Assessment of iron bioavailability and protein quality of new fortified blended foods in broiler chickens

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Abstract

Fortified-blended foods (FBFs), grain-legume porridges (most commonly corn and soy), are frequently used for food aid purposes. Sorghum and cowpea have been suggested as alternative FBF commodities because they are drought-tolerant, grown locally in food aid receiving countries, and are not genetically modified.

The objective of this thesis was to determine the protein quality and iron bioavailability of newly formulated, extruded FBFs in broiler chickens, which have been suggested as a good model for assessing iron bioavailability. Five FBFs were formulated to contain whey or soy protein to compare protein quality, sugar, oil, and an improved micronutrient premix. These included three white sorghum-cowpea FBFs; two were extruded with either whey protein concentrate (WSC) or soy protein isolate (WSC+SPI) added, one was non-extruded (N-WSC). Two others were white sorghum-soy (WSS) and corn-soy (CSB14) FBFs. Two additional white-sorghum cowpea FBFs were reformulated and "over-processed" to contain no sugar, less whey (O-WSC) or soy protein (O-WSC+SPI), and less oil, thus producing a less expensive FBF. Two studies were performed using prepared (Prep) or dry (Dry) FBFs, along with the United States Agency for International Development (USAID) corn and soy blend FBF, CSB+, fed to chickens for 3 and 2 weeks, respectively; food intake, body weights, hemoglobin, and hepatic iron were assessed.

In the Prep study, new FBFs significantly increased caloric and protein efficiency compared to CSB+, despite similar food intake and body weight gain. In the Dry study, CSB+ significantly decreased food intake and caloric efficiency, with the exception of O-WSC+SPI, and nonsignificantly reduced body weight gain and protein efficiency compared to new FBFs.

CSB+ significantly reduced hepatic iron content compared to all FBFs in the Dry study, and was nonsignificantly decreased compared to new FBFs in the Prep study.

In conclusion, sorghum and cowpea FBFs performed similarly to corn and soy FBFs, suggesting these commodities are suitable replacements for corn and soy. Soy protein isolate (WSC+SPI) was an effective alternative to whey protein concentrate (WSC), suggesting SPI can be a less expensive protein supplement in FBFs. Surprisingly, non-extruded sorghum and cowpea (N-WSC) was equally efficacious to extruded WSC. However, N-WSC did not meet viscosity requirements and is not precooked, which limits its viability as an FBF. O-WSC+SPI resulted in poorer outcomes compared to other FBFs, which suggests the protein quality of cowpea may be inferior and the inclusion of whey protein is needed in this formulation, as O-WSC with whey performed similarly to other FBFs. Overall, new FBFs, with the exception of O-WSC+SPI, resulted in improved food efficiency and hepatic iron outcomes compared to CSB+, suggesting they are of higher nutritional quality. However, further research is needed to refine and identify the best FBF formulations.

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Dedication

To the vulnerable populations for which this research is intended to help, may you one day never have to experience the burden of undernutrition.

Chapter 1 - Background Information

Protein-Energy Malnutrition

Prevalence

793 million people globally are still undernourished, despite a decrease of 216 million over the last decade (1). The United Nations Children's Fund (UNICEF) estimates that half of under-five child deaths are due to undernutrition (3 million per year; 2). Undernutrition includes protein-energy malnutrition (PEM), which encompasses a group of disorders that include marasmus (inadequate protein and calorie intake, characterized by emaciation), kwashiorkor (inadequate protein intake with adequate calorie intake, characterized by edema), wasting, stunting, and underweight as well as mild, moderate, or severe acute malnutrition (SAM; edema presence); these conditions collectively contribute to the global prevalence of undernutrition (3). Stunting is the irreversible cause of chronic malnutrition associated with impaired cognition and work performance (2). In 1990-2015, stunting prevalence declined from 39.6 percent to 23.2 percent, however this still equates to 156 million children (2). Wasting is a form of acute malnutrition due to rapid weight loss or inability to gain weight, characterized by low weight for height. There are 50 and 17 million children globally who are wasted and severely wasted, respectively (2). Most stunted and wasted children reside in Africa and Asia, where the annual GDP losses near 11 percent due to malnutrition (4).

Causes

Many factors contribute to undernutrition including lack of education, child feeding practices, clean water and sanitation, as well as hygiene, gender inequality, and lack of access to healthcare (4, 5). However, poverty is the primary cause of undernutrition, which explains why the majority of undernutrition primarily occurs in low and middle income countries (2, 6).

In regard to protein-energy malnutrition, children with edematous kwashiorkor and marasmic-kwashiorkor may not be able to maintain body protein breakdown as those with non-edematous marasmus might, resulting in less amino acid supply for plasma proteins (albumin) for nutrient transport and acute phase response to infection, leading to higher morbidity and mortality rates (7). Edema presence occurs from excess accumulation of fluid in extracellular spaces due to capillary fluid filtration exceeding reabsorption (8).

Another determinant of PEM may be the availability of protein from certain foods. Dietary utilizable protein, rather than crude protein intake, may provide a better reflection of protein inadequacy prevalence or population impact risk (9), therefore is it important to provide highly utilizable protein in PEM treatments. The costly inclusion of whey protein concentrate in PEM treatments such as in food aid products has been questioned (10), and there is still little evidence to support whether plant versus animal source proteins are more adequate for PEM treatment (11).

Protein quality measurements

The protein quality measurement previously recommended by FAO/WHO is the protein digestibility-corrected amino acid score (PDCAAS), which assesses protein quality based on the limiting amino acid score and its fecal digestibility to meet demands for amino acid utilization from dietary protein in a specific reference group (12). Limitations have been identified, which include the method overestimating product protein quality with antinutritional factors, poorly reflecting amino acid usage beyond the terminal ileum by using fecal digestibility, lack of distinction between higher quality proteins due to value truncation at 100%, and validity of reference patterns (12, 13). The digestible indispensable amino acid score (DIAAS) has been recommended to replace the PDCAAS to address these limitations (12). This method allows for

better individual amino acid digestibility measurements from foods using ileal digesta, does not use a truncated score, and uses better defined reference patterns (12).

Iron Deficiency Anemia

Prevalence

Undernutrition also includes micronutrient deficiencies (MNDs). MNDs are often called "hidden hunger" because they can cause significant health impairment without displaying acute symptoms, but chronically increase growth, cognition and disease risk (14). Iron, iodine, vitamin A, folate and zinc, are the most predominant MNDs; iron deficiency is the most prevalent globally (14). Two billion people worldwide are anemic (30%), half of anemia prevalence is due to iron deficiency (15, 16). Iron (Fe) is essential for oxygen transport and cellular respiration by being a component of hemoglobin, myoglobin, enzymes, and cytochromes, which ultimately allows for optimal growth and cognitive function (6). The global age-standardized anemia prevalence has decreased by 21 percent from 1990 to 2013, however iron deficiency anemia (IDA) only improved by 4 percent during the same timeframe (17). The most vulnerable populations at risk for IDA are children under five, pregnant women, and all women of reproductive age (15 – 49 years) (6, 15, 16), and in 2011 an estimated 42.6% of under-five children, 38.2% of pregnant women, and 29.0% of non-pregnant women were anemic, although this is not specifically for IDA, this still translates to 528.7 and 273.2 million women and children, respectively (18). Geographically, the highest rates of IDA are in Africa where 62.3% of children are anemic; in South-East Asia 48.7 and 41.5% of pregnant and non-pregnant women, respectively, are anemic (18). Iron deficiency anemia consequences include maternal death, impaired physical and cognitive development, increased morbidity and mortality risk in children, and decreased work productivity in adults (6, 16). Iron deficiency alone results in the

highest rates of anemia-related disability (17). In 1994, the median annual economic loss due to IDA in 10 developing countries was averaged at $16.78 per capita (19).

Causes

Iron deficiency anemia can be caused by numerous interconnected individual and societal determinants. Individual-level determinates include inadequate iron intake or poor bioavailability, along with increased iron requirements during growth (19). Children become vulnerable to IDA because of increased iron requirements due to rapid red cell expansion (19). Women of reproductive age lose iron via blood loss in menstruation and childbirth, and increased fetal and placental iron requirements during pregnancy (19). Parasitic infections and infectious diseases such as malaria, tuberculosis, and HIV are other individual-level anemia risk factors (16). Beyond acute biological and infectious disease burden, several chronic diseases such as chronic kidney disease, chronic heart failure, cancer, and inflammatory bowel disease have been linked to IDA (15).

Non-heme iron absorption and regulation

Iron is needed for either functional, storage, or transport purposes in the body. The primary use for functional iron is for hemoglobin (Hb), an iron containing protein in red blood cells responsible for transporting oxygen through circulation. There are two forms of iron, heme and non-heme, both are not highly bioavailable (12-25% and <5%, respectively) (6). Non-heme iron is primarily found in plant and iron-fortified foods, and recommended dietary allowances (RDAs) for iron are as high as 18 mg per day for women 19-50 years (20). It has been estimated that 1 to 2 mg of iron per day is lost due to enterocyte sloughing, daily dietary iron absorption aims to balance this loss (21). Iron recycling from senescent red blood cells also meets most of the iron needs in a healthy adult (22). Most dietary iron is found in the oxidized ferric form,

which has low bioavailability, and therefore is reduced to its ferrous form by duodenal cytochrome B (DcytB) (22). Ferrous iron is transported into the apical cell membrane of the enterocyte by divalent metal ion transporter 1 (DMT-1) (22). Iron is then either stored within cytoplasmic ferritin, or ferroportin (FPN1) exports iron across the basolateral membrane of the enterocyte and into circulation (22). In circulation, hephaestin oxidizes ferrous iron back to its ferric form to bind to plasma transferrin for transport and tissue-level utilization (22). Iron does not have regulated mechanisms for excretion, therefore absorption is controlled systematically by the liver hormone hepcidin, and at the tissue level (22).

Factors affecting iron absorption

Many factors affect iron absorption, primary factors include pH, inhibitors such as phytates and tannins, enhancers such as ascorbic acid, and competitors such as lead (23). Lower pH has been cited to increase iron absorption by enhancing its solubility (23). Inhibitors chelate iron and prevent absorption, however a systematic review reported this effect may be not so clear (24). Enhancers such as ascorbate reduce iron to its more available form, ferrous iron, thus increasing uptake, while competitors compete for the same uptake mechanism, thus decreasing absorption (23).

Measurements

Iron can be categorized into functional, circulating and storage iron (25). In conditions of negative iron balance, storage iron is first depleted (iron depletion), which then leads to depletion of circulating iron (iron deficiency), and finally iron anemia is present when functional iron is depleted further (25). Interventions for iron deficiency analysis, and thus iron assessment, can be divided into population vs individual level analysis. Often times, the cost required for assessing individual iron measurements is too high, therefore more feasible assessments aimed at the

population level are used. According to a WHO and CDC joint consultation on iron status assessment, hemoglobin concentration is the primary measurement for severity of iron deficiency at the population level (26). WHO criteria for anemia in adults, children 6 months to 6 years, and children 6 to 14 years are 12.5, 11.0, and 12.0 g/dL, respectively (27). Serum ferritin and transferrin receptor are biomarkers that allow for an accurate measurement of iron status at the individual level, however, ferritin levels increase during inflammatory states, such as in response to infectious disease in developing countries. Transferritin receptor does not increase during inflammatory states, which gives context to ferritin levels (26). In response to an intervention, serum ferritin is the best indicator of effect on iron deficiency and should be measured with hemoglobin (26).

Food Assistance

Global food assistance

In 2012, more than 5 million metric tons of food aid was delivered globally (28). Food assistance is used to combat undernutrition in emergency settings and among vulnerable populations including children, pregnant women, and those with tuberculosis or HIV/AIDS. Types of food assistance include cash or food vouchers, cash transfers for food, and local and regional purchase (29). Food aid distributed by the USA is delivered as in-kind food aid under Tittle II which provides the supply chain for procuring commodities, processing them, then shipping them to the recipient country (29). The United State Agency for International Development (USAID) is the largest provider of food assistance globally, and in 2015 provided over $2.5 billion in funding which included 1.2 million metric tons of in-kind food assistance (30). In 2012, Sub-Saharan Africa received 63% of food assistance, totaling 3.14 million metric tons (28).

Types of food aid products

Food aid products are meant to prevent, or treat, different types of undernutrition, primarily as a supplement to the regular diet, and consist of ready-to-use therapeutic foods (RUTFs), high energy biscuits, micronutrient powders (sprinkles), compressed food bars, and the most common product, fortified blended foods (FBFs) (31). RUTFs are lipid-based, nutrient-dense, peanut butter-like products used to treat moderate malnutrition, primarily in emergency settings (31). High energy biscuits, micronutrient powders, and compressed food bars are less commonly distributed, but provide a ready-to-eat protein and micronutrient fortified supplement when resources for food preparation are scarce (31).

Fortified blended foods

FBFs are cereal-legume based, micronutrient-fortified, partially precooked porridge products used to supplement adequate protein (31). FBFs were originally developed by the USA in the 1960s as a corn soy milk to supply 25% of the energy needs of preschool-aged children in developing countries (32). In the 1980s, the first corn soy blend (CSB) was introduced as a "one-size-fits-all" product for diverse population groups, and since then no significant updates have been made in its formulation and processing (32). The current most widely used USAID FBF, corn-soy blend plus (CSB+), incorporates heat-treated corn and soybeans with a vitamin-mineral premix and is recommended to be consumed with fortified vegetable oil (33). It is intended for complementary use by children 6-24 months, and pregnant and lactating women, to prevent micronutrient deficiencies, wasting, and stunting (33). It can also be used for the treatment of moderate malnutrition among 6-59 month old children (33). FBFs have been shown to be a more cost-effective option compared to an RUTF in the treatment of MAM (34).

Recommendations to improve FBFs

In 2011, the USAID/Tufts University Food Aid Quality Review (FAQR) Report was published to outline recommendations for improving food assistance provided by the US, specifically focusing on FBFs (35). The primary purpose for FBFs are that they should be "energy-dense and rich in micronutrients, easily digestible and palatable, and able to be prepared relatively quickly, i.e., with minimal cooking" (35). Therefore, the primary recommendations to improve FBFs were centered on these three product purposes. These improvements include upgrading the macro and micronutrient content, using more culturally acceptable food aid, considering alternate processing methods that improve nutritional efficacy, and improving the programming of food aid delivery and acceptability (35). To improve the macronutrient content, it was suggested that whey protein concentrate (WPC) be added to increase protein quality, and the fat content be increased by preparing CSB with fortified vegetable oil, thereby increasing the overall energy content (35). To address micronutrient content improvements, recommendations were made to reformulate certain levels of vitamins and minerals, including combining two forms of iron, NaFeEDTA and ferrous fumarate, to improve iron absorption (35), which is of particular interest. Sorghum was recommended as an alternative cereal to develop new cereal-based FBFs due to its "acceptability in Africa, its relatively low price, and its acceptability among host governments", as well as being used in combination with other pulses to produce a sorghum-pea blend, for example (35). Collaboration with industry to utilize different processing methods, such as extrusion, could improve FBF shelf life, nutrient availability and quality (35). CSB14 was introduced in this report to replace CSB13 as a model FBF that would meet the recommended improvements (35).

Commodities

Sorghum

Sorghum is the fifth most produced cereal globally (36), and the second most important cereal in Africa (37). The US is largest producer, however 90 percent of the world's sorghum area is in Africa and Asia (36). Sorghum, along with millet, provides energy to more than 300 million people in developing countries as part of their staple diet (37). Sorghum is drought-tolerant, and has been shown to have less dryland yield variation and sensitivity to environmental conditions compared to corn (38). It is widely accepted by most food aid receiving countries due to its typically cheaper price compared to corn, and it is not genetically modified which is desirable to many countries (38). Sorghum is primarily used for food in porridges, breads, and beverages, however in some countries it is mainly used for animal feed (36). The nutrient composition of sorghum is similar to corn (38), and could therefore be a feasible option to replace it in FBFs. Sorghum has also been proposed as a functional ingredient due to its ability to manage glucose and insulin levels in healthy adults (37).

Cowpea

Cowpeas, or black-eyed peas, are a grain legume primarily produced and consumed in Africa (63%) (39). Cowpea is drought-tolerant, and rich in protein (23-32%) and lysine (427 mg/g N) to complement sorghum or corn in a FBF (40).Compared to soy, cowpea contains lower total protein and fat content, but higher starch content (41). Cowpea can be intercropped with sorghum, and improves soil fertility and cropping systems through nitrogen fixation and its deep roots, which preserve moisture and stabilize soil (42).

Antinutritional factors

Antinutritional factors occur naturally in foods or are formed through protein processing, and are known for reducing protein digestibility, amino acid bioavailability and thus overall

protein quality of foods, as well as decreasing mineral bioavailability (43). In developing countries where there are less refined foods, antinutritional factors play an important challenge in improving macro and micronutrient availability from foods (43). Trypsin inhibitors, hemagglutinins, tannins, phytates, glucosinolates, gossypol, and uricogenic nucleobases are naturally occurring in legumes, cereals, oilseeds and certain protein products (43). Malliard reaction products, oxidized sulphur amino acids, D-amino acids, and lysinoalanine are formed during heat or alkaline treatments of protein products (43). Tannins and phytates are of particular interest due to their occurrence in potential FBF commodities, sorghum and cowpea. Sorghum and cowpea contain 0.5-72.0 and 1.4-10.2 g/kg tannin, respectively (43). Tannins in sorghum have been cited to decrease protein and amino acid digestibility by up to 23% in rats, pigs, and poultry (43). Sorghum also contains phytates (7 g/kg), which is comparable to corn, however cowpeas contain no phytates, which is a potential advantage for replacing it over soybean (26 g/kg) in FBFs (43). Phytates in foods have been cited to reduce protein and amino acid digestibility by up to 10% (43). Despite these disadvantages, tannins and phytates may have antioxidant, cancer fighting, and cardiovascular health benefits (44).

Extrusion processing

Extrusion processing involves moisture, pressure, high temperature, and mechanical shear applied to pre-conditioned starchy or proteinacious food to quickly cook and expand it through a die (45). This results in desirable enzyme denaturation, antinutritional factor (trypsin inhibitors, hemagglutinins, tannins, phytates) inactivation, final product sterilization, while retaining food color and flavor (45). It also increases protein digestibility, which is an important protein quality determinant especially in FBFs, with increasing extrusion temperature and animal protein feed ratio due to protein denaturation and inactivation of enzyme inhibitors, thereby

allowing increased digestive enzyme activity that results in improved protein bioavailability (45). Extruded products are also considered "precooked", and therefore don't take as much time or resources to prepare compared to traditional raw foods, which is desirable for improving FBFs. One potential drawback to extrusion processing is the retention of lysine, an essential and most limiting amino acid in cereal products, however increasing screw speed and reducing die diameter has been reported to enhance retention (45). Iron content of extrudates is usually increased due to metallic wear from the extruder screws (45). Collectively, mild extrusion conditions (high moisture content, low residence time, low temperature) results in higher amino acid retention, protein and starch digestibility, soluble dietary fiber, vitamin retention and mineral absorption, as well as decreased lipid oxidation (45).

Extrusion processing effects on protein digestibility and iron absorption

In general, protein digestibility is reported to improve due to extrusion processing, however decreasing available lysine content may be a concern. In one study with broiler chickens fed extruded versus non-extruded soybean meal, extruded soybean meal resulted in increased crude protein and amino acid digestibility, daily feed intake, average daily weight gain, and feed conversion ratio, suggesting extrusion improves nutritive value for broiler chicks (46). Feed moisture, screw speed, and barrel temperature had a linear effect on maize-mungbean extrudates for preferable functional properties including specific mechanical energy, bulk density, water absorption index, water solubility index, and degree of gelatinization, indicating locally available, affordable ingredients in low-income countries could use extrusion technology to produce nutritious weaning foods (47). In another study, the combination of micronized cowpea with extruded sorghum flour in a ready-to-eat porridge provided 40% of children's protein and lysine requirements, comparable to commercial maize-soy instant products

suggesting it could be used as a replacement (48). In extruded cassava-soy complementary porridge, the PDCAAS increased by 35 and 67% (with defatted or full fat soy), however available lysine was decreased in the extruded porridges by 12.5 and 16.7% compared to the same conventionally-cooked porridges (49). Extrusion processing increased *in vitro* protein digestibility in corn and lima bean flour blends (82%) compared to the raw flours (77%) (50). In extruded vs raw peas fed to rats, most amino acids were not affected by extrusion (including lysine), iron content was increased due to extruder screw wear (27%), and antinutritional factors were reduced by 5.9 to 98.3% (51). *In vitro* protein digestibility was significantly increased (4%), and when supplemented with required amino acids, rats fed extruded pea diet gained more weight and had higher PERs compared to rats fed raw pea diet (51). When whole grain red sorghum was extruded, protein digestibility increased 31% measured by an *in vitro* method, however available lysine was reduced by 25.4%; iron bioavailability was not affected (52).

Extrusion processing effects on iron bioavailability from foods are not as clear. In normal adults consuming either extruded or nonextruded wheat bran-flour in two separate test meals 2 weeks apart, no significant difference in iron retention was found after measuring iron absorption from meals (53). In 39 normal adults, four different cereal porridges (rice, maize, high extraction wheat, and low extraction wheat flours) produced by three different industrial processing methods (extrusion, roller-dried with sucrose, roller-dried without sucrose) were home-cooked and consumed and iron absorption was measured by Fe extrinsic tag technique; the type of industrial processing had no significant effect on iron absorption (54). In ileostomy subjects, mild extruded bran product consumption for two 4 day periods did not impact iron absorption compared to the corresponding non-extruded version when ileostomy contents were analyzed (55).

Chicken Model

Background

Rat and pig based models have traditionally been used as iron models to determine how iron outcomes could be expected to be impacted in humans. It has been suggested that the rat model for iron bioavailability may not be ideal because of some distinct physiological differences (56), and that the broiler chicken (Gallus gallus domesticus) might be a better *in vivo* model for assessing iron bioavailability from foods (57). It would be ideal to find a model that can accurately assess for iron absorption and protein quality; iron deficiency and PEM commonly co-occur, both have high prevalence rates (58). An accurate model may allow for better nutrition facilitation for food aid products, however, it is not clear whether the chicken model could be used for protein-quality assessment.

Methods for evaluating iron bioavailability

The primary methods for assessing iron bioavailability include radioiron or stable iron isotopes and postabsorption plasma iron measurements (59), the *in vitro* digestion/*Caco-2* cell model (60), and the animal hemoglobin depletion-repletion bioassay (59). Radioiron methods measure the amount of radioiron incorporated into red blood cells, and the difference between ingested and excreted radioiron from urine or feces to estimate absorption (59). Postabsorption plasma iron measures increases in plasma iron after oral iron administration (59). The advantage of these is that they can directly measure human iron bioavailability, however often times they are not feasible due to their time-intensive nature, cost, and radiation exposure (59).

For these reasons and more, non-human iron bioavailability studies are important and necessary due to their feasibility and cost-effectiveness. *In vitro*, Caco-2 cells differentiate and exhibit enterocyte-like features such as brush border microvilli and enzyme formation, and

increased uptake of ferrous rather than ferric iron (59, 60). Thus, the *in vitro* digestion/Caco-2 cell model is commonly used to simulate digestion and assess acute iron availability from foods by measuring ferritin formation (60). However, there are still important limitations to consider when extrapolating data from *in vitro* to *in vivo* human applications, such as limited gastrointestinal environment conditions, difficulty reproducing results, and inconsistent correlations to human outcomes that require confirmation (61).

Variations of the animal hemoglobin depletion-repletion model have been used to assess iron bioavailability. Usually, animals are fed an iron-deficient diet to develop anemia, then diets with the iron compound of interest are consumed to measure hemoglobin repletion relative to a reference source of iron (59). Rats have largely been used for this model, however pigs and chickens have also been used to mimic iron bioavailability *in vivo* (56, 62). Rats have been cited to absorb iron highly similar to humans (63), however this may be exaggerated due to large differences in energy expenditure for body size, lifespan, body proportion, and gastrointestinal morphology (56, 64). Pigs are more similar to humans in these aspects (56), however they are also more costly. More recently, chickens have been cited multiple times to respond as expected to dietary iron, and agree well with the *in vitro* digestion/Caco-2 cell model (57), and are more cost effective compared to rats and pigs.

Methods for evaluating protein quality

Some factors that influence protein quality assay procedure results include age and sex of animal, body weight, protein quantity and quality, food intake, other dietary components, husbandry, and environmental conditions (65).

Animal models primarily use a modified protein efficiency ratio (PER) to evaluate protein quality. Biological value (BV), net protein utilization (NPU), net protein ratio (NPR),

slope assay procedure, relative nitrogen utilization (RNU), and relative protein value (RPV) have also been proposed protein assays in rats (65). However due to intensive labor including urine and fecal sample collection, difficult measurements such as carcass nitrogen, preference against non-protein diet feeding, and overestimation of certain amino acid-deficient proteins, most of these assays are not feasible or routine (65). Ideal standard proteins for growth and maintenance that are similar to protein needs for humans are needed, but since this is not possible, selections must be made by test simplicity, economics, labor, and reproducibility (65).

In rats, the PER method has been commonly used for determining dietary protein quality (66). The PER assay is simply weight gain (g) divided by protein consumption (g) usually measured over a 28 day period (65). PER assays are effective and feasible due to their short-time period and low cost (65). A PER assay has been outlined in chickens using the same calculation, however only 14 days are needed to assess protein quality (67). The PER assay does have some limitations, including it not accounting for maintenance or potential complementary effects of two or more proteins during mixed feeding, however this is common among other bioassay procedures as well (65).

Gastrointestinal anatomy and physiology comparison between common models

Rat model

Animal gastrointestinal tract (GIT) anatomy is a main focus when considering feasibility of an animal model for human nutrition research. Rats are most similar to humans in their GIT morphology, however their main differences occur in their GIT gross anatomy and environment (68) due to their different life span, body size and proportion, which causes significant differences in food intake and energy expenditure (56).

Rodents have a less glandular stomach compared to humans (entire stomach is secretory), chickens, and pigs (69), and consists of two distinct regions separated by a limiting ridge that prevents the rat from retching (68). In humans, the small intestine contains watery chyme and folds in the luminal epithelium (plicae circulares); rats, do not have these folds (reducing surface area by 200-fold), their chyme is thick and chalky (68). Data normalized for body weight is an accurate correction when using rat models, where the relative gut surface area is four times less than in humans (68). The small intestine of humans, rats, pigs, and chickens is similarly divided into the duodenum, jejunum, and ileum and contain millions of villi designed to increase gut surface area and aid in nutrient absorption (56, 68-70), however in rats the jejunum makes up 90% of their small intestine compared to 38% in humans (68). Humans total intestinal length is 4.0-6.85 times longer compared to rats (calculated from Table 1.1). The rat cecum is large compared to the human cecum, which is poorly defined and continuous with the colon (69). In rats, the colon is not sacculated or long as compared to humans and pigs, and does not contain a sigmoid colon (69). The bile flow (mL bile per kg body weight each day) in rats is 2 to 42 times higher than that of humans since it is secreted continuously from the liver due to their lack of a gallbladder (69). Rats have higher GIT water content (7.8 fed, 3.2 mL fasted) compared to mice (0.98 fed, 0.81 mL fasted), and when normalized for body weight both have more water per kg body weight in GIT compared to humans which can especially affect drug dissolution and dispersion (71). In rats, intestinal pH levels have been reported to be lower than in humans, which can affect nutrient solubility and absorption (71). Throughout the small and large intestine, rats and mice have similar bacterial counts as compared to humans (69), however rats contain microorganisms in their stomach as well (68). Rats also practice coprophagy, which is important to note (56). Collectively, these differences may suggest that the human GIT can

absorb a higher volume of materials, and absorb them more quickly compared to rats (68), which is important to consider when extrapolating data between models.

Pig model

Primary similarities between pigs and humans are their digestion physiology, metabolic processes, nutritional requirements, transit time (56), and body size and proportion compared to rats or chickens, however there are still a few differences in pigs to take into consideration. The stomach of pigs is 2 to 3 times larger than that of humans, and they have a greater cardiac mucosa region that secretes mucus (69). The pigs stomach capacity (L) is 3.75 to 8 times larger than that of humans (69). Another distinct difference between the pig and human GIT is its spatial arrangement (56). In humans, the small intestine sits behind the large intestine, but in pigs it is on the right side of the abdomen (56). The large intestine in humans is more square-like, whereas in pigs it is in a spiral conformation (56). The total intestinal length of pigs is much longer than humans (2.2-4 times), therefore its intestinal weight is also (2.5 times), however when intestinal length is normalized to per kg bodyweight, they are similar (Table 1.1) (56). Differences in body fat percent and distribution may have different effects on iron regulation due to increased hepcidin levels seen in severely obese humans (56). Pigs also practice coprophagy, but not as frequently as rats (56). Compared to rats, pigs generally have more acidic pH values, but these values are comparable along the entire gastrointestinal tract (69). Pigs generally have more bacterial organisms per gram content of the GIT compared to rats, except for yeasts which occur in higher amounts overall in the GIT of rats (69).

Chicken model

A few of the main differences between avian and mammalian digestive systems are the former's modifications to aid flight, shortened intestinal tract, and lack of teeth and heavy jaw

muscles (70). Birds swallow their food whole, and the particles are later reduced by the ventriculus (gizzard; 70). Their sphincter-lacking esophagus transports food from the pharynx to the stomach, during this process, food can be stored in their crop, located in the cervical esophagus (70). Although birds are considered to be monogastrics, the proventriculus, along with the gizzard, act as a two chambered stomach in birds (70). The proventriculus is most identical to the mammalian stomach in that it is glandular; secrets mucus, hydrochloric acid, and pepsinogen, while the gizzard acts as the muscular stomach and mechanically digests food (70). Chickens contain paired ceca, which are unique to this model, that aid in additional small particle and fluid absorption (70). The ceca are thought to contain 87-97% urine since birds lack a urinary bladder (70). The chicken colon is mainly involved in water reabsorption, but has flat villi and few goblet cells relative to mammals (70). In chickens, digestive, excretory and reproductive waste is excreted through the cloaca, which is not present in mammals (70). Total rate of passage through the GIT varies due to diet and size of the animal. In chickens, the GIT can have a mean retention time of 5-9 hours when measured with insoluble markers (70), however in humans it is around 20 – 30 hours, which is most similar to pigs (69). The total intestinal length of humans is 3.6-4.4 timers longer compared to chickens (calculated from Table 1.1). Providing microbes in feed for poultry has been an accepted strategy to improve health, productivity, and weight gain (70), and therefore should be taken into consideration as well among models. Total lipid to protein ratios are similar in the chick, pigs, rat and mouse (0.5-0.6), which can affect fluidity of the membrane and thus absorption (69).

Table 1.1 Comparison of characteristics between models

Characteristics	Human[1]	Chicken[2]	Pig[1]	Rat[3]
Average mature weight (kg)	60 - 100	3.0	200 - 300	0.25[4]
Body length	1.8	0.46	1.25	0.17

(m)[5]				
Small intestinal length (m)	5.50 - 7.0	1.8	15 - 22	1.0 – 1.5
Large intestinal length (m)	1.5	0.13	4 - 6	0.2 – 0.3
Total intestinal length (m)	7 – 8.5	1.9	19 - 28	1.2 – 1.8
Total intestinal length for body length (m/m)	3.9 – 4.7	4.1	15.2 – 22.4	7.1 – 10.6
Small intestinal weight (g)	1040	73.6	2310	ND
Large intestinal weight (g)	590	5.1	1970	ND
Total intestinal weight for body weight (g/kg)	16.3 – 27.2	0.03	14.3 – 21.4	ND
Gastric pH	1.0 – 2.5; up to 5 (fed)[9]	4.65[8]	4.4[7]	3.2 – 3.9[6]
Small intestinal pH	6.2 – 7.9[9]	6.0 – 6.4[8]	6.1 – 6.7[7]	6.6[6]
GIT water content (mL)	35 ± 7[10]	ND	1546 (g)[7]	3.2 – 7.8[6]
Normal hemoglobin concentration (g/dL)	14 – 18 (males)[12]	10.1[15]	> 11.0[13]	11.0 – 19.2[14]
Normal hepatic iron content (µg/g)	2.0[11]	55[16]	ND	ND

ND: No data.

Adapted from [1](56), [2](70), [3](69), [4](68), [5](72), [6](71), [7](73), [8](74), [9](75), [10](76), [11](77), [12](78), [13](79), [14](80), [15](81), [16]Averaged values from standard iron diets reported in Tako et al. 2016 (64).

Current applications of the chicken model for human nutrition research

Iron bioavailability studies

Iron bioavailability of foods using the broiler chicken (Gallus gallus) model has been determined to assess single meal iron availability using a duodenal loop preparation and the stable isotope ^{58}Fe, as well as longer term iron availability from feeding trials; both have been shown to exhibit the appropriate response to dietary iron (57). Since 2010, ten primary research studies using the broiler chicken model to predict iron availability from foods have demonstrated that chickens respond appropriately to dietary iron levels by Hb concentration, total body Hb Fe, Hb maintenance efficiency (HME), liver ferritin and iron, and duodenal expression of proteins involved in iron reduction, uptake, and transport (DcytB, DMT-1, and ferroportin, respectively; (57, 62, 82-85, 85-87). This model has also been shown to mimic ferritin outcomes of *in vitro* digestion with the Caco-2 cell model (57, 62, 82-85, 85-87). However, it is worth noting that after normalizing data to the low iron diets in studies with similar diet formulations, the Caco-2 cell model exaggerated iron absorption between different dietary levels of iron as compared to other outcomes with chickens or pigs (82, 88, 89). Collectively, results show that chickens fed higher iron diets had increased or improved iron outcomes compared to chickens fed lower iron diets (57, 82, 83, 85-87, 90; Table 1.2). This is significant in that it supports the chicken model being sensitive to dietary iron levels and therefore exhibiting the appropriate response (57). These results also collectively agreed well with the *in vitro* digestion/Caco-2 cell model for ferritin formation (57, 82, 83, 85-87, 90). *In vivo* observations were made in two studies, and chickens agreed most with rats for certain iron outcomes such as liver ferritin and iron, and intestinal iron (91), exhibiting appropriate and similar responses to dietary iron compared to a well-known *in vivo* model, which is important for extrapolating data between *in vivo* models. In a recent review, two out of three feeding trials of standard or biofortified iron beans in chickens demonstrated nutritional benefit, which agreed with corresponding human efficacy studies fed

the same type of diet (64). Previously, the chicken model has been limited to agreement of response to dietary iron only *in vitro*, however this finding enhances its usefulness for extrapolating iron outcomes to humans (64). In conclusion, the broiler chicken model offers a rapid and cost-effective *in vivo* assessment of long term iron bioavailability from foods that can serve as a useful intermediary step to confirm *in vitro* results and advance experimental objectives to human efficacy studies (64).

Table 1.2 Summary of iron bioavailability studies performed using the chicken model for nutritional research

Reference	Models	N	Study Length (Days)	Intervention	Outcome	Comparison to chicken model
(91)	Rat, broiler chicken, Indian hill and common mynahs, turtledove	8	Not reported	Basal diet with 60-90 µg/g iron to evaluate intestinal and liver ferritin 1) Rats (control) 2) Chickens 3) Turtledoves 4) Indian hill mynahs 5) Common mynahs	Liver ferritin (µg/g wet wt.): 1) 969, 2) 914, 3) 634, 4) 1207, 5) 346 Liver ferritin saturation (%): 1) 12, 2) 11, 3) 9, 4) 28, 5) 34 Liver iron (µg Fe/g wet weight): 1) 63, 2) 52, 3) 31, 4) 179, 5) 78 Liver iron (µmol Fe/µmol ferritin): 1) 550, 2) 515, 3) 423, 4) 1252, 5) 1541 Intestinal ferritin (µg/g wet wt.): 1) 73, 2) 443, 3) 98, 4) 116, 5) 57 Intestinal ferritin saturation (%): 1) 13, 2) 14, 3) 9, 4) 31, 5) 21 Intestinal iron (µg Fe/g wet weight): 1) 5, 2) 34, 3) 5, 4) 20, 5) 6 Intestinal iron (µmol Fe/µmol ferritin): 1) 567, 2) 616, 3) 412, 4)	*In vivo*

					1401, 5) 958	
(92)	Broiler chicken, rat, dog, cat	20	14	Casein-dextrose based diet with graded levels of ferrous sulfate as hemin or hemoglobin iron, compared among rat, chick, dog, and cat.	Hemoglobin iron bioavailability was highest in chicks (93%) compared to rats (68%), dogs (90%) and cats (70%), while hemin was poorly available to rats and completely unavailable among other models.	*In vivo*
(83)	Broiler chicken, *in vitro* digestion/ Caco-2 cell culture model	12	28	Corn and biofortified or standard red mottled bean diets with low or high Fe: 1. High-Fe (54) 2. Low-Fe (42) (Fe content in µg/g)	*In vivo* (day 28): Body weight (g): 1) 684.3, 2) 599.9 Hb concentration (g/L): 1) 75.5, 2) 73.71 Total body Hb-Fe (mg): 1) 15.04, 2) 12.58 HME (%): 1) 15.9, 2) 17.6 Fe protein gene expression: 1) slightly lower than 2) Ferritin µg/g wet weight: 1) 425, 2) 409 Iron µg/g wet weight: 1) 48.1, 2) 39.5 Iron/ferritin µmol: 1) 68.5, 2) 59.8 *In vitro*: Ferritin (ng/mg of protein): 1) 15.7, 2) 11.2	*In vitro*
(90)[#]	Broiler chicken, *in vitro* digestion/ Caco-2 cell culture model	10	42	High and low-Fe bioavailability maize based diets with or without added Fe: 1. High + Fe (65)* 2. High (24) 3. Low + Fe (66)* 4. Low (23) (Fe content in µg/g) *supplemented as ferric citrate	*In vivo* (day 42): Body weight (g): No significant differences Hb concentration (g/L): 1) 97, 2) 82, 3) 87, 4) 67 Total body Hb-Fe (mg): 1) 16.49, 2) 13.79, 3) 14.52, 4) 10.73 HME (%): 1) 20.2, 2) 44.9, 3) 13.8, 4) 35.8 Fe protein gene expression:	*In vitro*

					1) < 3) < 2) < 4) Ferritin µg/g wet weight: 1) 650, 2) 435, 3) 645, 4) 355 Iron µg/g tissue: 1) 64.3, 2) 52.2, 3) 39.6, 4) 43.3 *In vitro*: Ferritin (ng/mg of protein): 1) 74.36, 2) 6.55, 3) 56.89, 4) 1.31 Fe concentration (µg/g sample): 1) 65.3, 2) 24.5, 3) 66.1, 4) 23.6	
(85)	Broiler chicken, *in vitro* digestion/ Caco-2 cell culture model	14	42	Corn and biofortified or standard Fe black bean diet: 1. Standard (39) 2. Biofortified (52) (Fe content in µg/g)	*In vivo* (day 42): [a]Hb concentration (g/L): 1) 68, 2) 78 [a]Total body Hb-Fe (mg): 1) 24.5, 2) 27 [a]HME (%): 1) 18, 2) 15 Fe protein gene expression: 1) = 2) Ferritin µg/g wet weight: 1) 282, 2) 293 Iron µg/g wet weight: 1) 27.2, 2) 33.1 Iron/Ferritin (µmol) 1) 39.8, 2) 45.6 *In vitro*: Ferritin (ng/mg of protein): 1) 2.97, 2) 2.75	*In vitro*
(86)	Broiler chicken, *in vitro* digestion/ Caco-2 cell culture model	12	42	Biofortified or standard pearl millet based diets: 1. High-Fe (78) 2. Low-Fe (22) (Fe content in µg/g)	*In vivo* (day 42): Body weight (g): From day 14, 1) > 2) [a]Hb concentration (g/L): 1) 75, 2) 70 Total body Hb-Fe (mg): 1) 25.6, 2) 14.4 [a]HME (%): 1) 13, 2) 57 Fe protein gene	*In vitro*

					expression: 1) < 2) Ferritin μg/g wet weight: 1) 285, 2) 277 Iron μg/g wet weight: 1) 25.2, 2) 19.3 Iron/Ferritin (μmol) 1) 34.5, 2) 29.7 *In vitro*: Ferritin (ng/mg of protein): 1) 2.46, 2) 1.47	
(87)	Broiler chicken, *in vitro* digestion/ Caco-2 cell culture model	14	42	Biofortified or standard cream seeded carioca bean based diets: 1. Biofortified (48) 2. Standard (33) (Fe content in μg/g)	*In vivo* (day 42): Body weight (g): From day 21, 1) > 2) [a]Hb concentration (g/L): 1) 85, 2) 81 [a]Total body Hb-Fe (mg): 1) 30.5, 2) 25 [a]HME (%): 1) 17, 2) 20 Fe protein gene expression: DMT-1: 1) < 2); others: 1) = 2) Ferritin μg/g wet weight: 1) 315, 2) 284 Iron μg/g wet weight: 1) 62.6, 2) 45.5 *In vitro*: Ferritin (ng/mg of protein): 1) 2.73, 2) 1.96	*In vitro*
(57)	Broiler chicken, *in vitro* digestion/ Caco-2 cell culture model	10	42 (start at 7 d, 49 total)	Corn-soy diet with adequate or deficient Fe content: 1) High-Fe (141)* 2) Low-Fe (51) (Fe content in μg/g) *supplemented as ferric citrate	*In vivo* (day 49): Body weight (g): 1) 2828, 2) 2647 Hb concentration (g/L): 1) 107, 2) 72 Total body Hb-Fe (mg): 1) 85, 2) 54 HME (%): 1) 11.8, 2) 21.9 Fe protein gene expression: 1) lower, 2) higher	*In vitro*

					Fe absorption: 1) 13.35, 2) 22.11 *In vitro*: Ferritin formation (ng ferritin/mg protein): 1) 14.78, 2) 5.18	
(82)	Broiler chicken, *in vitro* digestion/ Caco-2 cell culture model	6	49 (start at 7 d, 56 total)	Corn and white or red bean based diets with low or high Fe: 1) WB+Fe (179)* 2) WB (51) 3) RB+Fe (175)* 4) RB (47) (Fe content in µg/g) *supplemented as ferric citrate	*In vivo* (day 56): Body weight (g): 1) 3143, 2) 2859, 3) 2936, 4) 2649 Total body Hb-Fe (mg): 1) 84.1, 2) 74.4, 3) 73.5, 4) 61.0 HME (%): 1) 7.8, 2) 19.5, 3) 6.3, 4) 17.5 Fe protein gene expression: 1) < 2) < 3) < 4) Ferritin µg/g wet weight: 1) 1003, 2) 708, 3) 572, 4) 371 Iron µg/g wet weight: 1) 64.7, 2) 53.5, 3) 35.2, 4) 24.1 Iron/ferritin µmol: 1) 99.8, 2) 76.6, 3) 57.4, 4) 43.2 Liver ferritin iron saturation (%): 1) 16, 2) 14.5, 3) 11, 4) 9 *In vitro*: Ferritin (ng/mg of protein): 1) 71.84, 2) 11.84, 3) 51.78, 4) 2.97	*In vitro*

[a]Values estimated from graphs. [#]Article was retracted due to maize lines not being isogenic as portrayed, however data pertaining to available iron levels in maize lines are correct, and the *in vitro* and *in vivo* methods are valid and legitimate, and therefore the data cited is still correct and relevant for the purpose of this paper.

Note: For all Tako et al. studies, Fe: iron, Hb: Hemoglobin, HME: Hemoglobin maintenance efficiency calculated as $= \frac{Hb\ Fe\ (final) - Hb\ Fe\ (initial)}{Total\ Fe\ Intake\ (mg)} x\ 100$, (88, 93), where Hb Fe = total body Hb Fe calculated as $= BW\ (kg) x\ 0.085\ L \frac{blood}{kg} x\ Hb\ \left(\frac{g}{L} blood\right) x\ 3.35\ \frac{mg\ Fe}{g\ Hb}$. "Fe protein gene expression" includes DMT-1: divalent metal transporter 1 (Fe uptake transporter), DcytB: duodenal cytochrome B (reduces Fe at brush border), and ferroportin (Fe transport across enterocyte). Fe absorption: estimated from concentrations of stable isotope tracer (^{58}Fe) in whole blood relative to ^{56}Fe natural abundance concentration (57).

Limitations of the chicken model

Although the chicken model is useful for assessing iron bioavailability from foods due to its anatomy, size, growth rate, and low cost (57), it does have some noteworthy limitations. Up to date, the chicken model's response to dietary iron has primarily been studied directly with the *in vitro* digestion/Caco-2 cell model and therefore is limited in this aspect (57, 62, 82-85, 85-87). While this *in vitro* model has been shown to be useful for this application, it is still difficult to extrapolate this data to humans. Another limitation of the chicken model is their susceptibility to leg disorders, such as tibial dyschondroplasia, resulting in gait issues, which affects their eating patterns and thus weight gain due to decreases in locomotion (94, 95). Broiler chickens also have a higher mortality rate (4.8%) (96), compared to that usually found with rats or pigs, however in some studies preweaning mortality has been found to be higher in mice (5-20%) and swine (7-20%) (97).

Chapter 2 - Assessment of iron bioavailability and protein quality of new fortified blended foods in broiler chickens

Abstract

Iron deficiency and protein-energy malnutrition commonly co-occur in food aid receiving countries. Corn and soybean based fortified blended foods (FBFs) have been the primary food aid product provided by the United States to address these conditions. Sorghum and cowpea have been suggested as alternative FBF commodities because they are drought-tolerant, are grown in food aid receiving areas, and are not genetically modified. Extrusion processing has also been suggested to improve the quality of these FBFs. The primary objective of these studies was to determine protein quality and iron bioavailability of newly formulated sorghum, cowpea, soy, and corn-based FBFs, compared with the current USAID corn and soy blend FBF, CSB+, in broiler chickens, which have been suggested to be a good model for iron bioavailability. Two secondary objectives were to compare the protein quality of whey protein concentrate (WPC) to soy protein isolate (SPI) in FBFs, and to determine if reformulation and over-processing of FBFs could be equally efficacious, less expensive FBF options.

New FBFs consisted of extruded corn-soy (CSB14), white sorghum-soy (WSS), and white sorghum-cowpea (WSC); all containing WPC. Another extruded white sorghum-cowpea FBF (WSC+SPI) was similarly produced, but contained SPI rather than WPC. Additionally, two reformulated, over-processed white sorghum-cowpea FBFs (one with WPC: O-WSC, one with SPI: O-WSC+SPI), and a non-extruded sorghum-cowpea FBF (N-WSC) containing WPC were produced. Two studies were performed using prepared (Prep) or dry (Dry) versions of new FBFs, along with CSB+ and a control chicken diet. In the Prep study, nine groups of 8-day old broiler chicks (n = 10) consumed treatment diets for 21 days. In the Dry study, eight groups of 4-day old

broiler chicks (n = 24, control n = 23) consumed treatment diets for 14 days. Results were analyzed by one-way ANOVA with LSD test using SAS Studio 3.6.

In the Prep study, new FBFs significantly increased caloric and protein efficiency, and nonsignificantly increased body weight gained compared to CSB+, despite similar food intake. In the Dry study, CSB+ significantly decreased food intake and caloric efficiency, with the exception of O-WSC+SPI, and nonsignificantly reduced body weight gain and protein efficiency compared to new FBFs. CSB+ significantly and nonsignificantly reduced hepatic iron content compared to all FBFs in the Dry and Prep studies, respectively.

In conclusion, new FBFs, with the exception of O-WSC+SPI, resulted in improved food efficiency and hepatic iron outcomes compared to CSB+, suggesting they are of higher nutritional quality; sorghum and cowpea are suitable replacements for corn and soy, SPI is a viable alternative to WPC, and reformulated, over-processed WSC with WPC can be considered as a less expensive FBF option. However, further research is needed to refine and identify the best FBF formulations.

Background

Protein-energy malnutrition and iron deficiency continue to be the most common nutritional deficiencies globally (2, 15, 16). Fortified blended foods (FBFs), partially precooked grain-legume blends that are micronutrient fortified, have traditionally been used to treat these conditions (31), however they have not been consistently effective. The current most commonly used United States Agency for International Development (USAID) FBF is corn-soy blend plus (CSB+) (33). Recommendations have been made to improve FBFs, including utilization of different commodities that are drought-tolerant and locally available in food aid receiving countries, as well as using processing methods such as extrusion to improve nutritional quality of

FBFs (35). Sorghum and cowpea are suitable replacements for corn and soy in FBFs primarily due to their complementary amino acids (40), and high availability and consumption in food aid receiving countries (36, 39), which can promote local and regional procurement and thus improve agricultural market and nutritional outcomes in these areas (35). Extrusion processing, which involves moisture, high pressure, temperature, and mechanical shear to quickly cook food, has been shown to decrease antinutritional factors and thus improve protein and iron bioavailability from FBFs (45). This process also precooks the FBFs, requiring less time and resources to prepare which is beneficial in these areas. It has also been suggested that an animal source protein, such as whey protein concentrate (WPC), be included in FBFs to improve protein-energy malnutrition, such as stunting and wasting (35), however its costly inclusion has not been entirely supported or justified (10, 11). Plant protein, such as soy protein isolate (SPI), may be a less expensive option for inclusion in FBFs to obtain similar nutritional outcomes (12).

The chicken model has been suggested to be a good *in vivo* model for assessing iron bioavailability because its iron outcomes are consistent with the widely used *in vitro* digestion/Caco-2 cell model (57). It has also shown sensitivity to long term dietary iron levels by exhibiting expected responses to high and low iron diets (57). Rats have traditionally been the primary *in vivo* model for this application, and pigs have been common too, however due to the former's more efficient iron absorption due to large differences in energy expenditure for body size, lifespan, body proportion, and gastrointestinal morphology, and the latter being more costly (56), the chicken model becomes more useful due to its anatomy, size, growth rate, and low cost (57). However, its viability as an *in vivo* model for assessing iron bioavailability has been limited to mirroring this outcome only *in vitro*, and to the best of our knowledge, while a PER model has been established (67), the chicken model has not been used to assess protein quality of foods for

a human nutrition application. In a recent review two out of three feeding trials of standard or biofortified iron beans in chickens demonstrated nutritional benefit, which agreed with corresponding human efficacy studies with similar diets (64). Although this extrapolation is still limited, this further supports the application of the broiler chicken model; it ultimately offers a rapid and cost-effective *in vivo* assessment of long term iron bioavailability from foods that can serve as a useful intermediary step to confirm *in vitro* results and advance experimental objectives to human efficacy studies (64).

The primary objective of the two studies outlined in this paper was to determine the protein quality and iron bioavailability of new FBFs compared to a current USAID FBF, CSB+. Extruded sorghum, cowpea, corn, and soy FBFs were formulated according to USAID recommendations (35) along with a non-extruded sorghum-cowpea group to assess if sorghum and cowpea can be used as alternative commodities to corn and soy, and if extrusion processing is needed to result in similar or improved protein and iron outcomes. Another objective was to compare the protein quality of WPC to SPI inclusion in FBFs. Additionally, two reformulated, over-processed less expensive FBFs were developed to determine if this formulation can be a more cost-effective option to obtain similar protein and iron outcomes compared to other FBFs.

Methods

Animal safety and ethics

The Institutional Animal Care and Use Committee (IACUC) at Kansas State University approved all animal procedures (protocols 3717.2 and 3790).

Diet formulation and composition

Seven new FBFs were formulated based on USAID food aid recommendations (35) to compare to the current USAID FBF, CSB+ (Table 2.1). CSB+ was purchased from a USDA

producer (Bunge Milling, St. Louis, MO), which is prepared from heat treated corn and soybeans, with an added vitamin and mineral premix. To compare commodity types within the same FBF formulation, three white sorghum with cowpea (WSC, WSC+SPI, and N-WSC), one white sorghum with soy (WSS), and one corn with soybean (CSB14) blends were developed. FBFs were similarly produced by extruding grain and legume flours (with the exception of N-WSC), milling them to a powder, then adding sugar (15%), oil (9%), whey protein concentrate (WPC) 80% (Davisco Food International, Inc., Eden Prairie, MN) or soy protein isolate (SPI) 90% at 9.5% (ARDEX®F Dispersible 066-921, ADM, Decatur, IL), and vitamin and mineral premix, which was formulated according to USAID food aid recommendations (35), as 3.2% of FBFs (Research Products Company, Salina, KS). One sorghum and cowpea FBF was not extruded to determine the effects of extrusion processing (N-WSC), and the other two contained either an animal or plant protein source (WSC vs. WSC+SPI) to determine protein quality differences. Two additional sorghum-cowpea FBFs were similarly produced, however they were reformulated and over-processed with either whey or soy protein (O-WSC and O-WSC+SPI) to determine whether these FBFs meet viscosity requirements without the addition of sugar, and contain decreased oil (8.3%) and WPC or SPI (3%), resulting in a less expensive FBF that could maintain the same nutritional efficacy as the other FBFs. A 22% gamebird starter/grower diet (Country Lane, Orscheln Farm & Home, Moberly, MO) was fed to the control group to compare outcomes of FBFs with a normal chicken diet.

Iron forms and concentrations among CSB+, new FBFs, and the control chicken diet were different. The control chicken diet contained ferrous sulfate (41.5 mg/100g) almost 4 times higher than CSB+ and an average of 2.5 times higher than new FBFs. CSB+ and new FBFs contained sodium iron EDTA (NaFeEDTA) and ferrous fumarate at different concentrations.

NaFeEDTA was included due to its superior bioavailability compared to ferrous fumarate, therefore the combination of the two forms were expected to enhance iron bioavailability from FBFs (35).

Fortified blended food production

Sorghum-cowpea, sorghum-soy and corn-soy binary blends were extruded on a single screw extruder X-20 (Wenger Manufacturing Co., Sabetha, KS, USA) at the Kansas State University (KSU) Extrusion Lab. Normally processed binary blends that were extruded for WSC, WSC+SPI, WSS, and CSB14 FBFs shaft speed ranged from 497-564, and had an average dry feed rate of 171 kg/h, in-barrel moisture content of 24%, motor load of 74%, and specific mechanical energy of 299 kJ/kg. Over-processed binary blends that were extruded for O-WSC and O-WSC+SPI had a dry feed rate of 158 kg/h, in-barrel moisture content of 21%, motor load of 78%, and specific mechanical energy of 370 kJ/kg. Steam and water were added in the preconditioner at an average of 14 and 16%, respectively, for normally processed binary blends, and at 18 and 6%, respectively, for over-processed binary blends. Discharge temperature was maintained above 85°C, and the die had a single circular opening of 4.1 mm. After cutting, binary blend extrudates were dried using a double pass dryer/cooler (Series 4800, Wenger Manufacturing Co., Sabetha, KS, USA) operating at 107°C, where they were retained for 10 minutes, before being cooled for 5 minutes at room temperature. Cooled extrudates were milled using a hammer mill (Schutte Buffalo, NY, USA) fitted with a 315 µm screen and collected directly into 50 lb 3-walled paper bags and sealed until further use. The micronutrient premix, whey protein concentrate or soy protein isolate, and sugar were mixed into the extruded flours in steps to ensure mixing uniformity. Once dry ingredients were combined through this process, oil was added and mixed thoroughly to produce the final FBF product.

Diet and macronutrient analysis

FBFs were analyzed by AOAC official methods by the University of Missouri-Columbia Agricultural Experiment Station 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 (AOAC Official Method 975.44; 982.30 E(a,b,c), chp. 45.3.05, 2006).

Prepared FBFs' viscosity was assessed in duplicate using a Bostwick Consistometer (CSC Scientific Company, Inc., Fairfax, Virginia, USA). All new FBFs were prepared at 20% solids, CSB+ was prepared at 13.79% solids as directed (33). Water was brought to a boil and the FBF was slowly mixed in and left to boil for 1 minute with constant stirring. CSB+ and N-WSC were boiled for 5 minutes with constant stirring due to their partially and non-precooked characteristics, respectively. After 1 or 5 minutes, the FBFs were taken off of the hot plate and stirred for another 30 seconds before being covered with aluminum foil and set in a water bath for 10 minutes at 30°C. After 10 minutes, the FBF was weighed and the lost water, due to evaporation, was added back. The FBF sample was recovered and put back in the water bath for one hour at 30°C. Then, the FBF sample was weighed once more and water was added if there was any loss. The FBF was stirred and poured into the Bostwick Consistometer chamber, leveled off, and settled for 30 seconds. Then, the gate was opened and the FBF was allowed to flow for 1 minute until data were collected.

Study Design

Prepared FBF Study

Ninety 1-day old male broiler chicks were obtained from a commercial hatchery (Cobb-Vantress, Inc., Lafayette, TN), and arrived at the KSU Poultry Unit the same day. Upon arrival, all chicks were immediately placed in a 5 x 13 foot floor pen. Floor pens contained sufficient litter covering floor, and the environmental conditions consisted of a temperature controlled facility with 24-hour light provided. Initial temperature of the barn was set to 34°C and by the end of the study was decreased to 24°C. Chicks were fed a basic broiler starter diet (O.H. Kruse Feed Technology Innovation Center, Manhattan, KS) that consisted of corn and soybean meal with micronutrients for one week before beginning experimental diets. On day 8, chicks were randomized and allocated into 9 treatment diets on the basis of body weight, with 2 floor pen replications of 5 chicks per diet (n=10, 90 total). Animals were provided food and water (Fe content less than 0.11 μg/mL) *ad libitum*. Water was supplied by a uniform water source for the barn, and each pen had their own hanging tube nipple waterers. Normally, CSB+ is directed to be prepared at 13.79% solids, and new FBFs at 20% solids. However, due to the limited stomach capacity of the chickens, solids % had to be increased to make our FBFs more nutrient dense, and ensure they would be able to meet daily feed requirements outlined by the Cobb-Vantress, Inc. hatchery (98). Therefore, CSB+ was prepared at 1:2.55 solids to water, and new FBFs and control diet were prepared at 1:2 solids to water to account for this, while also maintaining the same difference between solids percentage of CSB+ and new FBFs to account for new FBFs increased solids when compared to CSB+. Due to their partially and non-precooked characteristics, CSB+ and N-WSC were boiled with water and stirred for 10 minutes in large turkey fryers to ensure complete cooking. For other FBFs, water was boiled together in a large turkey fryer, then was divided out and mixed into FBFs; room temperature water was added and mixed into the control diet. From days 8-23, chicks were given feed in small round plastic

containers with half lids (to keep chicks out of food) due to the small volume of food. Then on day 24, larger feeders were introduced that consisted of a 1/3 size foil steam table pan inside a wooden base and half covering. One days' worth of food for each group was prepared once per day; 2/3 was fed to chicks in the afternoon feeding (4:00pm), and 1/3 was refrigerated overnight in Tupperware containers, then fed to chicks in the morning feeding (7:30am). Therefore, chicks were fed twice per day and food intakes were measured daily. Chicks were weighed weekly as a replication group until study end when they were weighed individually. Total duration fed treatment diets was 21 days, however at study end animals were 28 days old; study length and sample size were originally based on a similar published study (57).

Dry FBF Study

Two hundred 1-day old male broiler chicks were obtained from a commercial hatchery (Cobb-Vantress, Inc., Lafayette, TN), and arrived at the KSU Poultry Unit the same day. Upon arrival, all chicks were immediately placed in 3.25 x 1.1 x 0.8 foot wire-bottomed battery brooder pens. Environmental conditions consisted of a temperature controlled facility (pens ranged from 26-29°C) with 24-hour light provided. Chicks were fed 22% gamebird starter/grower control diet (Country Lane, Orscheln Farm & Home, Moberly, MO) for four days before beginning experimental diets. On day 4, chicks were weighed and allocated into 8 treatment diets on the basis of body weight with 4 battery brooder pen replications of 6 chicks per diet (n=24, control n=23, 191 total). The control group originally contained 24 chicks like the other groups, however one chick had to be terminated a few days before study end due to an unexplained physical injury unrelated to the study regimen. Animals were provided food and water (Fe content less than 0.11 μg/mL) *ad libitum*. Water was supplied by a uniform water source for the facility. Feed intakes and body weights were measured weekly as a replication

group until study end where a small subset (n=6) from each group were weighed individually and then euthanized to collect blood and liver samples to assess iron outcomes; the remaining chicks were transferred for farm use. Prior to termination of the study, pens were randomized so that 6 chickens from each diet group would be selected for euthanization and sample collection. Total duration fed treatment diets was 14 days, however chicks were 18 days old at study end; study length and sample size were based on a previous PER study (67).

Average food intake and body weights for that replication group were readjusted to account for the loss of this chick. Food intake was averaged per chick, then the total intake for the five chicks in that replication was calculated and added together for each week. For body weights, since the removed chick was 12% smaller than the average of the other chicks in its group, we subtracted the proportioned amount of body weight from the initial and week 1 replication body weights to obtain more realistic values.

Data and sample collection

At termination of both studies, final individual weights of chickens were recorded (Prep: n=10, Dry: n=6). In the Prep study, gait scores were assessed by degree of impairment from 0 (none) to 5 (complete impairment) using criteria from a modified gait scoring system outlined previously (99). For hemoglobin analysis, in the Prep study blood was collected via the wing vein into 4 mL EDTA-K2 vacuolized tubes and in the Dry study via cardiac puncture into 2 mL EDTA-K2 vacuolized tubes. EDTA-K2 vacuolized tubes (BD and Company, Franklin Lakes, NJ) were immediately placed on ice and subsequently stored at 4°C for 6-7 and 2 days before analysis in the Prep and Dry studies, respectively. Following blood collection, chickens were euthanized by cervical dislocation. Liver tissue was collected, weighed, flash frozen in liquid nitrogen, and stored at -80°C. In the Prep study, both legs of chickens were collected and stored

at -20°C for future assessment of tibiae bone mineral density via Lunar PIXImus (GE Medical Systems) following manufacturer instructions.

Iron Quantification

Dietary and experimental water iron

Iron content of FBFs and control diet were analyzed in duplicate (n=9) (AACC method 40.70.01, AIB International, Manhattan, KS). Facility water samples were collected by turning on water faucets for 5 minutes before taking the sample. Samples were taken in duplicate and iron was assessed by both flamed atomic absorption spectrometry (AAS) and inductively coupled plasma optical emission spectrometry (ICP-OES). AAS can detect iron levels that are at least 0.11 μg/mL. AAS detected no iron in our samples, therefore it can be confirmed by this method that the iron content in our samples were lower than 0.11 μg/mL. ICP-OES can detect iron concentrations of at least 0.2 μg/mL, and also failed to detect iron in the water samples. These concentrations are much lower than a similar study that used broiler chickens with a water Fe concentration of 0.379 ± 0.012 μg/mL (57).

Hemoglobin

Hemoglobin samples were assessed in duplicate (Prep, n=10; Dry, n=6) using the QuantiChrom Hemoglobin Assay Kit (DIHB-250, BioAssay Systems, Hayward, CA), which is based on an improved Triton/NaOH method, following manufacturer instructions. Whole blood was diluted 100 fold with deionized H_2O (20 μL to 1980 μL). If duplicates were more than 25% different, a triplicate sample was analyzed.

Hepatic iron

Hepatic iron was determined in duplicate by wet ashing following procedures described previously (100) before quantification by inductively coupled plasma-optical emission

spectrometry (ICP-OES). All glassware were acid washed in a 6% nitric acid solution overnight before use. 1.0 g of hepatic tissue was placed into a 50 mL acid washed beaker, 10 mL of full strength trace metal grade nitric acid (Fisher) was slowly added, covered with a watch glass, and left for one hour for chemical decomposition. Samples were then placed on a hot plate and brought to boil, then gently refluxed for 2-3 hours until reduced to 1 mL. They were titrated to 10 mL with deionized H_2O in an acid washed volumetric flask by adding deionized H_2O to the sample in the beaker, swirling, and transferring to the flask. The final sample was then mixed again and transferred to a 15 mL sterile polypropylene tube and stored at room temperate until quantified in duplicate (Prep, n=10; Dry, n=6) by ICP-OES (Varian 720-ES, Agilent Technologies, Santa Clara, CA). If duplicates were more than 25% different, a triplicate sample was analyzed.

Tibiae bone mineral density

Right legs of chickens from the Prep study (n=10), were brought to room temperature before tibiae were dissected and bone mineral density (BMD) measured via Lunar PIXImus (GE Medical Systems). Four BMD measurements were taken due to reports of BMD varying across the regions of the tibia (101). The first measurement analyzed BMD of the entire tibia (Total BMD). The second measurement analyzed the diaphysis region (Diaphysis BMD), which was defined as the middle 50% region of the tibia (102). The third and fourth measurements analyzed the BMD of the proximal (Proximal BMD) and distal (Distal BMD) epiphyses, defined as the top and bottom 25% regions of the tibia (102). Total lengths of the tibiae were first measured, and regions were calculated from lengths and visibly marked. Tibiae were placed in the scanning area, and metal references were placed adjacent to marks so the region of interest could be adjusted on the PIXImus once scanned to obtain each BMD measurement.

Calculations

Caloric efficiency was calculated along with protein efficiency as an indicator of protein quality. Means of replication groups for each experimental diet were used.

$$\text{Caloric efficiency (weight gain per kcal consumed)} = \frac{weight\ gain\ (g)}{food\ intake\ (g)\ x\ kcal\ per\ gram\ of\ FBF}$$

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

Feed conversion ratio (FCR) was also calculated, which is widely used in poultry production to assess efficiency in converting feed into mass (103).

$$\text{Feed conversion ratio (grams feed intake per gram weight gain)} = \frac{feed\ intake\ (g)}{weight\ gain\ (g)}$$

Statistical analysis

Group differences were assessed using one-way ANOVA and least significant differences comparisons method at significance level $p<0.05$ using SAS Studio 3.6 (Cary, NC). Natural log transformation was used if the assumption of normality was violated.

Results

FBF analyzed composition and viscosity

Composition

CSB+ contained on average 6.5% fewer total kcals, 15.6% less protein, and was within 1.1% of carbohydrate content compared to new FBFs (Table 2.3). CSB+ contained similar fat content to both over-processed FBFs (O-WSC and O-WSC+SPI), and collectively these FBFs contained 20.7% less fat than non-extruded (N-WSC) and normally extruded FBFs (WSC, WSC+SPI, WSS, CSB14). O-WSC, O-WSC+SPI, and N-WSC provided less total kcal per 100g FBF, and the two over-processed FBFs contained less available lysine compared to other FBFs, but more than CSB+. CSB+ contained 36.5% less iron than new FBFs, and the control diet

contained markedly higher iron than new FBFs and CSB+. WPC versus SPI containing groups had comparable macro- and micronutrient compositions.

Viscosity

Required USAID Bostwick consistency values for corn-soy blend are 9 to 21 cm (104). Normally extruded sorghum-containing FBFs (WSC, WSC+SPI, and WSS) on average were 43.5% more viscous than corn-containing FBFs (CSB14 and CSB+; Table 2.4). Over-processing of sorghum and cowpea (O-WSC and O-WSC+SPI) allowed FBFs to reach viscosity requirements without the addition of sugar. N-WSC did not meet viscosity requirements; WSC and WSS slightly exceeded requirements.

Food intake, body weight, food efficiency, iron, and anthropomorphic outcomes

Prepared FBF Study

The control group significantly increased food intake and weight gain compared to all FBF groups (Table 2.5). WSS consuming group had significantly higher food intake compared to N-WSC, O-WSC+SPI, and WSC consuming groups.

The WSS consuming group had significantly increased body weight gain compared to all FBFs, with the exception of CSB14 and O-WSC. Of the sorghum-cowpea groups, O-WSC+SPI had significantly reduced body weight gain compared to O-WSC and WSC+SPI, but not WSC or N-WSC. CSB+ had reduced body weight gain compared to all FBFs, with the exception of WSC and O-WSC+SPI, despite having a higher food intake compared to many of the FBF groups.

The WSS consuming group had significantly higher final body weight compared to all FBFs, with the exception of CSB14. The O-WSC+SPI group had significantly decreased final body weight compared to WSS, CSB14, O-WSC, and WSC+SPI. With the exception of O-WSC+SPI, all other sorghum-cowpea FBFs had similar final body weights.

CSB+ significantly reduced caloric efficiency compared to all groups, by 24% compared to the next least efficient group, O-WSC+SPI. Caloric efficiency was significantly greater in the WSS consuming group than all the other FBF groups, except for N-WSC. N-WSC significantly improved caloric efficiency compared to O-WSC and O-WSC+SPI.

There was a significant reduction in protein efficiency in the CSB+ group compared to all other groups. Protein efficiency was significantly greater in the WSS consuming group than all groups, with the exception of N-WSC and CSB14. WSC+SPI was not significantly different than WSC, O-WSC, or N-WSC. However, O-WSC+SPI had significantly reduced protein efficiency compared all FBFs.

CSB+ consuming group had significantly higher food conversation ratio than all other FBF groups (37% more than next least efficient group, O-WSC+SPI), indicating they were the least efficient at converting feed to weight gain. The WSS consuming group had the most efficient feed conversion ratio; it was not significantly different than CSB14, N-WSC, WSC+SPI, and control groups.

There were no significant differences in hemoglobin concentration between groups (Table 2.6). The control group had significantly reduced hepatic iron levels compared to all groups, with the exception of CSB+. O-WSC+SPI consuming group had the highest hepatic iron levels, however it was not significantly higher than O-WSC and WSC+SPI groups. Liver weight as a percentage of body mass was significantly higher in the WSC consuming group compared to control, CSB14, and WSS groups.

Some chickens developed gait issues during the study, and thus gait scores were collected. N-WSC and WSC consuming groups had significantly increased gait scores compared to all other groups (Table 2.7). CSB14 and WSS groups also had gait scores that were

significantly greater than the other groups that did not have any impairment. To determine if this impairment was due to bone weakness in their legs, bone mineral densities were collected on right tibias. The WSS group had the highest total bone mineral density compared to other FBFs, but not significantly higher than WSC+SPI, O-WSC, and CSB14 groups. WSC had the lowest total bone mineral density, but not significantly lower than N-WSC, CSB+, and O-WSC+SPI. WSC+SPI had the highest total diaphysis bone mineral density compared to other FBFs, but not significantly higher than O-WSC, WSS, CSB14, and O-WSC+SPI. WSC had the lowest diaphysis bone mineral density, but not significantly lower than N-WSC, CSB+, and O-WSC+SPI. WSS had significantly higher proximal bone mineral density compared to all other FBFs. WSS had significantly higher distal bone mineral density compared to all other FBFs, with the exception of WSC+SPI and O-WSC. For all bone mineral density measurements, WSC had significantly reduced BMD, but not significantly different than N-WSC, CSB+, and O-WSC+SPI.

Dry FBF Study

The control group significantly increased food intake and weight gain compared to all FBF groups (Table 2.8). CSB+ consuming group ate significantly less total food compared to all other groups, with the exception of O-WSC+SPI. The WSS consuming group ate significantly more food compared to all other groups, with the exception of CSB14. Of the sorghum-cowpea groups, WSC consuming group ate significantly more than WSC+SPI, O-WSC, and O-WSC+SPI consuming groups.

CSB+ and O-WSC+SPI consuming groups gained significantly less weight than all other groups. WSS consuming group gained significantly more weight compared to all other groups, except for WSC.

CSB+ and O-WSC+SPI had significantly lower final body weights compared to all other FBFs. WSS and WSC groups had significantly increased final body weight compared to all other FBFs.

O-WSC, WSS, WSC, and WSC+SPI significantly increased caloric efficiency compared to CSB14, O-WSC+SPI, and CSB+. The CSB+ consuming group had significantly reduced caloric efficiency compared to all groups, with the exception of O-WSC+SPI.

There was a significant reduction in protein efficiency with O-WSC+SPI compared to all other groups, with the exception of CSB+. Protein efficiency was significantly greater in the O-WSC consuming groups than all groups, with the exception of WSS and WSC.

CSB+ consuming group had significantly increased (less efficient) FCR compared to all groups. Among new FBFs, O-WSC+SPI group had significantly higher FCR, with the exception of CSB14. The WSS group nonsignificantly reduced FCR among all FBFs, indicating it was the most efficient at converting feed into weight gain.

The CSB+ consuming group had significantly increased hemoglobin levels compared to all others groups (Table 2.9). There were no other significant differences between FBF groups' hemoglobin levels. The CSB+ consuming group had significantly reduced hepatic iron levels compared to all other FBF consuming groups. O-WSC+SPI consuming group had significantly higher levels compared to other groups, with the exception of WSC+SPI and O-WSC.

Overall Results

Overall, CSB+ and O-WSC+SPI trended towards reduced food efficiency outcomes compared to all other groups. In the Prep study, CSB+ nonsignificantly increased and decreased food intake and weight gained, respectively, compared to all groups. In the Dry study, with the exception of O-WSC+SPI, CSB+ significantly reduced both food intake and weight gained

compared to all groups. In the Prep study, CSB+ significantly reduced caloric and protein efficiencies, and significantly increased feed conversion ratio, compared to all groups. In the Dry study, CSB+ significantly reduced caloric efficiency (with exception of O-WSC+SPI), protein efficiency (with exception of WSC+SPI, O-WSC+SPI, and CSB14), and significantly increased feed conversion ratio compared to all groups. New FBFs, with the exception of O-WSC+SPI, performed similarly in both studies for food intake, weight gain, and food efficiency outcomes, although WSS trended towards improved outcomes.

Regarding iron outcomes, CSB+ significantly increased hemoglobin levels compared to all groups in the Dry study, however in both studies there were no significant differences between other groups. In both studies, O-WSC+SPI tended to nonsignificantly increase hepatic iron levels, while CSB+ nonsignificantly and significantly reduced levels in the Prep and Dry studies, respectively, compared to new FBFs which had similar levels.

Comparing FBFs to NRC recommendations

Due to significantly higher outcomes observed in the control group and gait issues identified in the Prep study, FBFs were compared with NRC requirements for protein, amino acid, and certain minerals. FBFs did not meet protein, certain amino acid, calcium, or phosphorus requirements for broiler chickens 0 to 21 days old, however calorie and iron requirements were exceeded by all FBFs (105; Table 2.10). CSB+ contained less lysine compared to other FBFs, which may have contributed to lower growth seen in the Dry study, however O-WSC+SPI had comparable nutrient content to other new FBFs, and its consumption resulted in outcomes similar to CSB+.

Discussion

In these studies, with the exception of O-WSC+SPI, new FBFs resulted in improved food efficiency and hepatic iron outcomes compared to the current USAID FBF, CSB+. New FBFs resulted in similar protein and iron outcomes, with the exception of O-WSC+SPI, suggesting sorghum and cowpea are suitable replacements for corn and soy, SPI is an effective alternative to WPC, and reformulated, over-processed FBFs with WPC can be considered as a less expensive FBF option.

Overall, CSB+ trended towards reduced food efficiency outcomes compared to new FBFs, with the exception of O-WSC+SPI. Although CSB+ contained lower caloric and protein content compared to new FBFs, all FBFs did not meet protein, but did exceed calorie and fat recommendations for broiler chickens 0-21 days, therefore it is not likely this slight decrease in protein content significantly reduced food efficiency outcomes that were observed. In a study with similar new FBFs and CSB+ fed to rats, CSB+ resulted in decreased food intake, growth suppression, and reduced caloric and protein efficiencies compared to other groups (100). Additionally, CSB+ inhibited growth in week 1 despite similar food intake with other groups (100); this suggests poorer food quality and digestibility in CSB+ compared to new FBFs. In another study with energy sufficient, but decreased lysine content in the diet, broiler chickens had significantly lower weight gain compared to other groups (106). Similarly, CSB+ contained sufficient energy but lower lysine compared to new FBFs, and significantly lower weight gain was observed in the Prep study compared to WSS and CSB14, and in the Dry study compared to all groups except for O-WSC+SPI; this suggests lower protein quality compared to new FBFs. Extrusion processing has been cited often to improve cereal and legume starch and amino acid digestibility (107-110), therefore lack of extrusion and reduced digestibility of CSB+ may have

been the primary factors that caused the increased difference between food intake and weight gain compared to other FBFs, and thus resulted in significantly reduced food efficiencies in the Prep study. However, it is important to note that the non-extruded FBF in the Prep study, N-WSC, performed similar to other new FBFs, and nonsignificantly improved weight gain while significantly improving food efficiencies compared to CSB+, despite it being non-precooked and thus requiring the same amount of boiling time to cook it like CSB+. This suggests that the nutritional quality of N-WSC is higher than CSB+, and that the absence of extrusion processing did not significantly affect the FBF's digestibility; although N-WSC did not meet viscosity requirements, thus limiting its viability. In another study feeding similar extruded FBFs to rats, phytate content was analyzed and extruded FBFs' phytate content was reduced by more than three times compared to CSB+ (100). In rats, amino acid bioavailability reduction has been observed with consumption of phytate containing foods due to effect on digestive enzyme activity (111), however this has only been observed to affect iron bioavailability in broiler chickens (85, 86). In addition, N-WSC and O-WSC performed comparably to other FBFs in the Prep study, and O-WSC even nonsignificantly improved food efficiencies in the Dry study, despite their potentially increased polyphenolic content due to non-extrusion and increase in phytate-containing grain and legume by volume, respectively, compared to other FBFs. Therefore, it is important to note but unlikely that differences in phytate content played a substantial role in the significant and nonsignificant reduction of food efficiencies in the CSB+ consuming group for both studies.

Along with CSB+, O-WSC+SPI significantly reduced food intake, body weight gain, final body weight, and food efficiencies in the Dry study, and in the Prep study consistently but nonsignificantly reduced these outcomes as well. However, the similarly reformulated, over-

processed FBF with WPC, O-WSC, significantly improved food efficiency outcomes in both studies compared to O-WSC+SPI. It is possible that for this particular formulation of FBF, SPI is not sufficient compared to WPC for improving these outcomes. However, the other SPI containing group, WSC+SPI, performed similarly in both studies to its identically formulated counterpart but with WPC, WSC, suggesting that in certain formulations of FBFs, soy protein is an effective alternative to whey protein. Additionally, this is supported in a review that found isocaloric, isonitrogenous animal source proteins were not superior to plant source proteins in enhancing linear growth, suggesting the costly inclusion of animal source proteins are not needed in FBFs (11). Therefore, in the case of O-WSC+SPI, it is maybe that since the majority of protein coming from cowpea flour that SPI could not make up for its lower protein quality like WPC.

Regarding iron outcomes, hemoglobin levels were not significantly different in the Prep study among all groups. However, in the Dry study, CSB+ significantly increased and reduced hemoglobin and hepatic iron levels, respectively, compared to all new FBFs. In one study, low ambient temperature (24-26°C from 1-7 days, 9-11°C from 8-21 days) expectedly increased hemoglobin levels due to oxidative stress of broiler chickens, and addition of a vitamin C supplement unexpectedly, but nonsignificantly, increased hemoglobin levels in 21-day-old broiler chickens compared to normal ambient temperature (29-31°C from 1-7 days, 24-26°C from 8-21 days) and non-supplemented groups (112). Although temperatures during this study were in the normal ambient temperature range, oxidative stress still may have been present in CSB+ chicks which caused the tissues' demand for oxygen, and thus increased need for mobilizing iron in hemoglobin rather than storage that was observed. In both studies, O-WSC+SPI significantly increased hepatic iron content compared to other FBFs, with the

exception of O-WSC and WSC+SPI. This was most likely due to markedly slower growth rates, and thus less demand for micronutrients; similar outcomes were observed in another study with CSB+ in rats (100). There were no observed signs that suggested micronutrient deficiencies, and their livers were not enlarged compared to other groups, suggesting iron toxicity was not likely a factor in their growth suppression.

In the Prep study, gait issues were present in N-WSC, WSC, CSB14, and WSS. WSC consistently nonsignificantly reduced BMD across all measurements, along with N-WSC, CSB+, and O-WSC+SPI, however the latter two groups did not have gait issues. The primary factors being cited for occurrence of leg disorders, including locomotion issues represented by high gait score, are rapid growth and weight gain, and decreased locomotor activity (95, 113, 114). However in this study, chickens impacted had slow growth and weight gain, and had large pens with food and water sources spread apart, thus requiring more walking. Collectively, locomotor activity was not restricted. In addition, control diet consuming groups had significantly increased growth and weight gain, more comparable to commercial broilers, but no gait issues were observed in this group. BMD is affected by age, sex, type of production, diet, and management (115), and tibia BMD has been cited to linearly increase with increasing levels of nonphytate phosphorus, and constant calcium content at 1.0% of diet (116). All FBFs did not meet NRC calcium (1000mg/100g) or phosphorus requirements (450mg/100g), and both mineral levels were the same across all new FBFs. It is not clear what caused gait issues in some FBF groups and not others, however certainly low calcium and phosphorus content in FBFs contributed to low BMD of chicks compared to control groups in this study.

Limitations

The locomotion impairment observed in some chicks in the Prep study may have caused decreased food intake or activity, and thus had a minor effect on overall outcomes for those groups. Although the ratio of solids between new FBFs and CSB+ remained the same as normally directed, the decreased solids of CSB+ could have contributed to the reduced food efficiencies that were seen. However, this also demonstrates the limitations of CSB+ in treating children for malnutrition in food aid programs due to their limited stomach capacity, and thus need for a more nutrient-dense FBF such as our new FBFs is important to consider (35). Both of these studies were short in duration, therefore results from this rapid growth period have to be translated with caution to humans. FBFs are normally meant to be consumed along with other foods, therefore the complementary feeding nature of FBFs to improve outcomes is limited in these studies.

Conclusions

In conclusion, sorghum and cowpea FBFs performed similarly to corn and soy FBFs, suggesting these commodities are suitable replacements for corn and soy. Soy protein isolate (WSC+SPI) was an effective alternative to whey protein concentrate (WSC), suggesting SPI can be a less expensive protein supplement in FBFs. Surprisingly, non-extruded sorghum and cowpea (N-WSC) was equally efficacious to extruded WSC, suggesting extrusion may not be necessary for improving protein and iron bioavailability from FBFs with this specific formulation. However, it should be noted that N-WSC did not meet viscosity requirements and requires cooking before consumption, thus limiting its viability. O-WSC+SPI resulted in poorer outcomes compared to other FBFs, which suggests the protein quality of cowpea may be inferior and the inclusion of whey protein is needed in this formulation, as O-WSC with whey performed

similarly to other FBFs. Therefore, reformulated over-processed FBFs with the inclusion of whey protein can be considered as a less expensive FBF option. Overall, new FBFs, with the exception of O-WSC+SPI, resulted in improved food efficiency and hepatic iron outcomes compared to CSB+, suggesting they are of higher nutritional quality. However, further research is needed to refine and identify the best FBF formulations.

Tables

Table 2.1 FBFs composition (%)

	Sorghum flour	Cowpea flour	Soy flour	Corn flour	Sugar	Whey protein concentrate	Soy protein isolate	Vegetable oil	Micro-nutrient premix
WSC, N-WSC	24.7	38.6	0	0	15	9.5	0	9.0	3.2
WSC + SPI	24.7	38.6	0	0	15	0	9.5	9.0	3.2
O-WSC	31.5	54.0	0	0	0	3.0	0	8.3	3.2
O-WSC+SPI	31.5	54.0	0	0	0	0	3.0	8.3	3.2
WSS	47.6	0	15.7	0	15	9.5	0	9.0	3.2
CSB14	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), tri-calcium phosphate (1.16), potassium chloride (0.17)

Control chicken diet based on label ingredients: Grain products, plant protein products, processed grain by-products, roughage products, vitamin supplements, minerals.

White sorghum-cowpea (WSC), Non-extruded WSC (N-WSC), WSC + soy protein isolate (WSC+SPI), Over-processed WSC (O-WSC), Over-processed WSC + soy protein isolate (O-WSC+SPI), White sorghum-soy (WSS), Corn-soy blend 14 (CSB14), Corn-soy blend plus (CSB+).

Table 2.2 New FBFs and CSB+ vitamin and mineral fortificant levels (mg/100g)

New FBFs		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)	8.0
Calcium D-Pantothenate (B_5)	3.646	Calcium D-Pantothenate (B_5)	1.6
Pyridoxine Hydrochloride (B_6)	0.752	Pyridoxine Hydrochloride (B_6)	1.0
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	90.0
Calcium (Tri-Calcium Phosphate)	279.08	Calcium (Tri-Calcium Phosphate)	452
Iron	13.0	Iron	6.5
Sodium Iron EDTA	1.47	Sodium Iron EDTA	1.12
Ferrous Fumarate	3.79	Ferrous Fumarate	2.44

Iodine (Potassium Iodide)	0.23	Iodine (Potassium Iodide)	0.04
Phosphorus (Tri-Calcium Phosphate)	290.97	Phosphorus (Tri-Calcium Phosphate)	290
Potassium (Potassium Monophosphate)	163.19	Potassium (Potassium Chloride)	140
Zinc Sulfate	5.50	Zinc Sulphate Monohydrate	5.0
Magnesium Oxide	9.47		
Sodium Chloride	225.67		

New FBFs: White sorghum-cowpea (WSC), Non-extruded WSC (N-WSC), WSC + soy protein isolate (WSC+SPI), Over-processed WSC (O-WSC), Over-processed WSC + soy protein isolate (O-WSC+SPI), White sorghum-soy (WSS), Corn-soy blend 14 (CSB14); CSB+: Corn-soy blend plus.

Table 2.3 Analyzed macronutrient, selected amino acid, and iron content

	WSC	WSC+SPI	N-WSC	O-WSC	O-WSC+ SPI	WSS	CSB14	CSB+
Total Calories (kcal/100g)	412.8	418.4	402.7	403.0	401.7	411.7	429.5	384.8
Carbohydrate (g/100g)	62.6	61.2	59.4	64.3	63.8	62.7	60.6	61.3
Protein (g/100g)	19.1*	19.8*	18.1*	17.9*	18.4*	19.5*	18.4*	15.8*
Fat (g/100g)	9.6	10.5	10.3	8.3	8.1	9.2	12.7	8.5
Ash (g/100g)	3.9	3.7	3.6	3.9	4.2	3.5	3.2	3.6
Crude Fiber (g/100g)	0.6	0.6	0.7	0.8	0.9	0.6	0.5	2.1
Moisture (g/100g)	4.3	4.3	7.9	4.8	4.7	4.5	4.8	8.8
Lysine (mg/g)	14.0	11.8	13.5	10.8	10.4	13.3	12.6	8.5*
Cysteine + methionine (mg/g)	6.3*	4.8*	6.0*	4.7*	4.4*	7.0*	6.5*	5.2*
Available lysine (mg/g)	13.4	11.2	13.1	9.7	9.5	12.8	12.0	8.2*
Iron (mg/100g)	17.2	16.8	16.2	16.5	16.8	15.9	16.0	10.5

White sorghum-cowpea (WSC), Non-extruded WSC (N-WSC), WSC + soy protein isolate (WSC+SPI), Over-processed WSC (O-WSC), Over-processed WSC + soy protein isolate (O-WSC+SPI), White sorghum-soy (WSS), Corn-soy blend 14 (CSB14), Corn-soy blend plus (CSB+).

*Lower than NRC requirements for broiler chickens 0-42 days (105).

Note: The control chicken diet is formulated to provide 230.2 kcal/100g* and contain 22 g/100g protein and 41.5 mg/100g iron; Macronutrient and micronutrient content analyzed in duplicate (macronutrients and amino acids,

AOAC official methods, University of Missouri-Columbia Agricultural Experiment Station Chemical Laboratories, Columbia, MO; iron, AIB International, Manhattan, KS).

Table 2.4 FBF viscosity outcomes

FBF	Time to Cook (min)	Average Bostwick Value (cm/min)
WSC	1	21.5
WSC+SPI	1	18.75
N-WSC	5	5
O-WSC	1	11
O-WSC+SPI	1	11.25
WSS	1	21.5
CSB14	1	13.25
CSB+ (13.79% solids)	5	10

New FBFs prepared at 20% solids.
White sorghum-cowpea (WSC), Non-extruded WSC (N-WSC), WSC + soy protein isolate (WSC+SPI), Over-processed WSC (O-WSC), Over-processed WSC + soy protein isolate (O-WSC+SPI), White sorghum-soy (WSS), Corn-soy blend 14 (CSB14), Corn-soy blend plus (CSB+).

Prepared FBF Study Outcomes

Table 2.5 Food intake, body weight, and food efficiencies (n=10)

	Control	WSC	WSC+SPI	N-WSC	O-WSC	O-WSC+SPI	WSS	CSB14	CSB+
Total Food Intake (g)	6120.2 ± 82.7^{a}	1759.2 ± 103.9^{b}	2217.8 ± 53.3^{bcd}	1946.6 ± 58.1^{bc}	2592.4 ± 61.3^{cd}	1928.4 ± 177.6^{b}	3023.3 ± 131.2^{d}	2445.9 ± 36.4^{bcd}	2989.3 ± 66.3^{d}
Total Weight Gained (g)	1065.0 ± 16.4^{a}	292.3 ± 23.6^{bd}	404.9 ± 10.9^{bc}	354.9 ± 19.0^{bcd}	418.9 ± 13.3^{bce}	265.6 ± 32.5^{d}	623.0 ± 22.4^{e}	459.6 ± 15.1^{ce}	295.6 ± 8.3^{bd}
Final Body Weight (g)	1165.6 ± 15.3^{a}	390.8 ± 23.5^{bc}	505.4 ± 11.8^{be}	456.2 ± 19.8^{bce}	518.6 ± 12.6^{be}	363.7 ± 32.9^{c}	723.7 ± 21.5^{d}	561.1 ± 14.3^{de}	396.3 ± 7.9^{bc}
Caloric Efficiency (g/kcal x 1000)	16.5 ± 0.0^{a}	9.1 ± 0.2^{bc}	9.7 ± 0.0^{bc}	10.1 ± 0.2^{be}	8.9 ± 0.1^{c}	7.6 ± 0.2^{d}	11.2 ± 0.1^{e}	9.8 ± 0.2^{bc}	5.7 ± 0.0^{f}
Protein Efficiency (g/g) x100	17.3 ± 0.0^{a*}	19.6 ± 0.4^{b}	20.5 ± 0.0^{bd}	22.5 ± 0.5^{cd}	20.1 ± 0.2^{b}	16.5 ± 0.5^{a}	23.6 ± 0.2^{c}	23.0 ± 0.4^{c}	14.0 ± 0.1^{e}
Feed Conversion	105.1 ±	107.0	98.7 ±	98.4 ±	111.1 ±	132.5 ±	86.9 ±	95.0 ±	181.2 ±

Ratio (g/g) x100	0.3[ab]	± 2.3[b]	0.2[ab]	2.2[ab]	0.9[b]	4.2[c]	0.7[a]	1.6[a]	1.2[d]

*Based on label values rather than analyzed values.

Data are mean ± SEM; values with different letters are statistically different ($p<0.05$).

White Sorghum-Cowpea (WSC), WSC + Soy Protein Isolate (WSC+SPI), Non-extruded WSC (N-WSC), Over-processed WSC (O-WSC), O-WSC + Soy Protein Isolate (O-WSC+SPI), White Sorghum- Soybean (WSS), Corn-Soybean Blend 14 (CSB14), Corn-Soybean Blend + (CSB+).

Table 2.6 Circulating and hepatic iron levels, and liver weight per body weight (n=10)

	Control	WSC	WSC+SPI	N-WSC	O-WSC	O-WSC+SPI	WSS	CSB14	CSB+
Hemoglobin (g/dL)	8.2 ± 0.5	10.0 ± 0.7	8.7 ± 0.6	9.4 ± 0.9	8.7 ± 0.5	8.3 ± 0.4	8.9 ± 0.7	8.9 ± 1.1	8.5 ± 1.4
Hepatic Iron (µg/g)	13.5 ± 1.4[a]	22.6 ± 3.6[bc]	27.5 ± 4.0[cd]	21.3 ± 1.6[bc]	30.1 ± 3.6[cd]	35.3 ± 4.2[d]	22.2 ± 2.8[bc]	24.5 ± 4.0[bc]*	18.9 ± 4.0[ab]
Liver Weight per Body Weight (%) x100	2.4 ± 0.2[a]	3.3 ± 0.4[b]	3.0 ± 0.1[abc]	3.0 ± 0.2[abc]	2.8 ± 0.2[abc]	3.2 ± 0.1[bc]	2.7 ± 0.3[ac]	2.6 ± 0.1[ac]*	2.8 ± 0.1[abc]

*n=9

Data are mean ± SEM; values with different letters are statistically different ($p<0.05$).

White Sorghum-Cowpea (WSC), WSC + Soy Protein Isolate (WSC+SPI), Non-extruded WSC (N-WSC), Over-processed WSC (O-WSC), O-WSC + Soy Protein Isolate (O-WSC+SPI), White Sorghum- Soybean (WSS), Corn-Soybean Blend 14 (CSB14), Corn-Soybean Blend + (CSB+).

Table 2.7 Gait scores and bone mineral density outcomes (n=10)

	Control	WSC	WSC+SPI	N-WSC	O-WSC	O-WSC+SPI	WSS	CSB14	CSB+
Gait Score (+1)	1.0 ± 0.0[a]	3.4 ± 0.6[b]	1.0 ± 0.0[a]	3.9 ± 0.6[b]	1.0 ± 0.0[a]	1.0 ± 0.0[a]	1.8 ± 0.6[ac]	2.1 ± 0.6[c]	1.0 ± 0.0[a]
Total BMD (g/cm^2) x100	21.1 ± 0.5[a]	8.4 ± 0.4[b]	11.7 ± 0.3[c]	9.1 ± 0.5[b]	11.6 ± 0.7[c]	9.6 ± 0.5[bd]	12.0 ± 0.6[c]	10.6 ± 0.3[cd]	9.2 ± 0.3[b]
Diaphysis BMD (g/cm^2) x100	25.6 ± 0.7[a]	9.3 ± 0.4[b]	12.1 ± 0.3[c]	9.3 ± 0.4[b]	12.1 ± 0.6[c]	10.3 ± 0.6[bc]	11.8 ± 0.6[c]	11.0 ± 0.4[c]	9.7 ± 0.3[b]
Proximal BMD (g/cm^2) x100	17.3 ± 0.3[a]	7.9 ± 0.5[b]	11.1 ± 0.4[c]	9.0 ± 0.6[b]	11.0 ± 0.6[c]	8.8 ± 0.5[b]	12.3 ± 0.5[d]	10.8 ± 0.3[c]	8.5 ± 0.2[b]
Distal BMD (g/cm^2) x100	19.1 ± 0.5[a]	8.0 ± 0.6[b]	11.8 ± 0.3[c]	8.8 ± 0.6[bd]	11.7 ± 0.8[c]	9.5 ± 0.6[bd]	12.1 ± 0.6[c]	9.8 ± 0.4[d]	9.3 ± 0.3[bd]

Gait Scores: increased by 1 to be able to analyze data in SAS. 1 = no impairment, up to 6 = complete lameness (99).

BMD: bone mineral density.

Data are mean ± SEM; values with different letters are statistically different ($p<0.05$).

White Sorghum-Cowpea (WSC), WSC + Soy Protein Isolate (WSC+SPI), Non-extruded WSC (N-WSC), Over-processed WSC (O-WSC), O-WSC + Soy Protein Isolate (O-WSC+SPI), White Sorghum- Soybean (WSS), Corn-Soybean Blend 14 (CSB14), Corn-Soybean Blend + (CSB+).

Dry FBF Study Outcomes

Table 2.8 Food intake, body weight outcomes, and food efficiencies

	Control n=23	WSC n=24	WSC+SPI n=24	O-WSC n=24	O-WSC+SPI n=24	WSS n=24	CSB14 n=24	CSB+ n=24
Total Food Intake (g)	889.5 ± 3.3^{a}	325.1 ± 2.5^{b}	286.5 ± 5.5^{c}	284.1 ± 2.7^{c}	209.4 ± 3.0^{d}	360.0 ± 6.4^{e}	327.9 ± 6.1^{be}	248.9 ± 2.4^{f}
Total Weight Gained (g)	575.3 ± 2.9^{a}	141.4 ± 1.0^{bd}	125.9 ± 3.4^{b}	126.9 ± 2.8^{b}	75.6 ± 1.7^{c}	163.1 ± 4.0^{d}	131.2 ± 4.6^{b}	79.0 ± 2.2^{c}
Final Body Weight (g)	663.6 ± 2.9^{a}	229.7 ± 1.1^{bd}	212.8 ± 3.6^{b}	215.1 ± 2.6^{b}	163.0 ± 2.1^{c}	251.3 ± 4.2^{d}	221.3 ± 4.8^{b}	167.4 ± 2.4^{c}
Caloric Efficiency (g/kcal) x1000	49.3 ± 1.3^{a}	17.6 ± 0.2^{b}	17.5 ± 0.1^{b}	18.4 ± 0.2^{b}	14.9 ± 0.1^{cd}	18.3 ± 0.2^{b}	15.5 ± 0.3^{c}	13.7 ± 0.3^{d}
Protein Efficiency (g/g) x100	51.6 ± 1.3^{a*}	38.0 ± 0.3^{bc}	37.0 ± 0.3^{be}	41.5 ± 0.5^{c}	32.7 ± 0.3^{d}	38.7 ± 0.5^{bc}	36.2 ± 0.8^{bd}	33.4 ± 0.6^{de}
Feed Conversion Ratio (g/g) x100	27.0 ± 0.4^{a}	38.3 ± 0.4^{bd}	38.1 ± 0.3^{bd}	37.5 ± 0.5^{b}	46.4 ± 0.5^{c}	36.9 ± 0.4^{b}	42.2 ± 0.9^{cd}	53.0 ± 1.0^{e}

*Based on label values rather than analyzed values.
Data are mean ± SEM; values with different letters are statistically different ($p<0.05$).
White Sorghum-Cowpea (WSC), WSC + Soy Protein Isolate (WSC+SPI), Over-processed WSC (O-WSC), O-WSC + Soy Protein Isolate (O-WSC+SPI), White Sorghum- Soybean (WSS), Corn-Soybean Blend 14 (CSB14), Corn-Soybean Blend + (CSB+).

Table 2.9 Circulating and hepatic iron levels, and liver weight per body weight (n=6)

	Control	WSC	WSC+SPI	O-WSC	O-WSC+SPI	WSS	CSB14	CSB+
Hemoglobin (g/dL)	9.2 ± 0.9^{a}	9.4 ± 0.1^{a}	8.5 ± 0.6^{a}	8.8 ± 0.3^{a}	8.6 ± 0.3^{a}	9.6 ± 0.4^{a}	9.5 ± 0.5^{a}	12.4 ± 0.4^{b}
Hepatic Iron (µg/g)	7.9 ± 1.1^{a}	17.6 ± 3.2^{b}	27.1 ± 4.5^{cd}	21.9 ± 2.0^{bc}	32.9 ± 4.1^{c}	20.7 ± 2.1^{bd}	16.4 ± 4.0^{b}	8.0 ± 0.7^{a}
Liver Weight per Body Weight (%) x100	2.6 ± 0.1^{a}	3.6 ± 0.1^{b}	3.4 ± 0.1^{b}	3.6 ± 0.2^{b}	3.2 ± 0.3^{b}	3.6 ± 0.1^{b}	3.3 ± 0.2^{b}	3.3 ± 0.2^{b}

Data are mean ± SEM; values with different letters are statistically different ($p<0.05$).

White Sorghum-Cowpea (WSC), WSC + Soy Protein Isolate (WSC+SPI), Over-processed WSC (O-WSC), O-WSC + Soy Protein Isolate (O-WSC+SPI), White Sorghum- Soybean (WSS), Corn-Soybean Blend 14 (CSB14), Corn-Soybean Blend + (CSB+).

Table 2.10 Comparison of NRC broiler chicken nutrient requirements to FBFs

	NRC 0-21 days	NRC 22-42 days	WSC	WSC +SPI	N-WSC	O-WSC	O-WSC +SPI	WSS	CSB 14	CSB+
kcal/100g	320	320	412.8	418.4	402.7	403.02	401.7	411.7	429.5	384.8
Amino Acids	**g/100g**									
Crude Protein	23	20	19.07	19.76	18.12	17.92	18.37	19.51	18.37	15.78
Arginine	1.25	1.1	0.89	1.31	0.87	1	1.14	0.92	0.82	0.98
Glycine + serine	1.25	1.14	1.44	1.65	1.35	1.38	1.46	1.54	1.37	1.29
Histidine	0.35	0.32	0.47	0.53	0.46	0.48	0.51	0.45	0.42	0.41
Isoleucine	0.8	0.73	0.99	0.9	0.95	0.81	0.8	1.06	0.98	0.66
Leucine	1.2	1.09	1.87	1.68	1.78	1.61	1.58	2.06	1.86	1.43
Lysine	1.1	1	1.34	1.18	1.35	1.08	1.04	1.33	1.2	0.82
Methionine	0.5	0.38	0.33	0.27	0.31	0.28	0.26	0.34	0.32	0.25
Methionine + cysteine	0.9	0.72	0.63	0.48	0.6	0.47	0.44	0.7	0.65	0.52
Phenylalanine	0.72	0.65	0.89	1.08	0.86	0.94	1.02	0.9	0.82	0.78
Phenylalanine + tyrosine	1.34	1.22	1.43	1.68	1.38	1.47	1.58	1.51	1.37	1.29
Proline	0.6	0.55	0.93	0.9	0.83	0.78	0.78	1.17	1.04	0.92
Threonine	0.8	0.74	0.97	0.7	0.91	0.71	0.65	0.98	0.92	0.57
Tryptophan	0.2	0.18	0.3	0.25	0.28	0.24	0.22	0.32	0.31	0.2
Valine	0.9	0.82	1.04	1	1	0.93	0.93	1.09	0.98	0.77
Selected minerals	**mg/100g**									
Calcium	1000	900	279.1	279.1	279.1	279.1	279.1	279.1	279.1	452
Nonphytate phosphorus	450	350	291	291	291	291	291	291	291	290
Iron	8	8	17.2	16.8	16.15	16.45	16.75	15.85	16.0	10.45

ND: No data. NRC requirements from (105).

Control chicken diet based on label values: 230.2 (kcal/100g), crude protein (22%), lysine (0.87%), methionine (0.43%), crude fat (2.5%), crude fiber (7.0%), calcium (1.0-1.1%), phosphorus (0.78%), salt (0.15-0.4%), sodium (0.01-0.3%); iron content analyzed in duplicate (41.5 mg/100g).

FBF protein and amino acid values analyzed in duplicate by University of Missouri-Columbia Agricultural Experiment Station Chemical Laboratories, Columbia, MO.

FBF iron content analyzed in duplicate by AIB International, Manhattan, KS.

FBF calcium and nonphytate phosphorus values based on Kansas State University proposed commodity specifications sheets.
White sorghum-cowpea (WSC), Non-extruded WSC (N-WSC), WSC + soy protein isolate (WSC+SPI), Over-processed WSC (O-WSC), Over-processed WSC + soy protein isolate (O-WSC+SPI), White sorghum-soy (WSS), Corn-soy blend 14 (CSB14), Corn-soy blend plus (CSB+).

Figures

Prepared FBF Study

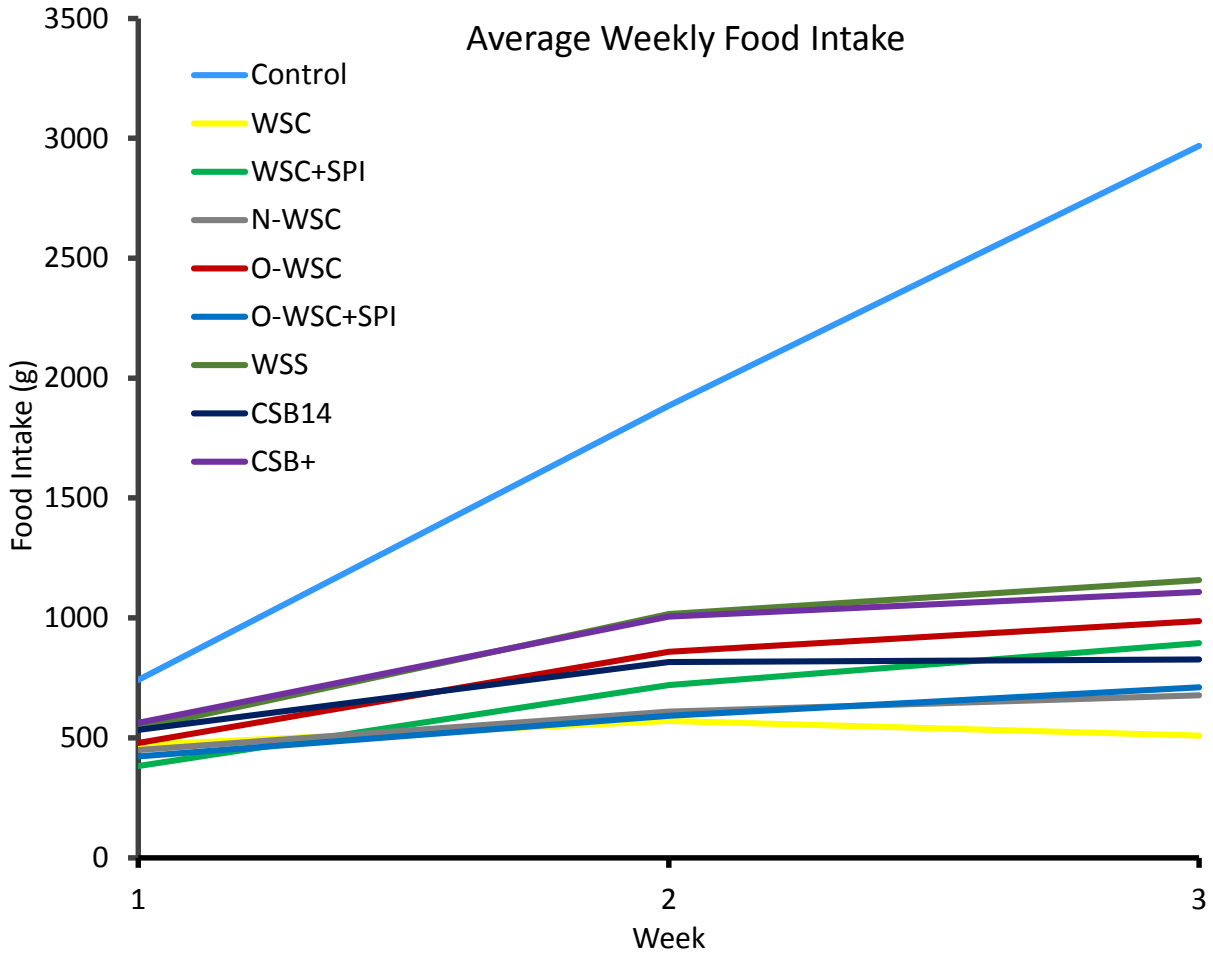

Figure 2.1 Average weekly food intake for Prep study (n=10).

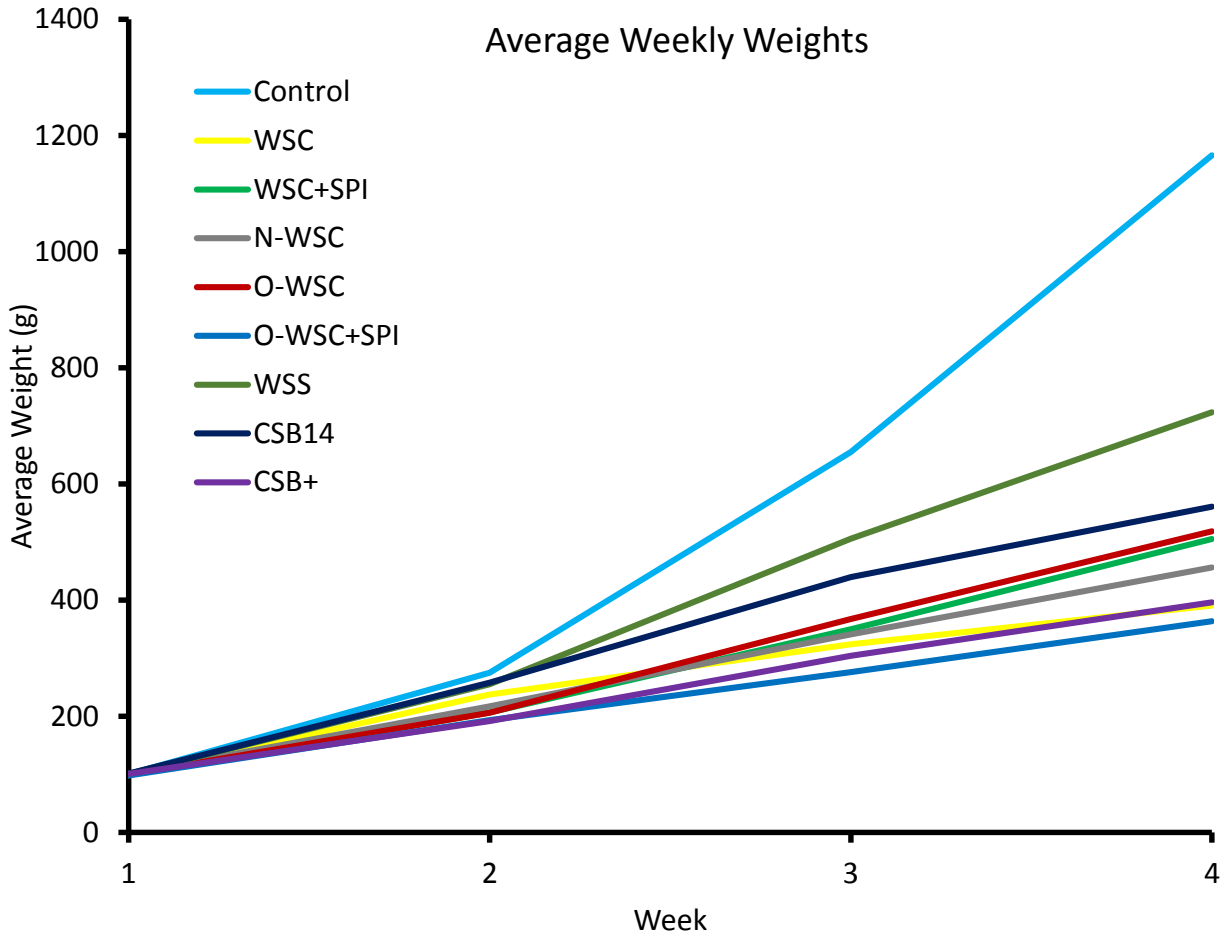

Figure 2.2 Average weekly body weights for Prep study (n=10).

Dry FBF Study

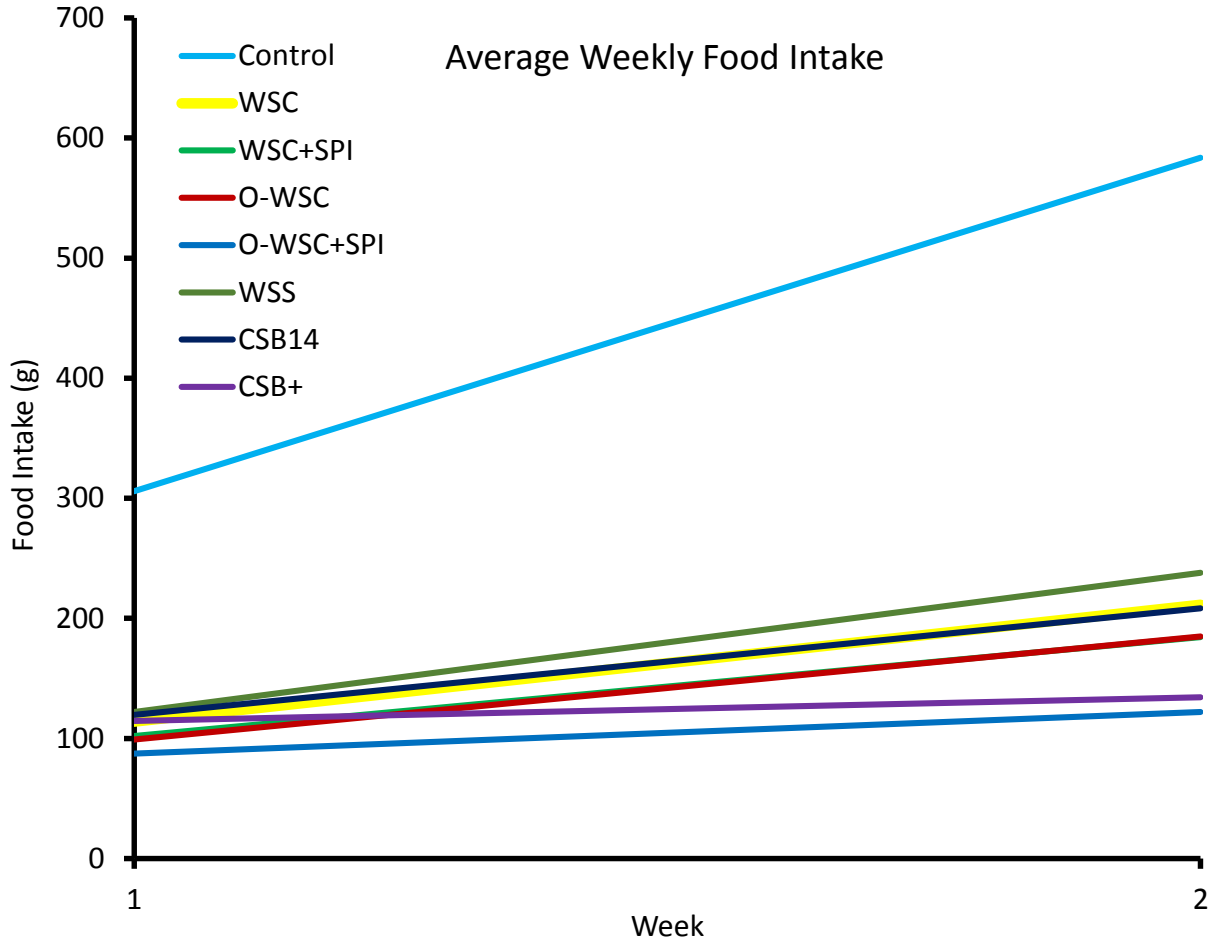

Figure 2.3 Average weekly food intake for Dry study (n=24, control n=23).

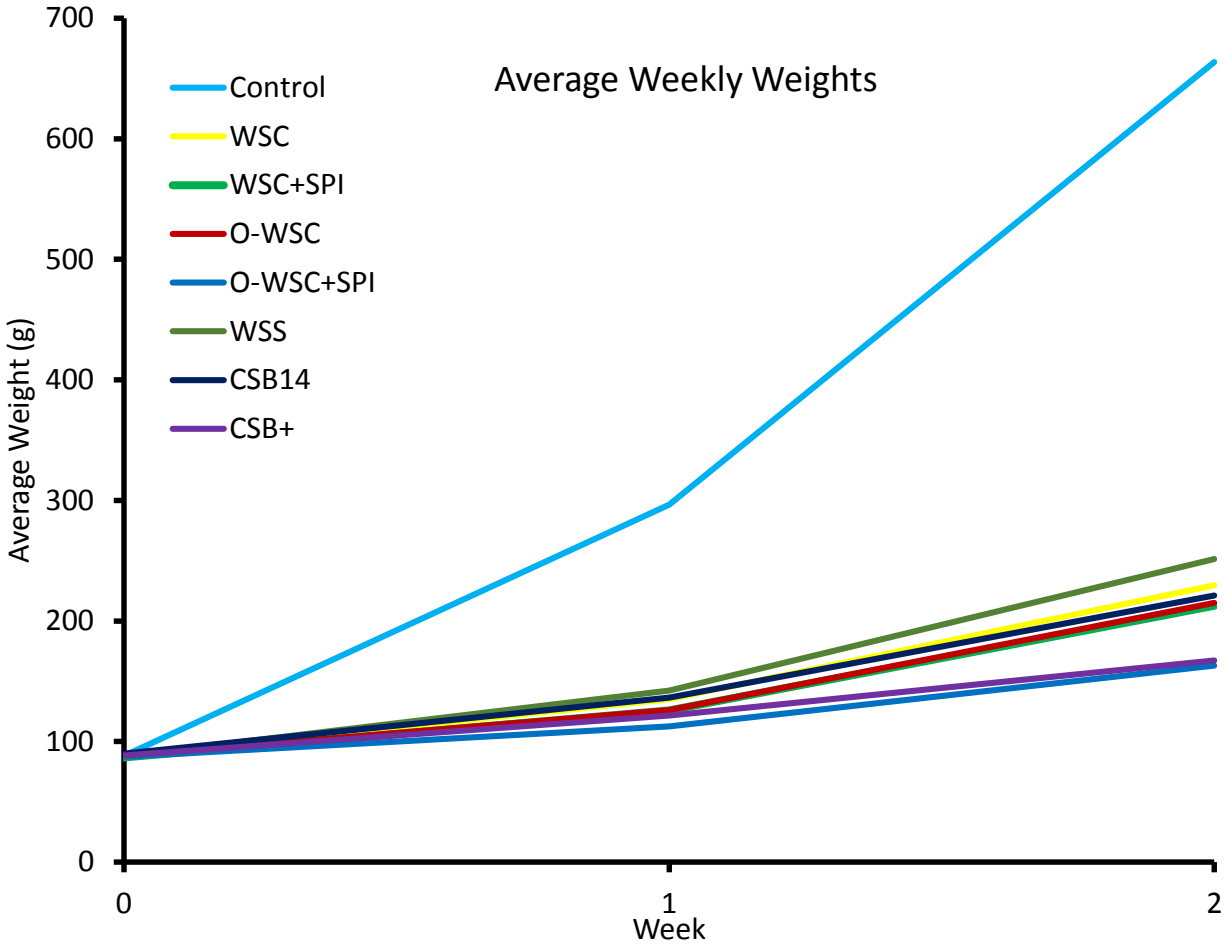

Figure 2.4 Average weekly body weights for Dry study (n=24, control n=23).

Chapter 3 - Final Conclusions and Future Directions

The assessment of new FBFs compared to CSB+ *in vivo* in the chicken model allowed us to observe the nutritional quality of these products in a cost-effective model, to further refine and identify better FBF formulations in the continued effort to improve undernutrition through these products. Although in the Prep study, the chicken model did not allow us to control for certain environmental conditions, in the Dry study chickens were more robust. In a potential future study comparing FBFs nutritional quality in chickens, it may be worthwhile to design the study to include two groups for each FBF; one group fed normal FBF, and one group fed FBF plus a supplement that would meet chicken nutrient requirements. This could prevent limitations, such as gait issues, to better compare protein quality and iron bioavailability outcomes of FBFs in a healthier chicken model.

As far as improving FBFs, suggestions made by the FAQR were supported in this study including sorghum and cowpea containing FBFs being effective alternatives to corn and soy, and extrusion processing improving nutritional quality compared to CSB+. Soy protein was also as efficacious to whey protein in one comparison group, deeming it a cost-effective protein supplement for improving growth outcomes. Additionally, less expensive options with reformulation and over-processing of sorghum and cowpea with whey protein were a novel formulation that proved to be equally efficacious, and can be considered to make a more cost-effective FBF. In a potential study, it would be interesting to look at increasing the level of soy flour to meet protein requirements, for example in a sorghum-soy FBF, without needing to add in any protein supplement, thus making an even more cost-effective FBF.

Although these studies were a small part in the overall mission to improve food aid, we still obtained very interesting and valuable results, and I am very grateful to have been able to

contribute to the overall goal of this project. I look forward to further research determining the most effective FBF formulations for ultimately treating, and ideally preventing, undernutrition in children and other vulnerable populations.

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