ORIGINAL ARTICLE

Effects of Polyphenols and Phytic Acid on Iron Bioavailability from Tef as Measured by an Extrinsic Radio iron (^{59}Fe) Tag Method

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ABSTRACT

Background: *Iron deficiency is the most common micronutrient deficiency. The major cause is an impaired absorption of non-heme iron as a result of interference of phytate and polyphenols. The aim of this study was to determine the effects of phytic acid and polyphenols on iron bioavailability in lactic fermented and unfermented cereal tef grains.*

Methods: *Iron bioavailability was measured using the extrinsic radioiron tag method. Radioiron (^{59}Fe) from the pepsin-pancreatin–bile salts digestion mixture which diffused across a 8000-to 10,000 molecular weight cut-off semipermeable membrane was used as an indicator of bioavailable iron.*

Results: *Brown tef contains high amount of polyphenols, 18-fold as much as in white tef flour. Preparation of white and brown cereal tef in to unfermented meals did not have a significant effect on iron bioavailability. Lactic fermentation of white tef increased iron bioavailability from about 4% to 45%. The increase in bioavailable iron was strongly related to the enzymatic degradation of phytic acid ($p<0.05$). The reduction of phytic acid and polyphenols was about 60 and 63%, respectively, in brown tef injera. Brown tef showed a minor increase in bioavailable iron after fermentation, ascribed to the inhibitory effect of polyphenols (both on iron and on enzymatic hydrolysis of phytic acid). Cereal tef–based unfermented meals were fond to contain high amount of total iron but of low bioavailability.*

Conclusion: *Estimation of the amount of bioavailable iron confirmed inadequate iron nutrition from such meals. Dietary modifications by traditional processing techniques such as lactic fermentation of cereal tef is, therefore, likely to improve iron nutrition significantly.*

Key words: Iron Bioavailability, Phytic Acid, Polyphenols, Tef.

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INTRODUCTION

Iron deficiency is the most common micronutrient deficiency in the world (1). Although the magnitude and distribution of anaemia in Ethiopia is not clearly known, a study on children 6-72 months old in a suburban area in north-western Ethiopia showed a prevalence rate of 47% (2). The major cause of iron deficiency is the impaired absorption of non-heme iron. During digestion, the non-heme iron in cereals enters a common pool where its absorption is influenced by various enhancers and inhibitors (3). Probably the most important inhibitor of non-heme iron absorption in cereals and legumes is phytate. Phytate is the principal storage form of phosphorus in plant foods, particularly cereals and legumes, and there is evidence that it may interfere with iron absorption, presumably by forming an insoluble iron-phytate complex (4). Other groups of substances in food considered to influence iron bioavailability negatively are phenolic compounds. Polyphenol compounds, particularly those occurring in the form of non-hydrolysable tannin, are widely present in cereals. The phenolic compounds are released from the food during digestion and can complex with iron in the gastrointestinal lumen making it less available for absorption (5). In this context, the presence of either of the inhibitors of non-heme iron absorption in foods such as cereals and legumes have been shown to decrease the bioavailability of dietary iron markedly (6).

Most Ethiopian staple diets are derived from cereal like t.f (*Eragrostis tef*), maize, sorghum, millet, barely and tubers like ensete (*Ensete ventricosum*). Among the cereals, the production and utilisation of tef is second only to maize in the country (7). In common with other cereals, tef is known to contain inhibitors of non-heme iron absorption such as phytate and polyphenol compounds (8). Although the iron content of many of the traditional Ethiopian diets including tef is very high, anaemia is prevalent among pre-school and school-age children, which may be attributed to poor bioavailability of iron from the diets (9). For more effective utilisation of the cereal tef, it is desirable to lower the concentration of the inhibitors of iron bioavailability. One possible approach of improving iron bioavailability is to reduce the phytic acid polyphenols content of foods by applying different food processing methods. Previous studies showed that the natural lactic fermentation lowered the concentrations of phytate and polyphenols and improved the availability of minerals from pearl millet flour (10).

In the present study, the effect of different food preparation methods on lowering the contents of phytic acid and polyphenolic compounds, and the effects of such reduction on iron bioavailability from tef diets using the extrinsic tag radioiron technique was reported. The underlying assumption of the extrinsic method is that if a small amount of a radioisotope of iron is added to the test meal as a soluble salt, it exchanges completely with the unlabelled non-heme iron pool in the meal. The per cent radioiron dialysed is calculated from the radioctivity present in the dialysate (11). The present method differs from the previous in that the pH adjustment from gastric to intestinal levels is gradual and reproducible, and only low molecular weight, soluble iron is used in the estimation of available iron (9,11,12).

MATERIALS AND METHODS

The study was conducted during 1996, in the former Ethiopian Nutrition Institute, Addis Ababa.

Reagents and materials: Pepsin solution was prepared from 16g of pepsin powder (porcine stomach mucosa, Sigma Chemical Co.) suspended in 20ml of 0.1M HCl and brought to 100ml with 0.1M HCl. Four grams of pancreatin (porcine pancreas,

Sigma Chemical Co.) and bile extract (25g, porcine, Sigma Chemical Co.) were dispersed in 0.1M $NaHCO_3$ and the mixture was brought to 1L with 0.1M $NaHCO_3$. Protein precipitant solution was prepared from 100g trichloroacetic acid, 100g hydroxylamine HCl and 100ml of concentrated HCl and dissolved in double distilled water and brought to a volume of 1L. Chromogen solution was prepared from 250mg bathophenanthroline sulfonate dissolved in 2M sodium acetate and brought to 1L with 2M sodium acetate.

^{59}Fe Radioactive: ^{59}Fe as ferric chloride in 0.1M HCl (0.01mg Fe/ml and 100 μCi/ml. Amersham International. UK) and

Dialysis tubing: MW cut-off, approximately 10,000 (Spectra /Por7. Spectrum Med. Indus., Houston. USA) were used.

Preparation and simulated digestion of test foods: White and brown tef (*Eragrostis tef*) grain varieties were procured from the local market in Addis Ababa. The grains were cleaned of dust, broken seeds and other foreign material and then ground to a fine powder with a Cyclotec sample mill (Tecator, Hogans, Sweden). Tef flour was prepared into *kitta* (unleavened flat bread), porridge, *atmit* (thin gruel) and *injera* as previously described (8). *Injera* was baked after 96 hr of fermentation. Samples of fermenting tef dough were pooled at 0, 24, 48, 72, 96 hr of fermentation for monitoring biochemical changes due to fermentation. Aliquots of the different meals were freeze-dried and the finely ground to a powder in a porcelain mortar.

Ten grams of the test samples were homogenised in 20ml double distilled water. The pH of the homogenate was adjusted to 2 with 6M HCl, spiked with $^{59}FeCl_3$ (1ml) and the final weight adjusted to 100g with 0.01M HCl. Pepsin suspension (3ml) was added to the homogenised food sample and incubated in a shaking water bath for 2 hr at 37°C. Duplicate 20g aliquots of the pepsin digest were transferred to conical flasks. Segments of dialysis tubing containing 25 ml double distilled water and an amount of $NaHCO_3$ equivalent to the titretable acidity (11) were put into each flask, and the flasks were incubated in a shaking water bath for 30 minutes at 37°C. Pancreatin-bile extract mixture (5ml) was then added, and the incubation continued for another 2hr. After pancreatic digestion, the dialysis bags were removed, rinsed with double distilled water, and the contents of conical flasks (weight of retentate) and dialysis bags (volume of diffusate) weighed. The contents of the dialysis sac and conical flasks were analyzed for bathophenanthroline reactive iron and ^{59} Fe activity.

Analytical methods:

Bathophenathroline reactive iron was measured in the dialysates immediately after pancreatin incubation using the method of Miller *et al.* (11). The total amount of iron in the dialysis bag (bathophenanthroline reactive) divided by the total amount initially present in the aliquot is defined as % dialysable iron (% DI) assayed colorimetrically. Radioactivity (as ^{59}Fe activity) in the dialysate and retentate samples was determined by counting aliquots in a Gamma Counter (Oak Field Instruments, Eyenesham, UK) following the method of Hazell and Johnson (13) and expressed as percentages. Both bathophenanthroline reactive iron (colorimetric) and racdioactivity (^{59}Fe) measurements were done to provide a double check on the percent dialiyzable iron and to determine the exchangeability of intrinsic and native iron. Protein was measured according to AOAC method (14). Total iron, and zinc after wet-acid digestion with nitric and perchloric acid mixture (HNO_3: $HClO_4$, 5:1v/v), were determined by atomic absorption spectroscopy (Varian, model Spectra A-20Q+, Mulgrave, Victoria, Australia). Phosphorous was estimated by

Fiske and Subbarrow method (15). Phytic acid was extracted with 0.2M HCl by continuous shaking for 3hr with a mechanical shaker and estimated colorimetrically (16). Total polyphenols in food samples were measured using the Vanillin Assay method with catechin as a standard (17).

Data were analysed by analysis of variance (ANOVA) to determine differences among treatments. Differences were considered statistically significant at $p<0.05$ (95% confidence limit).

RESULTS

The protein and calcium concentrations of both tef varieties were, however, similar. The iron content (µmol/100g) was 268 for white tef and 321 for brown tef (Table 1). Tef flour meals provided on average 352 kcal/100g meal. Expressed per unit of energy, the bioavailable iron density for white and brown tef corresponded to 0.187 and 0.183mg/100kcal, respectively. The phytic acid content of 100 g of the raw white and brown tef grains was 1.02 and 1.17µmol, respectively. Polyphenols were found in largest amounts ranging from 1575 mg/100g in brown tef to 80 mg/100g in white tef. Similarly, the iron: phytic acid molar ratio varied significantly, ($p<0.05$) between the two tef varieties.

The heat treatment during the preparation of *kitta* (unleavened flat bread), porridge or gruel had minor effect on the phytic acid content. Only small differences were observed between the raw cereals and prepared foods in phytic acid content. In contrast, polyphenols content was reduced significantly when flour of both tef varieties were used to prepare *kitta* or porridge. However, the higher reduction in polyphenols content (32 and 21%, respectively) was observed during the preparation of gruel from white and brown tef flour. As expected, the dialysability of iron of the two tef flour varieties as determined by radioactivity was, 4.17 and 3.63%, respectively. The difference between the two tef varieties was highly significant ($p<0.05$). Preparation of white and brown tef flour into porridge, gruel or *kitta* did not have a significant effect on iron dialysability (Table 1).

Table 1. Proximate composition of tef diets (per 100g). Ethiopian Nutrition Institute, Addis Ababa, 1996.

	White tef				Brown tef			
Description	Flour	*Kitta*	Porridge	Gruel	Flour	*Kitta*	Porridge	gruel
Protein, g	11.9	11.8	11.7	11.6	1.6	12.7	12.3	12.2
Calcium, g	76	74	75	73	62	68	60	65
Iron, µmol	267.9	285.7	294.6	285.7	321.4	330.4	339.3	339.3
Phosphorous, g	181	181	183	181	203	201	205	207
Polyphenols, g	0.08	0.06	0.06	0.05	1.58	1.30	1.24	1.07
Phytic acid, mM	1.02	1.02	1.01	1.01	1.17	1.16	1.67	1.55
Iron: phytate								
Molar ratio	0.263	0.280	0.292	0.283	0.275	0.285	0.293	0.295
Dailysable iron, %								
Colorimetric	3.84	3.93	3.86	3.91	3.26	3.34	3.41	3.55
Radioactivity	4.17	4.38	4.19	4.38	3.63	3.58	3.67	3.79
Energy, Kcal/100g	352.8	351.3	353.7	351.4	355.1	350.8	351.7	350.2
Bioavailable Fe density, mg/100kcal	0.187	0.179	0.175	0.184	0.183	0.181	0.186	0.191
BioavailableFe, mg	0.62	0.60	0.60	0.63	0.65	0.63	0.65	0.67

As a result of natural lactic fermentation, a significant reduction in phytic acid contents was observed in white tef flour (Fig.1).

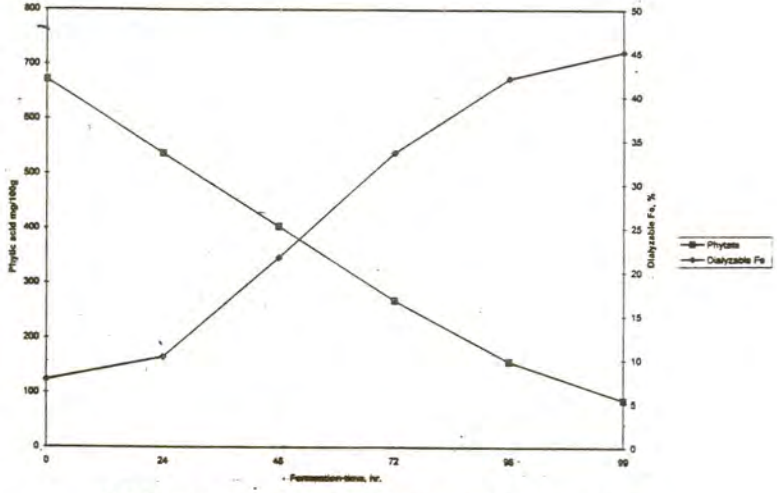

Fig. 1. Relationship between dialyzable Fe and amount of phytic acid in lactic fermented white tef

Consequently, the fermented dough had the lowest phytic acid content about 157mg/100 g (dry wt basis) in white tef at 96 hr under the fermentation conditions. Degradation of phytic acid after fermentation of the high-tannin brown tef flour and baking in to *injera* was about 60% (Fig. 2)

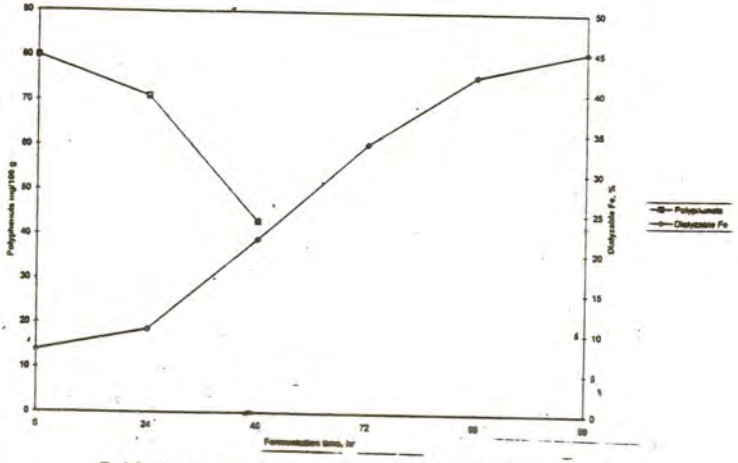

Fig. 2. Relationship between dialyzable Fe and amount of polyphenols in lactic fermented white tef

as compared to 87% reduction in low-tannin white tef flour. Lactic fermentation also decreased significantly ($p<0.05$) the concentration of polyphenols. Fermentation of white tef flour for 48 hr eliminated completely the content of polyphenols (Fig.3).

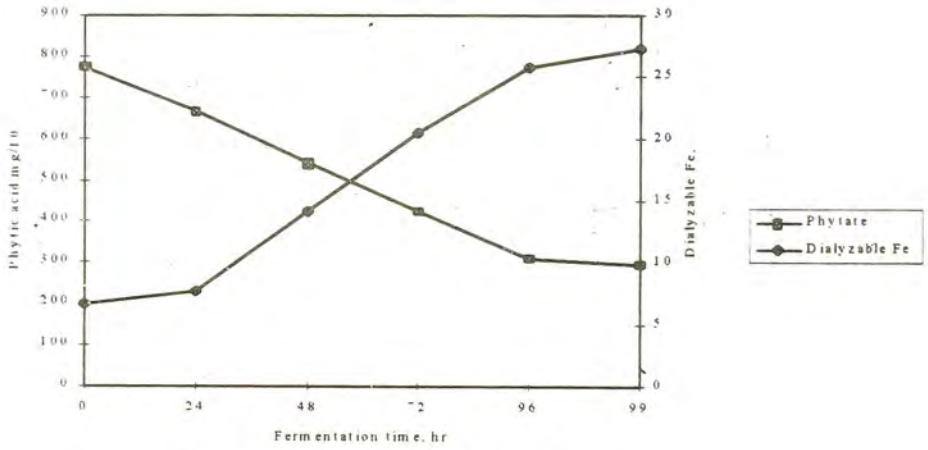

Fig. 3. Relationship betwen dialyzable Feand amount of phytic acid in luctic fermented browen tef

In brown tef. polyphenols content decreased from 45 % after 4 days of fermentation to 51% after baked to *injera* (Fig.4).

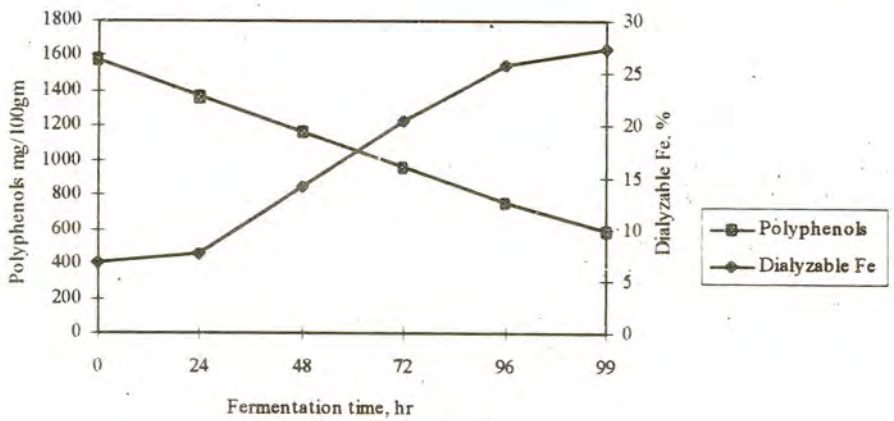

Fig. 4. Relationship between dialyzable Fe and amount of polyphenols in luctic fermented browen teff

Lactic fermentation of white tef flour samples resulted in a significant increase in dialyzable iron from about 4.17 to 45.2% as estimated by radioactivity (Fig.1). In contrast, the higher tannin brown tef flour resulted in lesser changes in dialyzable iron in comparison with the low-tannin white tef counterpart (Fig.2). In brown tef, iron dialysability increased by about four-fold after fermentation as determined by radioactivity. Analysis also indicated significant negative correlation between iron dialysability and phytate and polyphenols content in low tannin and high tannin tef varieties. Correlation coefficients for dialysable iron, phytic acid and polyphenols for fermented low-tannin white tef cereal by radioactivity was $r=-0.9608$ ($p<0.05$) and $r=-0.9711$ ($p<0.05$) respectively. For fermented brown tef, correlation coefficients for dialysable iron, phytic acid and polyphenols were $r=-0.9858$ ($p<0.05$) and $r=-0.9842$ ($p<0.05$) respectively This indicates that as the amount of the phytic acid and polyphenols decreases in fermenting tef, the estimated iron availability increased.

DISCUSSION

The total iron content in the collected tef samples was high compared to similar cereals like millet (18). Components of the low-tannin and high-tannin tef samples were not significantly different except for their phytic acid and polyphenols concentrations. The concentration of polyphenols in white tef varieties is similar to values reported for maize, rice and low-tannin sorghum (19, 20). Brown tef contains higher amount of polyphenols, 18-fold as much as in white tef flour and similar to pearl millet (20). This high amount of polyphenols in brown tef could be due to the presence of a pigmented layer under the seed coat as observed previously (21).

Cooking obviously deactivated the phytase enzyme in the flour, which resulted in significantly less degradation of phytic acid. Such treatments however, had minor effect on phytic acid concentration of the tef diets in agreement with previous observations (9). In contrast, the heat treatment during the preparation of unfermented tef meals reduction significantly ($p<0.05$) the concentration of polyphenols in these diets. Considering that meals were similar in composition and that the overall effect of the constituents had to be the same for all meals considered, the reduction in polyphenols, could be attributed to the effect of processing on this antinutrient. These losses were statistically significant ($p<0.05$). Our results are in agreements with studies on sorghum food, *ugali* (22). The apparent decrease in polyphenols during the preparation of *kitta*, porridge or gruel is most likely not due to an actual decrease in polyphenols but to a change in their solubility or chemical reactivity. It is known that during heat treatment some polyphenols may bind with proteins, the protein-bound polyphenols are normally not detectable by the vanillin method thus leading to a lower estimate of polyphenols in heat-treated samples (22).

The cooking and baking processes have shown not to increase non-heme iron availability in these unfermented tef diets. Considering the corresponding values of native iron dialysablity in low- and high-tannin tef flour of 4.17 and 3.63%, respectively, as estimated by radioactivity, it turns out that after heat treatments, native iron dialysability did not increase significantly ($p>0.05$).

Both colorimetric and radioactivity methods of determining iron dialysability in unfermented tef meals indicated the same trend (Table 1). Comparison of values for intrinsic (colorimetric) and

extrinsic (radioactive) iron measurements showed good agreement in most cases. indicating complete exchange of iron in the non-heme iron pool. However. radioactive values were slightly higher than colorimetric values. Apparently. for added inorganic iron to completely exchange with the non-heme iron pool it must be soluble under gastric conditions. The higher values for the extrinsic tag in the present study may be explained by noting that the radio-iron was added as $FeCl_3$ to meals at pH 2. Under these conditions. the tag would be expected to mix with non-heme Fe pool but not with the insoluble portion of the contaminant iron. Therefore. in cases where the contaminant iron is insoluble. the extrinsic tag reflects the availability of iron native from the food but not the insoluble contaminant iron.

The low- and high-tannin tef meals contained iron ranging between 268µmol to 339µmol. respectively. resulting in a molar ratio of iron to phytic acid of 0.263 to 0.295. However. there was theoretically enough phytic acid to bind all the iron because 1mole phytic acid can bind up to 6 mole ferrous iron. assuming that other metal ions or amino acid side chains had not occupied the phosphate biding sites. In an earlier study. phytic acid was shown to be a potent inhibitor of iron bioavailability until the phytic acid was reduced to <15 µmol in a 280g meal resulting in a molar ratio of iron to phytate >7:1(23). The high amount of phytic acid in the unfermented tef meals in the present study (molar ratio of iron to phytic acid of >0.263:1) would therefore be expected to inhibit iron bioavilibility.

The estimated energy requirement of preschool children is about1600kcal/day (24). With daily energy requirements which are provided by the unfermented tef diets. the total daily iron intake amounted to 68-81mg/day. Considering ≈3.6-4.1% bioavailable iron for absorption on the basis of our results from the *in vitro* analysis (Table 1) and bioavailable iron density ranging from 0.17 to 0.18 mg/100kcal. the absorbed iron corresponded to 0.12 and 0.25 mg/d. respectively. Although the iron content of these meals is well above the recommended dietary allowance (24) for preschool children (≈29-50mg/d). however. the meals resulted in absorbed iron values significantly below the required amount (≈1mg/d) of absorbed iron for a preschool child. A large part of the iron in the collected cereal tef samples thus. seemed to be contamination iron which was probably not available for absorption (i.e.. it is not soluble) and explains the extremely low percentage of bioavailable iron found in the cereal tef samples. In Ethiopia. a significant proportion of the high iron intake from the cereal tef has been found to be accounted for contamination with soil during the threshing under the hooves of cattle (25). These results show the need for dietary modifications to improve iron availability by traditional processing techniques such as lactic fermentation of cereals.

In previous studies. the pH of the unfermented cereal tef samples was about 6.8 and reached a pH of 4.0-3.8 after complete fermentation. thus passing through the interval of 5.0-4.5. believed to be optimal for cereal phytases (9, 26). In the fermented low-tannin white tef flour. there was about eight-fold decrease of phytic acid. Only in the case of brown tef (high tannin) the decrease of phytic acid was lower (about 2.6-fold). In spite of long fermentation time (96 hours) and acidity favorable for phytase activity this high amount of phytate could not be destroyed as it was the case with white tef flour. The comparison of the phytate content in the dough after fermentation and in *injera* indicates that some destruction of phytate takes place during the baking process.

when the temperature is still below the inactivating point of phytase.

Lactic fermentation of low-tannin cereal tef could strongly increase the availability of iron as estimated *in vitro*. The increase is mainly as a result of a corresponding degradation of phytate of the cereal by activating endogenous phytase. With the lactic fermentation process, it was possible to reduce the amount of phytic acid to < 0.13μmol/100g in white tef and 0.44μmol/100g in brown tef. The amount of soluble iron was then 24% in brown tef in comparison with about 45% in the low-tannin white tef. The results also indicated that the amount of phytic acid must be degraded >50% in the fermentation process before any significant effect is observed on iron dialysability. At levels around 300μmol/100g, the Fe dialysability in white tef dough was four times as high as in unfermented dough and, below 200μmol/100g, the iron solubility increased further. It therefore seems that the fermentation process *perse* had a promotive effect on iron dialysability, probably through the formation of organic acids as previously suggested (27).

Although lactic fermentation of high-tannin sorghum grains has been reported to eliminate most assayable polyphenols, it was less affected by fermentation in brown tef (10). However, the observed reduction in tannin content in fermented cereal tef flour has been attributed to the formation of an insoluble tannin-protein complex and not to the loss or degradation of tannin *per se*. Still, these complexes may have an inhibitory effect on iron dialysability.

The iron dialysability in the high-tannin cereal tef was less affected by fermentation compared to the low-tannin cereal tef. Brown tef is more resistant to phytate degradation, which could depend on the inhibitory effect of polyphenols on phytase activity. Polyphenols have been demonstrated to have an inhibitory effect on other seed enzymes (28). Even fermentation of the brown tef flour for 96 hr had less effect on phytate degradation. Therefore, the low dialysability of iron in high tannin brown tef is mainly due to the high phytate and the polyphenols concentrations. Polyphenols have been shown to have a marked inhibitory effect on non-heme iron bioavailability since a negative correlation between the bioavailability of iron from different food and their polyphenol contents was shown *in vitro* previously (27). Polyphenols in plant foods bind non-heme iron to form insoluble iron-polyphenol complexes that are poorly absorbed (27). The inhibitory effect is probably due to the polymerization of polyphenols with iron and to the subsequent formation of insoluble complexes.

Phytic acid and polyphenols have been recognized as potent inhibitors of iron bioavailability (6). Ionic iron forms complexes dietary constituents such as polyphenols and phytic acid, from which the iron cannot be removed for uptake into the gastrointestinal mucosal cells. Thus, traditional household method such as fermentation was demonstrated to significantly reduce the content of phytate in low-tannin cereal tef and led to the strongest increase in iron dialysability. Such fermented foods might contribute a significant portion of iron requirements to an individual. In Ethiopia, where iron fortification is not a possible option, this type of food may have a potential to improve the iron status for the population. On the other hand, lactic-fermented high-tannin brown tef will contribute less iron.

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