Improving iron and zinc bioaccessibility through food-to-food fortification of pearl millet with tropical plant foodstuffs (moringa leaf powder, roselle calyces and baobab fruit pulp)

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Abbreviations: AR – absolute requirement, db - dry basis, BFP – baobab fruit pulp, MLP – moringa leaf powder, PM – pearl millet, PVA – provitamin A, RCP – roselle calyx powder

ABSTRACT

Essential mineral (iron and zinc) deficiencies are still prevalent in the Semi-arid Tropics, where many people consume monotonous, predominantly cereal-based diets. This study aimed to evaluate the potential of including tropical plant foodstuffs high in iron and zinc (moringa leaves and roselle calyces) or mineral availability enhancers (baobab fruit pulp) in a pearl millet-based food containing a plant food-based provitamin A source, with the aim of preventing iron and zinc deficiencies in the Semi-arid Tropics. Mineral bioaccessibility was assessed by dialysability assay. Moringa, roselle and baobab considerably increased iron and zinc bioaccessibility when added at 10 parts:100 parts pearl millet (dry basis). These foodstuffs, increased the contribution to the absolute iron requirements of women of reproductive age by 2.5, 2.1 and 2.3 times for moringa, roselle and baobab, respectively and to their absolute zinc requirements by 2.4, 2.1 and 2.7 times, respectively. Combining these plant foodstuffs could contribute up to 28% and 41% of the women's absolute iron and zinc requirements, respectively, from a single meal. Moringa, despite having the highest iron content, when added at a very high level (30 parts:100 parts pearl millet) decreased bioaccessible iron and zinc, most probably primarily due to its high calcium content. Food-to-food fortification of staple cereal foods with moringa leaves, roselle calyces or baobab fruit pulp plus a provitamin A source can potentially sustainably improve iron and zinc bioavailability in the diets of at-risk communities in the Semi-arid Tropics.

Keywords: baobab; bioaccessibility; fortification; iron; moringa; roselle; zinc

Introduction

Micronutrient malnutrition affects about one-third of the world's population, with the Semi-arid Tropics having the highest micronutrient malnutrition scores in the world (Muthayya et al. 2013). Women of reproductive age, young children and the elderly are most susceptible. Despite widespread conventional food fortification and supplementation programmes, iron, zinc and vitamin A deficiencies, the most critical and widespread micronutrient deficiencies, are still prevalent (El Sheikha 2015). These deficiencies lead to impaired mental and physical development, reduced work productivity, increased risk for perinatal complications, morbidity from infectious disease and mortality (Muthayya et al. 2013).

A contributory factor is that a high proportion of people consume monotonous, predominantly cereal-based diets, which are poor sources of provitamin A and, when refined, also poor sources of iron and zinc (WHO and FAO 2006). Cereal foods are also low in enhancers of iron and zinc bioavailability and high in inhibitors (Lönnerdal 2000). Thus, although wholegrain cereals may contain adequate amounts of these minerals to meet daily requirements, the amounts available for absorption are generally low. In fact, often less than 5% of iron, and less than 10% of zinc is considered bioavailable for absorption from cereal-based foods (WHO and FAO 2006).

Pearl millet is a cereal of particular importance in the Semi-arid Tropics as it is well adapted to severe drought, poor soil fertility, and high temperatures (ICRISAT 2018). It is a major food staple for more than 90 million people, and since 1980 pearl millet grain production has increased by 143% in West and Central Africa and by 36% in India (ICRISAT 2018). However, all cereals contain antinutritional factors which negatively affect the bioaccessibility of iron and zinc (Raes et al. 2014). Iron and zinc bioaccessibility inhibitors include phytate, phenolic compounds, dietary fibre, and

calcium. Phytate, in particular, is a potent inhibitor of both iron and zinc absorption and is a contributory factor to iron and zinc deficiencies (Raes et al. 2014). Phenolic compounds, such as those bearing galloyl or catechol groups (ortho-dihydroxy phenolic compounds) and tannins can complex with iron and zinc, rendering the minerals unavailable for absorption (Raes et al. 2014). Fibre compounds may also form complexes with iron and zinc through both physical entrapment and electrostatic interactions. Furthermore, calcium, which is abundant in cereal products, can depress the absorption of iron and in the presence of phytate and can suppress zinc absorption (Lönnerdal 2000).

While cereals are high in such inhibitors, other dietary components such as organic acids (e.g. ascorbic acid and citric acid) and other small organic compounds are known to increase the amount of iron and/or zinc that is released from the food matrix through digestion and is solubilized in the gut lumen (Gibson 2006). Hence, these minerals are made more available for intestinal absorption and utilisation in the body. Ascorbic acid can overcome some of the inhibitory effects of phytate and phenolic compounds on non-haem iron bioaccessibility (WHO and FAO 2006), and citric acid has been shown to enhance zinc absorption (Gibson 2006). Such enhancers of mineral bioavailability are present in high concentration in many fruits and vegetables (Stadlmayr et al. 2013). Examples of such plant foodstuffs that are widely grown and consumed in semi-arid tropical countries that are high in iron and/or zinc or rich in enhancers of mineral availability include: moringa leaves (approx. 50 mg iron /100 g dry basis) (Moyo et al. 2011), roselle calyces (approx. 40 mg iron /100 g dry basis) (Zaman et al. 2017) and also rich in organic acids (Da-Costa-Rocha et al. 2014), and baobab fruit pulp, which is especially rich in citric acid, approx. 3300 mg/100 g dry basis (Tembo et al. 2017). Combining such locally available plant foodstuffs with staple

cereal foods (food-to-food fortification) has been advocated as a sustainable means of increasing iron and zinc contents and bioavailability (WHO and FAO 2006) and can be considered as form of micronutrient bio-fortification (El Sheikha 2015).

Hence, in this study moringa leaves, roselle calyces and baobab fruit pulp, together with mango and carrots as sources of provitamin A, were investigated as potential food-to-food fortificants of pearl millet to improve the iron and zinc status of at-risk populations in the Semi-arid Tropics.

Materials and methods

Raw materials

Whole grain pearl millet (PM) (*Pennisetum glaucum* L.) (cultivar Kuphanjala-2) was kindly provided by the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Bulawayo, Zimbabwe.

Moringa leaf powder (MLP) (*Moringa oleifera* Lam.), Roselle calyx powder (RCP) (*Hibiscus subdariffa* L.) and Baobab fruit pulp (BFP) (*Adansonia digitata* L.) products were obtained from Maria Production (Dakar, Senegal) and Free Work Service (Dakar, Senegal). The MLP, RCP and BFP products from the two suppliers were mixed homogenously on a 1:1 weight basis.

Mango-carrot premix, comprising 51% freeze-dried mango (*Mangifera indica* L.) and 49% freeze-dried carrot (*Daucus carota* L.).

Pearl millet processing

Whole millet grains were rubbed between gloved hands to remove glumes. The grain was further cleaned by sieving to remove foreign matter and quickly rinsed with

deionised distilled water, to remove dust contamination, and allowed to air dry at 24˚C for 24 hours. The grain was milled using a laboratory hammer mill (Falling Number 3100, Perten, Huddinge, Sweden) fitted with a 0.5 mm hole diameter stainless steel screen.

Deionised water was added to the PM flour in a ratio of 1:10, flour: water (w/w). The mixture was heated to 96˚C and maintained with constant stirring for 15 min. To preserve the porridge for analysis, it was left to cool to ambient temperature, after which it was frozen to -20˚C and freeze-dried. The freeze-dried porridge was crushed to a particle size that passed through a 0.5 mm hole diameter screen and stored at 10˚C.

Plant foodstuffs processing

The MLP, RCP, and BFP foodstuffs were milled using an air-cooled knife-type laboratory mill (A11 basic analytical mill, IKA, Staufen, Germany) and passed through a 0.5 mm hole diameter stainless steel screen. The resulting powders were stored at 10°C in double sealed, airtight plastic bags. The mango-carrot premixes were stored at -20°C in vacuum-sealed plastic bags, which were in turn wrapped in aluminium foil.

Porridge formulations

The porridge formulations consisted of 60 g freeze-dried mango-carrot mix:100 g freeze-dried PM porridge: 10 g sunflower oil (as a provitamin A (PVA) source); plus 10 g or 30 g of the plant foodstuffs (MLP, RCP, and/or BFP). The 30 g:100 g PM ratio was studied to establish whether there was an increased effect on mineral bioaccessiblity at higher levels of inclusion. However, it is recognised that such a very high level of food-to-food fortification would be uneconomical and could adversely affect the sensory properties of the foods. Based on this, various porridge formulations were formulated

(Table 1). The PVA source (mango-carrot premix plus sunflower oil) was included in the formulations to address underlying vitamin A deficiency and contributed approximately 970 µg retinoic acid equivalents (RAE) to each formulation (USDA Nutrient Database. 2018). Sunflower oil was added to provide a lipid source for provitamin A micellization (van Het Hof et al. 2000).

Mineral contents

Mineral contents (iron, zinc, calcium and phosphorus) of the foodstuffs were quantified using approved methods of the AOAC International (2000). Samples (500 mg) were digested using a combination of 65% nitric acid and 70% perchloric acid (5:2, v/v) at 2400˚C (AOAC method 935.13). Once digested, the samples were diluted to 50 ml, with deionised water. A GBC 905 atomic absorption spectrometer (Braeside, Australia) was used to quantify iron and zinc, according to AOAC method 999.10.

Calcium content was determined according to AOAC method 935.13. A solution of 1% lanthanum chloride heptahydrate and anhydrous nitric acid was used to inhibit the interaction of other elements with calcium (Giron 1973). A Perkin-Elmer, 5100 atomic absorption spectrometer (Walluf, Germany) was used.

Phosphorus content was measured colorimetrically (400 nm) according to AOAC method 965.17. The reaction of ammonium molybdate tetrahydrate and ammonium vanadate with phosphorus in the sample develops a yellow colour which is measured against a phosphorus standard.

Total phenolic contents

Total phenolic contents were determined by a modified Folin-Ciocalteu method, as described by Waterman and Mole (1994), with correction for the absorbance of the

extracts in the absence of the Folin-Ciocalteu reagent.

Profile of phenolic constituents

The phenolic composition was determined according to Apea-Bah et al. (2014), using a Waters Synapt G2 system comprising an Acquity ultra-performance liquid chromatograph (UPLC), equipped with a binary pump system (Waters, Milford, MA, USA). The UPLC system was coupled to a quadrupole time-of-flight mass spectrometer (QToF-MS, Waters) using an electrospray ionisation (ESI) source and a photodiode array (PDA) detector (Waters). Separation was performed using a Waters BEH C18 (100 x 2.1 mm, 1.7 µm) reversed phase column. The column injection volume was 2 µl with a flow rate of 0.3 ml/min, and the column was kept at 55ºC. Ionisation was in negative mode with a capillary voltage of 3 kV, and cone voltage of 15 V. Identification of phenolic compounds was done by comparison with external phenolic acid and flavonoid standards, as well as by comparison of mass and UV spectral data of phenolic compounds reported in the literature. Quantification was done by comparing integrated peak areas of phenolic compounds in the extracts at 280 nm with that of the standards. Sodium formate was used for calibration, and leucine enkephalin (molecular weight 555 Da) was used as lock mass for accurate mass determinations. Data were acquired using MassLynx v. 4.1 software (Waters).

Condensed tannin content

Condensed tannin content was determined by a modified Vanillin-HCl method, as described by Price et al. (1978), with correction for the absorbance of the extracts in the absence of the vanillin-HCl reagent.

Phytate content

Phytate content from purified samples was determined by indirect quantitative analysis of phytate, measured through colorimetry, as described by Frühbeck et al. (1995). Anion exchange chromatography was used to remove unbound phosphorus. The mineral and antinutrient levels of each porridge formulation were calculated from the mineral and antinutrient levels of each raw material.

Mineral bioaccessibility

Mineral bioaccessibility was measured by *in vitro* dialysability assay (Miller et al. 1981). The porridge formulations were subjected to *in vitro* digestion to simulate human gastric and intestinal digestion. The digestive enzymes used were pepsin (P-7000) and pancreatin (P-1750), plus bile extract (B-8631) (Sigma-Aldrich, Johannesburg, South Africa). The dialysis tubing used was Spectra/Por 7 (Ø = 20.4 mm) with a molecular weight cut-off (MWCO) of 10 kDa (GIC Scientific, Johannesburg, South Africa).

The mineral contents (iron, zinc and calcium) of the dialysates were determined directly (without digestion) by Inductively Coupled Plasma-Atomic Emission Spectroscopy (ICP-AES) according to method 200.7 of the United States Environmental Protection Agency (US EPA 1994). The difference in methodology from that used to determine the mineral contents of the foodstuffs was because in the bioaccessibility assay the porridges are subjected to simulated human digestion. The dialysate mineral analyses were performed by the Central Analytical Facilities of Stellenbosch University, South Africa.

Statistical analyses

Experiments were repeated, and analyses were carried out in duplicate for mineral contents, in triplicate for total phenolics and tannin contents, and in quadruplicate for phytate contents. These assays were replicated different numbers of times to obtain acceptable repeatability, a consequence of their relative complexity. For the bioaccessibility assay, each formulation was subjected to three gastric stages, which in turn were subjected to three intestinal stages, which in turn were analysed for mineral contents in duplicate. Values are presented per 100 g of dry matter. Bioaccessibility values were calculated per 100 g porridge product as consumed and data are presented as means ± 1 standard deviation (SD). The data were analysed using a one-way analysis of variance (ANOVA) with SPSS Statistics software (IBM, Armonk, New York) using Fisher's least significant difference (LSD) test to separate means, with a confidence level for significant differences at $p \leq 0.05$.

Results and discussion

Mineral contents

MLP and RCP were the only plant foodstuffs with a higher ($p \leq 0.05$) iron content than PM, approximately 7 and 6 times higher, respectively (Table 2). Moyo et al. (2011) reported that the iron content of moringa leaves was 54.2 mg/100 g, very similar to that reported here (58.4 mg/100 g). For roselle calyces, Zaman et al. (2017) reported an iron content of 40.5 mg/100 g, close to that found in this present work (47.1 mg/100 g). Concerning baobab fruit pulp, an iron content of 6.8 mg/100 g has been reported (Stadlmayr et al. 2013), close to value obtained in this work (13.8 mg/100 g). Apart from RCP, the zinc levels of all the plant foodstuffs were significantly lower ($p \leq 0.05$)

than in PM, with BFP and the mango-carrot premix containing less than half the level in PM. The MLP, RCP, BFP and mango-carrot premix had substantially higher calcium contents, compared to PM, but the phosphorus levels were all considerably lower.

Antinutrients

Phenolics

The total phenolic contents of MLP, RCP, and BFP were approximately 13, 9 and 11 times higher than PM, respectively (Table 2). Sreelatha and Padma (2009) reported 4581 mg/100 g total phenolics in mature MLP, similar to found in this study (4655 mg/100 g). Borrás-Linares et al. (2015) reported the total phenolic levels of deep red roselle varieties to range from 3000 to 10000 mg GAE/100 g. Lamien-Meda et al. (2008) reported total phenolic levels in baobab fruit pulp very similar to that reported here (3738 mg/100 g).

Phenolic acid and flavonoid profiling indicated that MLP, RCP, BFP and the mango-carrot premix contained approximately 0.66, 0.37, 0.01 and 0.05% total phenols (phenolic acids and flavonoids, excluding tannins), respectively (Table 3). Flavonoids comprised the highest portion of the phenolic compounds in MLP and BFP, whereas phenolic acids comprised the highest portion in RCP and the mango-carrot premix.

The total phenolics measured by the Folin-Ciocalteu assay (Table 2) were some 10 times higher than the total phenolic acids and flavonoids determined by UPLC (Table 3). This was probably due to the high content of organic acids with reducing power (Table 2), which would interfere with the Folin-Ciocalteu assay (Singleton et al. 1999). Thus, the total phenolic acids and flavonoids data determined by UPLC (Table 3) will be used in the subsequent discussion.

BFP and the mango-carrot premix contained negligible levels of phenolic acids and flavonoids (tannins not analysed) (Table 3). Condensed tannins, however, were only detected in the BFP (Table 2), with the levels being very similar to the 2220 mg/100 g reported by Umaru et al. (2007).

Phytate

RCP was the only plant foodstuff that contained significantly more ($p \leq 0.05$) phytate than PM, approximately 3 times more (Table 2). Ojokoh (2006) reported 2411 mg phytate/100 g for roselle calyces, approximately half that found in this present work. MLP, BFP and mango-carrot premix had considerably lower phytate contents than PM.

Mineral bioaccessibility (in vitro dialysability)

Iron bioaccessibility

The inclusion of all the plant foodstuffs at 10 parts:100 PM significantly increased ($p \leq 0.05$) the percentage and amount of bioaccessible iron as compared to PM+PVA. MLP with PM+PVA at 10 parts MLP:100 PM increased the percentage bioaccessible iron by 56%, resulting in a 149% increase in the amount of bioaccessible iron (Table 4). However, the inclusion of 30 parts MLP halved the percentage bioaccessibility compared to PM+PVA. Nevertheless, due to the high iron content of MLP (Table 2), inclusion at 30 parts still resulted in a 54% increase in the amount of bioaccessible iron, compared to PM+PVA. The reduction in the percentage iron bioaccessibility was probably due to the high level of calcium and also of phytate and phenolics in MLP. MLP contained approximately 0.7% total phenolics (Table 3) and 2115 mg/100 g calcium, 300 times of the level in PM (Table 2). Thus, porridges fortified at 30 parts MLP:100 PM had approximately 8 times the levels of these iron

bioaccessibility inhibitors than PM+PVA (Table 5). The inclusion of 10 parts MLP plus 10 parts RCP or BFP, or with 10 parts RCP plus 10 parts BFP significantly reduced ($p \leq 0.05$) the percentage bioaccessible iron compared to these formulations without MLP.

Not all phenolics chelate iron, and those that do have different binding capacities for iron (Andjelković et al. 2006). The metal chelating ability of phenolics is related to the presence of ortho-dihydroxy groups (phenolics bearing catechol or galloyl groups) (Khokhar and Apten 2003). MLP was particularly rich in chlorogenic acids (chlorogenic acid, neochlorogenic acid and cryptochlorogenic acid, a total of 215.4 mg/100 g) (Table 3). Notably, chlorogenic acid has been found to have high iron chelating capacity (Andjelković et al. 2006).

MLP was abundant in the flavonols rutin (172.6 mg/100 g) and quercetin and related compounds (199.6 mg/100 g), whereas these flavonols were virtually absent in the other plant foodstuffs (Table 3). It has been reported that quercetin has a strong iron reducing and chelating capacity which increases as pH decreases (Mira et al. 2002). The authors also reported that rutin has a moderate interaction with iron, although lower than quercetin, probably due to the fewer -OH groups in rutin. The fact that MLP contained the highest levels of specific phenolic compounds that have potent iron chelating abilities could, in part, explain why 30 parts MLP:100 PM adversely affected the iron bioaccessibility.

In contrast to MLP, the inclusion of 10 and 30 parts RCP:100 PM significantly increased ($p \leq 0.05$) both the percentage bioaccessible iron (by 42% and 55%, respectively) and the amount of bioaccessible iron (by 107% and 269%, respectively) (Table 4). As RCP was the plant foodstuff with the second highest iron content (47 mg/100 g) (Table 2), porridge formulations containing RCP had 1.5 and 2.4 times the

amount of iron (7 and 12 mg/100 g) compared to PM+PVA (5 mg/100 g) (Table 5). The porridge formulations containing RCP had the highest phytate contents (1.3 to 2.0 times higher than PM+PVA). Furthermore, porridge formulations containing RCP also had calcium contents that were 2.0-4.2 times higher than PM+PVA. It was also rich in chlorogenic acids (168.7 mg/100 g) (Table 3). However, despite the fact that the formulations containing RCP were high in these inhibitors (phytate, phenolics and calcium) iron bioaccessibility was always improved with its inclusion (Table 4). A probable reason why RCP had a positive effect on iron bioaccessibility is because of its high content of organic acids. RCP contained 2420 mg/100 g organic acids (Table 2), including citric acid, hydroxycitric acid, malic acid, ascorbic acid and tartaric acid (Da-Costa-Rocha et al. 2014) and 1781 mg/100 g hibiscus acid (Table 3). Probably because of this, RCP decreased the pH of the porridges from pH 4.82 to pH 3.76 and pH 3.06 with the inclusion of 10 and 30 parts RCP:100 PM, respectively (Table 4). Such organic acids are known to improve iron bioavailability (Lönnerdal 2000). They chelate iron through binding with carboxyl and hydroxyl groups, and thus, increase the solubility and iron availability.

Concerning BFP inclusion, at 10 and 30 parts BFP:100 PM iron bioaccessibility was greatly improved ($p \leq 0.05$), by 2.0 and 2.6 times for percentage bioaccessible iron and by 2.3 and 3.7 times for the amount of bioaccessible iron, respectively (Table 4). In fact, the inclusion of BFP at 10 parts gave the highest increase in percentage bioaccessible iron compared to the inclusion of MLP or RCP at this level. Iron bioaccessibility was improved, despite BFP having the lowest iron content of the plant foodstuffs and despite the formulations containing BFP having the highest level of tannins (Table 5). Adetola et al. (2019) attributed the improvement in iron bioaccessibility in cereal-based porridge by inclusion of BFP to its high contents of

citric acid and ascorbic acid, 3355 mg/100 and 140 mg/100 g dry basis, respectively. As stated, these organic acids are promoters of iron absorption (Lönnerdal 2000). In apparent contrast to the findings of this present study and those of Adetola et al. (2019), Gabaza et al. (2018) found % iron bioaccessibility was not improved when fermented pearl millet was enriched with baobab. The finding is probably due to the effect of the organic acids in baobab being obscured by fermentation, which is noted as a way of reducing the inhibitory effect of phytate on iron bioavailability in cereal foods (Raes et al. 2014).

Condensed tannins (proanthocyanidins), such as those in BFP, are known to adversely affect iron bioavailability primarily because they are potential chelators of iron, forming complexes with ferrous iron (Fe^{2+}), which renders the iron unavailable for absorption (Khokhar and Apenten 2003). Even though BFP contained the iron-chelating phenolics rutin, quercetin and related compounds and neochlorogenic acid, it also contained catechin (21.5% of total phenolics) (Table 3). Hart et al. (2015) reported that catechin, along with some other polyphenols, are strong, concentration-dependent promoters of iron bioavailability. Thus, the improved iron bioaccessibility from BFP observed in this present study may, in part, be due to it containing some polyphenols which promote iron bioavailability.

However, the improved iron bioaccessibility with the inclusion of BFP is probably mainly due to its high content of organic acids (3695 mg/100 g) (Table 2). In fact, BFP, like RCP, also decreased the porridge pH, from pH 4.82 to pH 4.38 and pH 3.90 for 10 and 30 parts BFP:100 PM, respectively (Table 4). BFP has been found to contain ascorbic, citric, and malic acids (at 466, 3300, and 2360 mg/100 g, respectively), as well as tartaric acid (174 mg/100 g) (Tembo et al. 2017). BFP also contained some hibiscus acid (23.7 mg/100 g) (Table 3). As stated, organic acids are

strong enhancers of iron bioavailability (Lönnerdal 2000). Furthermore, Cercamondi et al. (2014) proposed that organic acids in plant foodstuffs can overcome the inhibitory effects of phytates and tannins, and hence this would account for the improved iron bioaccessibility with BFP inclusion found in this present study.

The presence of mango and carrot, which are rich in provitamin A, may also have contributed to the improved iron bioaccessibility with the inclusion of the plant foodstuffs. Various provitamin A carotenoids have been reported to positively impact iron absorption from cereal-based meals (García-Casal et al. 1998). These authors reported that carotenoids promoted iron absorption from cereal-based meals to the extent of counteracting the negative effects of tannins on iron absorption. They proposed that beta-carotene may form a complex with iron which keeps the mineral soluble in the intestinal lumen and thus prevents the inhibitory effects of phytates and phenolics on iron absorption. While this effect may have negative consequences for provitamin A delivery, it would potentially limit the ability of tannins and phytates from adversely affecting iron bioaccessibility.

Zinc bioaccessibility

With the exception of the 30 parts MLP:100 PM formulation, the inclusion of all the plant foodstuffs with PM+PVA significantly improved ($p \leq 0.05$) both the percentage bioaccessible zinc (1.3 to 2.2 times) and the amount of bioaccessible zinc (2 to 3.6 times) (Table 4). When the inclusion level of MLP was increased from 10 parts to 30 parts, the percentage and the amount of bioaccessible zinc were approximately halved ($p \leq 0.05$). Furthermore, the inclusion of 10 parts MLP plus 10 parts BFP, and 10 parts RCP plus 10 parts BFP reduced ($p \leq 0.05$) the percentage and amount of bioaccessible zinc compared to these formulations without MLP. The probable reason that the

inclusion of MLP had this negative effect on zinc bioaccessibility is that it had by far the highest calcium content of the plant foodstuffs (Table 2). Calcium can co-precipitate with phytate and zinc forming insoluble Ca_4Zn_2-phytate complexes (Lönnerdal 2000). The author stated that calcium-bound phytate has a higher affinity for zinc than phytate alone, thereby decreasing dietary zinc bioaccessibility.

Concerning RCP, as with iron, the improved zinc bioaccessibility with the inclusion of RCP in the PM+PVA porridge was also likely due to its organic acids (Table 2). Most organic acids have positive effects on zinc bioaccessibility as soluble complexes with zinc are formed in the intestinal tract (Lönnerdal 2000). These complexes readily break down in the small intestine, thus, releasing mineral cations, bioaccessible for absorption. It is thought that the organic acids prevent the formation of insoluble zinc-phytate complexes (Gibson 2006).

The inclusion of BFP greatly improved ($p \leq 0.05$) the zinc bioaccessibility in all formulations. The percentage bioaccessible zinc from the 10 parts BFP:100 PM formulation was 2.0 times higher than from PM+PVA, and the amount of bioaccessible zinc was 2.7 times higher (Table 4). In fact, inclusion of 10 parts BFP gave the highest percentage and amount of bioaccessible zinc of all the porridge formulations at this level of inclusion. As with iron, zinc bioaccessibility improved despite the low zinc content and substantial levels of tannins in BFP and this is probably due to its high content of organic acids (Table 2).

Contribution to absolute (physiological) iron and zinc requirements (AR)

The absolute requirement for a mineral is equal to the sum of the daily basal losses of the mineral (via faeces, urine, skin and its appendages, milk, menstrual blood, and semen) plus the amounts of the mineral needed for growth (WHO/UNICEF/UNU

2001). For women of reproductive age, the absolute iron requirement is 1.46 mg/day (WHO/UNICEF/UNU 2001) and the absolute zinc requirement is 1.75 mg/day (Sandstead 2015). The contribution that 75 g (based on 250 g porridge portion of which 30% is nominal solids) of the various pearl millet porridge formulations could make to the iron and zinc AR for women of reproductive age was calculated.

Absolute bioaccessible mineral (per portion)

= Amount of bioaccessible mineral × portion weight.

Percentage contribution of porridge formulation to the AR

= Absolute bioaccessible mineral (per portion)/AR × 100.

MLP, RCP and BFP addition to PM+PVA at 10 parts:100 PM significantly improved ($p \leq 0.05$) the contribution to the AR for both iron and zinc (Figure 1). The inclusion of 10MLP+10RCP+10BFP parts:100 PM resulted in the highest improvement to the AR for iron (184%) and the inclusion of 10 parts BFP:100 PM gave the highest improvement to the AR for zinc (170%), when compared to PM+PVA. The inclusion of 10 parts RCP:100 PM increased the contribution to the AR for iron and zinc by 107% and 106%, respectively, compared to PM+PVA. The formulation which could provide the best contribution to the combined iron and zinc AR was PM+PVA+10RCP+10BFP and contributed 27% and 36% to the iron and zinc AR, respectively. This indicates that the iron and zinc status of women and other vulnerable groups consuming such food-to-food fortified porridges would be improved. However, the extent of improved status can only be predicted through an intervention study.

Conclusions

The inclusion of moringa leaves and roselle calyces, both which are high in iron and zinc, or baobab fruit pulp, notwithstanding its relatively low iron and zinc content, improve the iron and zinc bioaccessibility of cereal porridge containing a provitamin A source. The inclusion of moringa leaf powder at a very high level, despite its high iron content, does not improve iron bioaccessibility. This negative effect is probably primarily due to its high calcium content. The improvement in iron and zinc bioaccessibility by baobab fruit pulp and roselle calyces is probably due to their high content of mineral bioaccessibility-enhancing organic acids such as ascorbic acid and citric acid.

Fortification with moringa leaf powder, roselle calyces or baobab fruit pulp could contribute up to 28% and 41% to the absolute iron and zinc requirements, respectively, of women of reproductive age from a single meal. This is up to 3 times more than the cereal porridge containing pearl millet and a provitamin A source alone.

Food-to-food fortification of cereal staple foods with moringa leaves, roselle calyces or baobab fruit pulp, in the presence of a provitamin A source, is potentially a sustainable strategy to improve the iron and zinc bioavailability from cereal-based diets of communities in the Semi-arid Tropics.

Conflict of interest and funding

The authors report no conflicts of interest

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CAPTION TO FIGURE

Figure 1: Effects of fortifying pearl millet porridge + provitamin A (PVA) at 10 parts dried moringa leaves powder (MLP), roselle calyx powder (RCP) and baobab fruit pulp (BFP):100 pearl millet (PM) (db) alone and in combination on the contribution to the absolute (physiological) iron and zinc requirements of women of reproductive age

Tables

Table 1. Porridge formulations of freeze-dried pearl millet porridge (PM) and freeze-dried provitamin A source (PVA) plus dried moringa leaf powder (MLP), roselle calyx powder (RCP) and baobab fruit pulp (BFP) alone and in combination

Formulation	Composition (g/100 g)					
	Micronutrient rich plant foodstuffs			Base	Provitamin A source	
	MLP*	RCP*	BFP*	PM	Dried mango-carrot premix[a]	Sunflower oil
PM+PVA	0	0	0	100	60	10
PM+PVA + 10MLP	10	0	0	100	60	10
PM+PVA + 30MLP	30	0	0	100	60	10
PM+PVA + 10RCP	0	10	0	100	60	10
PM+PVA + 30RCP	0	30	0	100	60	10
PM+PVA + 10BFP	0	0	10	100	60	10
PM+PVA + 30BFP	0	0	30	100	60	10
PM+PVA + 10MLP + 10RCP	10	10	0	100	60	10
PM+PVA + 10MLP + 10BFP	10	0	10	100	60	10
PM+PVA + 10RCP + 10BFP	0	10	10	100	60	10

PM+PVA + 10MLP + 10RCP + 10BFP	10	10	10	100	60	10

Key: *Obtained from two different suppliers mixed homogenously on a 1:1 weight basis; [a]Dried mango-carrot premix consisted of 49% carrot and 51% mango.

Table 2. Mineral (iron, zinc, calcium and phosphorus) contents (mg/100 g, db[2]), total phenolic (mg CE[3]/100 g, db)[8], tannin (mg CE/100 g, db), and phytate (mg/100 g, db) levels of moringa leaves (MLP), roselle calyxes (RCP), baobab fruit (BFP), mango-carrot premix[4], and pearl millet (PM)

Plant foodstuffs	**Iron**	**Zinc**	**Calcium**	**Phosphorus**	**Total phenolics**	**Condensed tannins**	**Phytate**	**Total organic acids**[5]
MLP	$58.4^{a} \pm 4.9$[1]	$2.28^{b} \pm 0.15$	$2115^{a} \pm 56$	$284^{b} \pm 1$	$4655^{a} \pm 49$	-	$829^{bc} \pm 23$	$2255^{b} \pm 140$[6]
RCP	$47.1^{a} \pm 19.0$	$2.70^{a} \pm 0.14$	$970^{b} \pm 22$	$163^{c} \pm 43$	$3451^{b} \pm 289$	-	$4833^{a} \pm 799$	$2420^{b} \pm 30$[7]
BFP	$13.8^{b} \pm 16.1$	$1.24^{c} \pm 0.12$	$273^{c} \pm 27$	$53^{e} \pm 3$	$3738^{b} \pm 3$	$2286^{a} \pm 328$	$321^{c} \pm 26$	$3695^{a} \pm 79$[6]
Mango-carrot premix	$4.3^{b} \pm 0.3$	$1.23^{c} \pm 0.02$	$140^{d} \pm 1$	$138^{d} \pm 2$	-	-	$290^{c} \pm 85$	$600^{d} \pm 65$[6]
PM (whole grain, raw)	$7.5^{b} \pm 0.1$	$2.95^{a} \pm 0.08$	$7^{e} \pm 1$	$416^{a} \pm 10$	$353^{c} \pm 13$	-	$1360^{b} \pm 30$	$1057^{c} \pm 84$[6]

Key: [1]Values are the mean ± 1 standard deviation of samples analysed in duplicate (n=2) for minerals, two samples analysed in triplicate (n=6) for total phenolic and condensed tannin contents, and two samples analysed in quadruplicate (n=8) for phytate content. Mean values followed by different letter superscripts differ significantly according to Fisher's LSD test ($p \leq 0.05$); [2]db: dry weight basis; [3]CE: Catechin equivalents; [4]Mango-carrot premix composed of freeze-dried mango (51%) and carrot (49%); [5]Citric+tartaric+malic+ascorbic acid content; [6]from unpublished data; [7]Wong et al. 2002; – below limit of quantification; [8]measured by modified Folin-Ciocalteu method.

Table 3. Retention time, UV–visible absorption maxima, mass spectral characteristics and contents of phenolic acids and flavonoids identified in extracts from moringa leaves powder, roselle calyces powder, baobab fruit pulp and mango-carrot premix[a]

T_R	**λ_{max}**	**[M-H]-**	**MS/MS fragments**	**Proposed compound**	**Moringa leaves**	**Roselle calyces**	**Baobab fruit pulp**	**Mango-carrot premix**
(min)	(nm)	(m/z)			[mg/100 g, db ± SD (relative percentage to total flavonoids + phenolic acids)]	[mg/100 g, db ± SD (relative percentage to total flavonoids + phenolic acids)]	[mg/100 g, db ± SD (relative percentage to total flavonoids + phenolic acids)]	[mg/100 g, db ± SD (relative percentage to total flavonoids + phenolic acids)]
Anthocyanidins								
8.44	276, 526	595	300, 461, 175	Delphinidin-3-*O*-sambubioside	-	69.1^{a} ± 20.3 (18.8%)	-	-
9.71	280, 518	579	284, 285, 189, 127	Cyanidin-3-*O*-sambubioside	-	37.1^{b} ± 2.7 (10.1%)	2.8^{a} ± 1.2 (19.9%)	-
Total anthocyanidins					-	106.1^{b} ± 21.5 (29.0%)	2.8^{a} ± 1.2 (19.9%)	-
Flavan-3-ols								
8.2	280	289	245, 123 203	Catechin	-	-	3.0^{b} ± 1.5 (21.5%)	0.6^{a} ± 0.2 (1.4%)
13.72	270	319	127, 145,	Methylepigalloc	-	0.7^{a} ± 0.0 (0.2%)	-	-

T_R	λ_{max}	[M-H]-	MS/MS fragments	Proposed compound	Moringa leaves	Roselle calyces	Baobab fruit pulp	Mango-carrot premix
			189	atechin				
Total flavan-3-ols					-	0.7^{a} ± 0.0 (0.2%)	3.0^{b} ± 1.5 (21.5%)	0.6^{a} ± 0.2 (1.4%)
Flavonols								
12.86	352	595	300, 301	Quercetin-glucosyl xyloside	-	7.0^{a} ± 2.6 (1.9%)	-	-
13.86	352	609	300, 301, 189	Rutin	172.6^{b} ± 18.5 (26.0%)	7.7^{a} ± 2.0 (2.1%)	1.5^{a} ± 0.4 (10.7%)	0.7^{a} ± 0.2 (1.7%)
16.31	370	317	187, 125 209	Myricetin	-	6.9^{a} ± 1.2 (1.9%)	-	-
14.18	355, 285	463	300, 301	Quercetin-3-glucoside	133.5^{b} ± 15.5 (20.1%)	4.8^{a} ± 0.4 (1.3%)	3.3^{a} ± 1.2 (23.9%)	1.3^{a} ± 0.4 (3.1%)
19.62	366	301	151, 179, 189, 121	Quercetin	0.5^{a} ± 0.0 (0.1%)	3.6^{b} ± 0.4 (1.0%)	-	-
15.05	255, 353	505	300, 301, 271, 255	Quercetin glycoside malonate	65.6^{a} ± 7.1 (9.9%)	-	-	-

T_R	λ_{max}	[M-H]-	MS/MS fragments	Proposed compound	Moringa leaves	Roselle calyces	Baobab fruit pulp	Mango-carrot premix
Total flavonoids					372.2^{b} ± 41.1 (56.1%)	29.9^{a} ± 4.1 (8.2%)	4.8^{a} ± 1.7 (34.5%)	2.0^{a} ± 0.6 (4.8%)
Phenolic acids								
5.64	262	331	169, 125, 164	Galloyl glucose	0.4^{a} ± 0.0 (0.1%)	1.6^{b} ± 0.4 (0.4%)	-	14.3^{c} ± 0.6 (34.1%)
2.92	270	169	125	Gallic acid	-	5.3^{a} ± 1.3 (1.5%)	-	20.5^{b} ± 1.3 (49.0%)
8.68	324, 290sh	353	191	Chlorogenic acid	6.7^{b} ± 0.9 (1.0%)	53.1^{c} ± 5.5 (14.5%)	-	3.9^{ab} ± 0.5 (9.4%)
6.8	324, 290sh	353	191, 179, 135	Neochlorogenic acid	129.2^{c} ± 11.6 (19.5%)	70.3^{b} ± 4.6 (19.2%)	2.4^{a} ± 0.9 (17.0%)	0.2^{a} ± 0.2 (0.5%)
9.1	324, 290sh	353	173, 179 191, 135	Cryptochlorogenic acid	79.5^{c} ± 6.4 (12.0%)	45.3^{b} ± 6.3 (12.4%)	1.0^{a} ± 0.7 (7.1%)	0.4^{a} ± 0.1 (0.9%)
9.28		367		Methylchlorogenate isomer 1	24.4^{c} ± 0.7 (3.7%)	2.0^{b} ± 0.3 (0.5%)	-	0.0^{a} ± 0.0 (0.0%)
10.31		367		Methylchlorogenate isomer 2	-	1.4^{a} ± 0.2 (0.4%)	-	-

T_R	λ_{max}	[M-H]-	MS/MS fragments	Proposed compound	Moringa leaves	Roselle calyces	Baobab fruit pulp	Mango-carrot premix
13.16	280	367	135, 121, 245, 263, 179	Methylchlorogenate isomer 3	-	1.8^{a} ± 0.3 (0.5%)	-	-
8.11	312	337	163, 119	Coumaroylquinic acid	49.8^{b} ± 3.2 (7.5%)	9.0^{a} ± 1.1 (2.4%)	-	-
8.95	277	335	183, 139	5-*O*-cafeoylshikimic acid	-	9.8^{a} ± 0.5 (2.7%)	-	-
18.89	317, 285sh	312	148, 178 190, 297	N-feruloyltyramine	-	22.2^{a} ± 2.6 (6.1%)	-	-
8.88		179		Caffeic acid	1.6^{a} ± 0.2 (0.2%)	8.0^{b} ± 1.1 (2.2%)	-	-
Total phenolic acids					$1\,482.3^{c}$ ± 101.0 (43.9%)	231.6^{b} ± 18.9 (62.7%)	3.4^{a} ± 1.4 (24.1%)	39.3^{a} ± 2.1 (93.9%)
Total flavonoids and phenolic acids					663.9^{c} ± 63.0	366.5^{b} ± 38.3	14.0^{a} ± 1.5	41.9^{a} ± 2.5
Other organic acids								
1.21	<230	189	127; 189	Hibiscus acid	-	1781.2^{b} ± 98.2	23.7^{a} ± 4.5	-
Total organic acids					-	1781.2^{b} ± 98.2	23.7^{a} ± 4.5	-

Key: t_R = retention time; λ_{max} = UV–visible absorption maxima; $[M-H]^-$ = precursor ion; MS/MS = product ions; sh = shoulder; – = below limit of quantification. Values are means ± standard deviation of duplicates. Means in a row with different superscripts are significantly ($p \leq 0.05$) different from each other. Gallic acid derivative was calculated and expressed as gallic acid equivalents. Caffeic acid derivatives and coumaric acid derivatives were calculated and expressed as ferulic acid equivalents. All phenolic compounds were quantified from peaks detected at 280 nm. [a] = Mango-carrot premix composed of freeze-dried mango (51%) and carrot (49%).

Table 4. Effects of fortifying pearl millet porridge (PM) + provitamin A (PVA) at 10 and 30 g/100 g, db, pearl millet with dried moringa leaves powder (MLP), roselle calyx powder (RCP) and baobab fruit pulp (BFP) alone and in combination on iron and zinc bioaccessibility

Formulation	**Percentage bioaccessible iron** [% ± SD (percentage difference)]	**Amount of bioaccessible iron** [mg/100 g porridge, db ± SD (percentage difference)]	**Percentage bioaccessible zinc** [% ± SD (percentage difference)]	**Amount of bioaccessible zinc** [mg/100 g porridge, db ± SD (percentage difference)]	**pH of porridge**
PM+PVA	4.18^{b} ± 0.96 (0%)	0.189^{a} ± 0.043 (0%)	32.0^{ab} ± 12.0 (0%)	0.351^{a} ± 0.296 (0%)	4.82
PM+PVA + 10MLP	6.53^{ef} ± 1.32 (56%)	0.470^{de} ± 0.095 (149%)	53.0^{e} ± 8.4 (66%)	0.826^{de} ± 0.328 (135%)	4.90
PM+PVA + 30MLP	2.31^{a} ± 0.60 (-45%)	0.291^{b} ± 0.075 (54%)	26.4^{a} ± 6.1 (-17%)	0.403^{a} ± 0.244 (15%)	4.93
PM+PVA + 10RCP	5.95^{de} ± 1.06 (42%)	0.391^{cd} ± 0.069 (107%)	46.2^{de} ± 13.9 (44%)	0.725^{bcd} ± 0.348 (106%)	3.76
PM+PVA + 30RCP	6.50^{ef} ± 0.75 (55%)	0.696^{f} ± 0.080 (269%)	42.3^{cd} ± 3.4 (32%)	0.849^{cde} ± 0.068 (142%)	3.06
PM+PVA + 10BFP	8.28^{g} ± 0.76 (98%)	0.426^{cd} ± 0.039 (126%)	63.4^{f} ± 9.3 (99%)	0.947^{f} ± 0.396 (170%)	4.38
PM+PVA + 30BFP	10.82^{h} ± 0.70 (159%)	0.693^{f} ± 0.045 (267%)	68.8^{f} ± 6.9 (115%)	1.253^{g} ± 0.125 (257%)	3.90
PM+PVA + 10MLP + 10RCP	5.00^{bcd} ± 0.83 (20%)	0.464^{de} ± 0.077 (146%)	39.4^{bcd} ± 4.2 (23%)	0.738^{bc} ± 0.079 (110%)	4.03
PM+PVA + 10MLP + 10BFP	4.58^{bc} ± 0.75 (9%)	0.359^{bc} ± 0.059 (90%)	38.5^{bcd} ± 5.5 (20%)	0.697^{bc} ± 0.100 (98%)	4.65
PM+PVA + 10RCP + 10BFP	7.36^{fg} ± 1.41 (76%)	0.530^{e} ± 0.102 (181%)	54.0^{e} ± 7.7 (69%)	0.845^{ef} ± 0.380 (140%)	3.77

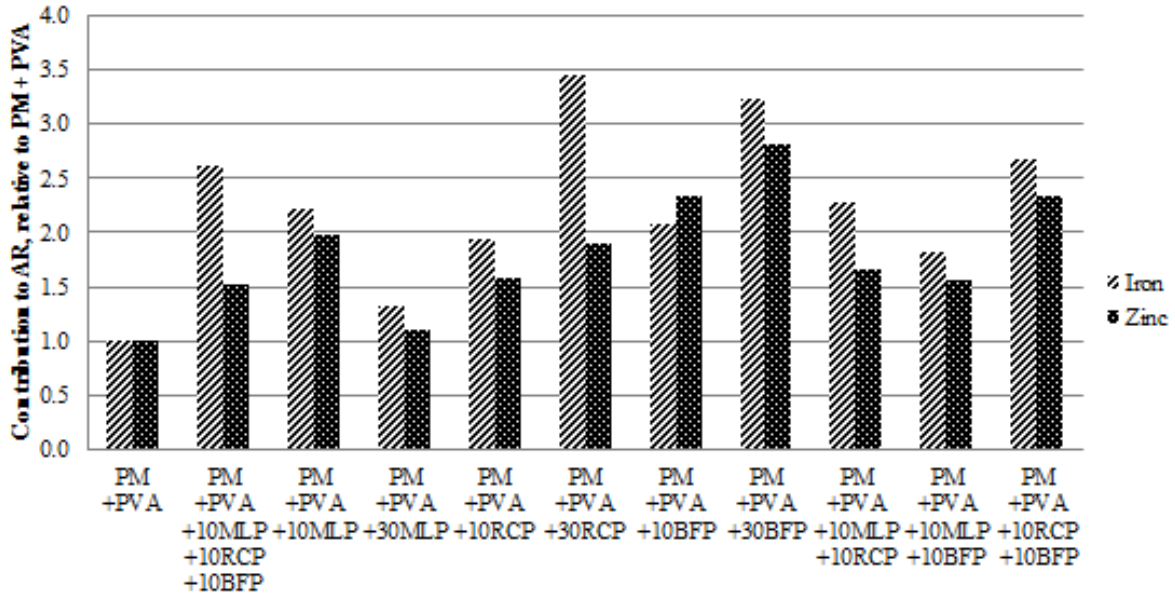
Formulation	**Percentage bioaccessible iron** [% ± SD (percentage difference)]	**Amount of bioaccessible iron** [mg/100 g porridge, db ± SD (percentage difference)]	**Percentage bioaccessible zinc** [% ± SD (percentage difference)]	**Amount of bioaccessible zinc** [mg/100 g porridge, db ± SD (percentage difference)]	**pH of porridge**
PM+PVA + 10MLP + 10RCP + 10BFP	5.41^{cd} ± 0.71 (29%)	0.536^{e} ± 0.000 (184%)	35.7^{bc} ± 3.0 (12%)	0.613^{b} ± 0.000 (74%)	3.78

Key: [1]Values are reported as mean ± 1 standard deviation; mean values followed by different letter superscripts differ significantly ($p \leq 0.05$). Each formulation was subjected to three gastric stages, which in turn were subjected to three intestinal stages, which in turn were analysed for mineral contents in duplicate (n = 18). [2]Numbers in brackets are the percentage difference in bioaccessibility compared to PM+PVA.

Table 5. Calculated mineral (iron, zinc, calcium) and antinutrient contents of each formulation

Formulation	**Mineral and antinutrient contents** (mg/100 g, db)					
	Iron	**Zinc**	**Calcium**	**Phytate**	**Phenolic acid[1]**	**Condensed tannin**
PM+PVA	5.05	1.84	46	716	32	0
PM+PVA + 10MLP	7.97	1.96	151	758	106	0
PM+PVA + 30MLP	13.81	2.19	363	840	254	0
PM+PVA + 10RCP	7.41	1.98	94	958	43	0
PM+PVA + 30RCP	12.12	2.25	191	1441	67	0
PM+PVA + 10BFP	5.74	1.91	59	732	32	114
PM+PVA + 30BFP	7.12	2.03	87	764	32	343
PM+PVA + 10MLP + 10RCP	10.33	2.09	200	999	117	0
PM+PVA + 10MLP + 10BFP	8.66	2.02	165	774	106	114
PM+PVA + 10RCP + 10BFP	8.10	2.04	108	974	44	114
PM+PVA + 10MLP + 10RCP + 10BFP	11.02	2.15	213	1015	118	114

Key: [1]Total phenolic content is based on UPLC values (Table 3).


Contribution to AR, relative to PM + PVA
4.0
3.5
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PM +PVA
PM +PVA +10MLP +10RCP +10BFP
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PM +PVA +10MLP +10BFP
PM +PVA +10RCP +10BFP
Iron
Zinc

Fig. 1