COLLEGE OF NATURAL and COMPUTATIONAL SCIENCES

CENTER FOR FOOD SCIENCE AND NUTRITION

Optimizing the folate content of injera using highly folate producing Lactic Acid Bacteria *Lactobacillus palantarum*; *Saccharomyces cerevisiae* and their combination

By: Genet Tesfaye

Advisors: Dr. Kaleab Baye

Dr. Christèle Humblot

A thesis submitted to the school of graduate studies of Addis Ababa University in partial fulfillment of the requirement for the Degree of Master of Science in Food Science and Nutrition.

April, 2019

DECLARATION

I, the undersigned, declare that this thesis is my original work and that all sources of materials used for the thesis have been dully acknowledged.

Signature;- Genet Tesfaye Date:- 8/4/2019

The thesis has been approved for submission by:

NAME OF SUPERVISORS SIGNATURES DATE

Dr. Kaleab Baye _____

Dr. Christèle Humblot _____

Acknowledgements

First I thank Almighty God his great help throughout my way. I would like to thank my advisors Dr. Kaleab Baye and Dr. Christèle Humblot for their effective and supportive supervision throughout the course of this research. I would like to thank Dr Aynadis Tamene for his supportive and humbleness advice in all tasks of my works.

I would like to express appreciation to everyone who participated in sensory evaluation and who provide me with invaluable feedback.

List of Abbreviations

DFE- Dietary Folate Equivalents

EPHI- Ethiopian Public Health Institute

FA- Folic acid

FAO- Food and Agricultural Organization

FDA- Food and Drug Administration

IES- Injera made using ersho as starter

ILPS- Injera made using *Lactobacillus plantarum* as starter

ISCS- Injera made using *Saccaromyces cerevisiae* as starter

IBS_1S- Injera made using *Lactobacillus plantarum* as starter (backslop1)

IBS_2S- Injera made using backslop1 as starter (backslop2)

IBS_3S- Injera made using backslop2 as starter (backslop3)

$IBS_{10}S$- Injera made using backslop9 as starter(backslop10)

ILSS- Injera made using a combination of *Lactobacillus plantarum* and *Saccaromycescerevisiae*

DES- Dough fermented using ersho as starter

DLPS- Dough fermented using *Lactobacillus plantarum* as starter

DSCS- Dough fermented using *Saccaromyces cerevisiae* as starter

DBS_1S- Dough fermented using *Lactobacillus plantarum* as starter (backslop1)

DBS_2S- Dough fermented using backslop1 as starter (backslop2)

DBS_3S- Dough fermented using backslop2 as starter (backslop3)

$DBS_{10}S$- Dough fermented using backslop9 as starter(backslop10)

DLSS- Dough fermented using a combination of *Lactobacillus plantarum* and *Saccaromycescerevisiae*

LAB- Lactic Acid Bacteria

MTHFR- Methylene tetrahydrofolate

NPE- Not possible establish

NTD- Neural Tube Defect

PABA- P- aminobenzoic acid

RDA- Recommended Dietary Allowances

THF- Tetrahydrofolic acid

WHO- World Health Organization

List of tables Page

List of Figures page

Table of contents

Abstract

Injera is an Ethiopian fermented pancake-like flatbread preferably made from teff. It involves a fermentation step of 3-4 days based on continuous backslopping. Starter cultures of *Lactobacillus plantarum* isolated from teff dough and *Saccharomyes cerevisiae* were tested singly or in combination for their ability to ferment teff dough for making injera and increase folate production. Folate concentration of dough and injera was determined by microbiological assay using *Lactobacillus rhamnosus* (ATCC 7469). The acceptability of injera was estimated by 30 adult healthy volunteers, using a 9 point hedonic scale. All of the starters showed an ability to ferment the dough in 3-4 days. Though the folate content of injera made with *Saccharomyces cerevisiae* was higher than other injera made with *Lactobacillus plantarum*, backslops of *Lactobacillus plantarum*, Ersho and combination of *Lactobacillus plantarum* and *Saccharomyces cerevisiae.*

Key words: Cereal, Fermentation, Folate, Lactic acid bacteria, Yeast.

1. Introduction

1.1. Background

Folate is a water-soluble B vitamin which is also known as vitamin B9. It naturally occurs in many chemical forms and is found in food, as well as in metabolically active forms in the human body. Folic acid is the major synthetic form found in fortified foods and vitamin supplements (Food and Nutrition Board, 1998). Everyone is encouraged to eat folate in their diet, but this is especially important for infants, children, women of childbearing age and breastfeeding women, in some countries they are advised to take a supplement containing 0.5 mg of folate/folic acid per day. Folate deficiencies lead to health problems such as neural tube defects (NTD) in the fetus or anemia. This, prevention of folate deficiencies has attracted major scientific and public health interest for years (Wagner, 1996).

Folate functions as a coenzyme in single-carbon transfers in the metabolism of nucleotides and amino acids. Folate primarily helps the body to make new cells, specifically by playing a role in copying and synthesizing DNA so that without folate, living cells cannot divide. The need for folate is higher when cell turnover is increased, such as neural development of fetus during pregnancy. It is also involved in the production of red blood cells, synthesis of purine, pyrimidines and amino acid (Wagner, 1996).

Dietary folates exist predominantly in the form of polyglutamates (containing several glutamate residues), whereas folic acid is in monoglutamate (containing one glutamate moiety) form.. In humans, polyglutamates must be deconjugated into monoglutamates to be metabolized within enterocytes, explaining their low bioavailability (~50%) compared to monoglutamates (~85%). Moreover, anti-folate components present in vegetables as well as exposure to heat and light during cooking and storage, all contribute to lowering folate availability. Differences in bioavailability prevent the establishing of recommendations. To adjust for the bioavailability differences between folate and folic acid, Dietary Folate Equivalents (DFE) are used. The DFE assumes that the bioavailability of folic acid added to foods is 1.7-fold greater than that of natural folate (mathematically, DFE = folate + 1.7 x folic acid) (Food and Nutrition Board, 1998).

The main strategies used to address the problem of vitamin deficiencies are supplementation, food fortification, and dietary diversification (Bhutta et al., 2013). Daily folic acid supplementation in pregnant women is recommended to reduce the risk of low birth weight, maternal anemia and NTD (WHO, 2012). In fortification programs, folic acid is added to wheat and/or maize flour at the industrial level to increase the folate intake of the whole population of a country to prevent folate deficiency. In 2015, flour fortification with folic acid was mandatory in 63 countries and authorized in four countries (Food Fortification Initiative, 2015). It has been estimated that folic acid consumption in appropriate amounts in the fortified products could prevent up to 75% of the most common NTD: spina bifida and anencephaly (Bell and Oakley, 2009).

However, food fortification programs are not always effective as Youngblood et al estimated that currently, less than 25.5% of the folic acid preventable NTD are actually prevented. In addition, it has been shown that the absorption of high amounts of folic acid can mask the symptoms of vitamin B12 deficiency, which may result in the progression of neuropathy to an irreversible point (FAO/WHO, 2005). Dietary diversification, by promoting the consumption of folate-rich foods such as green leafy vegetables, legumes, cereals etc, is a possible sustainable way of preventing folate deficiency (FAO/WHO, 2002).

Cereal-based foods are widely consumed as the primary staple foods in many countries of the world, especially in Africa (Tamena et al, 2018a). Their folate content, especially in whole grain is interesting (26 to 170µ/100g for cereal-based foods and wholegrain products, respectively) since cereals are consumed in high amounts (Saubade et al, 2017). Teff contains as much as 180µg of folates (Tamene et al, 2018a), and it is a staple food in Ethiopia and it is consumed daily by all the population (Baye et al., 2013). In Ethiopia, teff is consumed after fermentation. It has been demonstrated in other cereal-based fermented products such as sourdough rye bread that the use of microorganisms selected for their ability to produce folate is a possible way to increase folate content of the food. Almost all yeasts and some LAB are able to synthesis folates (Saubade et al 2017).

The aim of this study was to optimize the folate content of injera by making use of potential folate producing microorganism of LAB (*L.plantarum*) isolated from fermented teff dough, commercial yeast powder (*Saccharomyces cerevisiae*) and the combination of LAB and yeast.

1.1.1. Statement of the problems

In developing country like Ethiopia, the prevalence of anaemia and NTD (spinabifida) is still common and insufficient folate intake may explain it. When I said the prevalence of NTD (spinabifida) and anaemia has increased in Ethiopia, it is because I observed many children in hospitals having been born with this problem. From my observation, I realized that many pregnant mothers do not start taking folic acid supplementation at aproper time means in four weeks after they become pregnant. Usually, they start taking the tablet after four months of pregnancy. This makes the probability for the newly born children to be affected by this deseases high. More over, we do not have cereal products which are rich in folic acid in Ethiopia. It is clear that most of the Ethiopian people live on cereal foods especially, teff. Injera, which is made of teff is staple food for the majority of Ethiopian people. In the process of preparing teff to make injera , the folate content found in this grain is affected due to various factors, such as, light, oxidation,heat, etc. The main solution to overcome the problems mentioned above is increasing the content of folate which is found in diet. Therefore, this study is significant because it proves the possibility of increasing the folate content of injera by using insitu fortification by microbial fermentation. If the folate content in injera is increased, problem of the wider community can be addressed. In general, the research is important in addressing the problem of NTD (spinabifida) and aneamia, which affects many newly born children in Ethiopia.

1.2 Objectives of the study

1.2.1. General objective

To optimize the folate content of injera by making use of potential folate producing strain of LAB (*L.plantarum*) isolated from fermented teff dough, commercial yeast powder (*S. cerevisiae*) and the combination of LAB and yeast.

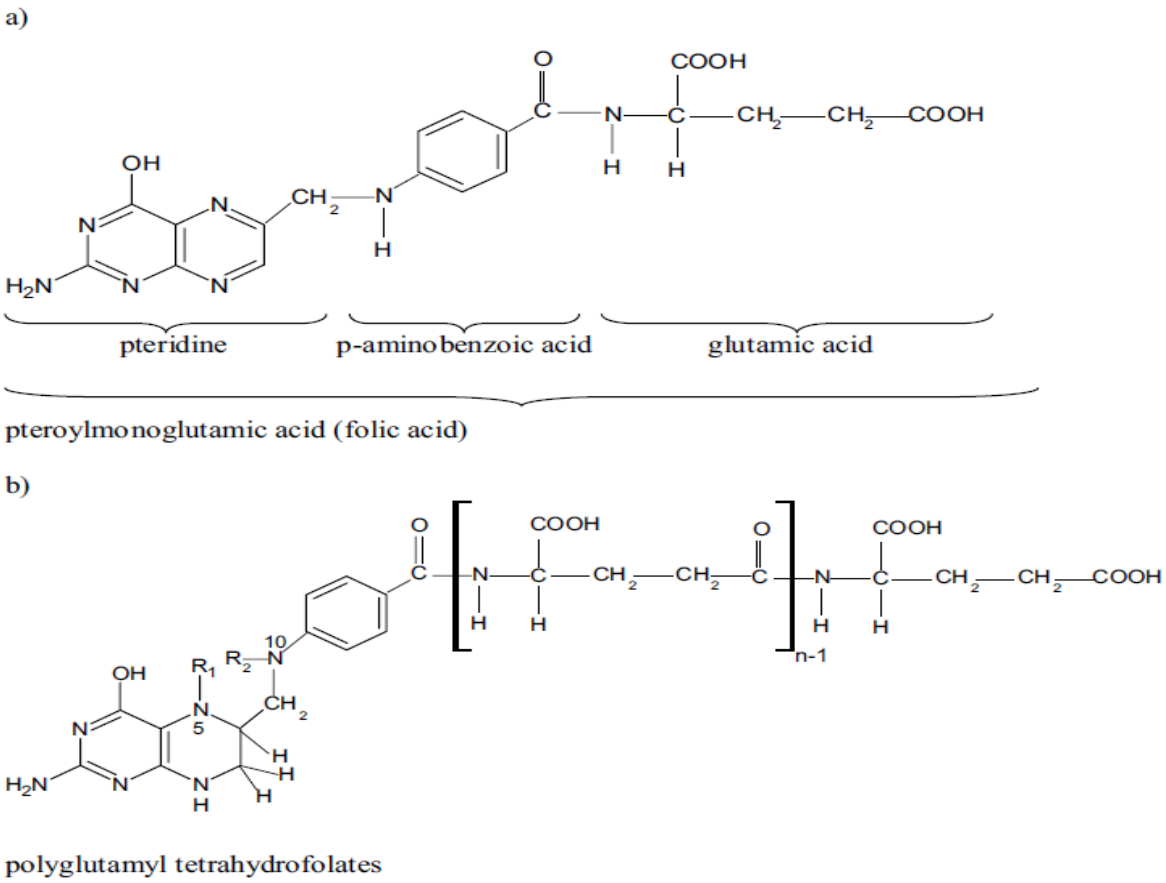
1.2.2. Specific objectives

- To determine the capacity of increasing folate content of injera by using folate producing microorganisms: LAB (*L.plantarum*), commercial yeast powder (*S.cerevisiae*) and the combination of the two.
- To evaluate the possibility of using continuous backsloping from a dough fermented with the selected microorganism *L.plantarum*.
- To evaluate the effect of absit on folate content of fermented teff dough.
- To evaluate the sensory profile of injera made by the strain, the yeast and the combination of strain and yeast.

2. Literature review

2.1. Folate

Folate is a generic term for a family of structurally related compounds that have a common pteroylglutamic acid core consisting of pteridine and *p*-aminobenzoic acid (PABA) linked to one or more glutamate molecules. These compounds include naturally occurring food folates as well as synthetic folic acid, the fully oxidized monoglutamate form used to supplement and to fortify foods. Folates are synthesized by plants and most microorganisms via the condensation of PABA with dihydropterin pyrophosphate, but are essential nutrients for mammals (Reed et al, 2006). The structure of folates can vary by the addition of a series of one to ten glutamate molecules in a chain, by reduction of the pteridine moiety to form dihydrofolic acid and tetrahydrofolic acid (THF), and by substitution of one-carbon units at the nitrogen atoms at the 5 or 10 positions or both (**Figure.1**). Folates function as coenzymes in the acceptance, oxidation/reduction and transfer of one-carbon units, and are particularly important in amino acid metabolism and in the synthesis of nucleic acids (Blancquaert et al, 2010). The coenzyme form is determined by substitution of one-carbon units, which include methyl (-CH3), methylene (-CH2-), methenyl (-CH=), formyl (-CH=O) or formimino (-CH=NH) groups, to the polyglutamate form of the tetrahydrofolicacidmolecule (Gregory, 1996).



Vitamer	R_1		R_2
tetrahydrofolate	-H		-H
5-methyltetrahydrofolate	$-CH_3$		-H
5-formyltetrahydrofolate	-HCO		-H
10-formyltetrahydrofolate	-H		-HCO
5,10-methylenetetrahydrofolate		$-CH_2-$	
5,10-methenyltetrahydrofolate		$-CH=$	

Fig.1. Structures of a) folic acid, and b) natural folate (polyglutamyl tetrahydrofolates). (Blancquaert et al, 2010).

All folates are sensitive to light, the stability of the different folates varies and depends on the chemical environment such as oxidants, pH and catalysts, even the type of buffer ions influence the vitamers. Folic acid, the form used for fortification and in the pharmaceutical industry, is more resistant to oxidative stress than reduced folate forms. The vitamin activity is lost if the C9-N10 bond is cleaved, but changes in the pteridine ring might also have an impact. Substitution to the N5 orN10 position or increases the stability so that the order of stability in aqueous solutions is 5-HCOH4folate

> 5-CH3-H4folate > 10-HCO H4folate > H_4folate. Oxidative cleavage leads to biologically inactive compounds (Gregory, 1996).

2.1.1 Dietary sources of natural folate

Folate is found in most unprocessed natural foods in the form of reduced polyglutamate derivatives. It is particularly concentrated in yeast, and in liver and other organ meats (Padalino et al, 2012). Among plant foods it is highest in green leafy vegetables, fresh oranges, asparagus, strawberries, peanuts and kidney and lima beans, however the levels in individual items are quite variable (Choumenkovitch et'al, 2002). The folate content of several foods is shown in Table 1.Folate from liver is in the form of pentaglutamyl conjugates (Padalino et al, 2012), while among plant folates the heptaglutamyl form dominates (Witthoft, 1999 and Padalino et al, 2012). These polyglutamate folates become bioavailable with their breakdown to pteroyl monoglutamates due to the action of conjugases present in vegetable and mammalian tissues, and in the human intestine (Reed et al, 2006).

Natural folates are vulnerable to oxidation, which is enhanced by heat, light and pressure in cooking or in other processing such as canning, leading to losses that may be over 55% , while losses through pasteurization of dairy products are often less than10 % (Witthoft et al, 2006). Processing may also deconjugate folates from vegetable leading to losses by diffusion into processing water. Orange juice is considered a major source of natural folate in the North American diet, but high losses of folate in frozen juices have been reported (Arcot et al, 2002).

2.2 Functions

Folate play as a coenzyme in single-carbon transfers in the metabolism of nucleotides and amino acids. Folate primarily helps the body make new cells, specifically by playing a role in copying and synthesizing DNA so that without folate, living cells cannot divide. The need for folate is higher when cell turnover is increased, such as neural development of fetus during pregnancy. It is also involved in synthesis of purine, pyrimidines and amino acid (Wagner, 1996).

Folate, with its different oxidation states, plays an essential role in biosynthetic pathways as a one-carbon donor or acceptor. One-carbon groups originate from the catabolism of serine, glysine, histidine or purines. Only 5,10-CH2-H_4folate, 10-HCO-H_4folate and 5-CH3-H_4folate act as direct C1 donors, whereas H4folate, 5,10-CH+-H_4folate and 5-HCO-H_4folate play important roles as acceptors and transferors of C1 groups (Jägerstadt and Jastrebova, 2013).

Folates are needed in the biosynthesis of DNA and RNA through the nucleotide synthesis cycle and in the metabolism of amino acids through the methylation cycle (Figure 2). One of the most important folate-dependent reactions is the conversion of homocysteine to methionine in the synthesis of S-adenosyl-methionine, an important methyl donor (Bailey and Gregory, 2006; Food and Nutrition Board, 1998).

Another folate-dependent reaction, the methylation of deoxyuridylate to thymidylate in the formation of DNA, is required for proper cell division. An impairment of this reaction initiates a process that can lead to megaloblastic anemia, one of the hallmarks of folate deficiency (Food and Nutrition Board, 1998).The methylation balance (SAM/SAH) is an important control point of many other reactions, such as methylation of DNA, and phospholipid and neurotransmitter synthesis (Wagner, 1995). Synthesis of H_4folate from 5-CH3-H_4folate is catalyzed by a vitamin B12 -dependent enzyme, methionine synthase. If vitamin B12 is lacking (for instance, due to pernicious anemia), a functional folate deficiency may occur, which leads to accumulation of 5-CH3-H_4folate and homocysteine (Hcy). Synthetic folic acid is reduced to H_4folate without the functions of methylenetetrahydrofolate reductase (MTHFR) and methionine synthase; its utilization does not require vitamin B12. Folic acid may thus mask the hematological clinical signs of vitamin B12 deficiency (Wagner, 1995).

2.3. Prevalence of folate deficiency

The global prevalence of folate deficiency is uncertain, owing to a lack of data. Only a few countries have national or even regional biochemical data on folate status. The recent review by WHO showed that the majority of data on the prevalence of folate and vitamin B12 deficiencies are derived from relatively small, local surveys, but these and national survey data from a few countries suggest that deficiencies of both of these vitamins may be a public health problem that could affect many millions of people throughout the world. Low blood concentrations of the vitamins occur across population groups and in countries in various stages of development. Inclusion of folate and vitamin B12 status

assessment in more representative national surveys is recommended, with standardization of the methods used to measure blood vitamin concentrations, and the application of universal cutoffs such as those proposed in this consultation (Hertrampf, 2003).

Inadequate intake of folate and vitamin B12 leads to low serum or plasma concentrations of both vitamins, and elevated plasma homocysteine. In addition, low folate intake causes low red blood cell folate, and elevated urinary or serum methylmalonic acid (MMA) occurs in vitamin B12 deficiency. Serum holotranscobalamin II is a more recently proposed additional measure of vitamin B12 status. Thus, several feasible and reasonably good indicators of status are available. Folate deficiency tends to be more prevalent in populations that have a high intake of refined cereals (which are low in folate) and a low intake of leafy greens vegetables and fruits (which are high in folate). Dietary surveys in India show that people eating predominantly cereal-based diets only consume about 75μg folate per day (Krishnaswamy et al, 2001). Prior to the introduction of mandatory wheat flour fortification with folic acid in 1998, about 15% of adult women in the United States were believed to have low serum and/or erythrocyte folate levels. Similarly, in Chile, where the consumption of white wheat flour is high, low serum and erythrocyte folate concentrations were common before the fortification of flour with folic acid (Hertrampf, 2003).
In European countries, the total prevalence of spina bifida and anecephaly is low due to folic acid supplementation , but in developing country like Ethiopia the prevalence is still common due to lack of Inadequate intake folate.

2.4. Cause and consequences of folate deficiency

Because of the central role of folate in cellular metabolism, an adequate supply of folate is crucial to the health of all mammals. This is critical in tissues with high rates of cell division or metabolism, and at times of high anabolic activity, such as in pregnancy and fetal development, in lactation and childhood development, when folate needs are increased. The signs of inadequate intake appear gradually and are first seen in the blood, as decreases in the concentration of plasma and erythrocyte folate, and a rise in plasma total homocysteine. As sufficient folate is not available for DNA synthesis, megaloblastic changes appear in the bone marrow and other tissues with high rates of cell division, and within weeks, anemia develops which is characterized by weakness, irritability and difficulty concentrating (Food and Nutrition Board, 1998).

Suboptimal folate intake is associated with a wide spectrum of health risks, including an elevated risk of neural tube defects, cleft lip and palate and other developmental disorders in offspring of women with low folate status, and preeclampsia and anemia in pregnancy, neuropsychiatric disorders increased risk of chronic degenerative disease, and stroke and a number of cancers, including neuroblastoma and leukemia and cancer of the breast, cervix, lung, pancreas, and in the GI tract, cancers of the esophagus and stomach and colorectal cancers. Perturbance of folate metabolism and absorption by allelic variations in genes coding for folate dependent also influences the effects of lower folate intake (Seshadri et al, 2002).

Sufficient folate intake is especially important during pregnancy and has important implications for maternal, foetal and neonatal health. Folate especially decreases the risk of neural tube defects (NTDs). NTDs are classified into ***spina bifida*** (opening in the vertebral column protecting the spinal cord), ***anencephaly*** (absence of a major portion of the brain and skull), and ***encephalocele*** (a hernia of part of the brain and the meninges). NTDs occur during the first four weeks of pregnancy when neural plate closes and forms the neural tube. There is genetic variation in NTD prevalence. Folate administration decreases elevated plasma homocysteine, which in several studies and meta-analyses has been proposed as a risk factor for cardiovascular disease, ischemic heart disease, and stroke (Brouwer et al, 1999). However, there is ongoing debate over whether homocysteine is causally related to cardiovascular disease or whether it is merely a cause or a marker of an unfavourable status underlying vascular disease or folate deficiency. Recent evaluation of the randomized trials has casted doubts over the role of homocysteine as a risk factor for cardiovascular disease (Wiliams et al, 2011).

However, folate seems to have dual modulatory effects on carcinogenesis depending on the dose, the form of folate, and the timing of folate administration. For instance, (Gopalan et al, 1999) found that low plasma folate concentration may protect against colorectal cancer, whereas a high intake of folate (attributable to supplemental folic acid) has been associated with an increased risk for breast cancer among postmenopausal women (Timmermans et al, 2011). Evidence for the protective effect of folate is strongest for colorectal cancer (Fekete et al, 2012). reported an inverse relationship between folate intake and the risk of colorectal adenomas or cancer in a case-control study. Large, prospective studies have also been able to demonstrate the protective effect of a sufficient folate intake. (Gibson et al, 2003).

2.5. Folate requirement

Traditionally, the dietary folate requirement was defined as the amount needed to prevent a deficiency severe enough to cause symptoms like anemia. The most recent RDA was based on the adequacy of red blood cell folate concentrations at different levels of folate intake, as judged by the absence of abnormal hematological indicators. Red cell folate has been shown to correlate with liver folate stores and is used as an indicator of long-term folate status Food and Nutrition Board, 2000). Because pregnancy is associated with a significant increase in cell division and other metabolic processes that require folate coenzymes, the RDA for pregnant women is considerably higher than for women who are not pregnant (Bailey and Gregory, 1999).

However, the prevention of neural tube defects (NTDs) was not considered when setting the RDA for pregnant women. Rather, reducing the risk of NTDs was considered in a separate recommendation for women capable of becoming pregnant, because the crucial events in the development of the neural tube occur before many women are aware that they are pregnant (Bailey, 1998).

The requirement of folate varies individually. However, recommendations for the population have to be set to meet the needs of most individuals. The Nordic Nutrition Recommendation (NNR) 2004 (NCM, 2004) identifies four intake levels for folate (Fig.2):

LI – The lower intake level, below this intake deficiency symptom can appear in some individuals.

AR – The average requirement for normal levels of folate in blood, serum and red blood cells.

RI – Recommended intake is based on AR and a safety margin.

UL – The upper limit is an estimate of the highest level of intake that carries no appreciable risk of adverse health effect.

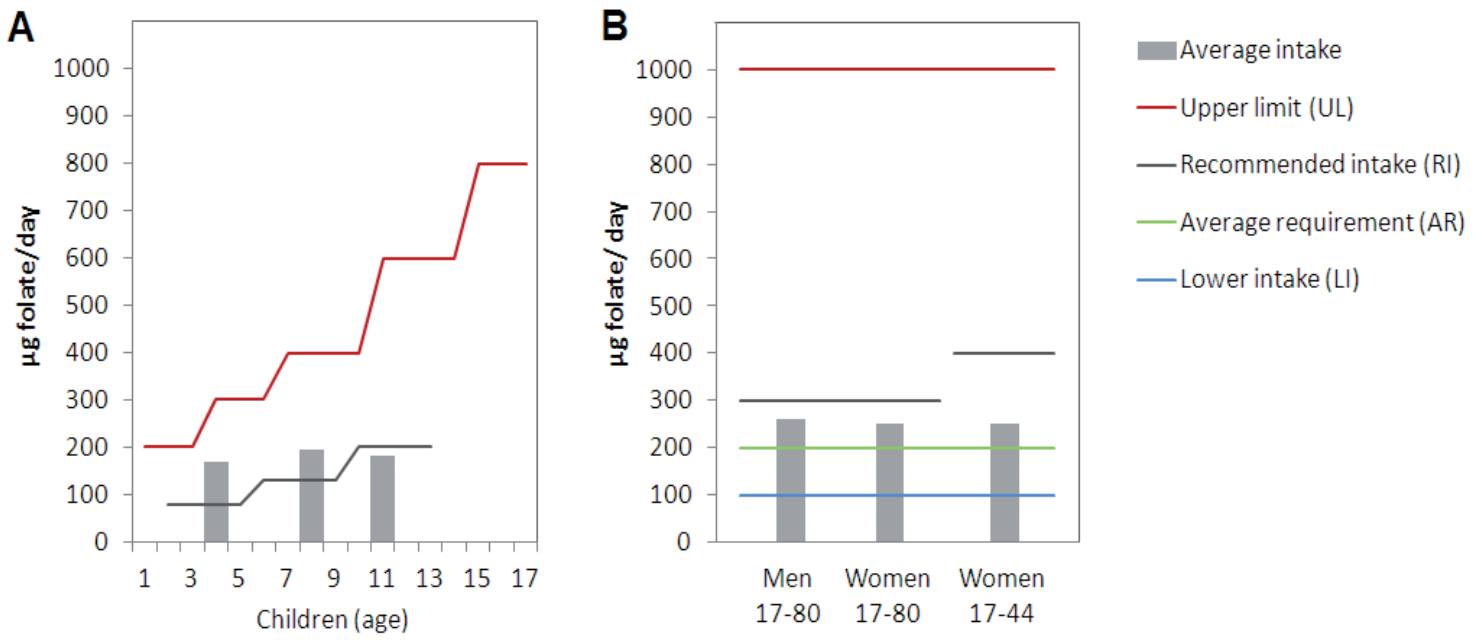


Figure.2. A. Folate intake for children - recommendations in NNR (NCM, 2005) and average folate intake. B. Folate intake for adults - recommendations in NNR (NCM, 2004) and average folate intake.

When the Food and Nutrition Board of the US Institute of Medicine set the new dietary recommendation for folate, they introduced a new unit, the Dietary Folate Equivalent (DFE) (Food and Nutrition Board, 2000). Use of the DFE reflects the higher bioavailability of synthetic folic acid found in supplements and fortified food compared to that of naturally occurring food folates (Bailey, 1998).

1 µg dietary folate equivalent (DFE) = 1 µg food folate

= 0.5 µg folic acid on an empty stomach

= 0.6 µg folic acid with meals or as fortified foods

For example, a serving of food containing 60 µg of folate would provide 60 µg of DFEs, while a serving of pasta fortified with 60 µg of folic acid would provide 1.7 x 60 = 102 µg of DFEs due to the higher bioavailability of folic acid. A folic acid supplement of 400 µg taken on an empty stomach would provide 800 µg of DFEs. It should be noted that DFEs were determined in studies with adults and whether folic acid in infant formula is more bioavailable than folates in mother's milk has not

been studied. Use of DFEs to determine a folate requirement for the infant would not be desirable (Food and Nutrition Board, 2000).

Inadequate folate intake leads to decreased serum folate, then decreased erythrocyte folate, a rise in homocysteine and megaloblastic changes in bone marrow and other rapidly dividing tissues. As depletion progresses, macrocytic cells are produced and macrocytic anaemia develops. Eventually, full-blown anaemia results in weakness, fatigue, irritability and palpitations. Folic acid supplementation in pregnancy can reduce both the occurrence and recurrence of neural tube defects in the newborn (Bower & Stanley, 1989). Indicators of folate requirement include erythrocyte, serum or urinary folate, plasma homocysteine and haematological status measures as well as clinical endpoints such as neural tube defects or chronic degenerative disease. Of these, erythrocyte folate is generally regarded as the primary indicator as it reflects tissue folate stores. For some age groups, erythrocyte folate is used in conjunction with plasma homocysteine and plasma or serum folate (Bower & Stanley, 1989).

Folate requirements increase substantially in pregnancy. This recommendation does not include consideration of additional needs to prevent neural tube defects as the neural tube is formed before most women know they are pregnant. The data indicate that maximal protection against NTD is obtained when the mother is consuming very high levels (5,000 μg) of folic acid as supplements, in the month preceding conception and in the first trimester (Konings et al 2001). Recommendations are based on evidence from controlled metabolic studies and a series of population studies. To estimate total folate requirement for lactation, the amount needed to provide sufficient breast milk folate (including a 50% bioavailability correction factor) was added to the EAR for adult women using the formula 0.78 L (volume) x 85 μg/L (concentration) x 2 (for bioavailability) = 133 μg/day (+ 320 μg/day).Fig. 2 lists the current RDAs for folate as micrograms (μg) of dietary folate equivalents (DFEs). The FNB developed DFEs reflect the higher bioavailability of folic acid than that of food folate. At least 85% of folic acid is estimated to be bioavailable when taken with food, whereas only

Table.1. Recommended Dietary Allowances (RDAs) for Folate (Food and Nutrition Board, 2000).

Age	Male	Female	Pregnant	Lactating
Birth to 6 months*	65 µg DFE*	65 µg DFE*		
7–12 months*	80 µg DFE*	80 µg DFE*		
1–3 years	150 µg DFE	150 µg DFE		
4–8 years	200 µg DFE	200 µg DFE		
9–13 years	300 µg DFE	300 µg DFE		
14–18 years	400 µg DFE	400 µg DFE	600 µg DFE	500 µg DFE
19+ years	400 µg DFE	400 µg DFE	600 µg DFE	500 µg DFE

* Adequate Intake (AI)

Based on these values, the FNB defined DFE as follows:

1 mcg DFE = 1 mcg food folate

1 mcg DFE = 0.6 mcg folic acid from fortified foods or dietary supplements consumed with foods

1 mcg DFE = 0.5 mcg folic acid from dietary supplements taken on an empty stomach

For infants from birth to 12 months, the FNB established an AI for folate that is equivalent to the mean intake of folate in healthy, breastfed infants in the United States.

2.6. Interventions for the prevention and control of folate deficiency

For the prevention and controls of folate deficiency the three general public health interventions are directed at ensuring adequate folate status through improving intake. These are: dietary diversification, supplementation, and fortification.

2.6.1. Dietary diversification

Dietary diversity previously has been defined as the number of different foods or food groups consumed over a given reference period. It encompasses both inter- and intra-level variety of foods groups for which increased availability, access to and consumption of adequate quantities and appropriate varieties of safe, good quality food is a prerequisite. Dietary diversity had long been recognized as an important component of diet quality (Ruel, 2003).

Acquiring all micronutrients from one or two food groups is not plausible and requires regular intake of several foods and food groups in sufficient quantity and variety to satisfy the nutritional needs. The example of four major micronutrients iron, vitamin C, folate, and zinc has been provided for reference (Indian Council of Medical Research, 2010). For a developing country like India which is predominantly cereal pulse-based vegetarian diet with minimal amounts of flesh foods, contrary to popular assumption, cereals appear to be the major source of iron and zinc, owing to the sheer volume of intake. For vitamin C, fruits and vegetables are the sole source, and for folate, mixtures of all food groups contribute (Gopalan et al, 1999).

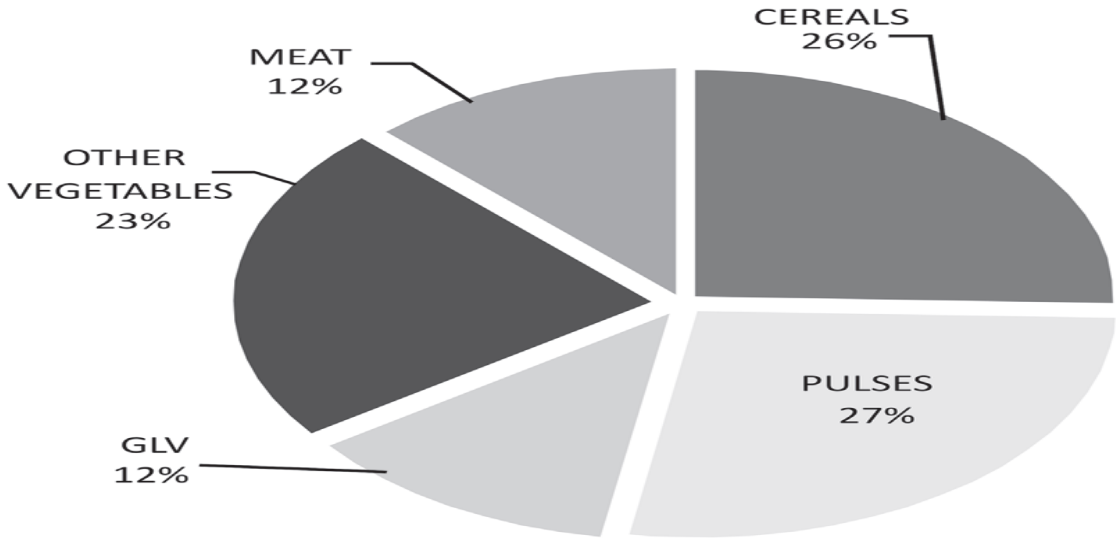


Figure.3. Contribution of different food groups in meeting the daily requirement of folate (Gopalan et al, 1999).

Strategies other than dietary diversification have the disadvantage of targeting the known factors. Addressing the "uncertainty of unknown" is an advantage for an approach aimed at diversification in the diet. Dietary diversification has an additional advantage of being more close to the population psyche and culture but also bears the challenge of breaking the inertia of habituation. Once made viable, dietary diversification is a strategy which is sustainable without external support and has the ability to simultaneously combat multiple micronutrient deficiencies (Tontisirin et al, 2002).

2.6.2. Supplementation programme

Folic acid (FA) supplementation is recommended worldwide in the periconceptional period for the prevention of neural tube defects. Due to its involvement in a number of cellular processes, its role in other pregnancy outcomes such as miscarriage, recurrent miscarriage, low birth weight, preterm birth (PTB), preeclampsia, abruption placenta, and stillbirth has been investigated (Jacques, 1999).

Supplementation with FA would therefore lead to different and more widespread effects during specific critical developmental windows in pregnancy, as demethylation and subsequent remethylation occur in the early embryo (Crider et al, 2013). In pregnancy an increase of folate breakdown products has been observed, in line with the extra demand due to the rapidly growing placenta and fetus (McPartlin et al, 2007).

A relevant scientific literature supports the role of FA in protecting against both the first occurrence and the recurrence of NTDs when used in the periconceptional period. For this reason expert committees worldwide have issued recommendations about supplementation with 0.4–1mg of FA, or 4-5mg if at higher risk of having a baby with NTD and some countries adopted a policy of food fortification with FA (Food and Drug Administration, 2016).

In the Cochrane review the issue of different doses, forms and schemes of folate supplementation in the studies available is reported. In this review it is confirmed that periconceptional (started before conception and continued until 12 weeks of gestational age) FA supplementation prevents NTDs, but there were insufficient data to evaluate the effects on other outcomes such as congenital cardiovascular defects, cleft lip and palate, miscarriages, or any other birth defects. FA supplementation may influence early placentation processes (Timmermans et al, 2011).

2.6.3. Fortification

Folic acid fortification is a process where folic acid is added to flour with the intention of promoting public health through increasing blood folate levels in the populace. In the USA, food is fortified with folic acid, only one of the many naturally-occurring forms of folate, and a substance contributing only a minor amount to the folates in natural foods (Smith, 2007).

The addition of folic acid to enriched grain products in the United States, was introduced in 1998, has since produced a substantial increase in average blood folate levels among women of childbearing age (Erickson et al, 1999-2000). This has resulted in the virtual elimination of low serum folate and the lowering of plasma homocysteine in the population at large (Jacques, 1999). The level of folic acid added (140μg/100 g flour) is unlikely to bring total folate intakes above the Tolerable Upper Intake Level (UL) of 1000μg per day in any life stage or gender group (Food and Nutrition Board, 2000), or to exacerbate or obscure problems caused by vitamin B12 deficiency. Studies such as these have obligated the governing bodies of many countries to advocate mandatory supplementation with folic acid (Lawrence, 1999).

The Food and Drug Administration noted that the uncertainties regarding the effects of chronic elevated exposure in children, whose requirements for folate are lower than those of adults. Further concerns include the potential to promote cancer and the recent hypothesis that exposure of the foetus to excess folic acid may favor the selection of the methylentetrahydrofolate polymorphism, associated with a range of debilitating illnesses. Natural folates (such as tetrahydrofolates produced by microorganism) do not cause "masking" of pernicious anemia that occurs at high concentrations of folic acid and should thus be considered as a viable alternative to folic acid fortification programs (Food and Drug Administration, 2016).

Based on the metabolic interactions between folate and vitamin B12, the FNB established a UL for the synthetic forms of folate (i.e., folic acid) available in dietary supplements and fortified foods (Table 3) (Food and Nutrition Board, 1998). The FNB did not establish a UL for folate from food because high intakes of folate from food sources have not been reported to cause adverse effects.

The ULs do not apply to individuals taking high doses of folic acid under medical supervision (Food and Nutrition Board, 1998).

Table.2: Tolerable Upper Intake Levels (ULs) for Folic Acid (Food and Nutrition Board, 1998).

Age	Male	Female	Pregnancy	Lactation
Birth to 6mths	NPE*	NPE*	NA*	NA*
7–12 months	NPE*	NPE*	NA*	NA*
1–3 years	300 µg	300 µg	NA*	NA*
4– 8 years	400 µg	400 µg	NA*	NA*
9–13 years	600 µg	600 µg	NA*	NA*
14–18 years	800 µg	800 µg	800 µg	800 µg
19+ years	1,000 µg	1,000 µg	1,000 µg	1,000 µg

* Not possible to establish (NPE*)

* Not applicable (NA*)

No adverse effects have been associated with consumption of the amounts of dietary folate equivalents normally found in foods or fortified foods. High supplemental intakes of folic acid have been shown to be related to adverse neurological effects in people with masking of B_{12} deficiency , General toxicity , increased carcinogenesis and adverse reproductive and developmental effects have also been reported (Cuskelly et al, 2007).

2.6.3.1. In-situ fortification

Another solution to improve the folate content of cereal-based staple foods would be to use in situ fortification by fermentation. In situ fortification is used to improve the nutritional quality of traditional fermented food. The production of folate by LAB during food fermentation has been validated in dairy products but the data on cereal based fermented foods (CBFF) are scarce. (Saubade et al, 2017).

2.7. Measurement of folate in foods

Microbiological assay has been considered to be one of the best and most versatile methods for determining food folates. *Lactobacillus (L.) rhamnosus* ATCC 7469 (formerly known as *L. casei*) is the most commonly used and most accepted organism for folate analysis of natural products. It responds to natural folate forms present in foods, and does not respond to pteroic acid, a common folate degradation product. *L. rhamnosus* has greater capacity for response to the γ-glutamyl folate polymers compared to the other assay organisms; however, its response is limited to no greater than three glutamates with much lower response to higher polymeric folates. Lack of response to the higher γ-glutamyl folate polymers requires treatment with pteroyl-y-glutamyl carboxypeptidase (folate conjugase, EC 3.4.19.9) in order to hydrolyze folate polyglutamates to folates with shorter glutamyl residues such as mono- or diglutamates, which can be utilized by the assay microorganism. Most current studies now determine food folate concentrations in response to growth of *L. rhamnosus* using 96-well microtiter plates (Vahteristo et al, 1997)).

2.8. Folate in cereals

2.8.1. Folate content of cereals and cereal products

Folate levels in cereal grains vary over a wide range among cereal species and cultivars. Folate content in cereal products depends on both initial grain content and on the milling level of the grain. Based on the information mainly on wheat, are unevenly distributed in cereal grain. Folate in wheat and rye have been studied the most. Their total folate content ranges from approximately 300 to 800µg/g dm. The comparison between different folate contents is difficult because of differences in the samples (e.g., one single cultivar, a mixed cultivar sample, cultivar not specified), sampling procedures (e.g., sampling from one or several grocery stores), storage and preparation of the samples and analytical methods (MA, HPLC or LC-MS). Considering their high consumption cereal grains and cereal products are an important source of natural folate. In Finland, bread and other cereal products accounted for as much as 29 and 33% of the total dietary intake for women and men, respectively (Helldán et al, 2013).

Folate contents differ markedly according to the grain species, cultivars, and growing conditions. In addition, sampling and analytical methods also vary. The use of grains requires various types of processing such as milling and fractionation, baking, and thermal treatments that have a significant effect on the folate contents of the end products (Helldán et al, 2013). .

2.9. Folate production by microorganisms

Fermentation with microbes has been used for many years in foods to increase the shelf life, to modify the texture, to contribute to flavour or to achieve alcoholic end products. In addition, microbes produce many nutritional components, such as vitamins. The ability of microbes to synthesise folate could be utilized for natural enrichment. Therefore, the interest in increasing the levels of natural folates by selecting high folate-producing food-grade microorganisms and to optimize processes in folate production is high. Folate-production ability has been studied mainly in milk-based media using LAB, propionic acid bacteria (PAB) and bifidobacteria (Rossi et al, 2011).

In addition, there are a few reports on folate production by yeasts. However, almost nothing is known about folate production by bacteria found in cereals endogenously and natural folate production by microbes using aqueous cereal matrices as the cultivation medium. Theoretically, it is possible to predict the ability for folate synthesis if the genome of the microbe is available. Folate de novo biosynthesis necessitates both the precursors DHPPP and pABA. Therefore, only those microbes that do have genes encoding the enzymes for the biosynthesis of DHPPP, chorismate and pABA are capable of folate production. It is known that among LAB, the genera *Lactococcus* and *Streptococcus* and, among bifidobacteria, the species *B. adolescentis* and *B. dentium* possess all the genes for both the shikimate pathway and chorismate conversion into pABA based on their sequenced genomes (Rossi et al, 2011).

Folate production by lactic acid bacteria

LAB are a heterogeneous group of microaerophilic, gram-positive organisms that ferment hexose sugars to produce primarily lactic acid. LAB can be classified as obligate homofermentative (synthesizing mainly lactic acid) or obligate hetero-fermentative (mainly lactic acid, acetic acid or ethanol, and CO2), besides having a key role in food fermentations, LAB are increasingly being paid attention to for the production of functional foods. Thus, their potential to produce folate has also

been observed. Common industrial LAB have been reported to produce folate in many studies. However, the ability of LAB to synthesise folate varies considerably among species. The variation may reflect the lack of the genes involved in folate biosynthesis, the strain differences and, finally, the effects of the different culture conditions (Capozzi et al, 2012). According to numerous reported studies, there is strong evidence for folate production in the majority of the strains of *S. thermophilus* and *L. lactis* (Sybesma et al, 2003). Their ability to produce folate may explain the folate content in fermented milk products such as yogurt. However, big differences have been noticed in the produced folate levels among individual strains of these bacteria (Ayad, 2007).

Folate production by yeasts

Yeasts play a significant role in many indigenous fermented foods where they are deliberately added or mainly introduced by spontaneous fermentation. Yeasts are a very heterogeneous group of unicellular fungi that differ largely in morphology as well as in physiology. Commercial baker's yeast *S. cerevisiae* and many other species have, for example, fermentative and respiratory metabolism, whereas most of the other species ferment only at a negligible rate or not at all (Kurzmann et al. 2011). *S. cerevisiae* itself is a rich source of folate, containing 24–35 μg/g dm of folate (Witthöft et al. 1999). In the previous reports, all of the studied yeast species produced folate . Hjortmo et al. (2005) showed big differences among yeast species in terms of folate production: the total folate content ranged from 40 to 145 μg/g dm cell mass. The best producers were strains of *S. cerevisiae* and closely related species. Several species showed a 2-fold or higher folate content as compared to the commercial strain of baker's yeast. In another study, Hjortmo et al. (2008c) also found a big difference between two species of *Saccharomyces*. Kariluoto et al. (2006a) showed that the studied yeasts excreted folate in negligible amounts into the medium.

3. Materials and Methods

3.1. Study area

The study was conducted in Addis Ababa University College of Natural and Computational Sciences. Center for Food Science and Nutrition laboratory.

3.2. Chemicals and raw materials

Sodium ascorbate, folic acid, Casei Medium, sodium chloride, CHES, hepes, mercatoethanol, nitrogen, protease, α-amylase, conjugase, MRS broth and MRS agar were the chemicals which were used for folate analysis, microorganism activation and anti-oxidation factor analysis. The raw material (tef grain) was obtained from Debre Zeit Agricultural Research Center, Ethiopia and divided in to eghite groups and used for making eghite different types of injera. During the process, flour was sampled for dry matter and folate measurement, dough were sampled for pH, dry mater and folate measurement and injera were sampled for dry matter, folate and sensory analysis.

3.3. Possibility to increase folate content of injera

3.3.1. Preparation of injera with traditional process

As we know there is no common standardized way of injera making process so, to come up with the knowledge from each sub-cities ten household was selected using simple random sampling technique and interviewing about traditional injera making process from their experience, based on the observed data the average result was taken and the following flowchart of traditional injera making process is developed.

Traditional Injera making processing. Injera can be prepared from widely used cereals by the people. It can be of from teff, barley, wheat, maize, sorghum or millet, but in this country teff is the most popular grain to make injera. Teff flour, water and starter (ersho) will be mixed. This was followed by the appearance of an acidic yellowish liquid on the surface of the dough at about 30-33 hours of fermentation. The liquid layer was discarded at the end of the first stage of fermentation. As soon as the liquid layer is poured off, about 10 % of the fermenting dough was mixed with three parts of water and boiled for 2 to 5 minutes. This is called "absit'', a dough enhancer, and it was mixed with the rest in the fermentation vat. This process signals the initiation of the second stage of fermentation. The boiled dough was mixed with the rest in the vat. Maximum dough-rising, which normally takes 30 minutes to $2^{1/2}$ hours, signals the termination of fermentation. At this stage the

fermenting dough was poured onto the hot flat pan, locally known as “mitad” for steam-baking into injera. Fig. 5 shows traditional “Injera” making procedure.

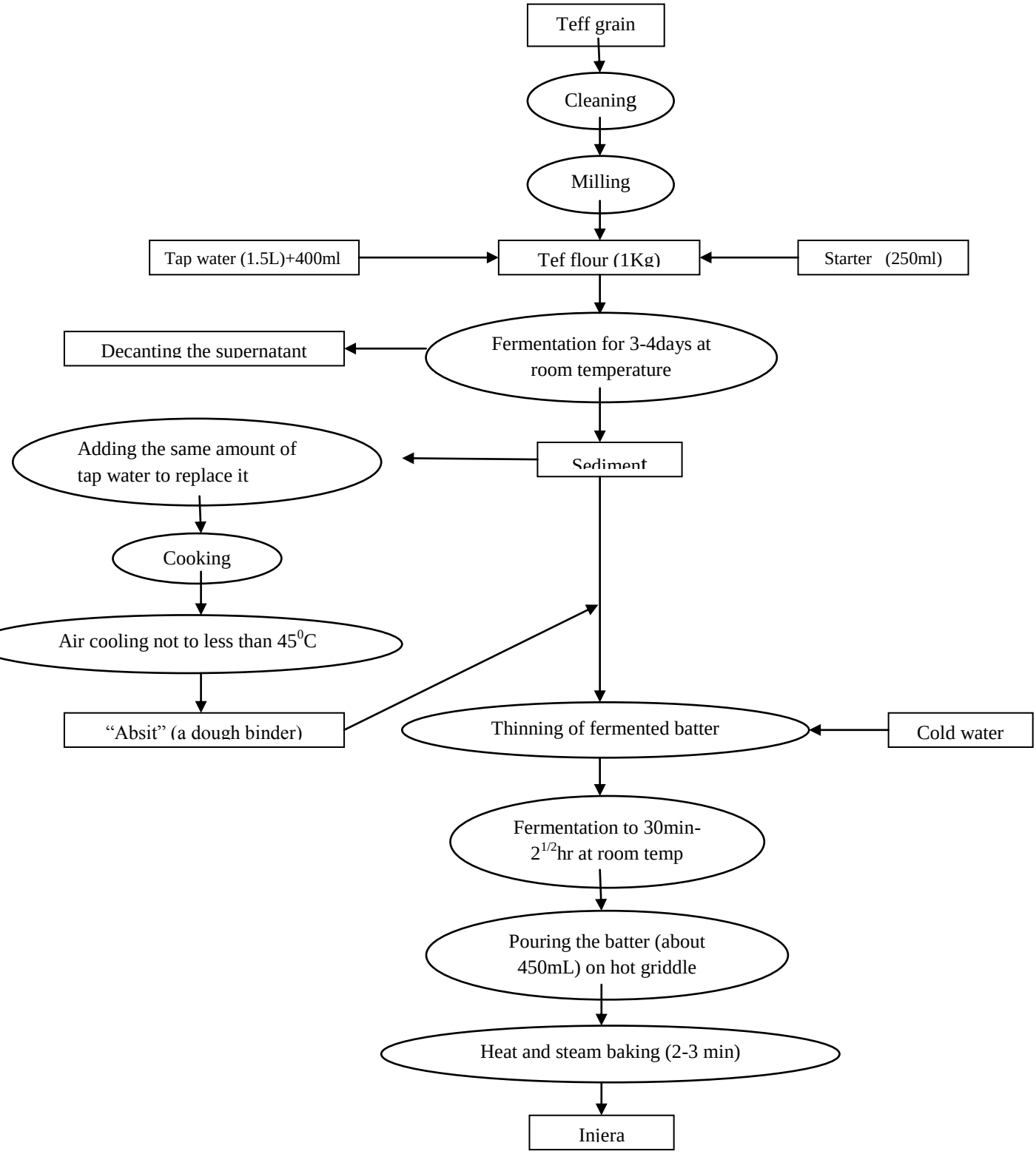


Fig.5. Flow diagram of traditional "injera" making procedure.

Starters:

- A residue of a previous batch (*Ersho*) obtained from the household
- *L. plantarum* isolated from tef dough
- Commercial powder yeast (*S. cerevisiae)*and Combination of *L. plantarum* and *S. cerevisiae*

3.3.2. Preparation of injera with folate producing microorganisms as starter

3.3.2.1. Preparation of injera using LAB (*L. plantarum*)

The objective of this process is to determine the capacity of increasing the folate content of tef injera by using *L. plantarum* isolated from tef dough as starter culture of fermentation.

Preparation of starter culture

The culture of folate producing LAB (*L. plantarum*) which was previously isolated from tef dough (Tamene et al, 2018) was used. The culture that had been stored at –80 °C in sterile cryo-tubes containing De Man, Rogosa, and Sharpe (MRS) agar (Merck, Schaffhausen, Switzerland) broth with 80 % (v/v) glycerol were cultivated by streaking on MRS agar and incubated aerobically at 30 °C for 48 h. A colony was picked from each pure culture plate, grown successively in MRS broth before centrifugation at 14000 x g/7 min. The pellets were washed with the same volume of physiological saline solution (0.9 % NaCl), centrifuged again and redistributed in physiological saline solution (0.9 % NaCl) of the same volume. This procedure achieved a culture preparation containing around 10^9 colony-forming units (cfu)/mL, checked as viable count on MRS agar.

injera making process

The traditional flow chart of making injera was adapted. Briefly; dough was prepared by mixing tef flour (1 kg), 1.5 L sterile tap water and 250 mL starter culture (0.36:0.54:0.09) (w/v/v). The 250 mL starter culture was prepared by mixing 125 mL saline containing the *L. plantarum* strain and 125 g tef flour (1:1) (v/g). Thereafter, 400 mL sterile tap water was carefully added to cover the surface of the batter and incubated for 4 days at room temperature (1^{st} phase fermentation). After 4 days of fermentation, liquid present on top of the batter was discarded and replaced by a similar amount of fresh tap water. Before baking injera from the fermented batter, 'absit' was prepared as in the traditional process. For this, the fermented batter was first thoroughly mixed with a spoon, before removing and adding a 250 mL portion to 750 mL boiling water (1:3) (v/v). The mixture (absit) was stirred, brought back to a boil, cooled to around 45 °C and transferred back to the remaining fermented batter. The batter was incubated further for 2 h at 25 °C until gas production was visible.

After 2 h of fermentation (2^{nd} phase fermentation), tap water (350 mL) was added to the dough. Portions of around 100 mL batter were poured on hot clay in a circular manner and heated for 2 min.

3.3.2.2. Preparation of injera using backslopping starter from dough fermented with LAB strain

The objective of this process was to evaluate the stability of the strain on the production of folate content of teff Injera. Four tef injera were prepared by triggering the fermentation with a back-slop (ersho) of the previous batch which was fermented using the strain *L. plantarum*. The four tef injera were fermented by backsloping up to 10 times. The samples were taken at round 1, 2, 3, and 10. The procedure followed was as in injera fermented with *L. plantarum*.

3.3.2.3. Preparation of injera using commercial bakery's yeast *S.cerevisie*

The objective of this process was to determine the capacity of increasing folate content of teff injera by using baker's yeast. Commercial yeast powder (*S. cerevisiae)* was used as starter culture to make injera. The same procedure was followed as with the others with some modification on starter culture preparation (figure 5). Briefly; dough was prepared by mixing tef flour (250 g), 375 mL sterile tap water and 62.5 mL starter culture. The 62.5 mL starter culture was prepared by mixing 31.25 mL sterile tap water with 0.62 g baker's yeast and 31.25 g tef flour (1:1) (v/w).

3.3.2.4. Preparation of injera using combination of bakery's yeast and LAB

The aim of this work was determine the capacity of increasing folate content of teff injera by mimicing natural fermentation. A combination of LAB (*L. plantarum*) and yeast (*S. cerevisiae*) was used as starter culture of fermentation during injera making. The same procedure was followed as in injera fermented with baker's yeast with the following modification on starter culture preparation; dough was prepared by mixing tef flour (250 g), 375 mL sterile tap water and 62.5 mL starter culture(0.36:0.54:0.09) (w/v/v). The 62.5 mL starter culture was prepared by mixing 31.25 mL solution containing *S. cerevisiae* and *L. plantarum* and 31.25 g tef flour (1:1) (v/w). The 31.25 mL solution with *S. cerevisiae* and *L. plantarum* was prepared by mixing 15.6 mL sterile tap water with 0.3 g baker's yeast and 15.6 mL saline solution containing respective number of *L. plantarum* (1:1) (v/v) (fig 6).

injera fermented with Ersho as starter cultures.

Inoculums from a residue of a previous batch *(*Ersho*)* were obtained following traditional fermentation from the household to represent natural fermentation. The same procedure was followed for the preparation of dough and injera using *Ersho*.

The raw material (tef grain) was obtained from Debre Zeit Agricultural Research Center, Ethiopia and divided in to seven groups and used for making seven different types of injera. During the process, flour was sampled for dry matter and folate measurement, dough were sampled for pH, dry mater and folate measurement, supernatant were sampled for pH and folate measurement and injera were sampled for dry matter, folate and sensory analysis. All of the samples were brought to the Center for Food Science and Nutrition of Addis Ababa University (AAU) laboratory.

3.4. ***Dry matter (DM) content***

The crucibles were dried in an oven at 105^0c for 1 hour and placed in desiccators to cool. The weights of the crucibles (W1) were determined. Five gram samples were weighed in the dry crucible (W2) dried at 105^0c for 3 hours and after cooling in desiccators to room temperature it is again weighed (W3). The moisture content was determined using the following Equation.

$$\textit{Moisture content in percent}\ (\%) = \frac{(\mathrm{W2}-\mathrm{W3})*100}{(W2-W1)}$$

Where, W1= weight of the crucible

W2= weight of crucible and fresh sample

W3= weight of crucible and dry sample

3.5. Determination of pH

The pH of dough was measured immediately after diluting with deionized water (1:1, v/v). The pH was measured with a pH meter after calibrating the instrument using pH 4.0 and 6.86 buffer.

3.6. Determination of folate content of fermented teff dough with different starter (before absit addition and after absit addition) and injera

The objective was to evaluate the effect of absit on the folate content of fermented teff dough, the feasibility of increasing the folate content of injera fermented with different starter and the capability of *L.plantarum* to produce folate during dough fermentation after backsloping.

The total folate content was determined using the reference microbiological assay, after trienzyme extraction (Kariluoto et al, 2001). 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.

Extraction and trienzyme treatment

For analyzing total folate with microbiological assay, samples (1-1.5 g), depending on the estimated folate content in each sample were first extracted in duplicate. Extraction was followed by tri-enzyme treatment (α-amylase, kidney conjugase and protease). After adjusting the pH to 4.9 using Hydrochloricacid 1ml of α-amylase and 100µl of conjugase were added and flush with nitrogen and incubated for 3hr at 37^0c in a shaking water bath. Then by adjusting the sample pH to 7.0 2ml of protease were added and flush with nitrogen and incubated for 1hr at 370c in a shaking water bath. This pretreatment made sample homogenization and pH adjustment easier. After inactivating the enzymes in a boiling water bath and cooling with ice, samples were filled to exact volume of 25mL with 0.5 % sodium ascorbate and directly analyzed with the microbiological assay.

Microbiological assay;

The assay was carried out on 96-well microtiter plates and the total folate content was determined based on the growth of folate-dependent strain *Lactobacillus rhamnosus* ATCC 7469 as the test organism and (6S)-5-formyltetrahydrofolate (5-HCO-H_4folate, calcium salt) (Merck, Schaffhausen, Switzerland) as the calibrant. Two dilutions were made from each sample extract using 0.5 % sodium ascorbate solution and eight levels of calibrant was used in each plate. 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. Readings were adjusted using sample blank.

3.7. Sensory acceptability

The acceptability and sensory profile for the prepared injera was estimated by 30 adult healthy volunteers, using a 9 point hedonic scale and quantitative descriptive analysis, respectively. The thirty judges or panelists of adult healthy volunteers were selected at random and they were instructed to evaluate all the seven injera on the basis of appearance, color, flavor, taste and texture and overall acceptability using a nine point hedonic scale where 1 = liked extremely and 9 = disliked extremely). Panelists were instructed to cleanse their mouth with water before testing the next sample. Test evaluation was conducted at Center for Food Science and Nutrition, Addis Ababa University.

3.8. Statistical Analysis

The data for consumer evaluation were analyzed using SPSSVersion 20 statistical software. Differences on the folate content among each injera were evaluated by analysis of variance (ANOVA). When the ANOVA is significant ($P < 0.05$), individual post-hoc test was performed among groups using Tukey's range statistic for pair wise comparisons. The total folate content of fermented teff -dough and injera presented as averages of replicate samples on a dry matter (dm) basis.

4. Results and Discussion

4.1. Results

4.1.1. Capability of *L.plantarum* to produce folate during dough fermentation after backsloping

Table.1. Total folate content of fermented teff dough before absit addition and after absit addition inµg/100g dry matter (DM)). A microbiologically determined folate content of teff flour is 89 µg/100 g DM.

Dough fermented with	Folate content of dough before absit addition (µg/100g DM	Folate content of dough after absit addition (µg/100g DM
DES	85.8	83.5
DLPS	106.0	103.3
DBS_1S	104.0	91.5
DBS_2S	121.0	94.13
DBS_3S	123.0	94.2
$DBS_{10}S$	135.0	105.0
DSCS	157.0	141.9
DLSS	113.0	108.2

DES = Dough fermented using *ersho* as starter

DLPS = Dough fermented using *L. plantarum* as starter

DBS_1S = Dough fermented using ILPS as starter (backsloping 1)

DBS_2S = Dough fermented using IBS_1Sas starter (backslopping 2)

DBS_3S = Dough fermented using IBS_2S as starter (backslopping 3)

$DBS_{10}S$= Dough fermented using IBS_9S as starter (backslopping 10)

DSCS = Dough fermented using *S. cerevisiae* as starter

DLSS = Dough fermented using a combination of *L. plantarum* and *S. cerevisiae*

Dough fermented with the leftover of a previous fermentation using *L. plantarum*was done for ten successive batches. Samples were taken after round 1, 2, 3 and 10) and total folate content was measured. The total folate content of dough fermented with backslop 2, 3 and 10 were found to be higher than dough fermented with *L. plantarum* and backslope1. This higher total folate amount in dough fermented with backslop 2, 3 and 10 than *L. plantarum* and backslope1 could be due to the adaptation and full domination of the strains of LAB to the fermenting environment as the batch of fermentation going longer and longer.

The use of backslopping (ersho) is a traditional way to produce injera. I have shown that after a first inoculation using folate-producing LAB, backslopping is efficient to maintain the high folate content of injera.

4.1.2. Effect of absit addition on the folate content of fermented teff dough with different starter

Absit used as a dough binder. It enhances gas formation, develop desired texture and consistency, as injera made without absit tends to be powdery and have fewer of the "eyes" which are so prized by Ethiopian consumers.In table 1 the folate content of dough fermented with all the different starter after absit addition is decreased when compared with dough fermented with different starter (*L.plantarum, S.cerevisiae and combination of S.cerevisiae and L. plantarum*) before absit addition.As folate are sensitive to heat, it is possible that in absit, which is cooked, the folate content decrease.Its addition dilute the folate content of the dough.

There are a few reported studies on the effect of cooking methods on folate retention in various foods, effect of processing on the folate content of fruit and vegetable and effect of baking method (Susanna et al, 2004).

However the effect of cooking absit on the folate content of fermented teff dough was not considered. In this regard, this study provides the first trial in addressing the effect of cooking absit on the folate content of fermented teff dough.

4.1. 3. Feasibility of increasing the folate content of injera fermented with different starter

Table.2. Total folate content of injera fermented with different starter in dry matter (µ/100g).

Injera fermented with	Folate content in (µg/100g DM)
IES	71.85
ILPS	81.9
IBS_1S	85.0
IBS_2S	85.5
IBS_3S	89.0
$IBS_{10}S$	90.0
ISCS	131.9
ILSS	101.11

IES = Injera made using *ersho* as starter

ILPS = Injera made using *L. plantarum* as starter

ISCS = Injera made using *S. cerevisiae* as starter

IBS_1S = Injera made using ILPS as starter (backsloping 1)

IBS_2S = Injera made using IBS_1Sas starter (backslopping 2)

IBS_3S = Injera made using IBS_2S as starter (backslopping 3)

$IBS_{10}S$ = Injera made using IBS_9S as starter (backslopping 10)

ILSS = Injera made using a combination of *L. plantarum* and *S. cerevisiae* as starter

Prior to this work, the feasibility of increasing the total folate content of injera using microbialstrain as starter culture was not checked. Therefore, this study was conducted to evaluate the feasibility of increasing folate content of tef injera using the folate-producing LAB isolated from injera dough, commercial yeast (*S. cerevisiae*) and combination of yeast and f LAB as starter culture. Injera fermented with yeast (*S. cerevisiae*) alone was found to have high amount of total folate content than injera fermented with LAB strain (*L. plantarum*), backslop 1, 2, 3, 10, mixture of *L.plantarum* and *S.*

cerevisiae and injera fermented with normal ersho.This was as expected because there was already high amount of total folate content in the dough which was fermented with *S. cerevisiae* (table 2). The result is also in agreement with the study reported on oat bran fermentation where *S. cerevisiae* was found to be the best folate producer (Korhola *et al.*, 2014).

A few studies have evaluated the effect of combining LAB with other folate producing microorganisms such as yeasts on the folate content of fermented foods. In this study we have found out that dough fermented with combination of yeast and LAB isolated from tef dough (*L. plantarum*) had similar amount of total folate than dough fermented with LAB or backslopes alone. This result is in agreement with a reported study by Korhola et al. (2014) who studied the production of folate in oat bran fermentation by yeasts and LAB where they reported that combination of *Lactobacilus rhamnosus* and *S. cerevisiae* produced somehow similar amount of folate to lactic acid bacteria alone.

4.1. 4. Sensory evaluation of injera made using different starters (*ersho, L. plantarum, S. cerevisiae and* combination of *L. plantarum* and *S. cerevisiae)*

Table.3. Sensory acceptability test for *injer*a made using different starters

Product	Color	Taste	Texture	Odor	Appearance	Overall acceptability
IES	2.8 ± 0.9^{b}	2.5 ± 0.7^{a}	2.5 ± 0.9^{b}	2.6 ± 1.1^{ab}	2.7 ± 1.3^{b}	2.5 ± 0.8^{b}
ILPS	1.5 ± 0.7^{a}	2.3 ± 0.6^{a}	1.8 ± 1.1^{a}	1.9 ± 0.8^{a}	1.5 ± 0.6^{a}	1.7 ± 0.7^{a}
ISCS	3.6 ± 0.7^{c}	3.8 ± 1.1^{b}	3.4 ± 0.9^{c}	3.5 ± 1.3^{b}	3.5 ± 0.9^{c}	3.6 ± 1.0^{c}
IBS_1S	1.5 ± 0.7^{a}	2.2 ± 0.9^{a}	1.6 ± 0.6^{a}	1.9 ± 1.29^{a}	1.5 ± 0.6^{a}	1.6 ± 0.7^{a}
IBS_2S	1.4 ± 0.8^{a}	2.4 ± 0.8^{a}	1.6 ± 0.9^{a}	1.7 ± 0.7^{a}	1.3 ± 0.5^{a}	1.7 ± 0.6^{a}
IBS_3S	1.4 ± 0.8^{a}	2.3 ± 0.6^{a}	1.5 ± 0.4^{a}	1.6 ± 0.5^{a}	1.5 ± 0.4^{a}	1.6 ± 0.4^{a}
ILSS	1.8 ± 0.6^{a}	2.4 ± 1.4^{a}	1.8 ± 0.7^{a}	2.9 ± 2.1^{b}	1.7 ± 0.7^{a}	2.1 ± 0.9^{ab}

Values are mean of thirty measurements ± standard deviation. Means followed by different letters in the same column are significantly different at $P < 0.05$

IES = Injera made using *ersho* as starter

ILPS = Injera made using *L. plantarum* as starter

ISCS = Injera made using *S. cerevisiae* as starter

IBS_1S = Injera made using ILPS as starter (backsloping 1)

IBS_2S = Injera made using IBS_1Sas starter (backslopping 2)

IBS_3S = Injera made using IBS_2S as starter (backslopping 3)

ILSS = Injera made using a combination of *L. plantarum* and *S. cerevisiae* as starter

After injera was prepared the seven different injera (injera made using Ersho, *L.plantarum*. *S.cerevisiae*, combination of *L.plantarum* and *S.cerevisiae*, Backslope 1, 2 and 3as starter culture) were coded in seven digit code and presented to 30 adult volentery food science and nutrition students to evaluate its sensory attribute (color, taste, texture, odor, appearance and overall acceptability) using 9 points hedonic scale (1= extremely liked and 9= extremely disliked).

4.1.4.1. Color

The mean score for the color of injera made using strain *L. plantarum* (ILPS), back-slop 1, 2 and 3 (IBS_1S, IBS_2S and IBS_3S) and were 1.5, 1.5, 1.4, 1.4 , respectively and the result show that the color of injera were extremely liked by panelists. The mean score for the color of injera fermented with combination of *L. plantarum* and *S. cerevisiae* (ILSS), *ersho* (IES) and *S. cerevisiae* (ISCS) alone were1.8, 2.8 and 3.6, respectively and the result show that the color of injera fermented with combination of *L. plantarum* and *S. cerevisiae* were verymuch liked by the panalists, injera made with ersho were moderately liked and the color of injera fermented with *S. cerevisiae* were slightly liked.

4.1.4.2. Taste

Taste is an important criteria for every product to be accepted by the users and the same is true for injera made using different starter. The recorded mean score of taste for injera fermented with *ersho*, *L. plantarum*, back-slops (1, 2 and3) and combination of *L. plantarum* and *S. cerevisiae* were below 2.5. So, the result shows that the taste of injera were verymuch liked by the panelists and the taste score of injera fermented with *S. cerevisiae* were 3.8, implicating that the taste was slightly liked by the panelists.

4.1.4.3. Texture

The mean score of texture for injera made using strain *L. plantarum* (ILPS), back-slop 1 and 2(IBS_1S and IBS_2S) and combination of *L. plantarum* and *S. cerevisiae* (ILSS) were above 1.6 and the result show that the texture of injera were very much liked by the panelists by comparison with injera made using ersho (IES) and *S. Cerevisiae* (ISCS), mean score of texture for injera made using backslope 3were 1.5 these shows the texture of injera were extremely liked by the panalists.

4.1.4.4. Odor

The mean score of odor for injera made using *L.plantarum*, backslops1, 2 and3 (ILPS, IBS_1S, IBS_2S and IBS_3S) were 1.5, 1.5, 1.3 and 1.5, respectively and this result show that the odor of injera were extremely liked by the panelists. The odor score of injera made using *S.cerevisiae* (ISCS) were 3.5 and the result show that the odor of this injera was moderately liked by the panelists.

4.1.4.5. Appearance

The mean score of appearance for injera made with L.plantarum. backslops1, 2 and 3(ILPS, IBS_1S, IBS_2S, and IBS_3S) were 1.5, 1.5, 1.3 and 1.5, respectively and this result show that the appearance of injera were very much liked by the panelists. The appearance score of injera made using *S.cerevisiae* (ISCS) were 3.5 and the result show that the appearance of injera fermented with *S.cerevisiae* were moderately liked by the panelists.

4.1.4.6. Overall acceptability

The overall acceptability result showed that injera fermented using *L.plantarum*, backslops1, 2 and 3 as starer (ILPS, IBS_1S, IBS_2S, and IBS_3S) were verymuch accepted whereas injera fermented with *S.cerevesiae* as starter (ISCS) was slightly acceptable by the panelists.

There are a few reported studies on isolation of folate producing microorganisms from cereal and non-cereal fermented products, estimation of folate production and evaluation of its bioavailability (Herrenen et al, 2010). However the effect of the strains on sensory quality of the fermented products was not considered. In this regard, this study provides the first trial in addressing the effect of using different starters in cereal fermentation both on the amount of total folate and sensory profile of the product. The study has clearly indicated that the isolated folate producing LAB (*L. plantarum*) could be used for the development of fermented foods like *injera* with better folate content and consumer preferences.

4. 2. Discussion

Lactic acid bacteria, previously isolated from tef dough have been found to be belongs to *Lactobacillaceae*. Folate production by one of the isolated LAB (*L. plantarum*) was examined. But the feasibility of increasing the total folate content of injera using this strain as starter culture was not checked. Therefore, this study was conducted to evaluate the feasibility of increasing folate content of tef injera using the isolated LAB as starter culture. The consistency of folate production of the strain from batch to batch fermentation was also assessed. Feasibility of making injera with improved total folate content using commercial yeast (*S. cerevisiae*) and combination of yeast and LAB as starter cultures during fermentation was also evaluated. The study also focused on evaluating sensory profile of the improved injera and the effect of absit addition on the folate content of fermented teff dough.

This study showed that tef dough fermented with yeast (*S. cerevisiae*) alone has high folate content than the other dough. This result is in lined with the previous reported findings that the bakers' yeast *S. cerevisiae* was found to be the best folate producers in various cereal fermentations (Kariluoto et al, 2006). A few studies have evaluated the effect of combining LAB with other folate producing microorganisms such as yeasts on the folate content of fermented foods. In this study I have found out that dough fermented with combination of yeast and LAB isolated from tef dough (*L. plantarum*) has similar amount of folate content of dough fermented with LAB or backslopes alone. This result is in agreement with a reported study by Korhola et al. (2010) who studied the production of folate in oat bran fermentation by yeasts and LAB where they reported that combination of *Lactobacilus rhamnosus* and *S. cerevisiae* produced somehow similar amount of folate to lactic acid bacteria alone.

Dough fermented with the leftover of a previous fermentation made by *L. plantarum* as backsloping for four successive batches (Backslope 1, 2, 3 and 10) were evaluated for total folate content. The total folate content of dough fermented with backslop 2, 3 and 10 were found to be high than dough fermented with *L. plantarum* and backslope1. This higher total folate amount in dough fermented with backslop 2, 3 and 10 than *L. plantarum* and backslope1 could be due to the adaptation and full domination of the strains of LAB to the fermenting environment as the batch of fermentation going longer and longer.

Injera fermented with yeast (*S. cerevisiae*) alone was found to have high amount of total folate content than the others. This was as expected because there was already high amount of total folate in the dough which was fermented with *S. cerevisiae*. The result is also in agreement with the study reported on oat bran fermentation where *S. cerevisiae* was found to be the best folate producer (Korhola et al, 2010). *Injera* fermented with *L. plantarum* has high amount of total folate than injera fermented with normal starter, *ersho* collected from the household.

Though the folate content of injera made with *S. cerevisiae* was high than the other injera and it was the least accepted product by the panalistes. But *injera* made with the LAB strain (*L. plantarum*), backslop 1, 2 and 3 were found to have high amount of total folate next to *S. cerevisiae* and it was the most preferred product by the panalistes.

There are a few reported studies on isolation of folate producing microorganisms from cereal and non-cereal fermented products, estimation of folate production and evaluation of its bioavailability (Laiño et al, 2012; Herrenen et al 2010). However the effect of the strains on sensory quality of the

fermented products was not considered. In this regard, my study provides the first trial in addressing the effect of using different starters in cereal fermentation both on the amount of total folate and sensory profile of the product. The study has clearly indicated that the isolated folate producing LAB (*L. plantarum*) could be used for the development of fermented foods like injera with better folate content and consumer preferences. It could be also used as a natural alternative to fortification and supplementation of the synthetic folic acid.

5. Conclusion and Recommendation

5.1. Conclusion

Finally, in this study the feasibility the isolated Lactic Acid Bacteria strain, (*L.plantarum*), bakery's yeast (*S.cerevisiae*) and the combination of *L.plantarum* and *S.cerevisiae* on increasing the folate content of teff injera using as starter culture was evaluated, the consistency of folate production of the strain *L.plantarum* from batch to batch fermentation and sensory profile of the improved injera was also assessed. Injera fermented with yeast (*S. cerevisiae*) alone was found to have high amount of total folate content than the others and it was the least accepted product by the panalistes. But *injera* made with the LAB strain (*L. plantarum*), backslop 1, 2 and 3 were found to have high amount of total folate next to *S. cerevisiae* and it was the most preferred product by the panalistes.

Due to this, this research show that Cereal-associated endogenous bacteria together with food-origin yeasts are promising as potential folate producers. This could open the door to novel food applications that are rich in folate produced by microbes. Therefore, tef injera, in addition of having health importance of being whole grain fermented product (Thompson, 1994), could be the potential source of useful LAB which can be used for prevention of folate deficiency.

5.2. Recommendation

It is recommended that using of this starter culture prepared from strain of LAB (*L.plantarum),*bakery's yeast *(S.cervesia)* and combination of *L.plantarum* and *S.cervesiae* in industrial production should improve the quality and uniformity of the final product as well as food safety by preserving typical sensory quality of the traditional fermented product and inhibiting the growth of undesirable microorganisms. The food industry should now take the next step to use this information for selecting folate-producing strains as part of their starter cultures in order to produce fermented products with elevated levels of this essential vitamin. Such products would provide economic benefits to food manufacturers since increased "natural" folate concentrations would be an important value-added effect without increasing production costs. Consumers would obviously benefit from such products since they could increase their folate intakes while consuming products that form part of their normal lifestyle.

6. Reference

Arcot, J., Wootton, M., Alury, S., Chan, H.Y., and Shrestha, A.K. (2002). Folate levels in twelve Australian wheats and changes during processing into bread. *Food Australia*,54, (18-20).

Ayad, EHE. (2007). Folate producing lactococci for dairy product innovation. *The International Agriculture Centre, Cairo, Egypt, 15,* (265-281).

Bailey, LB. (1998). Dietary reference intakes for folate: the debut of dietary folate equivalents. *Journal of Nutrition Review*, 56(10), 294-299.

Bailey, LB., & Gregory, JF. (1999). Polymorphisms of methylenetetrahydrofolate reductase and other enzymes: metabolic significance, risks and impact on folate requirement. *Journal of Nutrition*, 129 (5), 919-922.

Baye, K., Mouquet-Rivier, C., Icard-Vernière, C., Rochette, I., & Guyot, J. P. (2013). Influence of flour blend composition on fermentation kinetics and phytate hydrolysis of sourdough used to make *injera*. *Food Chemistry*, *138*(1), 430-436.

Bell, KN., & Oakley, GP. (2009). Update on prevention of folic acid-preventable spina bifida and anencephaly. *British Defects Research*, 85, (102-107).

Bhutta, Z.A., Salam, R.A., & Das, J.K. (2013). Meeting the challenges of micronutrient malnutrition in the developing world. *Britain Medicine Bull*, 106 (7–17).

Blancquaert, D., Storozhenko, S., Loizeay, K., De Steur, H., De Brouwer ,V., Viaene, J., Ravanel, S., Rebeille, F., Lambert, W., & Van Der Straeten, D. (2010). Folates and folic acid: from fundamental research toward sustainable health. *Critical Review in Plant Science*, 29, (14-35).

Bower,C.,& Stanley, FJ. (1989). Dietary folate as a risk factor for neural tube defects: evidence from a case-control study in Western Australia. *Medicine Journal of Australia*, 150, (613–9).

Brouwer, IA., van Dusseldorp, M., Thomas, CMG., Duran, M., Hautvast, JGAJ., Eskes, TKAB., & Steegers-Theunissen, RPM.(1999). Low-dose folic acid supplementation decreases plasma homocysteine concentrations: a randomized trial. *Journal of Clinical Nutrition*, 69, (99–104).

Capozzi, V., Russo, P., Dueñas, MT., López, P., & Spano, G. (2012). Lactic acid bacteria producing B-group vitamins: a great potential for functional cereals products. *Applied Microbiological Biotechnology*, 96,(1383-1394).

Choumenkovitch, SF., Selhub, J., Wilson, PW., Rader, JI., Rosenberg, IH., & Jacques, PF. (2002). Folic acid intake from fortification in United States exceeds predictions. *Journal of Nutrition*, 132,(2792-2798).

Crider, KS., Cordero, AM., Mulinare, J., Dowling, NF., & Berry, RJ. (2013). "Prenatal folic acid and risk of asthma in children: a systematic review and meta-analysis," in The *American Journal of Clinical Nutrition*, 98, (1272–1281).

Cuskelly, GJ., Mooney, KM., & Young, IS. (2007). Folate and vitamin B12: friendly or enemy nutrients for the elderly. Synthesis and utilisation of folate by yoghurt starter cultures and probiotic bacteria. *International Journal of Food Microbiology*, 80,(217-222).

Erickson, JD. (1999–2000). Folate status in women of childbearing age, by race/ethnicity – United States, Morbidity and Mortality Weekly Report, 2002, 51,(808– 810).

FAO/WHO. (2002).Human vitamin and mineral requirements, Bangkok, Thailand

Fekete, K., Berti, C., & Trovato, M. (2012). "Effect of folate intake on health outcomes in pregnancy: a systematic review and meta-analysis on birth weight, placental weight and length of gestation," *Nutrition Journal*, 75, (132-136).

Food and Drug Administration. (2016). food additives permitted for direct addition to food for human consumption; folic acid.*external link disclaimer Federal Register* 81(73), (22176-22183).

Food and Nutrition Board, Institute of Medicine. Folate. (1998). Dietary Reference Intakes: Thiamin, Riboflavin, Niacin, Vitamin B6, Folate, Vitamin B12, Pantothenic Acid, Biotin, and Choline. *Washington, D.C. National Academy Press*, 1, (196-305).

Gibson, RS., Yeudall, F., Drost, N., Mtitimuni, BM., & Cullinan, TR. (2003). Experiences of a community-based dietary intervention to enhance micronutrient adequacy of diets low in animal source foods and high in phytate: a case study in rural Malawian children. *Jounal of Nutrition*, 133(3992S–9S).

Gopalan, C., Rama Sastri, BV., Balasubramanian, SC., Rao, BS., Deosthale, YG., &Pant, KC. (1999). Nutritive Value of Indian Foods. Hyderabad: National Institute of Nutrition, Indian Medicine of council Research.

Gregory, J.F., Williamson, J., Liao, J.F., Bailey, L.B., &Toth, J.P. (1998). Kinetic Model of Folate Metabolism in Nonpregnant Women Consuming Folic Acid: Isotopic Labeling of Urinary Folate and the Catabolite Para-acetamidobenzoylglutamate Indicates Slow, Intake-Dependent, Turnover of Folate Pools. *Journal of Nutrition*, 128, (1896–1906).

Herranen, M., Kariluoto, S., Edelmann, M., Piironen, V., Ahvenniemi, K., Iivonen, V., Salovaara, H., & Korhola, M. (2010). Isolation and characterization of folate-producing bacteria from oat bran and rye flakes. *International Journal of Food Microbiology*, 142, (277-285).

Helldán, A., Kosonen, M., Tapanainen, H., Ovaskainen, ML., Raulio, S., & Virtanen, S. (2013). Finravinto 2012 -tutkimus - The National FINDIET 2012 Survey, THL, raportti 16/2013. Helsinki, Finland: *The National Institute for Health and Welfare*.

Hertrampf, E. (2003). Consumption of folic acid-fortified bread improves folate status in women of reproductive age in Chile. *Journal of Nutrition*, 133, (3166–3169).

Indian Council of Medical Research (ICMR). (2010). Nutrient Requirements and Recommended Dietary Allowances for Indians. A Report of the Expert Group of the Indian Council of Medical Research 2010. *Hyderabad: National Institute of Nutrition* .

Jacques, PF . (1999). The effect of folic acid fortification on plasma folate and total homocysteine concentrations. *New England Journal of Medicine*, 340,(1449–1454).

Jägerstad, M., & Jastrebova, J. (2013). Occurrence, stability, and determination of formyl folates in foods. *Journal of Agricultural Food Chemistry*, 61, (9758-9768).

Laiño, J. E., Zelaya, H., del Valle, M. J., de Giori, G. S., & LeBlanc, J. G. (2015). Milk fermented with selected strains of lactic acid bacteria is able to improve folate status of deficient rodents and also prevent folate deficiency. *Journal of Functional Foods*, *17*, 22-32.

Lawrence, JM . (1999). Trends in serum folate after food fortification. Lancet, 354, (915–916).

Kariluoto, MS., Vahteristo, LT., & Piironen, VI. (2001). Applicability of microbiological assay and affinity chromatography purification followed by high- performance liquid chromatography (HPLC) in studying folate contents in rye. *Journal of the Science of Food and Agriculture*, 81(9), (938-942).

Kariluoto, S., Aittamaa, M., Korhola, M., Salovaara, H., Vahteristo, L., & Piironen, V. (2006a). Effects of yeasts and bacteria on the levels of folates in rye sourdoughs. *International Journal Food Microbiology*, 106, (137-143).

Krishnaswamy, K., & Madhavan Nair, K. (2001). Importance of folate in human nutrition. *British Journal of Nutrition*, 85, (115–124).

Konings, EJM., Roomans, HHS., Dorant, E., Goldbohm, RA., Saris, WHM., & van den Brandt, PA. (2001). Folate intake of the Dutch population according to newly established liquid chromatography data for foods. *Journal of Clinical Nutrition*, 73, (765-776).

Laiño, JE., LeBlanc, JG., & Savoy de Giori G. (2012). Production of natural folates by lactic acid bacteria starter cultures isolated from artisanal Argentinean yogurts. Canada *Journal of Microbiology*, 58, (581-588).

McPartlin, J., Halligan, A., Scott, JM., Darling, M., & Weir, DG.(1993). "Accelerated folate breakdown in pregnancy," The Lancet, 8838, (148–149).

NNR. Nordic Nutrition Recommendations NNR 2004. Integrating Nutrition and Physical Activity; Nordic Council of Ministers: Stockholm, Sweden, 2005.

Padalino, M., Perez-Conesa, D., López-Nicolás, R., Frontela-Saseta, C., & Ros-Berruezo, G. (2012). Effect of fructooligosaccharides and galactooligosaccharides on the folate production of some folate-producing bacteria in media cultures or milk. *International Dairy Journal*, 27, (27-33).

Parazzini, F., Chiaffarino, F., Ricci, E., Improta, L., & Monni G. (2011). "Homocysteine, red cell, and plasma folate concentrations and birthweight in Italianwomen: results fromaprospective study," *Journal of Maternal-Fetal and Neonatal Medicine*, 3, (427–431).

Patring, J., Jastrebova, J., Hjortmo, S., Andlid, T., & Jägerstad, I. (2005). Development of a simplified method for the determination of folates in baker's yeast by HPLC with ultraviolet and fluorescence detection. *Journal of Agricultura Food Chemistry*, 53, (2406-2411).

Reed, MC., Nijhout, HF., Neuhouser, ML., Gregory, JE., Shane, B., James, SJ., Boynton, A., & Ulrich, CM. (2006). A Mathematical Model Gives Insights into Nutritional and Genetic Aspects of Folate-Mediated One-Carbon Metabolism. *Journal of Nutrition*, 136, (2653–2661).

Ruel, MT. (2003). Operationalizing dietary diversity: a review of measurement issues and research priorities. *Journal of Nutrition*, 133, (3911–3926).

Rossi, M., Amaretti, A., & Raimondi, S. (2011). Folate production by probiotic bacteria. Nutrients, 3, (118-134).

Saubade, F., Hemery, Y. M., Guyot, J. P., & Humblot, C. (2017). Lactic acid fermentation as a tool for increasing the folate content of foods. *Critical reviews in food science and nutrition*, *57*(18), 3894-3910.

Scott, J. (1984). "Catabolismof folates," in Folates and Pterins. JohnWiley, New York, NY, USA,1, (307–327).

Seshadri, S., Beiser, A., & Selhub, J. (2002). Plasma homocysteine as arisk factor for dementia and Alzheimer's disease. Medicine, 346, (476-483).

Smith, AD. (2007). "Folic acid fortification: the good, the bad, and the puzzle of vitamin B-12". *Journal of Clinical Nutrition*, 85 (1), (3–5).

Sybesma, W., Starrenburg, M., Tijsseling, L., Hoefnagel, MHN., & Hugenholtz J. (2003). Effects of cultivation conditions on folate production by lactic acid bacteria. *Applied Environmental Microbiology*, 69, (4542-4548).

Tamene, A., Baye, K., Kariluoto, S., Edelmann, M., Bationo, F., Leconte, N., & Humblot, C. (2018a). Folate in tef grain: quantification and effect of traditional processing of injera, a cereal fermented Ethiopian staple food. Unpublished

Timmermans, S., Jaddoe, VW., & Silva, LM. (2011). "Folic acid is positively associated with uteroplacental vascular resistance: the Generation R Study," Nutrition,Metabolism and Cardiovascular Diseases, 1, (54–61).

Thompson, L. U. (1994). Antioxidants and hormone- mediated health benefits of whole grains. *Critical Reviews in Food Science & Nutrition*, *34*(5-6), 473-497.

Tontisirin, K., Nantel, G., & Bhattacharjee, L. (2002). Food based strategies to meet the challenges of micronutrient malnutrition in the developing world. Proclim Nutrition Soc,61, (243–50).

Vahteristo, L., Lehikoinen, K., Ollilainen, V., & Varo, P. (1997a). Application of an HPLC assay for the determination of folate derivatives in some vegetables, fruits and berries consumed in Finland. *Journal of Food Chemistry*, 59, (589-597).

Wagner, C. (1996). Symposium on the subcellular compartmentalisation of folate metabolism. *Journal of Nutrition*, 126, (1228–1234).

Williams, PJ., Bulmer, JN., Innes, BA., & Pipkin, FB. (2011). "Possible roles for folic acid in the regulation of trophoblast invasion and placental development in normal early human pregnancy," *Biology of Reproduction*, 6, (1148–1153).

Witthöft, CM., Forssén, K., Johannesson, L., & Jägerstad, M. (1999). Folates - food sources, analyses, retention and bioavailability. *Journal of Nutrition*, 43, (138-146).

Witthoft, CM., Arkbage, K., Johansson, M., Lundin, E., Berglund, G., Zhang, JX., Lennernas, H., & Dainty, JR. (2006). Folate Absorption from Folate-Fortified and Processed Foods Using a Human Ileostomy Model. *British Journal of Nutrition*, 95, (181–187).

Young, CM., & Lin, MY. (2000). Folate levels in cultures of lactic acid bacteria. *International Dairy Journal*, 10, (409-413).