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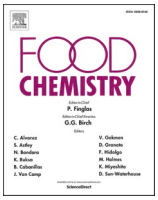

Bioaccessibility of iron in pearl millet flour contaminated with different soil types

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ABSTRACT

A controlled in-vitro experiment was conducted to determine the bioaccessibility of extrinsic soil iron in pearl millet contaminated with typical Malawian soils. Pearl millet was contaminated with soils at ratios typically encountered in real life. Iron concentrations of soil-contaminated flour increased such that soil-derived iron contributed 56, 83 and 91% of the total iron when the proportions of soil were 0.1, 0.5 and 1% (soil: grain w/w), respectively. When soils were digested alone, the concentration of bioaccessible iron differed depending on the type of soil. However, the concentration of bioaccessible iron in soil-contaminated flours did not exceed that of uncontaminated flour and there was no effect of soil type. This suggests that knowledge of the proportion of extrinsic soil iron in soil-contaminated grains would be useful for iron bioavailability estimations. Vanadium is a reliable indicator of the presence of extrinsic soil iron in grains and has potential applications in this regard.

1. Introduction

The inadvertent consumption of soil iron is common, especially in many small-holder farming communities of low-income countries. This is because cereal grains and other food groups, especially leafy vegetables that grow close to the ground are susceptible to contamination with soil dust in the field and during harvesting and processing. Rain splash may transfer soil particles to leaves and other plant tissues growing close to the ground (Gabaza, Shumoy, Muchuweti, Vandamme, & Raes, 2018b; Joy, Broadley, Young, Black, Chilimba, Ander, et al., 2015). In addition, small seeded cereal grains, such as tef (*Eragrostis tef*), sorghum (*Sorghum bicolor*) and millet (*Pennisetum glaucum*, *Eleusine coracana*) are typically processed by threshing on the ground using threshing sticks or under the hooves of cattle, depending upon the setting. Such grains are therefore associated with high levels of soil contamination due to the close proximity of grains with soil particles during post-harvest processing (Siyame, Hurst, Wawer, Young, Broadley, Chilimba, et al., 2013; Teklu, 2017). Although the mass of soil consumed via this route is small, iron concentration is orders of magnitude greater in soil (approximately 40,000 mg kg^{-1}) than in grain products (<100 mg kg^{-1}) such that minute amounts of extrinsic soil substantially increases the total iron concentration of the grains (Joy, et al., 2015).

High concentrations of iron in tef grains, ranging between 300 and 1,500 mg kg^{-1}, have been attributed to extrinsic soil iron contamination (Abebe, Bogale, Hambidge, Stoecker, Bailey, & Gibson, 2007; Shumoy, Lauwens, Gabaza, Vandevelde, Vanhaecke, & Raes, 2017). Similarly, a sample of 97 edible food items collected from across Malawi revealed high levels of soil contamination in grain and leafy vegetables with an estimated 33.8 % and 76.7 % of total iron derived from soil, respectively (Joy, et al., 2015). An analysis of grains of maize, sorghum and millets from several geographical locations in Zimbabwe also highlighted some unusually high iron concentrations from some locations which deviated by more than 400 % from mean grain iron concentrations; again this was attributed to extrinsic soil contamination (Gabaza, Shumoy, Muchuweti, Vandamme, & Raes, 2018b). Grains produced in small-holder farming communities are therefore likely to contain varied concentrations of extrinsic soil iron, and this will likely depend on post-harvest processing steps conducted, including winnowing, shelling, washing or soaking of grains and removal of bran during milling. Dietary iron supplies analyzed using food composition tables may underestimate dietary iron intake as iron concentration data from food composition tables is unlikely to capture the varied levels of extrinsic soil iron in different

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settings (Gibson, Wawer, Fairweather-Tait, Hurst, Young, Broadley, et al., 2015). Analysis of weighed diet composites for the assessment of dietary iron intakes in small-holder farming settings is thus recommended.

Despite high iron concentration in diets because of soil contamination, previous studies have found that dietary iron consumption and the iron content of cereal grains does not always reflect human iron status (Gibson, et al., 2015; Siyame, et al., 2013). A cross-sectional study by Siyame, et al. (2013) in Malawi showed that women of reproductive age from the Zombwe Extension Planning Area (EPA) had greater body iron concentrations than women from Mikalango EPA (5.3 vs 3.8 mg kg^{-1}) despite lower dietary iron intake of 16.6 mg day^{-1} compared to 29.6 mg day^{-1} for women from Mikalango EPA. Similarly, Dickinson, Rankin, Pollard, Maleta, Robertson, and Hursthouse (2014) also reported differences in the prevalence of iron deficiency anaemia among pregnant women from two contrasting geographical regions of Malawi despite similar dietary iron intakes. Two explanations related to bioavailability have been proposed to explain the dissonance between dietary iron intake and human iron status. Bioavailability refers to the amount of an ingested nutrient that is absorbable after a series of important physiological functions, namely, gastrointestinal digestion, absorption by intestinal cells and transport to body cells (Etcheverry, Grusak, & Fleige, 2012). First, the type of diet may influence the bioavailability of extrinsic soil iron, with bioavailability decreasing according to the concentration in the diet of phytic acid and other inhibitors of mineral absorption; diets of women in Zombwe were predominantly based on maize with high phytate, whereas diets of women from Mikalango were based on millets which are high in both phytate and polyphenols. Several in-vitro and in-vivo studies substantiate this assertion (Baye, Guyot, Icard-Vernière, Rochette, & Mouquet-Rivier, 2015; Gabaza, Shumoy, Muchuweti, Vandamme, & Raes, 2018a, 2018b; Hurrell, Reddy, Juillerat, & Cook, 2003). Secondly, the bioavailability of extrinsic soil iron in soil-contaminated cereal grains may also depend on the characteristics of the soil contaminating the grain; the soils of Zombwe are more acidic (median pH 5.2) than in Mikalango (predominantly alkaline Vertisols, median pH 7.8). When iron bioavailability of the different types of soils was measured using Caco-2 cells, the Zombwe soil induced the ferritin response whilst there was no response for the Mikalango soil (Gibson, et al., 2015).

Although it has been proposed that extrinsic soil iron may make a meaningful contribution to iron nutrition in settings where non-mechanised threshing practices are common, the extent of its exchangeability or equilibration with intrinsic iron or the factors influencing exchangeability remain elusive. Moreover, it is not clear whether the amount of soil iron that exchanges with the intrinsic iron is absorbed to the same extent as the intrinsic iron. A review by Harvey, Dexter, and Darnton-Hill (2000) of the evidence concerning the impact of ingesting extrinsic iron on human iron status indicated the challenges of predicting iron bioavailability from meals contaminated with extrinsic iron due to wide variation in the amount of extrinsic iron in diets and methodological limitations. Of the few studies reviewed, the amount of extrinsic iron was not directly measured. However, most of the studies seem to suggest that extrinsic iron from soil or other sources, if exchangeable, is absorbed to the same extent as intrinsic iron of the specific food matrix.

Considering the highly variable levels of reported soil contamination in cereal grains, the objective of this study was to determine the effect on iron bioaccessibility of extrinsic soil contamination in pearl millet flour by three types of Malawian soils, at differing proportions, in an in-vitro study. Unlike bioavailability, bioaccessibility refers to the amount of ingested nutrient that is potentially absorbable upon release of the nutrient from the food matrix after gastrointestinal digestion and is measured using in-vitro digestion techniques (Etcheverry, Grusak, & Fleige, 2012). In this manuscript, bioavailability will only be used to refer to the subject matter, while bioaccessibility will be used in cases where in-vitro measurements were undertaken. Pearl millet is an important crop for iron nutrition for many Asian and African populations (Tako, Reed, Budiman, Hart, & Glahn, 2015). Post-harvest processing of pearl millet involves threshing of the grain, making it susceptible to soil contamination. Extrinsic iron in grains can also be derived from milling and cooking equipment such that the use of specific indicators of the presence and source of contamination is important. The correlation of iron in edible portions of crops with specific heavy metals associated with soils i.e., titanium (Ti), aluminum (Al) and vanadium (V), is often used as a useful marker of the presence of extrinsic soil iron. Therefore, these three elements were evaluated to identify the most reliable indicator of extrinsic soil iron in soil-contaminated grains. An understanding of the iron bioaccessibility of soil-contaminated grains is crucial to our knowledge of bioavailable dietary iron supply in affected regions and it will also strengthen our understanding of the disparity between iron intake data and iron deficiency in developing countries, including spatial variation.

2. Materials and methods

2.1. Preparation of soil-contaminated pearl millet flour

Topsoil samples (0–20 cm) of three contrasting soil types were obtained from Malawi, as described by Ligowe, Young, Ander, Kabambe, Chilimba, Bailey, et al. (2020). A Vertisol (calcareous) was collected at Ngabu in Chikwawa (15° 33′ S and 35° 11′ E), an Oxisol (acidic) was obtained from Bembeke in Dedza (14° 21′ S and 34° 35′ E) and an Alfisol (moderately acidic) was obtained from Chitedze Research Station (13° 59′ S and 33° 35′ E) using hand hoes and spades. These three soil types are typical of Malawi soils with the Oxisol and Alfisol being the most prevalent soil orders. Pearl millet grain was obtained from a supermarket in Nottingham, UK. The pearl millet grain was carefully cleaned of all debris before being milled into a fine powder of < 40 μm using an ultra-centrifugal mill (ZM-200, Retsch, Haan, Germany). Air dried and sieved soil (<2 mm) was then mixed into the pearl millet flour and the soil-flour mixture was thoroughly ground and mixed using a pestle and mortar until the soil was not visible in the mixture. For each soil type, sufficient soil was added to the flour to achieve a final soil proportion of 1, 0.5 and 0.1 % (w/w) in the soil-flour mixture. The contamination levels were selected based on findings of Joy, et al. (2015) where a wide variation in soil contamination led to estimated soil iron contributions of up to 94 % of total iron in pearl millet grain. A total of nine soil-contaminated pearl millet flour (CPM) samples were produced, comprising: Alfisol-CPM, Oxisol-CPM and Vertisol-CPM at three different soil concentrations. A control sample of uncontaminated pearl millet was included. All analyses were performed in triplicate on subsamples.

2.2. Determination of iron bioaccessibility

Iron bioaccessibility was determined using the modified INFOGEST static in-vitro digestion method (Brodkorb, Egger, Alminger, Alvito, Assuncao, Ballance, et al., 2019) comprehensively described by Muleya, Young, and Bailey (2021). The modification involved isotopic labelling of reagent-derived iron with ^{57}Fe (greater than 95 % enrichment, Isoflex, San Francisco, USA) to discriminate between reagent-derived and sample-derived iron in the different sample matrices after digestion. This means ^{57}Fe was used as a tracer of reagent-derived iron, thereby enabling an accurate measurement of iron bioaccessibility. All reagents were procured from Merck Life Sciences UK ltd unless otherwise stated. The following enzymes were used: pepsin from porcine gastric mucosa (3,200–4,500 units/mg protein), α-amylase from *Bacillus* sp. (≥400 units/mg protein), pancreatin from porcine pancreas (8 × USP) and bovine bile. Master mixes of digestion fluids were prepared, namely: simulated salivary fluid (SSF complete), simulated gastric fluid (SGF complete), simulated pancreatin fluid (SPF complete) and simulated bile fluid (SBF complete) after adding the respective enzyme, $CaCl_2$ (only for

gastric and intestinal phase as it caused precipitation in the SSF), ^{57}Fe and Milli-Q water (18.2 MΩ cm) to achieve the required concentrations. The master mixes for each phase of digestion were placed in a shaking water bath at 20 °C, overnight, to allow for complete isotopic equilibration. After equilibration, the digestion fluids were placed on ice before commencing the digestion.

Digestion was performed in triplicate on CPM, control flour and the individual soil samples. Two sets of in-vitro digestions were performed: (i) stopped at the end of the gastric phase to determine the proportion of soluble iron released at this point and (ii) stopped at the endpoint of gastro-intestinal digestion to determine the proportion of soluble and bioaccessible iron. For each of the flour samples, 2.5 g of flour slurry (flour mixed with Milli-Q water to make a 30 % (w/v) dry matter slurry) was used. For each of the soil samples, 0.2 g of soil sample was used. The oral phase of digestion was initiated by adding 2.488 mL SSF complete, 0.012 mL 0.3 M $CaCl_2$ and adjusting pH to 7 using 1 M NaOH. The mixture was incubated at 37 °C, in a shaking water bath for 2 min. The gastric phase of digestion followed by adding 5 mL of SGF complete and the pH was adjusted to 3.0 using 1 M HCL followed by incubation for 120 min. Digestion was stopped at this point for the first set of samples and gastric supernatants were collected after centrifugation at 4,500 × g for 30 min and filtration through 5 μm syringe filters. For the second set of samples, after 90 min of gastric digestion, dialysis tubing (molecular weight cut-off 12.4 kDa, Sigma Aldrich, Dorset, UK) containing 17.5 mL of 0.05 M PIPES buffer (pH 6.7) was added to the digestion tubes and the tubes were incubated for a further 30 min. Intestinal digestion was done by adding 5 mL of SPF complete and 5 mL of SBF complete and adjusting the pH to 7 using 1 M NaOH where necessary. The tubes were incubated again for 2 h before being placed on ice for 15 min to stop enzyme activity. The dialysis membranes were removed and the dialysate (solution in the dialysis membranes representing the bioaccessible fraction) was carefully transferred into clean storage tubes. The remaining mixture was centrifuged (Thermo Fisher Scientific, GT 4R centrifuge) for 30 min at 4,500 × *g* and the supernatant (soluble-non-dialyzed fraction, SND) was separated from the pellet and further filtered through a 5 μm syringe filter. Digestion blanks, in which the in-vitro digestion was performed without food, were carried out alongside the samples to correct for reagent-derived iron. The fractions collected from the two sets of digestions, i.e., gastric supernatants, gastro-intestinal supernatants or SND and dialysate, were analyzed for iron using inductively coupled plasma-mass spectrometry (ICP-MS) as described in the next section.

2.3. Elemental analysis

For the flour samples, 0.2 g of sample was weighed into perfluoroalkoxy (PFA) vessels and 6 mL concentrated HNO_3 (PrimarPlus^{TM} grade) was added while for the in-vitro digested fractions, 2 mL of 50 % HNO_3 was added to 4 mL dialysate and 3 mL of concentrated HNO_3 was added to 3 mL of gastric or gastro-intestinal supernatants. The mixtures were heated in a closed vessel microwave heating system (Microwave Pro, Anton Paar GmbH, Graz, Austria) using a MF50 rotor at a microwave power of 1400 W for 40 min. Soil samples (0.2 g) were heated in 2 mL 65 % HNO_3, 1 mL 60 % $HClO_4$ and 2.5 mL 70 % HF on a programmable hot block as described by Ligowe, et al. (2020). The solutions were diluted accordingly to achieve an acid concentration of<5 % (v/v) using Milli-Q water prior to analysis using a triple quadrupole ICP-MS (iCAP TQ, Thermo-Fisher Scientific, Bremen, Germany). Isotopes measured included ^{56}Fe (native iron), ^{57}Fe (applied iron isotope), ^{27}Al (Aluminum), ^{48}Ti (Titanium) and ^{51}V (Vanadium). Conditions used for the ICP-MS analysis can be found in the supplementary information.

2.4. Data processing and analysis

For total elemental concentrations in the flour, measured concentrations of Fe, Al, Ti and V were converted to a gravimetric basis (mg kg^{-1}) based on the weights and volumes used for the analysis.

Processing of data to determine: (i) gastric and gastro-intestinal soluble Fe (Fe_{sol}; mg kg^{-1}) and (ii) bioaccessible Fe (Fe_{bio}; mg kg^{-1}), in both soils and flours were calculated according to Muleya, Young, and Bailey (2021). Gastric and gastro-intestinal Fe_{sol}(%) as well as Fe_{bio}(%) were subsequently obtained by expressing the respective concentrations relative to the total iron concentration in the raw material. Gastro-intestinal Fe_{sol} (mg kg^{-1}) and Fe_{sol} (%) is the sum of iron from the SND and dialysate fraction. Comparison of means was conducted in SPSS (IBM SPSS Statistics for Windows, Version 27, IBM Corp., Armonk, NY, USA) using two-way ANOVA ($p < 0.05$) for CPMs with soil type and soil proportion as factors. One-way ANOVA was used to compare means for soil samples. Tukey's Honest Significant Difference was used to identify differences within factors where applicable. Pertaining to Al, Ti and V concentrations in the uncontaminated and soil-contaminated pearl millet flour, scatter plots were constructed to examine the presence and strength of a correlation with iron concentrations.

3. Results and discussion

3.1. Iron concentration of soils and pearl millet flour contaminated with extrinsic soil iron

An analysis of the iron bioaccessibility of pearl millet flours contaminated with different types and proportions of typical Malawian soils was undertaken to assess the importance of extrinsic soil iron to iron nutrition. Table 1 shows the total iron concentration of the soils determined in the current study while some important characteristics of the contaminating soils, as determined by Ligowe, et al. (2020), are shown in **Table S1** (Supplementary data). The iron concentration of the Vertisol was significantly greater ($p < 0.05$) than that of the Alfisol and Oxisol which were statistically indistinguishable. The iron concentration of uncontaminated pearl millet flour was 47.1 mg kg^{-1}. Negligible extrinsic soil iron contamination was assumed as the iron concentration of the grains was within the expected range of intrinsic iron concentration in pearl millet grain (Kumar, Hash, Thirunavukkarasu, Singh, Rajaram, Rathore, et al., 2016). This also negated the need for a wet washing step with a dilute solution of hydrochloric acid, which is often required to ensure the absence of contaminant soil iron in grains. Contamination of flour with soil resulted in an increase in total iron concentration which was dependent on the type and proportion of soil added; there was a significant interaction between soil type and proportion after conducting a 2-way ANOVA ($p < 0.05$) (Table 1). The presence of only 0.1 % soil in the CPMs resulted in a doubling of the total iron concentration while soil proportions of 0.5 and 1 % increased total iron concentrations by more than 10-fold. This resulted in proportions of soil iron ranging between 54 and 57 % of the total iron for a soil contamination level of 0.1 %, and 78–88 % and 90–92 % for soil proportions of 0.5 % and 1 %, respectively. The large increase of iron concentration in the flour with minimal soil contamination underlines the relatively high iron concentration of soils. The inadvertent consumption of large amounts of extrinsic soil iron in grain-based foods is

Table 1
Iron concentration of pearl millet flour contaminated with different types and proportions of soils.

Soil proportion	Iron concentration (mg kg^{-1})		
	Alfisol-CPM	Oxisol-CPM	Vertisol-CPM
~0%	47.1 ± 0.24^{a}	47.1 ± 0.24^{a}	47.1 ± 0.24^{a}
0.1 %	101 ± 8.56^{b}	111 ± 0.64^{b}	110 ± 2.65^{b}
0.5 %	$215 \pm 26.7^{c,A}$	$278 \pm 10.5^{c,B}$	$377 \pm 19.6^{c,C}$
1 %	450 ± 17^{d}	531 ± 95^{d}	592 ± 63^{d}
Soils (g kg^{-1})	63.7 ± 3.9^{A}	68.3 ± 1.7^{A}	89.9 ± 3.9^{B}

CPM – contaminated pearl millet flour. Values with different small superscript letters within columns are significantly different. Values with different capital superscript letters across rows are significantly different. $p < 0.05$. $n = 3$.

thus likely to occur in many small-holder settings considering the high level of care and mechanical processing equipment required to prevent even minimal soil contamination.

3.2. Al, Ti and V concentrations as indicators of extrinsic soil iron in edible portions of crops

A strong positive correlation of iron to aluminum (Al), titanium (Ti), and vanadium (V) concentrations has led to the use of these elements as indicators of the presence of extrinsic soil iron in edible portions of crops (Gibson, et al., 2015; Siyame, et al., 2013). Fig. 1 confirms strong correlations (R^2 greater than 0.74) of iron to Al, Ti, and V concentrations in the soil-contaminated flour. The limited uptake and transport of these indicator elements to the edible portions of crops is such that their concentration in soil is many magnitudes greater than the concentration in the edible portions of crops (Gibson, et al., 2015; Joy, et al., 2015). A high concentration of these indicator elements in grains is therefore likely due to processes such as threshing practices described earlier, rather than systemic assimilation via root uptake. Of interest, however, was the markedly stronger correlation of iron to V concentration (R^2 = 0.97) compared to Al and Ti. This is in agreement with Joy, et al. (2015) who proposed that V is a reliable indicator of extrinsic soil iron in grains because (i) concurrent systemic uptake of V and iron by plants is unlikely and (ii) trivalent vanadium (V^{3+}) in soil isomorphically substitutes for iron (Fe^{3+}) within ferric oxides and vanadate (VO_4^{3-}) is adsorbed strongly on iron oxide surfaces. Based on this chemical relationship between iron and V in soils, Joy, et al. (2015) proposed an equation which can be used to determine the proportion of iron derived from soil (P_{Fe}) in cases where iron and V concentrations of both soil and soil-contaminated grain are available.

$$P_{Fe} = \frac{V_{plant} * Fe_{soil}}{V_{soil} * Fe_{plant}} \quad (1)$$

Where V_{plant} and Fe_{plant} are elemental concentrations in the plant, and V_{soil} and Fe_{soil} are concentrations in the soil.

However, in many analyses of grain and food composition, total elemental concentrations in the soil from which the grain was derived are not available suggesting the need for the development of models to estimate the proportion of soil iron in soil-contaminated grains based on elemental grain concentrations only. The construction of such a model would require analysis of large numbers of plant ingredients which are susceptible to soil contamination. Since V is a reliable indicator of the presence of extrinsic soil iron, its use in this regard has future potential applications when interpreting iron composition of foods. Therefore, when multi-element analysis of plant foods is done, where possible, concentration of V should also be determined to allow the estimation of intrinsic and soil-derived iron in the future.

3.3. Bioaccessibility of iron in soils

Fig. 2 shows the iron bioaccessibility results of the three soil types used in the current study which are typical of Malawi soils. In general, the Oxisol is highly acidic, while the Alfisol is moderately acidic, and the Vertisol is calcareous (**Table S1 – Supplementary data**). In addition, the Vertisol has greater proportions of soil organic carbon (%SOC), nitrogen and total exchangeable cations than the Alfisol and Oxisol. After gastric digestion, both the gastric Fe_{sol} (mg kg^{-1}) and Fe_{sol} (%) for the Vertisol was lower than that of the Alfisol and Oxisol. <0.01 % of iron was released after gastric digestion in all soils (Fig. 2). At the end point of digestion, gastro-intestinal Fe_{sol} (%) increased by more than 2-fold for all soils, with a significantly greater increase for the Oxisol. Despite higher gastro-intestinal Fe_{sol} (%) and Fe_{sol} (mg kg^{-1}) for the Oxisol than the Alfisol and Vertisol, there were no differences in Fe_{bio} (%) i.e. dialyzable iron of small molecular weight < 12.4 kDa, with an average of 0.012 % iron being available for absorption from all soil types. Since the

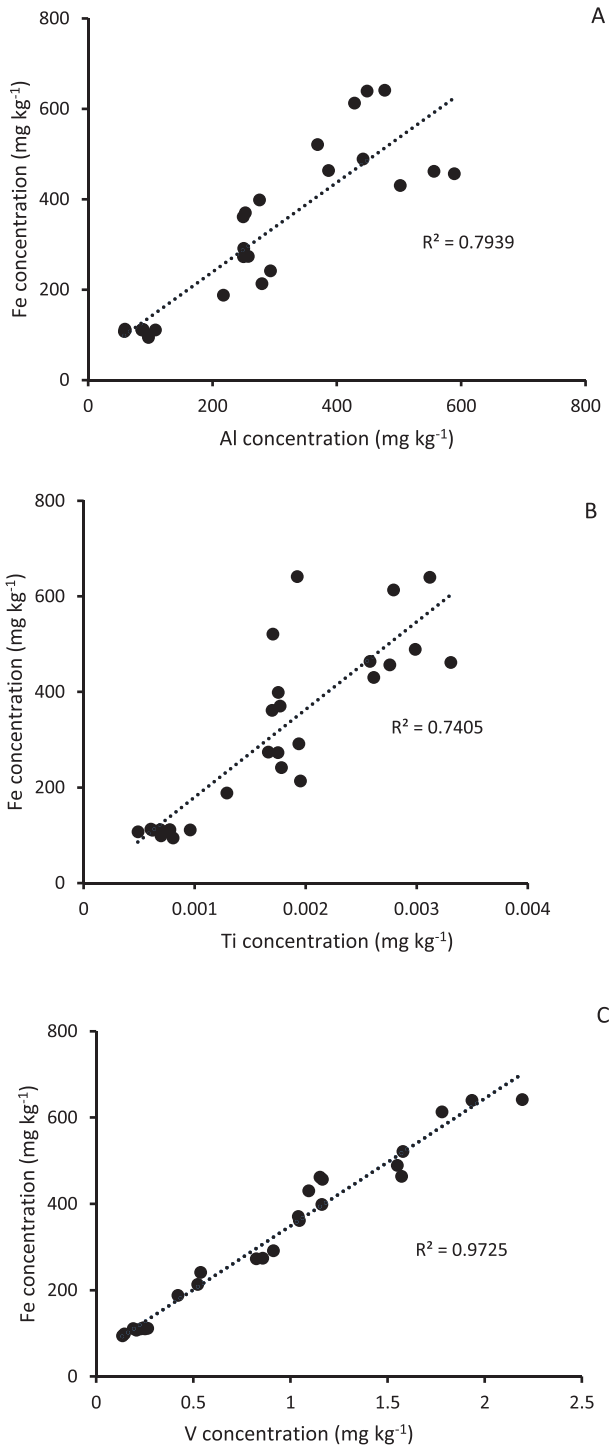

Fig. 1. Correlation of iron to Al (A), Ti (B) and V (C) concentrations in soil contaminated pearl millet flour.

Vertisol had a greater total iron concentration than the Alfisol and Oxisol, its Fe_{bio} (mg kg^{-1}), was therefore greater than the other soils. The results for the actual Fe_{bio} (mg kg^{-1}) of the soils are shown in **Table S2** – Supplementary data.

Only a small proportion of soil iron was released into the common 'non-haem iron pool' under conditions of gastro-intestinal digestion. The non-haem iron pool refers to the iron species derived from plant-based ingredients whose absorption is influenced by iron binding

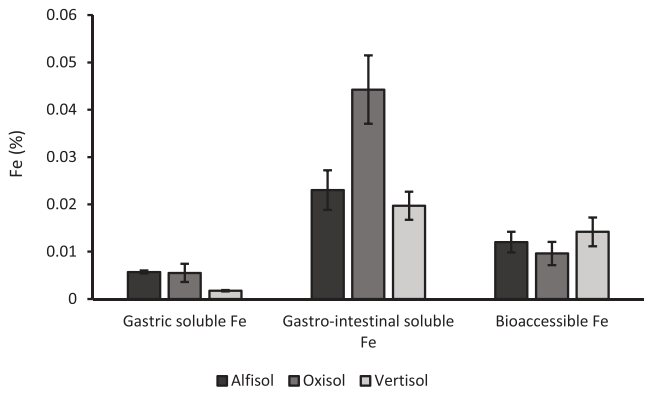

Fig. 2. Proportion of soluble and bioaccessible iron of soils digested without food after gastric and gastro-intestinal digestion. Bioaccessible iron refers to soluble, dialyzable iron of low molecular weight (<12.4 kDa) measured at the endpoint of gastro-intestinal digestion.

ligands, which can have either an inhibitory or enhancing effect on iron absorption. Soil iron is poorly soluble and its solubility is largely influenced by ferric oxides (Lindsay & Schwab, 1982; Uren, 1984). Indeed, the Oxisol had greater gastro-intestinal Fe_{sol} (mg kg^{-1} or %) than the Alfisol and Vertisol because of its greater ferric oxide concentration (**Table S1**). A surprising finding was the greater Fe_{bio} (mg kg^{-1}) of the Vertisol compared to the Oxisol and Alfisol (**Table S2**) which contradicts the findings from Gibson, et al. (2015) who reported ferritin formation when the in-vitro digesta of the acidic soil (Oxisol or Alfisol) were exposed to Caco-2 cells, but no ferritin formation in the case of the Vertisol. The authors concluded that grains contaminated with acidic soils may provide absorbable iron which could be important for iron nutrition. In our case, the Vertisol (calcareous) seems to provide more absorbable iron than both of the acidic soils although the Oxisol (highly acidic) clearly provided more soluble but not absorbable iron. This discrepancy could be a result of differences in methods used to assess bioaccessibility, in particular the time point at which a dialysis membrane is used. In the dialyzability assay used in the current study, a dialysis membrane with a buffer solution of pH 6.7 was inserted 30 min before the end of gastric digestion to facilitate gradual neutralization. It is possible that, during this pH transition, more dialyzable iron species could have been formed from the Vertisol than the Alfisol or Oxisol due to the high %SOC of the Vertisol. On the other hand, the dialysis membrane for the in-vitro digestion utilizing Caco-2 cells is inserted after pH is adjusted to 7. It is reasonable to conclude that the greater iron bioavailability of the acidic soil vs the calcareous soil observed by Gibson, et al. (2015) is likely to be a result of the superior solubility of iron in the acidic soil at pH 7 as observed in this study, rather than the existence of more bioaccessible iron species. Variations in the iron solubility and bioaccessibility of different types of soils have also been reported by other authors (Hallberg & Björn-Rasmussen, 1981; Latunde-Dada, 1992; Seim, Ahn, Bodis, Luwedde, Miller, Hillier, et al., 2013).

3.4. Bioaccessibility of iron in soil-contaminated pearl millet flour

As extrinsic soil iron contamination of grains is a common occurrence, especially in small-holder settings, it is essential to understand whether it modulates the bioaccessibility of iron in grains. It is also important to determine whether the differences in iron bioaccessibility across different soil types are apparent when soil is incorporated in grains in small amounts, which is typical of soil contaminated grains.

At the end of gastric digestion, Fe_{sol} (%) of uncontaminated pearl millet flour was 25.3 ± 0.12 % (**Figure S1** – Supplementary data). In terms of the CPMs, there was a decrease in Fe_{sol} (%) as the proportion of contaminating soil increased for all soil types. For example, for Alfisol-CPM, as the level of soil contamination increased from 0.1 % to 0.5 % and 1 %, Fe_{sol} (%) decreased from 13.8 ± 0.1 % to 8.23 ± 0.5 % and 2.88 ± 0.1 %, respectively. In turn, Fe_{sol} (mg kg^{-1}) slightly increased depending on the total iron of the soil CPM. As also seen in the gastric phase, at the end of gastro-intestinal digestion, as the soil proportion increased, Fe_{sol} (%) decreased from a mean of 33.3 % for the uncontaminated flour to as low as 4 % for CPM with 1 % soil (Fig. 3). However, Fe_{sol} (mg kg^{-1}) increased from 15.7 mg kg^{-1} to about 20.4 mg kg^{-1}, an increase of up to 30 % at the maximum contamination level of 1 % soil. In the case of the Alfisol and Vertisol-CPM, a soil proportion of 0.1 % did not significantly increase the Fe_{sol} (mg kg^{-1}). There was no further increase in Fe_{sol} (mg kg^{-1}) when soil proportion was increased from 0.5 % to 1 % across all soil types.

When the soils were digested without flour, there were clear differences in Fe_{sol} (mg kg^{-1}) under both gastric and gastro-intestinal conditions. The Vertisol released less iron than the Alfisol or Oxisol under gastric conditions but this was not apparent in the CPMs. On the other hand, more iron was released by the Oxisol under gastro-intestinal conditions but when the soils were digested together with pearl millet flour, the superior iron solubility of the Oxisol was only slightly apparent at low soil proportions of 0.1 %. Clearly, when soils were digested alone, their geochemical properties strongly determined the solubility of iron. In contrast, when soils were digested together with flour at soil proportions not exceeding 1 % as in the current study, the solubility of iron largely depended on the food matrix. While the release of iron from the soil may depend on geochemical characteristics, the proportion of iron remaining in solution after each phase of digestion is likely to depend on the interaction of the released iron with iron binders in the food matrix. The marginal increase in Fe_{sol} (mg kg^{-1}) across all the soil CPMs is consistent with the low solubility of soil iron and also validates the assertion that small amounts of soil iron are soluble and potentially absorbable (Gibson, et al., 2015).

As observed for Fe_{sol} (%), Fe_{bio} (%) decreased as soil proportion increased (Table 2). In general, Fe_{bio} (%) decreased from 3.99 ± 1.1 % for uncontaminated flour to about 0.4 % for CPM with 1 % soil. With each increase in soil contamination, Fe_{bio} (%) decreased by almost 50 %. Fe_{bio} (mg kg^{-1}) showed a tendency to increase with increasing soil proportion but this was not significant across all soil types implying that extrinsic soil iron did not reduce or increase the bioaccessibility of iron in pearl millet. Although there was a significant increase in Fe_{sol} (mg kg^{-1}) after gastro-intestinal digestion, this was not sufficient to substantially increase Fe_{bio} (mg kg^{-1}) in the pearl millet matrix.

Overall, Fe_{bio} (%) for soils digested alone were always lower than when digested with flour despite a lower amount of soil being digested and higher iron concentrations of the soils compared with the pearl millet flour. This agrees with findings from Seim, et al. (2013) who reported consistently lower iron bioavailability from geophagic soils than when the soils were combined with a white bean variety at higher soil: food ratios than used in the current study (1:16). As found in our study, when the soils were combined with white beans, iron bioavailability did not exceed that of the white beans alone, although 5 out of 16 of the soils caused a decrease. In another study by Seim, Tako, Ahn, Glahn, and Young (2016), there was no impact on iron status indicators when broiler chickens were fed geophagic soils daily at ratios of 1.0 g soil per kg body weight before being exposed to food. This also indicates that extrinsic soil iron had neither a negative nor a positive effect on iron nutrition. Some earlier studies suggested that extrinsic soil iron, in particular from geophagic soils consumed at much higher soil: food ratios than used in our study, may reduce the absorption of already bioavailable minerals (Hooda, Henry, Seyoum, Armstrong, & Fowler, 2002, 2004). At the low soil: food ratios used in the current study, there was no negative effect on the bioaccessibility of iron, nor of other elements such as zinc (data not shown).

The proportion of soluble iron that was bioaccessible after gastro-intestinal digestion in the individual soils compared with the CPMs may give some insight on the exchangeability of intrinsic food iron and extrinsic soil iron. Table 3 shows the proportion of Fe_{bio} (%) relative to

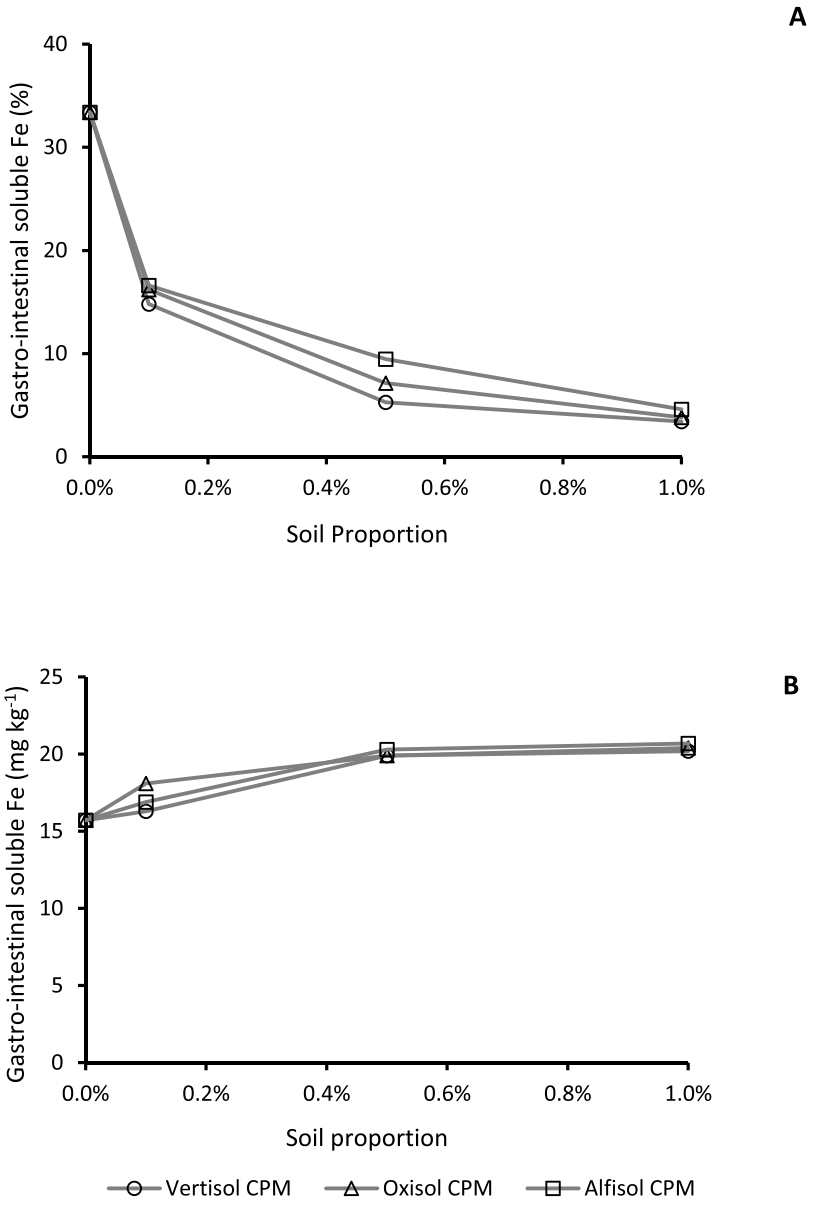

Fig. 3. Total gastro-intestinal soluble iron (%) – A and in mg kg^{-1} (B) for pearl millet flour contaminated with different types and proportions of soil. CPM – contaminated pearl millet flour.

Fe_{sol} (%) under gastro-intestinal conditions. A higher proportion of soluble iron from the Vertisol (71.5 %) was bioaccessible, followed by the Alfisol (52.1 %) and lastly the Oxisol (21.6 %). This clearly shows that the soluble iron species released by the soils are probably different, with the Vertisol releasing more dialyzable iron species. When pearl millet flour was digested without soil contamination, 12.0 % of soluble iron was bioaccessible. Pertaining to the CPMs, the proportion of bioaccessible iron in relation to soluble iron was not significantly different from that of the uncontaminated flour suggesting that iron bioaccessibility of CPMs was modulated by the pearl millet matrix rather than the soil contaminating it. In addition, the fact that the proportion of Fe_{bio} (%) in relation to Fe_{sol} (%) was consistent across all the soil types and contamination levels, shows that the small amount of soil iron that is soluble under gastro-intestinal conditions exchanges or equilibrates with the soluble intrinsic iron from the food and therefore, its bioaccessibility is the same as for the intrinsic iron.

The bioavailability of iron is lower in pearl millet than in other cereals such as maize because in addition to phytate, it also contains polyphenols which can form insoluble iron complexes at gastro-intestinal pH (Gabaza, Shumoy, Muchuweti, Vandamme, & Raes, 2018b). According to Tako, Reed, Budiman, Hart, and Glahn (2015), the bioavailability of iron from an iron-biofortified pearl millet variety was limited by the high levels of polyphenols. Bioaccessibility of extrinsic soil iron may therefore also depend on the type of contaminated grain matrix. Siyame, et al. (2013) asserted that extrinsic soil iron in maize-based diets is probably more bioavailable than from millet-based diets. Likewise, contaminant iron from milling equipment was not bioaccessible in pearl millet dishes from Burkina Faso while approximately 4 % of the extrinsic iron from a white sorghum variety was bioaccessible (Icard-Vernière, Hama, Guyot, Picq, Diawara, & Mouquet-Rivier, 2013). The bioaccessibility of extrinsic soil iron from colored varieties of grains such as tef, sorghum and finger millet is likely to be the same as that of

Table 2
Iron bioaccessibility of pearl millet flour contaminated with different types and proportions of soils.

Soil Proportion	Alfisol-CPM	Oxisol-CPM	Vertisol-CPM
	Bioaccessible iron (%)		
~0%	3.99 ± 1.1	3.99 ± 1.1	3.99 ± 1.1
0.1 %	1.91 ± 0.56	1.75 ± 0.1	1.95 ± 0.42
0.5 %	0.96 ± 0.24	0.78 ± 0.1	0.64 ± 0.1
1 %	0.41 ± 0.01	0.41 ± 0.1	0.40 ± 0.1
	Bioaccessible iron (mg kg^{-1})		
~0%	1.88 ± 0.51	1.88 ± 0.51	1.88 ± 0.51
0.1 %	1.94 ± 0.57	1.95 ± 0.12	2.15 ± 0.47
0.5 %	2.05 ± 0.51	2.17 ± 0.27	2.43 ± 0.34
1 %	1.82 ± 0.20	2.20 ± 0.28	2.37 ± 0.01

CPM – contaminated pearl millet flour. There was significant interaction between soil type and soil proportion for bioaccessible iron (%) and no significant differences across all soil types and soil proportion for bioaccessible iron (mg kg^{-1}), $p < 0.05$, $n = 3$. Bioaccessible iron refers to soluble, dialyzable iron of low molecular weight (<12.4 kDa) measured at the end point of gastro-intestinal digestion.

Table 3
Proportion of bioaccessible iron (%) in relation to total gastro-intestinal soluble iron in pearl millet flour contaminated with different types and proportions of soils and in individual soils.

Soil proportion	Alfisol-CPM	Oxisol-CPM	Vertisol-CPM
~0%	12.0 ± 3.66	12.0 ± 3.66	12.0. ± 3.66
0.1 %	11.5 ± 3.65	10.8 ± 0.33	13.2 ± 2.80
0.5 %	10.0 ± 2.02	10.8 ± 0.96	12.3 ± 2.34
1 %	8.79 ± 0.71	10.8 ± 1.42	11.8 ± 0.26
Individual soils	52.1 ± 1.50[b]	21.6 ± 2.97[a]	71.5 ± 4.61[c]

CPM – contaminated pearl millet flour. Values with different small superscript letters show significant differences across individual soils. There were no significant differences according to soil type or soil proportion for soil contaminated flours, $p < 0.05$, $n = 3$. Bioaccessible iron refers to soluble, dialyzable iron of low molecular weight (<12.4 kDa) measured at the end point of gastro-intestinal digestion.

pearl millet since these grains also contain high phytate and polyphenols (Gabaza, Shumoy, Muchuweti, Vandamme, & Raes, 2018b; Shumoy, Lauwens, Gabaza, Vandevelde, Vanhaecke, & Raes, 2017). Since the bioaccessibility of soluble soil iron is dependent on the food matrix, it is reasonable to conclude that extrinsic soil iron might contribute to iron nutrition under conditions that allow for improved bioaccessibility. For example, bioaccessibility of iron in maize contaminated with extrinsic iron from milling equipment was increased from 1.4 to 4.0 % after fermentation during the production of *mawè*, a fermented maize dish from Benin (Greffeuille, Kayodé, Icard-Vernière, Gnimadi, Rochette, & Mouquet-Rivier, 2011). Similarly, the addition of ascorbic acid can increase the bioaccessibility of extrinsic iron in contaminated foods (Derman, Bothwell, Torrance, Macphail, Bezwoda, Charlton, et al., 1982; Latunde-Dada, 1992).

It is apparent that extrinsic soil iron is not absorbed to the same extent as intrinsic food iron such that the use of recommended bioavailability estimates to calculate bioavailable iron is not appropriate for soil-contaminated grains. Our findings show that Fe_{bio} (mg kg^{-1}) of soil CPM is similar to that of uncontaminated flour. This means that when presented with soil-contaminated grains, it is important to know the intrinsic iron concentration as a starting point, then any effect of extrinsic iron on bioaccessible iron levels can be determined based on the level of soil contamination and type of food matrix or diet. The use of V as a marker for soil iron and a means of estimating the proportion of soil iron present in grains deserves further attention. The findings from this study are pertinent in the context of nutrition assessment and nutrition programming in low-income countries, specifically in identifying interventions that can be used to address the high prevalence of iron deficiency in these settings. The high level of unabsorbed iron in soil contaminated grains end up in the large intestine where it can exert adverse effects on the gut microbiota, which has been reported to regulate systemic iron homeostasis (Shumoy & Raes, 2021). Therefore, strategies aiming at increasing total iron intake, such as fortification and biofortification, may need to be carefully evaluated in these contexts.

4. Conclusions

Pearl millet flour contaminated with small amounts of soil (up to 1 % w/w) contained high levels of extrinsic soil iron contributing up to 92 % of the total iron. Bioaccessibility of extrinsic soil iron was dependent on the level of soil contamination, which influenced how much soluble iron was potentially available for absorption, and most importantly on the grain matrix, which appeared to control the bioaccessibility of the soluble iron. Soil contamination levels of up to 1 % (w/w) did not cause any changes in iron bioaccessibility as the pool of soluble iron was not sufficiently increased. Due to the reliability of V as an indicator of the presence of extrinsic soil iron in edible portions of crops, it can potentially be used to estimate the proportions of extrinsic soil iron in soil-contaminated grains provided an estimate of total Fe: V ratios in soil is available. This will be important for the interpretation of iron intake data of plant foods from regions where soil contamination is common and will assist in the implementation of sound nutrition programs that consider all sources of iron available in different settings.

CRediT authorship contribution statement

Molly Muleya: Methodology, Investigation, Formal analysis, Funding acquisition, Writing – original draft. **Scott D. Young:** Conceptualization, Methodology, Writing – review & editing, Funding acquisition. **Martin R. Broadley:** Writing – review & editing, Funding acquisition. **Edward J.M. Joy:** Writing – review & editing, Funding acquisition. **Prosper Chopera:** Supervision, Funding acquisition. **Elizabeth H. Bailey:** Conceptualization, Methodology, Writing – review & editing, Funding acquisition, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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