyzed to quantify cystatin SN before and after phytic acid supplementation at Week 0 and Week 4. One participant, whose iron bioavailability data was not obtained due to blood lysis in blood draws at 180 and 240 minutes, was excluded from analysis. Separately, salivary-phytic acid samples obtained after phytic acid consumption (stimulated saliva) were interacted with phytic acid *in vitro* by to verify phytate formation with cystatin SN. During this experiment, phytic acid was added to saliva obtained after meal consumption at a concentration of 0.00512 mg/100 µl saliva (physiological). Samples were shaken and allowed to incubate for five minutes, saliva was then centrifuged at 8000 RPM for 5 min, the supernatant was separated from precipitate, then spotted to ELISA 96 well plates.

Statistical analysis

Data were analyzed using SAS statistical software (SAS Studio version 3.6, Cary, North Carolina), statistical significance was set at $p < 0.05$, and are presented as mean ± standard deviation. Before analysis, all data were analyzed for normality and homogeneity of data in Q-Q plots and with Levene's test.

Variables that were non-normal were log transformed, and determined to be normal before analysis. Log-transformed variables were included in stepwise variable selection in adjusted model building (below). All log-transformed data were back-transformed for results presentation. Week 0 demographic and nutritional intake data were analyzed by analysis of variance (ANOVA).

Sample size

A sample size calculation determined that four participants would be needed to determine a change in incremental iron area under the curve (AUC) by 25% [9,34] (SAS studio version 3.6, Cary, NC) as statistically significant with 80% power and at an α-level of 0.05. With an anticipated dropout of 30%, it was determined that at least 6 participants were needed to enroll in the study.

Hematological outcomes analysis

Regression analysis of hematological outcomes

Unadjusted percentage of max iron absorption, ferritin, and hemoglobin were analyzed by linear regression at Week 0 and Week 4 to answer our second primary research question, which asked whether iron bioavailability or status would be affected with repeated phytic acid consumption. Multiple regression was used to adjust outcomes (% max iron absorption, incremental serum iron AUC, hemoglobin, ferritin) for repeated (participant) and random covariates after stepwise selection for variables. Significant differences for all outcomes were determined using least significant differences. *Post hoc* sample size analysis was conducted using t-tests with a power of 0.90 and α of 0.05.

Astringency and salivary protein outcomes analysis

Our third primary research question asked whether changes in salivary protein production were induced by phytic acid consumption over time, and whether there were correlations between salivary proteins and improved iron bioavailability before and after phytic acid supplementation. To answer these questions, proline-rich proteins were divided into type by retention times; histatins were excluded from analysis [29-32], peak mAu were recorded for each. Proportions of acidic salivary subtypes were further analyzed.

$$PRP\ subtype\ proportion = PRP\ type\ (area\ sum) \div Total\ PRP\ area$$

Or

$$PRP\text{: } Acidic\ salivary\ proteins = Total\ PRP\ area\ \div Total\ salivary\ protein\ area$$

Salivary proteins were analyzed by multiple factor ANOVA at Week 0 or Week 4; Pearson's product-moment correlations were used to analyze correlations between iron absorption, incremental AUC for serum iron, astringency ratings, and PRP types. Changes in participant cystatin SN concentrations were analyzed at Week 0 and Week 4 by ANOVA, and effect sizes were calculated using the equations:

Hedges' g (unbiased estimator of Cohen's d)[15]

$$= \boldsymbol{M1 - M2/Spooled}$$

Where $S_p = \sqrt{\frac{(ne-1)Se2+(nc-1)sc2}{ne+nc-2}}$ *and M1/M2 were the mean of experimental and control groups.*

95% confidence intervals were calculated using the equation:

$$g^* \pm 1.96(\sigma g\ *)$$

$\sigma g * = (\frac{ne+nc}{nenc} + \frac{g^*}{2(ne+nc)})$ *and* $g^{*=} g\left(1 - \frac{3}{4(ne+nc)-9}\right)$

Astringency perception, connections to salivary protein production, and iron bioavailability

We determined whether astringency perception was changed from Week 0 to Week 4 of phytic acid supplementation using Chi-square testing and Fisher's exact tests. Correlations between salivary protein production, iron bioavailability and astringency were analyzed by Pearson's product-moment correlations.

Results

Phase I: In vitro phytic acid salivary protein interaction

HPLC analysis of phytic acid-saliva interactions

To establish whether there was potential for meaningful mediation of iron-phytic acid chelation by salivary proteins, a single volunteer's saliva was interacted with phytic acid at various concentrations. HPLC results from non-pelleted salivary supernatant extracted after interaction showed progressive peak reduction at 40.3 minutes, suggesting preferential binding of phytic acid to this HPLC fraction (**Figure 6-2**).

Saliva-phytic acid pellet MALDI outcomes

Phytic acid-saliva pellets analysis resulted in peaks similar to those from purified protein fractions. Pellets were dissolved with tryptic digestion, indicating that the protein precipitated phytic acid. MALDI-TOF results found *in vitro* phytic acid supernatant sample peak reductions compared to control or acidified saliva (**Figure 6-3**). This lost fraction was recovered by tryptic digestion of MALDI-spots, also suggesting that phytic acid was directly precipitating specific protein fractions, rather than nonspecific precipitation of proteins due to pH reductions during experiments.

In vitro pellet tryptic digestion and purified protein fraction analysis

In vitro tryptic digestion of the phytic acid-salivary pellet dissolved the complex, suggesting that proline-mediated bonds were not pellet components, and thus the protein identified on HPLC was not likely a proline-rich protein. MALDI spots of purified whole saliva and *in vitro* phytic acid fractions collected at 40.3 minutes recovered the same protein peaks as were identified on MALDI after tryptic pellet digestion, suggesting that HPLC peak reductions were the same as

MALDI pellet components (**Figure 6-4**). To match peptide components to proteins, *in vitro* phytic acid sample peaks were removed from control samples before searching the database. The best matches were for cystatins S, SN & SA fragments. All cystatins without signal peptides were mapped to the data, the match with the best fit was for cystatin SN (49.6%).

Phase II: clinical arm results

Participant demographics

Participant ages ranged from 20-35, average age of participants was 26.2 ± 1.2 yrs. All participants were occasional (2-3 drinks/month) or moderate (2-3 drinks per week) alcohol consumers. Aside from one participant, who was vegan, and took vitamin B_{12} supplements, no participants took vitamin or mineral supplements during the study period. Average BMI of participants was 25.4 ± 5.8 kg/m^2 (range 19.7-29.8). Participant weights (kg) did not significantly change between Week 0 and Week 4.

Participant dietary intake

Mean caloric intake during the study was 2107 ± 672.8 kcal per day, 13.7± 3.2% of average caloric intake was from protein, 35.1 ± 12.1% and 51.2 ± 20.2 % came from fat and carbohydrates, respectively (**Table 6-1**). Average meat consumption ranged from 0-5.7 oz. per day. Average ascorbic acid intake exceeded the recommended daily value (RDA) by 15%, iron intake was on average 93% of the RDA. The average daily phytic acid intake was 863.8 ± 812.8 mg/ day (range 199.8 – 2388 mg per day), phytic acid supplementation more than doubled the typical dietary phytic acid intake for five of the seven participants.

Hematological indices and iron absorption

Unadjusted iron outcomes

Iron absorption was not significantly different from Week 0 to Week 4 by either % max iron absorption or iAUC for serum iron (**Table 6-2)**. The percentage of maximum iron absorption but not significantly different (p = 0.22, Tables 6-2, **Figure 6-5**) from Week 0 to Week 4. There were no significant differences in hemoglobin, ferritin, or CRP from Week 0 to Week 4 of the supplementation period (Table 6-2).

Individual level data show that 57% of participants in the study experienced reductions in hemoglobin values from Week 0 to Week 4 ranging from 2-5% (**Table 6-3**). Three of those participants also had reduced ferritin levels from Week 0 to Week 4, the reduction range was 8-37%. Four of seven participants experienced 1.1-4 times worse iron absorption measured by iAUC for serum iron at Week 4 compared to Week 0; of the three participants who had improved iron absorption, two also had reduced hemoglobin and ferritin levels.

Hematological covariate identification and adjustment

To test the impact of dietary and individual physiological differences (iron status, anthropometric, salivary protein) on iron bioavailability and status, we employed stepwise regression analysis to establish significant covariates to build an adjusted model for hematological outcomes. Covariates that were significantly associated with serum iron iAUC included dietary fat and proanthocyanidin trimer intake, rating of 0.07 mg/dl astringency, aPRP, and cystatin production (**Table 6-4**). Significant covariates associated with %max iron absorption included hemoglobin and proanthocyanidin monomers. Ferritin and hemoglobin

covariates were CRP, dietary protein intake, and rating of 0.2 mg/dl (highest) astringency rating (Table 6-4).

Significant covariates for each outcome measure were added to the linear regression for adjusted outcomes analysis. Following the full adjustment for significant covariates, % max iron absorption was significantly greater in Week 0 than Week 4 of the study ($p = 0.02$), but iAUC for serum iron was not statistically different from Week 0 to Week 4 (5019 ± 358.8 µg/dL*hr and 4793 ± 345.4 µg/dL*hr, respectively; $p = 0.32$, Table 6-4). There were no significant differences between Week 0 and Week 4 % max iron absorption, hemoglobin, or serum ferritin after adjusted outcomes analysis (Table 6-4).

Correlations between salivary protein subtypes, iron absorption, and dietary phytic acid intake

Week 0 % max iron absorption was not significantly correlated with acidic proline-rich protein (aPRP) and glycosylated proline-rich protein (gPRP) production (r = 0.535 and 0.48, respectively, *p*s > 0.22), while Week 4 absorption was significantly correlated with basic proline-rich protein (bPRP) production (r = 0.819; $p = 0.02$, **Table 6-5**). Cystatin production was not significantly correlated at Week 0 (r = 0.525; $p = 0.227$) or Week 4 (r = 0.269; $p = 0.56$, Table 6-5). Statherin was not-significantly correlated with % max iron absorption at Week 0 and Week 4. Dietary phytic acid intake was significantly correlated with bPRP (r = 0.89, $p = 0.007$), and cystatin (r = 0.79, $p = 0.03$) production at Week 0, and was not significantly correlated with % max iron absorption (r = 0.71, $p = 0.11$) or iAUC for serum iron (r = 0.77, $p = 0.07$) at Week 0.

Astringency testing

There were no significant differences in any astringency ratings from Week 0 to Week 4 of the study (*p*s > 0.18). At Week 0, there was a significant positive correlation between the 0.07 mg/dl (middle) astringency rating and iron bioavailability among participants ($r = 0.996$, $p < 0.001$, **Table 6-6**). Correlations at higher ratings of astringency were not significant. At Week 4, higher ratings of astringency perception with phytic acid consumption were significantly negatively correlated with iron absorption in all but the lowest concentration of alum powder, suggesting that lower ratings of astringency were associated with better iron absorption (Table 6-6).

Phase III: ELISA confirmation of purified protein peak findings and participant cystatin SN concentrations

Confirmation of cystatin SN-phytic acid interactions

To confirm cystatin SN-phytic acid interaction, HPLC fractions for control and *in vitro* phytic acid purified protein saliva samples collected at 40.3 minutes were assessed using a cystatin SN ELISA. Total cystatin in the control was 132 ng; 8 ng was recovered in the same fraction from 0.000512 mg/mL *in vitro* phytic acid interaction, suggesting that: 1) cystatin SN was the protein identified on HPLC and MALDI, and 2) that phytic acid interaction with saliva reduced cystatin SN in purified peak levels.

ELISA analysis of post-meal participant cystatin SN levels

Post-meal participant cystatin SN levels were significantly reduced after *in vitro* interaction with phytic acid ($p < 0.001$), confirming purified protein ELISA results that cystatin SN was precipitated from saliva with phytic acid (**Figure 6-6**). Cystatin SN salivary concentration did not increase 15 minutes after meal consumption at Week 0 ($R^2 = 0.89$, 95% CI [-0.55, 2.01], $p =$

0.23) and Week 4 (R^2 = 2.14, 95% CI [-0.48, 2.10], p = 0.19) in all measured participants. In participant salivary samples, fasting cystatin SN concentrations did not change with long-term phytic acid consumption (R^2 = -0.14, 95% CI: [-1.39, 1.09]), however, there was a non-significant increase in cystatin SN at Week 4 (effect size 0.24, 95% CI [-1.08, 1.40]). From HPLC results, cystatin SN production at Week 0 was negatively correlated with iron absorption (r = -0.977, p = 0.006), but was positively correlated with iron absorption from Week 0 to Week 4 (r = 0.996, p < 0.001).

Astringency ratings and cystatin SN levels

Cystatin SN levels were significantly negatively correlated with astringency ratings (r = -0.82, p = 0.046). There was no correlation between cystatin SN and bPRP at Week 0 among participants (r = -0.10; p = 0.86), but a positive correlation at Week 4 (r = 0.56, p = 0.07, **Figure 6-7**).

Discussion

The primary objectives of this study were to determine: 1) whether salivary proteins would interact with phytic acid *in vitro* 2) whether iron bioavailability, or markers or iron status would be affected with repeated phytic acid consumption, 3) whether there would be changes in salivary protein production with phytic acid consumption over time, and 4) whether salivary proteins could be associated with improved iron bioavailability during supplementation periods. *Post hoc* study objectives included determining whether cystatin SN could be correlated with improvements in iron bioavailability during the clinical study duration.

Phase I: *In vitro* phytic acid saliva analysis

The experimental hypothesis in phase I of this study was that salivary PRPs would bind to phytic acid salts *in vitro.* To the best of our knowledge, this is the first study that has explored

the effects of phytic acid supplementation on salivary proteins. This hypothesis was supported in that PRPs did not bind to phytic acid, instead, phytic acid formed complexes with another non-enzymatic salivary protein, cystatin SN. This finding was supported through tryptic and phytase digestion of phytate-saliva pellets formed after interaction, and co-analysis with supernatant fractions of whole and phytic-acid treated saliva. ELISA confirmation of cystatin SN in purified peak fractions and human participant saliva interacted *in vitro* with phytic acid suggests that this binding is consistent in a variety of participants.

The findings that PRPs do not bind to phytic acid is an important one. Tannin-PRP binding is specific [24], and bonds may not dissociate during digestion [19]. Because PRPs do not bind with phytic acid in addition to tannins, PRP mediated protection against tannin-iron chelation may be viable in phytic acid and tannin-rich foods.

Unlike other cystatin proteins, cystatin SN is only found in saliva. Its primary purpose is inhibition of cysteine proteolysis, but has been shown to be associated with enhanced tolerance of bitterness in infants and caffeine consumers [40,41], which is consistent with our findings that higher levels of cystatin SN were significantly correlated with lower ratings of astringency. In addition, induction of S-type cystatins with capscasin ingestion was found in rats, suggesting that these proteins may be stimulated with non-proteolytic oral irritants [42] like phytic acid.

Phase II: Clinical study arm

Hematological outcomes

The first hypothesis for the clinical arm was that repeated phytic acid consumption would not change iron bioavailability, hemoglobin, or ferritin values in participants over four weeks. Although Week 0 % max iron absorption was significantly greater than Week 4 in the covariate

adjusted model (Table 6-4), phytic acid supplementation did not change iron absorption, hemoglobin, or ferritin when consumed three times daily for a month in other outcomes both adjusted and unadjusted for covariates (Tables 6-3, 6-4). Interestingly, while %max iron absorption was adjusted for several dietary and non-dietary factors (Table 6-4), it was not adjusted for hemoglobin, which may explain predicted iron absorption differences from incremental AUC for serum iron (Table 6-4). Overall, these findings are in contrast to a similar study that found improvement in bioavailability of a high phytate meal after consuming a phytate diet for 8 weeks [9]. There is evidence that inulin improves iron bioavailability with phytic acid consumption over time [43], meaning that efficient adaptation may be due to combinations of homeostatic mechanisms, including changes in gut microbiota to resistant starch often found in phytate rich foods, rather than just with phytic acid alone.

Contrary to findings in single meal [3-5], and *in vitro Caco*-2 [44-46] models, we did not find that iron status, as indicated by ferritin or hemoglobin levels, was reduced with phytic acid supplementation over time. The current study findings are however, consistent with results from multiple rat studies [43,47-54]. Findings from single meal studies, and Caco-2 cell models, may not predict adaptive mechanisms employed by certain populations to antinutritional factors like phytic acid over time [55]. In support of this assertion, studies exploring long-term bioavailability in rat versus acute *Caco-2* bioavailability, phytic acid inhibited iron bioavailability in *in vitro,* but not *in vivo* models [56,57]. The *Caco-2* cell model may not accurately simulate nuances of digestion and absorption, such as salivary protein profiles and their effect on antinutritional factors and absorption.

Salivary protein production, correlations with iron bioavailability, and astringency

The second hypotheses were that there would be no changes in salivary protein production with phytic acid consumption over time, and that salivary protein production would not be correlated with improved iron absorption with phytic acid supplementation. These hypotheses were partially rejected; there were no significant changes in salivary proteins over time, but while *in vitro* binding of PRPs with phytic acid did not occur, bPRP were significantly correlated with improved iron bioavailability at Week 4 ($r = 0.819$, $p = 0.02$). Interestingly, bPRP levels significantly correlated with dietary phytic acid intake at Week 0, suggesting that individuals commonly consuming phytic acid rich foods may produce more bPRP compared to those who do not. These findings may suggest that repeated phytic acid consumption triggers bPRP production.

Perhaps supporting this idea, astringency ratings at Week 0 were positively correlated with iron bioavailability, while Week 4 astringency ratings were negatively correlated. A concurrent study from our lab group found that bPRP production is negatively correlated with astringency ratings as well, therefore these findings are consistent with previous bPRP-iron absorption correlations (unpublished data). Combined, these data suggest that in phytic acid naïve individuals, aPRP, gPRP, and total protein levels predict iron bioavailability in the short term, but that bPRP production better predicts iron bioavailability overall.

Part III*:* Cystatin SN and iron bioavailability

The *post hoc* hypothesis was that cystatin SN would not change with phytic acid consumption over time, and that cystatin SN production would not be correlated with iron bioavailability from our clinical trial results. There were no significant differences in cystatin SN

production after daily phytic acid supplementation over four weeks. Regardless, Week 0 cystatin SN and iron absorption were negatively correlated (r = -0.97, p = 0.006). At Week 0, cystatin SN was not correlated with bPRP production, which suggests that cystatin SN may predict suboptimal iron bioavailability independent of bPRP production in non-regular phytic acid consumers. It is possible that production of cystatin SN, in lieu of other salivary proteins or protective mechanisms, is inefficient in protecting against phytic acid-iron chelation. Thus, elevated cystatin SN levels may be a marker of inefficient phytic acid protection. We found that tryptic digestion easily destroyed cystatin-phytic acid complexes, questioning stability during digestion, which would likely be poor. It is possible that cystatin SN does not trigger non-salivary protective mechanisms to phytic acid as efficiently as other proteins. Higher levels of cystatin SN at Week 4 were positively correlated with bPRP production, which may explain the significant positive relationship between cystatin SN and iron bioavailability at Week 4.

Limitations

This study was conducted in a small sample as an arm of another study. Due to the variability in iron absorption among participants, statistical power to detect significant findings was limited, including differences in cystatin SN from Week 0 to Week 4 of the study (effect size 0.24, 95% CI [-1.08, 1.40]). In addition, we used phytic acid, rather than food-source phytates for the model. There is evidence that tannic acid may bind to salivary PRPs differently than condensed proanthocyanidins found in food [55], and it is reasonable to believe that there may be different effects from phytates consumed in food compared to a highly ionized, liquid form. Additionally, compared to a similar clinical trial [9], our participants' average Week 0 phytic acid consumption was higher (863 mg vs. 718 mg), more variable (199.8-2388 mg/day vs. 548-941

mg/day), and participant Week 4 consumption was greater (1913 vs. 1190 mg/day), which may have affected bioavailability over time. Our study only lasted four weeks, and although we anticipated that hemoglobin and ferritin would be impacted within this time frame, other studies have used longer supplementation periods that have produced significantly improved iron absorption from study start to end [9]. It may be that long-term phytic acid supplementation induces non-salivary mechanisms not assessed with our study. It is also important to note that while concentrations of cystatin SN did not change through the study, lack of measurement of salivary flow rate is a limitation in protein production quantification. Our study used a pre-determined 2 mL salivary sample (which was obtained over varying time spans), and thus participants with higher or lower salivary flow rates may have had different total cystatin production.

Lastly, the current study was conducted in iron sufficient, pre-menopausal women, with a nutrient replete and varied diet. In Malawain children, a high-phytate diet resulted in increased zinc excretion in sick, but not well children, highlighting the complexity of metabolism potentially based on inflammatory status [58]. Findings should be investigated in other populations of interest including children [40] and pregnant women [59], who have different salivary protein profiles than the current study population.

Future research

Future studies are needed to determine effects of salivary proteins on phytic acid mediated reductions in iron bioavailability. In addition, more studies are needed to explore the non-chelating protective effects of bPRPs on iron bioavailability, and the potential effects of cystatins throughout the GI tract including potential interactions between salivary proteins and

microbiota. Studies exploring consumption of foods with multiple antinutritional factors are needed to understand the interactions between compounds commonly co-consumed and salivary profiles, including secondary effects from salivary proteins on enterocyte mediated iron bioavailability. Finally, further research is needed to determine the effects of developmental and hormonal changes on salivary protective mechanisms on antinutritional factors and iron absorption.

Conclusions

To the best of our knowledge, this is the first time that salivary-phytic acid interactions have been investigated. Phytic acid does not specifically bind with PRPs, but does bind with cystatin SN, a non-proline-rich salivary protein. Iron bioavailability nor status were significantly affected with long term phytic acid consumption. Cystatin SN levels were significantly negatively correlated with iron absorption over time, suggesting that this protein may be an inefficient protein for protection against phytic acid, and production may identify individuals who do not adapt to phytic acid. Production of bPRP production positively influenced iron absorption with phytic acid consumption. Overall, these findings indicate that repeated phytic acid consumption may not negatively influence iron bioavailability and status, and that salivary proteins may help to protect against negative effects from phytic acid consumption.

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Author Contributions

ND conceived and conducted experiments, analyzed data, and wrote the manuscript. BK conceived and conducted experiments, analyzed data, and reviewed the manuscript. NF randomized participants to interventions, prepared supplements throughout the study, and edited the manuscript. KK analyzed salivary proteins on HPLC and edited the manuscript. SR conceived the experiment and edited the manuscript, MH conceived and edited the manuscript. JT conceived experiments and reviewed the manuscript; BL conceived and oversaw the experiment, analyzed data, edited, and reviewed the manuscript.

Tables

Table 6-1: Participant macronutrient, select micronutrient, proanthocyanidin, and phytic acid intake during the study duration

Outcome	Totals
	Mean ± SD
N	7
Caloric intake (kcal/day)	2107.7 ± 672.8
Protein (g/day)	72.4 ± 15.6
Fat (g/day)	82.2 ± 28.6
Carbohydrates (g/day)	279.2 ± 109.0
Meat (oz/day)	3 ± 1.8
Sugar (g/day)	132.2 ± 88.4
Fiber (g/day)	24.7 ± 13
Iron (mg/day)	16.8 ± 7.1
Ascorbic acid (mg/day)	69.5 ± 54.2
Zinc (mg/day)	11.3 ± 3.2
Copper (mg/day)	1.5 ± 0.66
Total proanthocyanidin intake (mg/day)	89.1 ± 45.5
Total phytic acid intake (mg/day)	863.8 ± 812.8

SD: standard deviation

Table 6-2: Week 0 and Week 4 iron bioavailability, status, and inflammatory indicators

	Week 0	Week 4
	Mean (95% CI)	Mean (95% CI)
% Max iron absorption	12.8 (-9.1, 34.1)	8.3 (-15.1-31.4)
iAUC (µg/dL*hr)	2027	1525
Hemoglobin (g/dl)	13.7 (13.4, 13.9)	13.4 (13.1, 13.6)
Ferritin (ng/mL)	48.9 (40.1, 57.6)	45.6 (36.8, 54.3)
CRP (mg/dL)	0.21 (-0.1, 0.49)	0.20 (-0.5, 0.94)

iAUC: incremental area under the curve for serum iron. CRP: C-reactive protein
No significant differences between Week 0 and Week 4 ($p < 0.05$)

Table 6-3: Hemoglobin, ferritin, % max iron absorption, iAUC for individual participants at Week 0 and Week 4

	Value	Week 0	Week 4
1	Hemoglobin (g/dL)	14.6	13.9
	Ferritin (ng/mL)	53.0	46.0
	% max iron	-25.8	0.0
	iAUC (µg/dL*hr)	-4845	-90
2	Hemoglobin (g/dL)	14.2	13.4
	Ferritin (ng/mL)	12.0	11.0
	% max iron	19.1	16.0
	iAUC (µg/dL*hr)	4725	4995
3	Hemoglobin (g/dL)	12.1	12.3
	Ferritin (ng/mL)	22.0	24.0
	% max iron	4.2	-7.4
	iAUC (µg/dL*hr)	510	-207
4	Hemoglobin (g/dL)	13.5	13.2
	Ferritin (ng/mL)	23.0	23.0
	% max iron	28.4	30.3
	iAUC (µg/dL*hr)	5325	4935
5	Hemoglobin (g/dL)	14.5	14.9
	Ferritin (ng/mL)	67.0	86.0
	% max iron	not obtained*	not obtained*
	iAUC (µg/dL*hr)	not obtained*	not obtained*
6	Hemoglobin (g/dL)	13.3	12.6
	Ferritin (ng/mL)	28.0	43
	% max iron	26.1	33.7
	iAUC (µg/dL*hr)	4365	7530
7	Hemoglobin (g/dL)	13.4	13.3
	Ferritin (ng/mL)	137.0	86
	% max iron	22.9	-22.9
	iAUC (µg/dL*hr)	2085	-8101

*Blood draws obtained at 180 and 210 minutes were severely lysed at Week 4 in this participant
iAUC: incremental area under the curve for serum iron

Table 6-4: Covariate adjusted estimation of iron bioavailability and status due to phytic acid supplementation

		B	SE *B*	β	t	*p*
% max iron absorption	Model				156.1	<0.0001
	Constant	-95.3	3.82	0	-24.96	<0.0001
	Week 0	4.59	1.4	0.11	3.31	0.02
	Fat intake	0.68	0.04	1.2	19.04	<0.0001
	Trimer PA	-2.95	0.20	-0.99	-14.59	0.023
	0.07 astringency	36.72	1.93	0.78	18.94	<0.0001
	aPRP	-46.97	4.84	-0.53	-9.71	0.0002
	Cystatin	0.0005	0.0001	0.25	5.00	0.004
AUC serum iron µg/dL*hr	Model				10.4	0.0028
	Constant	815.0	583.4	0	1.4	0.196
	Week 0	-68.12	64.2	-0.18	-1.1	0.32
	Hemoglobin	-60.6	44.9	-0.23	-1.35	0.58
	Monomer PA	16.35	3.1	0.88	5.35	0.0005
Hemoglobin (g/dL)	Model				5.95	0.016
	Constant	16.2	0.93	0	17.41	<0.0001
	Week 0	0.11	0.30	0.05	0.37	0.72
	0.2 astringency	-0.63	0.21	-0.56	-3.01	0.017
	bPRP	0.56	0.56	0.18	1.34	0.22
	CRP	-1.58	0.65	-0.50	-2.45	0.040
Ferritin (ng/dL)	Model				22.3	0.0002
	Constant	97.7	27.1	0	2.49	0.037
	Week 0	-5.09	6.91	-0.07	-0.74	0.48
	CRP	-66.3	14.79	-0.46	-4.48	0.002
	Kcal	-0.02	0.004	-0.42	-3.68	0.006
	Protein	-0.93	0.18	-0.50	-3.77	0.006
	Total PRP	174.02	35.2	0.34	2.67	0.028

Significance: $p < 0.05$ SE: standard error; CRP: C-reactive protein; PRP: proline-rich protein; AUC: area under the curve; aPRP: acidic proline-rich protein; bPRP: basic proline-rich protein PA: proanthocyanidin; Kcal: kilocalorie

Table 6-5: Correlations between proteins and iron bioavailability at Week 0 and Week 4 of phytic acid supplementation

Phase	Week 0/ Week 4	N		bPRP	aPRP	gPRP	statherin	cystatin	total
				r *p*	*r* *p*	*r* *p*	*r* *p*	*r* *p*	*r* *p*
phytate	Week 0	7	% max iron	0.334 (0.464)	0.525 (0.227)	0.48 (0.276)	0.246 (0.594)	0.525 (0.227)	0.537 (0.214)
	Week 4		%max iron	0.819* (0.02)	0.032 (0.946)	0.295 (0.52)	0.547 (0.204)	0.269 (0.56)	0.356 (0.434)

*$p < 0.05$; bPRP: basic proline-rich protein, aPRP: acidic proline-rich protein; gPRP: glycosylated proline-rich protein

Table 6-6: Correlations between astringency and iron absorption (1 = not astringent, 5 = extremely astringent)

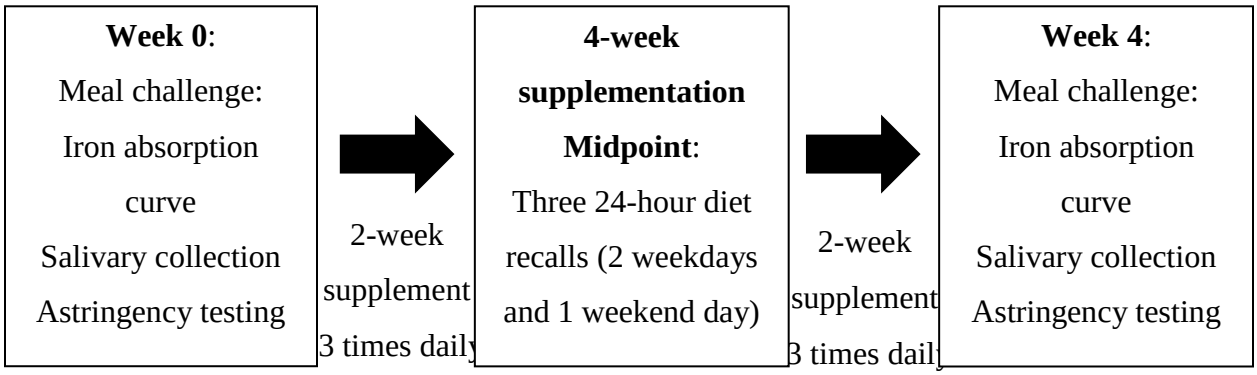
Dose (95% CI)	Week 0	Week 4
	Mean (95% CI) *r*	Mean (95% CI) *r*
0.03 mg/dL	1 (0.7, 1.3) 0.0	1 (0.7, 1.4) 0.0
0.07 mg/dL	2 (1.6, 2.4) 0.996*	1.8 (1.3, 2.3) -0.959*
1.5 mg/dL	2.9 (2.3, 3.4) 0.492	3.0 (2.3, 3.7) -0.84*
2.5 mg/dL	4 (3.4, 4.6) 0.553	4.2 (3.5, 4.9) -0.768*

Dose: astringency concentration testing at Weeks 0 and 4 of phytic acid supplementation.

*Denotes significant correlation, $p < 0.05$

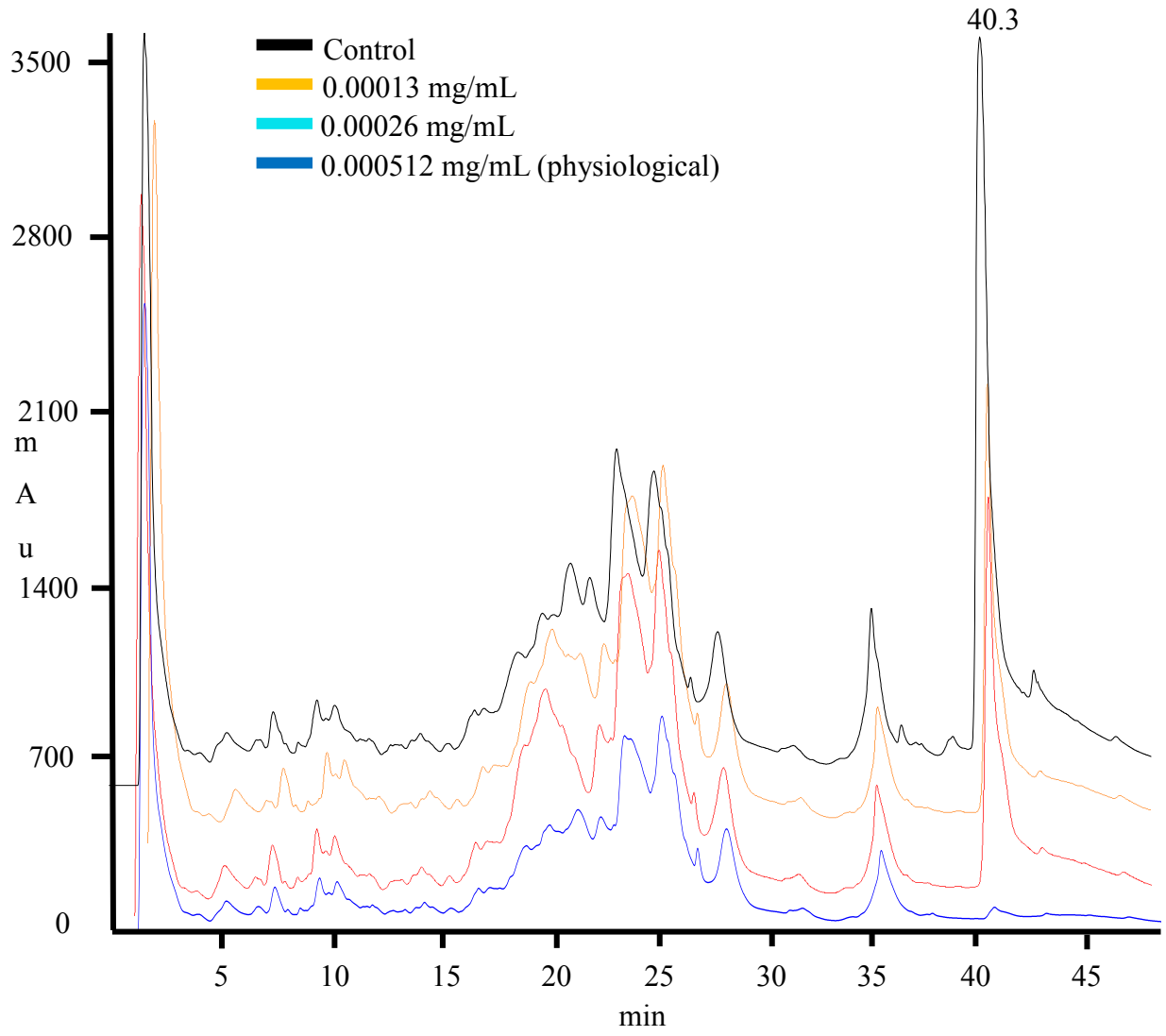
Figures

Figure 6-1: Study procedures



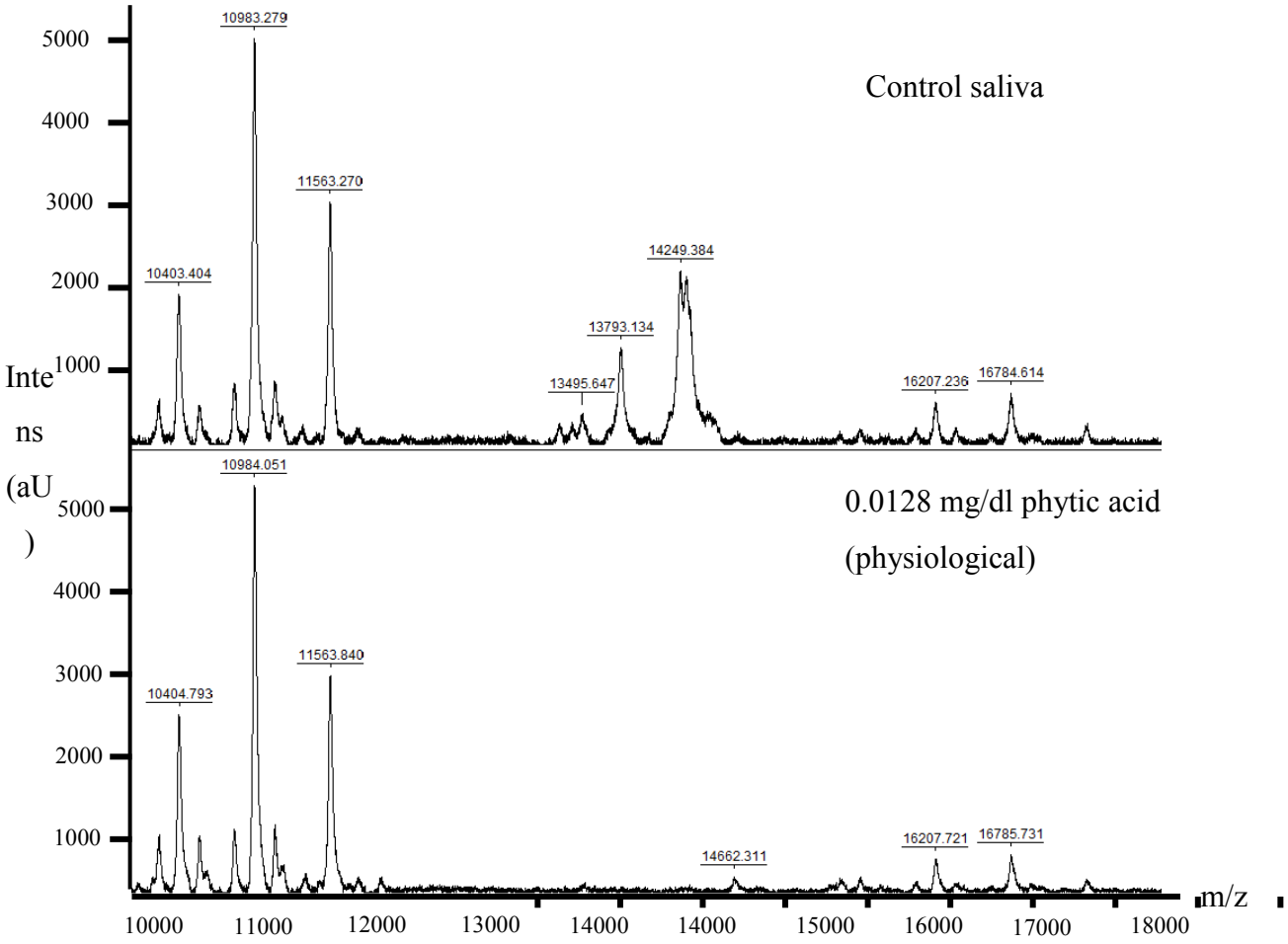
Week 0 and Week 4 meal challenges were employed to assess iron bioavailability and salivary protein changes before and after phytic acid supplementation period (four weeks). At midpoint, participants recorded three 24-hour dietary recalls.

Figure 6-2: *In vitro* phytic acid- salivary interactions



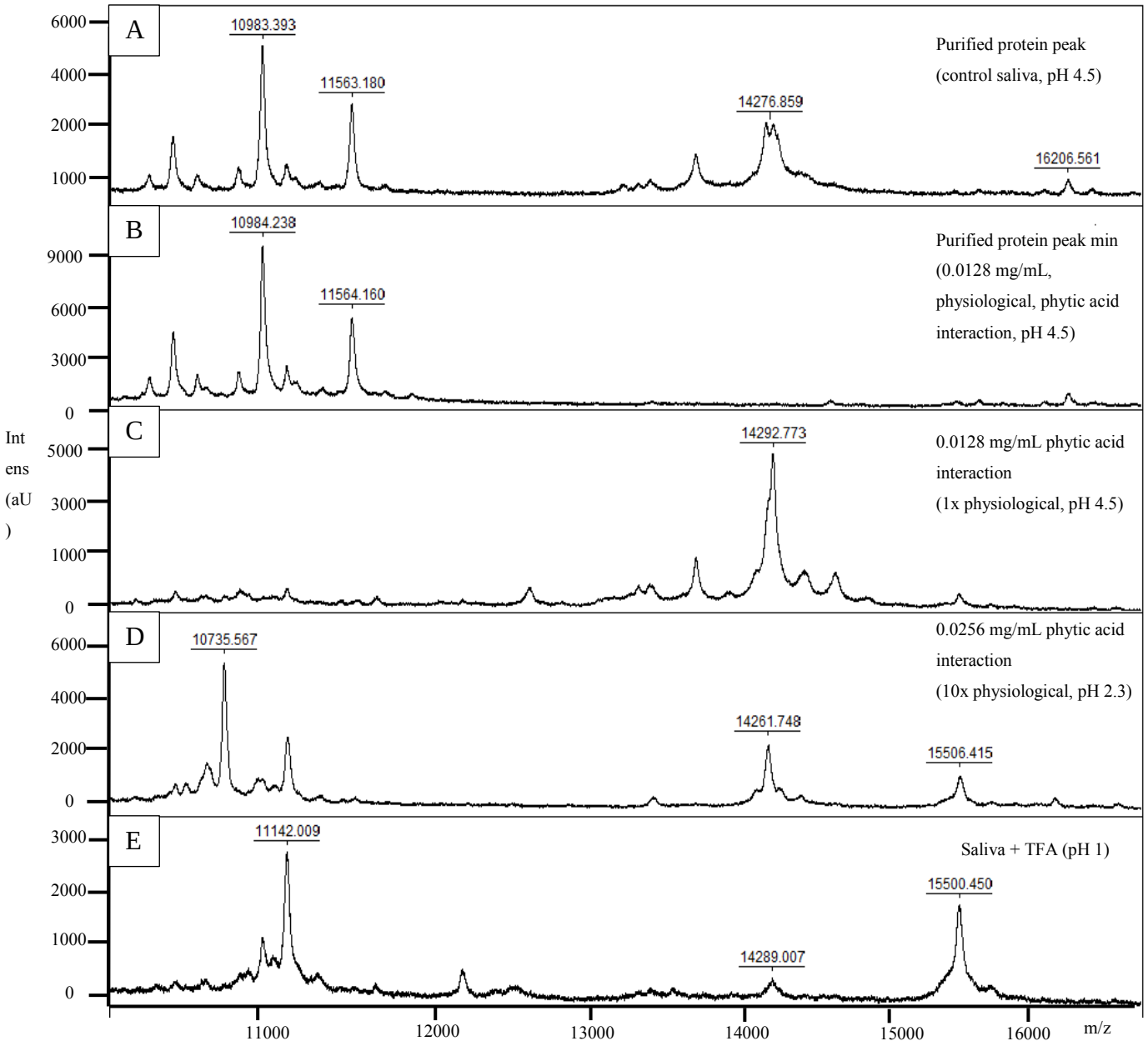
Progressive peak reductions were seen at 40.3 minutes with phytic acid concentration interaction independent of other peak reductions. At physiological doses (equivalent to phytic acid supplement in clinical study), there was total peak loss at 40.3 minutes.

Figure 6-3: MALDI-TOF whole saliva and phytic acid interacted salivary supernatant protein peaks



Control saliva at 40.3 minutes and saliva fraction at 40.3 minutes after addition of 0.0128 mg/mL phytic acid. Noted peak losses at 13000-14000 kDA.

Figure 6-4: MALDI-TOF whole saliva, phytic acid interacted supernatant purified protein peak, and pytate pellet digest

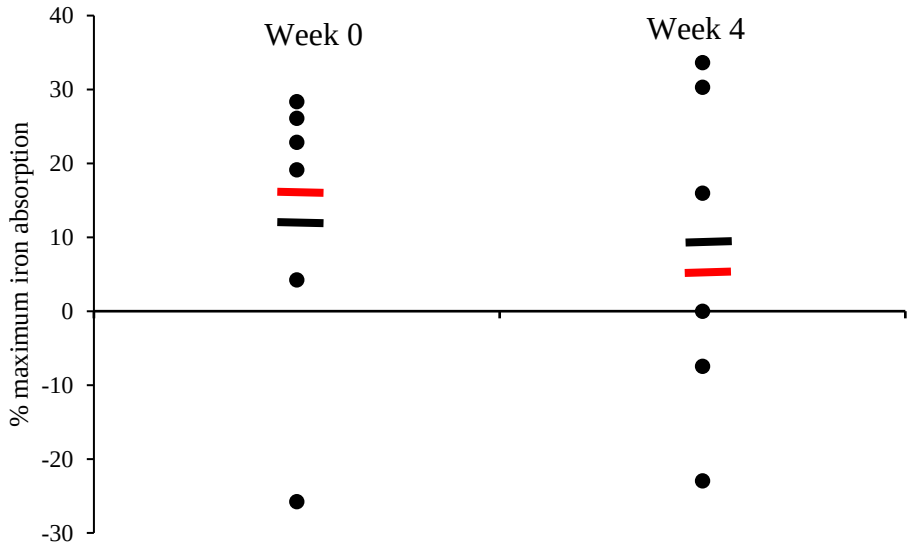


* mAu is correlated with signal strength, rather than concentration with MALDI-TOF analysis.

Salivary pellet trypsin digest and purified peak MALDI-TOF results: From purified peak collected at 40.3 minutes, there is peak loss seen in phytic acid (B) compared to control (A)

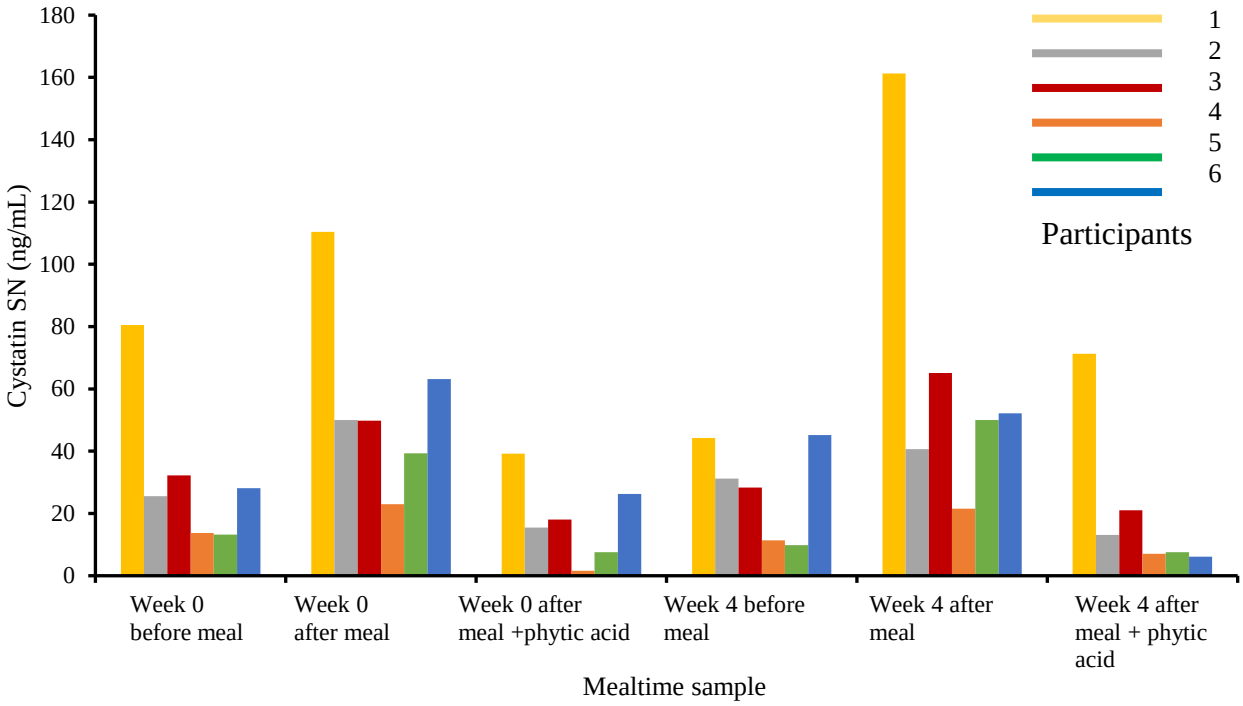
samples that is also found in phytic acid-saliva samples subjected to tryptic digestion (C-D). This peak was also not found in the pellet sample acidified with TFA (E), suggesting selectivity of phytic acid to this protein.

Figure 6-5: Iron bioavailability at Week 0 and Week 4 phytic acid postabsorptive meal challenges



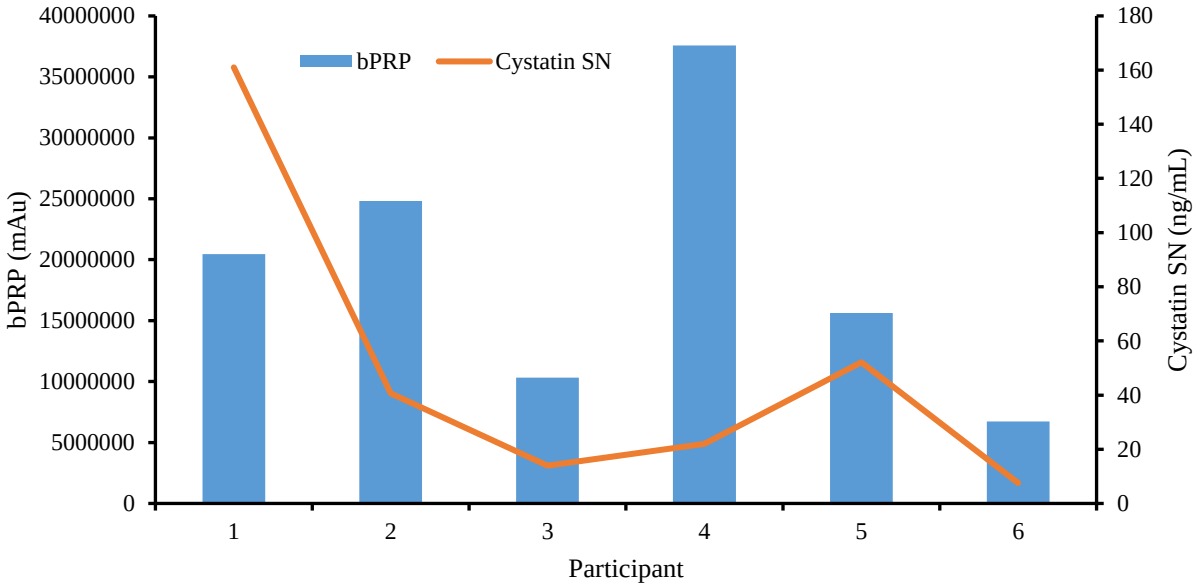
Unadjusted (black) and adjusted (red) iron absorption at Week 0 and Week 4 of supplementation. There was a significant reduction in covariate-adjusted iron absorption from Week 0 to Week 4 ($p = 0.02$). There were no significant differences in % max iron absorption from Week 0 to Week 4 in unadjusted ($p = 0.22$) models.

Figure 6-6: Cystatin SN before and after phytic acid meal challenges at Weeks 0 and 4



Cystatin SN ELISA results. Phytic acid *in vitro* interaction with saliva significantly reduced cystatin SN concentrations from saliva supernatant. There were no significant changes in cystatin SN production before (R^2 = -0.14, 95% CI: [-1.39, 1.09]), or after (R^2 = 0.24, 95% CI [-1.08, 1.40]) meals from Week 0 to Week 4.

Figure 6-7: Comparison of Week 4 bPRP and cystain SN quantities for individual participants



Week 4 bPRP and cystatin SN for each participant. There is a positive (r = 0.56, p = 0.07) between Week 4 bPRP and cystatin SN levels.

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Chapter 7 - CONCLUSION

Summary

Improving fortified-blended foods through nutritional formulation:

In vivo model findings

In vitro FBF quality evaluation was designed to look closely at nutritional aspects of FBF blends, rather than for the potential nutritional effects during childhood complementary feeding. This controlled animal model allowed us to see FBF nutritional qualities that might otherwise have been muted by dietary variety or socioeconomic factors that contribute to nutritional status. Our findings supported that animal growth (as a surrogate for protein quality), vitamin A, and iron outcomes were similar between newly formulated FBFs. In CSB+, a roasted corn soy blend, protein quality was notably reduced compared to extruded, protein quality enhanced and oil fortified blends. Specific aspects leading to protein quality improvement, including pre-cooking in extrusion, oil fortification, and protein isolates, have not been explored. Nevertheless, blend reformulation improved protein outcomes, regardless of commodity used, suggesting that formulation, rather than specific legumes or grains was necessary. These protein and micronutrient related outcomes may suggest that a variety of 'low quality' legumes or grains may be used as nutritious food sources when nutritional quality factors are accounted for in FBFs formulations.

Limitations and future directions

While using a rat model enabled new FBF evaluation in a controlled environment, several factors in the study design limit generalizability of results presented, especially in regards to protein quality and iron availability. Blends contained more iron than was necessary for growing rodents (nearly double AIN-93-G), making it is difficult to predict iron bioavailability for anemic children who could conceivably require more relative iron in their diets compared to rats from these findings. In addition, differences in protein quality due to extrusion versus roasting (in the case of CSB+) were not reflective of cooked FBFs, where CSB+ might be more fully 'cooked.' As noted above, it may be that protein availability changes with complete FBF cooking. Future directions for FBF evaluation could include using a rodent model where higher iron demand is present (perhaps with restricted intake), or a study design framed toward iron repletion with FBFs used in this work, could delineate FBF differences that could be important in the field. Beyond micronutrient availability, differences in plant and animal protein quality need to be further assessed, especially given the cost of animal protein and conflicting evidence regarding long-term effects of animal protein consumption on healthy weight and treatment of protein insufficiency.

Effects of tannins and phytates on iron bioavailability and status:

Concerns regarding the influence of antinutritional factors over protein quality and micronutrient availability from grain and legume based diets have been limiting factors in the use of sorghum and like grains for FBF formulation, despite in-country benefits (including local and regional procurement), and potential health benefits of tannins and phytic acid. A narrative literature review conducted as a part of this discussion suggests that limitations in current

research exploring these issues include the use of single meal studies, the fact that a majority of evidence comes from *in vitro* experiments, and the use of models with non-typically consumed tannin and phytate supplements that may not represent human metabolism found in grain and legume based diets. A systematic literature review as a part of this dissertation suggests that iron bioavailability adaptation to tannins is possible through production of salivary proline-rich proteins. This analysis considered factors involved in iron bioavailability like tannin size, nutrient digestion, and PRP production changes after tannin consumption. Several rat experiments included in this systematic review suggest that PRP production improved iron bioavailability after long-term tannin consumption, suggesting adaptation. Through use of multi-dose, long term supplements in a clinical trial, we approximated tannin and phytic acid supplemental influence on iron bioavailability over time.

Tannins

We assessed the influence of food-tannin (proanthocyanidin, condensed tannin) supplementation on iron bioavailability with normal dietary intake in adult women. Multi-concentration condensed tannin supplements did not change iron bioavailability or status with multi-meal supplementation over 4-week time periods in non-anemic adult women. In addition, basic salivary proline-rich proteins (bPRP) were positively correlated with iron bioavailability during tannin supplementation over time, consistent with hypothesized findings. Iron bioavailability and bPRP correlations increased from Week 0 to Week 4, suggesting that these proteins may be upregulated over time to improve iron bioavailability with tannin consumption.

Phytates

Overall, phytic acid consumption did not change iron bioavailability or status after a four-week supplementation period. Unlike tannin research, phytic acid intake has been previously explored in multi-meal studies, but salivary protein-phytic acid interaction has not. *In vitro* modeling suggests that phytic acid does not bind with salivary proline-rich proteins, but does bind specifically with cystatin SN, a cysteine protease-inhibitor. Correlations from ELISA analyses and clinical trial iron bioavailability testing suggests that cystatin SN was negatively, but bPRP were positively, correlated with iron bioavailability after the supplementation period. This is the first time that salivary protein influence on iron bioavailability with phytic acid consumption has been explored.

Findings Synthesis and Limitations

The preceding research studies suggest that neither tannin or phytic acid consumption influences iron status in healthy non-anemic women over time, even at concentrations more than double normal dietary intake. This research also suggests that certain salivary protein subtypes may improve iron bioavailability with chronic tannin or phytic acid consumption. Given that salivary protein production is genetically variable, and age-related, exploration of salivary protein production over the lifespan, and in anemic and non-anemic individuals, may lead to understanding of individualized nutritional outcomes. Understanding age and disease-related differences that contribute to tannin and phytic acid adaptation could better direct nutritional approaches to undernutrition including improved FBF formulation for complementary feeding, breastfeeding, or during pregnancy. Mechanisms outside of direct protein-tannin or protein-phytate chelation could help with understanding correlations between bPRP production and iron bioavailability with phytic acid challenges presented in study #5, since phytates did not bind with

PRPs *in vitro*. Future studies could include iron bioavailability assessment after dietary challenges with tannin and phytate rich meals, and possibly an analysis of the influence of fiber on tannin and phytate antinutritional effects. Targeting a cohort of healthy and anemic participants could help to identify individuals with different salivary or gut anatomy that may inhibit adaptation. Studies employing food tannin and phytate-rich foods both in and out of the diet may reinforce findings from this research. Understanding factors that affect iron bioavailability throughout the lifespan may elucidate differences in childhood and adult metabolic effects from tannins and phytates. Finally, one could envision studies exploring oral and gut isolated tannin or phytic acid meal challenges that could elucidate the direct impact of oral antinutritional exposure on iron absorption.

Future directions and development

Approaches to food aid formulation

While biases of the scientific community toward effectiveness of models or ideas in nutrition abound, this body of work illustrates the effectiveness of using multiple models to describe phenomena through nutritional research. Complexities in nutrition and metabolism, including nutrient interactions related to formulation, the human system, environmental, and social factors, necessitate a variety of research methods to describe and understand multifactorial issues encountered, for example, in effective food aid formulation. Developing food aid, and perhaps understanding nutrition in general, benefits from understanding nuances in the models for research that are employed to describe phenomena. Regarding the present work, nutritional quality of blends themselves, outcomes in field trial consumption, and clinical studies exploring

nuances of formulation describe a holistic picture of what ‘nutrition’ means outside of any single study alone.

Considering individual approaches to antinutritional factor mediated reductions in iron bioavailability

Understanding nuances from multiple model approaches to nutritional questions lends itself to moving forward into approaches favoring individualized or population specific nutrition. While our studies suggested that antinutritional factor intake influenced outcomes related to iron bioavailability; salivary proteins, for example, are also influenced genetically. Designing experiments that comprehensively look at the different factors in understanding antinutritional factors, salivary proteins, iron bioavailability genetics, epigenetics, and population differences will give context to the work found in models employed here. One could envision research approaches that would eventually explore interplay behind these factors, leading to a more comprehensive metabolic picture that could be employed in clinical and global undernutrition diagnostic and treatment modalities. Ultimately, targeted nutrition approaches may more effectively combat slowly improving issues, like iron deficiency, as compared to generalized knowledge.

Appendix A - Quality Assessment of Studies

(Each criterion assessed as having high, unclear, or low risk)

1.) Is there a clear purpose for study aims and research?
2.) Is there relevant background information presented?
3.) Methods assessment:
    a. Are methods employed appropriate?
    b. Were methods completely described or referenced?
        i. Studies described methods for allocation of samples or groups to treatments, if applicable
            1. Appropriate sample size or sample size justification

        ii. Are instruments used for data collection appropriate for outcomes assessed?
        iii. Methods allowed for randomization of study groups to minimize outcomes bias, if applicable
    c. Was the intervention independent of other changes (minimization or accounting of confounding variables)?
4.) Outcomes measurement
    a. Were all outcomes measured reported?
    b. Completeness of outcome data:
        i. Are data presented clearly related to methodological design?
        ii. Did studies, if applicable, account for attrition or negative outcomes to intervention or control groups?
        iii. Is analysis of data reported, and appropriate for study design?
        iv. Are data transformations accounted for?
5.) Conclusions:
    a. Do conclusions and discussion match findings presented?
    b. Do findings contribute to current theory or future practice research?

Appendix B - Biochemical Study Characteristics

Biochemical study Characteristics (references from Chapter 4)

Study	Topic	Tannin-non-heme iron binding (x) or tannin-PRP binding (o)	Tannic acid - Proanthocyanidin comparison	Tannins used	Size of tannins compared	Measurement of mechanism	Conditions for assay	Findings
(77)	Effect of AA or EDTA on polyphenol non-heme iron binding	x	X	tannic acid, gallic acid, catechin, coffee, tea		monomers	catechol/gallo yl equivalent measurement of non-heme iron binding capacity	900 µg/ml polyphenol bound less non-heme iron than at 40 µg/ml (<50% vs. 100%); tannic acid more predilection to non-heme iron binding ability vs. gallic acid or catechin
(51)	IB-8c (basic PRP) and GSE interactions influenced by polysaccharides in wine	o		proanthocyanidins	procyanidin tetramers, pentamers, gallate	dynamic light scattering (amount of precipitation)	31.2 mg/L tannin: 0.6-5mg/L IB8c and 3.12mg/L IB8c: 19.5-46.8 mg/L tannin; pH 5.0, 12% ethanol, 20C	20.8 uM needed for precipitation of PRPs. Plateau of PRP binding at 3.12 mg/L (tannin/IB8c molar ratio is 35); this means that at a given concentration of tannin, PRP are bound to a maxima (multiple PRP per tannin) and then decrease at a certain concentration- diffuse). At a higher tannin to protein ratio, there is also a plateau of precipitation. At increasing ionic concentrations, precipitation

								increases; explained by increasing hydrophobicity (polyphenol rings stack)
(54)	Affinity of GSE twoard PRP in whole saliva	o		proanthocyanidins	dimers, trimer,tetramer (average polymerization 3.2; mW 936)	HPLC, SDS-PAGE, tryptic digestion, Mass spec and top down analysis/MALDI-TOF	pH 5.0, 20C, 12% ethanol/saliva (0.00-1.5 mM GSE in saliva)	histatin, statherin, aPRP first bind, then bPRP and gPRP at higher concentrations (min @ 1.25)
(55)	binding affinity of different PRP to hydrolyzable vs. condensed tannins	o	x	Quebracho tannin vs. tannic acid	not noted	competitive binding assay at different concentrations of tannin	pH 7.4, 10 µg protein and tannin, 37C. Stability testing in HCL with or without pepsin for 210 minutes; 7.4 c/trypsin/chymotrypsin/carboxypeptidase	Plateau of precipitation at2- 3 µg of condensed tannin/tannic acid: more bound condensed tannin vs. tannic acid (40 µg /assay vs. 30 µg /assay. No glycosylated binding of condensed PRP, some of tannic acid, most binding to BPRP. At pH 2.0, 93% of complexes were insoluble (condensed tannin) vs. 71% hydrolyzable; small intestine c enzymes 72% insoluble vs. 53% hydrolyzable
(67)	comparison of bioavailability of tannins with and without PRP	o		tannic acid	tri, tetra, pentagalloyl glucose	Caco-2 cell transport with/without PRP presence	5-90 uM 5GG concentration	Increased concentration of 5GG decreased absorption, addition of Ib4 to fraction decreased

								absorption 8-9 fold (90uM 5GG). Precipitation of 5GG 4.5:10 5GG:IB4
(56)	concentration effects on binding; stereochemistry effects	o	x	epigallocatechin, gallic acid, 5GG	pentagalloyl glucose, monomers	tannin protein binding competition assay	PRP isolated from whole saliva as 0.1 µg/ml solution of saliva or with buffer (pH 9.6) with varying concentrations for 5-20 min.	higher proline content to bPRP and gPRP (0.11-1.22 µM displacement constants- which means that there was not much required to bind; vs. 0.54 um lowest in bacon and Rhodes 1998; in proanthocyanidin monomers to 350 µM for highest-hydrolyzable are low); galloylation decreases displacement constants
(46)	dimer (B2) vs. pentagalloyl glucose vs. trigalloylglucose vs. proanthocyanidin monomer vs. epicatechin vs. propyl gallate	o	x	see topic	see topic	NMR	40 mM B2, 50 mM PGG/epicatechin: 0.5ml 4mM PRP or 2mM PRP pH 3.8	N terminal proline shifts this is calculated by chemical shifts (binding site for tannin); the hydrolyzable tannins had H bonding (protons in tannins bound to protons of PRPs); not true of condensed tannins (non-heme iron binding is protonated); Ka B2 = 300/M vs.

PGG 241, TGG 28, epicatechin 39; increased ring structure and size leads to cross linking of polyphenols bound to PRP; does not occur in hydrolyzabl e tannins= also, larger size of polyphenol reduces N terminal sites that can bind on PRP but increase hydrophobic interactions (increasing precipitation), and the proline residues at the N terminal then functions to not only bind galloyl group, but to allow for secondary interactions with other galloly functions. Predominant force for binding is hydrophobic ; essential polyphenol site for binding is a proline residue with an amide bond and amino acid residue; polyphenols form multidentate bonds: the TGG PGG

								B2 polyphenol curves showed that there is cooperative binding between aromatic functions on polyphenols and the neighboring polyphenol sites that are on the PRP peptides; noted that polyphenols start to self-associate when bound: larger compounds more than smaller
(47)	B1, B3, C2 procyanidins and PRPs to look at the process of binding	o		B1/B2 dimers, C2 trimer	B1/B2 dimers, C2 trimer	NMR	pH 3.5; 0.5-20mM PRP (exp 1) 15.7mM tannin, 1.7mM protein	At below tannin concentration 10 mM the interaction is specific, then second phase at 10 mM there is a linear chemical shift with tannin addition (non-specific interaction where tannins exist in aggregates); longer proteins are better, due to wrapping of the PRP around the tannin. Again, Kd C2 > B1 > B3. PRP IB9 binds to 2-3 tannins each
(68)	types of tannins and the quality	o	x	proanthocyanidins	dimers (B1-9) and trimer C1, B2 3-O	nephelometry (stereochemistry)-	12% ETOH, pH 5.0 or 3.5; 760 µg procyanidin	Stabilization of precipitates at 40

	of binding with PRPs				gallate, epicatechin from GSE	tannin specific activities (TSA), nephlos turbidity units (NTU)	s, 48 µg PRP (15:1)	minutes; stereo specificity mattered for binding: c6 vs. c8 binding; gallic acid esterificatio n (Table 1: TSA) explored. NOTED THAT SMALL POLYPHE NOLS BIND BUT DO NOT CROSSLIN K; THAT THE PYRANIC RING STRUCTU RE OF FLAVAN 3-OL INCREASE S ABILITY TO INTERACT WITH PROTEINS. BIGGER POLYMER S BIND WITH MORE AFFINITY. ALSO, C8 BONDS > AFFINITY THAN C6 BONDS.
(48)	understandi ng effect of gallolylation , polymerizati on, and B ring hydroxylatio n on PRP binding	o	x	EgC, ECG, B2, B2 3-O gallate	dimers, monomers	ESI-MS	protein polyphenol 1:10, 24C, pH 3.2	Galloylation favors binding. Binding is specific to tannins (did not bind to compound similar in structure); binding in phases: 1) dissociation is minimal 2) log-linear dissociation 3) plateau (how strong is the bond): dimers >

							monomers; OH group on the B ring = improved stability. Importance of features: B ring hydroxylated < galloylation < degree of polymerization; procyanidins are mostly composed of epicatechin and catechin units
(57)	BPRP with various wavelengths against polyphenols	o	flavonoids, oligomeric flavan-3-ols	flavonoids, oligomeric flavan-3-ols	ESI-MS	1:1 peptide polyphenol conditions (gas) 3:1 peptide: polyphenol in 10% ETOH for 1 hour	C2 > B2 > quercetin > B1 > B4 > B3 > epicatechin > catechin; larger molecules display greater affinity; stereochemistry at C3 makes a difference (epicatechin (2R, 3R)/catechin (2R, 3S); dimers > monomers; C2 stereochemistry matters (2R > 2S); OH groups on the B cycle > not hydroxylated; B cycle branching position (B cycle bound to 2C of flavonoid skeleton greatest); increased length of the PRP peptide increases binding; they hypothesize

								d that OH groups and stereochemistry of the OH groups promoted H bonding, increasing interaction with PRPs
(65)	influence of gastric digestion on PRP tannin complexes	o		GSE	catechins, gallic acid, procyanidin dimers, trimers, tetramers, pentamers, hexamers/ galloyl derivatives	HPLC, in vitro digestion	saliva: wine ratio of 2:1 (well justified) pH 3.5; pepsin c pH 1.7 for 2 h	Tannin/SP complexes that are monomers, dimers, trimers are disrupted by gastric digestion. Tetramers, pentamers are more resistant; statherin, aPRP bind more potently, then gPRP, then bPRP
(49)	influence of EgCG on IB5	o		EgCG	EgCG	MS/ SAXS, DLS	pH 5.5 ; protein = .336mM (1-3.5 mg/ml), stimulated to mimic saliva	Threshold concentration of tannins, below this, the DLS intensities were the same as the pure protein solution; at threshold, there was an almost exponential increase in precipitation with increased concentration. At a higher concentration, it took much more EgCG to precipitate proteins; PRP can bind 1-8 tannins depending on the concentration, at higher concentration of tannin,

1 PRP can bind up to 14 tannin molecules meaning that the 'n' binding sites does not correlate to tannin binding at higher tannin concentrations because the tannin 'stacks'. E50 K 1-8 are similarly stable, greater than this is a different interaction (tannin stacking- hypothesized by authors); binding sites are independent and have free energy; found that even when not precipitated, PRP were binding a significant amount of tannin (up to 3 per molecule- could be why precipitate analysis does not show bPRP although these seem to bind more potently in other experiments - not hitting threshold); at precipitation, there is aggregation that may be attributed to

								the multidendate nature of tannins (crosslinking) that conserves PRP measures; the limitation in aggregation continuing are 'poison' PRP that do not carry enough tannins to continue aggregation (cannot crosslink); tannins move fairly easily on the PRP polypeptide chain, and thus will dissociate easily when population has not been met; in fact, at low ionic concentrations, tannins and PRP repel each other
(61)	influence of pH on tannin salivary protein binding	o	x	hydrolyzable and condensed extracts, catechin, tartaric acid, gallic acid	mixed in extracts, not specified	diffusion precipitation interaction assay	pH 3.5 vs. pH 7.0	pH 3.5 precipitates tannins > pH 7.0
(53)	influence of tannin binding on PRP conformation	o		EgCG	EgCG	mass spectrometry coupled with ion mobility	IB5 and 12% ETOH, pH 3.5; molar ratio of 1:20 IB5:EgCG	Poisson binding (continuous and independent binding in favor of 'non cooperative sticking process') binding 1-9 tannins; at binding of 1-7 tannins, IB5 structure is stable (in its still

							unstructured state) at 10 ligands, a more compact conformation is formed that is smaller than the unstructured protein; several tannins are required to stabilize the folded state. Folding is favored by H bonds between PRP and tannin OH groups on tannins that stack together
(66)	influence of polymerization on precipitation (as a surrogate of affinity)	o	GSE	monomers, dimers, trimers	HPLC DAD	37C incubation, 1,2,8 ml of saliva mixed with 40 ml GSE or 20/40 ml GSE sipped	increased tannin concentration increased precipitation; of LMW tannins, ECG precipitates effectively; at low-EGCG - protein ratios, binding is progressive in suspension without binding, at higher ratios, precipitates
(4)	glycosylation and influence on tannin binding	o	GSE	monomers and galloylated units	SDS-PAGE	10% ETOH, 20 µg purified protein or 40 µl saliva, different concentrations of tannins	20.57% of GSE had galloylated units; compared astringency trained and untrained individuals: gPRP more prevalent in trained saliva; 30% of tannins were

precipitated by the astringency trained individual (most remained in supernatant), 85% in the astringency untrained (all precipitated); precipitate was dose dependent and selective; the 'nature of the saliva' significantly predicted the precipitation of tannins as well; polymerization of tannins positively correlated with precipitation; however, trained saliva was able to precipitate smaller polymers than the untrained despite similar AMOUNTs of protein in saliva; bPRP most commonly precipitated and the trained participant had higher quantity than the untrained; there were no proteins in the untrained supernatant; there were abundant glycosylated

							PRP in the trained supernatant. PRP themselves (purified) are more effective at binding tannins than whole saliva however, PRP affinity greater than alpha amylase. At low tannin concentration, all polymers were bound, at higher concentration, selective to higher order polymers. Glycosylation seems to stabilize the complex to tannin outside of precipitation. At low tannin concentration, glycosylation may stay soluble by decreased hydrophobicity, while at higher concentrations, hydrophobicity is reduced, and the complex precipitates.
(42)	interactions of IB5 with EGCG	o	EgCG	EgCG	DLS, isothermal titration microcalorimetry, dichroism	pH 3.5 ionic strength at 100 mM (same as saliva mixed with wine) 6.4 or 12.8 mM EgCG solution with 0.25-2 mg/ml IB5	PRP are random and unfolded; At low protein concentration, no aggregates occurred until a high tannin ratio then would slowly start. At a next

								saturation, there was a rapid increase in aggregation and further increases in tannin led to immediate precipitation. If the solution was started at very high tannin concentration, immediate aggregation and precipitation. At a lower tannin ratio (more protein), the precipitation occurred at a lower threshold. 3 stages at low concentration: 1) saturation of protein residues with tannin 2) bridging of the proteins by tannin and hydrophobic stacking of tannins 3) aggregation and phase separation. Protein folds around the tannin and then later tannin bridges the condensed protein aggregates.
(58)	influence of tannin stereochemistry on PRP binding	o	GSE	monomer free; mostly catechin, epicatechin, epicatechin 3-O-gallate	ITC	10% ETOH, 40 µl saliva and different amounts of tannins	Individual salivary profiles differed greatly throughout the day and by individual;

								PRP came out of supernatant as tannin concentration increased. Glycosylation increased threshold for precipitation but did not impede affinity. Larger proanthyocyanidins precipitate first, LMWP stayed in supernatant longer.
(59)	influence of glycoprotein on binding	o		sorghum and Quebracho	multiple polymers of PACs		pH 4.8, methanol, acetate buffer	< binding to sorghum than Quebracho, deglycosylated proteins with less affinity suggesting that is important; glycosylation increased solubility (increased tannin binding in solution)
(62)	influence of pH, fructose, ethanol on precipitation of salivary proteins	x	x	tannic acid or wine	multiple polymers of PACs	SDS PAGE	25C with either tannic acid or wine (pH 3.6/3.6) with 2-4-6 g/L fructose, manoproteins, tartaric acid. 11% ETOH	Lower pH increased binding > decreased hydrophobicity and increased hydrophobic binding; this is more prominent in wine than in tannic acid (increase in 21.7 vs. 11, 9 respectively). ETOH/fructose negatively effects precipitation (disruption of H bonds between

								polyphenols and proteins; ETOH may change conformation of PRP and solubility of tannin and change reactivity; carbohydrate molecule CH2OH may also reduce binding sites to proteins by binding to NH3 proline surfaces on proteins; as pH decreases, wine more commonly precipitates before tannic acid
(50)	PRP alleviation of intestinal enzyme binding by tannins	o		EgCG	EgCG	in vitro digestion stability, absorbance for reactivity, SDS PAGE, HPLC	gastric juice pH 2.07 c pepsin; duodenal juice pH 7.8 c/ lipase, pancreatin	with PRP: IC50 of chymotrypsin, trypsin, lactase increased 2-3x; maximum pH effect of PRP at ~5-6, lower had increased protein binding, higher reduced (Figure 3); PRP bound and recovered EgCG >2x in duodenal digestion, no effect in gastric or control solutions; PRP are resistant to proteolytic digestion
(60)	saliva and hydrophobic	o	x	wine tannins	multiple polymers of PACs	TRAP	1:5 saliva to wine ratio with acidic	Moderate removal of polyphenol

	interactions with tannins						extraction (1% TFA)	with 1 ml saliva
(45)	binding mechanism tannins	o	x	EGCG, EGC, PGG	penta and tetra gallotannins	NMR, diffusion, dynamic light scattering	20mM polyphenol solutions 2mM PRP (mouse PRP); pH 3.8	Preferential binding to polyphenols at the proline residues; but interactions also occur at the arginine and phenylalanine side chains; polypeptides self-associate and so the number of binding sites does not equal the concentration of solution. The strongest self-association is PGG; ECG and EGCG same self-association suggesting that H bonding is not primary mode of self-association, but hydrophobic bonds are. Most binding sites on PRP can be filled simultaneously; arginine strengthens the bonds of tannin-phenylalanine/proline, but does not independently bind. Longer PRP 'wrap' around tannins (higher affinity); at

lower temp, multidendate binding. Binding affinity: PGG>TGG>TG>EGCG~EGG. Stages of binding: reversible hydrophobic binding of polyphenol that gives a soluble complex due to stacking, particularly of hydrophobic galloyl rings. 2 addition of polyphenol, and two peptides are crosslinked by two polyphenol protein interactions, and the complex becomes insoluble; this is different for different polyphenols, for example: 3:1 EGCG vs. 0.5:1 PGG 3) phase separation and aggregation of insoluble complexes determined by the surface charge (EGCG: pH 3.8; 50% monomers, 2.3/3 binding sites occupied (monomer and dimer binding) vs. PGG 64% monomeric,

								0.4/3 binding sites are occupied at precipitation.
(52)	type of polyphenol tannin binding	o		catechin, epicatechin, B2, C1	dimers, trimers, monomers	RPHPLC	pH 3.2 either 1:1 or 1:2 v/v mixture; 37C 90 min incubation	Flavone type and concentration of protein effect precipitation, but there is also a significant interaction between the compound and saliva; not all flavones behave the same way in saliva; C1 < epicatechin < catechin < B2; protein concentration> = more precipitation. Increased concentration of polyphenol = more precipitation. More protein had pronounced effect on polyphenols with less affinity (C1 and epicatechin); most pronounced reduction in affinity with smaller molecules. In lower concentrations of monomers, increased precipitation of protein
(64)	interaction of PRP with galloyl ring and pyrrolidine ring	x		tannic acid	monomers, dimers, trimers	NMR	1:0-1:5.6 ratio of PRP to polyphenol	Increase in temp or decrease in pH solubilize complexes. Changes in

							chemical shifts suggested binding rather than conformational changes; proline is key binding site. More complexes bound = less dissociation.
(63)	stereochemistry of tannins	o	EgCG	EgCG	NMR	pH 3.5; 100 mM and 5 mM NaCl	Ionic concentration changes conformations of PRP to be more structured at 100 mM, although both have unfolded conformation and there is a higher level of disorder in the PRP at 100 mM. On binding to EGCG, secondary structure of the protein was changed at 100mM but not 5 mM; NMR modeling suggests beta sheet structure of Proline residues after tannin binding; residual sites are preferential for binding
(43)	mechanism of binding	o	EgCG, B2, B23OG	dimers, EgCG	MS-SAXS	Interaction of IB5 with various tannins; water/ETOH 88:12 at pH 3.3. 10 µM IB5 to tannin solution for ratio of 1:10 protein: polyphenol	Polyproline helix and repeat proline sequences are most preferentially bound, they are surrounded by glycine and alanine which give

								more flexibility and establish increased number of H bonds. While PRP are unstructured, the polyproline regions are rigid and provide anchoring points to tannins. Shorter PRPs do not change conformation around tannins. Noted that extended tannin structure also precipitated binding
(44)	mechanism of binding	o		B3	Trimer	MS-circular dichroism	pH 3.5	tannin binding does not modify peptide folding; at low concentration, 34% TII helix, 66% extended and random colloid conformation; there are extended conformations with a type II helix with 7 residues (IB7); initial H bonding to proline residues; the PRP studied was amphiphilic, tannin binding to the hydrophilic face (H bonds)

Appendix C - Inclusion Criteria

1. Studies that include long-term effects of tannin consumption on PRP outcomes and non-heme iron bioavailability
2. Studies that include at least two key words, tannin AND salivary proline-rich protein OR non-heme iron
   AND

   3.) Studies that explore biochemical modeling for binding mechanisms of tannins and PRPs

   4.) Studies that explore binding affinity for PRP and tannins

   5.) Studies that compare mechanism of non-heme iron-tannin chelation to Tannin-PRP binding

Exclusion criteria:

1.) Validity studies for measurement tools
2.) Assessment studies of food tannin content or food non-heme iron content
3.) Studies that include surrogate PRP proteins (albumin, BSA, amylase, gelatin)
4.) Studies that explore PRP without tannin or non-heme iron binding
5.) Ruminant modeling
6.) Specialized conditions: cancer, Sjogren's syndrome, burning mouth syndrome, hemochromatosis
7.) Studies did not present comparative findings (descriptive or qualitative studies describing astringency or optical tongue without biochemical mechanisms/affinity studies)
8.) *In vivo* animal studies that did not explore non-heme iron bioavailability with PRP and tannins

Appendix D - Clinical Screening Questionnaire

Tannin-Dose Response Trial Screening Questionnaire

Name: DOB:

Height: Weight:

1.) Medical history:

Are you currently taking any prescribed medications? (please list the name, how often you take, and the dose)

Are you currently taking any non-prescribed medications, including multivitamins, herbs, mineral supplements, vitamin supplements? (please list the name, how often you take, and the dose)

Are you currently taking any iron supplementation (including prescribed or over the counter iron supplements)? (please list what you are taking, and your current dose)

Do you have any food allergies? (please list your food allergies, and reaction to the food)

Are you currently pregnant, planning to become pregnant, or breastfeeding?

Are you a current tobacco (smoking, chewing) user?

How often do you consume alcohol (circle one)?

Daily Weekly Monthly Never

If you consume alcohol, how much do you consume, per occasion? One drink equals 1 oz of liquor, 12 ounces of beer, or 4 oz of wine (circle one).

1 drink 2-3 drinks 3 or more drinks

Please check the following medical conditions that you may have:

History of gastrointestinal illness, including:

_____ Irritable bowel syndrome

_____ Irritable bowel disease (Chrohn's, Ulcerative Colitis)

_____ History of colostomy/colectomy

_____ Diagnosed condition of malabsorption (including diagnosis of chronic diarrhea)

_____ Other, please list:

History of oral illness, including:

_____ Sjogren's syndrome

_____ Burning mouth syndrome

_____ Active mouth bleeding, oral ulcerations

_____ Periodontal or gum disease

_____ Multiple or severe cavities

_____ Other, please list:

History of blood disorders, including:

_____ Sickle cell anemia

_____ Alpha or Beta Thalassemia

_____ Anemia of chronic disease

_____ Other, please list:

Appendix E - Effect of previous dose on dose-response

Hemoglobin:

Table of Hb by previous				
Hb(Hb)	previous(previous)			
Frequency	0	0.03	1.5	Total
drop	1	3	0	4
improve	1	3	1	5
maintain	0	0	2	2
Total	2	6	3	11

Statistic	DF	Value	Prob
Chi-Square	4	6.9667	0.1377
Likelihood Ratio Chi-Square	4	7.8869	0.0958
Mantel-Haenszel Chi-Square	1	5.2673	0.0217
Phi Coefficient		0.7958	
Contingency Coefficient		0.6227	
Cramer's V		0.5627	
WARNING: 100% of the cells have expected counts less than 5. Chi-Square may not be a valid test.			

Fisher's Exact Test	
Table Probability (P)	0.0173
Pr <= P	0.2251

Ferritin

Table of Ferritin by previous				
Ferritin(Ferritin)	**previous(previous)**			
Frequency	**0**	**0.03**	**1.5**	**Total**
drop	2	3	0	5
improve	0	2	3	5
maintain	0	1	0	1
Total	2	6	3	11

Statistic	DF	Value	Prob
Chi-Square	4	6.6000	0.1586
Likelihood Ratio Chi-Square	4	8.4281	0.0771
Mantel-Haenszel Chi-Square	1	1.2447	0.2646
Phi Coefficient		0.7746	
Contingency Coefficient		0.6124	
Cramer's V		0.5477	
WARNING: 100% of the cells have expected counts less than 5. Chi-Square may not be a valid test.			

Fisher's Exact Test	
Table Probability (P)	0.0216
Pr <= P	0.1775

% Max iron absorption

Table of iron_abs by previous				
iron_abs(iron abs)	previous(previous)			
Frequency	0	0.03	1.5	Total
drop	0	1	2	3
improve	2	5	1	8
Total	2	6	3	11

Statistic	DF	Value	Prob
Chi-Square	2	3.4375	0.1793
Likelihood Ratio Chi-Square	2	3.6651	0.1600
Mantel-Haenszel Chi-Square	1	2.9584	0.0854
Phi Coefficient		0.5590	
Contingency Coefficient		0.4880	
Cramer's V		0.5590	
WARNING: 100% of the cells have expected counts less than 5. Chi-Square may not be a valid test.			

Fisher's Exact Test	
Table Probability (P)	0.1091
Pr <= P	0.2061

iAUC:

Table of AUC by previous				
AUC(AUC)	previous(previous)			
Frequency	0	0.03	1.5	Total
drop	0	4	1	5
improve	2	2	2	6
Total	2	6	3	11

Statistic	DF	Value	Prob
Chi-Square	2	2.9333	0.2307
Likelihood Ratio Chi-Square	2	3.7009	0.1572
Mantel-Haenszel Chi-Square	1	0.1980	0.6563
Phi Coefficient		0.5164	
Contingency Coefficient		0.4588	
Cramer's V		0.5164	
WARNING: 100% of the cells have expected counts less than 5. Chi-Square may not be a valid test.			

Fisher's Exact Test	
Table Probability (P)	0.0974
Pr <= P	0.4156

Appendix F - Individual level iron absorption data

Participant	Value	0.03 g Week 0	0.03 g Week 4	0.25 g Week 0	0.25 g Week 4	1.5 g Week 0	1.5 g Week 4
1	Hemoglobin (g/dL)	12.9	12.9	13.8	13.8	13.6	13.6
	Ferritin (ng/mL)	30.0	28.0	27.0	36.0	35.0	44.0
	% max iron abs	1.7	2.7	9.2	9.6	-1.6	3.7
	iAUC (µg/dL*hr)	2.0	19.2	44.3	53.8	4.0	18.5
2	Hemoglobin (g/dL)	13.6	13.9	13.1	12.7	13.6	14.2
	Ferritin (ng/mL)	7.0	8.0	8.0	7.0	10.0	9.0
	% max iron abs	16.2	24.2	9.3	17.5	13.9	17.3
	iAUC (µg/dL*hr)	1208.6	652.8	789.9	1146.9	382.8	417.4
3	Hemoglobin (g/dL)	13.7	14.1	13.7	13.7	13.6	13.6
	Ferritin (ng/mL)	24.0	28.0	19.0	38.0	22.0	28.0
	% max iron abs	21.7	16.9	24.6	14.2	16.0	13.2
	iAUC (µg/dL*hr)	270.8	63.3	171.0	55.7	103.1	129.0
4	Hemoglobin (g/dL)	13.6	12.9	13.2	13.4	12.9	13.0
	Ferritin (ng/mL)	23.0	27.0	29.0	26.0	22.0	19.0
	% max iron abs	9.2	0.9	7.9	-6.5	11.5	-2.7
	iAUC (µg/dL*hr)	82.3	3.7	114.1	-49.6	141.8	-26.0
5	Hemoglobin (g/dL)	12.1	12.1	12.0	12.1	11.7	11.6
	Ferritin (ng/mL)	23.0	21.0	26.0	20.0	26.0	30.0
	% max iron abs	-11.5	0.9	-2.6	3.6	-7.1	2.1
	iAUC (µg/dL*hr)	0.0	-29.2	0.0	45.3	-60.5	27.6
6	Hemoglobin (g/dL)	14.0	13.2	13.1	12.9	13.2	12.7
	Ferritin (ng/mL)	38.0	38.0	27.0	24.0	27.0	43.0
	% max iron abs	23.9	22.2	23.9	25.0	18.9	15.6
	iAUC (µg/dL*hr)	123.1	201.2	214.1	253.1	331.9	53.4
7	Hemoglobin (g/dL)	14.0	14.7	14.9	14.2	15.0	15.5
	Ferritin (ng/mL)	88.0	70.0	94.0	66.0	111.0	109.0
	% max iron abs	3.8	10.3	6.0	15.8	1.0	2.2
	iAUC (µg/dL*hr)	7.9	35.0	10.8	26.0	-4.2	4.3
8	Hemoglobin (g/dL)	12.7	13.6	12.6	13.2	12.8	12.8
	Ferritin (ng/mL)	19.0	26.0	22.0	22.0	24.0	22.0
	% max iron abs	28.8	5.2	20.4	23.5	17.4	15.3
	iAUC (µg/dL*hr)	389.4	47.2	300.6	319.4	231.3	342.6
9	Hemoglobin (g/dL)	12.8	13.0	13.1	13.2	13.0	13.0
	Ferritin (ng/mL)	23.0	44.0	30.0	34.0	30.0	21.0
	% max iron abs	25.6	17.5	17.0	8.9	26.8	24.2
	iAUC (µg/dL*hr)	1770	600	1515	2835	1065	1440
10	Hemoglobin (g/dL)	12.9	13.6	13.9	13.8	13.1	12.9
	Ferritin (ng/mL)	14.0	18.0	17.0	19.0	14.0	22.0
	% max iron abs	9.4	19.2	9.0	12.9	22.7	15.4
	iAUC (µg/dL*hr)	62.8	327.2	91.9	91.3	336.3	85.5
11	Hemoglobin (g/dL)	12.8	12.6	13.0	14.1	14.3	14.4
	Ferritin (ng/mL)	100.0	157.0	95.0	118.0	119.0	142.0
	% max iron abs	10.9	-3.0	8.2	12.1	4.0	6.5
	iAUC (µg/dL*hr)	23.6	3.3	24.9	29.3	8.8	14.1

iAUC: incremental iron under the curve