Replacing electrolytic iron in a fortification-mix with NaFeEDTA increases both iron and zinc availabilities in traditional African maize porridges

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Highlights

• Maize meal was fortified with a multi-nutrient mix with electrolytic iron or NaFeEDTA.

• Iron & zinc availability (Caco-2 cells) from thick & fermented porridges was determined.

• Fermentation increased the iron & zinc availability from porridges, fortified or not.

• Adding NaFeEDTA compared to electrolytic iron increased both iron & zinc availability.

Abstract

While replacing electrolytic iron with NaFeEDTA in multi-micronutrient fortification-mixes is a popular option, there is no information about the effect on the iron and zinc availabilities in African staple foods. This study evaluated the effects of adding a multi-micronutrientfortification-mix, with no iron, electrolytic iron or NaFeEDTA on the

availabilities of iron and zinc from thick and fermented special-grade maize porridgesusing a Caco-2 cell model. Replacing electrolytic iron with NaFeEDTA significantly ($p \leq 0.05$) increased iron and, importantly zinc, availabilities in both the thick (2.16 vs. 1.45% and 2.51 vs. 2.29%, respectively) and fermented (3.35 vs. 2.66% and 3.04 vs. 2.61%, respectively) porridges. Some of the NaFeEDTA complexes perhaps partially dissociated because of pH changes duringsimulated digestion, binding with zinc and increasing its availability. NaFeEDTA in a multi-micronutrient fortification-mix, added to less refined, high phytate maize meal, would be more effective than electrolytic iron in addressing both iron and zinc deficiencies in low socio-economic populations of sub-Saharan Africa.

Key words: maize porridge, fortification, NaFeEDTA, Caco-2 cell uptake, zinc

Chemical compounds studied in this article

Ferric sodium ethylenediaminetetraacetate (PubChem CID: 30716); Iron (PubChem CID: 23925); Zinc oxide (PubChem CID: 14806); phytate (PubChem CID: 890)

1. Introduction

Despite the implementation of various nutritional interventions, including food fortification programmes, the developing world still suffers from multiple micronutrient deficiencies, including vitamin A, iodine, folate and, importantly, iron and zinc (Haddad *et al.,* 2015). These deficiencies are highly prevalent in nutritionally vulnerable groups such as women and children.

Examples include South Africa where a mandatory multi-micronutrient food fortification programme was implemented in 2003. While between 2005 and 2012iron deficiency among children under 5 years of age decreased from approximately 14% (Labadarios *et al.,* 2007) to 10% (Shisana *et al*., 2014), the prevalence of stunting (23%) among 1-3 year old children did not improve. This could have been for a variety of reasons including low bioavailability of fortified iron and, particularly, zinc, as stunting is often used as an indicator of zinc deficiency (Davidsson, Fontain and Hotz, 2007). In Kenya, it was found that iron fortification adversely affected the gut microbiome, increased pathogen abundance, and induced intestinal inflammation in infants (Jaeggi *et al*., 2015). Increased intake of iron promoted the growth and virulence of pathogenic enterobacteria (Jaeggi *et al*., 2015); most beneficial barrier bacteria do not require iron (Zimmermann et al., 2010).

In developing countries, electrolytic iron and zinc oxide are often used in fortification-mixes (Allen *et al*., 2006). This is because the effects of these compounds on the sensory quality of the food are low due to poor solubility, but it is possible to add more fortificant due to their low cost. However, this means fortified food contains large amounts iron that cannot be absorbed, but might increase the growth and virulence of pathogenic bacteria. Ferric sodium ethylenediaminetetraacetate (NaFeEDTA)is a popular fortificant because the iron is highly bioavailable, even in high phytate foods, so less has to be added. Also, addition of NaFeEDTA could increase zinc availability, but results from the limited research is contradictory with outcomes depending on the food vehicle (Bothwell and MacPhail, 2004).

Maize meal, consumed in the form of porridge, is one of the most important staple foods for the vulnerable populations in Africa (Smale, Byerlee and Jayne, 2011), and a popular choice for national food fortification programmes (Allen *et al*., 2006). Maize

flour, however, contains phytate, which inhibits the bioavailability of iron and zinc. Levels of phytate decrease as the grain is refined (Feil, 2001), and de-germed: most of the phytate is located in the germ (O'Dell, de Bowland & Koirtyohann, 1972). However, lower income houses, often at risk for nutrient deficiencies, consume less expensive, less refined (non-degermed) maize, which contain more phytate.

While iron fortification in general is a well-researched area, there are gaps regarding the bioavailability of fortificants from traditional African foods. There is no information comparing the bioavailability of electrolytic and NaFeEDTA from traditional African maize porridges, alone or in a multi-micronutrient mix. The aim of this study was to evaluate the effect of replacing electrolytic iron with NaFeEDTA, within a multi-micronutrient fortification-mix, on the iron and zinc availabilities from thick and fermented maize porridges.

The Caco-2 cell uptake model is a good assay for understanding more about the impact of dietary modulators on iron and zinc bioavailability (direction of effect) (Fairweather-Tait *et al*., 2005). Importantly, the Caco-2 cell model has been found to be the most useful experimental approach *in vitro* for iron availability, and the results can be used to make predictions about iron bioavailability *in vivo* (Fairweather-Tait *et al*., 2007).The Caco-2 cell model is also used because, compared to animal absorption and/or human bioavailability studies, large numbers of samples can be analysed at low cost and in a short period of time (Quintaes *et al.,* 2015).

The findings from this study will contribute towards the design of more effective fortification programmes in sub-Saharan Africa to address the unacceptable prevalence of iron and zinc deficiencies.

2. Materials and methods

2.1. Preparation of fortified maize porridges

Special-grade maize flour (79% extraction, non-de-germinated), kindly donated by Premier Mills (Pretoria, South Africa), was used to prepare the fortified maize meals. Three different fortification-mixes were kindly donated by DSM (Kempton Park, South Africa); base fortification-mix containing no iron fortificant and two containing either electrolytic iron or NaFeEDTA (Table 1). A joint FAO/WHO Expert Committee concluded the use of NaFeEDTA in food fortification programmes was safe because, when fortified at 15 mg/kg, it provides a daily iron intake (from NaFeEDTA) of 0.2 mg/kg bodyweight/ day or less (FAO/WHO, 2000).

Table 1: Multi-micronutrient fortification levels (per kg) of special grade maize meal

	Micronutrient fortificant	**Micronutrient content/ kg maize meal**
Fortification base[1]	Vitamin A palmitate[2] (activity: 75 000 mcgRE[2]/g)	2085 mcg RE
	Thiamine mononitrate (activity: 78% min) (mg)	2.19
	Riboflavin (mg)	1.69
	Nicotinamide/niacinamide (mg)	25.00
	Pyridoxine HCl (activity 81% min) (mg)	3.13
	Folic acid (90.5% min) (mg)	2.00
	Zinc oxide (activity: 80% min) (mg)	35.00
	Iron fortificants	**Iron / kg maize meal**
Iron fortification	Fortification base with no added iron fortificant (mg)	0.00
	Fortification base with electrolytic iron (activity: 98% min) (mg)	35.00
	Fortification base with NaFeEDTA (activity: 13% min) (mg)	15.00

[1]Fortification mix according to South African regulations (DOH, 2003)
[2]Retinol equivalents (RE) = 1 mcg retinol = 3.33 IU (International units) vitamin A

The fortification-mixes were added to10 kg of maize meal and mixed until homogenous. Representative sub-samples (n=4) were taken and analysed for minerals (Fe, Zn) to ensure homogenous distribution of the respective fortification-mixes.

2.2. Preparation of porridges

The thick and fermented maize porridges were prepared according to Kruger et al., (2012). In short, for the fermented maize meal, a starter culture of the unfortified maize meal was prepared by mixing flour and distilled water (1:2 w/w), and incubated at 25 ºC until the pH was less than 4 (≈36-48 hrs). The fermented flour samples were prepared by mixing maize meal, distilled water and the starter culture (1:2:0.1 w/w) and incubated as described (≈24-36 hrs).

For the porridge, distilled water was added to fermented or unfermented maize meal (1:10 w/w). The mixtures were heated to boiling and maintained, with constant stirring, for 15 min. The porridges were left to cool at room temperature (≈25°C), and then frozen at -20 ºC before being freeze-dried.

2.3. Analyses

2.3.1. Phytate content

Phytate was determined using an indirect quantitative anion exchange method (Frubeck, Alonso, Marz and Santidrian, 1995).

2.3.2. Mineral contents

The total mineral contents (Al, Fe, Zn, Mg, Ca, and P) were analysed using ion coupled plasma-optical emission spectrometry (ICP-OES), after acid digestion (Kruger, Taylor, Du, De Moura, Lönnerdal and Oelofse, 2013).

2.3.3. Iron and zinc availabilities

The iron and zinc availabilities were measured using a Caco-2 cell uptake model, as described by Kruger et al. (2013). In short, for digestion *in vitro,* crystallised, lyophilised, and essentially salt-free pepsin (porcine, 4200 U/mg), pancreatin, (P-1750) and bile extract (B-8631) (Sigma, Johannesburg, South Africa) were used.

Caco-2 cells were kindly donated by the Department of Pharmacology, North-West University(Potchefstroom, South Africa), and cultured in Dulbecco's Modified Eagle Medium (DMEM) with glucose, Earle's salts, and L-glutamine (Hyclone, Separations, Johannesburg, South Africa) containing 10% foetal bovine serum, which, was not heat-treated (Highveld Biological, Pretoria, South Afirca) and 1% antibiotics (Hyclone, Separations, Johannesburg, South Africa).

Differentiation of the cells was confirmed using scanning electron microscopy (SEM) to identify micro-villi (Figure 1). At 14 days post-confluence, cells were fixed with methanol, carbon-coated, and examined using a JSM-840 SEM (Jeol, Tokyo, Japan). Positive and negative controls were also analysed on each 24-well plate to confirm measure of the inhibitory/enhancing effects of modulators. All experiments were conducted between the 50-60th passages.

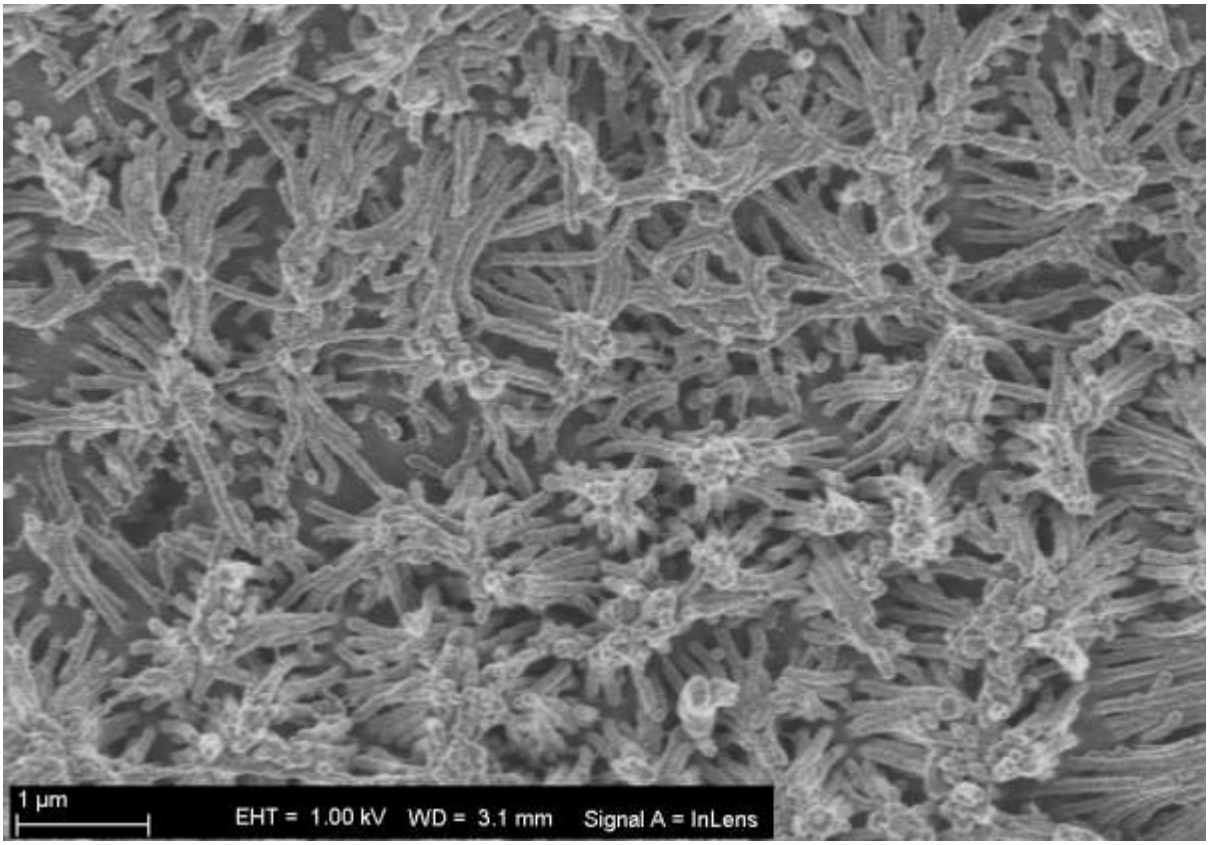

Figure 1: Scanning electron microscope image of micro-villi on differentiated Caco-2 cells

Iron and zinc radio isotope labelling was achieved by mixing digested maize porridges in equal parts with the same medium used to culture the cells, except only 2% bovine serum was added. ^{59}Fe and ^{65}Zn (Separations Scientific, Johannesburg, South Africa) in the form of $^{59}FeSO_4$ and $^{65}ZnCl_2$ were used. The samples were left overnight for the isotopes to exchange fully with the intrinsic iron and zinc.

The isotope labelled samples were applied to the Caco-2 cells, which were incubated for 6 hours, after which the maize:medium mix and cells were collected separately and counted in a Wizard^2 2470 automatic gamma counter (Wallac, Perkin Elmer, Johannesburg, South Africa). Results are presented as the percentage radioactivity in the cells, relative to the total activity in the well.

2.2. Statistical analyses

Statistica 12 (StatSoft, Johannesburg, South Africa)was used to analyse differences using one-way analysis of variance (ANOVA) and main effects ANOVA. Fisher's LSD post-hoc test was used to determine differences between specific means at a confidence level of 95% ($p \leq 0.05$). Phytate, total phenolic, and tannin contents were analysed in duplicate on two individually prepared samples (n=4). The iron and zinc Caco-2 uptake was determined in triplicate, on two individually prepared samples (n=6), and repeated using separately cultured sets of cells (total n=12).

3. Results and discussion

3.1. Mineral and phytate contents

The aluminium content of the special-grade maize meal was low and did not vary significantly ($p>0.05$)(Table 2). Aluminium contents greater than 1 mg/100 g have been found to be indicative of soil contamination (Kang and Priyadarshan, 2008). The iron (0.84-0.86 mg/100 g) and zinc (0.83 mg/100 g) contents of unfortified special-grade maize meal were lower than those reported previously (1.0 and 1.4 mg/100 g, respectively) (Kruger, Mongwaketse, Faber, van der Hoeven and Smuts, 2015). It has, however, been found that iron and zinc contents of whole maize varies between 1.1-8.7 and 1.5-4.9 mg/100 g, respectively (Maziya-Dixon, Kling, Menkir and Dixon, 2000; Ortiz-Monasterio, Palacios-Rojas, Meng, Pixley, Trethowan and Pena, 2007). Such variation in the whole grain would, certainly, affect the mineral content of refined maize meal; an early study estimated that in maize up to 40% of iron and 30% of zinc were located in the endosperm (O'Dell *et al.,* 1972).

Fortified iron and zinc levels (intrinsic mineral content subtracted from final fortified mineral content) were lower than the levels at which they were added for the zinc

Table 2: The mineral contents (mg/100 g, dry basis) of unfortified and fortified (Fe free, electrolytic Fe and NaFeEDTA) special grade maize meal and potential contribution a portion (100 g, as consumed) can make towards the recommended dietary allowances (RDA) of healthy adults

	Al	Fe	Zn	Ca	Mg	P
Unfortified	0.68[a](0.53)	0.84[a](0.13) [4-8%]	0.83[a](0.13) [7-9%]	4.07[a](0.80) [0.4%]	38.1[a](1.1) [5%]	118[a](3) [26-33%]
Fe free fortification mix	1.09[a](0.47)	0.86[a](0.09) [4-10%]	3.09[b](0.14) [25-34%]	4.42[a](1.28) [0.4%]	39.7[a](1.7) [5%]	122[a](6) [27-35%]
Electrolytic Fe fortification mix	1.09[a](0.39)	3.98[c](0.63) [18-41%]	2.78[b](0.51) [22-31%]	6.09[a](1.82) [0.5%]	41.0[a](4.3) [5%]	126[a](11) [28-36%]
NaFeEDTA fortification mix	0.89[a](0.68)	2.24[b](0.67) [10-23%]	3.06[b](0.08) [25-38%]	4.00[a](0.25) [0.4%]	39.3[a](0.8) [5%]	123[a](1) [77-35%]

Values expressed as means (n=4) and 1 SD in parenthesis
[abc]- Values in the same column with different superscripts, differ significantly (p≤0.05),
[] - In square brackets - percentage contribution a 100 g portion of porridge (as consumed) can make towards the RDA of healthy adults for iron (Institute of medicine [IOM], 2001) (women and men 18 and 8 mg/ day, respectively), zinc (women and men 8 and 11 mg/ day, respectively) (IOM, 2001), calcium (IOM, 1997) (women and men both 1000 mg/ day), magnesium (women and men both 700 mg/ day) (IOM, 1997) and phosphorus (women and men 310 and 400 mg/ day, respectively) (IOM, 1997).

(1.95-2.26 vs. 3.50 mg/100 g), electrolytic iron (3.13 vs. 3.50 mg/100 g), and NaFeEDTA (1.39 vs. 1.50 mg/100 g). This could not have been due to heterogeneous distribution, as the mineral contents of each maize meal were analysed four times using representative sub-samples. The reduced levels could be due to the small quantity of maize meal that was fortified (10 kg), compared with commercial fortification, which might result in greater losses during fortification, mixing, and storage.

Addition of electrolytic iron and NaFeEDTA increased the contribution a 100g-portion of maize meal porridge (as consumed) would make to the recommended dietary allowance (RDA) of healthy adults, approximately five and twofold, to 18-41% and

10-23%, respectively (Table 2).The added zinc also increased the contribution three fold to approximately 25-30% RDA. The maize meal could be a good source of phosphorus, where a 100g-portion would provide approximately 30% RDA. The calcium and magnesium contribution to the RDA would, however, be very low at 0.4-0.5 and 5%, respectively.

The phytate content of unfermented porridge (474 mg/100 g) (Table 3) was similar to that previously found for special-grade maize meal (488-648 mg/100 g) (Kruger *et al*., 2015). As expected, the fermentation significantly reduced phytate (57-75%, p≤0.05). Interestingly, there was a trend where the magnitude of phytate reduction in the fortified maize porridges was greater than that of the control, with the phytate content of the NaFeEDTA fortified porridge the lowest. This suggests the fermentation efficiency might increase with the nutrient content and availability.

Table 3: Phytate contents (mg/100 g, db) of thick unfortified and fermented unfortified and fortified (Fe free, electrolytic Fe and NaFeEDTA) maize porridges

	Phytate content
Unfermented unfortified	474^{c} (36)
Fermented unfortified	203^{b} (20)
Fermented Fe free fortified	166^{ab} (42)
Fermented Electrolytic fortified	166^{ab} (14)
Fermented NaFeEDTA fortified	118^{a} (42)

Values expressed as means (n=4) and 1 SD in parenthesis
^{abc}- Values with different superscripts, differ significantly (p≤0.05)

3.2. Iron and zinc availabilities

The addition of all three fortification-mixes significantly ($p \leq 0.05$) increased the iron and zinc availabilities in the thick maize porridges (Table 4). Adding either of the iron fortificant-mixes reduced the phytate:iron molar ratios approximately 3-5 fold, but not below the critical levels of 1 (Hunt, 2003) or above 10 (Saha, Weaver and Mason, 1994), beyond which iron bioavailability has been found to be seriously impaired.

Table 4: Iron and zinc availabilities and phytate molar ratios from unfortified and fortified (no Fe, electrolytic Fe and NaFeEDTA) special grade thick and fermented maize porridges as measured by a Caco-2 mineral uptake model

	Thick porridge	**Thick fermented porridge**	**LS mean values**
% iron availability [phytate:iron molar ratio]			
Unfortified	0.38^{a} (0.12) [48]	2.43^{de} (0.31) [20]	**1.50^{W}**
Fe free fortification mix	0.9^{b} (0.2) [46]	2.54^{e} (0.23) [16]	**1.79^{X}**
Electrolytic Fe fortification mix	1.45^{c} (0.17) [10]	2.66^{e} (0.39) [4]	**2.06^{Y}**
NaFeEDTA fortification mix	2.16^{d} (0.31) [18]	3.35^{f} (0.15) [4]	**2.81^{Z}**
LS mean values	**1.12^{W}**	**2.74^{X}**	
% zinc availability [phytate:zinc molar ratio]			
Unfortified	1.57^{a} (0.28) [57]	2.54^{c} (0.26) [24]	**2.13^{W}**
Fe free fortification mix	2.12^{b} (0.26) [15]	2.55^{c} (0.35) [5]	**2.27^{W}**
Electrolytic Fe fortification mix	2.29^{b} (0.23) [17]	2.61^{c} (0.38) [6]	**2.38^{W}**
NaFeEDTA fortification mix	2.51^{c} (0.22) [15]	3.04^{d} (0.41) [4]	**2.68^{X}**
LS mean values	**2.05^{W}**	**2.65^{X}**	

Values expressed as means (n=12) and 1 SD in parenthesis
^{abc}- Values of the same mineral with different superscripts, differ significantly ($p \leq 0.05$),
^{WXY}- Least Significant Mean values from main effects ANOVA with different superscripts in the same row/column, differ significantly ($p \leq 0.05$)
[] –Values in square brackets are the phytate:iron and phytate:zinc molar ratios

Adding the fortification-mix without any additional iron increased the availability of iron from the thick maize porridge. This suggests the multi-nutrient fortificants enhanced the availability of the intrinsic iron independently. The phytate:zinc molar ratios of all the fortified thick porridges (15-17) did not vary greatly, and were close to the critical range 10-15 (Saha *et al.*, 1994).

Iron availability from the thick maize porridge fortified with NaFeEDTA was the greatest. This was because EDTA 'protected' the minerals from the anti-nutrient influence of phytate during the simulated digestion (Bothwelland MacPhail, 2004).The EDTA:Fe molar ratio in this study was 0.62. It has been shown that a slightly higher ratio (0.66-1.00), in cereal foods, increased the iron bioavailability five-fold (Hunt, 2003).

The zinc availability from thick maize porridge fortified with the NaFeEDTA was significantly higher than that of other thick maize porridges (2.16 vs. 0.38-1.45%). EDTA is a hexa-coordinating complexing agent, binding to almost any metal (at different stability constants) with four carboxylate, and two tertiary amine groups (Bothwell and MacPhail, 2004). The stability constants for EDTA with ferric iron (25.1) and zinc (16.1) have been found to be high, resulting in stable bioavailable complexes. The complex stability is, however, pH dependant with the optimal pH for complexing at 1 and 4, for ferric iron and zinc, respectively (Bothwell and MacPhail, 2004). It is possible that the pH changes during digestion, from ≈6 (maize) to 2 (stomach digestion) and then ≈7 (intestinal digestion), that some of the NaFeEDTA dissociates and forms stable complexes with zinc.

Fermentation significantly increased ($p \leq 0.05$) both iron (2.74 vs. 1.12%) and zinc (2.65 vs. 2.05%) availabilities compared with the thick maize porridges (Table 4).

This is probably due to the highly significant ($p \leq 0.05$) reduction (57-75%) in phytate (Table 3). The phytate:iron molar ratios (Table 4) of electrolytic iron (4) and NaFeEDTA (4) fortified fermented porridges were reduced approximately 3-5 fold, compared to the thick porridges, well below the critical level established by Saha *et al*. (1994).The phytate:zinc molar ratios of all the fortified fermented porridges (4-6) were also well below the critical range of 10-15.

The iron and zinc availabilities in fermented porridge fortified with the NaFeEDTA were significantly higher ($p \leq 0.05$) than those of other fermented porridges (3.35 vs ≈2.5% and 3.04 vs ≈2.6%, respectively) (Table 4).Because phytate has a limited inhibitory effect on the availability of EDTA-mineral complexes (Bothwell and MacPhail, 2004), the increases were probably not due to phytate reduction during fermentation (Table 3). Also, while there were substantial differences in the phytate:iron (4-20) and phytate:zinc (6-24) molar ratios of the other fermented porridges (ratios both above and below the critical limits), there were no significant differences ($p > 0.05$) between the iron and zinc availabilities (Table 4). This reinforces the additional mineral availability enhancing effect of fermentation.

Lactic acid, produced by the microbes (lactic acid bacteria) involved in natural fermentation of cereals (Hurrell, Reddy, Burri and Cook, 2000), has been found to form stable soluble complexes with iron and zinc (Teucher, Olivares and Cori, 2004). Proulx and Reddy (2007) evaluated the effect of fortification (reduced iron, ferrous sulphate), fermentation and lactic acid on the iron solubility and availability (Caco-2 cell uptake model) from maize porridge. They found that a 24-hour fermentation, followed by porridge preparation, only reduced the phytate content by 11%, but increased iron uptake from ferrous sulphate and reduced iron fortified porridges 2.5 and 1.8 fold, respectively. Importantly, adding lactic acid to the unfermented

porridges resulted in a similar increase in iron solubility as fermentation. Also, the end point for maize fermentation was a pH below 4 (pH 3.64-3.91). It is possible that during fermentation at pH 4, optimal complex stability for Zn-EDTA, more zinc bound with EDTA, resulting in increased uptake from the fermented porridges.

Overall (main effects ANOVA), fermentation significantly ($p \leq 0.05$) increased both the iron (2.74% vs. 1.23%) and zinc (2.65% vs. 2.05%) availabilities (Table 4). However, the effect of different fortification-mixes was not the same for the iron and zinc availabilities. The iron availability was lowest for unfortified porridges (1.50%), but increased significantly with the addition of iron-free (1.79%), electrolytic iron (2.06%) and NaFeEDTA (2.81%) fortification-mixes. However, there was no overall effect on zinc availability following addition of the iron-free (2.27%) and electrolytic iron (2.38%) fortification-mixes, compared with the unfortified porridge (2.13%). This indicates that the added zinc had the same availability as intrinsic zinc in the maize flour. However, taking into consideration both porridges, addition of the NaFeEDTA significantly ($p \leq 0.05$) increased zinc availability (2.68%).

4. Conclusions

Fermentation decreases phytate content, and increases iron and zinc availabilities in both unfortified, and fortified maize porridges. Importantly, replacing electrolytic iron with NaFeEDTA in special-grade maize flour, not only increased the iron, but also the zinc availability from traditional porridges. This increased availability has the potential to alleviate high levels of iron and zinc deficiencies in rural areas where less refined, high phytate cereals are consumed.

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