NUTRIENT ENRICHMENT OF INJERA TO IMPROVE FOOD AND NUTRITION SECURITY IN ETHIOPIA

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Para la realización de esta tesis doctoral, Tigist Fekadu Markos obtuvo una beca del programa Mujeres por África.

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A mi familia, mi esposo y mis tres hijos.

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ABSTRACT

Micronutrient deficiency, including calcium deficiency, affects many people in Ethiopia due to the low level of nutrients in the diet and the wastage of food products. Injera is a staple food in Ethiopia which can be used as a carrier to provide micronutrients to the population. Eggshell, which is usually thrown as a waste product, has a high calcium content and it can potentially be used as a source of calcium. In addition, injera has a short shelf life which needs to be improved to be used in a food enrichment program.

The main objective of the thesis was to explore the possibility of using eggshell powder (EP) to enrich injera with calcium, one of the micronutrients needed in the Ethiopian diet. The specific objectives were i) to define the main quality parameters of injera, including physical, chemical, nutritional, microbiological and sensorial ones: ii) to investigate the effect of the addition of EP on the quality of the injera; iii) to determine conditions to extend the shelf life of enriched injera through refrigeration and modified atmosphere packaging and iv) to analyze the effect of the addition of EP on the acceptability of injera in Ethiopia, and to describe the sensory characteristics and identify the drivers of liking of the EP fortified injera.

The thesis has been divided in five chapters. In the very first one, a complete literature review was performed related to Ethiopian nutritional problems and the potential solutions through changes in the diet. The other four chapters are focused on the different specific objectives.

In chapter 2, injera processing was set up and injera quality parameters were defined. Quality parameters of injera included physicochemical, physical, microbial, and nutritional ones were determined. Similarly, injera batter quality parameters were also determined in this chapter. Sensory analysis research was described in chapter 5.

In chapter 3, fortification of injera with EP was conducted at two levels of EP (4.5% and 9% with respect to teff flour, respectively identified as I4.5 and I9.0) and then quality parameters of injera were analyzed. Enriching injera with EP improved total and bioavailable calcio and it affected slightly most quality parameters. Bioavailable calcium improved from 16.1 mg/100 g (control) to 742.7 mg/100 g (I4.5) and 1743.1 mg/100 g (I9.0). With the consumption of 200 g of injera, an adult person could meet 60% of the recommended dietary allowance (RDA) of Ca when using I4.5 flour and 140% when using I9.0 one.

Chapter 4 was focused on the shelf-life study of injera which was conducted by using a combination of modified atmosphere packaging (MAP) or atmospheric air and room temperature or refrigeration. The microbial quality of injera was assessed by measuring mold/yeast growth and total mesophilic. In addition, the physicochemical and physical qualities of injera were studied. The shelf-life study revealed that cold storage was the best for preserving injera for about 20 days without having any visual microbial growth but had a negative impact on injera texture likely due to starch retrogradation. The use of MAP showed to be a better preservation method but still it had a short shelf-life extension.

Finally, sensory analysis conducted in Hawassa, Ethiopia was described in Chapter 5. Thus, the sensory assessment was conducted on real consumers of injera in Ethiopia. The results showed that the injera prepared by fortifying with EP was accepted by the consumers. However, the liking of injera decreased when EP is added. The attributes that mostly affect the overall acceptance of injera were the grittiness and the eyes size being necessary the non-grittiness and medium eyes size in order not to decrease acceptability. The amount of added calcium that 50 % of consumers can detect was 7.44% for the eyes size and 2.5% for grittiness.

In conclusion, the current study showed that EP can be used for improving the calcium content of injera and to increase the bioavailable calcium in the Ethiopian diet. Further work has to be done for improving the quality parameters of injera that were affected by the addition of EP.

RESUMEN

La deficiencia en micronutrientes, incluido el calcio, afecta a una gran cantidad de personas en Etiopía debido al bajo nivel de nutrientes de la dieta y al deshecho alimentario. La injera es un alimento básico en Etiopía que podría ser utilizado como portador de micronutrientes para mejorar la dieta etíope. La cáscara de huevo, que normalmente se deshecha tiene un alto contenido en calcio y podría ser utilizada como fuente de este mineral. Por otra parte, la injera tiene una vida útil muy corta que necesita mejorar para ser utilizada dentro de un programa de enriquecimiento alimentario.

El objetivo principal de la tesis fue explorar la posibilidad de usar cáscara de huevo molida (EP) para enriquecer la injera con calcio, uno de los micronutrientes necesitados en la dieta etíope. Los objetivos específicos de la tesis fueron: i) Determinación y puesta a punto de los principales parámetros de calidad de la injera incluyendo parámetros físicos, químicos, nutritivos, microbiológicos y sensoriales; ii) estudiar el efecto de la adición de EP sobre la calidad de la injera; iii) aumentar la vida útil de la injera enriquecida mediante el uso de refrigeración y envasado en atmósfera modificada; y iv) analizar el efecto de la adición de EP en la injera sobre la aceptabilidad de la injera en una población local de Etiopía, y describir las características sensoriales e identificar los direccionadores de mejora de la aceptación de la injera enriquecida con EP.

Esta tesis se ha dividido por tanto en cinco capítulos. En el primero se realiza una revisión bibliográfica completa relacionada con la problemática nutricional etíope y las posibles soluciones a través de cambios en la dieta. Los otros cuatro capítulos están centrados en los distintos objetivos específicos experimentales.

Así, en el capítulo 2, se puso a punto la línea de procesamiento de injera y se definieron sus principales parámetros de calidad, tanto fisicoquímicos como físicos, microbiológicos y nutritivos. Los parámetros de calidad de la masa de la injera también se definieron en este capítulo. Los parámetros sensoriales se describen en el capítulo 5.

En el capítulo 3, la injera se fortificó con dos niveles de EP (4,5% y 9% de EP respecto a la harina de teff, respectivamente identificados como I4.5 e I9.0) y se estudió su efecto sobre los parámetros de calidad definidos en el capítulo 2. El enriquecimiento con EP mejoró tanto el contenido en calcio total como el calcio biodisponible y sólo afectó ligeramente a la mayor parte de los parámetros de calidad. El calcio biodisponible pasó de 16.1 mg/100g (control) a 742.7 mg/100 g (I4.5) o a 1743.1 mg/100 g (I9.0). Se concluyó que con el consumo de 200 gramos de injera, una persona adulta completa el 60% de las necesidades diarias de calcio (RDA) si utiliza harina I4.5 y el 140% si utiliza harina I9.0.

El capítulo 4 se centró en el estudio de vida útil de la injera enriquecida utilizando una atmósfera modificada (MAP) o aire atmosférico y manteniendo el producto a temperatura ambiente o bajo refrigeración. La calidad microbiológica de la injera se analizó midiendo el crecimiento de levaduras y hongos y mesófilos. Además, parámetros fisicoquímicos y físicos fueron incluido en el estudio. Los resultados indicaron que el frío permitió conservar microbiológicamente la injera durante más de 20 días, aunque tuvo un impacto negativo sobre la textura probablemente debido a la retrogradación del almidón. El uso de tecnología MAP mejoró la conservación, pero supuso un aumento de la vida útil corto.

Por último, se llevó a cabo un análisis sensorial de injera enriquecida en Hawassa, Etiopía, que se describe en el capítulo 5. Así, se llevó a cabo con consumidores reales de injera en Etiopía. Los resultados mostraron que la injera enriquecida con EP era aceptada por los consumidores. Sin embargo, el gusto por la injera disminuyó al adicionar EP. Los atributos que más afectan a la aceptación global fueron la “arenilla” y el tamaño de los ojos siendo necesaria la no “arenilla” y un tamaño medio de los ojos para no causar una disminución de la aceptación. La cantidad de calcio añadido que pueden detectar un 50% de los consumidores resultó ser de un 7.44% para el tamaño de los ojos y un 2.5% para la presencia de “arenilla”.

En conclusión, el presente estudio mostró que el EP puede usarse para mejorar el contenido de calcio de la injera y para mejorar el contenido en calcio biodisponible de la dieta en Etiopía. Se debe seguir trabajando para mejorar los parámetros de calidad de la injera que se vieron afectadas por la fortificación con EP.

CHAPTER 1
INTRODUCTION

CHAPTER 1. INTRODUCTION

Nutrition situation in Ethiopia

1.1.1 Undernutrition

Ethiopia is one of the most populated countries in Africa. Basic statistics about the Ethiopian population is as follows: the total population is about 115M; the fertility rate is 4.3 (live birth per women); the life expectancy is 67.8 years; infant mortality rate is 29.5 (infant death per 1000 live birth) and death of under five years of age is 44.0 (per 1000 live birth). Majority of the population, 78.7%, live in the rural part of Ethiopia being agriculture their main means of living. (World Bank, 2020).

Malnutrition, especially undernutrition, is a huge problem in Ethiopia affecting people's health and well-being. Before stating the nutrition situation in Ethiopia, it is important to define some terms used in this topic. Under nutrition includes stunting (height for age is low), under-weight (weight for age is low), wasting (weight for height is low) and inadequate intakes of vitamins and minerals. Stunting is the result of chronic or recurrent undernutrition that usually holds back children from reaching their physical and cognitive potential. Wasting indicates recent and severe weight loss, because inadequate food intake and/or because of infectious disease. A young child who is moderately or severely wasted has an increased risk of death, but treatment is possible. Underweight child may be stunted, wasted, or both.

Undernutrition exists in a higher percentage in under age five children in Ethiopia. The recent Ethiopian demographic and health survey (EDHS) report showed that the prevalence of stunting, underweight and wasting in under age five children is 37%, 21% and 7% (EPHI and ICF, 2021). The percentage of the children with those three forms of undernutrition are decreasing from time to time, shown in Table 1.1. However, according to the WHO classification based on the severity of the problem, the country is still under the classification of high prevalence rate for stunting problem (UNICEF, WHO and World Bank, 2019).

Table 1.1 Trends in the nutritional status of children under age five children

	EDHS data (%)			
	2005	**2011**	**2016**	**2019**
Stunted	51	44	38	37
Underweight	33	29	23	21
Wasted	12	10	10	7

Source: EDHS, 2019

In addition to the national level studies conducted, research done in the different parts of the country showed that undernutrition is an issue for Ethiopian children. Tariku *et al.*, (2014) found a very high prevalence of stunting, underweight and wasting in under age five children which were 60.6%, 31.1%, 12.6% respectively in Amhara region. A relatively similar level of stunting to that of the national data was found in a study conducted in Oromia region in children of 6-11 months old, 36%. However, the prevalence of underweight and wasting were higher, 41% and 13% respectively (Umeta *et al.*, 2003). A study conducted by Haileselassie *et al.* (2022) showed that the prevalence of stunting, underweight and wasting of under two age children were 41.4%, 21.6%, and 8.6% respectively. In another study, which was conducted in Southern Ethiopia, the prevalence of stunting in under two years children was very close to the national data which was 38% (Gunaratna, Moges and Groote, 2019).

The problem of undernutrition also appears in male and female adults. The EDHS 2016 data showed that 32.3% of men and 22.4% of women are undernourished (BMI, body mass index, < 18.5) (CSA, 2016). A study conducted in Gondar, Ethiopia, showed that underweight prevalence was relatively lower in men, 8.9% than in women, 14.5% (Amare *et al.*, 2012).

As it has been mentioned above, micronutrients deficiencies are among the forms of undernutrition. The minerals calcium, iron, zinc, iodine, and the vitamins A, C, B_{12}, folic acid are among the micronutrients which are important for the growth and the health of the body. Those nutrients deficiencies refer to micronutrients deficiencies. The deficiencies of the micronutrients usually can lead to hidden hunger in which the person may seem well nourished but in fact he/she could be malnourished. Unless some clinical or biochemical tests were done, the malnutrition problem may not be seen.

These micronutrients deficiencies are seen in the country Ethiopia widely. The 2019 EDHS assessed and presented some selected nutritional problems of under two years children and mothers. It presented data related to dietary intake of the children as a means to show that whether or not the children were getting the required nutrients. According to the finding, only 33% and 24 % of the 6-23 months old children consumed vitamin A and iron rich foods respectively during the 24 hours before the interview (EPHI and ICF, 2021).

In addition, the report showed that only 11% of 6-23 months old children met the minimum standards with respect to all three Infant and Young Child Feeding (IYCF) practices (breastfeeding status, number of food groups, and times they were fed during the day or night before the survey). Fourteen percent of children had an adequately diverse diet (i.e., they are given foods from the appropriate number of food groups), and 55% were fed the minimum number of times appropriate for their age (EPHI and ICF, 2021). A result for 6-23 months old children found by Haileselassie *et al.* (2022) for adequate diet diversity was only 2.3%

which was much below the national data and appropriate meal frequency prevalence was 53.7%, which was a very close value to that of the national data.

In addition to the national level data concerning the problem of micronutrients deficiency, different pocket studies were conducted in the country. A study conducted among infant and young children in the pastoral communities of Ethiopia showed that the children had inadequate nutrient intakes of vitamin A, vitamin C, iron, zinc and calcium (Mengistu *et al.*, 2017). In addition to the above mentioned nutrients, Haileselassie et al. (2022) found riboflavin, thiamin, niacin, and folate inadequate intake by children age 6-23 months old.

For pregnant women, the national survey only assessed nutrient supplementation intake. According to the data 40% of women with a child born in the last 5 years did not take any iron tablets during their most recent pregnancy and only 11% of women took iron tablets for 90 days or more (EPHI and ICF, 2021), showing that the majority of the women are at risk for iron deficiency anaemia.

A study conducted to compare the change in nutrient intake of pregnant and non-pregnant women showed that both groups of people had high prevalence of inadequate intake of the nutrients calcium, zinc, thiamin, niacin, folate and vitamin C. In some of the nutrients the prevalence reached until 99-100% like the case of calcium, niacin and folate (Asayehu *et al.*, 2017). Similarly, a study conducted in Sidama, Southern Ethiopia, on pregnant women showed that the prevalence of inadequate of the nutrients calcium and zinc were 99% and 74% respectively (Abebe *et al.*, 2008).

1.1.2 Causes and consequences of malnutrition

The causes of the malnutrition in Ethiopia are similar to other parts of the world. UNICEF (2013) has established three categories for the causes of malnutrition. Those are basic, underlining and immediate causes of malnutrition. Basic causes refer to sociocultural, economic and political context issues. Underlying causes refer to issues related to food insecurity, inadequate feeding practices and inadequate health services. Whereas, the immediate causes of the malnutrition refer to inadequate dietary intake and disease.

Malnutrition has a direct impact on the health and wellbeing of the society. It is highly associated with a nation mortality and morbidity. The health problems that happen due to the nutrients deficiencies includes (but are not limited to) blindness (vitamin A), rickets and osteomalacia (vitamin D), hemorrhagic disease (vitamin K), beriberi (vitamin B1), niacin (pellagra), pernicious anaemia (B12), megaloblastic anaemia (folate), scurvy (vitamin C), anaemia (iron), weak bone and teeth (calcium), retarded growth (zinc), and mental retardation (iodine) (Nutrition Society, 2009). Malnutrition, in addition, has an intergenerational effect in which a malnourished mother give birth to a malnourished child and the vicious cycle continue making it difficult to break it.

Moreover, malnutrition has impact on education, a country economy and development. This is due to the mentally retarded growth of the nation and the costs that put to treat the malnutrition problem. One of the major evidences of the consequences of malnutrition on the Ethiopian population was reported by the work which was done under the title of the cost of hunger in Ethiopia. It assessed the social and economic impact of child under nutrition in Ethiopia. According to the work, Ethiopia has lost 16.5% of GDP in the year 2009 as a result of child undernutrition. Undernutrition was associated with increased clinical episodes and with 24% of child mortalities. Stunting in children was associated with a higher grade repetition rate and school dropout. 67% of adults in Ethiopia suffered from stunting as children which affected their work potential. Lastly, an estimated 4.8 billion working hours were lost in 2009 due to absenteeism from the workforce as a result of nutrition-related mortalities (COHA, 2009).

1.1.3 Double burden of malnutrition

The world is now facing the double burden of malnutrition, which includes both under and over nutrition problem; the same is true for Ethiopia. The problem of over nutrition, which is manifested as overweight and obesity, is increasing from time to time. A review study conducted by Kassie, Abate and Kassaw (2020), showed that the prevalence of overweight was increased after the year 2015 which was found to be 22.55% as compared to the prevalence before 2015 which was found to be 17.49 %. A relatively similar result of overweight was found by the study conducted in Gondar, Ethiopia, which was 21.3%. The prevalence of overweight in men, 25.7%, was relatively higher as compared with the prevalence in women, 19.6% (Amare *et al.*, 2012). These increased weights are the main risk factors for the development of chronic diseases such as hypertension, diabetes and coronary heart diseases, in some situations which are observed to be the leading causes of mortality in nations.

1.2 Calcium

1.2.1 Food sources and health benefits of calcium

Calcium is a micronutrient, which is found in a human body at a high level as compared to other micronutrients. The adult human body contains, on average, slightly more than 1 kg of calcium. Almost all, 99%, of calcium is found in bone and teeth, whereas, only 1% of the calcium is found in blood and cells (Heaney, 2008). The importance of calcium for bone mass has been studied in old times on animals like cats, rats and dogs and had showed that when the animals put on low calcium diets they lose bone mass and when they put on calcium rich foods, recovery was noted (Heaney, 2008).

Calcium is naturally found in different food sources such as green vegetables (broccoli, mustard greens), legumes, milk and dairy products, and canned fish with bones (salmon, sardines) (Gharibzahedi and Jafari, 2017). Among those food sources, milk and milk products are considered the best because of the level of the amount present and the high bioavailability of calcium. Nowadays fortified foods and calcium supplementations are also in use to meet daily calcium requirement (Fructuoso *et al.*, 2021; Verstappen, 2022).

Calcium has a function being the principal cation of bones, comprising slightly less than 40% of the mass of the bone mineral and slightly less than 20% of the dry weight of bone. Calcium exists in the bones, in a mineral form that is usually characterized as hydroxyapatite, i.e., $Ca_{10}(PO_4)_6(OH)_2$. Other constituents of bone minerals include carbonate, citrate, potassium, and magnesium, among other ionic species (Heaney, 2008).

In addition to the function for bone and teeth formation, calcium helps to relax and contract the muscles; critical in nerve functioning, health of immune system, blood clotting, cell adhesion, hormone and neurotransmitter release, blood pressure regulation, glycogen metabolism, and cell proliferation and differentiation (Gharibzahedi and Jafari, 2017; WHO and FAO, 2006).

Calcium is important in the prevention of certain chronic diseases. Consumption of adequate amount of calcium has importance in preventing pre-eclampsia and related diseases (Hofmeyr, Duley and Atallah, 2007). Hypertension affected 30% of the world's population and is one of the main risk factor for mortality worldwide (Verstappen, 2022). Currently, the prevalence of hypertension is increasing in low- and middle-income countries. The prevalence of hypertension in Ethiopia ranges from 9.3 – 30.3% for population based studies and 7 -37% for institution based studies (Legese and Tadiwos, 2020). A review study conducted by Verstappen (2022) showed that increase in calcium intake has a positive effect in the prevention of hypertension. The same research showed that the effectiveness of the prevention of hypertension increased as the level of calcium intake increased from 1000 – 1500 mg to more than 1500 mg. And no effect was observed for the intake level of less than 1000 mg. The authors conclude that increasing calcium intake leads to small but significant reductions in both systolic and diastolic blood pressure in people with normal blood pressure. This finding confirms the positive health benefits of adequate calcium intake on the general population.

Similarly, a review study was conducted by Hofmeyr *et al.* (2018) about the relation of calcium supplementation in the prevention of pre-eclampsia (pregnancy related hypertension) of women. Pre-eclampsia is evident as high blood pressure and high protein in the urine. It is a major cause of death in pregnant women and new-born babies worldwide. Preterm birth (birth before 37 weeks) is often caused by high blood pressure and is the leading cause of new-born deaths, particularly in low-income countries. Evidence from randomised controlled trials shows that calcium supplements in high doses at least 1000 mg daily during pregnancy help prevent pre-eclampsia and preterm birth and lower the risk of a woman dying or having serious problems related to high blood pressure in pregnancy. This is particularly for women from communities with low dietary calcium and those at increased risk of pre-eclampsia, which is the case in Ethiopia (Hofmeyr *et al.*, 2018).

World health organization (WHO) has put a direction that women has to have calcium supplementation before they become pregnant so that to prevent the problem of preeclampsia (WHO, 2020). However, Ethiopia did not start calcium supplementation program for pregnant women which puts high risk for preeclampsia cases of pregnant women (Gebreyohannes *et al.*, 2021; Jikamo *et al.*, 2021). Supplementation in general has less compliance and adherence because of the requirement for purchase of relatively expensive pre-packed form, and the need of an effective distribution system (Shlobin *et al.*, 2021; WHO and FAO, 2006). It is not uncommon that women become pregnant without realizing they are pregnant. This issue may affect the fact that women need to start calcium supplementation before they become pregnant which could affect the effectiveness of calcium supplementation program. A research work in Ethiopia mentioned that calcium fortification could be a solution to such a problem since calcium is added to their normal food regardless of the pregnancy status. In addition, people will not feel that they are taking something which is like medicine which reduces their interest to take it.

Calcium intake is associated with bone health. Osteoporosis, a disease characterized by reduced bone mass and thus increased skeletal fragility and susceptibility to fractures, is the most significant consequence of a low calcium status (WHO and FAO, 2006). The effect of inadequate intake of calcium in children resulted in nutritional rickets (WHO and FAO, 2006). A major mechanism by which calcium is recognized to influence bone strength is through its effect on bone mass. Because bone functions as the calcium nutrient reserve, it follows inexorably that any depletion of that reserve (or failure to produce the genetically programmed skeletal mass during growth) will carry with it a corresponding decrease in bone strength. Additionally, it has recently been shown that, at prevailing calcium intakes, bone remodelling occurs at a rate considerably in excess of the need to repair fatigue damage (Parfitt, 2004; Heaney, 2003). Randomized controlled trials in humans have been published, establishing that elevating calcium intake enhances bone acquisition during growth, slows bone loss in postmenopausal women, and reduces fracture rates at spine, hip, and other extremity sites (Heaney, 2008).

In healthy individuals, bone mineral density increases until about 30 years of age, and thereafter begins to decline. Low intakes during childhood and adolescence can reduce peak bone density and thus increase the risk of osteoporosis in adulthood. Adolescence is characterized by rapid growth and development next to the period of infancy. Such remarkable physical growth and development significantly increases needs for nutrients like calcium. At this stage bone mass is acquired at much higher rates than other stages and making adolescence a crucial time for osteoporosis prevention. It is also important during childhood (as these are periods of rapid skeletal growth) to ensure adequate consumption of calcium (WHO and FAO, 2006). The other critical stage of life for taking adequate consumption of calcium is postmenopausal women and the elderly whose rate of bone loss is high (WHO and FAO, 2006). However, it is important to emphasize that adequate intake of calcium throughout the life is important to prevent the problem of osteoporosis. The age of onset and severity of osteoporosis depends not only on the duration of inadequate calcium intakes, but also on a number of other factors, such as oestrogen levels, vitamin D status and level of physical activity (WHO and FAO, 2006).

Another aspect of consuming adequate amount of calcium is its relationship with the prevention of fluorosis, a condition resulted due to toxic level consumption of the nutrient fluorine. Fluorosis affects teeth health, and it results in weak and stained teeth. As a consequence, bones become weak and in severe conditions fluorosis can lead to immobility. In Ethiopia, in the rift valley, water has a toxic level of fluoride. Therefore, consuming such kinds of water would affect the health of bones and teeth of the people. Research findings revealed that adequate intake of calcium prevents the problem of fluorosis and lessen its adverse effects (Patel *et al.*, 2017; Srivastava *et al.*, 2017).

1.2.2 Calcium requirement

The calcium requirement can be defined as the intake needed to support genetically programmed acquisition of bone during growth and to sustain acquired bone during maturity and the declining years of life. Because of differing absorption and retention efficiencies, individuals will inevitably have differing requirements for a skeletal endpoint. A recommended dietary allowance (RDA) is a figure designed to be at about the 95th percentile of individual requirements. Hence, if everyone in a population were ingesting at least as much as the RDA, it would ensure that 95% of the population would be getting as much as they need (Heaney, 2008). The RDA based on the North American and Western European data is shown in Table 1.2. Ethiopia does not have country specific RDA for any nutrient.

Table 1.2 Recommended calcium allowances based on North American and western European data

Group	Recommended intake (mg/day)
Infants and children	
0–6 months	
Human milk	300
Cow milk	400
7–12 months	400
1–3 years	500
4–6 years	600
Adolescents	
10–18 years	1300a
Adults	
Females	
19 years to menopause	1000
Postmenopause	1300
Males	
19–65 years	1000
65+ years	1300
Pregnant women (last trimester)	1200
Lactating women	1000

a Particularly during the growth spurt.
Source: WHO/FAO, 2004

1.2.3 Calcium inadequate intake and factors that increased the risks

There is not a practical biochemical indicator for determination of the status of calcium in human body. Since serum calcium is regulated by a complex homeostatic mechanism, intake of calcium, which makes it an unreliable indicator of calcium status. In this condition, comparing calcium intake with recommended nutrient intakes (RNIs) is commonly used to express calcium deficiency (WHO and FAO, 2006). Measurements of bone mineral density (BMD) and bone mineral content (BMC) have provided an alternative means of assessing the likely extent of calcium deficiency in some countries. Other approaches include measuring markers of bone resorption in urine or plasma, which tend to be higher in calcium deficient individuals. Such methods are, however, relatively expensive. (WHO and FAO, 2006). Therefore, most of the research work done in Ethiopia were focused on the assessment of the adequacy of the intake of calcium.

In Ethiopia, the level of Ca intake by children and adults, as it has been shown by different research works, was much lower than the recommended level. A work done Tezera, Whiting, & Gebremedhin (2017) showed that calcium intake by children was below the recommended level. A study conducted on under-five children in Jima, Oromia region, also in Ethiopia, showed that the majority of the study participants were taking calcium below the recommended level (Belachew *et al.*, 2005). Similarly, a recent study conducted by Haileselassie *et al.* (2022) showed that almost 100% of children aged 6-23 months old have inadequate intake of calcium. Other researchers showed that there was an inadequate intake of calcium by women and men (Amare et al., 2012; Asayehu, Lachat, Henauw, & Gebreyesus, 2017). Therefore, the studies showed that there is problem with calcium intake in Ethiopia.

Consumption of foods rich in calcium from dairy products was found to be very low in Ethiopia. The national food consumption survey conducted in the country showed that relatively higher level of dairy products consumed by children as compared with that of adults which was found to be much lower than 10% (EPHI, 2013). In contrary a study conducted by Haileselassie *et al.* (2022) showed dairy products consumption in children aged 6-23 months was very low which was only 9%. Intakes of calcium will almost certainly fall below the recommended levels where dairy product intake is low. Dairy products supply 50–80% of dietary calcium in most industrialized countries, while foods of plant origin supply about 25%. The calcium content and contribution from most other foods is usually relatively small (WHO and FAO, 2006).

Foods from plant sources are known for having constituents that affect the bioavailability of calcium and other nutrients. The strongest known inhibitor of calcium absorption is dietary oxalate, followed by the presence of phytates. Oxalate is not an important factor in most diets (although it is high in spinach, sweet potatoes and beans) but phytates are often consumed in large amounts, for instance, in legumes and wholegrain cereals (WHO and FAO, 2006). High level of phytates consumption is an issue in Ethiopia since the foods consumed by most of the population is whole grain cereal based and mostly not from animal sources.

Factors that put people in Ethiopia at risk in relation to the level of calcium include high consumption of coffee and sodium. High level of caffeine, found in coffee, and sodium consumption affects negatively calcium availability (Heaney, 2008). In Ethiopia, there is a high production and consumption of coffee (Deribew and Woldegiorgis, 2021; Kuma, Tamiru and Belachew, 2021) . The consumption of sodium is also high compared to the recommendation by WHO (Challa *et al.*, 2017; WHO, 2012). Therefore, it is important to make sure that the people consume adequate amount of calcium to minimize the negative effect of those substances.

Food sensitivity and allergy are among the issues that affect the nutrient deficiency problems of the current world. Milk allergy and lactose intolerance are one of the factors which played a role for calcium inadequate intake since people with those problems cannot consume milk which is one of the best sources of calcium. In such conditions where there is limited consumption of dairy products, development of a food which rich in calcium is crucial to fulfil the intake of recommended level of calcium. The current research is focusing to contribute to the problem of calcium inadequate intake by fortifying injera, the local staple food in Ethiopia.

1.3 Food fortification

1.3.1 Fortification and its advantages for nutrients utilization

Methods used to tackle micronutrient deficiencies, one of which is calcium, include food fortification, food supplementation, biofortification, and dietary diversity (Gunaratna, Moges and Groote, 2019; WHO and FAO, 2006). The method used in the current research is food fortification. Fortification is "the practice of deliberately increasing the content of an essential micronutrient, i.e. vitamins and minerals (including trace elements) in a food, so as to improve the nutritional quality of the food supply and provide a public health benefit with minimal risk to health" (WHO and FAO, 2006).

Fortification of a staple food has the advantage that affects everyone, including the poor, pregnant women, young children, and populations that can never be completely covered by social services. In addition, this fortification reaches at-risk groups, such as the elderly and those who have an unbalanced diet. Food fortification is usually socially acceptable, requires no change in food habits, does not alter the characteristics of the food, can be introduced quickly, can produce nutritional benefits for the target population quickly, is safe, and can be a cost-effective way of reaching large target populations that are at risk of micronutrient deficiency (WHO and FAO, 2006).

In addition, specifically in the case of calcium, fortification increased availability and utilization by the body. This is because the use of small amount of calcium at different times throughout the day is more effectively absorbed by the human body than using large amount of calcium at a time like in the case of calcium supplementation. Bioavailability of calcium has been shown to increase when it is taken with food instead of on an empty stomach (Rafferty, Walters and Heaney, 2007). The tradition of consuming injera at least twice a day in a small size portion instead of consuming a large amount at one time is advantageous with respect to the use of calcium by the body.

Calcium fortificants listed by WHO and FAO (2006) are white or colourless, have bland taste (except calcium tartarate), have low cost and have bioavailability close to natural foods which is 10% -30%. Those criteria are in line with the current research work in that the eggshell powder calcium has similar characteristics with the listed fortificants. Concerning the cost issue, eggshell powder is a substance which is considered as a waste and its cost can be considered very low.

The concerns of food fortification are related to the rise in production costs through expenses as initial equipment purchases, equipment maintenance, increased production staff needs and quality control and assurance facilities (WHO and FAO, 2006). However, household fortification can minimize the raised issue. A study conducted in Eastern Africa concerning the use of eggshell powder as a source of calcium showed that there is a potential to use it at a household level (Bartter *et al.*, 2018).

1.3.2 Injera as a food vehicle of calcium fortification

According to WHO and FAO (2006), to ensure that the target population will benefit from a food fortification programme, an appropriate food vehicle that is widely consumed throughout the year by a large portion of the population at risk of must be selected. The practice from other countries like United Kingdom and United States showed that cereals like wheat are the chosen vehicles for calcium fortification (WHO and FAO, 2006). Cereals are chosen to fortify calcium since they are staples and are consumed at a large amount accommodating large quantities of added calcium, which requires much more than other nutrients. In addition to cereals, other foods like juices, milk, yoghurt, soy milk and other beverages are also fortified with calcium (WHO and FAO, 2006).

There are limited practices of fortification of nutrients in Ethiopia, the most common one being fortifying salt with iodine at industry level. However, home fortification of multiple micronutrients has been used and seen to be effective when addressing micronutrients deficiency (Geletu, Lelisa and Baye, 2019). In addition, this practice has been found to be effective in the prevention of micronutrients deficiency in other low and middle income countries in Africa, Asia, Latin America, and the Caribbean (Suchdev *et al.*, 2020). A similar practice can be used for the case of fortification of injera with eggshell powder to tackle the problem of calcium deficiency. Injera is a flat bread which is prepared with the cereal teff, ***Eragrostis tef*** (Zucc.) Trotter, and sometimes by mixing teff with other cereals. Around two third of the Ethiopian people consume injera daily. In addition, injera is consumed equally by all age groups of people in the country. Therefore, fortifying injera with eggshell powder could be a successful means to tackle the problem of calcium deficiency which affects most Ethiopian people.

1.4 The cereal teff used for the preparation of injera

Teff is a tropical cereal cultivated mostly in Ethiopia and other countries adapted it for food consumption or animal feed (Bultosa, 2015). Teff plant look like a grass; the color of the most common teff grains are white, creamy white, light brown, or dark brown (Bultosa, 2015). Teff can be adapted to a wide range of environments, that is to moisture stress, high rainfall, different soil types, and a wide range of altitudes from near sea level to over 3000 m. Thus, it is a promising cereal for the current global problem related to climate change. Teff is known to have fewer disease and pest problems in the field than other cereals. In addition, the small size of the grain played a role to be less prone to attacks by pests during storage. Therefore, it can be safely stored for a long time under traditional storage conditions (Bultosa, 2015). Such characteristics of teff is crucial for countries like Ethiopia where there is huge amount of postharvest loss and food insecurity problem.

Regarding the nutritional quality of teff, the amount of the carbohydrate, protein, and fat are like other cereals. However, the protein composition shows teff is richer than the limiting essential amino acid lysine as compared with other cereals (Sharma and Chauhan, 2018). In addition, teff is a gluten free cereal which is important for those who have celiac disease or are gluten intolerant (Shumoy, Pattyn and Raes, 2018). Some researchers showed that teff has an advantage for diabetic people since it has low glycemic index carbohydrates which contributes to lowering of blood glucose level (Sharma and Chauhan, 2018). Since the grain is very small in size, the grain is milled with the husk leading to an advantage of being fiber rich food contributing to body weight control and prevention of chronic diseases like hypertension.

The grain is also known for having relatively higher amount of the minerals such as Fe and Ca as compared with the other common cereals like wheat, maize, barley and wheat (Baye, 2014). Researchers showed there was an improvement in anemia through the consumption of teff. However, there was a controversy related to the effect since the majority of the Fe is believed to come from soil contamination because the traditional processing method practiced in Ethiopia during harvesting usually makes Fe less bioavailable (Abebe *et al.*, 2007).

Teff has high phytate content of which causes binding of the minerals making them unavailable for absorption by the human body. The phytate content of whole red teff grain was found to be 675 mg/100g, while in whole white teff grain was 842 mg/100g (Abebe *et al.*, 2007). These high phyate levels of teff have been shown to affect the bioavailability of iron and zinc (Abebe *et al.*, 2007; Fischer *et al.*, 2014). Research has been conducted to assess the in-vitro bioavailability of iron and zinc of injera, but there was no finding concerning calcium (Shumoy *et al.*, 2017).

Injera is a fermented food that is circular in shape, having sour taste, soft texture, and many "eyes" (honeycomb like structure) on the top. It is prepared from different cereals but best quality injera is prepared from the cereal teff. The ingredients for injera are cereal flour, irsho and water. In the traditional preparation of injera, irsho, a backslide from the previous batter, will be added to the batter to be used as a starter culture and to help the batter fermentation.

1.5 Eggshell

1.5.1 Composition and preparation of eggshell powder

Eggshell from chicken egg is rich in calcium as a form of calcium carbonate. In addition, it has other nutrients such as Fe, Zn, Mg, P and protein (Schaafsma *et al.*, 2000; Hassan, 2015). Brun *et al.* (2013) found that one eggshell had a weight of 5.4 ± 0.79 g which contained 2.07 ± 0.18 g of Ca (381 ± 89 mg Ca/g eggshell). The organic matter was 16.1±4.6 g/100 g and the inorganic matter was 83.9±5.0 g/100 g chicken eggshell. The percentage of $CaCO_3$ in eggshell ranges from 93 to 97 (Kristl *et al.*, 2019). The result from a study by Aquipucho *et al.* (2020) showed that the calcium content in eggshell powder was 394.6 mg/g. Other researcher found the value of calcium in eggshell powder ranges of 385 – 401 mg/g which was close to the other two researchers (Schaafsma *et al.*, 2000) .

Not only eggshell powder is known for its high level of calcium, but it is also considered as having a high bioavailable calcium. According to Brun *et al.* (2013) calcium absorption from a diet containing chicken eggshell powder (45.6%±14.4%) was not different from a diet where $CaCO_3$ (39.9%±16.1%) was used as calcium supplement.

A piglet study was conducted by Schaafsma and Beelen (1999) to assess the digestibility of calcium from eggshell powder calcium and pure $CaCO_3$ based on two diets, casein based and soy protein isolate-based. The finding showed there was no significant difference in the digestibility of the two calcium source foods when casein-based foods were used. However, there was a significant difference when soy protein isolate-based diets were used, in which better digestibility was found for eggshell powder. Similarly, Daengprok *et al* (2003) showed that eggshell proteins help improve calcium absorption, in which total Ca transport across Caco-2 monolayers showed an increase of 64% in the presence of soluble eggshell matrix proteins.

Different researchers have shown that preparation of eggshell powder is simple and can be done at a household level. Simple households' utensils such as oven, and grinding machine that can be used to prepare eggshell powder. Brun *et al.* (2013) has prepared eggshell powder by using household utensils. The process of preparation includes drying, milling, and sieving. In addition, the dissolution of the powder was tested in the acid solutions of vinegar, lemon or orange juice for 48 h. Results showed that more amount of orange juice was required for dissolving the powder and smaller for vinegar. Aquipucho *et al.* (2020) prepared eggshell powder by collecting the eggshell from restaurants, dealerships, bakeries, food trucks, and so on. Washing and disinfecting with a 100-ppm sodium hypochlorite concentration for 5 min was the first step of the powder preparation. Then it continued with drying at 120 °C for 60 min, grinding and finished sieving through a mesh sieve with an opening of 106 μm.

A research in sub-Sahara Africa showed that there is a great potential of using eggshell powder as a calcium source with a low cost (Bartter *et al.*, 2018). This is because there was an indication of general acceptance of the approach and a willingness by the study participants to consider the incorporation of ground eggshells into their diets. In addition, 85% of rural communities in the sub-Saharan African countries keep poultry, making accessibility for the use of eggshell powder as a calcium source even if so far it has been considered as a waste.

1.5.2 Food uses of eggshell powder and its effect on food quality

Foodstuffs like breaded fried meat, bread, pizza, spaghetti, stew, rise with vegetables, and corn flour have already been fortified with eggshell powder (Brun *et al.*, 2013). The nutritional quality of those foods improved due to the eggshell powder addition. Mostly, there was no adverse effect of the addition of eggshell powder on food qualities. However, some researchers showed that its addition has affected some quality parameters.

Brun *et al.* (2013) tested the use of eggshell powder for different foodstuff at a household level by preparing the eggshell powder either by a mixer mill machine or a rolling pin in combination with a sieve. There was no difference on flavour qualities for all the food products tested based on the two milling methods used. However, there was a minimal change in the texture in most of the food products. When eggshell powder was prepared by a rolling pin the sensory panels detected a moderate change on stew and rice with vegetables; and unacceptable level on corn flour. In contrary, when mixer mill was used, the texture difference was minimal for the stew, rice with vegetables, and moderate for corn flour, whereas there was no difference for the other foods. This finding showed that the quality of food, especially texture, could be affected by particle size of eggshell powder.

The use of eggshell powder at a level of 3%, 6%, and 9% for the development of biscuit resulted in the improvement of total calcium to about 607, 1378 and 2175 mg/100g respectively, and the bioavailability turned into 26, 35 and 41%, respectively (Hassan, 2015). Results for sensory quality showed no significant effect on colour for all the products, and a significant effect on flavour, taste, and overall acceptability for 9% eggshell powder added product. The study conducted by Platon *et al.* (2020) on the quality of bread by the addition of eggshell powder at a level of 0.5% – 2% showed the process had improved the nutrient content, aging and flavor qualities, but the bread had a chewing inconvenience. In addition, bread with added eggshell powder presented elastic crumb, had uniform color and the knife blade stayed clean. Other study added nanopowdered eggshell in yoghurt and assessed the qualities at different storage times (Al Mijan, Choi and Kwak, 2014). Results showed that there was no significant adverse effect on some yogurt quality parameters such as pH, viscosity, lactic acid bacteria count, colour, and sensory evaluation.

1.5.3 Health benefits of eggshell powder calcium

An animal study which was conducted on ovariectomized rats showed that eggshell calcium in their diets increased their bone mineral density (BMD) as compared to the control groups indicating that eggshell calcium could be good solution for bone loss (Omi and Ezawa, 1998). A similar effect of improving BMD of lumbar spine and proximal tibia was observed in rats when comparing the use of eggshell powder calcium and pure $CaCO_3$ (Hirasawa, Omi and Ezawa, 2001). In both cases the addition of vitamin D improves the BMD. Similarly, Omelka *et al.* (2021) studied the effect of eggshell calcium and its combinations with vitamin D on ovariectomy-induced bone loss in a rat model of osteoporosis. The finding showed that the BMD of the experimental rat group increased as compared to the control group, showing that eggshell powder has a positive effect on preventing osteoporosis.

There are limited studies on the effect of the use of eggshell powder on human beings. Schaafsma and Pakan (1999) found that there was an increase in BMD of lumbar spine and hip of osteoporotic women after receiving 3 g of eggshell powder enriched dairy-based supplement, together with vitamin D and magnesium supplement. Another study done on human beings, healthy late postmenopausal women, showed that there was an increase in MBD of neck bone when eggshell calcium was used in the diet (Schaafsma *et al.*, 2002). Mulualem *et al.* (2021) provided eggshell powder supplementation (at a level of 1000 mg/day for six months) to women with a problem of fluorosis. The finding showed that the eggshell powder supplementation significantly reduced urinary fluorine excretion. A reduction in fluorosis symptoms were observed as compared to the control group thus providing evidence for using this dietary calcium source for mitigation of fluorosis.

1.6 Sensory assessment of injera

Sensory assessment of foods has been used as a quality parameter for determining their acceptability. The attributes which are assessed using the sensory assessment methods include appearance, taste, flavour, and texture. Among the quality parameters of injera, sensory quality plays also a great role. These qualities include appearance (colour, size, and distribution of eyes), texture (softness, hardness, elasticity, cohesiveness, and grittiness), taste (sourness), and odour.

The sensory qualities of injera were assessed by different researchers and most of them used hedonic scale. However, the method has a limitation to express the full range of sensory experience (Lim, 2011). Assefa *et al.* (2018) used sensory assessment to see the effect of different milling methods on injera qualities. He used a sensory panel based on 10 persons to assess the quality parameters of injera such as colour, taste, odour, texture (degree of softness), injera number of eyes, eye size, eye distribution (eye uniformity), and top and bottom surface (degree of being powdery and sticky); overall acceptability was also evaluated. A score sheet was prepared using the selected descriptors. Each one of the attributes was evaluated using a 10-point numerical scale (0–9) anchored on both sides with verbal descriptions (i.e., 0=unpleasant, 9=pleasant) to allow the panel to score the intensity on a framed common scale.

The sensory quality effect of addition of quinoa on teff injera preparation was studied by Agza, Bekele and Shiferaw (2018) using 5-point hedonic method. A panel based on 30 persons was used to determine the injera attributes of colour, taste, aroma, odours, rollability, eyes evenness, underneath colour and overall acceptability. Similarly, Ghebrehiwot *et al.* (2016) used five point hedonic scale to assess the quality of sorghum blended teff injera which were taste, appearance, texture and overall acceptability using 10 panelists. Hassen *et al.* (2018) assessed the qualities of injera such as appearance, taste, aroma, mouth feel and overall acceptability while testing different starter culture for the fermentation of injera.

1.7 Preservation of injera

Shelf life is defined as the duration of time a food product maintains hygienic, nutritional, and sensory qualities above established life. Sensory qualities include aroma, taste, appearance, and texture (firmness, initial bite, mouthfeel) (McDonough, Alviola and Waniska, 2015). The freshness of bread can be lost due to certain physico-chemical reaction called stalling. These process leads to the loss of aroma, increase in rigidity of bread crumb, loss of water from the surfaces which overall makes bread be felt as a drier and firmer product (Goesaert *et al.*, 2009).

Like other types of bread, the shelf life of injera is short. There is few research reported focused on the shelf life of injera. When injera is stored at room temperature and without using any preservation methods, the shelf life is 2-4 days (Ashagrie and Abate, 2012; Hassen, Mukisa and Kurabachew, 2018).

According to European Parliament and Council Directive (1995) preservatives are ‘substances which prolong the shelf life of foodstuffs by protecting them against deterioration caused by micro-organisms. Propionic acid, sorbic acid, benzoic acid and their salts sodium propionate, calcium propionate, potassium propionate, sodium sorbate, calcium sorbate, potassium sorbate, sodium benzoate, calcium benzoate and calcium benzoate are commonly used in the preservation of bread. Sorbic acid/ its salts and propionic acid / its salts are among the preservatives used in bread. Benzoic acid and its salts are also among chemical preservatives but their safe use is mentioned for other products like fruit and vegetables rather than for bread (Commission Regulation (EU), 2013).

Propionic acid and propionates (salts) are widely used because they do have more effect on fungi than in bacteria and do not have effect on yeast (Silva and Lidon, 2016). Even though the use of propionic acid and its salts are widely used for bread preservation, the research done by Ashagrie and Abate (2012) showed that calcium propionate was not effective on extending the shelf life of injera. Shelf life of injera with calcium propoinate was 4-5 days whereas that of control injera was 3-4 days (Ashagrie and Abate, 2012).

The use of sorbic acid and its salts is limited as compared with the use of propionic acid and its salts since therefore affects the activity of yeasts in leavened dough even though its effect on mold is wider. In addition, this preservative has some positive characteristics: is is odorless, or flavorless when appropriate amount of 0.3 % is used, being effective against mold, yeast and bacteria, do not appear to have relevant side effect, and its salt forms are more soluble in water (Silva and Lidon, 2016). Ashagrie and Abate (2012) used potassium sorbate for the preservation of injera and could be able to improve the shelf life from 3-4 days to 6-8 days with the use of 0.2% even though the safe level is mentioned to be 0.3 %. They have added the preservative into the injera batter just before cooking which lowered the effect of the preservative during the fermentation process.

Based on the European Union Commission Regulation of 2013 neither benzoate acid nor sodium benzoate are found under the list of the allowed additives for bread preservation. But its use is mentioned for fruits and vegetables. The use of benzoate and benzoic acid has been proved to extend the shelf life of injera 10-12 days. Benzoic acid is more effective in acidic foods and injera is acidic food in which its pH is less than or equal to four (Ashagrie and Abate, 2012). That could be the reason why the use of benzoic acid in injera was more effective as compared with the other preservatives. One of the side effect up on the use of this preservative is its nutritive health side effects (Silva and Lidon, 2016).

Even if the previous work has shown that the shelf life of injera can be improved by the use of chemical preservatives, looking for other options is important. This is because injera is a staple food in Ethiopia and consumed daily at least twice a day by most of the people, using chemical preservatives could put the Ethiopian consumers to some kinds of health risks. Use of modified atmosphere packaging (MAP) and cold storage could be used as alternative. MAP is a type of preservation method that uses modifying the natural composition of air to control or reduce the growth of undesirable microorganisms in food and to keep other qualities like freshness of the product. Packaging materials that have a high capacity to prevent gas diffusion into or out of the package are used. First food will be put into a package and then the package will be fleshed with one type of gas or a combination of different types of gases after removing the air present in the package. This technique help prevent the growth of aerobes such as molds, yeast, and aerobic bacteria (Ray, 2005).

The common types of gas used in MAP are CO_2 and N_2 which could be used at different levels. N_2 is used as an inert gas whereas CO_2 has a preservative effect on the growth of microorganisms. According to Kotsianis, Giannou and Tzia (2002) the level of CO_2 has to be 20% or greater to be effective against microbial spoilage. The determination of the level of the use of the different gases depends on the product type which needs to be preserved. A study conducted by Marcin and Agnieszka (2019) proved the use of MAP at a level of 30% CO_2 and 70% N_2 was optimal storage condition for preserving bread with amaranth flour. The method allowed to preserve the qualities of bread more as compared with the use of

100% N_2 which was studied in the same research work. The qualities of bread such as volume, water content, brightness, and texture were better preserved when 30% CO_2 and 70% N_2 was used. Similarly, the use of 30% CO_2 and 70% N_2 improved the shelf life of cheesecake from 11 days to 40 days (Nicola et al., 2016). Els et al., (2018) used MAP for preservation of par-baked wheat bread at using a gas composition of 70% CO_2 and 30% N_2 and were able to extend the shelf life of the bread to about 13 days. Daphne and James (2003) used as high as 100% CO_2 to study the growth of *Clostridium botulinum* in yeast and chemically leavened crumpet.

Besides the use of different levels of CO_2 and N_2, the type of packages used has an influence on the shelf life. According to Smith (1993), food packages are required to have multi-functions in terms of chemical, physical, and biological alterations of the food to prolong its shelf life. Chemically, the packages should be able to control and/or prevent oxidation and some other chemical reactions, such as hydrolytic rancidity and Maillard reaction, in food products. The packages should maintain the moisture level in the products which is a critical parameter controlling bread staling and changes in texture and physical appearance. Most importantly, microbial growth in food during storage is a serious problem and a major cause to shorten food shelf life. Packages that can inhibit microbial proliferation are currently in demand (Settakorn, and Piyachat, 2018).

The packaging materials commonly used in the system of modified atmosphere packaging (MAP) are laminated film made of one layer of polyamide (nylon) and one layer of polyethylene (PA/PE), laminated films of PVDC/cellophane/PVDC/PE, laminated films of OPA and OPA/PE, and combinations of metalized polymers (Pasqualone, 2019). Els et al., (2018) used plastic bags (PA/ PE/20/70) (PA: polyamide; PE: polyethylene) (for air packaging) or packaged under modified atmosphere (MA) with a Tray Sealer using a gas composition of 70% CO_2 and 30% N_2. The breads were MA-packaged in a tray made of

PP/EVOH/PP (PP: polypropylene; EVOH: ethylene vinyl alcohol) and sealed with a cover film of OPA/PE/EVOH/PE/PP (OPA: orientated polyamide).

In any case, research work to preserve injera using MAP is limited. There was research work conducted on injera using vacuum packaging to improve the shelf life and staling (Terefe et al., 2022). In this research, injera was packed under vacuum using polyethylene bag and studied for 15 days to determine the microbial load, moisture content, pH, and color "L" value (lightness), visible mold growth, and sensory quality evaluation. According to the research, exclusion of oxygen from the package has prolonged the storage duration of injera without visible mold growth to more than 15 days. However, the sensory acceptability of injera was basically affected due to the crumbling effect of the packaging method.

1.6 References

Abebe, Y. *et al.* (2007) 'Phytate, zinc, iron and calcium content of selected raw and prepared foods consumed in rural Sidama, Southern Ethiopia, and implications for bioavailability', *Journal of Food Composition and Analysis*, 20(3–4), pp. 161–168. doi: 10.1016/j.jfca.2006.09.003.

Abebe, Y. *et al.* (2008) 'Inadequate intakes of dietary zinc among pregnant women from subsistence households in Sidama, Southern Ethiopia', *Public Health Nutrition*, 11(4), pp. 379–386. doi: 10.1017/S1368980007000389.

Agza, B., Bekele, R. and Shiferaw, L. (2018) 'Quinoa (Chenopodium quinoa, Wild.): As a potential ingredient of injera in Ethiopia', *Journal of Cereal Science*. Elsevier, 82(June), pp. 170–174. doi: 10.1016/j.jcs.2018.06.009.

Al Mijan, M., Choi, K. H. and Kwak, H. S. (2014) 'Physicochemical, microbial, and sensory properties of nanopowdered eggshell-supplemented yogurt during storage', *Journal of Dairy Science*. Elsevier, 97(6), pp. 3273–3280. doi: 10.3168/jds.2013-7367.

Amare, B. *et al.* (2012) 'Nutritional status and dietary intake of urban residents in Gondar, Northwest Ethiopia', *BMC Public Health*, 12(1). doi: 10.1186/1471-2458-12-752.

Aquipucho, K. *et al.* (2020) 'Production of Bread With Eggshell Powder and the Increase of Calcium Content in the Body', *Carpathian Journal of Food Science and Technology*, 12(1), pp. 80–88. doi: 10.34302/crpjfst/2020.12.8.

Asayehu, T. T. *et al.* (2017) 'Dietary behaviour, food and nutrient intake of women do not change during pregnancy in Southern Ethiopia', *Maternal and Child Nutrition*, 13(2), pp. 1–10. doi: 10.1111/mcn.12343.

Ashagrie, Z. and Abate, D. (2012) 'Improvement of Injera Shelf Life Through the Use of', *African Journal of Food, Agriculture, Nutrition and Development*, 12(5), pp. 6409–6423.

Assefa, Y. *et al.* (2018) 'Influence of milling type on tef injera quality', *Food Chemistry*, pp. 155–160. doi: 10.1016/j.foodchem.2018.05.126.

Bartter, J. *et al.* (2018) 'Use of chicken eggshell to improve dietary calcium intake in rural sub-Saharan Africa', *Maternal and Child Nutrition*, 14(February), pp. 1–10. doi: 10.1111/mcn.12649.

Baye, K. (2014) 'Synopsis : Teff : nutrient composition and health benefits', *Ethiopia Strategy Support Program*, (October), pp. 2013–2014.

Belachew, T. *et al.* (2005) 'Calcium deficiency and causation of rickets in Ethiopian children', *East African Medical Journal*, 82(3), pp. 153–159. doi: 10.4314/eamj.v82i3.9273.

Brun, L. R. *et al.* (2013) 'Chicken eggshell as suitable calcium source at home', *International Journal of Food Sciences and Nutrition*, 64(6), pp. 740–743. doi: 10.3109/09637486.2013.787399.

Bultosa, G. (2015) *Teff: Overview*. 2nd edn, *Encyclopedia of Food Grains: Second Edition*. 2nd edn. Elsevier Ltd. doi: 10.1016/B978-0-12-394437-5.00018-8.

Challa, F. *et al.* (2017) 'Urinary sodium excretion and determinates among adults in Ethiopia: Findings from National STEPS survey', *Ethiopian Journal of Health Development*, 31(Specialissue1), pp. 370–377.

COHA (2009) *The Cost of Hunger in Ethiopia Implications for the Growth and Transformation of Ethiopia*. doi: 10.13140/RG.2.2.15147.23841.

Commission Regulation (EU) (2013) 'Amending Annex II to Regulation (EC) No 1333/2008 of the European Parliament and of the Council by establishing a Union list of food additives', *Official Journal of the European Union*, (February 1998), pp. 1–5. Available at: https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CONSLEG:2006R1881:20100701:EN:PDF%0Ahttps://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A01985L0374-19990604&qid=1604918047856.

CSA, and I. (2016) *Ethiopian Demographic and Health Survey, Addis Ababa, Ethiopia, and Rockville, Maryland, USA: CSA and ICF*. doi: 10.2147/ahmt.s148434.

Daengprok, W. *et al.* (2003) 'Chicken Eggshell Matrix Proteins Enhance Calcium Transport in the Human Intestinal Epithelial Cells, Caco-2', *Journal of Agricultural and Food Chemistry*, 51, pp. 6056–6061.

Daphne Phillips Daifas and James P. Smith. (2003). Challenge studies with Proteolytic *Clostmdium botulinum* in yeast and chemically leavened crumpets packaged under modified atmospheres.

Deribew, H. A. and Woldegiorgis, A. Z. (2021) 'Acrylamide levels in coffee powder, potato chips and French fries in Addis Ababa city of Ethiopia', *Food Control*. Elsevier Ltd, 123(October 2020), p. 107727. doi: 10.1016/j.foodcont.2020.107727.

Els Debonne, Filip Van Bockstaele, Manon Van Driessche, Ingrid De Leyn, Mia Eeckhout, and Frank Devlieghere.. (2018). Impact of par-baking and packaging on the microbial quality of parbaked wheat and sourdough bread. Food Control 91 (12-19).

EPHI (2013) *Ethiopia National Food Consumption Survey*, *Ethiopian Public Health Institute*.

Ethiopian Public Health Institute (EPHI) and ICF (2021) *Ethiopia Mini Demographic and Health Survey 2019: Final Report*. Available at: https://dhsprogram.com/pubs/pdf/FR363/FR363.pdf.

European Parliament and Council Directive (1995) 'European Parliament and Council Directive No 95/2/EC of 20 February 1995 on food additives other than colours and sweeteners', *Official Journal of the European Union*, L61(3600), pp. 1–40. Available at: https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX%3A31995L0002.

Fischer, M. M. *et al.* (2014) 'Phytic acid degrading lactic acid bacteria in tef-injera fermentation', *International Journal of Food Microbiology*. Elsevier B.V., 190, pp. 54–60. doi: 10.1016/j.ijfoodmicro.2014.08.018.

Fructuoso, I. *et al.* (2021) 'An overview on nutritional aspects of plant-based beverages used as substitutes for cow's milk', *Nutrients*, 13(8), pp. 9–12. doi: 10.3390/nu13082650.

Gebreyohannes, R. D. *et al.* (2021) 'Association of dietary calcium intake, total and ionized serum calcium levels with preeclampsia in Ethiopia', *BMC Pregnancy and Childbirth*. BioMed Central, 21(1), pp. 1–7. doi: 10.1186/s12884-021-04005-y.

Geletu, A., Lelisa, A. and Baye, K. (2019) 'Provision of low-iron micronutrient powders on alternate days is associated with lower prevalence of anaemia, stunting, and improved motor milestone acquisition in the first year of life: A retrospective cohort study in rural Ethiopia', *Maternal and Child Nutrition*, 15(3), pp. 1–9. doi: 10.1111/mcn.12785.

Gharibzahedi, S. M. T. and Jafari, S. M. (2017) 'The importance of minerals in human nutrition: Bioavailability, food fortification, processing effects and nanoencapsulation', *Trends in Food Science and Technology*. Elsevier Ltd, 62, pp. 119–132. doi: 10.1016/j.tifs.2017.02.017.

Ghebrehiwot, H. M. *et al.* (2016) 'Nutritional and sensory evaluation of injera prepared from tef and Eragrostis curvula (Schrad.) nees. flours with sorghum blends', *Frontiers in Plant Science*, 7(JULY2016), pp. 1–8. doi: 10.3389/fpls.2016.01059.

Goesaert, H. *et al.* (2009) 'Amylases and bread firming - an integrated view', *Journal of Cereal Science*. Elsevier Ltd, 50(3), pp. 345–352. doi: 10.1016/j.jcs.2009.04.010.

Gunaratna, N. S., Moges, D. and Groote, H. De (2019) 'Biofortified maize can improve quality protein intakes among young children in southern Ethiopia', *Nutrients*, 11(1), pp. 1–15. doi: 10.3390/nu11010192.

Haileselassie, M. *et al.* (2022) 'The influence of fasting on energy and nutrient intake and their corresponding food sources among 6-23 months old children in rural communities with high burden of stunting from Northern Ethiopia', *Nutrition Journal*. BioMed Central, 21(1), pp. 1–15. doi: 10.1186/s12937-022-00759-z.

Hassan, N. M. M. (2015) 'Chicken Eggshell Powder as Dietary Calcium Source in Biscuits', *World Journal of Dairy & Food Sciences*, 10(2), pp. 199–206. doi: 10.5829/idosi.wjdfs.2015.10.2.1152.

Hassen, Y. *et al.* (2018) 'Evaluation of Yeast and Lactic Acid Bacteria Starter Cultures for the Production of Rice Injera', *Journal of Food Processing & Technology*, 09(03). doi: 10.4172/2157-7110.1000721.

Hassen, Y., Mukisa, I. M. and Kurabachew, H. (2018) 'The Effect of Selected Chemical Preservatives and Starter Cultures on the Sensory Characteristics and Shelf Life of Rice Injera', *Journal of Bioprocessing & Biotechniques*, 08(01). doi: 10.4172/2155-9821.1000318.

Heaney, R. P. (2003) 'Is the paradigm shifting?', *Bone*, 33(4), pp. 457–465. doi: 10.1016/S8756-3282(03)00236-9.

Heaney, R. P. (2008) 'Calcium', in *Principles of Bone Biology*. 3rd edn. Academic Press, pp. 1697–1710.

Hirasawa, T., Omi, N. and Ezawa, I. (2001) 'Effect of 1α-hydroxyvitamin D3 and egg-shell calcium on bone metabolism in ovariectomized osteoporotic model rats', *Journal of Bone and Mineral Metabolism*, 19(2), pp. 84–88. doi: 10.1007/s007740170045.

Hofmeyr, G. J. *et al.* (2018) 'Calcium supplementation during pregnancy for preventing hypertensive disorders and related problems', *Cochrane Database of Systematic Reviews*, 2018(10). doi: 10.1002/14651858.CD001059.pub5.

Hofmeyr, G. J., Duley, L. and Atallah, A. (2007) 'Dietary calcium supplementation for prevention of pre-eclampsia and related problems: A systematic review and commentary', *BJOG: An International Journal of Obstetrics and Gynaecology*, 114(8), pp. 933–943. doi: 10.1111/j.1471-0528.2007.01389.x.

Jikamo, B. *et al.* (2021) 'Cultural adaptation and validation of the Sidamic version of the World Health Organization Quality-of-Life-Bref Scale measuring the quality of life of women with severe preeclampsia in southern Ethiopia, 2020', *Health and Quality of Life Outcomes*. BioMed Central, 19(1), pp. 1–9. doi: 10.1186/s12955-021-01872-z.

Kassie, A. M., Abate, B. B. and Kassaw, M. W. (2020) 'Prevalence of overweight/obesity among the adult population in Ethiopia: A systematic review and meta-analysis', *BMJ Open*, 10(8), pp. 1–10. doi: 10.1136/bmjopen-2020-039200.

Kristl, M. *et al.* (2019) 'Evaluation of calcium carbonate in eggshells using thermal analysis', *Journal of Thermal Analysis and Calorimetry*, 138(4), pp. 2751–2758. doi: 10.1007/s10973-019-08678-8.

Kotsianis I.S., Giannou V. and Tzia. C. (2002). Production and packaging of bakery products using MAP technology Trends in Food Science & Technology 13(319–324).

Kuma, M. N., Tamiru, D. and Belachew, T. (2021) 'Hemoglobin Level and Associated Factors among Pregnant Women in Rural Southwest Ethiopia', *BioMed Research International*, 2021. doi: 10.1155/2021/9922370.

Legese, N. and Tadiwos, Y. (2020) 'Epidemiology of hypertension in ethiopia: A systematic review', *Integrated Blood Pressure Control*, 13, pp. 135–143. doi: 10.2147/IBPC.S276089.

Lim, J. (2011) 'Hedonic scaling: A review of methods and theory', *Food Quality and Preference*. Elsevier Ltd, 22(8), pp. 733–747. doi: 10.1016/j.foodqual.2011.05.008.

Marcin A Kurek and Agnieszka Krzemin´ska. (2019). Effect of modified atmosphere packaging on quality of bread with amaranth flour addition. Food Science and Technology International 26(1) 44–52. 10.1177/1082013219864197 journals.sagepub.com/home/fst

McDonough, C. M., Alviola, J. N. and Waniska, R. D. (2015) *Preservatives: Extending Shelf Life and Shelf Stability*. 1st edn, *Tortillas: Wheat Flour and Corn Products*. 1st edn. Elsevier Ltd. doi: 10.1016/B978-1-891127-88-5.50009-8.

Mengistu, G. *et al.* (2017) 'Energy and nutrient intake of infants and young children in pastoralist communities of Ethiopia', *Nutrition*. Elsevier Inc., 41, pp. 1–6. doi: 10.1016/j.nut.2017.02.012.

Mulualem, D. *et al.* (2021) 'Efficacy of calcium-containing eggshell powder supplementation on urinary fluoride and fluorosis symptoms in women in the ethiopian rift valley', *Nutrients*, 13(4). doi: 10.3390/nu13041052.

Nicola Secchi, Costantino Fadda, Manuela Sanna, Paola Conte, Alessandra Del Caro, Pasquale Catzeddu, Antonio Piga. (2016). Effectiveness of modified atmosphere packaging and ovine whey powder in extending the shelf life of whey cheesecakes. LWT - Food Science and Technology journal homepage: www.elsevier.com/locate/lw

Nutrition Society (2009) *Introduction to Human Nutrition*, *Sports Nutrition*. Edited by M. J. Gibney et al. WILEY BLACKWELL. doi: 10.1002/9781118692318.ch1.

Omelka, R. *et al.* (2021) 'The effects of eggshell calcium (Biomin H®) and its combinations with alfacalcidol (1α-hydroxyvitamin D3) and menaquinone-7 (vitamin K2) on ovariectomy-induced bone loss in a rat model of osteoporosis', *Journal of Animal Physiology and Animal Nutrition*, 105(2), pp. 336–344. doi: 10.1111/jpn.13458.

Omi, N. and Ezawa, I. (1998) 'Effect of Egg-Shell Ca on Preventing after Ovariectomy and doing moderate physical activity are necessary been sufficient in Japan, the Ca intake in particular has never been sufficient (Ministry of Health and Welfare 1997). The Ca intake has nearly re', *Journal of Home Economics*, 49(3), pp. 277–282.

Parfitt, A. M. (2004) 'What is the normal rate of bone remodeling?', *Bone*, 35(1), pp. 1–3. doi: 10.1016/j.bone.2004.03.022.

Pasqualone, A. (2019). Bread packaging: Features and functions. In Flour and Breads and Their Fortification in Health and Disease Prevention, 211–222. https://doi.org/10.1016/B978-0-12-814639-2.00017-4

Patel, Prerna *et al.* (2017) 'Association of dental and skeletal fluorosis with calcium intake and serum Vitamin D concentration in adolescents from a region endemic for fluorosis', *Indian Journal of Endocrinology and Metabolism*, 21(1), pp. 190–195. doi: 10.4103/2230-8210.196013.

Platon, N. *et al.* (2020) 'White bread fortified with calcium from eggshell powder', *Revista de Chimie*, 71(7), pp. 299–306. doi: 10.37358/RC.20.7.8248.

Rafferty, K., Walters, G. and Heaney, R. P. (2007) 'Calcium fortificants: Overview and strategies for improving calcium nutriture of the U.S. population', *Journal of Food Science*, 72(9). doi: 10.1111/j.1750-3841.2007.00521.x.

Schaafsma, A. *et al.* (2000) 'Mineral, amino acid, and hormonal composition of chicken eggshell powder and the evaluation of its use in human nutrition', *Poultry Science*. Poultry Science Association, Inc., 79(12), pp. 1833–1838. doi: 10.1093/ps/79.12.1833.

Schaafsma, A. *et al.* (2002) 'Positive effects of a chicken eggshell powder-enriched vitamin–mineral supplement on femoral neck bone mineral density in healthy late post-menopausal Dutch women', *British Journal of Nutrition*. Universidad Publica de Navarra, 87(3), pp. 267–275. doi: 10.1079/bjn2001515.

Schaafsma, A. and Beelen, G. M. (1999) 'Eggshell powder, a comparable or better source of calcium than purified calcium carbonate: Piglet studies', *Journal of the Science of Food and Agriculture*, 79(12), pp. 1596–1600. doi: 10.1002/(SICI)1097-0010(199909)79:12<1596::AID-JSFA406>3.0.CO;2-A.

Schaafsma, A. and Pakan, I. (1999) 'Short-term effects of a chicken egg shell powder enriched dairy-based products on bone mineral density in persons with osteoporosis or osteopenia.', *Bratislavské lekárske listy*, 100(12), pp. 651–656.

Settakorn Upasen, and Piyachat Wattanachai. 2018. Packaging to prolong shelf life of preservative-free white bread. Heliyon, 4:e00802

Sharma, K. and Chauhan, E. S. (2018) 'Nutritional composition , physical characteristics and health benefits of teff grain for human consumption : A review', *The Pharma Innovation Journal*, 7(10), pp. 3–7.

Shlobin, N. A. *et al.* (2021) 'Folate fortification and supplementation in prevention of folate-sensitive neural tube defects: a systematic review of policy', *J Neurosurg Pediatr*, 27(March), pp. 294–310. doi: 10.3171/2020.7.PEDS20442.J.

Shumoy, H. *et al.* (2017) 'Traditional fermentation of tef injera: Impact on in vitro iron and zinc dialysability', *Food Research International*. Elsevier, 102(June), pp. 93–100. doi: 10.1016/j.foodres.2017.09.092.

Shumoy, H., Pattyn, S. and Raes, K. (2018) 'Tef protein: Solubility characterization, in-vitro digestibility and its suitability as a gluten free ingredient', *LWT - Food Science and Technology*. Elsevier, 89(November 2017), pp. 697–703. doi: 10.1016/j.lwt.2017.11.053.

Silva, M. M. and Lidon, F. C. (2016) 'Food preservatives - An overview on applications and side effects', *Emirates Journal of Food and Agriculture*, 28(6), pp. 366–373. doi: 10.9755/ejfa.2016-04-351.

Smith J.P. (1993). Bakery products, in: R.T. Parry (Ed.), Principles and Applications of Modified Atmosphere Packaging of Foods, Springer, Boston, MA.

Srivastava, A. K. *et al.* (2017) 'Prevalence of dental fluorosis and the role of calcium supplementation in its prevention', *Journal of Medical Sciences (Faisalabad)*, 17(4), pp. 156–161. doi: 10.3923/jms.2017.156.161.

Suchdev, P. S. *et al.* (2020) 'Home fortification of foods with multiple micronutrient powders for health and nutrition in children under two years of age', *Cochrane Database of Systematic Reviews*, 2020(2). doi: 10.1002/14651858.CD008959.pub3.

Tariku, B. *et al.* (2014) 'Prevalence and Risk Factors of Child Malnutrition in Community Based Nutrition Program Implementing and Nonimplementing Districts from South East Amhara, Ethiopia', *OALib*, 01(03), pp. 1–17. doi: 10.4236/oalib.1100425.

Terefe A., Emire S.A., Gemede H.F., & Woldegiorgis A.Z. (2022). Improvement of Injera Shelf Life and Staling through Vacuum and Nonvacuum Polyethylene Packaging: Their Synergistic Effect with Chemical Preservative. Journal of Food Quality. https://doi.org/10.1155/2022/8972355.

Tezera, F., Whiting, S. J. and Gebremedhin, S. (2017) 'Dietary calcium intake and sunlight exposure among children aged 6-23 months in Dale Woreda, Southern Ethiopia', *African Journal of Food, Agriculture, Nutrition and Development*, 17(3), pp. 12427–12440. doi: 10.18697/ajfand.79.16510.

Umeta, M. *et al.* (2003) 'Factors associated with stunting in infants aged 5-11 months in the Dodota-Sire District, Rural Ethiopia', *Journal of Nutrition*, 133(4), pp. 1064–1069. doi: 10.1093/jn/133.4.1064.

UNICEF (2013) *Improving child nutrition.*, *UNICEF*.

Verstappen, A. (2022) 'Calcium Supplementation for the Prevention of Primary Hypertension: Summary of a Cochrane review', *Explore*. Elsevier Inc., 18(1), pp. 131–132. doi: 10.1016/j.explore.2021.10.006.

WHO (2012) *Guideline: Sodium intake for adults and children*, *World Health Organization*. Available at: http://apps.who.int/iris/handle/10665/77985%5Cnhttp://www.ncbi.nlm.nih.gov/pubmed?term=Sodium%5BTitle%5D AND intake%5BTitle%5D AND adults%5BTitle%5D AND children%5BTitle%5D AND WHO%5BTitle%5D%5Cnhttp://www.ncbi.nlm.nih.gov/pubmed?term=Sodium%255BTitle%255D AND.

WHO and FAO (2006) *Guidelines on food fortification of foods with micronutrients*, *Unscn.Org*. Available at: http://www.unscn.org/layout/modules/resources/files/fortification_eng.pdf.

World Bank (2020) 'World Bank 2020 Report'. doi: https://data.worldbank.org/indicator/SP.POP.TOTL.FE.ZS?locations=ET.

World Health Organization (2020) *Calcium supplementation before pregnancy for the prevention of pre-eclampsia and its complications*. Available at: https://apps.who.int/iris/bitstream/handle/10665/331787/9789240003118-eng.pdf?ua=1.

Yetneberk, S. *et al.* (2004) 'Effects of Sorghum Cultivar on Injera Quality', *Cereal Chemistry*, 81(3), pp. 314–321. doi: 10.1094/CCHEM.2004.81.3.314.

CHAPTER 2.
QUALITY PARAMETERS OF INJERA

CHAPTER 2. QUALITY PARAMETERS OF INJERA

2.1 Abstract

Injera is a fermented flat bread, which is prepared by the cereal teff, *Eragrostis tef* (Zucc.) Trotter. Most researchers described the quality of injera based on sensory assessment, even though some quality parameters were studied instrumentally. Therefore, the objective of the current research was to define a wide range of injera and injera batter quality parameters. Injera was prepared based on three different ingredients: flour, water, and starter culture. The quality parameters analyzed were physicochemical, physical, microbial, and nutritional. Physicochemical parameters included pH, pH, acidity, water activity and moisture content. Physical quality included reological quality, texture profile analysis, color and eye quality. Microbial quality had to do with lactic acid bacteria and yeast. Finally nutritional quality was focused on bioavailable Ca content. Through this research a large variety of quality parameter of injera were defined which was critical for the rest of the chapters of this thesis.

Key words: Injera, quality parameters, physicochemical, physical, microbial, and nutritional

2.2 Introduction

Injera is a fermented flat bread, which is prepared by the cereal teff, ***Eragrostis tef*** (Zucc.) Trotter. Injera is also prepared by mixing teff with other cereals such as maize, wheat, rice, sorghum, and barley. Some researchers also tried to use legumes or root crops by having a purpose either to improve the nutritional content of injera or to minimize the cost of injera. However, injera is considered best quality when it is prepared from only teff. Injera is characterized by being flat, soft, round, rollable, slightly sour, and havening a lot of evenly distributed openings (called eyes which are honey combe structure) on the top. The eyes are formed due to the production of gas during fermentation and baking. The ingredients for injera are cereal flour, irsho (a backslide from the previous batter) and water. In the traditional preparation of injera, irsho, a backslide from the previous batter, will be added to the batter to be used as a starter culture and to help the batter fermentation. Few research were done to do improvement on the fermentation process by replacing irsho with a commercially available starter culture so that to have injera product which is consistent in its quality (Fischer *et al.*, 2014; Hassen *et al.*, 2018).

The first step in injera preparation is to mix all the ingredients to make batter and then letting it to ferment for about 24-72 h at room temperature (25 ^{0}c). The fermentation hour depends on the level of sourness required, if a sourer injera is needed the fermentation can be done for 48-72 h but if sweet injera needed the fermentation will be done for 24 h only. After that, part of the fermented batter will be thinned with water and cooked in boiling water for 5-10 min (this is called absit) depending on the amount of the absit prepared, more cooking time required if the amount of the absit is large. The objective cooking absit, gelatinized batter, is primarily to bring about cohesiveness of the batter and secondly to provide easily fermentable carbohydrate to leaven the injera. It also promotes the growth of mesophilic microorganisms by raising the fermentation temperature to≈30°C (Yetneberk *et al.*, 2004). The gelatinized batter will be cooled to ≈45^{0}C - 60 ^{0}C and added back to the fermenting dough. Then it will be kept further for about 2-3 hours to be fermented further before cooking (Yetneberk *et al.*, 2004). Additional water may be added to fermented batter to get the required consistency; this depends on the initial water added to prepare the batter. If initially more water has been added, no need at the last stage to add more water. The fermented batter will be poured in a circular manner onto a hot clay griddle, covered, and baked for approximate 2-3 min. The baked injera will then be removed and kept in an airtight container (Yetneberk *et al.*, 2004).

Most researchers studied the quality of injera based on sensory assessment, even if some quality parameters were studied instrumentally (Yetneberk *et al.*, 2004; Abebe *et al.*, 2007; Cherie *et al.*, 2018; Hassen *et al.*, 2018). The qualities of injera such as titratable acidity (TTA), texture of injera which assess hardness, springiness, and cohesiveness, aw, injera eye quality based on certain eye area and circularity and bioavailability of calcium were not addressed in other research works. In addition, the qualities of injera batter such as aw, moisture content, color, TTA, and viscosity were not addressed by other researchers with the exception of microbial quality and pH of batter. Therefore, the objective of the current research was to study a wide range of injera and injera batter quality parameters. This will in turn help to give an input for standardization of injera that will enable production at industrial level and be traded at a big level.

2.3 Materials and methods

2.3.1 Materials

White teff flour was provided by Blonk Quality Ingredients (Spain). Eggshell powder (EP) was provided by Eggnovo S.L. (Spain). The starter cultures *Saccharomyces cerevisiae* and *Lactobacillus plantarum* subsp. *plantarum* were purchased from Valencia University CECT (Spanish Type Culture Collection). Enzymes and bile for in vitro digestibility study were bought from Sigma-Aldrich (St Louis, MO, USA). Analytic standard reagents were obtained from Panreac, Spain (HCl, KCl, $NaHCO_3$, NaCl, $MgCl_2(H_2O)_6$, and 65 % HNO_3); Sigma-Aldrich, Japan ($(NH_4)_2CO_3$, $CaCl_2(H_2O)_2$, $LaCl_3$)); Merck, Spain (NaOH, and 0.1 N NaOH); and J. T. Baker, Spain (KH_2PO_4). Medias and chemicals used for microbiology were purchased from OXID, France (MRS broth, MRS agar, and dehydrated culture media for microbiology); Panreac, Spain (agar technical ingredient for microbiology, and Glucose D (+), cycloheximide and chloramphenicol; VWR life science (Bacterial ultra-pure yeast extract, and tryptone peptone (peptone from casein) for microbiology; and VWR chemicals, from Belgium (Buffered peptone water, and PCA.

2.3.2 Injera processing

Lactic acid bacteria strain *Lactobacillus plantarum 1170 (L. plantarum),* and yeast strain *Saccharomyces cerevisiae 748* were used for fermentation during injera preparation. Yeast extract dextrose peptone (YEPD) agar was used to culture S. *cerevisiae* strain. YEPD agar was prepared by putting 16 g of D-glucose, 8 g of agar, 2 g of trypton peptone and 2 g of yeast in 400 ml of sterilized water and then mixed using magnetic stirrer. Sterilization of the media was done in an autoclave at a temperature of 121 ºC for 15 minutes. Man -Rogosa-

Sharpe (MRS) agar which was used to culture *Lactobacillus plantarum* was prepared by mixing 26.92 in 400 ml of sterilized water and sterilizing it at 121 ºC. Single strains of *S. cerevisiae* and *L. plantarum* were cultured on YEPD agar and MRS agar respectively, and then were incubated at 30 ºC for 48 h. The strains were then successively sub-cultured in YEPD broth and MRS broth at 30 ºC for 24 h and then for 48 h in a new broth. Pellets of yeast and lactic acid bacteria were collected by centrifugation at 10000 × g for 15 min at a temperature of 20 ºC using SIGMA 3K30 Laborzentrifugen GmbH (Germany) centrifuge machine. Sterilized water added to the pellets and then the centrifugation done again to clean the broth. Finally, before use, the starter cultures were solubilized with small amount of sterilized water and then put to a refrigerator at a temperature of 5 ºC for two days until the concentration was determined. After refrigeration the concentration was determined by making ten-fold dilutions and culturing them in their respective agar media. The process of starter culture was shown in figure 2.1.

Injera was prepared based on three different ingredients: flour, water, and starter culture. Before starting the processing, teff flour was sterilized in a hot dry air by an auto cooking machine RATIONAL SCC 61/06 (Germany) at 190 ºC for 6 minutes following the procedure described by (Baye *et al.*, 2015) and cooled to room temperature.

Injera was prepared following the procedure shown in Figure 1. Firstly, to make the batter, 1100 g of teff flour and starter cultures (to reach 10^{7} CFU /ml in the final batter) along with sterilized ultrapure water to reach a final weight of 2360 g were placed into a Thermomix TM-5 (Vorwerk, Germany) to be mixed and kneaded at 3 rpm for 5 min at room temperature. Once the batter was obtained, it was placed in metal pot and the top was covered with 400 ml sterilized ultra-pure water to prevent mold growth.

Fermentation was performed in a convective oven at 25 ºC (room temperature in Ethiopia) for 48 h. After that, to prepare the *absit* 150 g of fermented batter were taken and mixed well for two minutes then cooked in 450 ml of boiling water for 10 min by continual steering with a spoon. The *absit*, at approximately 60 ºC, was added back to the fermenting batter and mixed well with a spoon. After that, the mixture was kept at 25 ºC for about 2 h to be fermented further before cooking. Approximately 100 ml of the fermented batter was poured in a spiral manner onto an injera WASS Electronics Digital Mitad Grill, covered, and baked for 2 min at a cooking temperature of 121 ºC. The baked injera was then removed and kept in an airtight container at room temperature.

The samples used for quality parameters analysis were taken immediately after preparing the batter (B0), at 24 h (B24), 48 h (B48) and 50 h (B50) of fermentation of the batter. Sampling times were chosen based on the work described by (Misci *et al.*, 2021). At each sample time, 200 g of the batter were taken and used for analysis. Finally, a cooked injera (INJ) analysis was performed. Sample collection is indicated in Figure 2.1.

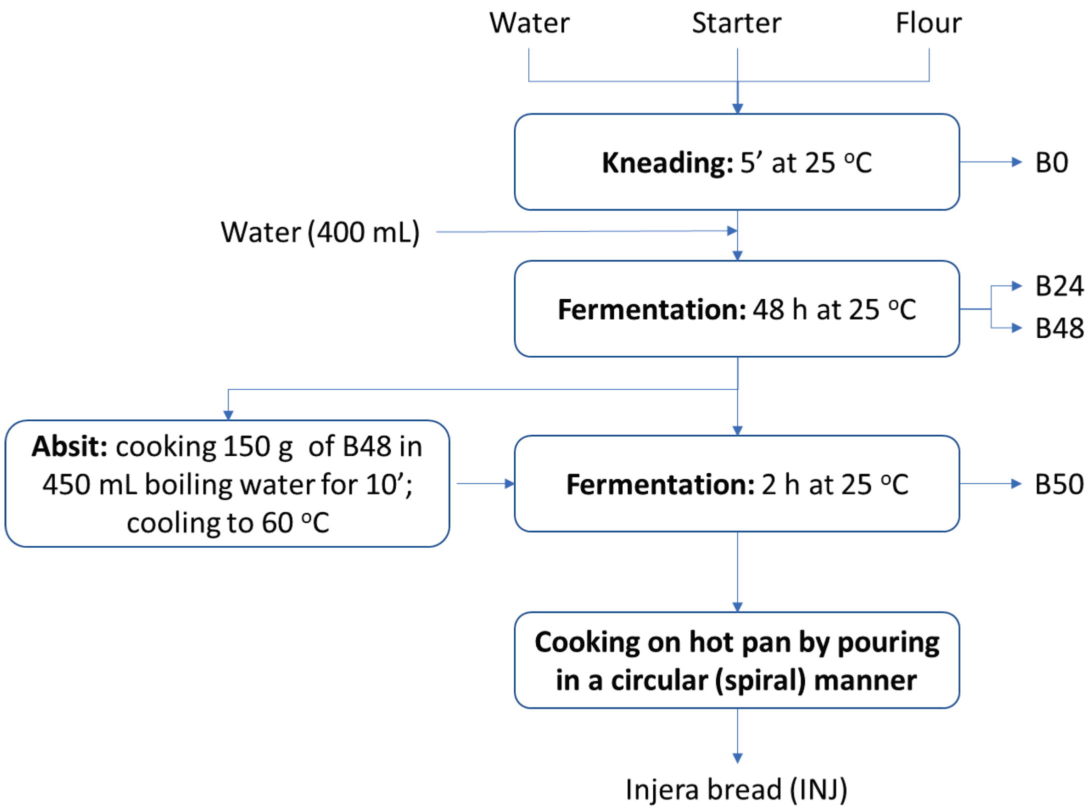

Figure. 2.1 Injera cooking flowchart. B0, B24, B48, B50 refer to batter samples at 0, 24, 48, & 50 hours of fermentation.

2.3.3 Determination of physicochemical parameters

2.3.3.1 pH and titratable acidity

The pH of batter was determined using a Crison Basic 20 pH meter by directly inserting the electrode into the batter. Titratable acidity of batter was determined following the method by Harth, Van Kerrebroeck and De Vuyst (2016). 10 g of batter sample was taken and mixed with 90 ml of deionized water using magnetic stirrer. The solution was then titrated with 0.1 M NaOH to an end point pH of 8.5. The volume of NaOH (in milliliters) used for each titration was recorded and used to calculate the titratable acidity of the batter.

The pH of injera was measured according to AOAC Official Method 945.42 (AOAC, 2005b). Injera samples (10 g) were taken and cut with a coffee grinder machine for 30 seconds, mixed with 100 ml of distilled water that was boiled and cooled at 25 ºC, mixing continued for 30 minutes at 25 ºC, and then settled for 10 minutes. pH of the supernatant was immediately measured after decanting it into a beaker. The solution prepared to determine the pH of injera was used to determine the titratable acidity of injera by titrating it with 0.1 M NaOH to a pH of 8.5.

2.3.3.2 Moisture content and water activity

Moisture content: The moisture content of batter and injera was determined by gravimetrically. Five grams of sample was dried at 105 ºC for 24 h. The dried sample then was put an in desiccator to prevent any gain of moisture and then weighed in a weighing scale. Loss in weight was recorded as moisture content of batter and injera.

Water activity: The water activity of batter and injera was measured by weighing 2 g of the samples in the measuring plastic cup and recording the result. The measurement was always done at 25 ºC by the machine LabMaster-Aw (Novasina AG; Lachen, Switzerland).

2.3.4 Physical parameters

2.3.4.1 Rheological properties of batter

The rheological properties of batter were analyzed using rotatory viscosimeter Haake RV1 by means of a flow curve consisting of a ramp-up step from 0-500 s^{-1} during 90 s, a holding time of 30 s at 500 s^{-1} and a ramp-down step from 500-0 s^{-1} in 90 s. The Ostwald de Waele rheological model was applied to the ascending ramp flow curve, obtaining the consistency index (K), the flow behavior index (n) and dynamic viscosity (η) of the batter at a 100 s^{-1} shear gradient (Equation 1 and Equation 2). Measurements were done in five repetitions.

$$\tau = K \cdot \dot{\gamma}^{n}$$ Equation 1 (Ostwald de Waele model of flow curve)

$$\eta = K \cdot \dot{\gamma}^{n-1}$$ Equation 2 (Ostwald de Waele model of viscosity curve)

Where: τ is the shearing stress (Pa); K is the consistency index ($Pa \cdot s^{n}$); $\dot{\gamma}$ is the shear gradient (s^{-1}); n is the flow behavior index (dimensionless); and η is the dynamic viscosity (Pa·s)

2.3.4.2 Texture Profile Analysis (TPA) of injera

TA.XTi plus texture analyzer (Stable Micro Systems; Surrey, UK) was used to measure the mechanical properties of injera through a TPA. Five circular samples (ø 5 cm) were placed together one on top of the other under a flat probe (P/75 compression platen) attached to the testing machine (Kim, Kwak and Jeong, 2017). The injera samples were compressed until reaching 75 % of its initial height, using a 0.049 N trigger force, and a test speed of 2 $mm \cdot s^{-1}$. Samples were left to rest for 5 s before the second compression. After testing, the hardness, springiness, and cohesiveness were calculated by using the software Exponent v6.1.16. Measurements were done in five repetitions.

2.3.4.3 Color

Color was assessed using the DigiEye Imaging System equipment (VeriVide Ltd.; UK). Five repetitions were done for both batter and injera samples, and CIE L*, a*, and b* coordinates were obtained as described by Etxabide, Kilmartin and Maté (2021).

2.3.4.4 Eye formation

Eye qualities of injera were assessed using the softwares DigiEye v.2.8.0.2 (VeriVide Ltd.; UK) and ImageJ v.1.52a (National Institutes of Health, USA) on injera pictures previously taken. DigiEye was used to calculate the proportion between the dark brown color, which represent the craters (eyes), and the light color proportion, which represent the remaining part (surface). Mean size (area) and eye diameters were obtained by means of ImageJ after image calibration, color to gray transformation and thresholding using the "MaxEntropy" algorithm. Binary function "Fill Holes" was also used to avoid artifacts derived from nested eyes. Five repetitions of the measurements were carried out.

2.3.5 Microbial analysis

Yeast, lactic acid bacteria, and total mesophilic counts were assessed to study the microbial properties of batter, and injera. Samples were analyzed in duplicate using 10 g following the procedure described by Harth, Van Kerrebroeck and De Vuyst (2016). To assess the growth of the microorganisms, 0.1 ml of properly diluted sample of batter or injera was spread on pre-dried agar medias. MRS agar with 0.1 g/L of cycloheximide or YEPD with 0.1 g/L of chloramphenicol was used to assess the growth of lactic acid bacteria or yeast and molds respectively. The growth of total aerobic mesophilic bacteria was assessed on PCA at 30°C for 72 h (Harth, Van Kerrebroeck and De Vuyst, 2018). The analysis was done two times and triplicate plates were used.

2.3.6 Nutritional Quality

2.3.6.1 Total Ca

Total Ca of injera was analyzed following the AOAC official method 985.35 (AOAC, 2005a). Dry ashing method was first used to destroy organic matrix in a muffle furnace at 525 ºC for 10 h. 1 M HNO_3 was used to aid in the ashing process and to solubilize white ash and to make dilutions. Total Ca was analyzed by inductively coupled plasma atomic emission spectroscopy (ICP - AES) at Universidad de Zaragoza, Spain. The amount of Ca was calculated and expressed as mg Ca/100 g of dried injera. The analysis of total Ca was carried out in duplicate with two samples of injeras processed independently.

2.3.6.2 Bioavailable Ca

A static *in vitro* simulation of gastrointestinal food digestion nature protocol developed by Brodkorb *et al.* (2019) was used to perform *in vitro* digestion of injera and thus to measure bioavailable Ca. Simulated stock solutions were prepared and stored at -20 ºC until use (Table 1). *In vitro* digestion of the injera at oral (2`), gastric (2 h), and intestinal phase (2 h) was made by incubating in Mini Shaker (VWR, USA) at 200 rpm and at 37 ºC, all detail is shown in Table 2.

After the digestion process, centrifugation of the sample was carried out using SIGMA 3K30 Laborzentrifugen GmbH (Germany) centrifuge machine at 3500 x g at 4 ºC for 1 h as described by Cámara *et al.* (2005). The supernatant was collected and placed into a 15 ml Falcon and sent to Universidad de Zaragoza for bioavailable Ca analysis. The analysis of total Ca was carried out in duplicate with two samples of injeras processed independently.

Table 2.1. Volume of electrolyte stock solutions of digestion fluids for 400 ml diluted with water

Solution	**Stock concentrations (M)**	**Volume (mL) to be taken for electrolyte solution preparation 400 ml (for each fluid)**		
		SSF	**SGF**	**SIF**
KCl	0.5	15.1	6.9	6.8
KH_2PO_4	0.5	3.7	0.9	0.8
$NaHCO_3$	1.0	6.8	12.5	42.5
NaCl	2.0	-	11.8	9.6
$MgCl_2(H_2O)6$	0.15	0.5	0.4	1.1
$(NH_4)_2CO_3$	0.5	0.06	0.5	-
$CaCl_2(H_2O)_2$	0.3	0.025	0.005	0.02
pH adjustment solutions				
HCl	6	0.09	1.3	0.7
pH		7	3	7

Table 2.2. In vitro digestion of injera

Digestion phase	Oral (SSF) pH 7	Gastric (SGF) pH 3	Intestinal (SIF) pH 7	
Food or digest	5 g of control injera	10 mL from oral phase	20 mL from gastric phase	
Electrolyte stock solutions (mL)	4	8	8	
$CaCl_2(H_2O)_2$ (0.3 M) (mL)	0.025	0.005	0.04	
Enzymes	Salivary amylase	Pepsin	Trypsin in pancreatin	Bile salts
Enzyme activity (U/mL) or bile concentration (mM) in total digesta (final volume in milliliters at each digestion phase, see row below)	75 U/mL	2000 U/mL	100U/mL	10 mM
Specific activity (U/mg), concentration (bile) mmol/g	5 U/mg	3500 U/mg	6 U/mg	0.667 mmol/g
Concentration of enzyme/bile solution (mg/mL)	200	0.019	133.3	6.25
Volume of enzyme/bile to be added (mL)	0.75	0.667	5	3
Type of injera sample	I0	I0	I0	
H_2O (mL)	0.129	1.168	3.86	
HCl (5 M) for pH adj. (mL)	-	0.16	-	
NaOH (5 M) for pH adj. (mL)	0.096	-	0.1	
Final volume (mL)	10	20	40	

2.4 Results and Discussion

2.4.1 Physicochemical parameters

The pH of batter at different processing stages and pH of injera are shown in Figure 2.2. The result showed that the pH of batter decreased as the fermentation times increased. However, there was no significant difference on pH of batter at fermentation times of 48 h and of 50 h and for injera. The maximum pH was found in the B0 (batter at 0 h fermentation time) samples. The pH of batter after 48 h of fermentation (B3) was found to be 4.1 a value comparable with pH reported by other researchers which was ranged from 3.4 to 4.1 as for similar products Fischer *et al.* (2014). The pH of injera was 4.1 which was similar to the pH reported in previous research wherein the pH value fell between 3.4 and 4.5 (Ashagrie, and Abate, 2012).

TTA of batter upon increase of fermentation time and injera is shown in Figure 2.3. As the fermentation time increased there was an increase in the TTA of batter. However, no significant effect was observed between samples of B48 and B50. This could be the short time of fermentation between the two samples. The highest TTA of batter was found at time 48 h fermentation which was 12% and the lowest was for time 0 h fermentation which was found to be 3% and the value for injera was 6.4%.

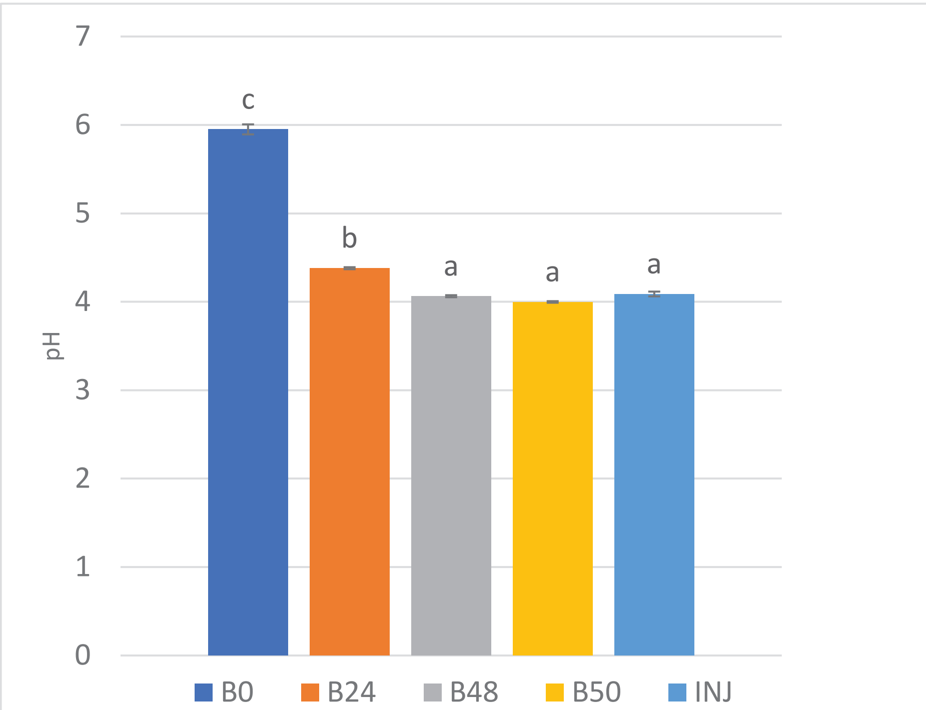

Figure 2.2 pH of batter (B) after 0, 24, 48, and 50 h of fermentation and injera (INJ). Mean and standard error bars (n=6). Letters show differences based on fermentation time.

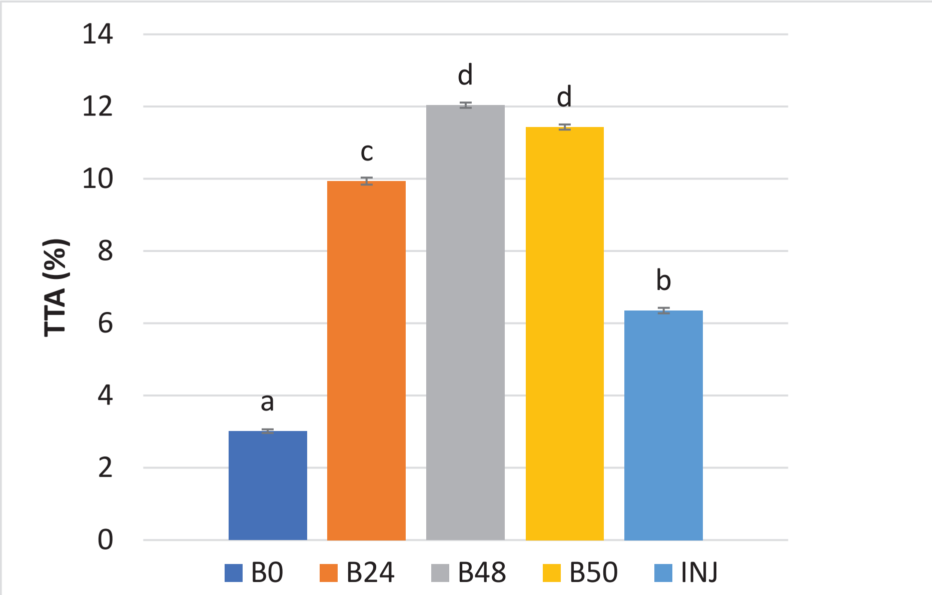

Figure 2.3 TTA of batter (B) after 0, 24, 48, and 50 h of fermentation and injera (INJ). Mean and standard error bars (n=6). Letters show differences based on fermentation time.

Moisture content and water of batter and injera are shown in Table 2.3. An increase in the moisture content of batter has been observed as the fermentation time increase. However, the increase was not related to the fermentation time, it rather related to the addition of water in the process. The minimum and maximum moisture content of batter was found to be 57.6 ± 0.2% (B0) and 71.5 ± 0.2% (B50) respectively. Average moisture content of injera was 60.5 ± 0.8%, similar to injera moisture contents found by Cherie *et al.* (2018).

The result of the water activity showed no significant differences as the time of fermentation increased and for injera and was very high for all the samples. This fact is critical for the potential shelf life of this product. In all cases, water activity was above 0.95 allowing the growth of most microorganisms.

2.4.2 Rheological properties of batter

The viscosity (η100), consistency index (K), and flow behavior index (n) of batter are shown in Table 2.3. As a result of fermentation and addition of water in the process, viscosity dropped noticeably after 24–48 h, and again, after the addition of *absit* which was the secondary fermentation step (B50). A similar pattern was observed in the evolution of the consistency index in time (Table 2.3). There was a drastic decrease of K due to the fermentation. The consistency index for the batter at 0 h (B0) were in the range of cake batter (17–40 Pa sn) previously reported (Noorlaila *et al.*, 2020). The evolution of the flow behavior index (n) of the batter is shown in Table 3. At 0 h the non-Newtonian behavior was more making the batter more sensitive to shearing. This effect seemed to disappear after the 24–48 h period of fermentation, in which this parameter significantly decreased. Finally, after the addition of *absit*, the batter recovered its initial non-Newtonian character, likely due to an internal relaxation of the structure of the starch granules (gelatinization).

2.4.3. Texture of injera

The texture parameters of injera obtained from TPA are shown in Table 2.4. The hardness, springiness, and cohesiveness of injera were found to be 90.8 N, 0.73 and 0.53 respectively. Recently, Yasin (2021) reported firmness of injera to be 3.13 N using a sharp blade cutting probe. The value found for hardness (a comparable term for firmness) in the current research is relatively higher due to the different method used to assess hardness of injera (shearing vs compression). Formerly, Yetneberk *et al.* (2004) conducted another study to evaluate flexion properties of injera using a three-point bending rig; the maximum bending force obtained was 0.14 N. Given the different experimental setup, results cannot be compared.

2.4.4. Color

The color of batter with different fermentation time, as well as the color of injera are shown in Table 2.4. The L* of batter significantly increased as the fermentation time increased. Among batter samples, the lowest L* value was for B0, 73.8 ± 0.3, and the highest was for B50, 79.3 ± 0.3. A relatively similar L* value of injera was found in the current research compared with another research value of 54.95 (Cherie *et al.*, 2018). Among the batter samples, the lowest a color value was found for B48 and B50 samples whereas the highest value was found for B24 sample. As the fermentation time increased the b color characteristics shown an increase in value.

2.4.5. Eye formation

The visual quality of injera is determined by the number and size of eyes on its surface. Injera eye parameters related to eye quality are shown in Table 2.4 by presenting the values for total area (%), individual eye area (mm^2), eye diameter (mm) and eye circularity (minor/major axis ratio). Sensory analysis has been used by different researchers to determine injera eye qualities which were expressed as number of eyes and eye size (Yetneberk *et al.*, 2004; Agza, Bekele and Shiferaw, 2018). Image analysis can be a good approach to sensory perception of eye properties of injera. Thus, Yasin (2021) used the software Injera Eyes, version 1.0.0.0 to determine the number of injera eyes in a fixed surface of 1550 × 1550 pixels, from which an "eye density" can be obtained. Our current research assessed the proportion of dark brown color area (number of pixels darker than a certain threshold) as an approach to the sensory perception of global "eye proportion" perceived by consumers. Complementarily, the present work aimed to obtain a deeper understanding about the morphology of the eyes studying its symmetry and size.

Table 2.3. Physicochemical, physical, rheological and microbial qualities of batter and injera.

Variables	**Fermentation Time**				
	0 h	**24 h**	**48 h**	**50 h**	**Injera**
Moisture content (%)	57.6 (0.2)	65.3 (1.2)	66.0 (0.1)	71.5 (0.2)	60.5 (0.8)
aw (mean)	0.99 (0.0)	0.99 (0.0)	0.99 (0.0)	0.99 (0.0)	0.99 (0.0)
Rheology					
K (Pa·sn)	20.3 (2.8) ^{c}	3.9 (0.1) ^{b}	3.1 (0.2) ^{b}	0.57 (0.0) ^{a}	
N	0.63 (0.0)	0.52 (0.0)	0.56 (0.0)	0.63 (0.0)	
Viscosity (η100; *Pa ·s*)	3.6 (0.1)	0.42 (0.0)	0.4 (0.0)	0.1 (0.0)	
Color					
L	73.8 (0.3) ^{b}	78.4 (0.1) ^{c}	78.9 (0.3) ^{cd}	79.3 (0.3) ^{d}	53.4 (0.1) ^{a}
a	4.2 (0.1) ^{b}	4.7 (0.1) ^{c}	3.9 (0.2) ^{a}	3.8 (0.2) ^{a}	7.1 (0.2) ^{d}
b	16.7 (0.1) ^{a}	17.6 (0.2) ^{b}	18.3 (0.3) ^{c}	18.1 (0.2) ^{c}	19.1 (0.5) ^{d}
Microbial quality					
LAB	7.2 (0.1) ^{a}	9.6 (0.1) ^{b}	9.6 (0.0) ^{b}	9.5 (0.1) ^{b}	ND
TMC	7.4 (0.1) ^{a}	9.6 (0.1) ^{b}	9.6 (0.1) ^{b}	9.5 (0.1) ^{b}	ND
Yeast	6.9 (0.0) ^{a}	7.8 (0.0) ^{b}	7.8 (0.0) ^{b}	7.8 (0.1) ^{b}	ND

Moisture content and aw as mean (SD) of n = 6 observations of batter at times of 0 h (B1) (B2), 24 h, (B3)48 h, (B4)50 h fermentation of batter and the final product injera (INJ). Statistical analysis: ANOVA. Different letters in the same raw show differences with fermentation time.

Table 2.4. Injera eye, texture of injera and nutritional qualities of injera

Eye quality	**Total eye area (%)**	**Individual eye area (mm^2)**	**Eye diameter[1] (mm)**	**Eye circularity (minor/major axis ratio)**
	38.92 (2.5)	5.87 (0.7)	2.84 (0.2)	0.70 (0.0)
Texture of injera	**Hardness (N)**	**Springiness**	**Cohesiveness**	
	90.8 (4.1)	0.73 (0.1)	0.53 (0.0)	
Nutritional content of injera	**Total Ca (mg/100 g d.b.)**	**Bioavailable Ca (mg/100 g d.b.)**	**Percentage of bioavailable Ca (%)**	
	235.9 (1.7)	16.1 (0.4)	6.8	

Injera eye qualities: [1]Mean Feret diameter. Result of injera eye quality as mean (SD) of n = 10 observations of injera.

2.4.6 Microbial quality

The evolution of the growth of lactic acid bacteria, total aerobic mesophilic bacteria and yeast is shown in Table 2.3. When the fermentation time increased from 0 h to 24 h a significant increase on microbial population was found. However, no significant differences were observed when the fermentation time increased from 24 h onwards. After 24 h fermentation, lactic acid bacteria and total aerobic mesophilic bacteria counts were found to be higher than those of other research work in which natural fermentation (no inoculation) was used (Fischer *et al.*, 2014). Hassen *et al.* (2018) also indicated that lactic acid bacteria and yeast did not grow as much when natural fermentation was used as compared with controlled fermentation. In addition, it is likely that flour sterilization performed in our research could have contributed to facilitate microbial growth during fermentation.

2.4.7 Nutritional quality

The nutritional quality of injera related to its calcium content is shown in Table 2.4. The level of total Ca in injera was 235.9 mg/100 g (dry basis, d.b.). Total Ca content in injera in the current research was high as compared with another research finding that presented 61 mg/100 g (d. b.) (Cherie *et al.*, 2018). This could be due to the different processing methods of injera. In other research, after the last stage of fermentation, surface water on top of the batter was thrown. This practice may have decreased the total Ca since some of it may have been lost with the discarded water. In addition, total Ca in teff flour used in this study can be also considered higher than the one reported in the literature (141–188 mg/100 g d/b) (Kibatu, Chacha and Kiende, 2017; Shumoy and Raes, 2017). These differences are likely based on varietal difference of teff grain. Anyhow, the values of Ca in teff were found to be higher as compared to other cereals such as maize (16 mg/100 g), wheat (15.2–39.5 mg/100 g), sorghum (5.0–5.8 mg/100 g) and rice (23 mg/100 g) expressed on d.b (Baye, 2014). Thus, teff flour and injera could be a potentially better Ca source than other cereals.

However, the percentage of bioavailable Ca for injera was found to be 6.8% which can be considered very low. The reason for the low level of bioavailable Ca in injera is likely due to the high content of phytates and fiber (Baye, 2014). Anti-nutrients bind micronutrients and make them unavailable for absorption by human body. A study conducted on the bioavailability of Ca in spinach showed it was also very low (1.7%) due to the high content of anti-nutrient oxalate which binds Ca and makes it unavailable for absorption (Cámara *et al.*, 2005). An in-vitro study on the bioavailability of other minerals such as iron and zinc on injera showed that the high level of phytates also affected their bioavailability (Shumoy *et al.*, 2017). In light of all these facts, it is important not only to increase Ca in the diet but also to ensure there is an increase in its bioavailability.

If it is assumed that the average amount of injera consumed by an Ethiopian adult is about 200 g (wet basis; w.b.), considering that the moisture content of injera is about 60%, the intake of bioavailable Ca with injera would be 12.9 mg/day when injera is included in the diet. According to FAO/WHO (2005) the RDA and Upper Intake Level (UL) of Ca range from 1000 to 3000 mg/day. Therefore, the amount of Ca intake can be considered inadequate.

In addition, Ethiopian Great Rift Valley areas are highly affected by fluorosis which has affected bone and teeth health. In this region, it is especially important to maintain high levels of Ca intake to reduce the health consequences related to fluorosis (Patel *et al.*, 2017; Srivastava *et al.*, 2017). Moreover, the life expectancy of Ethiopians is increasing; thus, there are more elderly people in the country. This group is highly susceptible to osteoporosis and likely to be at risk in the future also, if the diet does not change. Thus, there is a need to increase Ca in the diet of the population to reduce the risk of osteoporosis among the elderly, now and in the future.

2.5 Conclusions

The current research characterized the qualities of batter and injera. Even if injera is one of the most important staple foods in Ethiopia, commercialization of the product based on specified quality parameters has not been set so far by Ethiopian quality standard agency. Therefore, the current research findings can give inputs for the agency in the way of commercializing injera. In addition, the research has revealed that there is a need to fortify injera so that to increase its contribution to the nutritional Ca requirement of people by increasing level of bioavailable Ca.

2.6 References

Abebe, Y. et al. (2007) 'Phytate, zinc, iron and calcium content of selected raw and prepared foods consumed in rural Sidama, Southern Ethiopia, and implications for bioavailability', Journal of Food Composition and Analysis, 20(3–4), pp. 161–168. doi: 10.1016/j.jfca.2006.09.003.

Agza, B., Bekele, R. and Shiferaw, L. (2018) 'Quinoa (Chenopodium quinoa, Wild.): As a potential ingredient of injera in Ethiopia', Journal of Cereal Science. Elsevier, 82(June), pp. 170–174. doi: 10.1016/j.jcs.2018.06.009.

AOAC (2005a) 'Mineral in Infant Formula, Enteral Products, and Pet Foods', Official methods of analysis of AOAC international, 514, pp. 1–3.

AOAC (2005b) 'pH of bread', Official methods of analysis of AOAC international, 02.

Ashagrie, Z. and Abate, D. (2012) 'Improvement of Injera Shelf Life Through the Use of', African Journal of Food, Agriculture, Nutrition and Development, 12(5), pp. 6409–6423.

Assefa, Y. et al. (2018) 'Influence of milling type on tef injera quality', Food Chemistry, 266(November 2017), pp. 155–160. doi: 10.1016/j.foodchem.2018.05.126.

Baye, K. (2014) 'Synopsis : Teff : nutrient composition and health benefits', Ethiopia Strategy Support Program, (October), pp. 2013–2014.

Baye, K. et al. (2015) 'Enzymatic degradation of phytate, polyphenols and dietary fibers in Ethiopian injera flours: Effect on iron bioaccessibility', Food Chemistry. Elsevier Ltd, 174, pp. 60–67. doi: 10.1016/j.foodchem.2014.11.012.

Brodkorb, A. et al. (2019) 'INFOGEST static in vitro simulation of gastrointestinal food digestion', Nature Protocols. Springer US, 14(4), pp. 991–1014. doi: 10.1038/s41596-018-0119-1.

Cámara, F. et al. (2005) 'Bioaccessibility of minerals in school meals: Comparison between dialysis and solubility methods', Food Chemistry, 92(3), pp. 481–489. doi: 10.1016/j.foodchem.2004.08.009.

Cherie, Z. et al. (2018) 'Optimization and modeling of teff-maize-rice based formulation by simplex lattice mixture design for the preparation of brighter and acceptable injera', Cogent Food & Agriculture. Cogent, 4(1), p. 1443381. doi: 10.1080/23311932.2018.1443381.

Etxabide, A., Kilmartin, P. A. and Maté, J. I. (2021) 'Color stability and pH-indicator ability of curcumin, anthocyanin and betanin containing colorants under different storage conditions for intelligent packaging development', Food Control, 121. doi: 10.1016/j.foodcont.2020.107645.

FAO/WHO Expert Consultation (2005) 'Human Vitamin and Mineral Requirements', Geneva : World Health Organization, (2nd ed), p. 341 p. Available at: http://apps.who.int/iris/handle/10665/42716.

Fischer, M. M. et al. (2014) 'Phytic acid degrading lactic acid bacteria in tef-injera fermentation', International Journal of Food Microbiology. Elsevier B.V., 190, pp. 54–60. doi: 10.1016/j.ijfoodmicro.2014.08.018.

Harth, H., Van Kerrebroeck, S. and De Vuyst, L. (2016) ‘Community dynamics and metabolite target analysis of spontaneous, backslopped barley sourdough fermentations under laboratory and bakery conditions’, International Journal of Food Microbiology. Elsevier B.V., 228, pp. 22–32. doi: 10.1016/j.ijfoodmicro.2016.04.011.

Harth, H., Van Kerrebroeck, S. and De Vuyst, L. (2018) ‘Impact of process conditions on the microbial community dynamics and metabolite production kinetics of teff sourdough fermentations under bakery and laboratory conditions’, Food Science and Nutrition, 6(6), pp. 1438–1455. doi: 10.1002/fsn3.690.

Hassen, Y. et al. (2018) ‘Evaluation of Yeast and Lactic Acid Bacteria Starter Cultures for the Production of Rice Injera’, Journal of Food Processing & Technology, 09(03). doi: 10.4172/2157-7110.1000721.

Kibatu, G., Chacha, R. and Kiende, R. (2017) ‘Determination of Major, Minor and Trace Elements in Tef Using Portable Total X-Ray Fluorescence (TXRF) Spectrometer’, EC Nutrition, 1, pp. 51–59.

Kim, S., Kwak, H. S. and Jeong, Y. (2017) ‘Effect of Water Roux Starter (Tangzhong) on Texture and Consumer Acceptance of Rice Pan Bread’, Journal of Texture Studies, 48(1), pp. 39–46. doi: 10.1111/jtxs.12204.

Misci, C. et al. (2021) ‘Fermentation as a tool for increasing food security and nutritional quality of indigenous African leafy vegetables: the case of Cucurbita sp.’, Food Microbiology. Elsevier Ltd, 99. doi: 10.1016/j.fm.2021.103820.

Noorlaila, A. et al. (2020) ‘The effects of xanthan gum and hydroxypropylmethylcellulose on physical properties of sponge cakes’, Journal of the Saudi Society of Agricultural Sciences. King Saud University, 19(2), pp. 128–135. doi: 10.1016/j.jssas.2018.08.001.

Patel, Prerna et al. (2017) ‘Association of dental and skeletal fluorosis with calcium intake and serum Vitamin D concentration in adolescents from a region endemic for fluorosis’, Indian Journal of Endocrinology and Metabolism, 21(1), pp. 190–195. doi: 10.4103/2230-8210.196013.

Shumoy, H. et al. (2017) ‘Traditional fermentation of tef injera: Impact on in vitro iron and zinc dialysability’, Food Research International. Elsevier, 102(June), pp. 93–100. doi: 10.1016/j.foodres.2017.09.092.

Shumoy, H. and Raes, K. (2017) ‘Tef: The Rising Ancient Cereal: What do we know about its Nutritional and Health Benefits?’, Plant Foods for Human Nutrition. Plant Foods for Human Nutrition, 72(4), pp. 335–344. doi: 10.1007/s11130-017-0641-2.

Srivastava, A. K. et al. (2017) ‘Prevalence of dental fluorosis and the role of calcium supplementation in its prevention’, Journal of Medical Sciences (Faisalabad), 17(4), pp. 156–161. doi: 10.3923/jms.2017.156.161.

Yasin, U. A. (2021) ‘Textural and Eye Qualities of Injera Prepared from Quality Protein Maize and Teff Blended Flours’, International Journal of Nutrition and Food Science, 10(4), pp. 78–85. doi: 10.11648/j.ijnfs.20211004.11.

Yetneberk, S. et al. (2004) ‘Effects of Sorghum Cultivar on Injera Quality’, Cereal Chemistry, 81(3), pp. 314–321. doi: 10.1094/CCHEM.2004.81.3.314.

CHAPTER 3.

EFFECT OF FORTIFICATION WITH EGGSHELL POWDER ON INJERA QUALITY

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CHAPTER 3. EFFECT OF FORTIFICATION WITH EGGSHELL POWDER ON INJERA QUALITY

3.1 Abstract

In Ethiopia there is a severe calcium nutrient deficiency problem. Cereals are appropriate vehicles for food fortification along with eggshell powder (EP), a waste product, which contains a high amount of Ca. The objective of the current study was to enrich injera (Ethiopian flat bread from cereal teff) with EP to improve Ca content and to assess its effect on quality parameters. Injera was prepared by adding 4.5% and 9% EP to the teff flour (injera I4.5 and I9.0, respectively). The fortification resulted in significantly increased bioavailable Ca from 16.1 mg/100g (control) to 742.7 mg/100 g (I4.5) and 1743.1 mg/100 g (I9.0). With the consumption of 200 g of injera, an adult could meet 60% of the recommended dietary allowance (RDA) of Ca from I4.5 and 140% of Ca RDA from I9.0. The addition of eggshell powder affected pH, titratable acidity, color and texture. However, there was no significant effect on moisture content, aw, viscosity, microbial quality, and eye characteristics. Current research revealed that fortification of injera by EP could be a good source of total and bioavailable Ca without significant deleterious effects on the key physico-chemical quality parameters of injera.

Key words: calcium, eggshell powder, bioavailability, injera, quality parameters

3.2 Introduction

Calcium is one of the important micronutrients being that it is necessary for the normal functioning of the human body. This nutrient is a major component of bones and teeth and participates in many physiological processes of human body (Gharibzahedi & Jafari, 2017). In addition, Ca plays an important role in the prevention of chronic diseases such as high blood pressure (Hofmeyr, Duley, & Atallah, 2007). The Ca deficiency causes osteoporosis in adults, and rickets in children (Pettifor, 2004).

Milk and milk products are excellent sources of Ca. However, fulfilling Ca requirements from food sources is very difficult in areas where there is famine and drought which affect the production of livestock and their products in countries like Ethiopia. As a consequence, the level of Ca intake by the children has been below the recommended level (Tezera, Whiting, & Gebremedhin, 2017). Similarly, other researchers showed that there was an inadequate intake of Ca by women and men (Amare et al., 2012; Asayehu, Lachat, Henauw, & Gebreyesus, 2017).

In Ethiopia, the Great Rift Valley areas are commonly affected by fluorosis due to the high level of fluorine in the water. People affected by fluorosis have weak bones and weak and stained teeth. Studies have shown the consumption of an adequate amount of Ca to help decrease fluorosis (Patel et al., 2017).

Food fortification has been proven to be effective to increase Ca intake. Cereal-based foods are usually the target food to be used to fortify Ca since they are consumed in great quantities; thus a higher level of Ca can be added (Cilla, López-García, & Barberá, 2018) to the diet. $CaCO_3$ is a common Ca source for food fortification in cereals-based foods because its addition usually does not have a significant effect on the taste and appearance of the final food product (Rafferty, Walters, & Heaney, 2007).

Injera (flat bread) is a staple food consumed equally by all age groups and by two-third of the Ethiopian population. The cereal teff, *Eragrostis tef* (Zucc.) Trotter, is a grass-type cereal that is used to cook injera. Teff has a nutrient composition similar to other cereals although it has higher iron and Ca contents (Shumoy, & Raes, 2017). In addition, since teff is consumed as a whole grain, injera contains high amounts of fiber which reduce the absorption capability of minerals.

Teff injera quality is judged by consumers considering appearance and taste. Color, texture, and eyes (a honeycomb structure on top of injera) are among the quality parameters which are critical for their acceptance (Yasin, 2021; Yetneberk, Kock, Rooney, & Taylor, 2004). Sour, soft, cohesive, and light colored injera is highly demanded.

Eggshell contains high amounts of bioavailable Ca in the form of $CaCO_3$ (Brun, Lupo, Delorenzi, Di Loreto, Loreto, & Rigalli 2013) it was shown that EP contained 97% solids, out of which 98% is $CaCO_3$ (Daengprok Daengproka, Garnjanagoonchorna, & Mineb, 2002). One gram of EP contains 360-400 mg of Ca (Schaafsma et al., 2000). Trials suggested that bioavailable Ca content in EP was estimated to be 45% (Brun, Lupo, Delorenzi, Di Loreto, Loreto, & Rigalli 2013). Animal studies showed that EP has a higher content of absorbable Ca than commercial $CaCO_3$ (Omi & Ezawa, 1998).

Research findings showed that food products like bread, sausage, pizza, spaghetti, or stew were fortified with eggshell powder thus improving the Ca content of these products (Brun, Lupo, Delorenzi, Di Loreto, Loreto, & 2013). In addition, the sensory evaluation presented an overall acceptability of the products, even though texture quality was affected. Eggshell, a waste product, is easily accessible by most Ethiopian households; and thus, it can be used as a low-cost Ca source with also contributes to a circular economy.

Even though micronutrient deficiencies are among the most important nutritional problems in Ethiopia, so far there is no mandatory food fortification program designed to be a solution to tackle the problem. Fortifying injera, with eggshell powder (EP), could be used to address the Ca deficiency problem and contribute to improving the health of Ethiopians.

The objective of this research was to fortify injera with eggshell powder and to assess its effect on some injera quality parameters, including physicochemical, physical, microbiological, and nutritional quality. As far as we know, there are no studies previously conducted related to the development of fortified injera with EP and its effect on injera quality.

3.3 Materials and methods

3.3.1 Materials

White teff flour was provided by Blonk Quality Ingredients (Spain). Eggshell powder (EP) was provided by Eggnovo S.L. (Spain). The starter cultures *Saccharomyces cerevisiae* and *Lactobacillus plantarum* subsp. *plantarum* were purchased from Valencia University CECT (Spanish Type Culture Collection). Enzymes and bile for in vitro digestibility study were bought from Sigma-Aldrich (St Louis, MO, USA). Analytic standard reagents were obtained from Panreac, Spain (HCl, KCl, $NaHCO_3$, NaCl, $MgCl_2(H_2O)_6$, and 65 % HNO_3); Sigma-Aldrich, Japan ($(NH_4)_2CO_3$, $CaCl_2(H_2O)_2$, $LaCl_3$)); Merck, Spain (NaOH, and 0.1 N NaOH); and J. T. Baker, Spain (KH_2PO_4). Medias and chemicals used for microbiology were purchased from OXID, France (MRS broth, MRS agar, and dehydrated culture media for microbiology); Panreac, Spain (agar technical ingredient for microbiology, and Glucose D (+), cycloheximide and chloramphenicol; VWR life science (Bacterial ultra-pure yeast extract, and tryptone peptone (peptone from casein) for microbiology; and VWR chemicals, from Belgium (Buffered peptone water, and PCA.

3.3.2 Injera processing

YEPD agar and broth were used to culture S. *cerevisiae* strain while MRS agar and broth for *Lactobacillus plantarum.* Culturing of the strains was first made on their respective agar medias and incubated at 30 ºC for 48 h. The strains were then cultured in their respective broth at 30 ºC for 24 h and then for 48 h in a new broth. Pellets of each strain were collected by centrifugation at 10,000 × g for 15 min at a temperature of 20 ºC using a SIGMA 3K30 Laborzentrifugen GmbH (Germany) centrifuge machine. Sterilized water was added to the pellets before the centrifugation was performed again to eliminate the broth. Finally, before use, the starter cultures were solubilized in small amount of sterilized water and then placed in refrigerator at 5 ºC for two days. After refrigeration the concentration was determined by making ten-fold dilutions and culturing them in their respective agar media.

Injera formulation was based on three different components: flour, water, and starter culture. Before starting the processing, teff flour and EP were sterilized in a hot dry air by an auto cooking machine RATIONAL SCC 61/06 (Germany) at 190 ºC for 6 minutes following the procedure described by Baye *et al.*, (2015) and cooled to room temperature.

Three different types of injera were prepared based on teff flour fortified with EP at three different levels: i) Control Injera (I0) based on 100% teff flour with no EP, ii) Injera I4.5 based on flour with 95.5% (w: w) teff flour and 4.5% EP and iii) Injera I9.0 based on flour with 91% (w: w) teff flour and 9% EP. The experiment was performed two times for each formulation. The level of EP added to the flour was selected to achieve the RDA of an adult person by consuming 200 g of injera, assuming that EP contained 50% (I4.5) or 25% (I9.0) of bioavailable Ca (FAO/WHO, 2005).

Injera was prepared following the procedure shown in Figure 1. Firstly, to make the batter, 1,100 g of the corresponding flour (teff flour and EP) and starter cultures (to reach 10^{7} CFU /ml in the final batter) along with sterilized ultrapure water to reach a final weight of 2360 g were placed into a Thermomix TM-5 (Vorwerk, Germany) to be mixed and kneaded at 3 rpm for 5 min at room temperature. Once the batter was obtained, it was placed in metal pot and the top was covered with 400 ml sterilized ultra-pure water to prevent mold growth.

Fermentation was performed in a convective oven at 25 ºC (room temperature in Ethiopia) for 48 h. After that, to prepare the *absit* (starter used as leaven) 150 g of fermented batter were taken and mixed well for two minutes then cooked in 450 ml of boiling water for 10 min by continual steering with a spoon. The *absit*, at approximately 60 ºC, was added back to the fermenting batter and mixed well with a spoon. After that, the mixture was kept at 25 ºC for about 2 h to be fermented further before cooking. Approximately 100 ml of the fermented batter was poured in a spiral manner onto an injera WASS Electronics Digital Mitad Grill, covered, and baked for 2 min at a cooking temperature of 121 ºC. The baked injera was then removed and kept in an airtight container at room temperature.

The samples used for quality parameters analysis were taken immediately after preparing the batter (B0), at 24 h (B24), 48 h (B48) and 50 h (B50) of fermentation of the batter. Sampling times were chosen based on the work described by (Misci et al., 2021). At each sample time, 200 g of the batter were taken and used for analysis. Finally, a cooked injera (INJ) analysis was performed. Sample collection is indicated in Figure 3.1.

3.3.3. Physicochemical parameters

3.3.3.1 pH and titratable acidity

pH of batter was determined using a Crison Basic 20 pH meter by directly inserting the electrode into the batter. Titratable acidity of batter was determined based on lactic acid following the method by Harth, Van Kerrebroeck, & De Vuyst (2016). The pH of injera was measured according to AOAC Official Method 945.42 (AOAC, 2005b). The solution, prepared to determine the pH of injera, was also used to determine its titratable acidity.

3.3.3.2 Moisture content and water activity

Moisture content of batter and injera was determined gravimetrically. Water activity of batter and injera was measured by LabMaster-Aw (Novasina AG; Lachen, Switzerland) at 25 ºC.

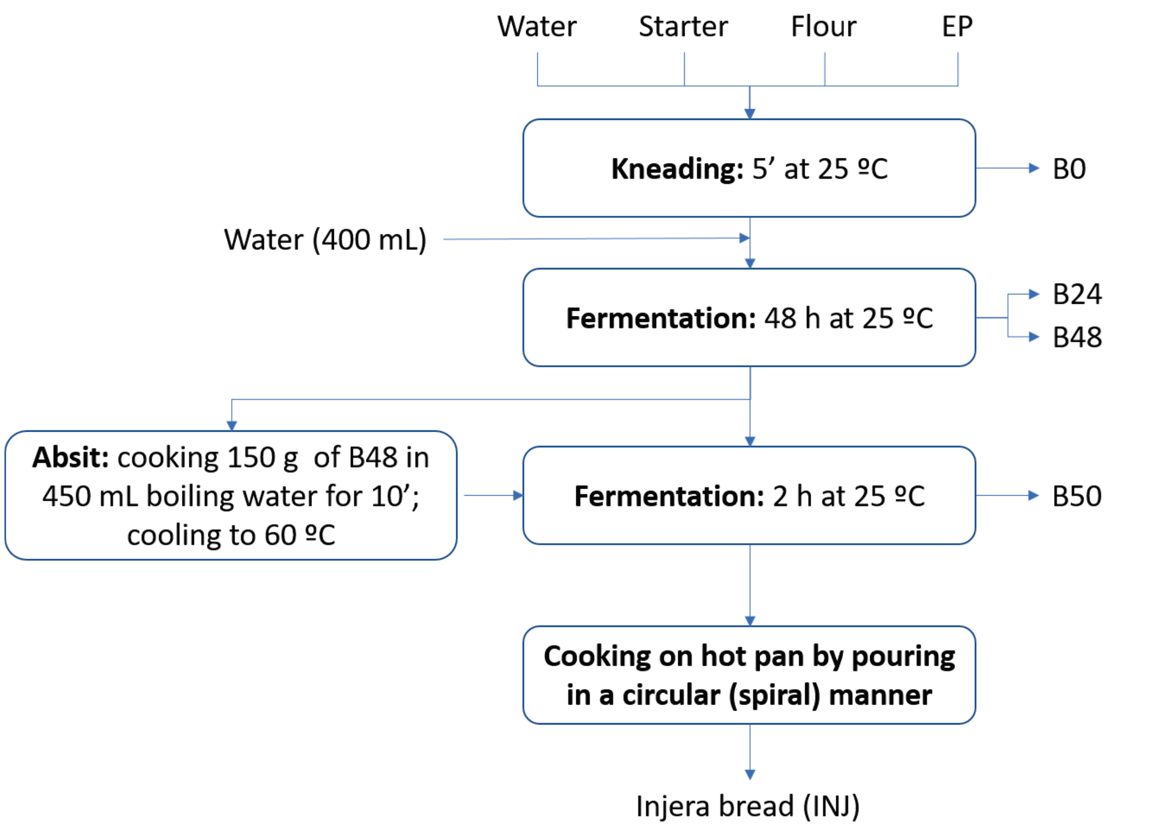

Figure. 3.1. Injera cooking flowchart. EP refers to eggshell powder; B0, B24, B48, B50 refer to batter samples at 0, 24, 48, & 50 hours of fermentation.

3.3.4. Physical parameters

3.3.4.1 Rheological properties of batter

The rheological properties of batter were analyzed using rotatory a viscosimeter Haake RV1 by means of a flow curve consisting of a ramp-up step from 0-500 s^{-1} during 90 s, a holding time of 30 s at 500 s^{-1} and a ramp-down step from 500-0 s^{-1} in 90 s. The Ostwald de Waele rheological model was applied to the ascending ramp flow curve, obtaining the consistency index (K), the flow behavior index (n) and dynamic viscosity (η) of the batter at a 100 s^{-1} shear gradient (Equation 1 and Equation 2). Measurements were done in five repetitions.

$$\tau = K{\cdot}\dot{\gamma}^{n}$$ Equation 1 (Ostwald de Waele model of flow curve)

$$\eta = K{\cdot}\dot{\gamma}^{n-1}$$ Equation 2 (Ostwald de Waele model of viscosity curve)

Where: τ is the shearing stress (Pa); K is the consistency index ($Pa{\cdot}s^{n}$); $\dot{\gamma}$ is the shear gradient (s^{-1}); n is the flow behavior index (dimensionless); and η is the dynamic viscosity (Pa·s)

3.3.4.2 Texture Profile Analysis (TPA) of injera

TA.XTi plus texture analyzer (Stable Micro Systems; Surrey, UK) was used to measure the mechanical properties of injera through a TPA. Five circular samples (ø 5 cm) were placed together one on top of the other under a flat probe (P/75 compression platen) attached to the testing machine (Kim, Kwak, and Jeong, 2017). The injera samples were compressed until reaching 75 % of its initial height, using a 0.049 N trigger force, and a test speed of 2 $mm{\cdot}s^{-1}$. Samples were left to rest for 5 s before the second compression. After testing, the hardness, springiness, and cohesiveness were calculated by using the software Exponent v6.1.16. Measurements were done in five repetitions.

3.3.4.3 Color

Color was assessed using the DigiEye Imaging System equipment (VeriVide Ltd.; UK). Five repetitions were done for both batter and injera samples, and CIE L*, a*, and b* coordinates were obtained as described by Etxabide, Kilmartin, & Maté (2021). The color difference (ΔE*) values for fortified injera (I4.5 and I9.0) with respect to control (I0) were calculated according to Eq. 3 as described by Garzon, Skendi, Lazo-Velez, Papageorgiou, & Rosell (2021).

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \qquad \text{Equation 3}$$

3.3.4.4 Eye formation

Eye qualities of injera were assessed using the softwares DigiEye v.2.8.0.2 (VeriVide Ltd.; UK) and ImageJ v.1.52a (National Institutes of Health, USA) on injera pictures previously taken. DigiEye was used to calculate the proportion between the dark brown color which represent the craters (eyes) and the light color proportion which represent the remaining part (surface). Mean size (area) and eye diameters were obtained by means of ImageJ after image calibration, color to gray transformation and thresholding using the "MaxEntropy" algorithm. Binary function "Fill Holes" was also used to avoid artifacts derived from nested eyes. Five repetitions of the measurements were carried out.

3.3.5 Microbial analysis

Yeast, lactic acid bacteria, and total mesophilic counts were assessed to study the microbial properties of batter, and injera. Samples were analyzed in duplicate using 10 g following the procedure described by Harth, Van Kerrebroeck, & De Vuyst, (2016). To assess the growth of the microorganisms, 0.1 ml of properly diluted sample of batter or injera was spread on pre-dried agar medias. MRS agar with 0.1 g/L of cycloheximide or YEPD with 0.1 g/L of chloramphenicol was used to assess the growth of lactic acid bacteria or yeast and molds respectively. The growth of total aerobic mesophilic bacteria was assessed on PCA at 30°C for 72 h (Harth, Van Kerrebroeck, & De Vuyst, 2018). The analysis was done two times and triplicate plates were used.

3.3.6 Nutritional Quality

3.3.6.1 Total Ca

Total Ca of injera I0, I4.5, and I9.0 was analyzed following the AOAC official method 985.35 (AOAC, 2005a). Dry ashing method was first used to destroy organic matrix in a muffle furnace at 525 ºC for 10 h. 1 M HNO_3 was used to aid in the ashing process and to solubilize white ash and to make dilutions. Total Ca was analyzed by inductively coupled plasma atomic emission spectroscopy (ICP - AES) at Universidad de Zaragoza, Spain. The amount of Ca was calculated and expressed as mg Ca/100 g of dried injera. The analysis of total Ca was carried out in duplicate with two samples of injeras processed independently.

3.3.6.2 Bioavailable Ca

A static *in vitro* simulation of gastrointestinal food digestion nature protocol developed by Brodkorb et al., (2019) was used to perform *in vitro* digestion of injera and thus to measure bioavailable Ca. Simulated stock solutions were prepared and stored at -20 ºC until use (Table 3.1). *In vitro* digestion of the injera at oral (2`), gastric (2 h), and intestinal phase (2 h) was made by incubating in Mini Shaker (VWR, USA) at 200 rpm and at 37 ºC, all detail is shown in Table 3.2.

After the digestion process, centrifugation of the sample was carried out using SIGMA 3K30 Laborzentrifugen GmbH (Germany) centrifuge machine at 3500 x g at 4 ºC for 1 h as described by Cámara et al. (2005). The supernatant was collected and placed into a 15 ml Falcon and sent to Universidad de Zaragoza for bioavailable Ca analysis. The analysis of total Ca was carried out in duplicate with two samples of injeras processed independently.

3.3.7 Statistical analysis

Analysis of variance (ANOVA) was performed, and multiple mean comparison was done by Tukey's Honestly Significant Difference (HSD) test at $p < 0.05$ using SPSS version 27 (SPSS Inc., Chicago, IL, USA).

Table 3.1. Volumes of electrolyte stock solutions of digestion fluids for a volume of 400 ml diluted with water

Solution	**Stock concentrations**	**Volume (mL) to be taken for electrolyte solution preparation 400 ml (for each fluid)**		
	(M)	**SSF**	**SGF**	**SIF**
KCl	0.5	15.1	6.9	6.8
KH_2PO_4	0.5	3.7	0.9	0.8
$NaHCO_3$	1.0	6.8	12.5	42.5
NaCl	2.0	-	11.8	9.6
$MgCl_2(H_2O)_6$	0.15	0.5	0.4	1.1
$(NH_4)_2CO_3$	0.5	0.06	0.5	-
$CaCl_2(H_2O)_2$	0.3	0.025	0.005	0.02
pH adjustment solutions				
HCl	6	0.09	1.3	0.7
pH		7	3	7

Table 3.2. In vitro digestion of injera

Digestion phase	Oral (SSF), pH 7			Gastric (SGF) pH 3			Intestinal (SIF) pH 7		
Food or digest	5 g of control injera			10 mL from oral phase			20 mL from gastric phase		
Electrolyte stock solutions (mL)	4			8			8		
CaCl2(H2O)2 (0.3 M) (mL)	0.025			0.005			0.04		
Enzymes	Salivary amylase			Pepsin			Trypsin in pancreatin	Bile salts	
Enzyme activity (U/mL) or bile concentration (mM) in total digesta (final volume in milliliters at each digestion phase, see row below)	75 U/mL			2000 U/mL			100U/mL	10 mM	
Specific activity (U/mg), concentration (bile) mmol/g	5 U/mg			3500 U/mg			6 U/mg	0.667 mmol/g	
Concentration of enzyme/bile solution (mg/mL)	200			0.019			133.3	6.25	
Volume of enzyme/bile to be added (mL)	0.75			0.667			5	3	
Type of injera sample	I0	I4.5	I.9	I0	I4.5	I9.0	I0	I4.5	I9.0
H_2O (mL)	0.129	0.195	0.208	1.168	0.9	0.758	3.86	3.865	3.869
HCl (5 M) for pH adj. (mL)	-	-	-	0.16	0.428	0.57	-	-	-
NaOH (5 M) for pH adj. (mL)	0.096	0.03	0.017	-	-	-	0.1	0.095	0.091
Final volume (mL)	10			20			40		

3.4 Results and Discussion

3.4.1 Physicochemical parameters

The **pH** of batter at different processing stages and pH of injera are shown in Figure 3.2. Results indicate that the addition of EP had a significant effect on the pH of both batter and injera. The higher the amount of EP added, the higher the pH. In addition, and was expected for all studied formulations, pH of batter decreased significantly with increased fermentation time. However, there was no significant difference on pH of batter at fermentation times of 48 h and of 50 h. For all formulations, the maximum pH was found in the B0 (batter at 0 h fermentation time) samples. pH of injera was 4.1 in I0 but increased to 5.7 and 6.3 for I4.5 and I9.0 respectively.

The pH of I0 batter after 48 h of fermentation (B3) was found to be 4.1 a value comparable with pH reported by other researchers which was ranged from 3.4 to 4.1 as for similar products (Fischer, Egli, Aeberli, Hurrell, & Meile, 2014). However, the pH of the I4.5 and I9.0 batters after fermenting for 48 h (5.0 and 5.4 respectively) were significantly higher than pH of I0 batter due to the presence of $CaCO_3$. The pH of Control Injera was 4.1 which was similar to the pH reported in previous research wherein the pH value fell between 3.4 and 4.5 (Ashagrie, & Abate, 2012). The pH of I4.5 and of I9.0 were 5.7 and 6.3 respectively, which was significantly higher than pH of I0. The increased pH of enriched injera will likely affect its characteristic sour taste and may increase the sweetness due to the presence of eggshell powder. In addition, the increased pH of enriched injera may have an implication on its shelf life since it would allow the growth of higher number of microorganisms.

The effect of EP addition on the TTA of batter and injera is shown in Figure 3.3. As the fermentation time increased there was an increase in the TTA of the three types of batter.

However, no significant effect was observed between samples of B48 and B50 in any batter. The TTA of control batter was the highest at all stages ranging from 3 % at the beginning of the fermentation to 11.4 % after fermenting for 50 h. Results also indicated that the higher the content of EP, the lower the TTA because of the neutralizing effect of $CaCO_3$. As observed for the decrease in the TTA of injera as the level of the eggshell powder increased also may have an implication on the shelf life of injera because an increase in acidity prevents the growth of microorganisms that cause spoilage.

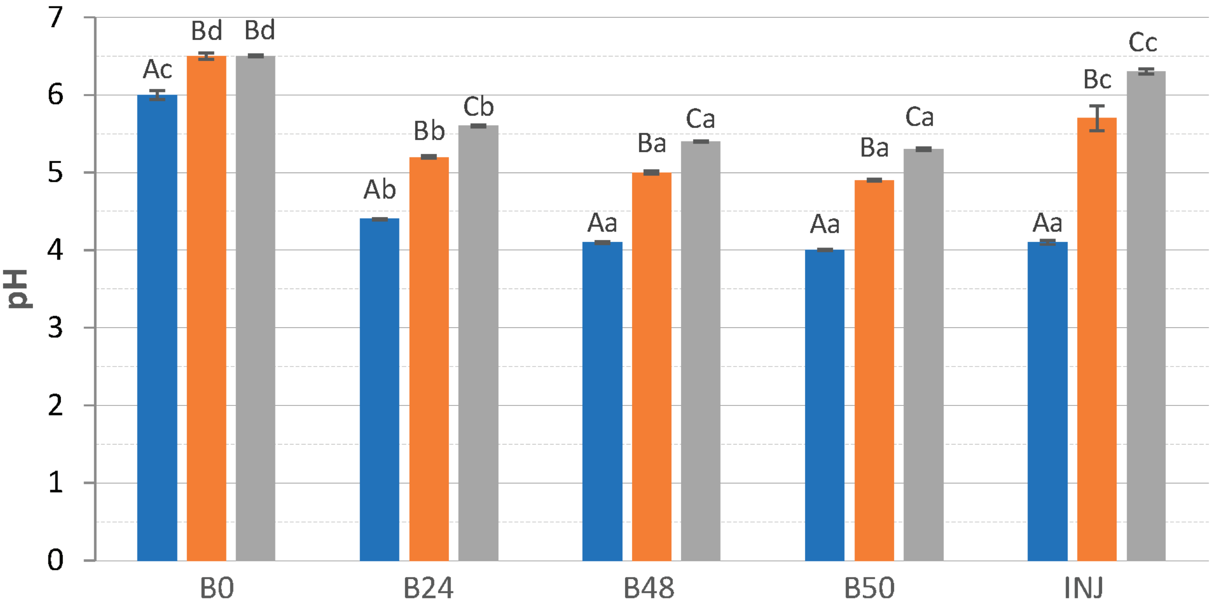

Figure 3.2. Effect of eggshell powder on pH of batter (B) after 0, 24, 48 and 50 h of fermentation and injera (INJ). I0: teff flour without eggshell powder (Control); I4.5: teff flour with 4.5% eggshell powder; I9.0: teff flour with 9.0% eggshell powder. Mean and standard error bars (n= 6). Capital letter shows difference based on formulation and small letter based on fermentation time.

The **moisture content** and **water activity** of batter or injera was not affected significantly due to the addition of EP. The maximum moisture content of batter was found to be 71.5 ± 0.2 % (B50). Average moisture content of injera was 60.5 ± 0.8 %, similar to injera moisture contents found by Cherie, Ziegler, Gemede, Woldegiorgis, & Yildiz (2018).

Water activity of batter was very high in all formulations and at all stages, even for injera. This fact is critical for the potential shelf life of this product. In all cases, water activity was above 0.95 allowing the growth of most microorganisms.

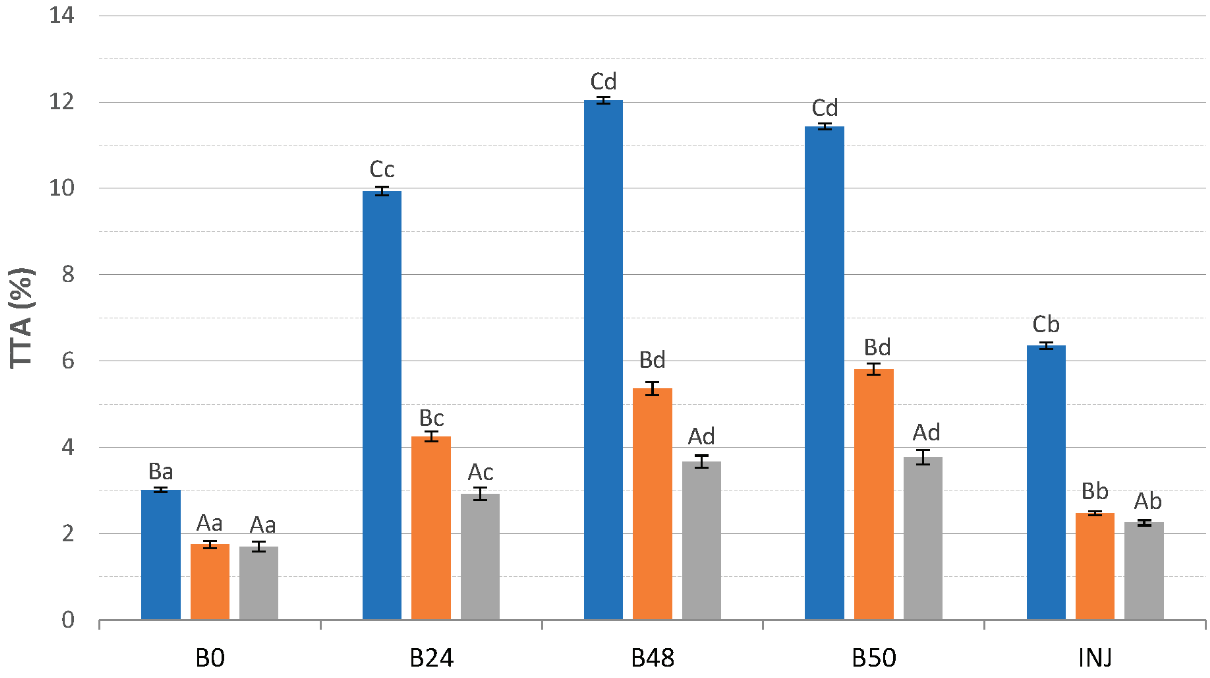

Figure 3.3. Effect of eggshell powder on total titratable acidity of batter (B) after 0, 24, 48 and 50 h of fermentation and injera (INJ). I0: teff flour without eggshell powder (Control); I4.5: teff flour with 4.5% eggshell powder; I9.0: teff flour with 9.0% eggshell powder. Mean and standard error bars (n= 6). Capital letter shows difference based on formulation and small letter based on fermentation time.

3.4.2 Rheological properties of batter

The viscosity (η_{100}), consistency index (K), and flow behavior index (n) of batter are shown in Figure 3.4.

The addition of eggshell powder significantly reduced the viscosity of batter at the beginning of fermentation process (Figure 3.4A). As a result of fermentation and addition of water in the process, viscosity dropped noticeably after 24-48 h, and again, after the addition of *absit* and the secondary fermentation step (B50). The reduction of viscosity due to the fermentation was so influential that the effect of the addition of EP was unnoticeable.

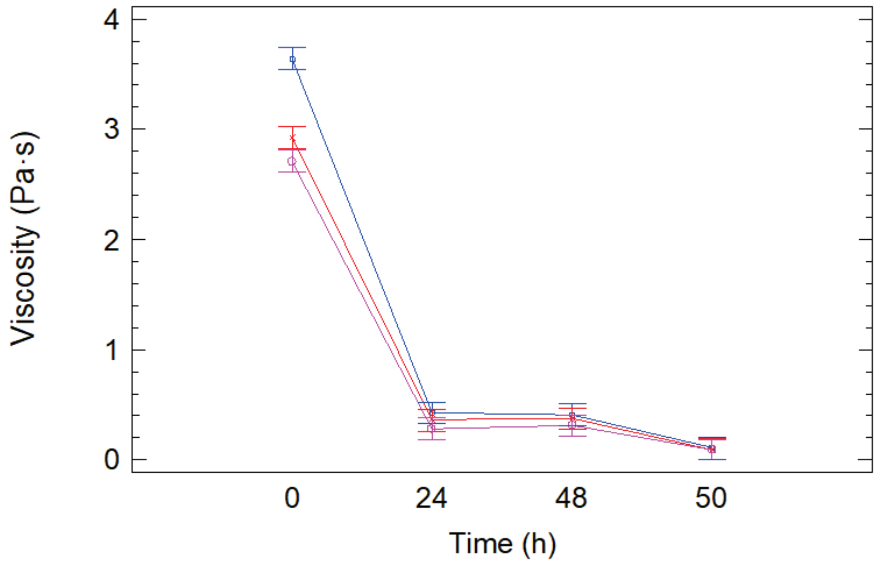

Figure 3.4A. Effect of eggshell powder addition on batter viscosity** ($\eta_{100}; Pa{\cdot}s$) **during the fermentation process. I0: teff flour without eggshell powder (Control -o-); I4.5 teff flour with 4.5% eggshell powder (-o-); I9.0: teff flour with 9.0% eggshell powder (-o-).

A similar pattern was observed in the evolution of the consistency index in time (Figure 3.4B). The presence of EP only had a significant effect on K at time 0 h. After that there was a drastic decrease of K due to the fermentation which reduced differences among treatments. The consistency index for the batter at 0 h (B0) were in the range of cake batter (17 - 40 Pa s^n) previously reported (Noorlaila, Hasanah, Asmeda, & Yusoff, 2020).

The evolution of the flow behavior index (n) of the batter is shown in Figure 3.4C. Just after the addition of the eggshell powder, significant differences among the pseudoplastic (n < 1) character of all the treatments were observed. At 0 h the non-Newtonian behavior was clearly associated to the proportion of added eggshell powder, making the batter more sensitive to shearing. This effect seemed to disappear after the 24-48 h period of fermentation, in which this parameter significantly decreased for all formulations. Finally, after the addition of *absit*, the batter recovered its initial non-Newtonian character, no matter the level of eggshell powder addition, likely due to an internal relaxation of the structure of the starch granules (gelatinization).

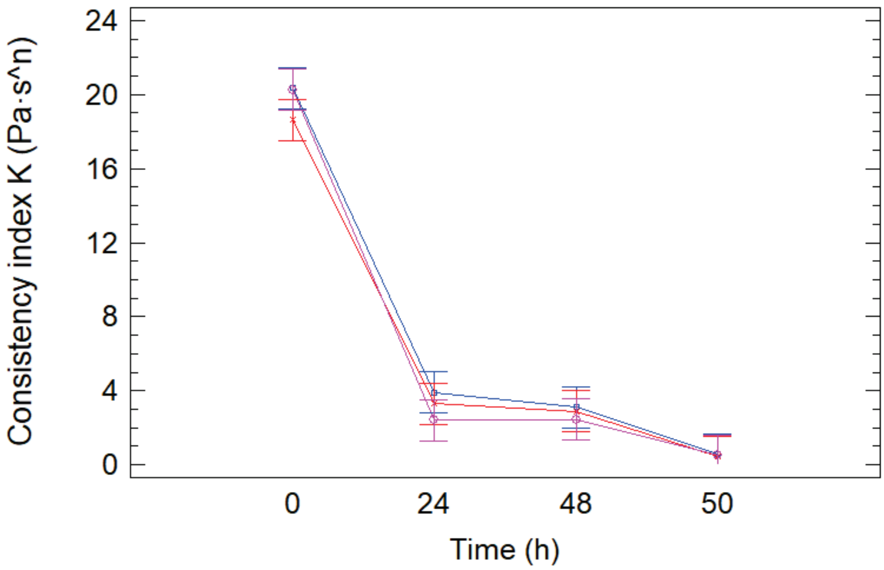

Figure 3.4B. Effect of eggshell powder addition on injera batter consistency index (K; Pa·s^n) during the fermentation process. *I0: teff flour without eggshell powder (Control -o-); I4.5 teff flour with 4.5% eggshell powder (-o-); I9.0: teff flour with 9.0% eggshell powder (-o-).*

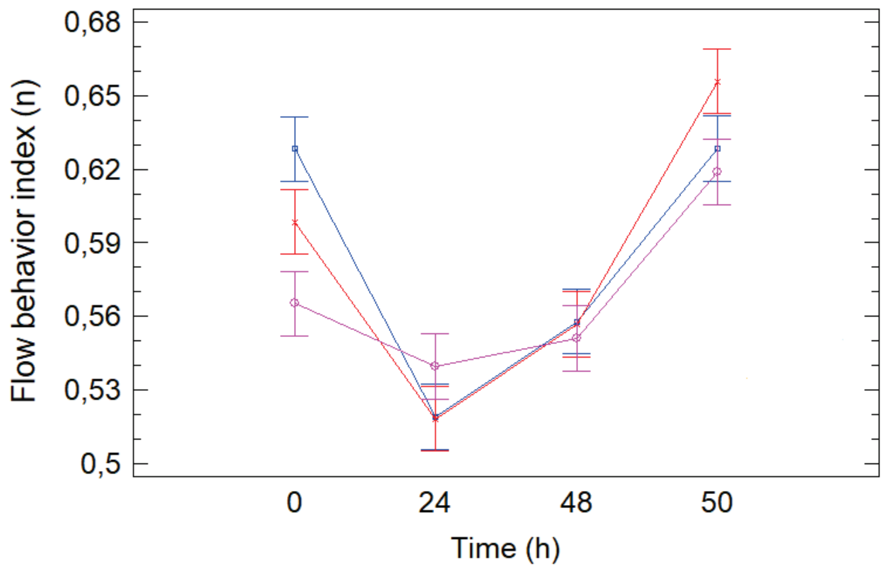

Figure 3.4C. Effect of eggshell powder addition on injera batter flow behavior index (n; dimensionless) during the fermentation process. *I0: teff flour without eggshell powder (Control -o-); I4.5: teff flour with 4.5% eggshell powder (-o-); I9.0: teff flour with 9.0% eggshell powder (-o-).*

3.4.3 Texture of injera

The texture parameters of injera obtained from TPA are shown in Table 3.3. The hardness of injera was affected by the addition of the eggshell powder although springiness and cohesiveness were not affected. The hardness of injera decreased as the level of eggshell powder increased from control injera (I0) to I4.5 and further to I9.0. Similar to our research, the hardness of bread decreased as the level of eggshell powder increased (Chilek et al., 2018). In addition, Bassett et al. (2014) found a decrease in the hardness of bread in the lower crust after Ca fortification with $CaCl_2$ and $CaCO_3$.

Recently, Yasin (2021) reported firmness of injera to be 3.13 N using a sharp blade cutting probe. The value found for hardness (a comparable term for firmness) in the current research is relatively higher due to the different method used to assess hardness of injera (shearing vs compression). Formerly, Yetneberk, Kock, Rooney, & Taylor (2004) conducted another study to evaluate flexion properties of injera using a three-point bending rig; the maximum bending force obtained was 0.14 N. Given the different experimental setup, results cannot be compared.

Table 3.3. Effect of eggshell powder on texture of injera. I0: Control (injera without eggshell powder); I4.5: injera with 4.5% eggshell powder; I9.0: injera with 9.0% eggshell powder

Treatments	Hardness (N)	Springiness	Cohesiveness
I0	90.8 (4.1) ^{C}	0.73 (0.1) ^{A}	0.53 (0.0) ^{A}
I4.5	82.8 (3.5) ^{B}	0.74 (0.0) ^{A}	0.53 (0.0) ^{A}
I9.0	76.1 (5.2) ^{A}	0.72 (0.0) ^{A}	0.50 (0.0) ^{A}

Result of texture analysis as mean (SD) of n = 5 observations of batter and injera. Statistical analysis: ANOVA, significant difference at $P < 0.05$. Difference in capital letters in the same column show significant difference based on addition of eggshell powder.

3.4.4 Color

The color of batter with different fermentation time, as well as the color of injera are shown in Table 2. Decreases in the lightness (L*) of batter was observed as the level of eggshell powder increased; however, there was no pattern in the characteristics of a* and b* parameters of the batter. There was also a decrease in the lightness of injera as the level of eggshell powder increased from I0 (53.4 ± 0.1) to I4.5 (51.5 ± 0.1) or to I9.0 (51.9 ± 0.1). No significant difference was found between I4.5 and I9.0 injera.

A relatively similar L* value of control injera was found in the current research compared with another research value of 54.95 (Cherie, Ziegler, Gemede, Woldegiorgis, & Yildiz, 2018). Chilek et al. (2018) studied the effect of EP on L* of wheat bread and found that lightness of bread decreased as the level of EP increased which is consistent with our study. A similar finding was observed by Bassett et al. (2014) when replacing 50 % NaCl with $CaCO_3$ and $CaCl_2$ observing that the color of wheat bread was darker. The difference in the lightness of injera due to the addition of EP could be due to the difference in the particle size of teff flour and EP powder (Chilek et al., 2018).

Arufe et al. (2018) stated that if ΔE* is greater than 3, a difference in color can be detected by the naked eye. In the current research, for I4.5, and I9.0 injera no visual color difference was observed compared with I0 since ΔE*; the value was below 3.

3.4.5 Eye formation

The visual quality of injera is determined by the number and size of eyes on its surface. Injera eye parameters related to eye quality are shown in Table 3.5. Results indicated that the addition of EP only significantly affected eye circularity and did not affect the rest of the eye quality parameters. Total eye area (darker color) was not modified by eggshell powder addition, allowing normal gas bubble formation and subsequent reticulation process. The same conclusions were derived from the analysis of individual eye area and mean eye diameter.

Table 3.4. The effect of eggshell powder on the color of batter and injera of batter (after 0, 24, 48 and 50 h of fermentation) and injera (INJ). I0: Control (injera without eggshell powder); I4.5: injera with 4.5% eggshell powder; I9.0: injera with 9.0% eggshell powder.

		I0	**I4.5**	**I9.0**
	B0	73.8 (0.3) Bb	73.6 (0.6) Bb	72.9 (0.7) Ab
L*	B24	78.4 (0.1) Cc	76.2 (0.9) Bc	75.0 (0.5) Ac
	B48	78.9 (0.3) Ccd	76.8 (0.7) Bc	75.8 (0.1) Ac
	B50	79.3 (0,3) Cd	76.9 (0,8) Bc	75.7 (0,4) Ac
	INJ	53.4 (0.1) Ba	51.5 (0.1) Aa	51.9 (0.1) Aa
	B0	4.2 (0.1) Ab	4.0 (0.2) Ab	4.2 (0.2) Ab
a	B24	4.7 (0.1) Bc	3.8 (0.3) Ab	3.7 (0.3) Aa
	B48	3.9 (0.2) Aa	3.8 (0.2) Ab	3.8 (0.4) Aab
	B50	3.8 (0.2) Ba	3.2 (0.2) Aa	3.5 (0.3) Aa
	INJ	7.1 (0.2) Bd	7.2 (0.2) Bc	6.8 (0.2) Ac
	B0	16.7 (0.1) Ca	17.3 (0.1) Bb	17.6 (0.2) Ac
	B24	17.6 (0.2) Cb	16.5 (0.3) Ba	16.0 (0.4) Aa
b	B48	18.3 (0.3) Bc	17.5 (0.5) Ab	16.9 (0.8) Aab
	B50	18.1 (0.2) Bc	17.1 (0.4) Ab	17.4 (1) ABc
	INJ	19.1 (0.5) Cd	18.4 (0.5) Bc	16.6 ± 0.9 Aab
	B0	-	5.2	5.9
	B24	-	2.6	3.8
ΔE*	B48	-	2.3	3.2
	B50	-	2.7	3.7
	INJ	-	2.4	1.9

Result of color as mean (SD) of n = 5 observations of batter and injera. Statistical analysis: ANOVA, significant difference at $P < 0.05$. Difference in small letters in the column show difference based on fermentation time. Difference in capital letters in the same row show significant difference based on addition of eggshell powder.

However, injera fortified with 4.5% eggshell powder showed a significant lower circularity (more irregular shape), probably related with small differences in the speed of manual spreading of the batter over the hot plate during the cooking process.

Table 3.5. Effect of eggshell powder addition on injera eye quality parameters. I0: Control (injera without eggshell powder); I4.5: injera with 4.5% eggshell powder; I9.0: injera with 9.0% eggshell powder.

	Treatment	Mean (±SD)
Total eye area (%)	I0	38.92 (2.5) A
	I4.5	40.18 (2.1) A
	I9.0	40.97 (2.1) A
Individual eye area (mm²)	I0	5.87 (0.7) A
	I4.5	5.79 (0.8) A
	I9.0	5.61 (0.8) A
Eye diameter[1] (mm)	I0	2.84 (0.2) A
	I4.5	2.86 (0.2) A
	I9.0	2.78 (0.2) A
Eye circularity (minor/major axis ratio)	I0	0.70 (0.0) B
	I4.5	0.67 (0.0) A
	I9.0	0.69 (0.0) B

[1]Mean Feret diameter. Result of injera eye quality as mean (SD) of n = 5 observations of injera. Statistical analysis: ANOVA, significant difference at $P < 0.05$. Difference in capital letters in the same column show significant difference based on addition of eggshell powder.

Sensory analysis has been used by different researchers to determine injera eye qualities which were expressed as number of eyes and eye size (Agza, Bekelea, & Shiferaw 2018; Yetneberk, Kock, Rooney, & Taylor, 2004). Image analysis can be a good approach to sensory perception of eye properties of injera. Thus, Yasin (2021) used the software Injera Eyes, version 1.0.0.0 to determine the number of injera eyes in a fixed surface of 1550 x

1550 pixels, from which an "eye density" can be obtained. Our current research assessed the proportion of dark brown color area (number of pixels darker than a certain threshold) as an approach to the sensory perception of global "eye proportion" perceived by consumers. Complementarily, the present work aimed to obtain a deeper understanding about the morphology of the eyes studying its symmetry and size.

3.4.6 Microbial quality

The evolution of the growth of lactic acid bacteria, total aerobic mesophilic bacteria and yeast is shown in Table 3.6. The results indicated the addition of EP did not have significant effect on the microbial quality. The differences in pH and acidity among batters of the different formulations and the presence of $CaCO_3$ did not significantly affect microbial evolution.

For all formulations, when the fermentation time increased from 0 h to 24 h a significant increase on microbial population was found. However, no significant differences were observed when the fermentation time increased from 24 h onwards.

After 24 h fermentation, lactic acid bacteria and total aerobic mesophilic bacteria counts were found to be higher than those of other research work in which natural fermentation (no inoculation) was used (Fischer, Egli, Aeberli, Hurrell, & Meile, 2014). Hassen, Mukisa, Kurabachew, & Desalegn (2018) also indicated that lactic acid bacteria and yeast did not grow as much when natural fermentation was used as compared with controlled fermentation. In addition, it is likely that flour sterilization performed in our research could have contributed to facilitate microbial growth during fermentation.

Table 3.6. Effect of addition of eggshell powder on the microbial population of batter (after 0, 24, 48 and 50 h of fermentation) and injera. I0: Control (teff flour without eggshell powder); I4.5: teff flour with 4.5% eggshell powder; I9.0: teff flour with 9.0% eggshell powder.

		I0	**I4.5**	**I9.0**
	B0	7.2 (0.1) ^{A}	7.1 (0.1) ^{A}	7.2 (0.1) ^{A}
Lactic acid bacteria	B24	9.6 (0.1) ^{B}	9.6 (0.0) ^{B}	9.7 (0.3) ^{B}
(log cfu/g)	B48	9.6 (0.0) ^{B}	9.6 (0.1) ^{B}	9.6 (0.0) ^{B}
	B50	9.5 (0.1) ^{B}	9.5 (0.0) ^{B}	9.5 (0.0) ^{B}
	INJ	ND	ND	ND
	B0	7.4 (0.1) ^{A}	7.3 (0.1) ^{A}	7.4 (0.1) ^{A}
Total aerobic mesophilic	B24	9.6 (0.1) ^{B}	9.6 (0.1) ^{B}	9.8 (0.3) ^{B}
(log cfu/g)	B48	9.6 (0.1) ^{B}	9.6 (0.0) ^{B}	9.6 (0.0) ^{B}
	B50	9.5 (0.1) ^{B}	9.6 (0.0) ^{B}	9.5 (0.0) ^{B}
	INJ	ND	ND	ND
	B0	6.9 (0.0) ^{A}	7.0 (0.1) ^{A}	7.0 (0.2) ^{A}
Yeast (log cfu/g)	B24	7.8 (0.0) ^{B}	7.8 (0.1) ^{B}	7.7 (0.1) ^{B}
	B48	7.8 (0.0) ^{B}	7.8 (0.0) ^{B}	7.8 (0.0) ^{B}
	B50	7.8 (0.1) ^{B}	7.8 (0.1) ^{B}	7.8 (0.1) ^{B}
	INJ	ND	ND	ND

Microbial growth as mean (SD) at fermentation times of 0 h (B1) (B2),24 h, (B3)48 h, (B4)50 h and injera (INJ). Statistical analysis: ANOVA, significant difference at $P < 0.05$. Different in letters in the same column show difference based on fermentation time.

3.4.7 Nutritional quality

The effect of the addition of EP on both total and bioavailable Ca of injera is shown in Table 3.7. The level of total Ca in injera increased from 235.9 mg/100 g (dry basis, d.b.) in control injera to 2157.9 mg/100 g (d.b.) and 4397.4 mg/100 g (d.b.) for I4.5 and I9.0 respectively. This was expected due to the large $CaCO_3$ content in EP.

Total Ca content in injera I0 in the current research was high as compared with another research finding that presented 61 mg/100 g (d.b.) (Cherie, Ziegler, Gemede, Woldegiorgis, & Yildiz, 2018). This could be due to the different processing methods of injera. In other research, after the last stage of fermentation, surface water on top of the batter was thrown. This practice may have decreased the total Ca since some of it may have been lost with the discarded water.

Table 3.7. Effect of eggshell powder on total and bioavailable calcium of injera. I0: Control (teff flour without eggshell powder); I4.5: teff flour with 4.5% eggshell powder; I9.0: teff flour with 9.0% eggshell powder.

	Total Ca (mg/100 g db)	Bioavailable Ca (mg/100 g)	Percentage of bioavailable Ca (%)
I0	235.9 (1.7) ^{A}	16.1 (0.4) ^{A}	6.8 ^{A}
I4.5	2157.9 (38.3) ^{B}	742.7 (48.9) ^{B}	34.4 ^{B}
I9.0	4397.4 (313.7) ^{c}	1743.1 (164.0) ^{c}	39.6 ^{B}

Total, bioavailable and percent of bioavailable Ca as mean (SD) of n = 2 observations of injera. Statistical analysis: ANOVA, significant difference at P < 0.05. Different in letters in the same column show difference based on addition of eggshell powder.

In addition, taking into account injera I0 formulation, total Ca in teff flour used in this study can be also considered higher than the one reported in the literature (141-188 mg/100 g d/b) (Kibatu, Chacha, & Kiende, 2017; Shumoy, & Raes, 2017). These differences are likely based on varietal difference of teff grain. Anyhow, the values of Ca in teff were found to be higher as compared to other cereals such as maize (16 mg/100 g), wheat (15.2 - 39.5 mg/100 g), sorghum (5.0 - 5.8 mg/100 g) and rice (23 mg/100 g) expressed on d.b (Baye, 2014).

Thus, teff flour and injera could be a potentially better Ca source than other cereals. However, the percentage of bioavailable Ca for the control injera was found to be 6.8 % which can be considered very small. The reason for the low level of bioavailable Ca in injera is likely due to the high content of phytates and fiber (Baye, 2014). Anti-nutrients bind micronutrients and make them unavailable for absorption by human body. A study conducted on the bioavailability of Ca in spinach showed it was also very small (1.7 %) due to the high content of anti-nutrient oxalate which binds Ca and makes it unavailable for absorption (Cámara, Amaro, Barberá, & Clemente, 2005). An in-vitro study on the bioavailability of other minerals such as iron and zinc on injera showed that the high level of phytates also affected their bioavailability (Shumoy et al., 2017). In light of all these facts, it is important to not only increase Ca in the diet but to ensure there is also an increase in its bioavailability.

When EP was included in the formulation, bioavailable Ca increased from 16.1 mg/100 g (d.b.) for injera I0, to 742.7 and 1743.1 mg/100 g for injera I4.5 and I9.0 respectively (Table 5). This meant that the percentage of bioavailable Ca increased from 6.8 % to almost 40 % in both fortified formulations. It is possible that the amount of added Ca in the form of $CaCO_3$ was not affected by the antinutrients that are present in injera. It is also possible that the binding capacity of the phytates was saturated when Ca content was high enough. The high percentage of bioavailable Ca with respect to the total Ca is an indication that eggshell is an efficient source of Ca for fortified diets.

If it is assumed that the average amount of injera consumed by an Ethiopian adult is about 200 g (wet basis; w.b.), considering that the moisture content of injera is about 60 %, the intake of bioavailable Ca with injera would be 12.9 mg/day when injera I0 is included in the diet. If injera I4.5 is consumed instead, the amount of bioavailable Ca would be 594.2 mg/day; and if injera I9.0 is used the amount would turn into 1394.5 mg/day. According to (FAO/WHO, 2005) t he RDA and Upper Intake Level (UL) of Ca range from 1000 to 3000 mg /day. Thus, with the consumption of 200 g of injera, an adult will get 60% of the minimum daily requirement of Ca when consuming injera I4.5 and 140% of the total RDA of Ca when consuming injera I9.0. Extra Ca intake is known to minimize the risk of chronic diseases such as hypertension if the upper intake limit is not reached. In addition, Ethiopian Great Rift Valley areas are highly affected by fluorosis which has affected bone and teeth health. In this region, it is especially important to maintain high levels of Ca intake to reduce the health consequences related to fluorosis (Patel et al., 2017; Srivastava et al., 2017). Moreover, the life expectancy of Ethiopians is increasing; thus, there are more elderly people in the country. This group is highly susceptible to osteoporosis and likely to be at risk in the future also, if the diet does not change. Thus, there is a need to increase Ca in the diet of the population to reduce the risk of osteoporosis among the elderly, now and in the future. The use of eggshell powder as an additive in the process of injera could be part of the solution.

In addition, it is recommended that for Ca availability and utilization, instead of taking a large amount of Ca at a time it is better to take smaller amounts at different times throughout the day. Bioavailability of Ca has been shown to increase when it is taken with food instead of on an empty stomach (Rafferty, Walters, & Heaney, 2007). The tradition of consuming injera at least twice a day in a small size portion instead of consuming a large amount at one time is advantageous with respect to the use of Ca by the body.

3.5 Conclusions

Even though the total Ca in injera is high, its contribution to the nutritional Ca requirement is minimal since the level of bioavailable Ca is very low in this foodstuff. The current research revealed that the addition of eggshell powder is an excellent source of total and bioavailable Ca. Adding 4.5% or 9% of eggshell powder to the teff flour drastically increased the absolute and relative amounts of bioavailable Ca. Furthermore, even though the addition of eggshell powder significantly affected both pH and acidity of the batter and injera, it did not significantly affect microbial evolution in the batter, nor the physical quality parameters of injera including texture, eye parameters and color. The addition of EP to injera formulation can be considered a new tool to improve the Ethiopian diet. Clinical studies are needed to confirm the actual benefit of the enrichment. In addition, sensory acceptability of injera with EP for Ethiopian population will have to be addressed in the future.

3.6 References

Agza, B., Bekele, R., & Shiferaw, L. (2018). Quinoa (Chenopodium quinoa, Wild.): As a potential ingredient of injera in Ethiopia. Journal of Cereal Science, 82(January), 170–174. https://doi.org/10.1016/j.jcs.2018.06.009

Amare, B., Moges, B., Moges, F., Fantahun, B., Admassu, M., Mulu, A., & Kassu, A. (2012). Nutritional status and dietary intake of urban residents in Gondar, Northwest Ethiopia. BMC Public Health, 12(1). https://doi.org/10.1186/1471-2458-12-752

AOAC. (2005a). Mineral in Infant Formula, Enteral Products, and Pet Foods. Official Methods of Analysis of AOAC International, 514, 1–3.

AOAC. (2005b). pH of bread. Official Methods of Analysis of AOAC International, 02.

Arufe, S., Della Valle, G., Chiron, H., Chenlo, F., Sineiro, J., & Moreira, R. (2018). Effect of brown seaweed powder on physical and textural properties of wheat bread. European Food Research and Technology, 244(1), 1–10. https://doi.org/10.1007/s00217-017-2929-8

Ashagrie, Z. and Abate, D. (2012). Improvement of Injera Shelf Life Through the Use of. African Journal of Food, Agriculture, Nutrition and Development, 12(5), 6409–6423.

Bassett, M. N., Pérez-Palacios, T., Cipriano, I., Cardoso, P., Ferreira, I. M. P. L. V. O., Samman, N., & Pinho, O. (2014). Development of Bread with NaCl Reduction and Calcium Fortification: Study of Its Quality Characteristics. Journal of Food Quality, 37(2), 107–116. https://doi.org/10.1111/jfq.12079

Baye, K. (2014). Synopsis : Teff : nutrient composition and health benefits. Ethiopia Strategy Support Program, October, 2013–2014.

Brodkorb, A., Egger, L., Alminger, M., Alvito, P., Assunção, R., Ballance, S., Bohn, T., Bourlieu-Lacanal, C., Boutrou, R., Carrière, F., Clemente, A., Corredig, M., Dupont, D., Dufour, C., Edwards, C., Golding, M., Karakaya, S., Kirkhus, B., Le Feunteun, S., … Recio, I. (2019). INFOGEST static in vitro simulation of gastrointestinal food digestion. Nature Protocols, 14(4), 991–1014. https://doi.org/10.1038/s41596-018-0119-1

Brun, L. R., Lupo, M., Delorenzi, D. A., Di Loreto, V. E., & Rigalli, A. (2013). Chicken eggshell as suitable calcium source at home. International Journal of Food Sciences and Nutrition, 64(6), 740–743. https://doi.org/10.3109/09637486.2013.787399

Cámara, F., Amaro, M. A., Barberá, R., & Clemente, G. (2005). Bioaccessibility of minerals in school meals: Comparison between dialysis and solubility methods. Food Chemistry, 92(3), 481–489. https://doi.org/10.1016/j.foodchem.2004.08.009

Cherie, Z., Ziegler, G. R., Fekadu Gemede, H., Zewdu Woldegiorgis, A., & Yildiz, F. (2018). Optimization and modeling of teff-maize-rice based formulation by simplex lattice mixture design for the preparation of brighter and acceptable injera. Cogent Food & Agriculture, 4(1), 1443381. https://doi.org/10.1080/23311932.2018.1443381

Chilek, T. Z. T., Kairuaman, N. A., Ahmad, F., Wahab, R. A., Zamri, A. I., & Mahmood, A. (2018). Development of white bread fortified with calcium derived from Eggshell powder. Malaysian Applied Biology, 47(6), 29–39.

Cilla, A., López-García, G., & Barberá, R. (2018). In vitro bioavailability of iron and calcium in cereals and derivatives: A review. Food Reviews International, 34(1), 1–33. https://doi.org/10.1080/87559129.2016.1210631

Daengprok, W., Garnjanagoonchorn, W., & Mine, Y. (2002). Fermented pork sausage fortified with commercial or hen eggshell calcium lactate. Meat Science, 62(2), 199–204. https://doi.org/10.1016/S0309-1740(01)00247-9

Etxabide, A., Kilmartin, P. A., & Maté, J. I. (2021). Color stability and pH-indicator ability of curcumin, anthocyanin and betanin containing colorants under different storage conditions for intelligent packaging development. Food Control, 121. https://doi.org/10.1016/j.foodcont.2020.107645

FAO/WHO Expert Consultation. (2005). Human Vitamin and Mineral Requirements. Geneva : World Health Organization, 2nd ed, 341 p. http://apps.who.int/iris/handle/10665/42716

Fischer, M. M., Egli, I. M., Aeberli, I., Hurrell, R. F., & Meile, L. (2014). Phytic acid degrading lactic acid bacteria in tef-injera fermentation. International Journal of Food Microbiology, 190, 54–60. https://doi.org/10.1016/j.ijfoodmicro.2014.08.018

Garzon, R., Skendi, A., Antonio Lazo-Velez, M., Papageorgiou, M., & Rosell, C. M. (2021). Interaction of dough acidity and microalga level on bread quality and antioxidant properties. Food Chemistry, 344, 128710. https://doi.org/10.1016/j.foodchem.2020.128710

Gharibzahedi, S. M. T., & Jafari, S. M. (2017). The importance of minerals in human nutrition: Bioavailability, food fortification, processing effects and nanoencapsulation. Trends in Food Science and Technology, 62, 119–132. https://doi.org/10.1016/j.tifs.2017.02.017

Harth, H., Van Kerrebroeck, S., & De Vuyst, L. (2016). Community dynamics and metabolite target analysis of spontaneous, backslopped barley sourdough fermentations under laboratory and bakery conditions. International Journal of Food Microbiology, 228, 22–32. https://doi.org/10.1016/j.ijfoodmicro.2016.04.011

Harth, H., Van Kerrebroeck, S., & De Vuyst, L. (2018). Impact of process conditions on the microbial community dynamics and metabolite production kinetics of teff sourdough fermentations under bakery and laboratory conditions. Food Science and Nutrition, 6(6), 1438–1455. https://doi.org/10.1002/fsn3.690

Hofmeyr, G. J., Duley, L., & Atallah, A. (2007). Dietary calcium supplementation for prevention of pre-eclampsia and related problems: A systematic review and commentary. BJOG: An International Journal of Obstetrics and Gynaecology, 114(8), 933–943. https://doi.org/10.1111/j.1471-

0528.2007.01389.x

Kibatu, G., Chacha, R., & Kiende, R. (2017). Determination of Major, Minor and Trace Elements in Tef Using Portable Total X-Ray Fluorescence (TXRF) Spectrometer. EC Nutrition, 1, 51–59.

Kim, S., Kwak, H. S., & Jeong, Y. (2017). Effect of Water Roux Starter (Tangzhong) on Texture and Consumer Acceptance of Rice Pan Bread. Journal of Texture Studies, 48(1), 39–46. https://doi.org/10.1111/jtxs.12204

Misci, C., Taskin, E., Dall'Asta, M., Fontanella, M. C., Bandini, F., Imathiu, S., Sila, D., Bertuzzi, T., Cocconcelli, P. S., & Puglisi, E. (2021). Fermentation as a tool for increasing food security and nutritional quality of indigenous African leafy vegetables: the case of Cucurbita sp. Food Microbiology, 99. https://doi.org/10.1016/j.fm.2021.103820

Noorlaila, A., Hasanah, H. N., Asmeda, R., & Yusoff, A. (2020). The effects of xanthan gum and hydroxypropylmethylcellulose on physical properties of sponge cakes. Journal of the Saudi Society of Agricultural Sciences, 19(2), 128–135. https://doi.org/10.1016/j.jssas.2018.08.001

Omi, N., & Ezawa, I. (1998). Effect of Egg-Shell Ca on Preventing after Ovariectomy and doing moderate physical activity are necessary been sufficient in Japan , the Ca intake in particular has never been sufficient (Ministry of Health and Welfare 1997). The Ca intake has nearly re. Journal of Home Economics, 49(3), 277–282.

Patel, P., Patel, P., Zulf, M., Yagnik, B., Kajale, N., Mandlik, R., Khadilkar, V., Chiplonkar, S., Phanse, S., Patwardhan, V., Joshi, P., Patel, A., & Khadilkar, A. (2017). Association of dental and skeletal fluorosis with calcium intake and serum Vitamin D concentration in adolescents from a region endemic for fluorosis. Indian Journal of Endocrinology and Metabolism, 21(1), 190–195. https://doi.org/10.4103/2230-8210.196013

Pettifor, J. M. (2004). Nutritional rickets: deficiency of vitamin D, calcium, or both? The American Journal of Clinical Nutrition, 80(6 Suppl), 1725–1729. https://doi.org/10.1093/ajcn/80.6.1725s

Rafferty, K., Walters, G., & Heaney, R. P. (2007). Calcium fortificants: Overview and strategies for improving calcium nutriture of the U.S. population. Journal of Food Science, 72(9). https://doi.org/10.1111/j.1750-3841.2007.00521.x

Schaafsma, A., Pakan, I., Hofstede, G. J. H., Muskiet, F. A. J., Van Der Veer, E., & De Vries, P. J. F. (2000). Mineral, amino acid, and hormonal composition of chicken eggshell powder and the evaluation of its use in human nutrition. Poultry Science, 79(12), 1833–1838. https://doi.org/10.1093/ps/79.12.1833

Shumoy, H., Lauwens, S., Gabaza, M., Vandevelde, J., Vanhaecke, F., & Raes, K. (2017). Traditional fermentation of tef injera: Impact on in vitro iron and zinc dialysability. Food Research International, 102(June), 93–100. https://doi.org/10.1016/j.foodres.2017.09.092

Shumoy, H., & Raes, K. (2017). Tef: The Rising Ancient Cereal: What do we know about its Nutritional and Health Benefits? Plant Foods for Human Nutrition, 72(4), 335–344. https://doi.org/10.1007/s11130-017-0641-2

Srivastava, A. K., Singh, A., Tripathi, P., & Mathur, A. (2017). Prevalence of dental fluorosis and the role of calcium supplementation in its prevention. Journal of Medical Sciences (Faisalabad), 17(4), 156–161. https://doi.org/10.3923/jms.2017.156.161

Tezera, F., Whiting, S. J., & Gebremedhin, S. (2017). Dietary calcium intake and sunlight exposure among children aged 6-23 months in Dale Woreda, Southern Ethiopia. African Journal of Food, Agriculture, Nutrition and Development, 17(3), 12427–12440. https://doi.org/10.18697/ajfand.79.16510

Hassen Y, Mukisa IM, Kurabachew H and Desalegn B. (2018). Evaluation of Yeast and Lactic Acid Bacteria Starter Cultures for the Production of Rice Injera. Journal of Food Processing & Technology, 09(03). https://doi.org/10.4172/2157-7110.1000721

Yasin, U. A. (2021). Textural and Eye Qualities of Injera Prepared from Quality Protein Maize and Teff Blended Flours. International Journal of Nutrition and Food Science, 10(4), 78–85. https://doi.org/10.11648/j.ijnfs.20211004.11

Yetneberk, S., De Kock, H. L., Rooney, L. W., & Taylor, J. R. N. (2004). Effects of Sorghum Cultivar on Injera Quality. Cereal Chemistry, 81(3), 314–321. https://doi.org/10.1094/CCHEM.2004.81.3.314

CHAPTER 4.

SHELF-LIFE STUDY OF EGGSHELL POWDER FORTIFIED INJERA

CHAPTER 4. SHELF-LIFE STUDY OF EGGSHELL POWDER FORTIFIED INJERA

4.1 Abstract

Injera is a staple bread consumed by most people in Ethiopia. Even though injera is a perishable food, there was not much work done to improve its shelf life except by use of chemical preservatives or by the use vacuum packaging. The objective of the current research was therefore to improve the shelf life of injera using modified atmosphere packaging and cold storage and to assess its effect on injera qualities. Injera was prepared by adding 4.5% and 9% EP to the teff flour (injera I4.5 and I9.0, respectively). Injera samples were analyzed for pH, water activity, moisture content, color, texture, and microbiology during 20 days of storage. The findings of the study revealed that temperature, atmospheric condition and time did not affect factors like aw and moisture content. However, those factors affected pH, texture and microbial growth on stored injera. Cold storage has been found to be most effective on prevention of microbial growth. However, it affected negatively the texture of injera, since storage time made increase hardness of injera. The use of MAP resulted in significant effect on controlling microbial growth. However, no significant effect was observed when MAP was used for fortified injera.

Key words: Injera, eggshell powder, MAP, cold storage, shelf life

4.2 Introduction

Injera, like other breads, is a staple food which is commonly consumed by most people in Ethiopia. Even though injera is a perishable food, there was not much work done to improve its shelf life except by use of chemical preservatives (Ashagrie, Z. and Abate, 2012; Hassen et al., 2018). Terefe et al. (2022) has studied the shelf-life improvement of injera by the use of vacuum packaging, air packaging, combination of vacuum packaging and chemical preservatives and combination of air packaging and chemical preservatives. The condition of injera preparation has not changed over time. Women are still the main cook and industrialization of injera is at an infancy stage. Injera cooking is done on daily basis which makes hard for women to be involved in its preparation and for those people who are involved in its commercialization. There are good starts for marketing injera not only for domestic consumption but also for export level. For both markets controlling injera shelf life is crucial. The importance of improving the shelf life of injera is not only the benefit for business owners, but also very important for Ethiopian women who are involved in cooking injera every 2-4 days.

Shelf life of injera is very short, from two to four days (Ashagrie, Z. and Abate, 2012; Hassen et al., 2018). One of the drawbacks of the short shelf life of injera is that it is very likely that injera contributes to increase food waste. In countries like Ethiopia, where there is limited production of foodstuff and it is not enough to support the needs of the nation, if there is a great loss of the food due to food wastage, it will aggravate the food and nutrition security problem. In some countries the household bakery products waste account to 31%; however, with the use of proper food packaging it is believed that the waste can be minimized (WRAP, 2008). At the same time, it is estimated that roughly one-third of food produced globally is lost or wasted along the food chain (Ivanova et al., 2020). In sub-Saharan African countries, where almost one in every four people is undernourished and lacks adequate food for a healthy and productive life, it is estimated that close to 40% of food produced is lost or wasted (Bremner, 2017; Sheahan and Barrett, 2017). Data related to injera or in general bakery products in Ethiopia is lacking. However, few research work did present the data of certain kinds of food waste. For example, a study done by Parmar *et al.* (2017) showed that there is 34-54% cassava postharvest waste in Ethiopia.

Injera is not so different from other bread types in relation to its perishability. Similar with other breads, injera shelf life is 2-4 days if no preservatives are used. Even though injera is widely consumed by the people in Ethiopia, not much work has been done so far to improve its shelf life. There is not packaging practice for injera except that it is covered with polyethylene bag right after cooking which helps maintain the softness of the product for a day or so. However, since the product is not packed, the staling problem occurs mainly losing its freshness is a problem. As the storage time increased, injera become harder to eat. Apart from the staling problem, microbial spoilage, mainly mold growth, has impact on the less shelf life of injera. Among the research work related to the microbial study of injera shelf life, Ashagrie, Z. and Abate (2012) and Hassen et al. (2018) studied the way for improving the shelf life of injera by using chemical preservatives focusing on prevention of the growth of yeast and mold.

Ashagrie, Z. and Abate (2012) had studied the spoilage of injera due to mold growth. According to them, three types of fungal species belonging to the genera of *Penicillium*, *Aspergillus* (*Aspergillus niger*) and *Rhizopus* species were responsible for injera spoilage. The study stated that small white fungal colonies (visible to the naked eye) appeared on injera starting on the 4th day of storage. In addition, it showed that *Penicillium* and *Rhizopus* were more dominant in spoiling injera at lower temperature, while *Aspergillus niger* grew much faster as temperature gets higher (25-32 ºC), however; neither of them grew at 4 ºC. the work by Terefe et al. (2022) had determined the total aerobic bacterial count and yeast and mold growth in injera under the different packaging system and the use of chemical preservatives.

Studies from bread showed that microbial growth such as yeast, molds, and bacteria are the ones affecting the shelf life of bread (Pasqualone, 2019). A study by Gerez et al., (2009) showed that mould growth is by far the most important microbiological shelf-life limiting factor of bread products, with Penicillium spp., Aspergillus spp. and Fusarium spp. being the most dominant species. Spoilage of bread can also be caused by chalk yeasts (Deschuyffeleer et al., 2011). Spore forming bacterial spoilage can also be observed in bread (Debonne et al., 2018).

Short shelf life of bread is related to the loss of freshness due to staling. The problem exists due to physicochemical changes which gives rise to increasing crumb firmness and crumbliness due to moisture loss and starch retrogradation (Pasqualone, 2019). Water migration plays an important part for bread quality and its distribution among the bread components. Bread with crust loses its freshness quickly while bread without crust stays fresh longer (Bechtel et al., 1953; Besbes et al., 2014). However, the effect of the presence of the crust is exerted on the exchange of water between the crumb, the crust and the atmosphere and not on starch retrogradation. According to Ronda et al. (2011), starch retrogradation and water loss have effects of the same intensity on the increase in bread firming. In addition, increase in firmness, there is taste and aroma alterations during staling due to oxidative phenomena (Pasqualone, 2019).

People in Ethiopia have developed strategies to minimize food waste due to microbial spoilage (mold growth) and loss of freshness of injera. Firstly, the part of the injera which has a visible mold growth is removed; secondly, injera without visible mold growth isheated and used to prepare ‘firfer’ (a traditional food from injera served as a meal). People also give the mold grown injera for animal consumption or throw it away. Those mentioned practices can have a health risk due to the spoiled food which increase the country burden.

Use of proper packaging is among the means of preservation of bread which is useful to retard moulding and oxidation and it is helpful to maintain freshness of the bread (Pasqualone, 2019). The main aim of packaging is protecting food from degradative factors such as light, oxygen, water vapor, molds, yeasts, bacteria, and insects. Another goal of packaging, indeed, is delaying bread drying out, which involves the use of films with low water vapor permeability. Although starch retrogradation is the real cause of crumb-hardening, a higher moisture level is perceived as freshness. Traditionally, packaging materials had to be as inert as possible (so-called passive packaging), and bread was protected by barrier film made of polymers with low gas permeability, coupled with a modification of internal atmosphere (Pasqualone, 2019).

The packaging materials commonly used in the system of modified atmosphere packaging (MAP) are laminated film made of one layer of polyamide (nylon) and one layer of polyethylene (PA/PE), laminated films of PVDC/cellophane/PVDC/PE, laminated films of OPA and OPA/PE, and combinations of metalized polymers (Pasqualone, 2019). Upasen, & Wattanachai (2018) used single LDPE layer and three layers of LDPE laminated with O-nylon for preserving chemical free white bread under the use of MAP. The research by Debonne et al. (2018) used a tray made of PP/EVOH/PP and sealed with a cover film of OPA/PE/EVOH/PE/PP to pack bread by using MAP with gas composition of 70% CO_2 and 30% N_2.

Bread molding can be inhibited by reducing as much as possible the oxygen concentration in the headspace of the package. The use of vacuum packaging, however, is uncommon for bread because its alveolar and soft structure would collapse, especially in those bread types with soft surface. Modified atmosphere packaging (MAP), instead, consists of displacing atmospheric air by a gas mixture commonly composed of carbon dioxide (CO_2) and nitrogen. The latter works as an inert filling gas; that is it is used for displacing O_2 and prevents package collapse (Karin et al., 2005). MAP allows the reduction of oxygen concentration in the headspace of the package to <1%. Furthermore, CO_2 has a fungistatic activity that can slow mold formation. CO_2 alters the permeability and functionality of mold cell membrane, with negative effects on nutrient uptake and absorption, and lowers the intracellular pH, altering enzyme and metabolic activities (Pasqualone, 2019).

Research works related to the preservation of injera either with or without the fortification with EP are limited. In addition, those limited research works used chemicals for preserving injera in which that may impose negative health effect on the consumers. Thus, the objective of the current research was to increase the shelf life of injera (EP fortified and non-fortified) using modified atmosphere packaging and cold storage and to assess its effect on injera qualities.

4.3 Materials and methods

4.3.1 Materials

White teff flour was provided by Blonk Quality Ingredients (Spain). Eggshell powder (EP) was provided by Eggnovo S.L. (Spain). The starter cultures *Saccharomyces cerevisiae* and *Lactobacillus plantarum* subsp. *plantarum* were purchased from Valencia University CECT (Spanish Type Culture Collection). Medias and chemicals used for microbiology were purchased from OXID, France (MRS broth, MRS agar, DRBC, and dehydrated culture media for microbiology); Panreac, Spain (agar technical ingredient for microbiology, and Glucose D (+), cycloheximide and chloramphenicol; VWR life science (Bacterial ultra-pure yeast extract, and tryptone peptone (peptone from casein) for microbiology; and VWR chemicals, from Belgium (Buffered peptone water, and PCA.

4.3.2 Methods

4.3.2.1 Injera processing

As described in chapter 2, lactic acid bacteria strain *Lactobacillus plantarum 1170 (L. plantarum),* and yeast strain *Saccharomyces cerevisiae 748* were used for fermentation during injera preparation. Yeast extract dextrose peptone (YEPD) agar was used to culture S. *cerevisiae* strain. YEPD agar was prepared by putting 16 g of D-glucose, 8 g of agar, 2 g of trypton peptone and 2 g of yeast in 400 ml of sterilized water and then mixed using magnetic stirrer. Sterilization of the media was done in an autoclave at a temperature of 121 ºC for 15 minutes. Man -Rogosa-Sharpe (MRS) agar which was used to culture *Lactobacillus plantarum* was prepared by mixing 26.92 in 400 ml of sterilized water and sterilizing it at 121 ºC. Single strains of *S. cerevisiae* and *L. plantarum* were cultured on YEPD agar and MRS agar respectively, and then were incubated at 30 ºC for 48 h. The strains were then successively sub-cultured in YEPD broth and MRS broth at 30 ºC for 24 h and then for 48 h in a new broth. Pellets of yeast and lactic acid bacteria were collected by centrifugation at 10000 × g for 15 min at a temperature of 20 ºC using SIGMA 3K30 Laborzentrifugen GmbH (Germany) centrifuge machine. Sterilized water added to the pellets and then the centrifugation done again to clean the broth. Finally, before use, the starter cultures were solubilized with small amount of sterilized water and then put to a refrigerator at a temperature of 5 ºC for two days until the concentration was determined. After refrigeration the concentration was determined by making ten-fold dilutions and culturing them in their respective agar media.

Injera was prepared based on three different ingredients: flour, water, and starter culture. Injera was formulated by adding EP at two levels and having control injera (C) without the addition of EP. S45 injera constituted 4.5 % EP and 95.5% teff flour, and S9 injera constituted 9% EP and 90% teff flour. Before starting the processing, teff flour was sterilized in a hot dry air by an auto cooking machine RATIONAL SCC 61/06 (Germany) at 190 ºC for 6 minutes following the procedure described by (Baye *et al.*, 2015) and cooled to room temperature.

Injera was prepared following the procedure shown in Figure 2.1 of chapter 2. Firstly, to make the batter, 1,100 g of teff flour and starter cultures (to reach 10^{7} CFU /ml in the final batter) along with sterilized ultrapure water to reach a final weight of 2360 g were placed into a Thermomix TM-5 (Vorwerk, Germany) to be mixed and kneaded at 3 rpm for 5 min at room temperature. The 1.100 g flour constituted the three types of injera at the different level of EP and teff flour that means it was either all the 1.100 g only teff (C) or containing teff and EP in the case of I4.5 and I9.0 injera. Once the batter was obtained, it was placed in metal pot and the top was covered with 400 ml sterilized ultra-pure water to prevent mold growth.

Fermentation was performed in a convective oven at 25 ºC (room temperature in Ethiopia) for 48 h. After that, to prepare the *absit* 150 g of fermented batter were taken and mixed well for two minutes then cooked in 450 ml of boiling water for 10 min by continual steering with a spoon. The *absit*, at approximately 60 ºC, was added back to the fermenting batter and mixed well with a spoon. After that, the mixture was kept at 25 ºC for about 2 h to be fermented further before cooking. Approximately 100 ml of the fermented batter was poured in a spiral manner onto an injera WASS Electronics Digital Mitad Grill, covered, and baked for 2 min at a cooking temperature of 121 ºC. The baked injera was then removed and kept in an airtight container at room temperature. Injera samples then cooled for an hour and heat-sealed with Ethylene-vinyl alcohol (EVOH) using heat-sealing machine making the atmosphere in the package to be air and MAP with a concentration of 30% CO_2 and 70% N_2. When MAP was used for packaging, the atmospheric air composition in the packages were evacuated first and then back flushed with a mixture of $C0_2$ and N_2 with a level of 30% and 70% respectively.

4.3.2.2 Experimental design

In order to perform the experiments, 75 g samples of injera I, I4.5 or I9.0 were places in PP plastic trays sealed with a PP/EVOH/PP cover films. An ILPRA tray sealing machine to incorporate the corresponding atmosphere into the package headspace. Four storage conditions (two temperatures and two atmosphere) were included in the study: i) air at 25 ºC (AIR 25), ii) MAP (30% CO_2, 70% N_2) at 25 ºC (MAP 25), iii) air at 5 ºC (AIR 5), and iv) MAP (30% CO_2, 70% N_2) at 5 ºC (MAP 5). Samples were analyzed on days 0, 3, 7, 13 and 20. Each sampling day 2 packages of injera (75 g X2) were used for the analysis. Firstly, the composition of the headspace was analyzed, and then quality parameters were studied including pH, water activity, moisture content, color, texture, and microbiology. The whole research was repeated twice for all preservation methods and the three types of injera.

4.3.2.3 Atmospheric composition of packaging headspace

Prior to the analysis of the injera quality in each time, the atmospheric composition of the headspace was checked. To do this, the Dansensor CheckMate 3 – AMETEK MOCON gas headspace checker was used.

4.3.2.4 Physicochemical parameters

pH: pH of injera was measured according to AOAC Official Method 945.42 (AOAC, 2005b). Injera samples (10 g) were taken and cut with a coffee grinder machine for 30 seconds, mixed with 100 ml of distilled water that was boiled and cooled at 25 ºC, mixing continued for 30 minutes at 25 ºC, and then settled for 10 minutes. pH of the supernatant was immediately measured after decanting it into a beaker.

Moisture content: The moisture content of injera was determined by gravimetrically. Five grams of sample was dried at 105 ºC for 24 h. The dried sample then was put an in desiccator to prevent any gain of moisture and then weighed in a weighing scale. Loss in weight was recorded as moisture content of injera.

Water activity: The water activity of injera was measured by weighing 2 g of the samples in the measuring plastic cup and recording the result. The measurement was always done at 25 ºC by the machine LabMaster-Aw (Novasina AG; Lachen, Switzerland).

4.3.2.5 Physical parameters

Texture Profile Analysis (TPA) of injera: TA.XTi plus texture analyzer (Stable Micro Systems; Surrey, UK) was used to measure the mechanical properties of injera through a texture profile analysis. Circular samples were cut by means of a sharp stainless-steel hole borer of 3 cm diameter and one cut injera sample was used each time for analysis. Cylindrical 36 mm diameter probe attached to the testing machine was used. The injera samples were compressed until reaching 75 % of its initial height, using a 0.147 N trigger force and a pre-test, test and post-test speeds of 5 mm/s, 0.5 mm/s and 10 mm/s respectively. Samples were allowed to rest 5 s between the two consecutive compressions of TPA. After testing, mechanical parameters hardness, springiness and cohesiveness were calculated by means of dedicated macro subroutines with the software Exponent v6.1.16. Measurements were done with three different 3 cm circular samples repetitions.

Color: Color was assessed using the DigiEye Imaging System equipment (VeriVide Ltd.; UK). Five repetitions were done for injera samples, and CIE L*, a*, and b* coordinates were obtained as described by Etxabide, Kilmartin and Maté (2021). The color difference (ΔE*) values for fortified injera (I4.5 and I9.0) with respect to control (I0) was calculated according to Equation 4.1.

$$\Delta E = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \qquad \text{Equation 4.1}$$

4.3.2.6 Microbial analysis

Total mesophilic count, and yeast and mold growth were assessed to study the microbial properties of injera. Samples were analyzed in duplicate by taking 10 g and mixing with 90 ml of 0.1% sterilized buffered peptone water using a stomacher bag for 2 minutes and then tenfold dilutions were made. To assess the growth of the microorganisms, 0.1 ml of properly diluted sample of injera was spread on pre-dried agar medias. To assess the growth of yeast and molds Dichloran-rose Bengal Chloramphenicol agar (DRBC) was used. The incubation of the media was done for five days to assess the growth of yeast and mold. However, if no growth was observed, the incubation of the media was continued for two more days. The growth of total aerobic mesophilic bacteria was assessed on plate count agar (PCA). The incubation of the media was done at 30 °C for 72 h to count the colonies. The analysis was done two times and triplicate plates were used (Harth, Van Kerrebroeck and De Vuyst, 2018).

4.3.3 Statistical analysis

Analysis of variance (ANOVA) was performed, and multiple mean comparison was done by Tukey's Honestly Significant Differences (HSD) multiple rank test at $p < 0.05$ using SPSS version 27 (SPSS Inc., Chicago, IL, USA).

4.4 Result and discussion

4.4.1 Composition of atmospheric gas in the package

The atmospheric composition of the package was measured before opening it to analyze the injera qualities. Figures 4.1 and 4.2 showed the evolution of the concentration of O_2 and CO_2 inside the packages respectively.

Figure 4.1 showed how the CO_2 in the package changed as the preservation days increased. When the injera was kept in MAP 5 and MAP 25, the CO_2 level decreased as the storage time increased for all the three types of injeras (control, I4.5, and I9.0). A similar finding was observed by the work of Secchi et al. (2017) in which the level of CO_2 was decreased as the preservation time was increased. The researcher used a combination of MAP 70/30 for N_2/CO_2 and atmospheric air packaging to preserve whey cheesecake. The CO_2 level in the MAP packed injera was above 20% even if it had showed a decrease. This level is the minimum level to prevent the growth of aerobic spoilage microorganisms like molds which are considered to be the main cause of injera spoilage (Smith et al., 2004). For injera packed under AIR no significant difference has been seen as the preservation time was increased in all the three types of injera.

Figure 4.2 showed how the O_2 in the package varied with the increased preservation time for all the three formulations of injera. In packaging with AIR 25 and AIR 5, there was not significant differences in the level of oxygen with increased preservation time. However, when injera was preserved using MAP 25, and MAP 5, there was a slight increase in the level of oxygen with increased preservation time.

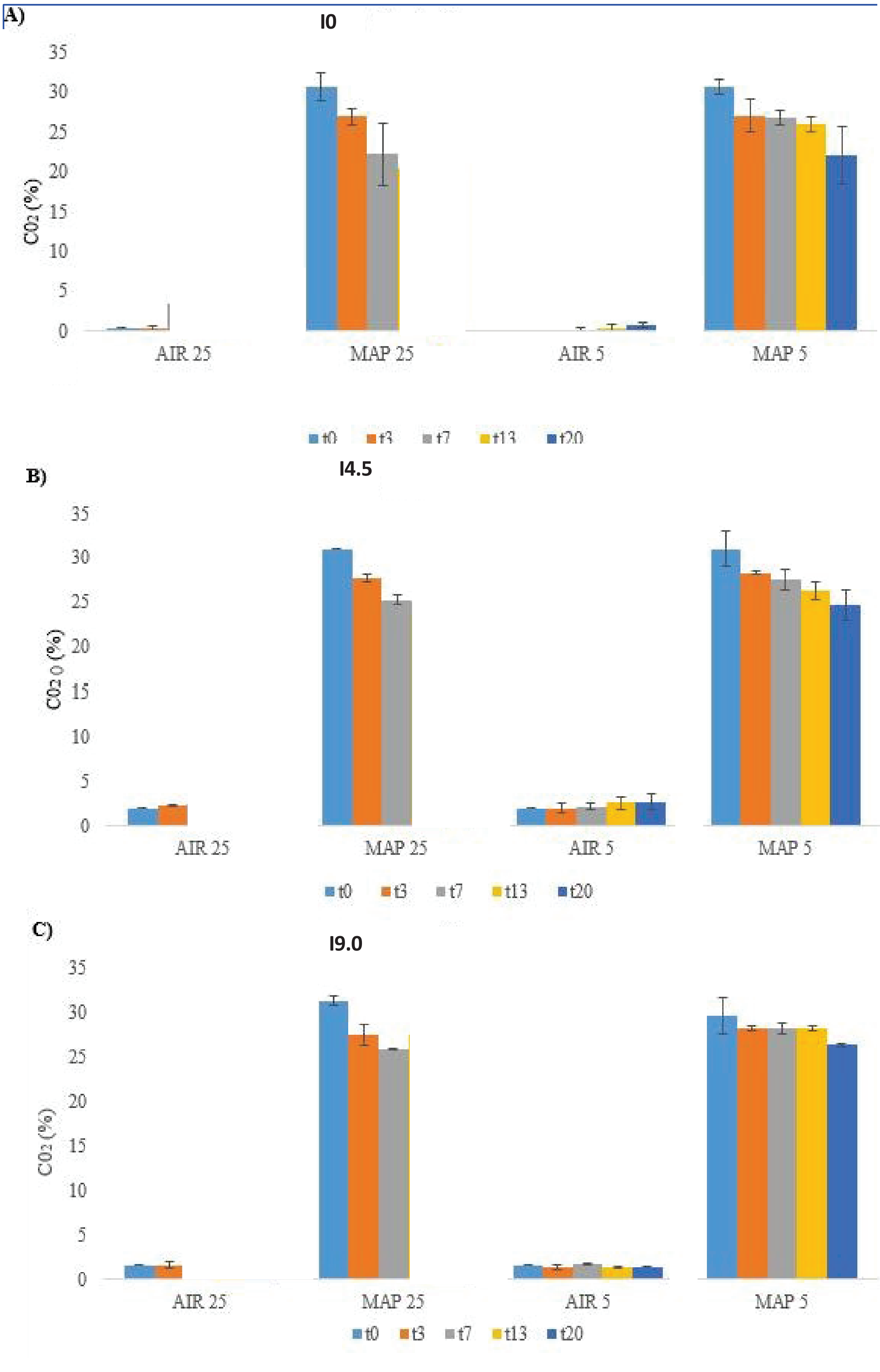

Figure 4.1 Evolution of CO_2 content in the package of I0, I4.5, and I9.0 injera

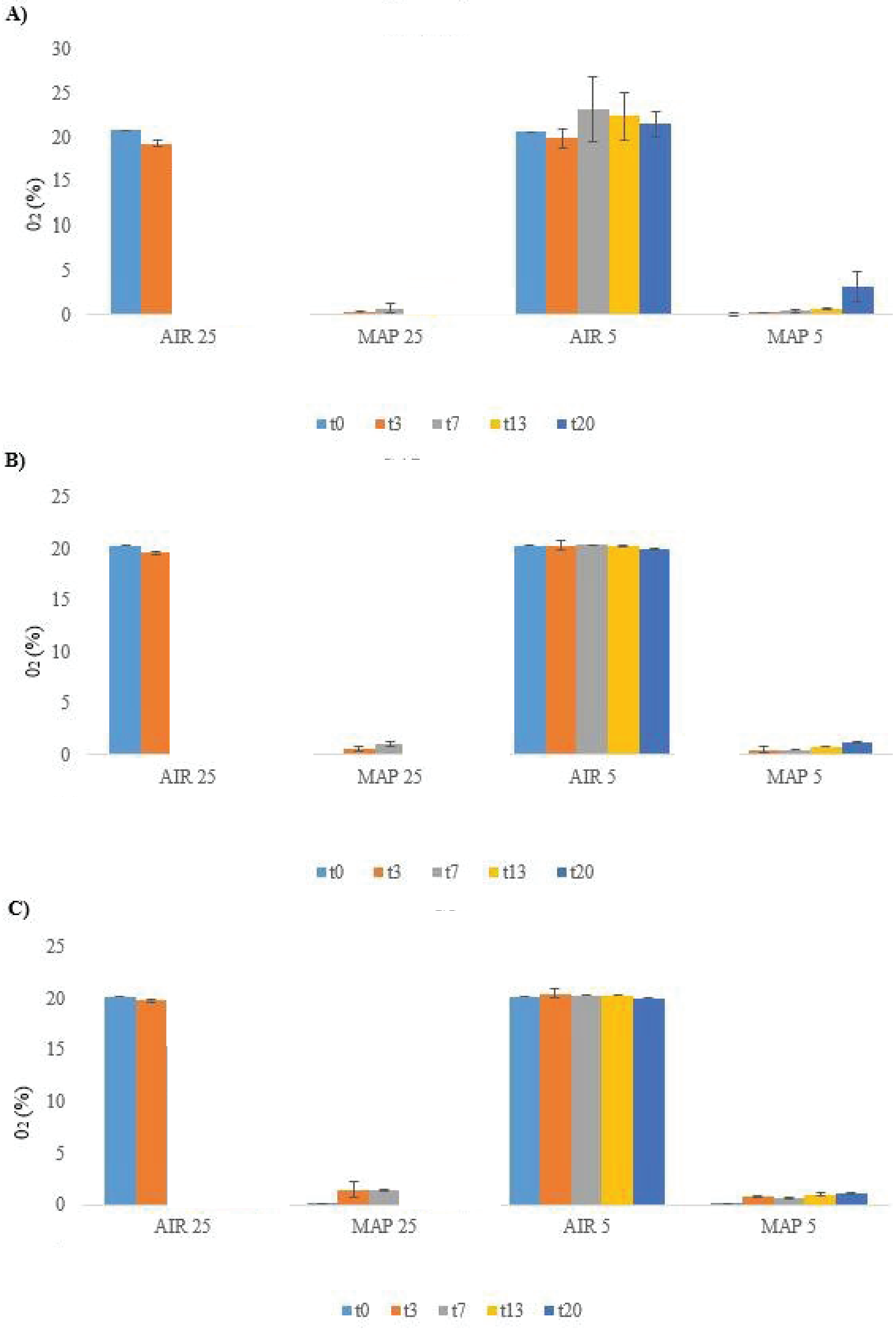

Figure 4.2 Evolution of O_2 content in the package of I0, I4.5, and I9.0 injera

The change in the composition of the headspace air, which had led to a slight increase in the O_2 level and decrease in the CO_2 level when packed under MAP, may be related to the packaging method. Research works confirmed that even for a high barrier film of the package, there is always a change in headspace composition due the transmission of gas from the environment (Smith et al., 2004).

4.4.2 Physicochemical qualities of injera

pH: the change in pH of injera for the three formulations upon increased preservation time has been shown in Table. 4.1. The result of pH of control injera and I4.5 injera indicated that the pH didn't change significantly as the days of preservation increased.

Table 4.1 Effect of preservation methods and time on pH injera. I0: injera without eggshell powder; I4.5: injera with 4.5% eggshell powder; I9.0: injera with 9.0% eggshell powder.

Preservation time		AIR 25 ˚C	MAP 25 ˚C	AIR 5˚C	MAP 5 ˚C
0	**I**	4.0 (0.3) Aa	4.0 (0.3) Aa	4.0 (0.3) Aa	4.0 (0.3) Aa
3		4.2 (0.1) Aa	4.2 (0.1) Aa	4.2 (0.1) Aa	4.3 (0.0) Aa
7			4.2 (0.1) Aa	4.2 (0.1) Aa	4.2 (0.1) Aa
13				4.1(0.1) Aa	4.2 (0.2) Aa
20				4.1 (0.1) Aa	4.2 (0.0) Aa
0	**I4.5**	5.7 (0.1) Aa	5.7 (0.1) Aa	5.7(0.1) Aa	5.7 (0.1) Aa
3		6.0 (0.2) Aa	6.0 (0.2) Aa	6.0 (0.1) Aa	6.0 (0.3) Aa
7			5.9 (0.1) Aa	5.9 (0.0) Aa	5.9 (0.0) Aa
13				5.8 (0.0) Aa	5.9 (0.1) Aa
20				5.8 (0.1) Aa	5.7 (0.0) Aa
0	**I9.0**	6.1 (0.1) Aa	6.1 (0.1) Aa	6.1 (0.1) Aa	6.1 (0.1) Aa
3		6.1 (0.1) Aa	6.1 (0.0) Aa	6.1 (0.0) Aa	6.0 (0.1) Aa
7			6.5 (0.1) Ab	6.5 (0.0) Ab	6.4 (0.1) Ab
13				6.6 (0.2) Ab	6.5 (0.2) Ab
20				6.5 (0.0) Ab	6.4 (0.2) Ab

Result of pH as mean ± SD of n = 6 observations of injera. Statistical analysis: ANOVA, significant difference at $P < 0.05$. Difference in small letters in the column show difference based on preservation time. Difference in capital letters in the same row show significant difference based on preservation method.

The research work done by Daifas et al., 1999 on crepe showed that there was no change in the pH upon increased preservation time when MAP or atmospheric air were used. However, there was increase in pH of I9.0 injera as the preservation time increased. This could be due the addition of EP to I9.0 injera at a higher level which has contributed to the higher level of Ca in injera. The change in the pH among the different preservation methods was not significant in all the three types of injera.

aw: the change in aw of injera for the three formulations upon increased preservation time is shown in Table. 4.2. Results indicated that for all injera types and preservation treatments and times difference in aw among the different preservation methods was not significant. Secchi et al. (2017) the aw of the product has not been changed significantly. A study conducted by Vlášek, Langová, & Štencl (2013) showed that gluten free bread had minimal aw changes during storage condition of atmospheric air, N_2 and CO_2 and has stated the reason could be due to the fact that gluten free bakery being less hygroscopic than common bakery products.

Table 4.2 Effect of preservation methods and time on aw injera. I0: injera without eggshell powder; I4.5: injera with 4.5% eggshell powder; I9.0: injera with 9.0% eggshell powder.

Preservation time		**AIR 25 ˚C**	**MAP 25 ˚C**	**AIR 5˚C**	**MAP 5 ˚C**
0	**I0**	0.97 (0.01)	0.97 (0.01)	0.97 (0.01)	0.97 (0.01)
3		0.97 (0.02)	0.96 (0.01)	0.97 (0.01)	0.95 (0.01)
7			0.97 (0.01)	0.95 (0.02)	0.95 (0.01)
13				0.98 (0.00)	0.98 (0.01)
20				0.95 (0.00)	0.97 (0.01)
0	**I4.5**	0.95 (0.01)	0.95 (0.01)	0.95 (0.01)	0.95 (0.01)
3		0.95 (0.02)	0.95 (0.01)	0.94 (0.01)	0.94 (0.01)
7			0.97 (0.01)	0.96 (0.01)	0.97 (0.01)
13				0.96 (0.01)	0.95 (0.01)
20				0.92 (0.00)	0.93 (0.01)
0	**I9.0**	0.92 (0.01)	0.92 (0.01)	0.92 (0.07)	0.92 (0.01)
3		0.93 (0.01)	0.93 (0.02)	0.92 (0.03)	0.92 (0.01)
7			0.95 (0.01)	0.97 (0.04)	0.94 (0.00)
13				0.95 (0.19)	0.96 (0.02)
20				0.95 (0.02)	0.95 (0.00)

Result of aw as mean (SD) of n = 6 observations of injera. Statistical analysis: ANOVA, significant difference at $P < 0.05$.

Aw of control injera was similar with increased time of preservation however for I4.5 and I9.0 injera the aw seem to be increased with increased preservation time.

Moisture content: the change in moisture content of injera for the three formulations upon increased preservation time has been shown in Table. 4.3. The result showed that in all the treatments the moisture content showed no significant change as the days of preservation increased. The change in the moisture content among the different preservation methods was not significant. According to Smith et al., (2004) moisture loss or moisture gain in bakery products can be minimized if proper packaging like polyethylene is used. With the exception of time 20 preservation which showed a relatively higher level of moisture content in the three types of injera either for MAP 5 or AIR 5 condition. A study conducted by Vlášek, Langová, & Štencl (2013) showed that gluten free bread had minimal moisture content changes during storage condition of atmospheric air, N_2 and CO_2 and has stated the reason could be due to the fact that gluten free bakery being less hygroscopic than common bakery products.

Table 4.3 Effect of preservation methods and time on moisture content (g/100g w.b.). I0: injera without eggshell powder; I4.5: injera with 4.5% eggshell powder; I9.0: injera with 9.0% eggshell powder.

Preservation time (days)		AIR 25 ºC	MAP 25 ºC	AIR 5 ºC	MAP 5 ºC
0	**I0**	59.29 (1.69)	59.29 (1.69)	59.29 (1.69)	59.29 (1.69)
3		59.72 (1.83)	60.11 (1.12)	61.2 (1.11)	59.41 (1.09)
7			58.95 (2.61)	60.62 (0.81)	59.02 (1.74)
13				59.74 (1.31)	63.36 (2.70)
20				63.21 (4.27)	63.31 (4.28)
0	**I4.5**	60.75 (0.35)	60.75 (0.35)	60.75 (0.35)	60.75 (0.35)
3		60.03 (1.56)	58.47 (3.12)	59.44 (0.7)	60.75 (1.56)
7			59.55 (2.50)	58.57 (0.71)	59.30 (2.76)
13				61.15 (0.83)	58.19 (2.16)
20				59.27 (4.62)	63.00 (0.18)
0	**I9.0**	61.23 (1.92)	61.23 (1.92)	61.23 (1.92)	61.23 (1.92)
3		60.38 (1.84)	57.56 (5.14)	59.92(1.79)	60.02 (2.3)
7			61.47 (0.72)	57.11(1.86)	57.74 (3.13)
13				59.11 (1.44)	59.72 (2.9)
20				63.34 (6.07)	59.34 (3.9)

Result of moisture content as mean (SD) of n = 6 observations of injera. Statistical analysis: ANOVA, significant difference at $P < 0.05$.

4.4.3 Physical qualities of injera

Texture of injera: The evolution of hardness of the different types of injeras is shown in Figure 3. For control injera hardness was found to be lower in treatments at 25 ºC than at 5 ºC. for the AIR 25 treatment, storage times 0 and 3 showed a significantly higher hardness than times 7 and 13. However, the injera with MAP 25 didn't show significant differences among storage times. When injera was preserved at 5 ºC, there was a significant increase in hardness with increased preservation time for both AIR and MAP. Evolution of hardness was similar for the other two types two types of injera formulations (I4.5 and I9.0). When 25 ºC was used, hardness was very stable with time and when 5 ºC was used as storage temperature, a significant increase in hardness was observed.

Stalling of bread occur when moisture migrates from swollen starch to gluten or it can also be related to the degree and rate of crystallization of mainly the non-linear amylopectin fraction (Smith et al., 2004). A study conducted by Bosmans et al. (2014) on parbaked bread crumb showed that there was an increase in firmness as the storage time increased when bread was stored either at 23 ºC or at 4 ºC. However, bread stored at 4 ºC showed a higher firmness increase rate as compared to those stored at 23 ºC. The rate of staling has been related to starch retrogradation process in which at 4 ºC storage temperature had led to largest increase in amylopectin crystal formation. Similarly, a study by Aguirre et al (2011) showed that at 4 ºC storage temperature there was the highest starch retrogradation rate as compared to 25 ºC, and -18 ºC storage temperature of bread.

The finding of the current study on injera hardness is in line with the above mentioned research works. This is because the finding showed that injera stored at 5ºC showed increase in hardness whereas injera stored at 25 ºC showed no significant difference on the hardness.

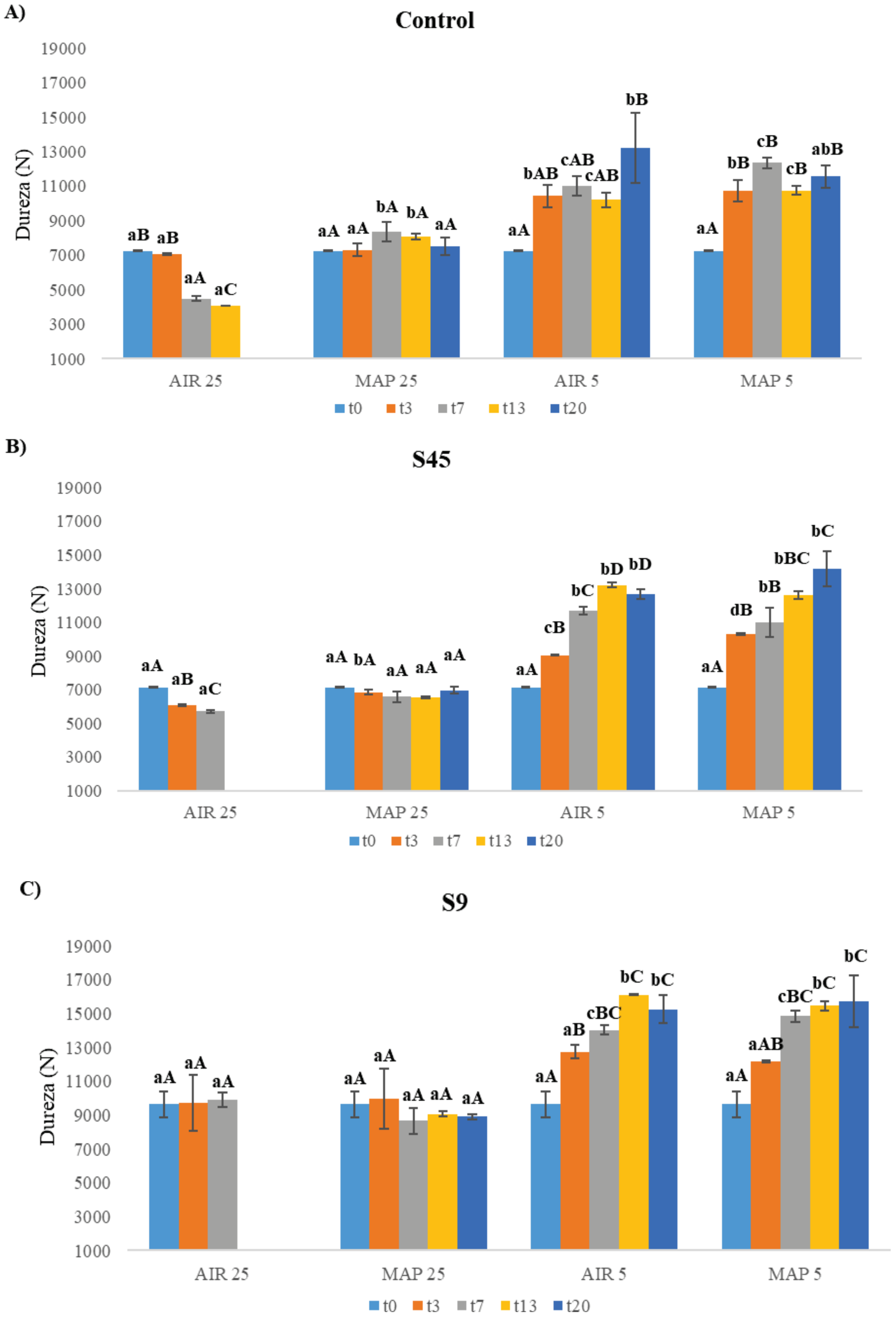

Figure 4.3: Effect of preservation methods on injera hardness. I0 (A), I4.5(B), I9.0(C). The lowercase letters indicate significance with respect to preservation methods and capital letters with respect to time of preservation.

4.4.4 Microbial quality of injera

The count of total mesophilic aerobic microorganisms is shown in Table 4.4. The result showed that for control injera, when stored at 5 ºC, there was no growth of mesophilic aerobes throughout the preservation time regardless of atmospheric condition. However, when injera was stored at 25 ºC, there was growth of mesophilic microbes and the result showed significant differences. Thus, among treatments at storage time 7, the AIR 25 treatment showed almost twice as many mesophilic aerobes as the treatment with MAP 25 which showed the effectiveness of a reduced O_2 atmosphere. In the latter treatment, mesophilic aerobic microorganisms increased exponentially throughout the preservation time of injera.

In the I4.5 injera, Table 4.4 showed that the MAP 5 treatment did not showed growth of mesophilic aerobic microorganisms (TMC). The AIR 5 treatment showed TMC growth at storage times 13 and 20. The injera stored at a temperature of 25 ºC, from time 3 on, showed microbial growth. Injera I9.0 stored at 5 ºC had microbial growth from day 13 on. In the case of storage at 25 ºC, microbial growth started at day 3, being similar values in both atmospheric compositions.

The count of yeasts and molds is shown in Table 4.5. The result showed that for control injera, when stored at 5 ºC, there was no growth of yeast and mold throughout the preservation time regardless of atmospheric condition. However, when injera was stored at 25 ºC, there was growth of yeast and mold and the result showed differences. Thus, at AIR 25 storage, yeast and mold growth at time 7 was 3.6 log/g whereas for the MAP 25 there was no growth until time 13. The difference in the growth of the mold yeast showed the effectiveness of a reduced O_2 atmosphere in the package.

Table 4.4: Effect of preservation methods and time on total mesophilic aerobic count (log/g) of injera. I0: injera without eggshell powder; I4.5: injera with 4.5% eggshell powder; I9.0: injera with 9.0% eggshell powder.

Preservation Time		AIR 25 °C	MAP 25 °C	AIR 5°C	MAP 5 °C
0	I.0 TMC	ND	ND	ND	ND
3	(log/g)	ND	ND	ND	ND
7		6.1(1.3) Aa	3.6 (0.2) Ba	ND	ND
13		-	4.3 (0.8) ab	ND	ND
20		-	5.1 (0.4) b	ND	ND
0	I4.5 TPC	ND	ND	ND	ND
3	(log/g)	4.3 (1.4) Aa	5.5 (0.0) Aa	ND	ND
7		6.1 (0.7) a	6.3 (0.2) b	ND	ND
13		-	7.0 (0.0) Ac	4.3 (0.0) b	ND
20		-	-	5.0 (0.1) a	ND
0	I9.0 TPC	ND	ND	ND	ND
3	(log/g)	6.2 (0.5) A	6.0 (0.7) A	ND	ND
7		7.3 (0.2) A	7.1 (0.2) A	ND	ND
13		-	7.7 (0.6) B	5.2 (0.0) Aa	4.2 (0.0) Aa
20		-	-	4.8 (0.0) Aa	4.9 (0.4) Aa

Result of total mesophilic aerobic count as mean (SD) of n = 4 observations of injera. Statistical analysis: ANOVA, significant difference at $P < 0.05$. Difference in small letters in the column show difference based on preservation time. Difference in capital letters in the same row show significant difference based on preservation method.

For the I4.5 injera, Table 4.5 showed that AIR 5 and MAP 5 treatments showed no growth of yeasts and molds throughout the preservation times. The injera stored at a temperature of 25 ºC, from time 3 on, showed microbial growth. Injera I9.0 showed growth of yeasts and molds at day 20. In the case of storage at 25 ºC, like injera I4.5, microbial growth started at day 3 of storage time, showing significant differences on day 7.

In this study, 3 factors were analyzed on microbial quality of injera, that is, temperature, atmospheric composition and injera pH. Temperature was the most important factor, thus when the storage temperature was 5 ºC, there was limited growth of both mesophilic and yeast and mold. Similarly, the effect of pH on the growth of the microorganisms has been observed, in which as the pH increased there was increase in the growth of the microorganisms.

Table 4.5: Effect of preservation methods and time on yeast and mold count (log/g) of injera. I0: injera without eggshell powder; I4.5: injera with 4.5% eggshell powder; I9.0: injera with 9.0% eggshell powder.

Preservation time		AIR 25	MAP 25	AIR 5	MAP 5
0	**I0 YM**	ND	ND	ND	ND
3	(log/g)	ND	ND	ND	ND
7		3.6 (0.0)	ND	ND	ND
13		-	3.9 (001) ^{a}	ND	ND
20		-	4.5 (0.4) ^{a}	ND	ND
0	**I4.5 YM**	ND	ND	ND	ND
3	(log/g)	4.5 (0.5) ^{Aa}	4.1 (0.7) ^{Aa}	ND	ND
7		5.0 (1.5) ^{Aa}	5.1 (0.2) ^{Aa}	ND	ND
13		-	5.6 (0.4) ^{A}	ND	ND
20		-	-	ND	ND
0	**I9.0 YM**	ND	ND	ND	ND
3	(log/g)	4.0 (0.2) ^{Aa}	3.6 (0.1) ^{Aa}	ND	ND
7		5.3 (1.3) ^{Ba}	3.7 (0.1) ^{Ab}	ND	ND
13		-	6.5 (0.1) ^{B}	ND	ND
20		-		3.7 (02) ^{A}	3.9 (0.1) ^{A}

Result of yeast and mold count as mean ± SD of n = 4 observations of batter and injera. Statistical analysis: ANOVA, significant difference at $P < 0.05$. Difference in small letters in the column show difference based on preservation time. Difference in capital letters in the same row show significant difference based on preservation method.

Research work that used MAP or cold storage for improving the shelf life of injera is limited. Terefe et al (2022) used non vacuum packaging and vacuum packaging with or without chemical preservatives to improve the shelf life of injera. According to the research, injera preserved under the vacuum packaging method combined with the use of chemical preservative showed the least microbial growth. The main limitation of the use of vacuum packaging method was the loss in the sensory acceptability of the product because of the crumbling effect of the vacuum packaging.

Other researchers were used only chemical preservatives to improve the shelf life of injera (Hassen, Mukisa, and Kurabachew, 2018; Ashagrie and Abate, 2012). The work of Ashagrie and Abate (2012) showed that the shelf life of injera was increased to 12 days by the use of the chemical preservatives. On the other hand, Hassen, Mukisa, and Kurabachew, (2018) increased shelf life of injera to 10 days.

A relatively longer shelf life of bakery products like crumpet has been observed from other research works when MAP was employed, and the bread was stored at room temperature (Smith, Jackson, and Ooraikul, 1982). However, the level of CO_2: N_2 used during the preservation of crumpet was in a ratio of 3:2 which showed a relatively higher level of CO_2 used as compared with the current research on injera preservation. Even for bread which used chemical preservatives a relatively longer shelf life was obtained as compared with injera shelf life. This could be related to the characteristics of injera in which it has a high surface area to volume ratio which can contribute to a larger level of microbial contamination and growth.

Bakery products with porous texture like crusty rolls are susceptible to mold growth because their structure makes difficult to eliminate all O_2 from the headspace. And this has nothing to do with the type of the package used, since even with packaging film of low 0_2 permeability, the growth of molds were observed since the amount of O_2 accumulated is sufficient for its development. Therefore, in such kind of products, the use of fungistatic agent or the elimination of headspace O_2 is recommended. Injera is a product with a highly porous texture as that of crusty roll and similar problem related to mold growth has been observed in the current research (Table 4.5).

The nature of mold showed that it is capable to grow in a wide range of environment such as pH (2-8), aw (as low as 0.60), temperature (-5 to 70 °C). However, molds grow best at room temperature, and prefer acidic pH. Besides, they are strictly aerobes, making them unable to grow without some available O_2. Therefore, the use of MAP for the control of the growth of molds is important. However, products like injera with a porous texture may accumulate O_2 making difficult the control of O_2 in the packed food, even with a very low O_2 permeability film.

A 10% increase in CO_2 concentration in the atmosphere inside the package, together with a 5.5°C reduction in storage temperature, doubled the mould-free shelf life of bread and cake. A decrease in temperature also have effect on minimizing the growth of yeast. Addition of preservatives such as sorbates, benzoates, and parabens is quite effective in retarding the growth of yeasts. Similarly, in the current research on injera, it has been shown that on increase in preservation time was achieved when cold storage was used.

In crumpet trial, CO_2 inhibited mould growth, but it took a minimum of 41% CO_2 to retard other microbial activity and delay swelling of the package by 2 weeks. Increasing CO_2 in the headspace to 50% appeared to be adequate for suppression of mould growth and other microbial activities for more than 2 weeks. The mold free shelf life of crumpet was approximately 4 days but when MAP of 60 % CO_2 was used the shelf life was increased for a month at ambient temperature (Smith et al., 2004). In the current research when MAP was used, the increase in the preservation time was not like the one observed in the crumpet research. This could be related to the relatively low level of head space CO_2 used to preserve injera.

4.5 Conclusions

The findings of the study revealed that temperature, atmospheric condition and time did not affect factors like aw and moisture content. However, they affected pH, texture, and microbial quality on the stored injera. Cold storage has been found to be most effective on prevention of microbial growth although, it affected the texture of injera, since storage time increased its hardness. The use of MAP was found to have a significant effect on the preservation of injera due to preservation of microbial growth for control injera. However, no significant effect was observed on the use of MAP for I4.5 and I9.0 injera.

Further work has to be done by increasing the level of CO_2 used in MAP or by adding anti-staling enzymes to improve the quality lost (softness) on the cold storage. The other recommendation is the combined use of MAP with preservatives at a lower level. Injera is a flat bread with high level of aw, therefore affected by high level of microbial growth. Adding ingredients which can lead to minimizing the aw of injera could be the other option to improve injera shelf life. Furthermore post sterilization of packed injera could also be used as a means to improve injera shelf life.

4.6 References

Aguirre J.F., Osella C.A., Carrara C.R., Sa´nchez H.D., & Buera M. P. (2011). Effect of storage temperature on starch retrogradation of bread staling. Starch. 63, 587–593 DOI 10.1002/star.201100023.

Ashagrie, Z. and Abate, D. (2012). Improvement of Injera Shelf Life Through the Use of. *African Journal of Food, Agriculture, Nutrition and Development*, *12*(5), 6409–6423.

Baye, K. *et al.* (2015) 'Enzymatic degradation of phytate, polyphenols and dietary fibers in Ethiopian injera flours: Effect on iron bioaccessibility', *Food Chemistry*. Elsevier Ltd, 174, pp. 60–67. doi: 10.1016/j.foodchem.2014.11.012.

Daifas, D. P., Smith, J. P., Blanchfield, B., & Austin, J. W. (1999). Growth and Toxin Production by Clostridium botulinum in English-style Crumpets Packaged Under Modified Atmospheres. In *Journal of Food Protection* (Vol. 62, Issue 4). http://meridian.allenpress.com/jfp/article-pdf/62/4/349/1670962/0362-028x-62_4_349.pdf

Degirmencioglu, N., Göcmen, D., Inkaya, A. N., Aydin, E., Guldas, M., & Gonenc, S. (2011). Influence of modified atmosphere packaging and potassium sorbate on microbiological characteristics of sliced bread. *Journal of Food Science and Technology*, *48*(2), 236–241. https://doi.org/10.1007/s13197-010-0156-4

Deschuyffeleer, N., Audenaert, K., Samapundo, S., Ameye, S., Eeckhout, M., & Devlieghere, F. (2011). Identification and characterization of yeasts causing chalk mould defects on par-baked bread. Food Microbiology, 28(5). https://doi.org/10.1016/j.fm.2011.02.002

Debonne E., Bockstaele F.V., Driessche M.V., Leyn I.D., Eeckhout M., Devlieghere F. (2018). Impact of par-baking and packaging on the microbial quality of parbaked wheat and sourdough bread. Food Control, 91, 12-19. https://doi.org/10.1016/j.foodcont.2018.03.033.

Hassen, Y., Mukisa, I. M., & Kurabachew, H. (2018). The Effect of Selected Chemical Preservatives and Starter Cultures on the Sensory Characteristics and Shelf Life of Rice Injera. *Journal of Bioprocessing & Biotechniques*, *08*(01). https://doi.org/10.4172/2155-9821.1000318

Gerez, C. L., Torino, M. I., Rollan, G., & Font de Valdez, G. (2009). Prevention of bread mold spoilage by using lactic acid bacteria with antifungal properties. Food Control, 20(2). https://doi.org/10.1016/j.foodcont.2008.03.005

Suhr, K.I & Nielsen, P.V. (2005). Inhibition of Fungal Growth on Wheat and Rye Bread by Modified Atmosphere Packaging and Active Packaging Using Volatile Mustard Essential Oil. In *Journal of Food Science* (Vol. 70). www.ift.org

Legand, J. D., & Voysey, P. A. (1991). Yeast spoilage of bakery products and ingridients. *Journal of Applied Bacteriology*, *70*, 361–371. https://doi.org/002188479100056W

Pasqualone, A. (2019). Bread packaging: Features and functions. In *Flour and Breads and Their Fortification in Health and Disease Prevention*, 211–222. https://doi.org/10.1016/B978-0-12-814639-2.00017-4

Secchi, N., Fadda, C., Sanna, M., Conte, P., del Caro, A., Catzeddu, P., & Piga, A. (2017). Effectiveness of modified atmosphere packaging and ovine whey powder in extending the shelf life of whey cheesecakes. *LWT*, *75*, 373–378. https://doi.org/10.1016/j.lwt.2016.09.018

Smith, J. P., Daifas, D. P., El-Khoury, W., Koukoutsis, J., & El-Khoury, A. (2004). Shelf Life and Safety Concerns of Bakery Products - A Review. In *Critical Reviews in Food Science and Nutrition*. 44 (1), 19–55. https://doi.org/10.1080/10408690490263774

Smith J.P., Jackson E.D., and Ooraikul B. (1982). Microbiological Studies on Gas-Packaged Crumpets. Journal of Food Protection, 46 (4), 279-283.

Terefe A., Emire S.A., Gemede H.F., & Woldegiorgis A.Z. (2022). Improvement of Injera Shelf Life and Staling through Vacuum and Nonvacuum Polyethylene Packaging: Their Synergistic Effect with Chemical Preservative. Journal of Food Quality. https://doi.org/10.1155/2022/8972355.

Upasen S., & Wattanachai P. (2018). Packaging to prolong shelf life of preservative-free white bread. Heliyon. 4. e00802. doi: 10.1016/j.heliyon.2018. e00802.

Vlášek V., Langová J., & Štencl J. (2013). Efect of modifed atmosphere packaging on stability of three kinds of bread," Acta Universitatis Agriculturae et Silviculturae Mendelianae Brunensis, 61 (6). 1881–1887. http://dx.doi.org/10.11118/actaun201361061881.

WRAP. (2008). The food we waste. In *WRAP* (Vol. 2, Issue July).

CHAPTER 5.

SENSORY ANALYSIS OF EGGSHELL POWDER FORTIFIED INJERA

CHAPTER 5. SENSORY ANALYSIS OF EGGSHELL POWDER FORTIFIED INJERA

5.1 Abstract

A study of sensory assessment is important when a new food is developed or when an existing food is modified to determine if the modification has led to a difference. Injera was developed by fortifying it with eggshell powder (EP) to improve the level of Ca. The objective of the current study was to assess the sensory quality of EP fortified injera by obtaining the sensory characterization, studying consumers' overall acceptability and identifying drivers of liking for the new product. Check all that apply (CATA) question was carried out with consumers (n = 60) to characterize the samples. Hedonic scores combined with ideal product characterization were employed to obtain information about product characteristics that increase or decrease acceptability. In addition, Just Noticeable Difference (JND) method was used to determine at what level consumers detect a difference in the attributes between the control injera and the EP fortified injera. The findings from the sensory analysis revealed that the injera prepared by fortifying with EP was accepted by the consumers. However, the liking of injera decreased as the level of EP increased. Grittiness and the eyes size were the more influential attributes for the acceptance. In order not to decrease acceptability, non-grittiness and medium eyes size should be characteristics of the products. The JND study showed that the amount of added calcium that 50 % of consumers can detect was 7.44% for the eyes size and 2.5% for grittiness.

Key words: Injera, eggshell powder, CATA, check-all-that-apply, Just Noticeable Difference

5.2 Introduction

The problem of under nutrition is affecting many people in Ethiopia, especially micronutrients deficiency has impacts on peoples' health (Abebe et al., 2008). The problem of Ca deficiency, as it has been assessed by inadequate calcium (Ca) intake from diet, has been affecting people in Ethiopia (Haileselassie *et al.*, 2022). There are no fortification or supplementation programs which are set at a country level to tackle Ca deficiency, even if there are some steps used to face the problem of other micronutrients such as iron (iron-folate supplementation program for pregnant women), iodine fortification (for the general population), multiple micronutrients supplementation (for children under five years old) (Geletu et al., 2019).

One of the risk factors for the Ca deficiency problems in Ethiopia is inadequate milk consumption by the people (EPHI, 2013). The other issues are high consumption of dietary components that inhibit Ca absorption like phytates, consumption of high level of sodium, and high consumption of caffeine (Challa et al., 2017; Deribew & Woldegiorgis, 2021). In addition, there are health risk issues like high fluoride consumption in the rift valley areas which the problem could be reversed with adequate consumption of calcium (Mulualem et al., 2021; Patel et al., 2017). Nowadays chronic diseases are increasing and affecting peoples' health. Pre-clampsia is one of it in which it affects more pregnant women with inadequate consumption of Ca (Hofmeyr et al., 2007; World Health Organization, 2020). The problem is highly prevalent in developing countries. Therefore, development of a food which is rich in Ca content is very important to tackle the problem related to Ca deficiency for the country.

Sensory assessment of injera were conducted by researchers and most of them used hedonic scale (Yassin et al., 2018; Yetneberk et al., 2004). Yassin et al (2018) used nine-point hedonic scale to study consumer acceptability of rice injera. Yetneberk et al (2004) used ten-point scale to obtain a descriptive profile of barley injera. The hedonic scale method is considered simple, and it is used widely to understand the acceptance of food. However, the method has a limitation in which it cannot express full range of sensory experience. It is used to express only the liking of the products but not considering which attribute has led to the more liking or less liking.

Knowing directions for formulating products that aligned as closely as possible with consumer's expectations is key in a new product development. For this purpose, hedonic evaluation combined with the check-all-that-apply (CATA) method and ideal product (IP) have proven to be very useful tools to obtain information about product characteristics that increase or decrease acceptability (Ares et al., 2014; Ares and Jaeger, 2015; O´Sullivan 2017). CATA method is useful to get a rapid product profile from consumers by using question format. The method consists on to provide a list of descriptors (words or phrases) and asks consumers to check the ones that can appropriately describe their experience with the sample being evaluated. The combined use of CATA with hedonic method is very common and, in the method, the same untrained consumers can be used to evaluate the same food samples both by CATA and hedonic methods (Marín-Arroyo and Bonilla, 2022).

On the other hand, it is important to know what is the amount of the added ingredient that consumers can detect. Just noticeable difference (JND) is the amount of stimuli change that is necessary for the observer to note that the stimulus has become increased or decreased (Lawless, 2013). The method has been used in case of reducing an ingredient in a formula or in case of increasing an ingredient in a formula (Orellana-Escobedo et al., 2012).

The current research has developed injera fortified with eggshell powder EP to improve the Ca content so that to contribute to the minimization of Ca deficiency problems. The work of Fekadu et al. (2022) showed some physico-chemical quality parameters of injera have been affected when injera is fortified with calcium. In food, knowing its sensory characteristics is of great importance. In the present research, the hedonic scores combined with CATA questions and ideal product (IP) were employed to assess overall acceptability, describe the sensory characteristics, and identify the drivers of liking of the EP fortified injera. In addition, the JND method was used to determine the minimum level of EP which can be detected by the consumers.

5.3 Materials and methods

5.3.1 Participants

A panel of 60 individuals were recruited in Hawassa, Ethiopia. Hawassa university students, lecturers and workers and people from outside the university community who are consumers of injera were selected for the study. The selection was also done based on their interest and availability to participate in the study. Their ages were 20 – 60 years old (41% 20 – 30 years old, 30% 31 – 40 years old, 20% 41 – 50 years old and 8% 51 – 60 years old). Male to female ratio by % was 57/43.

5.3.2 CATA and hedonic study samples

Three injera samples were prepared based on the level of EP fortification. The formulations investigated were I0 (100% teff flour and 0% EP), I4.5 (95.5% teff powder and 4.5% EP) and I9.0 (91% teff flour and 9% EP). Injera was prepared following the method described in Fekadu et al. (2022). One big injera of 300 g was cooked and cooled for 2-3 hours before serving to assessors.

5.3.3 CATA and hedonic study test conditions

The cooked injera was cut into eight pieces and each judge received a piece served on a plastic plate labelled with three-digit random numbers. Samples were presented at room temperature (25 ºC). Samples were presented monadically (one at a time), and each judge received the samples in a different order following Latin square design (Williams, 1949), to avoid sample position and first order carry-over effects. Mineral water was provided for rinsing the mouth in between testing. All samples were tested by all the assessors. Bias was minimized by presenting the samples in a similar way. That means similar plastic plates were used and the cutting of the sample injera was made in a similar way.

5.3.4. CATA and hedonic study sensory tests

First, participants were asked to indicate their overall liking/disliking using a horizontal labelled 9-point hedonic scale ranging from "dislike extremely" to "like extremely" (Peryam and Girardot, 1952).

After completing the hedonic evaluation, participants were asked to select, from a CATA questionnaire with 20 sensory characteristics of injera, those that best described the sample that they had tasted, with no limit on the number of items that could be selected. The words and phrases included in the questionnaire were light brown, medium brown, dark brown, small eye size, medium eye size, large eye size, less elastic, medium elasticity, too elastic, less cohesive, medium cohesiveness, dry, soft, grittiness in the mouth, no gritty, less sour, medium sourness, very sour, characteristic odor, and strange odor. Those attributes were used to express the quality of injera, and other researchers used them to describe the quality of injera (Yassin et al., 2018; Yetneberk et al., 2004). To minimize the bias of the judge's responses due to the position of the descriptors in the checklist, each participant received the questionnaire with the descriptors in a different order (Ares, and Jaeger, 2015; Meyners and Castura, 2014) established by using Williams' Latin square design.

Finally, the consumers were asked to check all the attributes they considered appropriate to describe the ideal injera. This time the selection of the attribute of the ideal injera were made without tasting the injera samples. Latin Square method was used for the presentation order of the terms used in the study.

5.3.5. CATA and hedonic study statistical analysis

A one-way analysis of variance (ANOVA) followed by a multiple mean comparison Tukey's Honestly Significant Difference (HSD) test at $p < 0.05$ was performed on the hedonic evaluations data, considering sample as fixed source of variation and consumer as a random effect, using SPSS version 27 (SPSS Inc., Chicago, IL, USA). For the analysis, the following scores were assigned to each point scale descriptor: 1-dislike extremely, 2-dislike very much, 3-dislike moderately, 4-dislike slightly, 5-neither like nor dislike, 6-like slightly, 7-like moderately, 8-like very much, 9-like extremely.

The analysis of the CATA data and ideal product were performed by XLSTAT statistical software (Addinsoft, Paris, France). A Cochran's Q test followed by a *post hoc* multiple-pairwise-comparisons Sheskin critical-difference test (Sheskin, 2011) was applied to the binary data obtained by the CATA questionnaire, to investigate whether there were significant differences between products of eggshell powder added injera with that of control injera.

A multiple correspondence analysis (MCA) was performed on the contingency table obtained by counting the number of consumers that checked each term to describe each sample. The Hellinger distance was used to explore the degree of relationship between samples and attributes (Hellinger, 1909). Only attributes with a *p*-value below the 0.1 threshold were included in the MCA analysis.

A principal component analysis (PCA) was applied to the correlation coefficients between attributes and liking scores to obtain the sensory profile of injera samples.

Finally, a penalty analysis (Ares et al., 2014; Meyners and Castura, 2014) was performed with the liking scores and CATA and ideal product data. Penalty analysis analyze the drop in overall liking associated with a deviation from the ideal (attribute checked for the ideal product but not for the sample, checked for the sample but not for the ideal product) for each attribute from the CATA questionnaire. Only attributes considered as deviating from the ideal for at least 20 % of the consumers were considered.

5.3.6 JND study samples

Injera samples were prepared by adding EP to teff flour for the target injera and without adding EP for the control injera. The levels of the target injera developed based on a multiple of 1.5 those were I1 (1% EP and 99% teff flour w/b), I1.5 (1.5% EP and 98.5% teff flour w/b), I2.3 (2.3% EP and 97.7% teff flour w/b), I3.4 (3.4% EP and 96.6% teff flour w/b), I5.1 (5.1% EP and 94.9% teff flour w/b), I7.6 (7.6% EP and 92.4% teff flour w/b). Injera was prepared following the method describe in Fekadu et al. (2022). One big injera of 300 g was cooked and cooled for 2 - 3 hours before serving to assessors.

5.3.7 JND study test conditions

The cooked injera was cut into eight pieces and each judge received a piece served on a plastic plate labelled with three-digit random numbers. Samples were presented to the consumers in a pairwise. Six pairs of injera samples (one control injera and the other being the control or each of the target injera) following randomization method. Random presentation of samples was different for each panelist. The assessors were asked to try each sample and to answer the paired samples whether they were the same or different based on the quality parameters of injera. Terms used related to sensory characterization of injera were color, eyes, softness, grittiness, taste, and odor. Samples were presented to the consumers at room temperature (25 ºC). Mineral water was presented for rinsing the mouth in between testing. Bias was minimized by presenting the samples in a similar way. That means similar plastic plates were used and the cutting of the sample injera was made in a similar way.

5.3.7. JND study statistical analysis

Data were analyzed by calculating the relationship between the proportion of correct responses and the content of stimulus of each sample.

5.3.8 Location

All the sensory evaluations were conducted at Hawassa university school of nutrition, food science and technology sensory lab, Ethiopia. The lab had appropriate ventilation, partitioned area, neutral color/background, artificial day light illumination, and minimal traffic and free from distractions, noise, and odors.

5.3.9 Ethical and security aspects

Written consent was obtained from each participant prior to their participation in the study.

5.3.10 Limitation of the study

The study was conducted on Ethiopian adults; therefore, it may not be possible to generalize the study for other ethnics or age groups.

5.4 Result and discussion

5.4.1 Overall liking of injera

The overall liking of injera decreases with EP addition. The mean and deviation values for injeras I0, I4.5, and I9.0 were 7.5 (1.5), 6.3(1.9), and 5.9(2.2) respectively. There was a statistically significant difference (p value < 0.05) between the control injera (I0) and the EP fortified injera (I4.5 and I9.0). However, no significant difference was found between I4.5 and I9.0 injera.

5.4.2 Consumers sensory description of the injera samples

Contingency table of the counts of the number of assessors that checked each attribute for each sample and for the ideal injera is shown in Table 5.1. The ideal injera was describe by more than half of the assessors as soft (90.0 %), not gritty (80.0 %), having medium elasticity (68.3 %), medium eye size (65.0 %), characteristic odor (63.3 %), light brown (56.6 %) and having medium cohesiveness (55.0 %). In the EP fortified injera samples, the desirable attributes found by more than half of the assessors were soft (85.0 % for I9.0 and 73.3 % for I4.5), characteristic odor and medium elasticity (60.0 % in all cases), and medium eye size (53.3 % for I9.0). The sample used as control (I.0) was described very similar to the ideal.

Table 5.1 Contingency table of the counts of the number of assessors that checked each attribute for each sample.

Product	Light brown	Medium brown	Dark brown	Small eye size	Medium eye size	Large eye size	Less elastic	Medium elasticity	Too elastic	Less cohessive	Medium cohesiveness	Dry	Soft	Grittiness in the mouth	No Gritty	Less sour	Medium sourness	Very sour	Characteristic odor	Strange odor
Ideal	34	18	4	10	39	5	6	41	7	18	33	2	54	4	48	24	28	4	38	6
I.0	34	24	2	15	33	8	15	40	4	24	26	3	54	14	38	17	32	7	37	14
I4.5	20	31	7	27	25	6	16	36	6	25	22	12	44	36	17	41	13	5	36	16
I9.0	23	33	3	15	32	12	18	36	6	23	27	5	51	48	8	42	15	0	36	15

I4.5 = injera with 4.5% EP, I9.0 = injera with 9.0% EP, Ideal = ideal injera, and I.0 = control injera.

The Figure 5.1 showed a graphical representation of elicitation difference for the I4.5, I9.0 and I.0 (control) injera compared to the ideal. The figure showed, for each attribute, if each product was similar or different from the ideal product. The more for a given attribute the product is similar to the ideal product, the closer the line will be to 0. Therefore, control injera had attributes very close to zero showing that it is very similar to the ideal product. Few attributes in the I4.5 and I9.0 injera such as grittiness, less sour, medium brown were far from the ideal product. The attributes of no grittiness, medium sourness and light brown were to the negative side showing that the attributes were not present enough.

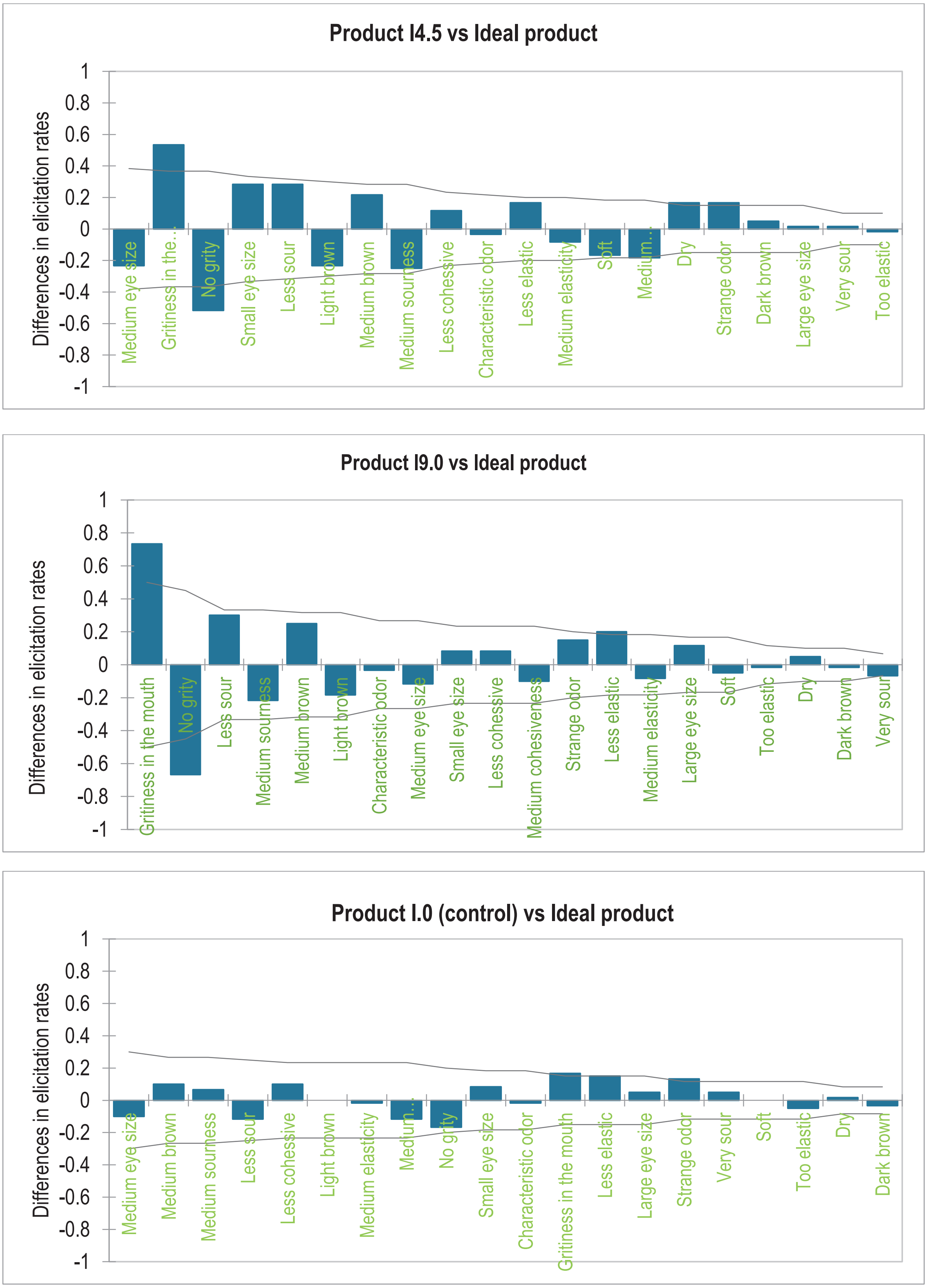

Figure 5.1. Differences in elicitation rates for each product compared to the ideal. I4.5 = injera with 4.5% EP, I9.0 = injera with 9.0% EP, Ideal = ideal injera, and I.0 = control injera.

The attributes of the three types of injera (I0, I4.5 and I9.0) were compared based on Cochran Q test followed by the Sheskin multiple comparisons test (see **Table 5.2**.). Among the 20 injera attributes that were used to assess the injera quality, the assessors perceived differences in nine of them. The determinants of differences between samples fortified with EP (I4.5 and I9.0) and control (I.0) were the texture attributes related with grittiness in the mouth and the taste attributes related with sour.

Table 5.2 Frequency selection (%) for attributes for which a statistically significant difference ($p < 0.05$) among samples was found by using Chochran's Q test.

Attributes	Ideal	I.0	I4.5	I9.0
Soft	90.0	90.0 (b)	73.3 (a)	85.0 (ab)
No gritty	80.0	63.3 (b)	28.3 (a)	13.3 (a)
Light brown	56.7	56.7 (b)	33.3 (a)	38.3 (ab)
Medium sourness	46.7	53.3 (b)	21.7 (a)	25.0 (a)
Less sour	40.0	28.3 (a)	68.3 (b)	70.0 (b)
Small eye size	16.7	25.0 (a)	45.0 (b)	25.0 (a)
Grittiness in the mouth	6.7	23.3 (a)	60.0 (b)	80.0 (c)
Very sour	6.7	11.7 (b)	8.3 (ab)	0.0 (a)
Dry	3.3	5.0 (a)	20.0 (b)	8.3 (ab)

Difference in letters (a,b,c) within a row indicate that products differ significantly ($p < 0.05$) according to the multiple-pairwise-comparisons Sheskin critical-difference procedure. I4.5 = injera with 4.5% EP, I9.0 = injera with 9% EP, I.0 = control injera.

The grittiness in the mouth was selected the most in the I9.0 injera then comes the I4.5 injera and the least grittiness was chosen in I0 injera. This attribute has been observed in such a pattern due to the addition of EP, I4.5 and I9.0, which has a relatively coarse characteristic as compared to the teff flour injera. In I9.0 injera the highest level of eggshell powder was added. This finding was in line with the work by Brun et al. (2013) which the texture characteristics of food was changed as EP was added to food products.

The other attribute for injera which showed a significant difference among the three injera types was the taste. Medium sourness was more frequently chosen for the I0 injera as compared with I4.5 and I9.0. The sourness characteristics of I0 injera was very close to that of the ideal injera. No one stated that I9.0 was a very sour injera, and relatively more people selected that I0 injera was sourer than I4.5 injera. The sourness of injera was affected by the addition of eggshell powder which was in line with a study by Fekadu et al. (2022). This was due to the high level of $CaCO_3$ in EP that has affected the pH of the food.

The lightness of injera was the other characteristic of injera that had shown a significant difference among the injera types. Control injera was found to be light brown as compared with the I4.5 injera. The effect of eggshell powder on injera color quality was in accordance with the work by Chilek et al. (2018) in which the lightness of white bread was affected due to the addition of eggshell powder. The previous work on injera which had assessed the color showed that the lightness of injera had been affected due to the addition of EP Fekadu et al. (2022).

The dryness and softness of injera has also been affected by the addition of eggshell powder. I0 injera was found to be less dry and softer as compared to I4.5 injera. Small eye size was observed more frequently in I4.5 as compared with the I0 and I9.0. The work by Fekadu et al. (2022) showed that there was a difference in the circularity of the injera but not the size of injera eyes.

5.4.3 Representation of the samples

To illustrate the association between the samples and the terms as well as the similarities and differences between the samples, a multiple correspondence analysis (MCA) was carried out on the contingency table to obtain a sensory map of the three-injera types. The first two dimensions on the map explained 95.83% of the experimental data variability, representing 84.12 the dimension 1 (F1) and 11.71 % the dimension 2 (F2). The MCA in Fig. 5.2 showed that the EP fortified samples were located in a different quadrant from the control sample and the Ideal product. The ideal product had characteristics to be soft, light brown, medium sourness and no gritty. According to the panelists, these characteristics of injera were considered the factors of good quality injera. The reference product, I0, characteristics were very close to the ideal product.

Both the ideal product and the reference product lay at the positive value of the second dimension. The product I9.0 lays to the negative value of the first and second dimension and has characteristics of grittiness in the mouth and less sour. Whereas product I4.5 lays to the negative value of the first dimension and positive value of the second dimension and has the characteristics of small eye size, and dry.

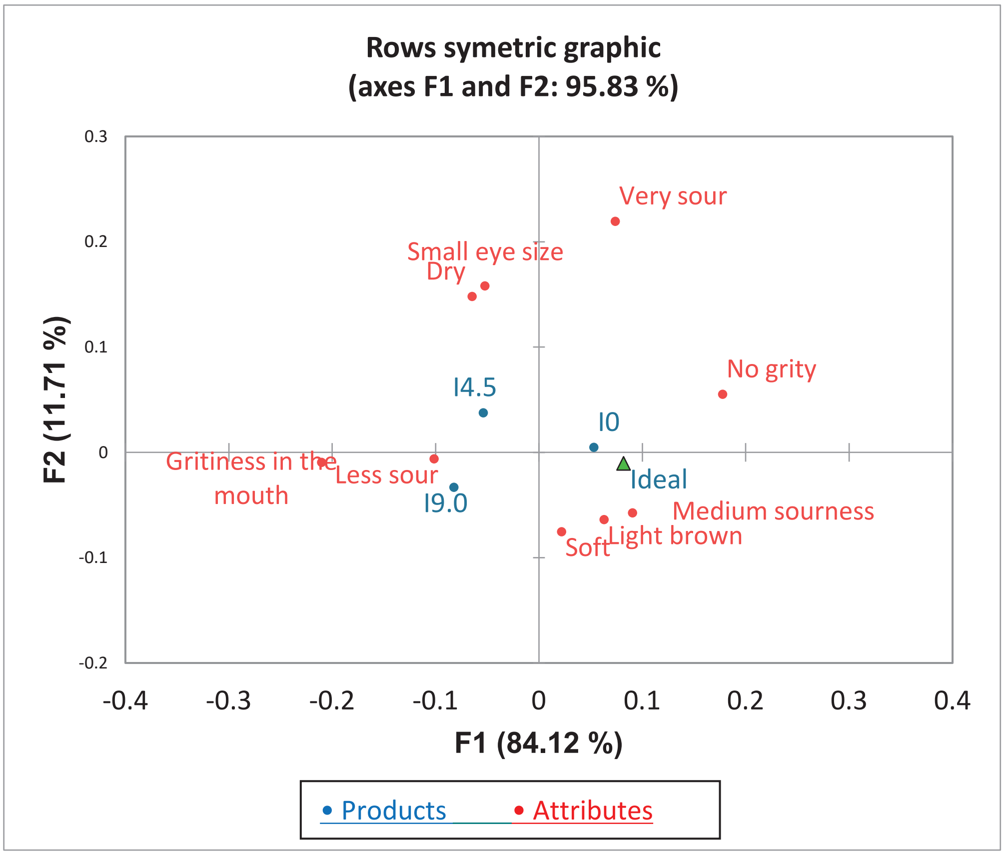

Figure 5.2 Representation of the samples and the terms in the first and second dimensions of the multiple correspondence analysis of the frequency table of the CATA question. I4.5 = injera with 4.5% EP, I9.0 = injera with 9.0% EP, Ideal = ideal injera, and I.0 = control injera.

5.4.4 Principal component analysis

Principal component analysis (figure 5.3) showed that the liking of injera was associated to the attributes soft, medium sourness, elasticity and eye size and no gritty. The characteristics of injera such as grittiness in the mouth, large or small eye size, less sour or too elastic are less liked by the consumers.

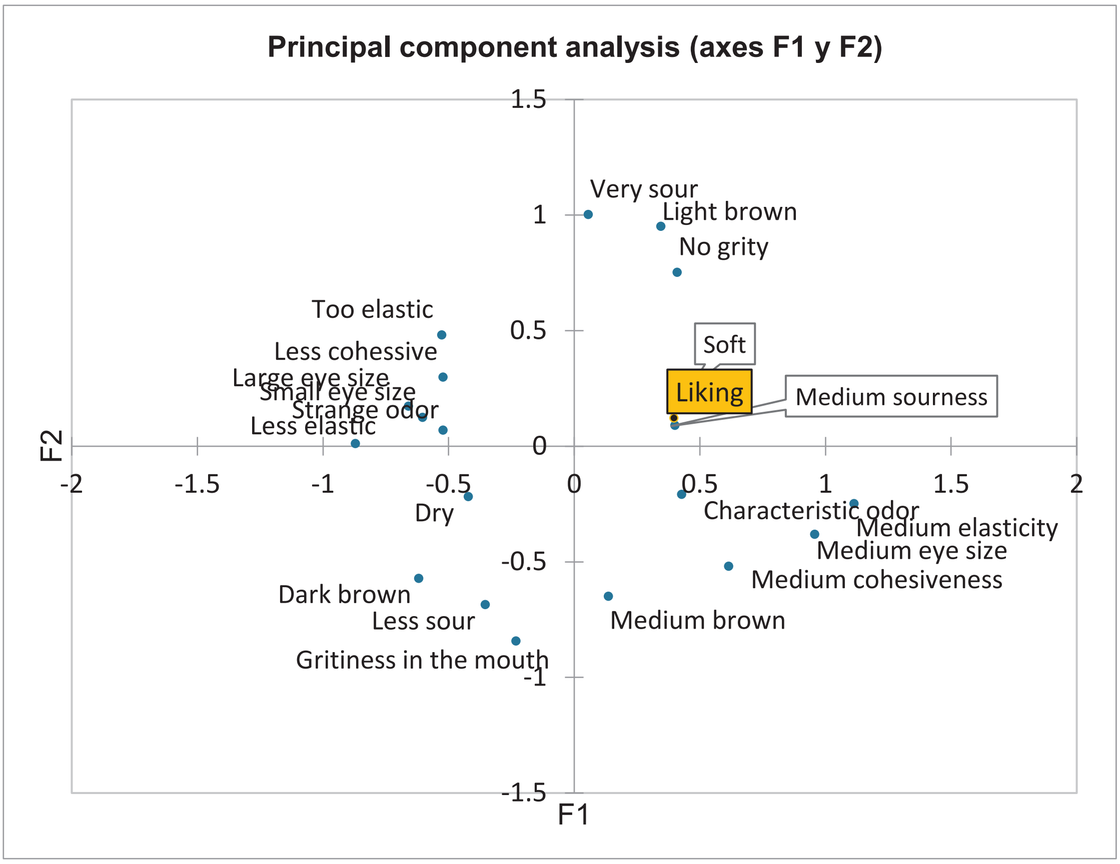

Figure 5.3 Principal Component analysis.

5.4.5 Penalty analysis

Penalty analysis was performed since the CATA study included an ideal product and liking score for the real products. The penalty analysis can be used to determine the decrease in overall liking associated with a deviation from the ideal for each attribute. The analysis is based on the gaps between the real products and the ideal, and the impact on liking scores. The "must have" attributes

(attributes that must be present in order not to cause a large decrease in liking) were identify by analyzing attributes missing in the injera product (I0, I4.5 and I9.0) whereas they exist in the ideal product (P(No)/I(Yes)) (see Appendix 1). A second analysis of the attributes missing in the ideal but present in the real product (P(Yes)/I(No)) permitted the identification of the "must not have" attributes (attributes whose presence results in a drop in liking (see Appendix 2).

Fig 5.4 showed the attributes that positively (highlighted in blue) or negatively (highlighted in red) impact the average liking scores. It is seen that no gritty, medium eye size and medium sourness were drivers of liking, irrespective of whether it was used for the ideal product, if no gritty, medium eye size and medium sourness were elicited the average liking was substantially higher than if there were those attributes. Grittiness in the mouth and small eye size were clearly shown to be a negative driver for liking.

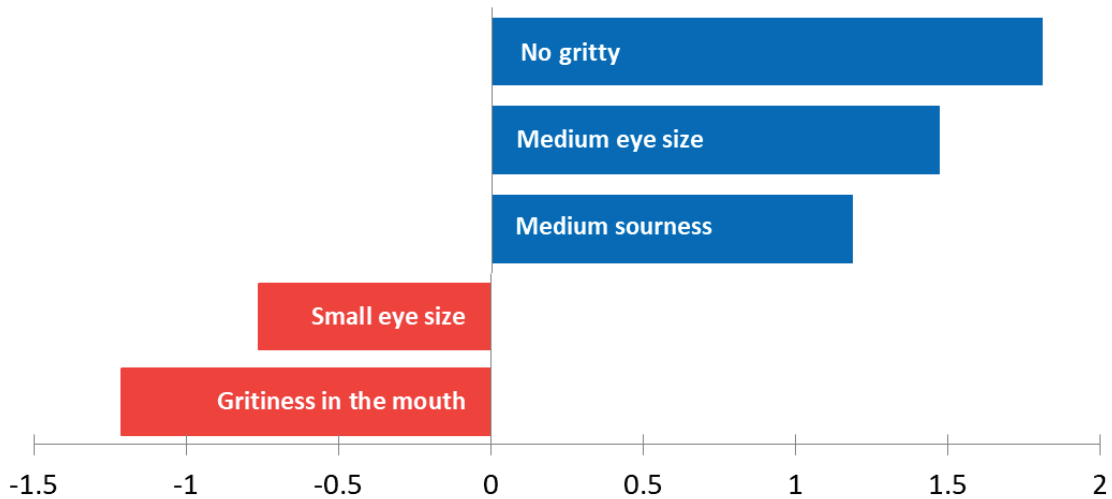

Figure 5.4. Penalty analysis. Attributes with a significant ($p < 0.05$) positive (blue color) or negative (red color) impact on the average liking scores of injera.

Table 5.3 showed the attributes by classifying them as necessary, has no influence, indifferent, and have negative effect in the product. To decide the inclusion of the attributes in one of these groups, their impact on the acceptability and the percentage of consumers (20 % or higher) for whom there was an inconsistency (attribute selected in the ideal product and not in the real product or vice versa) were consider. The necessary characteristics were that the injera should not have the attribute gritty and the eye size has to be medium. This characteristic of injera grittiness is sometimes observed in injera preparation when there was contamination of the grain with sand or soil during harvesting and when the cleaning was not done properly before the process of milling. Therefore, the sensory panel classified having the characteristics of grittiness in the mouth as the characteristic that was not the necessary attribute in injera quality. Small eye size is the other characteristics of injera which have negative effect on the product. The study showed that the majority of injera characteristics have fallen under the indifferent.

Table 5.3 Summary table of the attributes of injera

Necessary	Has no influence	Indifferent	Negative
Medium eye size	Light brown	Medium brown	Small eye size
No gritty	Medium cohesiveness	Dark brown	Grittiness in the mouth
		Less elastic	
		Too elastic	
		Less cohesive	
		Dry	
		Less sour	
		Medium sourness	
		Very sour	
		Strange odor	

Being light brown in the color of injera was considered as has no influence characteristic in the current study. Color is one of the attributes that are considered as one of the factors for determining injera quality. Now a day's people are changing their attitude towards the color of injera. For many years, the light brown color was the one preferred by so many people. However, people are now becoming more health concerned since the light brown colored injera, which was prepared from the white teff variety, has relatively less level of some minerals as compared to the brown injera, which is prepared either from a mixed type teff or from a dark brown teff. This could be the reason that in the current research the light brown color became the attribute under the class of the does not have influence characteristics. Does not harm attributes were dry, less sour, medium sourness and very sour. These characteristics were classified as such maybe because of the influence of personal choice on those qualities of injera.

Those that had a negative impact on injera quality were small eye size and grittiness in the mouth. Eye size was related to the fermentation process of injera. Since eye size was considered as an attribute that can affect negatively the injera quality, solutions related to fermentation has to be developed. This could be increasing the fermentation time or increasing the level of starter culture that can speed up the fermentation process and have influence positively the size of injera eyes.

The addition of the eggshell powder had created grittiness in injera, which negatively affected the acceptability of the injera. Research done by Brun et al. (2013) mentioned that particle size of eggshell powder influences the texture of food. As the eggshell powder became very fine, its effect on the texture of food was minimal. Therefore, preparing a very small particle size eggshell powder during the injera preparation could be a solution to tackle the negative effect of the addition of eggshell powder on the grittiness characteristics of injera.

5.4.6. JND results

The results for the analysis of JND for injera qualities of color, eyes, softness, grittiness, taste, and odor is shown in Fig. 5.5. The result of the JND study for color showed that when the level of eggshell powder was 1%, it was detected by 27% of the panels. As the level of eggshell powder increased to 1.5%, the change in the color of injera was detected by 35% of the panels. When 7.6% of eggshell powder was added, the difference in color was detected by 75% of the panelist.

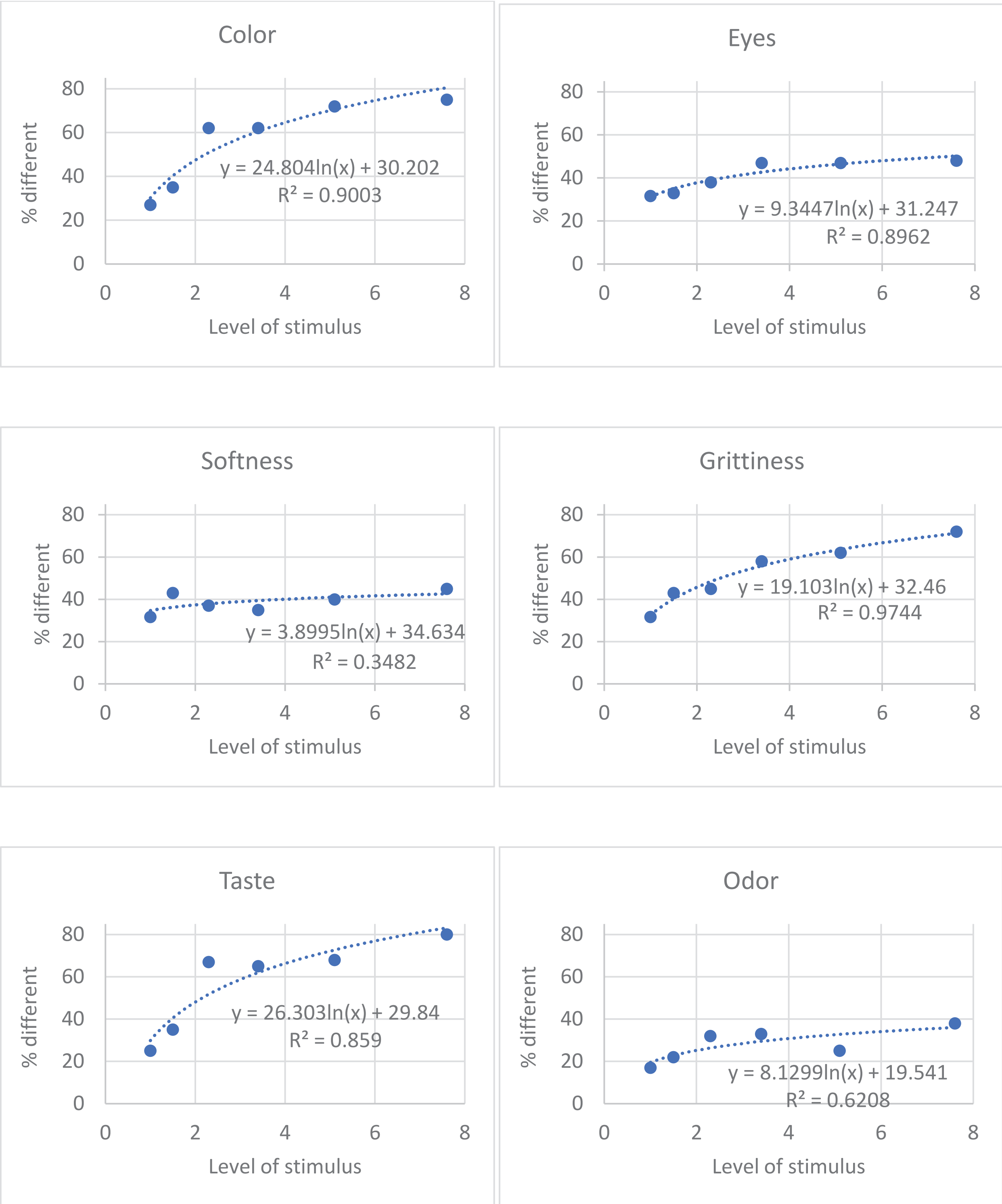

Figure 5.5 Relationship between the proportion of correct responses and the content of stimulus of each sample. Equation in each graph was obtained by data logarithmic adjustment.

The result of the JND study for eyes showed that when the level of eggshell powder was 1%, the difference in the eyes of injera was detected by 32% of the panelist. Not much difference was observed in the detection when the level of eggshell powder was increased to 1.5%. As the level of eggshell powder increased 2.3 %, the change in the color of injera was detected by 38% of the panelists. When 7.6% of eggshell powder was added, the difference in color was detected by 48% of the panelists. The qualities of injera eyes, softness, and odor the result of the analysis showed that even when eggshell powder was added at a level of 7.6% they were not detected by 50% of the panelists.

5.5 Conclusions

The injera prepared by fortifying with EP was accepted by the consumers although they like more unfortified injera than EP fortified one. The sensory assessment of injera revealed that the attributes light brown, small eye size, dry, soft, grittiness in the mouth, no gritty in the mouth, medium sourness and sour were significantly different attributes among the injera products. However, the most important attributes that determined the overall liking of injera were grittiness in the mouth, no gritty, small eye size, and medium eye size. Grittiness in the mouth and small eye size affect the injera liking negatively whereas no grittiness in the mouth, medium eye size, and medium sourness affects injera liking positively. The JND study showed that the minimum amount of EP to be added to consumers detect a difference was 7.44% for the eyes size and 2.5% for grittiness.

Work must be done to improve the attributes of injera such as grittiness in the mouth, small eye size, less sour and dry since injera with such attributes were less liked by the consumers. For the grittiness in the mouth, a very fine eggshell powder can be tried to avoid such characteristics of injera upon addition of eggshell powder. In addition, to that some researchers have used acids, citric acid from lemon and vinegar, as a means for the improvement of the texture of the products.

The small eye size and being less sour are linked with the fermentation process. Therefore, increasing the fermentation time so that to obtain the acidity of injera comparable with that of the control injera could be a solution. Further work must be done to improve those attributes of injera which have been affected by the addition of eggshell powder.

5.6 References

Abebe, Y., Bogale, A., Hambidge, K. M., Stoecker, B. J., Arbide, I., Teshome, A., Krebs, N. F., Westcott, J. E., Bailey, K. B., & Gibson, R. S. (2008). Inadequate intakes of dietary zinc among pregnant women from subsistence households in Sidama, Southern Ethiopia. *Public Health Nutrition*, *11*(4), 379–386. https://doi.org/10.1017/S1368980007000389

Ares, G.; Dauber, C.; Fernández, E.; Giménez, A.; Varela, P. Penalty analysis based on CATA questions to identify drivers of liking and directions for product reformulation. *Food Qual. Prefer.* **2014**, *32*, 65–76. https://doi.org/10.1016/j.foodqual.2013.05.014.

Ares, G.; Jaeger, S. Check-All-That-Apply (CATA) questions with consumers in practice: Experimental considerations and impact on outcome. In *Rapid Sensory Profiling Techniques*; Woodhead Publishing Series in Food Science, Technology and Nutrition; Woodhead Publishing: Kent, UK, 2015; pp. 227–245.

Belachew, T., Nida, H., Getaneh, T., Woldemariam, D., & Getinet, W. (2005). Calcium deficiency and causation of rickets in Ethiopian children. *East African Medical Journal*, *82*(3), 153–159. https://doi.org/10.4314/eamj.v82i3.9273

Brun, L. R., Lupo, M., Delorenzi, D. A., di Loreto, V. E., & Rigalli, A. (2013). Chicken eggshell as suitable calcium source at home. *International Journal of Food Sciences and Nutrition*, *64*(6), 740–743. https://doi.org/10.3109/09637486.2013.787399

Challa, F., Tadesse, Y., Mudie, K., Taye, G., Gelibo, T., Bekele, A., Sileshi, M., Getahun, T., Geto, Z., Ayalkebet, A., Nagasa, B., Teklie, H., Taddele, T., Getachew, T., Feleke, Y., Guta, M., Shiferaw, F., Gebremichael, M., Girma, Y., … Bekele, A. (2017). Urinary sodium excretion and determinates among adults in Ethiopia: Findings from National STEPS survey. *Ethiopian Journal of Health Development*, *31*(Specialissue1), 370–377.

Chilek, T. Z. T., Kairuaman, N. A., Ahmad, F., Wahab, R. A., Zamri, A. I., & Mahmood, A. (2018). Development of white bread fortified with calcium derived from Eggshell powder. *Malaysian Applied Biology*, *47*(6), 29–39.

Deribew, H. A., & Woldegiorgis, A. Z. (2021). Acrylamide levels in coffee powder, potato chips and French fries in Addis Ababa city of Ethiopia. *Food Control*, *123*(October 2020), 107727. https://doi.org/10.1016/j.foodcont.2020.107727

EPHI. (2013). Ethiopia National Food Consumption Survey. In *Ethiopian Public Health Institute* (Vol. 3).

Fekadu, T., Cassano, A., Angós, I., & Maté, J. I. (2022). Effect of fortification with eggshell powder on injera quality. *Lwt*, *158*, 113156. https://doi.org/10.1016/j.lwt.2022.113156

Geletu, A., Lelisa, A., & Baye, K. (2019). Provision of low-iron micronutrient powders on alternate days is associated with lower prevalence of anaemia, stunting, and improved motor milestone acquisition in the first year of life: A retrospective cohort study in rural Ethiopia. *Maternal and Child Nutrition*, *15*(3), 1–9. https://doi.org/10.1111/mcn.12785

Haileselassie, M. *et al.* (2022) 'The influence of fasting on energy and nutrient intake and their corresponding food sources among 6-23 months old children in rural communities with high burden of stunting from Northern Ethiopia', *Nutrition Journal*. BioMed Central, 21(1), pp. 1–15. doi: 10.1186/s12937-022-00759-z.

Hassen, Y.; Mukisa, I.M.2; Kurabachew, H. and Desalegn B.B. (2018). Evaluation of Yeast and Lactic Acid Bacteria Starter Cultures for the Production of Rice Injera. *Journal of Food Processing & Technology*, *09*(03), 1–6. https://doi.org/10.4172/2157-7110.1000721

Hellinger, E. (1909). Neue Begründung der Theorie quadratischer Formen von unendlichvielen Veränderlichen. Journal für die reine und angewandte Mathematik, 136, 210–271.

Hofmeyr, G. J., Duley, L., & Atallah, A. (2007). Dietary calcium supplementation for prevention of pre-eclampsia and related problems: A systematic review and commentary. *BJOG: An International Journal of Obstetrics and Gynaecology*, *114*(8), 933–943. https://doi.org/10.1111/j.1471-0528.2007.01389.x.

Lawless, H. T. (2013). *Quantitative Sensory Analysis Psychophysics Models and intelligent Design* (First). John Wiley & Sons Ltd.

Marín-Arroyo, M.R.; González-Bonilla, S.M. Sensory Characterization and Acceptability of a New Lulo (*Solanum quitoense* Lam.) Powder-Based Soluble Beverage Using Rapid Evaluation Techniques with Consumers. *Foods* **2022**, *11*, 3129. https://doi.org/10.3390/foods11193129

Meilgaard, M.C., Civille G.V. and Carr B.T. (2007) Sensory Evaluation Techniques. 4th Edition, CRC Press, U.S.A.

Meyners; Castura, J.C. Check-All-That-Apply Questions. In *Novel Techniques in Sensory Characterization and Consumer Profiling*; CRC Press: Boca Ratón, FL, USA, 2014; pp. 272–301.

Mulualem, D., Hailu, D., Tessema, M., & Whiting, S. J. (2021). Efficacy of calcium-containing eggshell powder supplementation on urinary fluoride and fluorosis symptoms in women in the ethiopian rift valley. *Nutrients*, *13*(4). https://doi.org/10.3390/nu13041052

Orellana-Escobedo, L., Ornelas-Paz, J. J., Olivas, G. I., Guerrero-Beltran, J. A., Jimenez-Castro, J., & Sepulveda, D. R. (2012). Determination of Absolute Threshold and Just Noticeable Difference in the Sensory Perception of Pungency. *Journal of Food Science*, *77*(3). https://doi.org/10.1111/j.1750-3841.2011.02589.x

O´Sullivan, M.G. *A Handbook for Sensory and Consumer-Driven New Product Development*, 1st ed.; Elsevier: Cambridge, UK, 2017; pp. 3–218.

Patel, P., Patel, P., Zulf, M., Yagnik, B., Kajale, N., Mandlik, R., Khadilkar, V., Chiplonkar, S., Phanse, S., Patwardhan, V., Joshi, P., Patel, A., & Khadilkar, A. (2017). Association of dental and skeletal fluorosis with calcium intake and serum Vitamin D concentration in adolescents from a region endemic for fluorosis. *Indian Journal of Endocrinology and Metabolism*, *21*(1), 190–195. https://doi.org/10.4103/2230-8210.196013

Peryam, D. R., & Girardot, N. F. (1952). Advanced taste-test method. Food Engineering, 24, 58–61.

Peryam, D. R., & Pilgrim, F. J. (1957). Hedonic scale method of measuring food preference. Food Technology, 11, 9–14.

Sheskin, D. *Handbook of Parametric and Nonparametric Statistical Procedures*, 5th ed.; Chapman and Hall/CRC: London, UK, 2011.

Williams, E. Experimental designs balanced for the estimation of residual effects of treatments. *Aust. J. Sci. Res. B* **1949**, *2*, 149–168. https://doi.org/10.1071/CH9490149.

World Health Organization. (2020). *Calcium supplementation before pregnancy for the prevention of pre-eclampsia and its complications*. https://apps.who.int/iris/bitstream/handle/10665/331787/9789240003118-eng.pdf?ua=1

Yetneberk, S., de Kock, H. L., Rooney, L. W., & Taylor, J. R. N. (2004). Effects of Sorghum Cultivar on Injera Quality. *Cereal Chemistry*, *81*(3), 314–321. https://doi.org/10.1094/CCHEM.2004.81.3.314

GENERAL CONCLUSIONS

GENERAL CONCLUSIONS

The current research set up methodologies to quantify quality parameters of injera. Those parameters were physicochemical, physical, microbial and nutritional. The findings could give inputs for Ethiopian quality standard agency and other concerned bodies about quantifying the quality parameters of injera for commercializing it. In addition, the research has revealed that there is a need to fortify injera so that to improve its contribution to the nutritional Ca requirement of people by increasing the level of bioavailable Ca.

Fortification of injera with EP has been shown to increase significantly both total and bioavailable Ca. As a consequence, EP enriched injera can be considered as a new tool to improve the Ethiopian diet. Further clinical studies are needed to confirm the actual benefit of the fortification. However, some injera quality parameters like pH and acidity were affected. Further work is required to maintain the quality of injera unaffected.

The shelf life study has revealed that cold storage has been found to be the most effective tool on prevention of microbial growth although, it affected the texture of injera, since storage time increased its hardness. The use of MAP was found to have a significant effect on decreasing the microbial growth for control injera. However, no significant effect was observed on the use of MAP for EP fortified injera. Further work has to be done to effectively preserve injera by considering factors like changing the level of CO_2 used in MAP or adding anti-staling enzymes.

The sensory assessment of injera showed that injera fortified with EP at a level of 4.5% and 9.0% were accepted by the consumers although the liking is lower when EP has been added than in the unfortified injera. The most important attributes that determined the overall liking of injera were grittiness in the mouth and eyes size. Grittiness in the mouth and small eye size affected the injera liking negatively whereas no grittiness in the mouth and medium eye size were necessary in order not to decrease acceptability. The JND study showed that the amount of added calcium that 50 % of consumers can detect was 7.44% for the eyes size and 2.5% for grittiness. It can be concluded that injera developed by fortifying with EP at different levels were accepted by the consumers showing that EP can be potentially used for improving the nutritional deficiency problem existed in Ethiopia due to Ca deficiency. However, further work must be done to improve those attributes of injera which have been affected by the addition of EP.

Appendix 1

Penalty analysis. Summary of the frequencies with which presence in the ideal but not in the real product (P(No)|(Yes)) and presence in the ideal and real product (P(Yes)|(Yes)) occurs for each attribute, mean drops in liking between the two situations and significance.

Variable	Level	Frecuency %	Mean (Liking)	Effect on the average	P-value	Significant	Penalty
Light brown	P(No)I(Yes)	27.22%	6.286	0.129	0.943	No	
	P(Yes)I(Yes)	29.44%	6.415				-0.175
Medium brown	P(No)I(Yes)	11.11%	7.350	-0.438			
	P(Yes)I(Yes)	18.89%	6.912				0.460
Dark brown	P(No)I(Yes)	6.67%	7.167		<0,0001	No	0.000
	P(Yes)I(Yes)	0.00%			<0,0001	No	0.000
Small eye size	P(No)I(Yes)	10.00%	6.944	-0.111			
	P(Yes)I(Yes)	6.67%	6.833				0.315
Medium eye size	P(No)I(Yes)	30.56%	5.836	1.422	0.000	Sí	
	P(Yes)I(Yes)	34.44%	7.258				1.097
Large eye size	P(No)I(Yes)	6.67%	7.167	-2.500			
	P(Yes)I(Yes)	1.67%	4.667				-1.904
Less elastic	P(No)I(Yes)	2.78%	6.000	1.308			
	P(Yes)I(Yes)	7.22%	7.308				0.829
Medium elasticity	P(No)I(Yes)	17.22%	5.581	1.387			
	P(Yes)I(Yes)	51.11%	6.967				0.876
Too elastic	P(No)I(Yes)	8.33%	7.200	-1.700			
	P(Yes)I(Yes)	3.33%	5.500				-1.075
Less cohessive	P(No)I(Yes)	11.11%	6.700	-0.347			
	P(Yes)I(Yes)	18.89%	6.353				-0.229
Medium cohesiveness	P(No)I(Yes)	21.67%	6.359	0.541	0.387	No	
	P(Yes)I(Yes)	33.33%	6.900				0.542
Dry	P(No)I(Yes)	2.78%	6.400	0.600			
	P(Yes)I(Yes)	0.56%	7.000				0.464
Soft	P(No)I(Yes)	13.33%	5.750	1.025			
	P(Yes)I(Yes)	76.67%	6.775				1.013
Grittiness in the mouth	P(No)I(Yes)	1.11%	9.000	-3.800			
	P(Yes)I(Yes)	5.56%	5.200				-1.418
No Gritty	P(No)I(Yes)	47.78%	5.907	1.748	<0,0001	Sí	
	P(Yes)I(Yes)	32.22%	7.655				1.647
Less sour	P(No)I(Yes)	13.89%	6.520	-0.563			
	P(Yes)I(Yes)	26.11%	5.957				-0.787

Variable	Level	Frecuency %	Mean (Liking)	Effect on the average	P-value	Significant	Penalty
Medium sourness	P(No)I(Yes)	27.78%	6.560	1.146	0.022	Sí	
	P(Yes)I(Yes)	18.89%	7.706				1.439
Very sour	P(No)I(Yes)	5.56%	7.300	0.200			
	P(Yes)I(Yes)	1.11%	7.500				0.972
Characteristic odor	P(No)I(Yes)	16.67%	6.033	0.919			
	P(Yes)I(Yes)	46.67%	6.952				0.775
Strange odor	P(No)I(Yes)	2.78%	6.000	0.692			
	P(Yes)I(Yes)	7.22%	6.692				0.165

Appendix 2

Penalty analysis. Summary of the frequencies with which no presence in the ideal nor in the real product (P(No)|(No)) and no presence in the ideal but yes in the real product (P(Yes)|(No)) occurs for each attribute, mean drops in liking between the two situations and significances.

Variable	Level	Frecuency %	Mean (Liking)	Effect on the average	P-value	Significant	Penalty
Light brown	P(No)I(No)	30.00%	6.741				0.288
	P(Yes)I(No)	13.33%	6.875	0.134			
Medium brown	P(No)I(No)	40.00%	6.236				-0.505
	P(Yes)I(No)	30.00%	6.407	0.171	0.881	No	
Dark brown	P(No)I(No)	86.67%	6.519				-0.147
	P(Yes)I(No)	6.67%	6.167	-0.353			
Small eye size	P(No)I(No)	58.33%	6.695				0.375
	P(Yes)I(No)	25.00%	5.933	-0.762	0.039	Sí	
Medium eye size	P(No)I(No)	19.44%	6.200				-0.421
	P(Yes)I(No)	15.56%	6.750	0.550			
Large eye size	P(No)I(No)	78.89%	6.697				0.750
	P(Yes)I(No)	12.78%	5.478	-1.219			
Less elastic	P(No)I(No)	70.00%	6.635				0.320
	P(Yes)I(No)	20.00%	6.000	-0.635	0.099	No	
Medium elasticity	P(No)I(No)	20.56%	6.486				-0.066
	P(Yes)I(No)	11.11%	6.150	-0.336			
Too elastic	P(No)I(No)	82.78%	6.577				0.222
	P(Yes)I(No)	5.56%	5.600	-0.977			
Less cohessive	P(No)I(No)	48.89%	6.625				0.168
	P(Yes)I(No)	21.11%	6.421	-0.204	0.860	No	
Medium cohesiveness	P(No)I(No)	36.67%	6.364				-0.277
	P(Yes)I(No)	8.33%	6.333	-0.030			
Dry	P(No)I(No)	86.11%	6.626				0.626
	P(Yes)I(No)	10.56%	5.842	-0.784			
Soft	P(No)I(No)	3.89%	5.571				-1.007
	P(Yes)I(No)	6.11%	5.909	0.338			
Grittiness in the mouth	P(No)I(No)	44.44%	7.225				1.235
	P(Yes)I(No)	48.89%	6.011	-1.214	<0,0001	Sí	
No Gritty	P(No)I(No)	17.22%	5.935				-0.729
	P(Yes)I(No)	2.78%	8.200	2.265			
Less sour	P(No)I(No)	30.56%	7.218				0.978
	P(Yes)I(No)	29.44%	6.358	-0.860	0.061	No	

Variable	Level	Frecuency %	Mean (Liking)	Effect on the average	P-value	Significant	Penalty
Medium sourness	P(No)I(No)	38.89%	5.943				-0.975
	P(Yes)I(No)	14.44%	6.577	0.634			
Very sour	P(No)I(No)	87.78%	6.506				-0.266
	P(Yes)I(No)	5.56%	6.100	-0.406			
Characteristic odor	P(No)I(No)	22.78%	6.122				-0.540
	P(Yes)I(No)	13.89%	6.440	0.318			
Strange odor	P(No)I(No)	72.22%	6.708				0.608
	P(Yes)I(No)	17.78%	5.875	-0.833			