Quantification of folate in the main steps of traditional processing of tef *injera*, a cereal based fermented staple food

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Highlights

Tef flour had an average folate content of 59 µg/100 g of dry matter content.

Fermentation increased batter folate content up to 148% compared with that of flour.

Thermal treatment (baking) always reduced folate content.

Abstract

Injera is an Ethiopian fermented flatbread preferably made from whole grain cereal (tef). Tef it is increasingly used to produce gluten-free pasta and bread, but the folate content of teff and products made from it remains unknown. Given that folate deficiencies lead to several health disorders, the aim of this study was to quantify folate in each of the three main steps of traditional processing of tef injera. Total folate contents of tef flour, fermented batter and injera were determined through microbiological assays using Lactobacillus rhamnosus (ATCC 7469). Folate content of tef flour was 8.7 µg/100 g of dry matter content, which is in the same range as the richest cereals like oats. The increase in folate content due to fermentation was highly variable (60-148%). Cooking always led to folate losses, with a maximum of 52.8%. Altogether, injera processing increased folate retention between 38.0 and 121.8%. Folate content of injera was 14.3 µg/100 g on fresh weight-basis. Tef Injera can contribute up to 10% of the recommended nutrient intake of folate for children aged 1 -3 and women of reproductive age. Although the folate content of tef is already high, future studies should focus on optimizing the folate content of injera.

Key words: Fermentation, Folate, *Injera*, Tef.

1. Introduction

Every year, inadequate folate intake predisposes women to birth complications like neural tube defects (Moore *et al*., 2003). Inadequate maternal folate status has also been associated with low infant birth weight, preterm delivery and fetal growth retardation (Scholl and Johnson, 2000). Megaloblastic anemia and elevated blood concentrations of homocysteine have also been linked to folate deficiencies (Bailey and Gregory, 2006) and (Ho et al., 2011). Although animal source foods (liver, kidney, chicken giblets, egg yolk), and vegetable source foods (pulses, and dark-green leafy vegetables) are rich sources of folate, regular consumption of some of these foods is limited in low and middle income countries (LMIC). Instead, diets are predominantly based on cereal and pulses (Lee et al., 2013). Despite cereal and pulses non-negligible folate content, combined with the limited availability and access to folic acid fortified foods, and the low compliance/adherence to folic acid supplementation during pregnancy, a significant proportion of the population in LMIC is at risk of folate deficiency and its adverse effects (McLean et al., 2008; Haidar, 2010).

In Africa, the preparation of many cereal-based staple foods includes a fermentation step (Humblot and Guyot, 2008). *Injera* is a staple food that is widely consumed in Ethiopia (Baye et al., 2013), and is often prepared from tef (*Eragrostis tef*), an ancient cereal crop indigenous to Ethiopia (Yetneberk *et al*., 2004). Tef is becoming popular worldwide thanks to its nutritional profile (gluten-free, high dietary fibre content, high iron content etc.). It is increasingly used in health food to produce gluten-free pasta and bread (Zhu, 2018). But to the best of our knowledge, its folate content has never been estimated. Fermentation and thermal treatment - baking - the two main processes used to prepare injera may have an effect on the folate content of *injera*. For example, baking has been found to cause up to 25% folate losses in wheat and rye sour batter breads (Kariluoto *et al*., 2004 and Gujska and Majewska, 2005). Unlike heat treatment, household fermentation can either increase or decrease the initial folate content of the flour (Saubade *et al*., 2017a). During fermentation of food products, yeasts and some bacteria have been found to increase the folate content of the original raw material. But the folate originally present in the cereal or that resulting from microbial synthesis can also be consumed by other bacteria (LeBlanc et al., 2007; Moslehi-Jenabian *et al*., 2010; Holzapfel, 2002 Keagy *et al*., 1975 and Kariluoto *et al*., 2004). Thus, the final folate content of the fermented food is a balance between production and consumption by microorganisms.

Information on folates in tef *injera* is rare, and the effect of fermentation and baking in households is largely unknown. This is unfortunate because daily intake and hence the risk of folate deficiency cannot be estimated without precise knowledge of the folate content of widely consumed foods like *injera*. A better understanding of the dynamics of folate retention after tef fermentation and of the extent of folate losses caused by thermal treatment when *injera* is prepared in the household should help optimize the preparation of *injera* by increasing the production and retention of folate.

The aim of this study was to quantify folate in the main steps of traditional processing of tef *injera*. The folate content of tef flour was quantified to complete the food composition table in Ethiopia, which currently does not include folate. The traditional preparation of tef *injera* in urban households was characterized in detail and the effect of fermentation and thermal treatment on folate content evaluated. Finally, the contribution of tef *injera* to meeting folate requirements was estimated.

2. Material and methods

2.1. Chemicals

All the chemicals used in this study were purchased from Sigma-Aldrich Chemie GmbH, Switzerland.

2.2. Sampling of tef flour, batter and *injera*

Detailed observations of the traditional *injera* making process were made in 20 selected households (where *injera* is traditionally prepared) in Addis Ababa. Since there are 10 sub-cities in Addis Ababa, two households were selected for observation in each sub-city, which resulted the flow diagram presented in Fig. 1. Briefly, the process begins by milling whole tef grain into flour, mixing 4–5 kg of tef flour and with 5–6 L of tap water. Fermentation is started by inoculation by backslopping using 1 L of leftover (called *ersho*) from a previous successful spontaneous traditional fermentation. The mixture is then allowed to ferment for an average of 3–4 days at room temperature (called 1st stage fermentation). After fermentation, the liquid present on top of the batter (the supernatant) is discarded and replaced with the same volume of fresh tap water. Then, a portion equivalent to 1/11th of the fermented batter (1 L) is mixed with 3 L of tap water, boiled for 10 min and allowed to cool to~45 °C. The resulting product is called *absit*, it serves as a batter binder and is added back to the fermented batter, which is allowed to ferment for an additional 2-3 h (2nd stage fermentation). Finally, 450 mL of the fermented rather liquid batter is poured onto a hot clay griddle, covered, and baked for 1–2 min. The resulting flat bread is called *injera*.

Samples of tef flour (n=60), batter (n=60), and *injera* (n=60) were collected from the 20 households on three separate occasions (referred to as sampling occasion 1, 2 and 3) at intervals of approximately one month, giving a total of 180 samples for the experiment. The samples were collected from each household using a simple random sampling technique. The samples were collected aseptically and placed in sterile plastic bottles covered with aluminum foil to protect them from direct light and transported back to the laboratory in an ice box. The dry matter (DM) content of all three types of samples (flour, batter and *injera*) and the pH of the batter samples were determined immediately. DM content was determined by drying the samples at 105 °C in open dishes to constant weight. The remaining samples were stored at −20 °C for further folate analysis. All the samples of tef flour collected from the 20 households were mixtures of red and white teff varieties.

2.3. Determination of pH

pH was measured using a fresh aliquot of the batter immediately after diluting with deionized water (1:1, v/v) and compared with data in the literature.

2.4. Effect of processing

The effect of traditional household, i.e. fermentation, thermal treatment (baking), and of *injera* processing as a whole on the total folate content of tef *injera* were evaluated and are expressed as percentage retention.

2.5. Contribution of tef *injer*a to the recommended nutrient intake (RNI) of folate

Based on the data gathered in the Ethiopian National Food Consumption survey conducted in 2013 (EPHI, 2013), we estimated the contribution of tef *injera* to the recommended nutrient intake (RNI) of folate for children aged 1-3, and women of reproductive age. These population groups were selected because they are at an increased risk for folate deficiency.

2.6. Folate analysis

The total folate contents of tef flour, batter and *injera* were determined using the reference microbiological method, after tri-enzyme extraction (Kariluoto, 2004). All analytical procedures were carried out under yellow or subdued light. Alternatively, aluminum foil was used to cover the samples and calibrants. Sample extracts were kept under nitrogen atmosphere.

2.6.1. Extraction and tri-enzyme treatment

For the analysis of total folate using the microbiological assay, samples weighing 1 to 1.5 g, depending on the estimated folate content in each sample, were extracted in triplicate (Kariluoto and Piironen, 2009). Extraction was followed by tri-enzyme treatment (α-amylase, hog kidney conjugase and protease) with some modifications: 200 µL of α-amylase was added in the extracted samples and allowed to settle for 30 min before the pH was adjusted to 4.9 using HCl. This pretreatment facilitated sample homogenization and pH adjustment. Hog kidney conjugase (1 mL) and 800 µL of α-amylase were then added to the samples. Hog kidney conjugase was prepared from fresh hog kidneys according to Gregory *et al*. (1984). Its activity was tested according to Vahteristo *et al*. (1996). After the enzymes were inactivated in a boiling water bath and cooled on ice, the samples were brought to exactly 25 mL with 0.5% sodium ascorbate and directly analyzed with the microbiological assay.

2.6.2. Microbiological assay

Ninety-six-well microtiter plates were used for the assay and the total folate content was determined based on the growth of folate-dependent strain *Lactobacillus rhamnosus* ATCC 7469 as the test organism and 5-CHO-H_4 folate as the calibrant. Two dilutions were made from each sample extract using 0.5% sodium ascorbate solution and eight levels of calibrant (0–80 pg/well) in each plate. The plates were incubated for 18 h at 35 ºC and turbidity was measured with a microplate reader (Multiskan EX; Labsystems, Helsinki, Finland) at 595 nm. The performance of the method was confirmed by analyzing a blank sample. Certified CRM 121 reference material was analyzed as a quality control in each set of samples. A control chart previously constructed by Kariluoto *et al*. (2004) was used for the folate content of the reference material (certified value 500-700 ng/g DM), and a coefficient of variation (CV) $< 10\%$ among analytical replicates was considered acceptable.

2.7. Statistical analysis

Statistical analysis of folate was computed using SPSS version 20. The folate analyses were carried out in triplicate and the average values and standard deviations were calculated. Differences between means of folate values in tef flour, batter and *injera* were evaluated using one way-analysis of variance (ANOVA) and Tukey's post hoc test. Differences in means were considered statistically significant with a p-value ≤ 0.05.

3. Results

3.1. pH of the batter

Measured at 25 ºC, the pH of the batter samples ranged from 2.8 to 5.6 with an average of 3.54 ± 0.4.

3.2. Folate content of tef flour, batter and *injera*

Microbiologically determined folate contents ranged from 31 to 89 µg/100 g DM in tef flour (n=60), 26 to 82 µg/100 g DM in batter (n=60) and 21 to 61 µg/100 g DM in *injera* (n=60) (Figure 2).

The average total folate content of *injera* (39 ± 8 µg/100 g DM) was significantly lower than that of the batter (52 ± 12 µg/100 g DM) and of the flour (59 ± 11 µg/100 g DM; $P<0.05$). High variability was observed among the samples produced by the same household (data not shown). To see if the folate content of samples produced by different households differed, we also compared the households by pairs. After fermentation, only the samples taken from one household had higher folate contents than the other 19 households, and it was the same household where the folate content of the original tef flour was also higher (data not shown).

3.3 Effect of fermentation

Percentage retention of folate after fermentation was calculated by comparing the amount of folate content in tef flour and in the fermented batter on dry matter basis (Figure 3A). A folate retention value > 100% showed that fermentation increased folate content whereas retention values < 100% indicated folate consumption/losses. Folate retention after fermentation of tef batter ranged from 59 to 148% (Figure 3A).

Folate retention of more than 100 % was observed in 12/60 cases. In 9/60 cases, retention of about 100% was recorded, while in the remaining cases retention was less than 100%.

3.4 Effect of thermal treatment

In all the households, on all three sampling occasions, the folate retention value due to thermal treatment was less than 100%, it ranged from 47 to 96% with average folate retention of 67.7% (Figure 3B).

3.5 Effect of *injera* processing

The folate retention value due to *injera* processing as a whole ranged from 38 to 97%, showing that the folate content in the final product (*injera*) was almost always lower than the folate content of the original ingredient (tef flour) (Figure 3C). One sample (out of the 60) showed folate retention > 100% (122%, household 5, sampling occasion 2).

3.6 Contribution of tef *injera* to folate requirements (RNI).

Microbiologically determined folate contents of tef *injera* per fresh weight ranged from 7.1 to 20.1 µg/100 g, with an average folate content of 14.3 µg/100 g. The contribution of *injera* to the RNI of folate in the two population groups (children aged from one to three and women of reproductive age) was estimated and the results are listed in Table 1. *Injera* consumption ranged between 23 and 66 g/day for children and between 131-202 g/day for women (EPHI, 2013). Using this consumption data, we calculated that folate intake from tef *injera* contributes a maximum of 10% of the RNI for both the children and the women of reproductive age.

4. Discussion

To our knowledge, this is the first study to evaluate the folate content of tef flour, fermented tef batter, and tef *injera* to determine the fate of folate in the preparation of this cereal-based fermented

Ethiopian food. Our study shows that the whole grain cereal (tef) flour is a relatively good source of folate. The effect of the fermentation step in *injera* processing was highly variable but increased folate content in some cases, whereas baking invariably led to losses. Using available levels of *injera* consumption (EPHI, 2013), we calculated that tef *Injera* contributes up to 10% of the daily folate requirements of vulnerable groups like young children and women of reproductive age.

The average total folate content of tef flour (52.1 µg/100 g DM) was higher than that reported for other cereals including oats, rice, whole wheat flour and maize (30–40 µg/100 g) but was slightly lower than values reported for sorghum flour (77.0 µg/100 g) (Hager *et al*., 2012). It is worth noting that our observations of high intra- and interhousehold variability in tef folate content were made possible by repeated and representative sampling. Several factors like the mixture of tef varities, the milling conditions and the duration and conditions of storage could partly explain the observed variability (Monks *et al*., 2013; Czarnowska and Gujska, 2012), and such high variability in folate content is common. For example, highly variable folate contents have been reported for oats (49.5–60.4 µg/100 g DM), wheat (36.4–77.4 µg/100 g DM) and rye (64–93 µg/100 g DM (Shewry *et al*., 2008; Piironen *et al*., 2008; Kariluoto *et al*., 2001).

Recent studies suggest that fermenting cereals has the potential to increase folate content, and that its effectiveness as a strategy to combat folate deficiencies should be further explored (Saubade *et al*., 2017b). The possibility of increasing folate by fermentation was indeed confirmed in our study, as cases in folate retentions of more than 100% were observed. Our results suggest that in these cases of fermentation, folate producing microorganisms dominated those that do not produce or that consume folate. Which microorganisms and which conditions led to folate production in these cases of fermentation warrants further detailed investigations, but this observation already confirms the potential of tef fermentation to produce folate.

The pH of the fermented batter samples was 3.5 ± 0.4, which is consistent with data in the literature on tef *injera* fermentation (Baye *et al*., 2013; Fischer et al., 2014; Yigzaw *et al*., 2004).

For any produced folate to be of use, it will need to survive the baking temperature. Folate is heat labile and can also interact with oxygen in the presence of light (Sotiriadis and Hoskins, 1982). Folate retention relative to the fermented batter was highly variable (47–96%), possibly explained by the difference in *injera* baking conditions (time-temperature). Folate losses due to baking are widely reported (Hefni and Witthöft, 2011; Kariluoto *et al*., 2004), but the fact that more than 90% of the folates were retained in some examples of baking *injera* suggests that this process could be optimized to minimize folate losses.

Some of the folate present in the original flour and synthesized by microorganisms responsible for fermentation can be lost during heat treatment, as reported in previous studies (e.g. Saubade *et al*., 2017a). This means the final folate content of cooked products can be lower than the folate content of the original raw material. In this study, we also showed that the folate content in the final product (*injera*) was almost always lower than the folate content of the original tef flour (Fig. 3C). However, in one case, the final cooked products had higher folate content than the original flour. It has been shown that fermentation can lead to such high folate production that even after cooking, the final products have higher folate contents than the original flour (Kariluoto *et al.*, 2004). This suggests that if appropriate selection is used and microorganisms responsible for folate production are used, it will be possible to increase the amount of folate in the final fermented and baked products. This has been accomplished in other traditional fermented foods made from other cereals such as pearl millet or rye (Greppi *et al*., 2017; Kariluoto *et al.*, 2006).

According to the U.S. Food and Drug administration (FDA, 2016), foods that contribute 10% or more of the daily folate requirements can be considered as good sources of folate. Based on portion size estimates obtained from the national food consumption survey, the maximum folate content in our tef *injera* (20.1 µg/100 g per fresh weight) samples could contribute up to 10% of the RNI of children (1–3 years) and woman of reproductive age. Considering that the national food consumption survey was conducted during the lean season (June–September) when food consumption can be lower (EPHI, 2013), the folate contribution to the RNI is likely underestimated. Assuming two servings (~350 g/serving) of *injera* are consumed per day by women of reproductive age, tef *injera* could contribute up to 25% of the RNI. It is worth noting that *injera* is always consumed with a stew, and that depending on the nature of the stew (e.g. legume based), additional folate intakes may be expected.

Several limitations need to be taken into consideration when interpreting our findings. First, although different types of cereals are used to make *injera* (Baye *et al*., 2015), we focused only on tef *injera*. Second, the extent to which discarding the supernatants contributed to folate loss was not quantified making it difficult to ascribe losses to material loss or possible consumption by microorganisms during fermentation. Third, although the present study revealed that fermentation can increase folate content, evaluating the responsible microorganisms and the conditions that enable folate production were beyond the scope of the present study. However, studies addressing these issues are now underway in our laboratory.

Notwithstanding the above limitations, our study quantified the folate content of a widely consumed staple in Ethiopia for the first time. In addition, the study has advanced our understanding of the possible effect of *injera* processing (fermentation and thermal treatment) on folate content.

Conclusion

Tef is a relatively good source of folate and fermenting it to prepare *injera* can increase or decrease its folate content, while baking invariably leads to folate losses. The increase in folate content we observed in some cases, could be attributed to production by the microorganisms involved in fermentation. Reduced folate content could be the result of folate consumption by other microorganisms, or losses due to discarding the supernatant. As fermentation was not controlled, we recorded the net folate content resulting from a balance between the consumption and production of folate by microorganisms. This finding also points to the need for further investigation of the conditions that favor folate production through fermentation while minimizing losses due to thermal treatment (baking). Further studies should also identify the microorganisms (yeast and bacteria) responsible for the folate production during fermentation and their use as a starter culture for the preparation of folate-enriched *injera* should be evaluated. Studies addressing these issues are underway in our laboratory.

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Disclosure of interest

The authors report no conflict of interest.

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Table 1 Calculated contribution of tef *injera* to folate requirements

Population group	**Age (years)**	**RNI** (μg/day in DFEs)[a]	***Injera* intake** (g/day)[b]		**Contribution to RNI (%)**
Children	1-3 years	150	Minimum	23.0	2.2
			Maximum	108.1	10.3
			Average	66.1	6.3
Women	19-45	400	Minimum	130.7	3.7
			Maximum	291.2	10.4
			Average	202.4	7.2

[a] Recommended nutrient intakes (RNIs) are from WHO, 2004; DFEs, Dietary folate equivalents
[b] Estimated using consumption values obtained from the Ethiopian National Food Consumption Survey (EPHI, 2013)

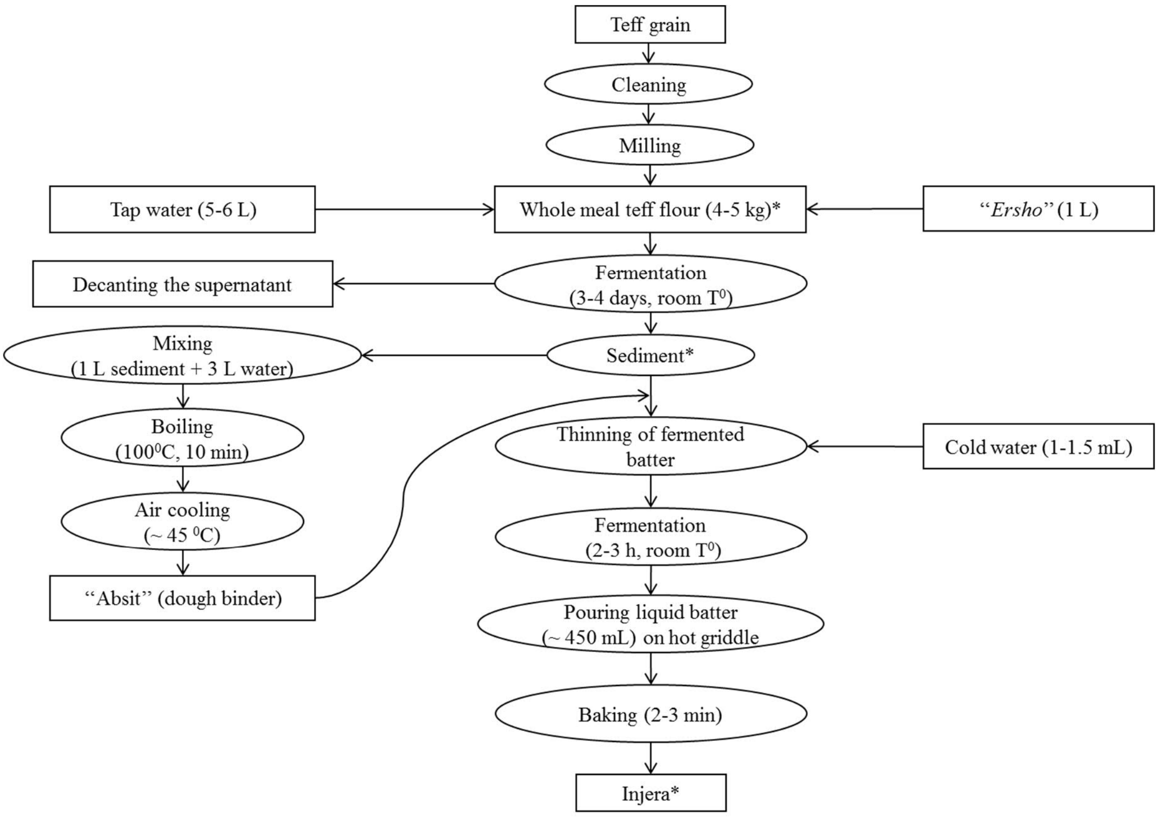

Figure 1. Flow diagram of the traditional *injera* making process observed in Ethiopian urban households.

*samples were taken for folate analysis

"*Ersho*": inoculum used for *injera* making, it is a leftover from a previous successful spontaneous traditional fermentation.

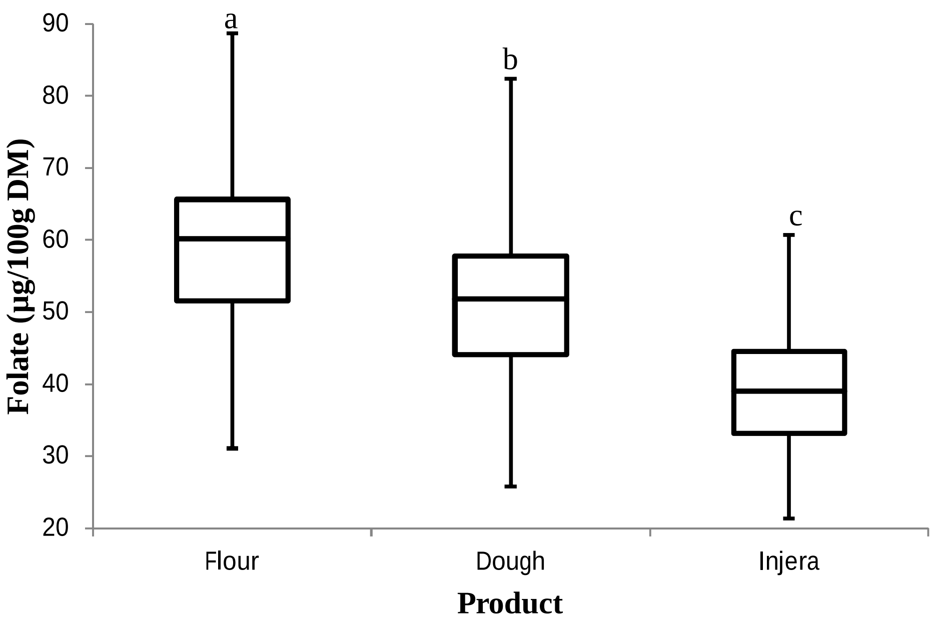

Figure 2. Box plot showing the distribution of total folate content of tef flour (n=60), fermented batter (n=60) and *injera* (n=60) in *µg*/100 g DM

The lower edge of the box corresponds to the 25th percentile, the upper edge to the 75th percentile, and the line across the middle corresponds to the median (50th percentile). The vertical lines extending outside the box represent the full range of observations.

DM, Dry matter basis; different superscript letters indicate a statistically significant difference ($p < 0.05$).

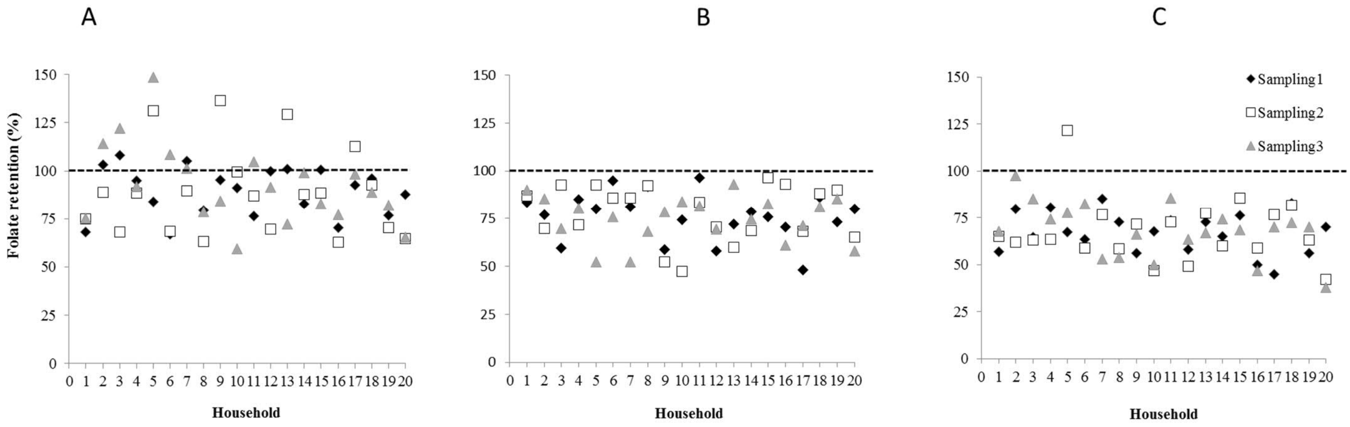

Figure 3. Folate retention (%) due to fermentation, thermal treatment and *injera* processing

A: Folate retention (%) after 1st stage fermentation; Folate retention (%) = $Folate_{flour}/Folate_{batter}$ x 100.

B: Folate retention (%) during cooking; Folate retention (%) = $Folate_{batter}/Folate_{injera}$ x 100.

C: Folate retention (%) due to *injera* processing; Folate retention (%) = $Folate_{flour}/Folate_{injera}$ x 100.