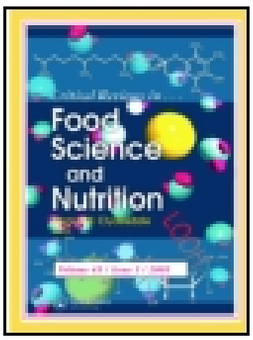

Critical Reviews in Food Science and Nutrition

ISSN: 1040-8398 (Print) 1549-7852 (Online) Journal homepage: http://www.tandfonline.com/loi/bfsn20

Advantages and limitations of in vitro and in vivo methods of iron and zinc bioavailability evaluation in the assessment of biofortification program effectiveness

Desirrê Morais Dias, Neuza Maria Brunoro Costa, Marilia Regini Nutti, Elad Tako & Hércia Stampini Duarte Martino

To cite this article: Desirrê Morais Dias, Neuza Maria Brunoro Costa, Marilia Regini Nutti, Elad Tako & Hércia Stampini Duarte Martino (2017): Advantages and limitations of in vitro and in vivo methods of iron and zinc bioavailability evaluation in the assessment of biofortification program effectiveness, Critical Reviews in Food Science and Nutrition, DOI: 10.1080/10408398.2017.1306484

To link to this article: http://dx.doi.org/10.1080/10408398.2017.1306484

Accepted author version posted online: 17 Apr 2017.

Advantages and limitations of *in vitro* and *in vivo* methods of iron and zinc bioavailability evaluation in the assessment of biofortification program effectiveness

Desirrê Morais Dias[1,*], Neuza Maria Brunoro Costa[2], Marilia Regini Nutti[3], Elad Tako[4], Hércia Stampini Duarte Martino[1]

[1]Department of Nutrition and Health, Federal University of Viçosa, Viçosa, Minas Gerais, Brazil

[2]Department of Pharmacy and Nutrition, Center for Exact, Natural and Health Sciences, Federal University of Espirito Santo, Alto Universitario, 29500-000, Alegre, ES, Brazil

[3]EMBRAPA Food Technology, Rio de Janeiro, Brazil - Leader of the Brazilian Biofortification Network

[4]USDA/ARS, Robert W. Holley Center for Agriculture and Health, Cornell University, Ithaca, NY 14853, USA

[*]Corresponding author. Address: Department of Nutrition and Health, Universidade Federal de Viçosa, Avenida PH Rolfs s/n, 36570-900, Viçosa -MG, Brazil. Tel.: +55 31 3899 1276. E-mail: desirremorais@hotmail.com

Abstract

Biofortification aims to improve the micronutrient concentration of staple food crops through the best practices of breeding and modern biotechnology. However, increased zinc and iron concentrations in food crops may not always translate into proportional increases in absorbed zinc (Zn) and iron (Fe). Therefore, assessing iron and zinc bioavailability in biofortified crops is imperative to evaluate the efficacy of breeding programs. This review aimed to investigate the advantages and limitations of *in vitro* and *in vivo* methods of iron and zinc bioavailability evaluation in the assessment of biofortification program effectiveness. *In vitro*, animal and

isotopic human studies have shown high iron and zinc bioavailability in biofortified staple food crops. Human studies provide direct knowledge regarding the effectiveness of biofortification, however, human studies are time consuming and are more expensive than *in vitro* and animal studies. Moreover, *in vitro* studies may be a useful preliminary screening method to identify promising plant cultivars, however, these studies cannot provide data that are directly applicable to humans. None of these methods provides complete information regarding mineral bioavailability, thus, a combination of these methods should be the most appropriate strategy to investigate the effectiveness of zinc and iron biofortification programs.

Keywords

Caco-2 cells, animal models, clinical trials, biofortification, mineral bioavailability.

ACCEPTED MANUSCRIPT

INTRODUCTION

Biofortification aims to improve the micronutrient concentration of staple food crops through the best practices of breeding and modern biotechnology. The most commonly targeted micronutrients include iron, zinc, and provitamin A carotenoids because of the high prevalence of deficiencies of these micronutrients in children and women of childbearing age (Bouis et al., 2011; La Frano et al., 2014). Biofortification may reach malnourished rural populations who may have limited access to supplements and commercially fortified foods (Saltzman et al., 2012).

However, the enhancement of zinc and iron concentrations in staple food crops may not translate into a proportional increase in absorbed zinc and iron since absorption inhibitors or enhancers may be present. Therefore, it is necessary to measure not only mineral concentration in enhanced crops but also bioavailability (Tako et al., 2011).

Several *in vitro* and *in vivo* models have been identified as appropriate for estimating human absorption and metabolism (La Frano et al., 2014). *In vivo* analyses of how food components are absorbed through the intestinal wall into the circulatory system to exert their biological effects are not easy to perform. Thus, *in vitro* studies represent useful tools to simulate the conditions of the alimentary tract and enable more detailed research into cell metabolism under controlled conditions. Moreover, this approach is more ethical than research conducted on experimental animals (Wlodzimierz; Olejnik, 2004).

Finally, as advances in plant breeding are achieved, it is important to determine how effectively micronutrient content increments are utilized by humans, in addition to their public health benefits and sustainability of these benefits. Thus, this review aimed to identify the

advantages and limitations of *in vitro* and *in vivo* methods of iron and zinc bioavailability evaluation to assess the effectiveness of biofortification programs.

METHODS

A search was performed in the PubMed, Medline and Science Direct databases for articles published between 2000 and 2016 related to zinc and iron bioavailability in biofortified foods. Combinations of the following keywords were used: “iron”, “zinc”, “biofortified foods”, “biofortification”, and “bioavailability”. To identify studies not included in the initial search, a reverse search was conducted using the reference lists in the identified articles. *In vitro* (Caco-2 cells) and *in vivo* (human and animal models) studies regarding zinc and iron bioavailability in biofortified foods were included. Each article selected for inclusion in this review was critically analyzed.

RESULTS

The search strategy resulted in the selection of 25 articles from 1556 articles researched (903 articles on iron and 653 articles on zinc) (Figure 1). Eight references evaluated more than one mineral or evaluated one mineral by more than one method. Five references evaluated iron bioavailability using both *in vitro* and *in vivo* models (Tako et al., 2011; Tako et al., 2013; Tako et al., 2014; Tako et al., 2015a; Tako et al., 2015b). One reference evaluated zinc and iron bioavailability using human and Caco-2 cell models as well as iron bioavailability using a rat model (Vaz-Tostes et al., 2015). Furthermore, one reference assessed iron and zinc bioavailability in humans (Kodkany et al., 2013), and another study assessed iron and zinc bioavailability in Caco-2 cells (Jou et al., 2012). Thus, 13 articles were found that evaluated zinc bioavailability in biofortified crops (animal studies: 3; *in vitro* studies: 4; human studies: 6), and

23 articles were found that evaluated iron bioavailability in biofortified crops (animal studies: 8; *in vitro* studies: 7; human studies: 8), for a total of 36 articles on iron and/or zinc bioavailability.

Methods of mineral bioavailability evaluation

In vitro *model: Caco-2 cell culture*

In vitro models of human tissues are gaining importance because of their relevance and wide applicability. The combination of cell culture and *in vitro* digestion models presents an alternative for studies that are frequently challenging to conduct in humans and animals because of ethical concerns (Payne et al, 2012).

Caco-2 cells are isolated from colon adenocarcinomas and mimic the typical characteristics of the human small intestinal epithelium (Brito et al., 2013; Wlodzimierz; Olejnik, 2004). This model can be used in *in vitro* Fe bioavailability studies combined with *in vitro* digestion methods and the high-throughput measurement of Caco-2 cell ferritin formation as a measure of Fe uptake (Yun et al., 2004). Caco-2 cell ferritin is a sensitive and clear marker of cell Fe uptake since cells produce ferritin proportionately in response to increases in intracellular iron (Ariza-Nieto et al., 2007).

Iron and zinc bioavailability may be assessed through the determination of nutrient uptake and transport by Caco-2 cells. Iron uptake may be estimated by ferritin formation or ^{59}Fe uptake (a radioisotope of iron). In contrast to ferritin formation, which is an indicator of iron uptake, there are no biomarkers of zinc uptake. Metallothionein, a cytoplasmic protein that stores zinc, has been used as an indicator of zinc uptake. However, this protein may also bind and store other metals, such as copper, selenium, cadmium, mercury, silver, and arsenic (Bell; Vallee, 2009). Thus, metallothionein is not specific for zinc, which makes its application as a biomarker for

zinc bioavailability questionable (Etcheverry et al., 2012). Therefore, the combination of isotopic techniques with Caco-2 cells should be a better approach to estimate zinc absorption *in vitro* because similar results have been obtained from animal and human studies (Jou et al., 2012).

Iron uptake may be estimated by ^{59}Fe uptake (a radioisotope of iron). Whereas, zinc bioavailability studies have used an extrinsic labeling with ^{65}Zn. Extrinsic ^{65}Zn equilibrates with intrinsic Zn in complex food matrices of animal and plant origin after *in vitro* digestion (Jou et al., 2012).

Thus, many studies have used this *in vitro* method to estimate the iron and zinc bioavailability from biofortified foods (Tables 1 and 2).

Animal models

Different approaches may be used to evaluate zinc- and iron-biofortified foods as dietary mineral sources for humans. The most appropriate model is to directly evaluate different biofortified foods in human studies. However, these studies are expensive and time consuming. An alternative approach is to perform bioavailability studies on animals (Petterson et al. 2008; Carlson et al. 2014).

Iron bioavailability may be assessed in rats and pigs via the hemoglobin repletion assay reported by Perks and Miller (1996) to obtain a relative measure of iron absorption. Blood is sampled at the initiation of the feeding period to measure the initial hemoglobin concentrations. The animals are subsequently fed experimental diets for 2 to 5 weeks, after which blood samples are obtained for the determination of the final hemoglobin concentrations. The feed intake is measured throughout the period to calculate the iron intake. The blood volume is estimated from

the body weight, and it is used to calculate the hemoglobin repletion efficiency (HRE), which is an indicator of iron bioavailability (Petterson et al, 2014).

Moreover, various techniques have been developed to assess mineral retention and absorption *in vivo*. One of the simplest methods for indirectly measuring the absorption of ingested iron and zinc is to assess fecal and urinary excretion levels and then differentially calculate the absorption/retention based on the ingested dose (Petterson et al. 2008). However, the main pathway of zinc and iron excretion is endogenous, which limits the utility of this method because it may underestimate the bioavailability of these micronutrients.

Another option involves the use of stable radioisotopes. Whole-body counting is performed to determine the retention level, and individual tissues may be assayed to determine the distribution patterns throughout the body. Isotope absorption is subsequently determined by measuring the changing isotopic ratios in tissue, blood, and/or urine against the more abundant, natural isotopic form (Griffin, 2002).

Summaries of studies that have investigated zinc and iron bioavailability using these animal models are presented in Table 1 (iron studies) and Table 2 (zinc studies).

Human studies

The human studies that have evaluated iron and zinc bioavailability in biofortified foods have used radioactive and stable isotopes. This technique discriminates the amounts of the micronutrient provided by the diet from endogenous forms, which enables a more accurate measurement of bioavailability. Iron and zinc have been both intrinsically and extrinsically labeled. Intrinsic labeling is the biological incorporation of an isotope into a plant during its growth, whereas extrinsic labeling is the addition of an isotope to food prior to ingestion

(Fairweather-Tait et al., 2005). Extrinsic labeling may be used for non-heme iron (present in plant foods) and zinc absorption studies in humans (Jou et al., 2012).

These techniques are also useful to investigate the potential efficacy of different iron and zinc compounds for use in food fortification and mineral supplements (Hotz; Brown, 2004). Furthermore, they may be useful to assess the efficiency of mineral biofortification programs.

Iron bioavailability studies have used isotopically labeled $^{58}FeSO_4$ and $^{57}FeSO_4$, and the amounts of ^{57}Fe and ^{58}Fe isotopic labels have been analyzed in blood samples (Petry et al., 2012; Petry et al., 2014; Cercamondi et al., 2013; Kodkany et al., 2013). For zinc bioavailability, human studies have utilized the stable isotopes ^{67}Zn, ^{68}Zn, and ^{70}Zn, and the amounts of the isotopic labels have been analyzed in urine (Rosado et al., 2009; Islam et al. 2013; Brni´c et al. 2015; Chomba et al., 2014). These human studies are summarized in Table 1 (iron studies) and Table 2 (zinc studies).

Iron bioavailability evaluation

In vitro *studies*

Iron (Fe) deficiency is the most prevalent nutrient deficiency, affecting approximately 40% of the world's population, particularly women and children in developing countries (WHO, 2008; Muthayya et al., 2013). Strategies for reducing the prevalence of iron deficiency include the distribution of Fe supplements, food fortification and the diversification of diets. The common bean (*Phaseolus vulgaris L.*), one of the staple food crops targeted for nutritional enhancement by HarvestPlus, is an attractive candidate for Fe biofortification because there is genetic variability in Fe concentration, and it is possible to increase the Fe concentrations in beans (Welch et al., 2000). Furthermore, the Fe concentrations in beans are high relative to those

in other crops, therefore, beans may deliver substantial amounts of Fe (Tako et al., 2008). In this context, the CIAT (International Center for Tropical Agriculture, Cali, Colombia) has developed biofortified beans that contain up to 100 μg Fe/g bean, which represents a substantial increase over that in standard beans (Blair et al., 2010).

Two studies have used the *in vitro* method with Caco-2 cells to evaluate the iron bioavailability of biofortified red mottled beans (Tako et al., 2011) and carioca beans (Tako et al., 2015b) from the CIAT. In two studies, higher ferritin concentrations were found in cells exposed to the Fe-biofortified bean than in cells exposed to the standard Fe bean. These findings indicate increased amounts of bioavailable Fe in the Fe-biofortified beans. In contrast, Vaz-Tostes et al. (2015) did not identify differences in ferritin concentrations between common beans (PE) and the targeted bean for mineral biofortification (PO) (PO: 13.1 ± 1.4 and PE: 13.6 ± 1.4 ng mg^{-1} protein).

Maize (*Zea mays* L.) and pearl millet have also been used in a biofortification program as a strategy to increase iron intake in an at-risk population. Maize is widely consumed in developing countries and provides energy, vitamins and minerals. Thus, maize is an attractive candidate for Fe biofortification (Cannon et al., 2011; Tako et al., 2013). An *in vitro* digestion/Caco-2 cell culture model employed by Tako et al. (2013) showed higher amounts of bioavailable iron in biofortified maize than in common maize. Similarly, Tako et al. (2015a) identified increased amounts of bioavailable Fe in high-Fe pearl millet. Thus, maize and millet are promising vehicles to alleviate Fe deficiency in human populations where these foods are major dietary staples.

Biofortified rice has also been investigated for iron bioavailability in the Caco-2 cell model. Biofortified rice had more bioavailable iron than the control. Moreover, this result was even more pronounced in the presence of ascorbic acid, which is reported to be the most efficient promoter of iron absorption (Trijatmiko et al. 2016).

Animal studies

Despite the rat model presents differences from humans regarding Fe absorption, hemoglobin depletion/repletion studies are widely used to assess the relative bioavailability of Fe in foods (Tako et al., 2009). In this context, two studies have used this model to evaluate iron bioavailability in biofortified foods. In these studies, the authors identified increased iron bioavailability in beans targeted for iron biofortification (Dias et al., 2015; Vaz-Tostes et al., 2015). However, rats are more efficient than humans at iron absorption from plant foods since they produce phytase and vitamin C, which led these studies to overestimate the iron bioavailability in plant foods (Sant'ana et al., 2006).

Therefore, poultry may be a suitable model for the measurement of iron bioavailability because of their quick response to iron deficiency (Tako; Glahn, 2010; Tako et al., 2011). The modern broiler chicken is a fast-growing animal that is sensitive to dietary deficiencies of trace minerals such as Fe and they have limited ability to synthesize ascorbic acid (Tako et al., 2011; Tako; Bar; Glahn, 2016). Physiological adaptations to iron deficiency may occur over time, thus, animals must be monitored for signs of anemia. In addition, this model has very good agreement with the results obtained from *in vitro* Caco-2 cells. Moreover, the combination of this animal model with the *in vitro* Caco-2 cell model has been effective for testing the bioavailability of iron in food crops (Tako et al, 2014; Tako; Glahn, 2011; Tako et al, 2009).

Therefore, a group of researchers has investigated iron bioavailability by combining *in vitro* (Caco-2 cells) and *in vivo* (*Gallus gallus*) models. In this experimental model, two groups of animals are compared, including one group fed iron-free diets (which leads to iron deficiency) and a second group provided with the test foods as a dietary iron source. Blood variables (hemoglobin) and the gene expression of proteins related to iron metabolism are evaluated to determine the iron bioavailability. In this way, these authors have compared the iron bioavailability of biofortified red beans (Tako; Glahn, 2011), biofortified black beans (Tako et al, 2014), biofortified maize (Tako et al, 2013) and pearl millet (Tako et al, 2015a) with those of their conventional counterparts. They identified an increase in iron bioavailability by increasing blood hemoglobin and liver ferritin and reducing the gene expression of divalent metal transporter 1 (DMT-1), duodenal cytochrome b (Dcytb) and ferroportin in the animal duodenum (Tako; Glahn, 2011; Tako et al, 2014; Tako et al, 2013; Tako et al, 2015).

Piglets have been used as a model for iron bioavailability studies because of similarities in gastrointestinal anatomy and physiology between pigs and humans. Pigs, similar to humans, are truly omnivorous, and the digestive and metabolic processes in pigs are similar to humans (Patterson et al., 2008). Moreover, pigs readily consume monotonous diets that may be formulated to simulate the human diets common in resource-poor regions of the world. Furthermore, iron deficiency develops rapidly in young pigs (Tako et al., 2009). This model was used by Tako et al. (2009) to assess the iron bioavailability of iron-biofortified and standard black beans consumed in a maize-based diet in a 5-week feeding study. Hemoglobin regeneration efficiency, which represented a measure of iron bioavailability, did not differ between the groups, which indicates that although the biofortified beans contained increased the

concentrations of iron, the bioavailable iron in these beans was equal to that in the standard beans.

Human studies

Current iron biofortification research programs have focused on increasing the iron concentration of staple crops, such as wheat, maize, rice, beans, and pearl millet (Nestel; Bouis, 2006). Thus, these studies have evaluated the iron bioavailability in these biofortified foods (Table 1).

Petry et al. (2012) utilized an iron stable isotope to elucidate the potential of common beans as a biofortification vehicle for iron. This study was conducted in women with a low iron status. They observed that iron absorption from the high-iron bean was 40% lower than that from the normal-iron bean, which resulted in equal amounts of iron absorbed. In addition, when beans were combined with other meal components in multiple meals, high polyphenol concentrations had no negative impact on iron absorption. However, the quantity of iron absorbed from composite meals with high-iron beans was not different from that absorbed from meals with normal-iron beans, which indicates that efficacious iron biofortification may be difficult to achieve in beans rich in phytic acid (PA) and polyphenols.

Similarly, in an intervention study with high-iron beans, Vaz-Tostes et al. (2015) determined there were no changes in iron nutritional status in preschool children after high-iron bean intake (ferritin, 41.2 ± 23.2 and 28.9 ± 40.4 μg L^{-1}; hemoglobin, 13.7 ± 2.2 and 13.1 ± 3.2 g dL^{-1}, respectively). In contrast, Petry et al. (2014), using a stable isotope technique to assess the effect of PA on iron bioavailability in iron-biofortified beans (the beans were grown from certified

seeds at CIAT), reported a higher quantity of absorbed iron from this variety than from the control bean.

Furthermore, they promoted bean dephytinization, and an increase in the quantity of iron absorbed from the biofortified bean was identified that was higher than that from the control bean. Thus, the authors concluded that the PA decreases iron bioavailability in iron-biofortified beans, and a high PA concentration is an important impediment to the optimal effectiveness of bean iron biofortification (Petry et al., 2014). However, PA is required for plant growth, thus, its reduction may lead to decreased productivity.

Because PA decreases iron bioavailability in beans, one study investigated whether low-PA beans provide more bioavailable iron than iron-biofortified beans. A multiple-meal crossover design with 25 young women was performed that utilized stable iron isotopes to assess iron absorption. The amount of bioavailable iron in low-PA beans did not differ from that available in the biofortified beans, however, the amount of bioavailable iron in the biofortified beans was >50% higher than that in the control beans (Petry et al., 2016).

Haas et al. (2016) conducted a randomized controlled trial to compare the efficacy of iron-biofortified beans (Fe-Beans) relative to standard unfortified beans (Control-Beans) in improving the iron status in iron-deficient women. Iron status was assessed via measurements of hemoglobin, serum ferritin, soluble transferrin receptor, and body iron. Fe-Beans were associated with significantly greater increases in hemoglobin, serum ferritin and body iron than Control-Beans. For every 1 g of Fe consumed from beans during the study, there was a significant 4.2-g/L increase in hemoglobin. Thus, the consumption of iron-biofortified beans significantly improved the iron status of iron-deficient women.

Another food targeted for iron biofortification is pearl millet, which has been reported to contain 7-8 mg/100 g of Fe (Hama et al, 2012; Harvestplus, 2009), approximately double the iron content of other major cereal staples (Cercamondi et al., 2013). Thus, two studies have assessed the iron bioavailability in pearl millet (Cercamondi et al., 2013; Kodkany et al., 2013). Cercamondi et al. (2013) used stable iron isotopes to evaluate the potential of iron-biofortified millet to provide additional bioavailable iron compared with conventional millet and post-harvest iron-fortified millet in women with marginal iron status. It was reported that the total absorbed iron from biofortified millet was higher than that from conventional millet. Furthermore, the quantity of total absorbed iron from the post-harvest iron-fortified millet was higher than that from the conventional and iron-biofortified millet. Thus, although the fractional absorption of iron from biofortification was lower than that from fortification, iron-biofortified millet should be highly effective in combating iron deficiency in millet-consuming populations.

The same result was obtained by Kodkany et al. (2013), who used a stable isotope in iron-deficient children. They found a higher total amount of absorbed iron from biofortified pearl millet than from conventional pearl millet. In addition, the absorption of iron from the biofortified millet exceeded the physiological requirement (0.54 mg/d) for this age group (Kodkany et al., 2013).

Rice has been targeted for iron biofortification and was one of the first crops biofortified by HarvestPlus (Haas et al., 2005). The International Rice Research Institute (IRRI) recently developed a variety of rice for experimental use that has 400–500% more iron, after processing and cooking, than conventional varieties (Gregorio et al., 2000). Thus, one study (Hass et al., 2005) investigated the efficacy of consuming this biofortified rice in Filipino women at risk of

iron deficiency. They used a randomized, controlled, double-blind and longitudinal (9 months) intervention trial. The study analyzed two groups (low-iron rice and high-iron rice) and two groups of subjects (anemic and non-anemic women). The biofortified and conventional rice produced no differences in blood hemoglobin or ferritin in the anemic women. However, in the non-anemic women, blood hemoglobin and ferritin were higher in the biofortified rice group than in the conventional rice group. Thus, the consumption of biofortified rice, without other changes in the diet, may be efficacious to improve the iron stores of women with iron-poor diets.

Zinc bioavailability evaluation

In vitro *studies*

Using a stable isotope, ^{65}Zn, in a Caco-2 model, Jou et al. (2012) compared zinc bioavailability in undermilled and polished biofortified rice and undermilled conventional rice. They showed that zinc absorption from biofortified rice, either undermilled or polished, was twofold higher than that from conventional rice. In addition, the molar ratio of phytate to zinc was lower in biofortified rice (19:1) than in the common varieties (35-46:1), which may result in increased zinc bioavailability in biofortified rice. In addition to biofortification, foliar Zn fertilization produced significant increases in Zn retention, transport and uptake efficiency in Caco-2 cells (Wei, Shohag; Yang, 2012). However, the latter study did not utilize stable isotopes, it analyzed zinc concentrations in the cells, which may overestimate zinc absorption.

It has been shown that both biofortification and rice genotype may influence zinc bioavailability in rice. Wei et al. (2012) compared *in vitro* Zn bioavailability between three genotypes of Zn-fortified germinated brown rice and normal germinated brown rice. They found higher percentages of Zn absorption by Caco-2 cells from the Zn-fortified germinated brown rice

than from the normal germinated brown rice, and there was bioavailability variation among the tested rice genotypes.

Beans were also assessed in the Caco-2 cell model in one study (Vaz-Tostes et al., 2015). In this study, two bean varieties were evaluated: the conventional BRS Pérola (PE) (20.47 mg/kg Zn) and a variety targeted for mineral biofortification, BRS Pontal (PO) (26.1 mg/kg Zn). However, in contrast to rice, there was no difference in zinc uptake between the conventional bean and the bean targeted for mineral biofortification (PO: 15.9 ± 1.5 and PE: 15.5 ± 3.5 μmol mg^{-1} protein).

Animal studies

Rodent models are suitable for the assessment of zinc bioavailability. For zinc, the rat pup model is the most appropriate model because young rats do not have intestinal phytase activity (La Frano et al., 2014). Jou et al. (2012) utilized this model to test Zn bioavailability in five varieties of rice. Four varieties represented several of the most highly produced varieties in Bangladesh (i.e., BR-28, BR-29, BR-11, and Paijam) (between 14 and 19 mg Zn/kg), and one variety represented a Zn-biofortified line (IR-68-1-44) (35 mg/kg of Zn in polished form) developed at the IRRI (Los Baños, Philippines).

Rats were fed a radiolabeled diet that contained ^{65}Zn. The radioactivity in the stomach, perfused intestine, perfusate, liver, carcass, and cecum-colon was measured via gamma counting. The absorbed Zn was expressed as fractional absorption and was calculated as radioactivity in the carcass, liver, kidney, and perfused small intestine as a percentage of the total recovery. The results indicated that the absorbed zinc from the biofortified rice was twice as high as that from the common rice. However, the phytate:zinc molar ratio was lower in biofortified rice (19:1) than

in common varieties (35-46:1), which may result in increased zinc bioavailability in biofortified rice (Jou et al., 2012).

Similarly, Welch et al. (2000) used a rat model to determine the zinc bioavailability in the same genotype of the previously described zinc-biofortified rice (IR68144) and zinc-biofortified beans from CIAT. Furthermore, they used the same methodology as Jou et al. (2012), which included the radioisotopes ^{65}Zn and ^{59}Fe. Bioavailability was calculated from the amount of radiolabeled zinc retained in the rats over a 10-day period, as determined each day via a whole-body gamma spectrometry assay. The results demonstrated that increasing the amount of zinc in enriched rice grains and beans significantly increased the amount of bioavailable zinc. These findings also support the contention that the selection of traits in bean and rice genotypes that enrich the zinc concentration in their seeds and grains will provide more bioavailable zinc to target populations dependent on these foods as a major source of zinc in their diet.

Pigs have also been used to evaluate zinc bioavailability. This model is known to be a good model for humans. Carlson et al. (2012) fed two groups of pigs with zinc-biofortified wheat and beans for seven days. They collected and weighed the urine and feces during the balance period. However, the authors were not able to calculate reliable zinc bioavailability values using this model because the animals had low zinc intake, which represents a limitation of this type of study. Moreover, the balance technique was not appropriate for estimating zinc bioavailability because the main pathway of zinc excretion is endogenous. Recently, two studies proposed a new biomarker of zinc status in a poultry model that can be used in human zinc status studies, the erythrocyte linoleic acid:dihomo-γ-linolenic acid (LA:DGLA) ratio. An elevation in the 18:2ω6:20:3ω6 ratio may be a sensitive marker for zinc deficiency because this mineral is

required by Δ^6-desaturase, which converts 18:2ω6 to 20:3ω6 (Reed et al., 2014; Knez et al., 2016).

Human studies

In a study of Bangladeshi children, Islam et al. (2013) used the dual-isotope tracer ratio technique to calculate the total absorbed zinc from high-zinc rice. The zinc intake from the conventional rice-based diet was 1 mg less than that from the high-zinc diet, however, the total absorbed zinc from these diets was not significantly different. This finding was a result of the lower fractional absorption (20.1%) from the high-zinc rice than from the conventional rice (25.1%) and the increased phytate content present in the high-zinc rice.

Using a double-isotope tracer ratio method in adults, Brni´c et al. (2016) reported similar absorbed fractions of zinc from biofortified rice and fortified rice. Thus, rice biofortification is likely to be as good as post-harvest zinc fortification as a strategy to combat zinc deficiency.

Rosado et al. (2009) compared zinc absorption from high-zinc-biofortified wheat and control wheat, which were extracted at high (95%) and moderate (80%) levels, respectively. To assess the fractional absorption of zinc, they used a dual-isotope tracer ratio technique in women. Both extraction rates resulted in similar reductions in the zinc and phytate contents, suggesting that the benefits of high-zinc wheat are not reduced by milling. Zn absorption was 31% (95% extraction group) and 33% (80% extraction group) higher from the Zn-biofortified wheat than from the control. These findings suggest that Zn absorption from the same quantities of wheat flour is higher for Zn-biofortified wheat than for wheat with a more typical Zn concentration.

The bioavailability of zinc in biofortified pearl millet has been investigated in one human study with non-zinc-deficient children (Kodkany et al., 2013). Zinc consumption was 5.8 mg/d

from biofortified pearl millet and 3.3 mg/d from regular pearl millet. Using stable isotope extrinsic labeling of zinc, the amount of absorbed zinc from biofortified millet was found to be higher than that from control millet, although the fractional absorption was less than expected.

Another food target for zinc biofortification is maize, particularly in countries with a higher consumption of this cereal. Therefore, Chomba et al. (2014) used the dual-isotope tracer technique in young children to compare the zinc absorption from control maize, zinc-biofortified maize and zinc-fortified maize. They found that the total daily absorption of zinc from the biofortified maize was higher than that from the control maize, however, it did not differ from that from the fortified maize. Thus, biofortified maize intake meets zinc requirements and provides an effective dietary alternative to regular maize for this vulnerable population.

These studies assessed zinc bioavailability in biofortified foods using isotopic techniques. However, in addition to these techniques, one study used plasma zinc and erythrocyte zinc determination to investigate the beneficial effects of beans targeted for biofortification on improving zinc nutritional status in preschool children (Vaz-Tostes et al., 2015). Thus, these researchers performed a nutritional intervention with the Pontal bean, which is a target variety for the mineral biofortification program of the Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA), Brazil. There were no differences in zinc nutritional status after Pontal bean consumption (plasma zinc: 119.2 ± 24.5 and 133.9 ± 57.7 μg dL^{-1}; erythrocyte zinc: 53.5 ± 13.8 and 59.4 ± 17.1 μg g^{-1}, respectively). Nonetheless, this type of study provides feedback for biofortification programs to produce beans with higher mineral bioavailability.

Correlation among *in vitro* (cell culture), animal and human studies: advantages and limitations

No single bioavailability method is ideal for all micronutrients, and all bioavailability methods present advantages and limitations (Table 3). The required equipment, costs of labor and animals, and funds available all play important roles in determining the method of choice. The selection of a method to use in determining micronutrient bioavailability in plant foods requires the consideration of several issues that may affect the results obtained. Several particularly important issues include intrinsic versus extrinsic labeling of the plant material, the bioavailability model to use (*in vitro* or animal species), the micronutrient status of the experimental subjects, and the levels of anti-nutrients and promoter substances in the test plant food and test meals (Welch et al., 2000).

Human studies provide the most applicable results because they are capable of considering host factors, disease states, and physiological changes during digestion. Therefore, these results may be interpreted more directly and used to assess the true absorption of nutrients (La Frano et al, 2014). However, human studies remain difficult to perform because of the social and ethical considerations that govern the invasive medical procedures necessary in accessing the human large intestine, thus, human studies are primarily limited to fecal sample analyses (Payne et al, 2012).

The use of radioactive and stable isotopes in human studies is an alternative to invasive methods and it enables the discrimination of the dose of micronutrients provided by the diet and endogenous forms, which provides a more accurate measurement of bioavailability. However, the use of isotopes has limitations because of the risk of radioactivity exposure, costs, complexity and labor-intense procedures required (Au; Reddy, 2000).

Therefore, animal models represent an alternative to human studies because they may provide useful information regarding *in vivo* bioavailability, particularly in the dissection and analysis of individual tissues to provide a whole-body assessment of absorption (La Frano et al, 2014).

Rats may be used to provide relative estimates of bioavailable iron and zinc in plant foods (Whelch et al., 2000). However, because of their quick response to micronutrient deficiencies, including low iron status, poultry may represent a suitable model to measure iron bioavailability (La Frano et al., 2014; Tako; Bar; Glahn, 2016). The poultry model has found a useful niche as an intermediate test of *in vivo* iron bioavailability studies in preparation for subsequent human studies (Tako; Bar; Glahn, 2016). In addition, the *Gallus gallus* model has been used in numerous studies aimed at assessing iron bioavailability, absorption and status *in vivo*, specifically to assess the effectiveness of iron-biofortified crops to deliver more absorbable iron to maintain or improve iron status (Tako et al., 2014; Tako et al., 2015; Tako; Bar; Glahn, 2016).

Piglets are also a good model for iron bioavailability studies because of their similarities to humans with respect to gastrointestinal anatomy and physiology. However, differences in body fat content may translate into differences in nutrient absorption because of the increased expression of hepcidin, which may inhibit iron absorption (Frazer; Anderson, 2005).

The *in vitro* cell model has also been used to investigate the mineral bioavailability and it is especially important for investigating interactions between minerals and enhancers or inhibitors, such as the effect of polyphenolics and phytic acid. However, it lacks communication with other organs that are involved in the regulation of nutrient absorption *in vivo* (Pigeon et al., 2001; Roetto et al., 2003; Scheers et al., 2014).

Relevance of *in vitro* and *in vivo* studies to evaluate the effectiveness of food biofortification programs

Biofortification involves the development of micronutrient-enhanced staple crop varieties via traditional breeding practices or modern biotechnology (Nestel et al., 2006). Biofortification is potentially more sustainable and cost-effective than conventional fortification, and it implicitly targets low-income households in remote areas with a substantial daily consumption of a limited number of food staples and limited access to commercially marketed fortified foods (Meenakshi et al., 2010; Bouis et al., 2011). However, mineral biofortification improves zinc and iron status only if the additional amounts of these minerals provided by the biofortified crop are bioavailable (Cercamondi et al., 2013). Thus, prior to the implementation of an intervention and in addition to agronomic traits, environmental factors, and variability in micronutrient concentrations, bioavailability is a major concern (La Frano et al., 2014).

Bioavailability, which represents the amount of a nutrient that is accessible for utilization in normal physiological functions, metabolism and storage, may be enhanced or inhibited by the presence of food components and food-processing techniques. Therefore, the amount of a mineral that is present in food and available for absorption must be investigated to properly estimate the minimum amount of that mineral that breeders must achieve and to predict the success of these interventions. Therefore, the measurement of zinc and iron absorption from biofortified crops is an important first step prior to demonstrating the efficacy of these crops in improving the status of these minerals (Bouis et al., 2011; La Frano et al., 2014).

Since the nutrient absorption by the body is a prerequisite for the prevention of micronutrient deficiencies the change in the prevalence of mineral deficiencies with the long-term intake of

biofortified staple foods must be directly measured. Therefore, human studies that demonstrate the impact of biofortified crops on the micronutrient status are required to provide evidence to support the release of biofortified crops (Bouis et al., 2011).

Zinc and iron bioavailability is an important factor that has been considered in biofortification programs. Thus, studies that evaluate the effectiveness of these programs have focused on this evaluation. *In vitro* studies have been used as a means to screen, rank, and categorize cultivars and foods, substantial numbers of genetic variants, food-processing effects, and influencers of absorption, as well as to direct attention to factors that may deserve further investigation *in vivo* (Etcheverry et al., 2012; La Frano et al., 2014).

Approximately 78% of the iron bioavailability assays reported higher iron absorption from biofortified foods than from conventional foods (Table 1). While, about 62% of the zinc bioavailability studies reported higher zinc absorption from bioforitified foods than from conventional foods (Table 2). These results indicate that zinc- and iron-biofortified foods may represent an effective strategy to combat nutritional deficiencies in populations at nutritional risk.

Conclusion

In vitro and *in vivo* studies have provided knowledge regarding zinc and iron bioavailability in biofortified foods and assessments of the effectiveness of mineral biofortification programs. Thus, these studies have reported higher amounts of absorbed zinc and iron from biofortified foods than from similar conventional foods. These results are likely due to the presence of higher amounts of Zn/Fe in these biofortified foods than in conventional foods.

The *in vitro* Caco-2 cell model cannot provide data directly applicable to humans because it cannot simulate all the physiological and metabolic responses of the organism. However, this model may be used as a useful preliminary screening method to identify promising plant cultivars to be tested in *in vivo* bioavailability assays. Furthermore, this method enables the investigation of interactions between zinc and iron and enhancers or inhibitors of their absorption at different concentrations in a shorter time and at a lower cost than the *in vivo* models.

The utilization of *in vivo* models is necessary to assess the physiological alterations caused by biofortified foods. Animal models enable the dissection and analysis of individual tissues to provide a whole-body assessment of absorption and an understanding of the gene expression alterations caused by biofortified food intake. Isotopic techniques in human studies have also been used to assess zinc and iron absorption rates, and they provide a more accurate measurement of bioavailability.

Human studies also provide direct knowledge regarding the effectiveness of biofortification because they enable the assessment of alterations in zinc and iron nutritional status. However, human studies are longer and more expensive than *in vitro* and animal studies. Thus, the combination of *in vitro* (Caco-2 cells), animal and human studies is most appropriate to investigate zinc and iron bioavailability and the effectiveness of biofortification programs. Therefore, it should be acceptable to perform screening in a cell culture assay as an initial step and subsequently investigate the best variety of foods identified in the cell culture study in animal studies. These biofortified varieties could then be tested in human studies.

References

Ariza-Nieto, M. et al. (2007). Screening of iron bioavailability patterns in eight bean (Phaseolus vulgaris L.) genotypes using the Caco-2 cell in vitro model. *J. Agric. Food Chem* 55, 7950–7956.Au, A.P., Reddy, M.B. (2000). Caco-2 cells can be used to assess human iron bioavailability from a semipurified meal. *The Journal of nutrition*, 130(5): 1329-1334.

Bell, S. G., Vallee, B. L. (2009). The metallothionein/thionein system: an oxidoreductive metabolic zinc link. *Chembiochem*, 10(1): 55-62.

Blair, M.W. et al. (2010). Registration of high mineral common bean germplasm lines NUA35 and NUA56 from the red mottled seed class. *J Plant Regis*, 4:1-5.

Bouis, H. E. et al (2011). Biofortification: a new tool to reduce micronutrient malnutrition. *Food and nutrition bulletin*, 32(1 suppl1): S31-S40.

Bouis, H. E., Welch, R. M. (2010). Biofortification—a sustainable agricultural strategy for reducing micronutrient malnutrition in the global south. *Crop Science*, 50(Supplement_1): S-20.

Brnić, M. et al. (2016). Zinc Absorption by Adults Is Similar from Intrinsically Labeled Zinc-Biofortified Rice and from Rice Fortified with Labeled Zinc Sulfate. *The Journal of nutrition*, 146(1): 76-80.

Cannon, E.K. et al. (2011). POPcorn: an online resource providing access to distributed and diverse maize project data. *Int J Plant Genomics*, 92:30–35.

Carlson, D. et al. (2012). Bioavailability of trace elements in beans and zinc-biofortified wheat in pigs. *Biological trace element research*, 150(1-3): 147-153.

Cercamondi, C. I. et al. (2013). Total iron absorption by young women from iron-biofortified pearl millet composite meals is double that from regular millet meals but less than that from post-harvest iron-fortified millet meals. *The Journal of nutrition*, 143(9): 1376-1382.

Chomba, E. et al. (2015). Zinc absorption from biofortified maize meets the requirements of young rural Zambian children.*The Journal of nutrition*, 145(3): 514-519.

Dias, D. M. et al. (2015). Rice and Bean Targets for Biofortification Combined with High Carotenoid Content Crops Regulate Transcriptional Mechanisms Increasing Iron Bioavailability.*Nutrients*, 7(11): 9683-9696.

Etcheverry, P., Grusak, M.A., Fleige, L.E (2012). Application of *in vitro* bioaccessibility and bioavailability methods for calcium, carotenoids, folate, iron, magnesium, polyphenols, zinc, and vitamins B(6), B(12), D, and E. *Front Physiol*. 3: 1-22.

Fairweather-Tait, S. et al (2005). The usefulness of in vitro models to predict the bioavailability of iron and zinc: a consensus statement from the HarvestPlus expert consultation. *International journal for vitamin and nutrition research*, 75(6): 371-374.

Frazer, D.M., Anderson, G.J (2005). Intestinal iron absorption and its regulation. *Am J Physiol Gastrointest Liver Physiol*. 289:G631–G635.

Gregorio, G. B. et al. (2000). Breeding for trace mineral density in rice. *Food and Nutrition Bulletin*, 21(4): 382-386.

Griffin, I. J. (2002). Using stable isotopes and isotope ratio mass spectrometry to study mineral metabolism in humans Invited Lecture. *Journal of Analytical Atomic Spectrometry*, 17(9): 1186-1193.

Haas, J. D. et al. (2005). Iron-biofortified rice improves the iron stores of nonanemic Filipino women. *The Journal of nutrition*, 135(12): 2823-2830.

Haas, J. D. et al. (2016). Consuming Iron Biofortified Beans Increases Iron Status in Rwandan Women after 128 Days in a Randomized Controlled Feeding Trial. *The Journal of Nutrition*, 146(8): 1586-1592.

Hama, F. et al. (2012). Potential of non-GMO biofortified pearl millet (Pennisetum glaucum) for increasing iron and zinc content and their estimated bioavailability during abrasive decortication. *International Journal of Food Science & Technology*, 47(8): 1660-1668.

HarvestPlus. Iron pearl millet; 2009 [cited 2012 Oct]. Available from: http://www.unscn.org/layout/modules/resources/files/HarvestPlus_Pearl_Millet_Strategy.pdf.

Hotz, C., Brown K.H. (2004) International zinc nutrition consultative group (IZiNCG) technical document #1. Assessment of the risk of zinc deficiency in populations and options for its control. *Food Nutr Bull*, 25: S99 –S199.

House, W.A (1999). Trace element bioavailability as exemplified by iron and zinc. *Field Crops Res*. 60: 115–141.

Islam, M. M. et al. (2013). Total zinc absorption from a diet containing either conventional rice or higher-zinc rice does not differ among Bangladeshi preschool children. *The Journal of nutrition*, 143(4): 519-525.

Jou, M. Y. et al. (2012). Biofortification of rice with zinc: assessment of the relative bioavailability of zinc in a Caco-2 cell model and suckling rat pups. *Journal of agricultural and food chemistry*, 60(14): 3650-3657.

Knez, M., Stangoulis, J. C., Zec, M., Debeljak-Martacic, J., Pavlovic, Z., Gurinovic, M., Glibetic, M. (2016). An initial evaluation of newly proposed biomarker of zinc status in humans-linoleic acid: dihomo-γ-linolenic acid (LA: DGLA) ratio. *Clinical Nutrition ESPEN*, 15: 85-92.

Kodkany, B. S. et al. (2013). Biofortification of pearl millet with iron and zinc in a randomized controlled trial increases absorption of these minerals above physiologic requirements in young children. *The Journal of nutrition*, 143(9): 1489-1493.

La Frano, M. R. et al. (2014). Bioavailability of iron, zinc, and provitamin A carotenoids in biofortified staple crops. *Nutrition reviews*, 72(5): 289-307.

Muthayya, S. et al (2013). The global hidden hungerx indices and maps: an advocacy tool for action. *PLoS One*, 8:7860. 2.

Patterson, J. K. et al. (2008). The pig as an experimental model for elucidating the mechanisms governing dietary influence on mineral absorption. *Experimental Biology and Medicine*, 233(6): 651-664.

Perks, S. M., Miller, D. D. (1996). Adding ascorbic acid to iron-fortified cow's milk does not enhance iron bioavailability to piglets. *Nutrition Research*, 16(6): 969-975.

Petry, N. et al (2016). In Rwandese Women with Low Iron Status, Iron Absorption from Low-Phytic Acid Beans and Biofortified Beans Is Comparable, but Low-Phytic Acid Beans Cause Adverse Gastrointestinal Symptoms. *The Journal of nutrition*, 146(5): 970-975.

Pigeon, C. et al. (2001). A new mouse liver-specific gene, encoding a protein homologous to human antimicrobial peptide hepcidin, is overexpressed during iron overload. *Journal of biological chemistry*, 276(11): 7811-7819.

Reed, S., Qin, X., Ran-Ressler, R., Brenna, J. T., Glahn, R. P., Tako, E. (2014). Dietary zinc deficiency affects blood linoleic acid: Dihomo-γ-linolenic acid (LA: DGLA) ratio; a sensitive physiological marker of zinc status in vivo (Gallus gallus). *Nutrients*, 6(3): 1164-1180.

Roetto, A. et al. (2003). Mutant antimicrobial peptide hepcidin is associated with severe juvenile hemochromatosis. *Nature genetics*, *33*(1).

Rosado, J. L. et al. (2009). The quantity of zinc absorbed from wheat in adult women is enhanced by biofortification. *The Journal of nutrition*, 139(10): 1920-1925.

Sant'ana, L.F.R, Cruz, A.C.R.F., Costa NMB (2006). Biodisponibilidade de ferro de uma multimistura em uma dieta de arroz e feijão. *J Braz Soc Food Nutr* 31:1–14.

Scheers, N. M. et al. (2014). Proposing a Caco-2/HepG2 cell model for in vitro iron absorption studies. *The Journal of nutritional biochemistry*, 25(7): 710-715.

Stein, A.J. (2010). Global impacts of human mineral malnutrition. *Plant Soil*. 335: 133–154.

Tako, E. et al. (2011). Biofortified red mottled beans (Phaseolus vulgaris L.) in a maize and bean diet provide more bioavailable iron than standard red mottled beans: Studies in poultry (Gallus gallus) and an in vitro digestion/Caco-2 model. *Nutrition journal*, 10(1): 2-10.

Tako, E. et al. (2009). Biofortified black beans in a maize and bean diet provide more bioavailable iron to piglets than standard black beans. *The Journal of nutrition*, 139(2): 305-309.

Tako, E. et al. (2013). High bioavailablilty iron maize (Zea mays L.) developed through molecular breeding provides more absorbable iron in vitro (Caco-2 model) and in vivo (Gallus gallus). *Nutrition journal*, 12(1): 1.

Tako, E. et al. (2014). Polyphenolic compounds appear to limit the nutritional benefit of biofortified higher iron black bean (Phaseolus vulgaris L.). *Nutrition journal*, 13(1): 1.

Tako, E. et al. (2015) (a). Higher iron pearl millet (Pennisetum glaucum L.) provides more absorbable iron that is limited by increased polyphenolic content. *Nutrition journal*, 14(1): 1.

Tako, E. et al. (2015) (b). Studies of cream seeded carioca beans (phaseolus vulgaris L.) from a Rwandan efficacy trial: in vitro and in vivo screening tools reflect human studies and predict beneficial results from iron biofortified beans. *PloS one*, 10(9): e0138479.

Tako, E., Bar, H., Glahn, R. P. (2016). The Combined Application of the Caco-2 Cell Bioassay Coupled with In Vivo (Gallus gallus) Feeding Trial Represents an Effective Approach to Predicting Fe Bioavailability in Humans. *Nutrients*, 8(11): 732.

Tako, E., Glahn, R. P. (2010). White beans provide more bioavailable iron than red beans: studies in poultry (Gallus gallus) and an in vitro digestion/Caco-2 model. *International Journal for Vitamin and Nutrition Research*, 80(6): 416.

Tako, E., Glahn, R. P. (2011). Iron status of the late term broiler (Gallus gallus) embryo and hatchling. *Int J Poul Sci*, 10(1): 42-48.

Tako, E., Glahn, R. P. (2011). White beans provide more bioavailable iron than red beans: studies in poultry (Gallus gallus) and an in vitro digestion/Caco-2 model. *Int J Vitam Nutr Res*, 81: 1-14.

Thakkar, S. K. et al. (2009). Impact of style of processing on retention and bioaccessibility of β-carotene in cassava (Manihot esculanta, Crantz). *Journal of Agricultural and food chemistry*, 57(4): 1344-1348.

Trijatmiko, K. R. et al. (2016). Biofortified indica rice attains iron and zinc nutrition dietary targets in the field. *Scientific reports*, 6.

Vaz-Tostes, M. D. G. et al. (2016). Evaluation of iron and zinc bioavailability of beans targeted for biofortification using in vitro and in vivo models and their effect on the nutritional status of preschool children. *Journal of the science of food and agriculture*, 96(4): 1326-1332.

Wei, Y. et al. (2012). Effect of zinc sulfate fortification in germinated brown rice on seed zinc concentration, bioavailability, and seed germination. *Journal of agricultural and food chemistry*, 60(7): 1871-1879.

Wei, Y., Shohag, M. J. I., Yang, X. (2012). Biofortification and bioavailability of rice grain zinc as affected by different forms of foliar zinc fertilization. *PloS one*, 7(9): e45428.

Welch, R.M. et al. (2000). Genetic selection for enhanced bioavailable levels of iron in bean (Phaseolus vulgaris L.) seeds. *J Agric Food Chem*. 48:3576–80.

White, P.J. et al (2005). Biofortifying crops with essential mineral elements. *Trends in Plant Science* 10: 586–593.

WHO: Worldwide Prevalence of Anaemia 1993–2005. WHO Global Database on Anaemia. Geneva: World Health Organization; 2008.

Wu, C. et al. (2010) Uptake, translocation, and remobilization of zinc absorbed at different growth stages by rice genotypes of different Zn densities. *J Agric Food Chem* 58: 6767–6773.

Yun, S. et al. (2004). An in vitro digestion/Caco-2 cell culture system accurately predicts the effects of ascorbic acid and polyphenolic compounds on iron bioavailability in humans.*The Journal of nutrition*, 134 (10): 2717-272.

Table 1 Iron (Fe) bioavailability in biofortified foods

REFERENCE	TESTED FOODS	STUDY DESIGN	RESULTS
In vitro *studies*			
Tako et al. (2011)	High-Fe red mottled beans (71 mg/Kg Fe) and control red beans (49 mg/Kg Fe)	Caco-2 cell assay	Increased amounts of bioavailable Fe in the -biofortified red mottled beans. Fe-biofortified red mottled beans.
Tako et al. (2015a)	High-Fe pearl millet (84.9 μg/g Fe) and low-Fe pearl millet (25.9 μg/g Fe)	Caco-2 cell assay	Increased amounts of bioavailable Fe in the high-Fe pearl millet.
Tako et al. (2015b)	Fe-biofortified carioca beans (106 mg/Kg Fe) and standard carioca beans (58 mg/Kg Fe)	Caco-2 cell assay	Increased amounts of bioavailable Fe in the Fe-biofortified carioca beans.
Vaz-Tostes et al. (2015)	Common bean (52.43 mg/kg Fe) and high-Fe bean (60.62 mg/kg Fe)	Caco-2 cell assay	No differences were identified in ferritin concentrations between common beans and beans tagged for mineral biofortification.
Tako et al. (2013)	High-Fe-bioavailability maize (21 mg/Kg Fe) and low-Fe-bioavailability maize (20 mg/Kg Fe)	Caco-2 cell assay	Higher amount of bioavailable iron from the biofortified maize than from the common maize.
Tako et al. (2014)	Fe-biofortified (88 mg/Kg Fe) and standard black beans (59 mg/Kg Fe)	Caco-2 cell assay	Low Fe bioavailability in Fe-biofortified beans and standard beans.

Trijatmiko et al. (2016)	Fe-biofortified polished rice (8.2 mg/Kg Fe) and wild-type rice (2-3 mg/Kg Fe)	Caco-2 cell assay	Higher amount of bioavailable iron in the biofortified rice than in the control.
Animal models			
Dias et al. (2015)	High-Fe Pontal beans (75.2 mg/Kg Fe)	Rat model using the hemoglobin depletion-repletion method	Higher iron bioavailability in beans targeted for iron biofortification.
Vaz-Tostes et al. (2015)	Common bean (52.43 mg/kg Fe) and high-Fe bean (60.62 mg/kg Fe)	Rat model using the hemoglobin depletion-repletion method	Higher iron bioavailability in beans targeted for iron biofortification than in the control.
Tako et al. (2011)	High-Fe red mottled beans (71 mg/Kg Fe) and control red beans (49 mg/Kg Fe)	Poultry model via hemoglobin maintenance efficiency	Increase in iron bioavailability in high-Fe red mottled identified by increases in blood hemoglobin and liver ferritin.
Tako et al. (2014)	Fe-biofortified (88 mg/Kg Fe) and standard black beans (59 mg/Kg Fe)	Poultry model via hemoglobin maintenance efficiency	The Fe bioavailability in Fe-biofortified beans was lower than that in standard beans.
Tako et al. (2013)	High-Fe-bioavailability maize (21 mg/Kg Fe) and low-Fe-bioavailability maize (20 mg/Kg Fe)	Poultry model via hemoglobin maintenance efficiency	Increase in iron bioavailability in high-Fe bioavailability maize by increasing the blood hemoglobin and liver ferritin.

Tako et al. (2015a)	High-Fe pearl millet (84.9 μg/g Fe) and low-Fe pearl millet (25.9 μg/g Fe)	Poultry model via hemoglobin maintenance efficiency	Increase in iron bioavailability in high-Fe pearl millet identified by increases in blood hemoglobin and liver ferritin.
Tako et al. (2009)	Fe-biofortified (106 mg/Kg Fe) and standard black beans (71 mg/Kg Fe)	Pig model via hemoglobin maintenance efficiency	Hemoglobin regeneration efficiency did not differ between biofortified beans and standard beans.
Tako et al. (2015b)	Fe-biofortified carioca beans (106 mg/Kg Fe) and standard carioca beans (58 mg/Kg Fe)	Poultry model via hemoglobin maintenance efficiency	Increase in iron bioavailability in Fe-biofortified carioca beans identified by increases in blood hemoglobin and liver ferritin.
Human studies			
Petry et al. (2012)	High-Fe beans (9.1 mg/Kg Fe) and control beans (5.2 mg/Kg Fe)	Stable isotope in women with low iron status	Fe absorption from the high-iron bean was lower than from the normal-iron bean, which resulted in equal amounts of iron absorbed.
Vaz-Tostes et al. (2015)	High-Fe beans (60.62 mg/kg Fe)	Intervention study with preschool children using ferritin and hemoglobin analysis	No changes in Fe nutritional status in preschool children before and after the consumption of high-iron beans.
Petry et al. (2014)	Fe-biofortified beans (88 mg/Kg Fe), control beans (54 mg/Kg Fe) and dephytinized	Stable isotope technique in women	Higher quantity of absorbed iron from biofortified beans than from control beans. The dephytinized biofortified

	biofortified beans		beans presented higher absorbed iron than the control beans.
Cercamondi et al. (2013)	Fe-biofortified pearl millet (88 mg/Kg Fe), regular millet (25 mg/Kg Fe) and post-harvest Fe-fortified millet (40 mg/Kg Fe added to the regular millet)	Stable iron isotopes in women with marginal iron status	The total Fe absorbed from the biofortified millet was higher than that from the conventional millet. Moreover, the total Fe absorbed from the post-harvest Fe-fortified millet was higher than that from the conventional and biofortified millet.
Kodkany et al. (2013)	Fe-biofortified pearl millet (124 mg/Kg Fe) and control millet (46.5 mg/Kg Fe)	Stable isotope in iron-deficient children	The total amount of absorbed Fe was significantly higher for biofortified millet than for regular millet.
Haas et al. (2005)	Fe-biofortified rice (3.2 mg/Kg Fe) and control rice (0.57 mg/Kg Fe)	Randomized, controlled, double-blind, longitudinal, intervention trial in anemic and non-anemic women	No differences in blood hemoglobin or ferritin between the biofortified and conventional rice in the anemic women. In the non-anemic women, hemoglobin and ferritin were higher in the biofortified rice group than in the conventional rice group.
Haas et al. (2016)	Fe-biofortified beans (86 mg/Kg Fe) and standard unfortified beans (50 mg/Kg Fe)	Randomized controlled trial in Fe-deficient women; iron status was assessed via	The Fe-Bean group had significantly greater increases in hemoglobin, serum ferritin and body iron than the standard unfortified bean group.

		hemoglobin, serum ferritin and body iron	
Petry et al. (2016)	Fe-biofortified beans (99 mg/Kg Fe), low-phytic acid beans (70 mg/Kg Fe) and control beans (5.2 mg/Kg Fe)	Multiple-meal crossover design with young women using stable iron isotopes	The amount of bioavailable iron in low-phytic acid beans did not differ from that in the biofortified beans; however, that in the biofortified beans was >50% higher than that in the control beans.

Table 2 Zinc (Zn) bioavailability in biofortified foods

REFERENCE	TESTED FOODS	STUDY DESIGN	RESULTS
In vitro *studies*			
Jou et al. (2012)	Zn-biofortified rice (undermilled: 42.5 mg/Kg; polished: 35.5 mg/Kg) and conventional rice (undermilled: ~20.3 mg/Kg; polished: ~17.5 mg/kg Zn)	Caco-2 cell assay using a stable isotope	Zn absorption was twice as high in the biofortified rice (undermilled or polished) as in the common rice.
Wei, Shohag and Yang (2012)	High-Zn polished rice (~27.02 mg/kg Zn) and control rice (22.92 mg/kg Zn)	Caco-2 cell assay	Increased Zn retention, transport and uptake efficiency.
Wei et al. (2012)	Zn-fortified germinated brown rice (~59.9 mg/kg Zn) and normal germinated brown rice (~22.9 mg/kg Zn)	Caco-2 cell assay	Higher percentages of Zn bioavailability in Zn-fortified germinated brown rice than in normal germinated brown rice.
Vaz-Tostes et al. (2015)	Common bean (20.47 mg/kg Zn) and Pontal bean (a targeted	Caco-2 cell assay	The zinc uptake from the common bean and Pontal bean

	variety for mineral biofortification) (26.1 mg/kg Zn)		was similar.
Animal models			
Jou et al. (2012)	Zn-biofortified rice (undermilled: 42.5 mg/Kg; polished: 35.5 mg/Kg) and conventional rice (undermilled: ~20.3 mg/Kg; polished: ~17.5 mg/kg Zn)	Rat model using the isotopic technique	The absorbed zinc from the biofortified rice was twice that of the common rice.
Welch et al. (2000)	10 varieties of Zn-biofortified rice (range from 35.09 to 60.5 mg/kg Zn) and 24 genotypes of zinc-biofortified beans from CIAT (range from 30.42 to 62.51 mg/kg Zn)	Rat model using the isotopic technique	Increasing the amount of zinc in enriched rice grains and beans significantly increased the amount of zinc bioavailable to rats.
Carlson et al. (2014)	Zn-biofortified wheat (42.8 mg/kg Zn), control wheat (15.6 mg/kg/Zn), Zn-biofortified beans (41.4 mg/kg Zn) and common	Pig model using the balance technique (urine and feces)	The authors could not calculate reliable zinc bioavailability values in this model because the animals had very low zinc intake.

	beans (29.9 mg/kg Zn)		
Human studies			
Islam et al. (2013)	High-zinc rice (26.0 mg/Kg Zn) and conventional rice (13.5 mg/kg Zn)	Dual-isotope ratio in Bangladeshi children	The total absorbed zinc from high-zinc rice and conventional rice was similar.
Brni´c et al. (2016)	Zn-biofortified rice (22 mg/Kg Zn) and control-fortified rice (8 mg/kg Zn)	Double isotope in adults	The fractional absorption of zinc from biofortified rice was similar to that from zinc-fortified rice.
Rosado et al. (2009)	High-zinc-biofortified wheat (41.3 mg/Kg Zn) and control wheat (23.6 mg/Kg Zn).	Dual-isotope tracer ratio technique in women	Zn absorption from the Zn-biofortified wheat was higher that than from the control wheat.
Kodkany et al. (2013)	Biofortified pearl millet (84.1 mg/Kg Zn) and control pearl millet (43.7 mg/Kg Zn)	Stable isotope in non-Zn-deficient children	The amount of absorbed Zn from the biofortified millet was higher than that from the control millet.
Chomba et al. (2014)	Zn-biofortified maize (43 mg/Kg Zn), control maize (21 mg/Kg Zn) and fortified maize (60 mg/Kg)	Dual-isotope tracer ratio in young children	The total absorption of Zn from the biofortified maize was higher than that from the control maize; however, it did not differ from

			the total absorption from the fortified maize.
Vaz-Tostes et al. (2015)	Common bean (20.47 mg/kg Zn) and Pontal bean (a targeted variety for mineral biofortification) (26.1 mg/kg Zn)	Nutritional intervention in preschool children. Evaluation by plasma zinc and erythrocyte zinc determination	There were no differences in zinc nutritional status following Pontal bean consumption.

Table 3 Advantages and limitations of methods used to assess iron and zinc bioavailability

METHODS	ADVANTAGES	LIMITATIONS
In vitro (Caco-2 cells)	✓ It is less costly and less time- and work-intensive.	✓ It cannot simulate all physiological and metabolic responses of the human body.
	✓ It enables a substantial number of breeding lines to be compared in a single experiment.	✓ There is variability in protocols.
	✓ It is a better indicator of bioavailability than the solubility method.	✓ There are changes in intestinal epithelial permeability because of modifications in transporters and metabolic enzyme expression in carcinoma cells.
	✓ It simulates gastric and intestinal digestion of food.	✓ There are no biomarkers of zinc uptake.
	✓ It enables the identification of genetic markers for iron bioavailability.	✓ It lacks communication with other organs.
In vivo		
Animal models	✓ It enables the analysis of individual tissues to provide a whole-body assessment of absorption.	✓ No animal model exactly simulates the physiological responses of humans.
	✓ There are faster physiological responses than in humans.	✓ Food intake, energy expenditure, body proportions, intestinal morphologies and enteric microbiota are different from those of humans.
	✓ The poultry model has a quick response to micronutrient deficiency.	✓ Rats endogenously synthesize ascorbic acid and phytase.
	✓ Piglets have similarities to humans with respect to gastrointestinal anatomy and physiology.	✓ The body fat content of pigs differs from that of humans.
		✓ Animals practice coprophagy.
Human model	✓ It provides the most applicable	✓ There is a risk of radiation

	results.	exposure, and studies are costly and complex.
	✓ It assesses the true absorption of nutrients from foods.	✓ Stable isotopes are costly, and the procedures required are labor-intensive.
	✓Stable isotopes allow discrimination between the dose of the micronutrient provided and endogenous forms of the micronutrient, allowing for a more accurate measurement of bioavailability.	✓Studies are difficult to perform because of the social and ethical considerations that govern invasive medical procedures.
	✓ It is possible to assess alterations in zinc and iron nutritional status, allowing a direct assessment of the effectiveness of biofortification.	

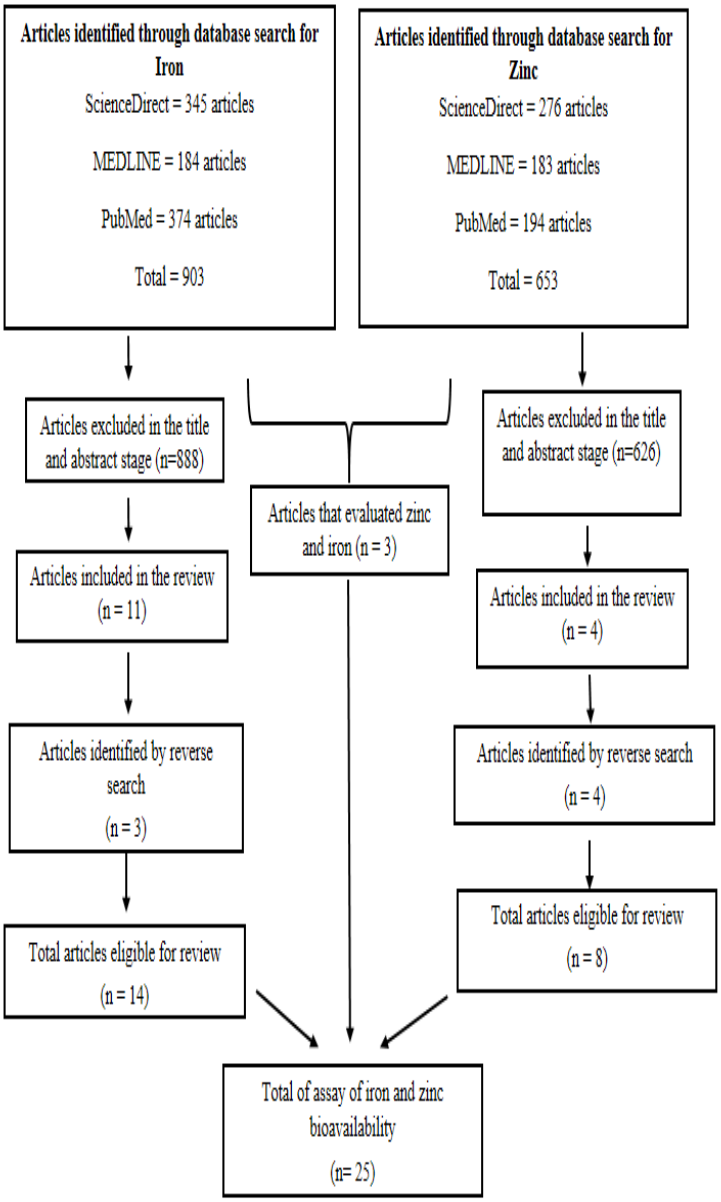

Figure 1. Search and selection of articles