Promotor   **Prof. dr. ir. Katleen Raes**
                      Department of Food Technology, Safety and Health, Faculty of
                      Bioscience Engineering, Ghent University - Campus Kortrijk, Belgium

Examination Committee

Chairman   **Prof. dr. ir. Monica Höfte**
                      Department of Plants and Crops, Faculty of Bioscience Engineering,
                      Ghent University, Belgium

Secretary   **Prof. dr. ir. John Van Camp**
                      Department of Food Technology, Safety and Health, Faculty of
                      Bioscience Engineering, Ghent University, Belgium

                      Prof. dr. ir. Koen Dewettinck
                      Department of Food Technology, Safety and Health, Faculty of
                      Bioscience Engineering, Ghent University, Belgium

                      dr. ir. Filip Van Bockstaele
                      Department of Food Technology, Safety and Health, Faculty of
                      Bioscience Engineering, Ghent University, Belgium

                      Prof. dr. John Taylor
                      Department of Food Science, University of Pretoria, Pretoria, South
                      Africa

                      Prof. dr. Dominique Bounie
                      Department GBIAAL Boulevard Langevin, Polytech Lille, Université
                      de Lille Sciences et Technologies, Lille, France

Dean   **Prof. dr. ir. Marc Van Meirvenne**
Rector   **Prof. dr. Ir. Rik Van de Walle**

COMPOSITIONAL AND NUTRITIONAL PROPERTIES OF TEF AND TEF-BASED FOOD PRODUCTS

HABTU SHUMOY

Thesis submitted in the fulfilment of the requirements for the degree of Doctor (PhD) in

Applied Biological Sciences: Food Science and Nutriti

Dutch translation of the title:

Samenstelling en nutritionele eigenschappen van tef en levensmiddelen op basis van tef

Refer this thesis as: Shumoy H., 2018. Compositional and nutritional properties of tef and tef-based food products. PhD dissertation, Faculty of Bioscience Engineering, Ghent University, Belgium.

Financial support

Acknowledgements

Dedication

I dedicate this PhD to my parents who decided to send me to school while they were a struggling agrarian family in a poor village where many people of my age did not get same chance as I did.

Habtu Shumoy Abraha

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AA Amino acid

ABTS (2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)

AUC Area under curve

BW Buckwheat

CE Catechin equivalent

CV Coefficient of variance

D Dialyzable

DAD Photodiode array detector

dm Dry matter

DPPH 2,2-diphenyl-1-picrylhydrazyl

eGI Estimated glycemic index

ELISA Enzyme-linked immunosorbent assay

FAO Food and Agriculture Organization

FCs Flavonoid contents

FG Free glucose

FN Falling number

FRAP Ferric reducing antioxidant power

GAE Gallic acid equivalent

GI Glycemic index

GOPOD Glucose oxidase/peroxidase

HI Hydrolysis index

HPMC Hydroxypropyl methylcellulose

In Insoluble

IVPD *In vitro* protein digestibility

LOD Limit of detection

LOQ Limit of quantification

mPa.s Millipascal second

Nd Not detected

PA Phytic acid

PCs Phenolic compounds

RDS Rapidly digestible starch

RS Resistant starch

SDS Slowly digestible starch

SGF Simulated gastric fluid

SIF Simulated intestinal fluid

SND Soluble nondialyzable

SSF Simulated salivary fluid

TFA Trifluoroacetic acid

TFC Total flavonoid content

TE Trolox equivalent

TPC Total phenolic content

TS Total starch

WHO World Health Organization

WW Whole wheat

LIST OF FIGURES

LIST OF TABLES

GENERAL INTRODUCTION AND RESEARCH FRAMEWORK

Tef [*Eragrostis tef* (Zucc.) Trotter] is a cereal crop and its use as human food is mostly confined to its origin, Ethiopia, where it is currently highly cultivated and diversified without however, any technological application in the contemporary food industry. Indeed, in Ethiopia tef has been considered as an ordinary cereal and as food of the poor while wheat, rice and oat based products got higher social value and a premium price until very recently. The worldwide increasing importance of tef started mainly after its seed touched in the Netherlands in 2003 and subsequent research findings which unveiled that tef is an ideal food for people with celiac diseases.

Tef is milled as whole flour (Bultosa, 2007) resulting in a high fiber content which is associated with positive health significance (Baye, 2014). The physiological benefits of fiber during the simulated gastrointestinal digestion as a prebiotic food is already established. It is also well documented that phenolic compounds (PCs) in cereals are concentrated in the bran part which makes consumption of food products prepared from whole flour an important source of PCs. However, the co-existence of the PCs with fiber raises a question on the bioaccessibility of PCs. In cereals, PCs are found as soluble and bound forms with the bound PCs accounting for the majority of the phenolic content. Food processing techniques involving biological processes such as fermentation has been suggested to release the bound PCs in the food matrix and increase their bioaccessibility during simulated gastrointestinal digestion.

The bran of cereals also contains the majority of the minerals which makes whole flours as an essential source of dietary minerals. Moreover, whole flours are also known to contain high amounts of phytic acid (PA), and tannins besides phenolic compounds. It is an established fact that PA, tannins and PCs bind dietary minerals such as iron (Fe) and zinc (Zn) that decrease their bioaccessibility. Traditional food processing techniques like fermentation has been suggested as a means of enhancing dietary mineral bioaccessibility.

Nutritionally, cereals are the dominant source of carbohydrates in the human diet, providing the major source of energy. Carbohydrates account for more than 75% of the mass of mature cereals with starches accounting for the main part of it. The digestion and nutritional properties of carbohydrates is highly influenced by starch type and dietary fiber content. Starch is composed of amylose and amylopectin and its gelatinization properties and the consequent gastrointestinal digestion is highly affected by its amylose/amylopectin ratio. High amylopectin (waxy) starches

are relatively more digestible resulting in a high glycemic index (GI) due to their branched structure which ease the action of hydrolytic enzymes. The desirability of highly or slowly digestible starch depends on the intended use of the starchy food product or the intended health or nutritional outcomes that consumers need. Based on digestibility, starches can be categorized as resistant starch (RS), slowly digestible starch (SDS) and rapidly digestible starch (RDS). Starches with higher proportions of RS and SDS may result in a lower GI which is desirable particularly for diabetic people and those of in diet control in general. Cereals with higher fiber content have an added value when lower GI food product is needed as fiber is composed mainly of non- starch cell wall polysaccharides which are resistant for digestion.

Cereals account for a significant amount of the global food protein supply, largely in developing countries where consumers essentially rely on cereal consumption. Quality of cereal proteins in terms of digestibility and amino acid composition remain an important issue for people mainly depending on plant based diets. The availability of gluten and other protein allergens in many cereals is also another key concern for celiac disease patients and those people sensitive to certain protein allergens.

From a technological application aspect, it has been suggested that ancient gluten-free cereals can be used for the same purpose as wheat flour, without however, verification of their suitability on their product quality aspects. The processing of ancient cereals such as tef has remained mostly traditional and their consumption is limited to specific countries or communities. Due to this, the impact of the traditional processing of tef on the overall nutritional and health outcomes of the resulting food product in question is not well studied.

Recently, interest in tef consumption among consumers in Western countries is increasing. Tef is now included in the list of gluten-free foods of 'Celiac Diseases Foundation' and 'Celiac Support Association'. Unlike its growing global demand, the knowledge on the compositional and nutritional benefits of tef food products, other than some indication as gluten-free, is rather inadequate. Overall, there is limited literature regarding its compositional and nutritional properties from the perspectives of phenolic content and antioxidant capacity, minerals content and bioaccessibility, starch digestibility and protein characterization. Therefore, this PhD was designed to realize a holistic view of compositional and nutritional properties of this cereal by addressing the following objectives:

1. Assess the phenolic contents and antioxidant capacity of tef and its main traditional food products (chapter 2)

2. Investigate its mineral content and their bioaccessibility as affected by fermentation (chapter 3)

3. Evaluate starch digestibility and glycemic index of its major traditional and conventional food products (chapter 4)

4. Characterize its storage protein and assess its suitability as a gluten-free ingredient (chapter 5)
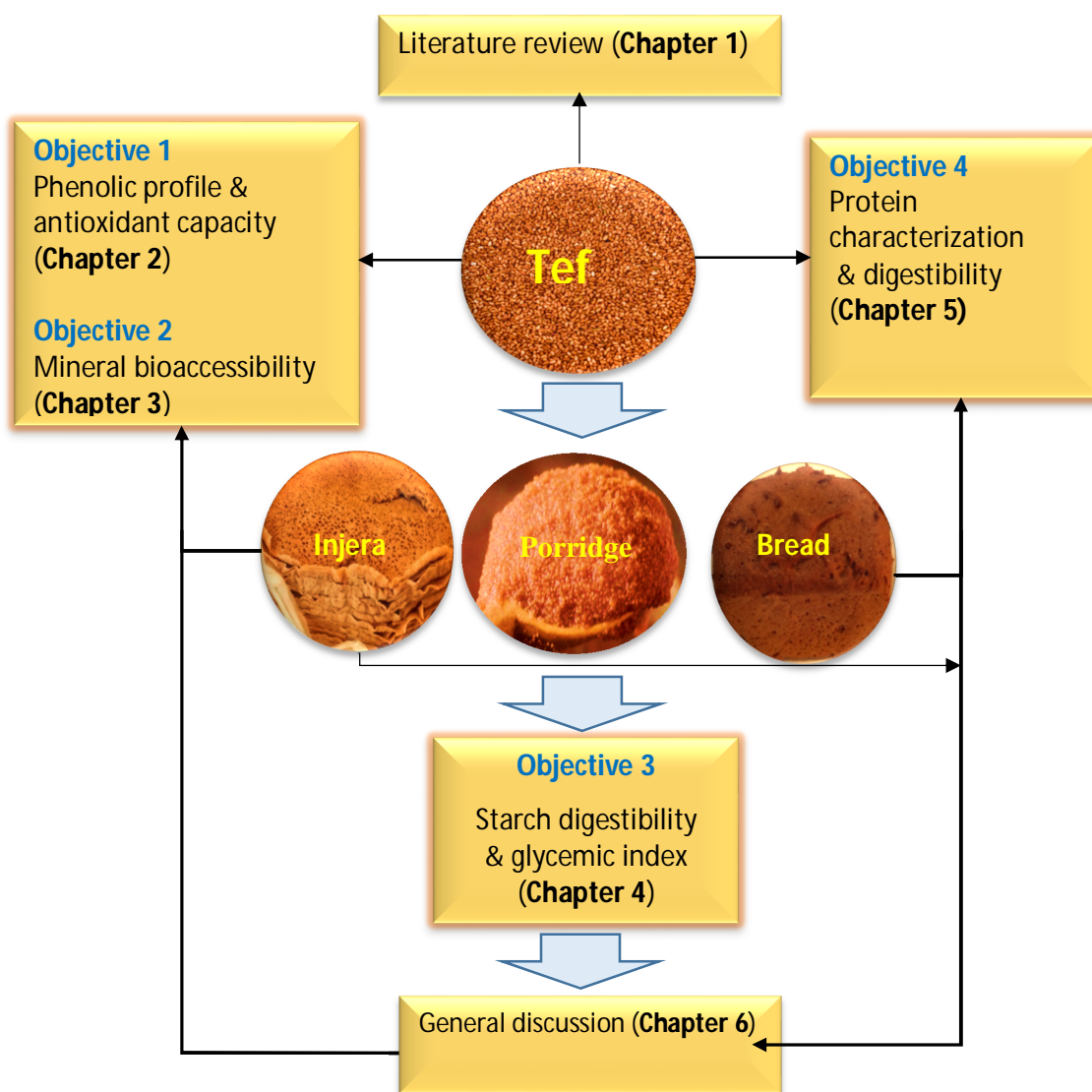
Figure 0.1 Thesis framework

SUMMARY

This PhD mainly focused on the evaluation of tef cereal in terms of its composition and nutritional properties. For this purpose, two sets of tef flour were procured. The first set contained 7 pure tef varieties of brown and white seed color and used for the experiments in chapters 2, 3, 4 (part 4.1) & 5. The second set containing two types of unknown varieties or mixed tef (brown and white seed color), are used in chapter 4 (part 4.2). We believe that this work will contribute to the realization of the blessings of this uniquely small sized but nutritionally condensed cereal among consumers and will indirectly benefit to all parties involved in tef business, *i.e.* tef farmers worldwide and millions of poor Ethiopian peasants in particular, in a way to create a more global market.

The expedition of this PhD was started by conducting a comprehensive literature study (chapter 1) to clearly identify the knowledge gaps of this cereal as a food. In this literature review, tef was compared to other common cereals and pseudocereals with regard to its chemical composition. The major findings of this review were that tef actually contains high Fe and Ca contents, although the controversy still remains if the iron in tef is intrinsic or coming from a possible contamination with soil during harvesting. It was clear that tef is also relatively rich in fiber but at same time it also contained high amounts of antinutrients such as phytic acid and phenolic compounds. This chapter also pointed out that tef protein contains all the essential amino acids and particularly a high lysine content. The starch of tef flour is composed mainly of slowly digestible and resistant starch and a lower GI of typical cereal products. Finally, this review also identified research gaps in terms of mineral bioaccessibility, proportion of soluble and bound PCs and their antioxidant capacity, starch digestibility and GI, protein digestibility and solubility characteristics, not only tef flour but also tef-based food products.

Chapter 2 deals with the phenolic content and antioxidant capacity of tef and its major food product, *injera*. The main objective was to investigate the variability among different tef varieties and to evaluate proportions of the soluble and bound phenolic compounds and the effect of fermentation on the content and bioaccessibility of the PCs. All the seven tef varieties had more than 84% of their total phenolic content (TPC) in the bound form, which contributed to more than 80% of the total antioxidant capacity as measured by FRAP (ferric reducing antioxidant power) and DPPH ((2,2-diphenyl-1-picrylhydrazyl)). It was clear that the brown tef varieties showed a higher TPC and antioxidant capacity compared to their white counterparts. Catechin

followed by rosmarinic and ferulic acid were the major phenolic compounds of the soluble phenolic fraction, while ferulic followed by rosmarinic and *p*-coumaric acid was mainly present in the bound fraction.

The effect of fermentation and cooking of a traditional tef food product, *injera*, on the proportion of soluble and bound phenolics and their corresponding antioxidant capacity was investigated. The traditional fermentation significantly increased the TPC and antioxidant capacity of the *injera* (Table 0.1). More interesting was the increase in proportion of the soluble phenolics while a decrease for bound phenolics was noticed. Many of the phenolic compounds detected in the flour were detected in their corresponding *injera*. Corresponding to their flours, *injeras* of the brown tef varieties showed higher total phenolic and antioxidant capacity signifying the superiority of these varieties as a potential source of healthy food products.

In an attempt to draw a complete picture of the phenolic and antioxidant capacity of tef referring to their health importance, a simulated static *in vitro* digestion of the tef *injeras* was performed to investigate the bioaccessibility of the PCs (part 2.3). An increase of TPC during fermentation and baking processes was not clearly reflected in the bioaccessibility of the phenolic compounds (Table 0.1).

Table 0.1 Effect of fermentation on inhibitors of mineral bioaccessibility

Parameter	Fermented vs tef flour
TPC	↗
Soluble phenolics	↗
Bound phenolics	↘
Bioaccessibility of PCs	Expected ↗ not well demonstrated
Antioxydant capacity	↗
Phytic acid	↘
Bioaccessibility of Fe	↗ (not for all species)
Bioaccessibility of Zn	↗

As is seen in chapter 1, tef is endowed with higher Fe content compared to other cereals and a fairly high Zn content but their bioaccessibility is not yet well documented. Therefore, the bioaccessibility of Fe and Zn from tef *injeras* was studied in chapter 3. The Ethiopian traditional fermentation of *injera* caused a significant decrease of the phytic acid, resulting in an increase of Fe and Zn bioaccessibility, although this was not observed in all the varieties (Table 0.1). In this

chapter, it became clear that a high Fe content does not necessarily result in high amount of bioaccessible Fe, particularly if the Fe co-existed with high antinutrients.

The other focus of this PhD was to characterize the starch digestibility properties of tef and its food products. In chapter 4, the starch digestibility and resulting estimated glycemic index (eGI) of major traditional tef food products (*injera* and porridge) and a conventional bread were investigated. Each of the tef varieties showed a decrease in resistant and slowly digestible starches while an increase in rapidly digestible starch after baking/cooking (part 4.1) (Table 0.2). Our *in vitro* experiment shows that freshly prepared *injera* and porridge may not be a good alternative for diabetic people as they exhibited high starch digestibility and resulted in medium to high eGI. In parallel, we also studied the starch digestibility and eGI of a conventional tef bread as affected by the addition of sourdough and the storage time (part 4.2). Similar to part 4.1, the baking process caused a significant decrease in resistant and slowly digestible starches while the rapidly digestible starch increased. The sourdough addition increased the eGI of fresh breads. The starch digestibility and resulting eGI were highly dependent on the storage time of the breads. As the duration of bread storage increased, their starch digestibility and eGI showed a significant decrease.

Table 0.2 Effect of processing on starch digestibility and eGI

Parameter	Processed (baked, fermented) vs. tef flour				Stored vs fresh bread
	Bread vs. flour	sourdough Bread vs. flour	Porridge vs. flour	Injera vs. flour	
Resistant and slowly digestible starch	↘	↘	↘	No uniform pattern among varieties	↗
Rapidly digestible starch and eGI	↗	↗	↗	↗ ↗	↘

In thought of a broad approach to the compositional and nutritional properties of tef, we also investigated the protein properties in respect to its total protein content, digestibility, solubility, SDS-PAGE molecular distribution and suitability as a gluten-free food for celiac disease patients (chapter 5). Tef has a total dietary protein content comparable to other cereals but its protein has fairly high digestibility properties. The Osborne protein solubility showed low recovery of storage protein. The SDS-PAGE molecular distribution of the total protein and storage proteins

tef varieties showed similarities among the varieties but were different from the SDS-PAGE molecular distribution wheat. This study also confirmed that tef is gluten-free and is in compliance with the European Commission Food Safety Regulation Directive, thus it can be used as safe ingredient for the gluten-free food formulation. In this chapter, the possible presence of allergenic protein compounds were highlighted and it became clear that tef may contain some protein allergens, however, more specific investigations to confirm the presence of these allergens is still needed.

The overall PhD study and its future perspectives are discussed in chapter 6. The PhD study showed that tef has a high antioxidant capacity, phenolic and mineral (Fe and Zn) contents with moderate bioaccessibility. This study also clearly showed that the eGI of fresh tef bread, *injera* and thick porridge can be classified as medium or high in the international GI table. Finally, it was shown that tef is gluten-free and has highly digestible protein.

SAMENVATTING

In dit doctoraatswerk gaat de focus voornamelijk uit naar de evaluatie van het graan tef in termen van de samenstelling en nutritionele eigenschappen. Om dit doel te bereiken werden 2 sets of tef bloem gebruikt. Een eerste set bestond uit bloem van 7 zuivere tefvariëteiten met een witte of bruine zaadkleur, en werd gebruikt voor de proeven uitgevoerd in hoofdstuk 2, 3, 5 en deel 4.1). Een tweede set bestond uit bloem afkomstig van 2 types niet-gedefinieerde variëteiten of gemengd tef (bruine en witte zaadkleur), en werd gebruikt in deel 4.2. De resultaten van dit werk zullen verder bijdragen tot kennis van dit uniek, klein maar nutritioneel belangrijk graan bij de consumenten, en zal indirect voordelen geven aan alle partijen betrokken in tefhandel nl. tefproducenten wereldwijd en in het bijzonder miljoenen arme Ethiopische boeren, zodat een meer globale vermarkting gecreëerd kan worden.

Het doctoraatsonderzoek werd gestart met een uitgebreide literatuurstudie (hoofdstuk 1), zodat de tekorten aan kennis rond het gebruik van dit graan als levensmiddel konden geïdentificeerd worden. In dit literatuuroverzicht werd tef vergeleken met andere gekende granen en pseudogranen in termen van chemische samenstelling. De belangrijkste resultaten van de review waren dat tef hoge gehaltes aan ijzer en calcium bevat in vergelijking met andere (pseudo)granen, alhoewel nog steeds onduidelijkheid bestaat of het ijzer intrinsiek ijzer is of ijzer afkomstig van mogelijke contaminatie met de grond tijdens het oogsten. Ook werd duidelijk dat tef relatief rijk is aan vezels, maar terzelfdertijd ook hoge gehaltes aan antinutritionele componenten bevat zoals fytinezuur en fenolische componenten. Dit hoofdstuk duidde ook aan dat eiwit in tef het essentieel aminozuurprofiel heeft en in het bijzonder rijk is aan lysine. Het zetmeel in tef bestaat voornamelijk uit traag verteerbaar en resistent zetmeel en een lagere glycemische index in de graanproducten. Tot slot toonde deze review de onderzoeksmogelijkheden aan wat betreft mineralenbiobeschikbaarheid, aandelen oplosbare en gebonden fenolische componenten en hun antioxidatieve capaciteit, zetmeelverteerbaarheid en glycemische index, eiwitverteerbaarheid en oplosbaarheidseigenschappen van het eiwit, niet alleen van tef als graan maar ook van voedingsproducten bereid met tefbloem.

Hoofdstuk 2 bespreekt het gehalte aan fenolische componenten en de antioxidatieve capaciteit van tef en zijn belangrijkste voedingsproduct, *injera*. Het hoofddoel was om de variabiliteit tussen verschillende tefvariëteiten te onderzoeken en de aandelen aan oplosbare en gebonden fenolische componenten in kaart te brengen. Ook het effect van fermentatie op het gehalte en

biotoegankelijkheid van de fenolische componenten werd bekeken. Bij alle 7 de tefvariëteiten waren meer dan 84% van de totale fenolische componeten aanwezig onder vorm van gebonden fenolische componenten, welke ook bijdragen tot meer dan 80% van de totale antioxidatieve capaciteit, zoals gemeten met de FRAP (ferric reducing antioxidant power) en DPPH ((2,2-diphenyl-1-picrylhydrazyl) methode. Het werd duidelijk dat de bruine tefvariëteiten een hogere TPC gehalte en antioxidatieve capaciteit hadden in vergelijking met de witte variëteiten. Catechine, gevolgd door rosmarinezuur en ferulazuur waren de belangrijkste fenolische componenten in de oplosbare fenolische fractie, terwijl ferulazuur gevolgd door rosmarinezuur en p-coumarinezuur de belangrijkste in de gebonden fractie waren.

Het effect van fermentatie en bakken bij de productie van een traditioneel tef levensmiddel, *injera*, op het aandeel oplosbare en gebonden fenolische componenten en de daarbijhorende antioxidant capaciteit werd onderzocht. Het traditionele fermentatieproces zorgde voor een significante toename in TPC en antioxidatieve capaciteit van *injera* (Table 0.3). Opmerkelijk was dat er een toename in het aandeel oplosbare fenolische componenten werd waargenomen, samen met een daling aan gebonden fenolische componenten. Een groot aantal van de fenolische componenten aanwezig in de bloem werd ook teruggevonden in de *injera*. Analoog aan de resultaten op de bloem, vertoonden *injeras* gemaakt met bruine tef variëteiten een hoger gehalte aan totale fenolische componenten en een hogere antioxidatieve capaciteit dan deze bereid met witte tefvariëteiten. Dit wijst nogmaals op de betere geschiktheid van deze bruine variëteiten als mogelijke bronnen voor het maken van gezonde voedingsproducten.

In een poging om een volledig beeld van de fenolische componenten en de antioxidatieve capaciteit van tef te krijgen in relatie tot gezondheidsaspecten, werd een gesimuleerde statische *in vitro* vertering uitgevoerd op de tef *injeras*. Dit had als doel om de biotoegankelijkheid van de fenolische componenten te onderzoeken (deel 2.3). Een toename in totale fenolische componenten gedurende het fermentatie en bakproces werd niet duidelijk weerspiegeld in de biotoegekelijkheid van de fenolische componenten (Tabel 0.3).

Zoals besproken in hoofdstuk 1, wordt tef gekenmerkt door de hoogste gehaltes ijzer in vergelijking met andere granen, en het bevat ook vrij hoge zinkgehaltes. De biotoegankelijkheid van deze mineralen in tef is echter niet goed beschreven. Daarom werd in een experiment de biotoegankelijkheid van ijzer en zink in tef *injeras* onderzocht (hoofdstuk 3). De traditionele ferementatie van *injera*, zoals uitgevoerd in Ethiopia, veroorzaakte een significante daling in fytinezuur. Dit had als gevolg dat de biotoegankelijkheid van ijzer en zink significant toenam

(Tabel 0.3), alhoewel dit niet in alle variëteiten kon waargenomen worden. Het werd ook duidelijk dat hoge Fe gehaltes niet noodzakelijk leidt tot hoge gehaltes biotoegankelijk Fe, zeker wanneer Fe samen aanwezig is in de matrix met antinutritionele factoren.

Table 0.3 Effect van fermentatie op inhibitors van mineralenbiobeschikbaarheid

Parameter	Gefermenteerde *injera* vs tefbloem
TPC	◂
Oplosbare fenolische componenten	◂
Gebonden fenolische componenten	↘
Biobeschikbaarheid van fenolische componenten	verwachte ↗ niet duidelijk aantoonbaar
Antioxidatieve capaciteit	◂
Fytinezuur	↘
Fe biobeschikbaarheid	◂ (niet voor alle variëteiten)
Zink Biobeschikbaarheid	◂

Een andere focus in dit doctoraatsonderzoek was het karakteriseren van de zetmeelverteerbaarheid van tef en de afgeleide voedingsproducten. In hoofdstuk 4 wordt de zetmeelverteerbaarheid en de daarbijhorende glycemische index van de belangrijkste tef voedingsproducten, *injera* en pap, en van tefbrood besproken. Bij elk van de variëteiten werd een daling in resistent en traag verteerbaar zetmeel waargenomen, terwijl een toename in snel verteerbaar zetmeel opgemeten werd na de verhittingsstap (koken/bakken) (deel 4.1) (Tabel 0.4). Een *in vitro* proef toonde aan dat vers bereide *injera* en pap geen goede alternatieven zijn in het dieet van diabetici, aangezien deze producten een hoge zetmeelverteerbaarheid vertoonden, en dus een middelmatige tot hoge glycemische index hadden. Ook werd de zetmeelverteeraarheid en glycemische index bepaald van tefbrood, en de impact van zuurdesem en bewaarperiode werd hierop onderzocht (deel 4.2). Analoog als waargenomen in deel 4.1, zorgde het bakproces voor een significante daling in resistent en traag verteerbaar zetmeel, terwijl het snel verteerbaar zetmeel toenam. Zuurdesem toediening had geen effect op de zetmeelverteerbaarheid, noch op de glycemische index. De zetmeelverteerbaarheid en daarbijhorende glycemische index werden voornamelijk beïnvloed door de bewaartijd van de broden, met de laagste verteerbaarheid en glycemische index opgemeten bij de broden welke het langst bewaard werden.

Table 0.4 Effect van processing van tef op zetmeelverteerbaarheid en geschatte glycemische index

Parameter	Processing (bakken, fermentatie) vs. tef boem				Bewaard vs vers brood
	Brood vs. bloem	Zuurdesem brood vs. bloem	Porridge vs. bloem	Injera vs. bloem	brood
Resistent en traag verteerbaar zetmeel	↘	↘	↘	Geen uniform patroon voor de verschillende variëteiten	↗
Snel verteerbaar zetmeel en geschatte glycemische index	↗	↗	↗	↗	↘

Met als doel om een brede inschatting te maken van de nutritionele en gezondheidsvoordelen van tef, werden ook de eiwiteigenschappen onderzocht, meer specifiek wat betreft verteerbaarheid, oplosbaarheid, moleculair gewichtsverdeling op basis van SDS-PAGE en de mogelijkheid om tef te gebruiken in de productie van glutenvrije voedingsproducten (hoofdstuk 5). Tef heeft vergelijkbare totale eiwitgehaltes met de andere granen, maar het eiwit in tef heeft vrij goede verteringseigenschappen. De Osborne eiwitoplosbaarheid vertoonde een lage recovery van de opslageiwitten in tef. De moleculaire distibutie van het totaal eiwit alsook van de opslageiwitten, zoals bepaald vi SDS-PAGE, vertoonde overeenkomsten tussen de variëteiten maar waren verschillend van de moleculaire distributie bekomen voor tarwe-eiwitten. Deze studie bevestigde dat tef een glutenvrij graan is en dus voldoet aan de 'European Commission Food Safety Regulation Directive'. Het kan bijgevolg gebruikt worden als ingrediënt voor de bereiding van glutenvrije levensmiddelen. In hoofdstuk 5 werd ook de mogelijke aanwezigheid aangetoond van andere eiwitten met allergene eigenschappen. Hieruit werd duidelijk dat tef mogelijks enkele eiwitallergenen kan bevatten, maar dit gegeven moet in de toekomst verder onderzocht worden.

Een overzicht van de bekomen resultaten en toekomstperspectieven betreffende tef en de tefgebaseerde voedingsproducten wordt beschreven in hoofdstuk 6. Samengevat kan gesteld worden dat dit doctoraatsonderzoek aangetoond heeft dat tef een hoge antioxidante capaciteit,

hoge gehaltes aan fenolische componenten en mineralen (ijzer en zink) bevat, echter met een matige biotoegankelijkheid. Deze studie toonde ook duidelijk aan dat de glycemische index van vers tef brood, *injera* en pap tot de klasse van middelmatige tot hoog glycemische waarden behoort, dit volgens de internationale tabel van glycemische waarden. Tot slot werd er aangetoond dat tef een glutenvrij graan is met een hoog verteerbare eiwitten, waarbij globuline het belangrijkste opslageiwit is.

CHAPTER 1: TEF: THE RISING ANCIENT CEREAL: WHAT DO WE KNOW ABOUT ITS NUTRITIONAL AND HEALTH BENEFITS?

CHAPTER 1: TEF: THE RISING ANCIENT CEREAL: WHAT DO WE KNOW ABOUT ITS NUTRITIONAL AND HEALTH BENEFITS?

1.1 Abstract

Tef [*Eragrostis tef* (Zucc.) Trotter], an ancient cereal mainly produced in Ethiopia, is increasingly getting higher acceptance in the global market. This review covers the nutritional significance of tef as compared to other common cereals and pseudocereals with emphasis on starch digestibility, protein content, and iron and zinc bioavailability and antioxidant capacity. Tef is a gluten-free cereal which has high micro- and macro- nutritional profile and is becoming globally popular in the healthy grain food chain. There are significantly conflicting reports of iron content of tef ranging from 5-150 mg/100 g dm flour. Tef food products are prepared from whole flour and are rich in mineral, fiber and antioxidants among the most important nutrients. The Ethiopian traditional fermentation of *injera* preparation process could reduce majority of the PA but no significant change to mineral bioavailability was observed. This review pointed out that more studies on *in vitro* and *in vivo* starch digestibility, protein characterization, amylase and protease inhibitors and enzymes in general, mineral bioavailability, antioxidant capacity of known tef varieties are needed to further explore the nutritional and possible health significance of tef.

1.2 Introduction

Tef [*Eragrostis tef* (Zucc.) Trotter], a cereal crop with very small kernel (Fig. 1.1), is believed to have originated in Ethiopia, where it currently shows major diversity and highest production (Endalew, 2017). It has been cultivated in the horn of Africa for at least for 2000 years and the earliest known agricultural systems in this region date back to the Pre-Aksumite period (800–400 BC). The word tef might have been derived from the Semitic *thaf*, which is applied in Yemen to indicate a wild harvested cereal. Indeed, tef is also called as *thaf* and *thafi,* in two Ethiopian languages, *Tigrigna and Afaan Oromo*, used by two Ethiopian ethnic groups *Tigrie* and *Oromo*, respectively.
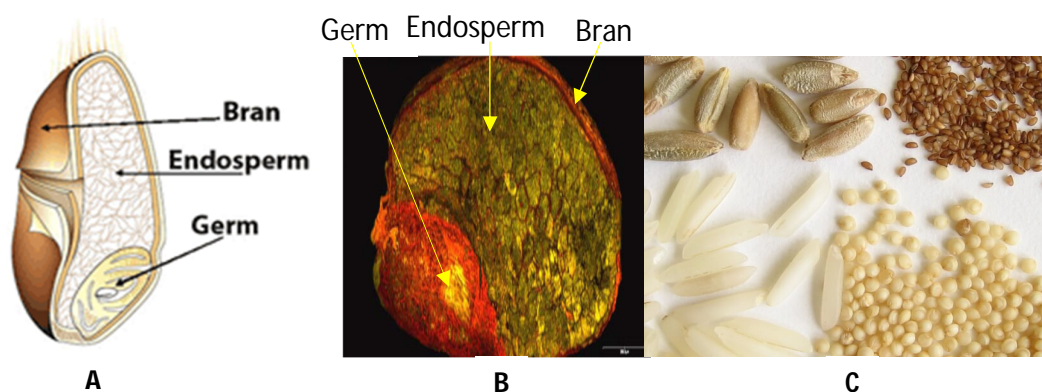
Figure 1.1 structural and size comparison of tef with other cereals
A: Wheat kernel longitudinal section, B: tef kernel longitudinal section, C: Upper left: rye kernels; upper right: tef; lower left: long grain rice; lower right: amaranth. Source: A, (http://www.deltamill.org/flour/sorting.html); B: (Gebremariam et al., 2012); C: https://slicesofbluesky.com/teff-worlds-smallest-grain/.

Tef accounts for 20% (2.7 million hectares) of all the cultivated area in Ethiopia; grown by 6.3 million subsistence farmers with a national total production of 4.5 million tonnes in the year 2016 (Endalew, 2017). Compared to other cereal crops, tef is a lower risk crop to drought as it can withstand adverse weather conditions which makes it a preferred crop by the rain fed subsistence agriculture for Ethiopian farmers. However, it has one of the lowest yield among cereal crops with only 1.3 tonnes/hectare (Demeke and Marcantonio, 2013). Although Ethiopians prefer to consume tef but only middle and high income people can have access to the grain due to its high increase of price from time to time in recent years compared to other cereals such as maize, wheat, barley and sorghum. Tef consumption in Ethiopia contributes to a gain of approximately 600 kcal/day in urban areas but only about 200 kcal/day in the rural areas where

alternatively consumption of other cheap cereals dominate (Demeke and Marcantonio, 2013). Due to high global market demand, tef production has shown a sharp increase by 122% in Ethiopia, between 2005-2016 cropping years and this increase was achieved mainly due to 37% expansion of area under cultivation and 64% increase yield/hectare by the use of improved agricultural practices. Although, there is high market demand of tef worldwide currently, the government of Ethiopia banned tef export since 2006 but there are big efforts underway to intensify and mechanize production and resume the export trade. The price of tef at the local retail markets in Ethiopia is less than €1/kg (still high in price compared to other cereals and expensive for Ethiopians living in Ethiopia) which is much lower compared to about €6/kg in western countries (http://www.glutenvrijmeel.nl)

Tef can grow under wide and diverse agro-ecologies. It grows best between altitudes of 1500 and 2500 meters with an annual rainfall of 750-850 mm and a temperature range of 10-27°C though it can also grow in much more varied areas with rainfall up to 1200 mm (Bekabil et al., 2011). The length of growing period ranges from 60 to 180 days (depending on the variety and altitude) with an optimum of 90 to 130 days (Table 1.1) and it is harvested when the vegetative and reproductive part is turned to a yellowish color (Bekabil et al., 2011; Tefera et al., 2001). Tef varieties differ in color from milky white to dark brown. There are 33 released improved tef varieties (Table 1.1) (Ethiopian Agricultural Transformation Agency, 2013). However, as Ethiopia is the center of diversity for tef, it is believed that there are hundreds of landrace tef varieties in the hands of farmers throughout the country. For example, Mengesha (1966) reported that he collected as much as 124 tef varieties (for research purpose) from the major tef producing regions of Ethiopia. Landrace tef varieties are those traditionally selected (protected from mixing with other varieties and not conventionally breed) by farmers and suitable for particular agroclimatic conditions.

In Ethiopia, tef is mainly used to make *injera* (Fig 1.2A) and porridge (Fig 1.2B) (Umeta and Faulks, 1988; Yohannes et al., 2013). Injera is defined as a pancake-like fermented /sour flat bread usually prepared from Tef flour. The front side of a good quality injera has uniformly spaced honeycomb-like pores traditionally called "eyes", formed due to the penetration of escaping gas that is produced during fermentation and baking, whereas the bottom surface of *injera* is smooth and shiny. Tef porridge is made by cooking the mixture of tef flour with water and can vary from thick to thin gruel depending on the preference of consumers.

Thick porridge is mainly for adults and children who can chew their food. In the past, it was believed that thin gruel is good for infants, however, with the ongoing health and nutrition education, feeding thicker porridge has been promoted due to its condensed nutritional advantage over the watery/bulky thin gruel.

Table 1.1 List of released improved tef varieties in Ethiopia from 1970-2012

No.	List of Varieties	Technical Recommendations			Productivity[a]
		Altitude[b]	Annual RF[c]	Maturity[d]	
1	DZ-01-99	1500-2400	300-700	80-130	1.7-2.2
2	DZ-01-196	1500-2400	200-700	80-113	1.4-1.6
3	DZ-01-354	1600-2400	300-700	85-130	1.7-2.2
4	DZ-CR-787	1800-2500	400-700	90-130	1.7-2.2
5	DZ-CR-44	1800-2500	400-700	125-140	1.7-2.2
6	DZ-CR-82	1700-2000	300-700	112-119	1.8-2.2
7	DZ-CR-387	1500-2500	300-700	80-113	2.0-2.2
8	DZ-01-1278	2200-2300	700-800	125-140	2.7
9	DZ-CR-37	1500-2200	150-200	82-90	1.4-1.9
10	DZ-CR-255	1700-2000	300-700	114-126	1.6-2.2
11	DZ-CR-974	1400-2400	150-700	76-138	2.0-2.5
12	DZ-01-146	1450-1850	660-1025	78-85	1.55
13	DZ-CR-358	1400-2400	150-700	75-137	1.8-2.4
14	DZ-01-1281	1850	600	73-95	1.017
15	DZ-01-1285	1900-2200	300-700	104-118	1.8-2.5
16	DZ-01-1681	1600-1900	300-500	84-93	1.6-1.9
17	DZ-01-254	1450-1850	660-1025	68-100	1.6
18	PGRC/E 205396	No data	900-1200	85-110	11.4
19	DZ-01-1821	1450-1850	660-1025	78-85	1.51
20	DZ-01-1868	2000-2600	>600	108	1.63
21	ACC 205953	1450-1850	660-1025	79	1.79
22	DZ-CR-387	1450-1695	690-965	62-83	1.4
23	KENA	1850-2400	1000-1200	110-134	1.3-2.3
24	DZ-01-3186	1800-2600	1230	92-117	1.6-2.2
25	DZ-01-1880	1850-2500	1000-1200	132	1.4-2.0
26	Ho-Cr-136	1600-1700	500-850	63-87	1.2
27	DZ-01-2423	2000-2600	>600	105	1.68
28	DZ-01-899	2000-2500	1000-1200	118-137	1.6
29	Lakech (RIL 273)	1450-1850	660-1025	90	1.3-1.8
30	DZ-01-2675	1800-2500	997-1200	112-123	1.6-2.0
31	Simada (RIL 295)	Nd	300-700	88	1.0
32	DZ-Cr-285	Nd	Nd	88	1.0
33	DZ-Cr-409	Nd	Nd	84-89	Nd

Source: (Ethiopian Agricultural Transformation Agency, 2013). [a](Tonnes/hectare), [b](masl)-meters above sea level); [c](mm)-millimeter, [d](days), Nd: No data.

Figure 1.2 Tef *injera* (A) and porridge (B)
Source: Creative Commons.

In combination with other flours or alone, tef is also used to make conventional bread, gluten-free 'sprits' (Dutch shortcake cookie), gluten-free (sponge) cake, gluten-free Kanos (Dutch almond fingers) and Dutch almond tartlets, pancakes, and gluten-free portugeesjes (Dutch frangipane cakes). Tef has been gaining popularity mainly owing to its gluten-free nature (Dekking et al., 2005; Hopman et al., 2008) and extraordinarily high mineral content in particular iron (Alaunyte et al., 2012; Baye et al., 2014). It is processed as whole flour which makes it a good source of phenolic compounds, fiber and minerals (Adom and Liu, 2002; Mellado-Ortega and Hornero-Mendez, 2015; Omoba et al., 2015; Shumoy and Raes, 2016).

Tef has been introduced to different parts of the world through various institutions and individuals. The Royal Botanic Gardens, Kew, imported seed from Ethiopia in 1866 and distributed it to India, Australia, the USA and South Africa (Ketema, 1997). Different individuals also introduced tef into many countries *i.e.* Skyes in 1911 to Zimbabwe, Mozambique, Kenya, Uganda, Tanzania; Burt Davy in 1916 to California (USA), (where it is currently cultivated in several states such as Montana, South Dakota and Idaho) to Malawi, Zaire, India, Sri Lanka, Australia, New Zealand and Argentina; Horuitz in 1940 to Palestine (Ketema, 1997). Recently, 12 pure tef varieties that could perform well in cold climates were also introduced from Ethiopia to the Netherlands by the Soil and Crop Improvements Company and currently (Andersen and Winge, 2012). The Netherlands is becoming known as the tef center and as the major producer of tef for the European and beyond markets.

In Ethiopia, tef is mostly preferred to make *injera* only because of its excellent quality *with* desirable texture. Tef *injera* can last up to three days at room temperature without compromised organoleptic quality. From a nutritional viewpoint, tef had always been considered as an ordinary cereal throughout history. The nutritional and health importance of tef was started to be realized globally mainly after its seed touched in the Netherlands in 2003 and the subsequent

groundbreaking research findings by Dekking (Dekking et al., 2005) and Hopman (Hopman et al., 2008) again from the Netherlands who proved that tef actually was a healthy food at least for people with celiac diseases due its gluten free nature. Currently, it is getting a huge global market and its price is skyrocketing, however, data on its nutritional and health benefits is still far from complete. Recently, tef has been included in the list of gluten free foods of 'Celiac Diseases Foundation' and 'Celiac Support Association'. The inclusion in these lists could have also boosted the chance of tef to get more niche market by the celiac diseases affected population. However, it is not yet clear if tef has other unique nutritional or functional properties making it extremely expensive compared to other gluten free cereals such as maize, sorghum and rice in the Western countries. In fact, Abebe and Ronda (Abebe and Ronda, 2014) broadly stated that interest in the food application of tef has increased in the recent years because of its good nutritional qualities and absence of gluten.

Research on the nutritional, health and functional properties of tef has been increased probably due to its global acceptance and the interest among consumers to know more about the nutritional, health benefits and functional properties of this cereal, has also increased. The purpose of this review was to summarize the existing literature on tef concerning its nutritional and health significance. The existing literatures dealing with tef grain proximate composition and nutritional benefits are presented and compared to other common cereals and pseudocereals and finally major research gaps are pointed out.

1.3 Phenolic Content and Antioxidant Capacity

Whole cereals naturally contain high amounts of PCs. Indeed, phytochemicals, such as phenolic acids, phytosterols, alkylresorcinols, lignans and folate are predominantly found in the bran than in the interior parts of grains (Mattila et al., 2005). Shumoy and Raes (2016) reported a TPC of seven different tef varieties grown in Ethiopia in the range of 263-448 mg gallic acid equivalent (GAE)/100 g dm. Relatively lower TPC that ranged from 126 to 219 mg GAE/100 g were also reported (Forsido et al., 2013; Kotaskova et al., 2016). The low TPC reported by Forsido et al. (2013) only constituted methanolic phenolic extracts or only soluble form of PCs while the reports in both the studies of Shumoy and Raes (2016) and Kotaskova et al. (2016), it is as a summation of both soluble and bound phenolic extracts. In both of these two studies brown colored seed coat tef varieties showed higher TPC than the white varieties.

Unlike to report of Shumoy and Raes (2016) that revealed more than 84% of the phenolic compound as bound, Kotaskova et al. (2016) showed only 31% of the PCs as bound. However, similar to the reports of Shumoy and Raes (2016), Adom and Liu (2002) revealed higher proportions of bound phenolic contents in different cereals such as corn 85% and 91%, oats 75% and 61%, wheat 75% and 93%, rice 87% and 65%, respectively. The reason for the wide difference of TPC and the proportion of soluble and bound phenolics in tef could be attributed to the difference in tef varieties grown in different locations and methodological differences. Gallic acid, vanillic acid, caffeic acid, syringic acid, trans-*p*-coumaric acid, *m*-coumaric acid, ferulic acid, rutin, protocatechuic acid, cinnamic acid and quercetin were detected and quantified in tef flour (Kotaskova et al., 2016; Shumoy and Raes, 2016). Trans-*p*-coumaric followed by ferulic acids and ferulic acid followed by catechin were the most dominant phenolic acid in the soluble and the bound extracts, respectively (Kotaskova et al., 2016) whereas catechin followed by rosmerinic acid and ferulic followed by rosmerinic acids in the soluble and bound extracts, respectively (Shumoy and Raes, 2016).

There is scarce information on how different food processing techniques affect the contents of total, soluble and bound phenolic contents of tef based food products. However, it was reported that a traditional sourdough fermentation to make a pancake known as *injera* has significantly increased the TPC by as much as 31-54% (Shumoy et al., 2017). In this study, the proportion of soluble phenolic extracts has increased while that of bound decreased after the fermentation. The increase of the soluble PCs could be due to the action of endogenous and microbial enzymes initiated during the fermentation, which leads to the release of bound phenolics. Indeed several yeasts and LAB, which are also involved in tef fermentation, are capable of synthesizing enzymes like esterases, xylanases, and phenoloxidases that in turn are capable of breaking down ester linkages to release bound PCs in the form of soluble PCs (Ajila et al., 2011). The increase in the soluble phenolic proportion following the traditional fermentation would mean that an increase in the bioavailability of the PCs. FRAP and DPPH value in the range of 42-79 µmol Fe^{2+}/g dm and 25-142 µmol trolox equivalent (TE)/g dm, respectively were reported in different tef varieties (Shumoy and Raes, 2016). ABTS (2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) and DPPH radical scavenging capacity that ranged from 3-6 and 2-5 µmol TEAC/100 g dm, respectively were also revealed (Kotaskova et al., 2016). White wheat flour breads prepared by substitution of 10%, 20% and 30% by tef flour exhibited a significant increase of total antioxidant capacity

from 1.4 to 2.4 mM TEAC/100 g (Alaunyte et al., 2012) indicating that tef could be a good source of antioxidants which fight against free radicals that cause chronic diseases.

1.4 Minerals

The ash and mineral contents of tef and other common cereals and pseudocereals is shown in Table 1.2. Tef could be a good source of dietary minerals, particularly Fe, Zn and Ca, however, there are concerns that the minerals in tef are from direct soil contamination which can be evidenced from the strangely wider range (5-150 mg/100 g) in Fe content. Due to this, some researchers tried to measure the inherent iron content by washing the grains with acid containing deionized water (Areda et al., 1993). The results obtained in different studies still vary significantly and contradict to one another. As presented in Table 1.2, Baye et al. (2014) reported 80.1 and 31.6 mg/100 g of iron content for unwashed and washed tef, respectively. Iron content of >150 and 37 mg/100 g of unwashed red tef and white tef, respectively was also reported (Abebe et al., 2007) while much lower iron contents (8.5 mg/100 g) of unwashed tef were revealed by Hager et al. (2012). If the higher iron content in tef is to be ascribed to soil contamination during threshing and other activities on the field, some questions should be clarified. Why is the difference of iron content so large between brown and white tef (Abebe et al., 2007) provided that they have similar size and passed through similar agricultural practice? Why are the results reported by different researchers contradict to one another like unwashed samples appear to contain less iron than the washed ones? For instance, there are reports of iron content (mg/100 g dm) 6 and 5, respectively of unwashed white and brown tef (Almgard G., 1963) and in that range of 12-19 in different tef varieties grown in a controlled system where at least the tef kernels were not in direct contact with soil (Mengesha, 1966). Moreover, acid containing deionized water washed tef resulted a relatively higher iron contents of 21.5 (white tef) and 21.5 (brown tef) mg/100 g dm (Hofvander, 1968). According to these contradictory reports, the iron content of tef is still controversial and it is difficult to conclude whether the higher iron content is derived from soil contamination or not. This may create fundamental uncertainties if tef has to be used as source of iron to solve iron deficiency anemia and other iron deficiency related diseases.

In most cereals, PA is the major phosphate storage compound and a major chelator of nutritionally indispensable micronutrients such as Fe, Zn, Ca, Mg and Mn (Bhati et al., 2014).

Table 1.2 Ash[a] and mineral[b] contents of tef, common cereals and pseudocereals

Minerals	Common cereals and Pseudocereals								
	Tef[1*]	Wheat[2*]	WW[3*]	Rice[4*]	Maize[5*]	Sorghum[6*]	Oat[7*]	Quinoa[8*]	BW[9*]
Ash	2-3	0.5-1	0.5-2	0.5-0.6	0.5-2	0.5-1	1-3	2-3	2-3
Fe	5-150	1-3	3-5	0.4-0.7	2-4	1-11	2-5	5-6	2-4
Ca	79-180	15-34	35-37	28-58	6-138	5-13	25-58	40-70	18-41
Cu	1-3	0.1-0.2	0.4-1	0.2-0.3	0.2-2	0.01-0.4	0.3-0.6	0.6-0.7	0.5-1
Mg	170-187	26-93	90-137	25-38	93-127	31-165	44-235	161	231-251
K	380- 427	107-174	363-460	76-112	287-381	145-363	195-566	563-855	402-577
P	400-429	104-108	235-357	115-105	241-272	87-289	165-734	457-462	319-347
Mn	4-9	0.7-1	3-4	1-3	0.4-0.5	0.2-2	0.3-6	2-4	0.1-2
Na	7-16	2-4	0.1-2	2-5	4-35	1-6	2-4	3-5	1-11
Zn	2-5	0.7-2	2-3	1-2	2-3	0.5-2	1-4	3-4	2-3

[1](Baye, 2014), (Bultosa, 2007), (Abebe et al., 2007), (Baye et al., 2014), (Bultosa and Taylor, 2004a), (Mamo and Parsons, 1987) ; [2](Baye, 2014), (Heshe et al., 2015); [3](Baye et al., 2014), (Beloshapka et al., 2016); [4](Baye, 2014); [5](Baye, 2014) (Food and Agriculture Organization (FAO) and World Health Organization (WHO), 1989), (Edwardson, 1996); [6](Baye, 2014), (Baye et al., 2014), (Awadalkareem et al., 2008); [7](Baye, 2014); [8](Baye, 2014), (Wang et al., 2014), (James, 2009); [9](Baye, 2014), (Wang et al., 2014), (Rosell et al., 2016); *(USDA, 2016); WW, whole wheat, BW, buckwheat. [a](g/100 g dm), [b](mg/100 g dm).

The storage form of PA is known as phytate or phytin, which is mainly found in the bran. High contents of PA 675-1544 mg/100 g dm of tef flour, similar to that of white sorghum, red sorghum, barley and wheat were reported (Abebe et al., 2007; Baye et al., 2014; Umeta et al., 2005). The wide range of PA content in the reports could be attributed to differences of the source of tef as there is high positive correlation in the phosphorus content of the soil and phytic phosphorus in grains.

Several food processing steps have been described to lower the PA content. During fermentation and baking, endogenous and exogenous microbial phytases degrade the PA and increase the bioavailability of essential minerals (Schlemmer et al., 2009). Tef *injera* baked after 96 hours of backslop fermentation showed a decrease in PA by 73-80 % in white and brown tef (Urga and Narasimha, 1997). On the other hand, it was stated a decrease of PA of *injera* from 1050 to 340 mg/100 g representing a degradation rate of 68% when inoculated with *L. buchneri* MF58 but of 42% with that of backslop fermentations (25°C, 48 hours) (Fischer et al., 2014). Greffeuille et al. (2011) reported that the adverse effect of PA on iron absorption of plant-based food product can only be eliminated if the PA content is decreased to a level of less than 100 mg/100 g dm.

Iron absorption can be improved in plant-based foods, if the molar ratio of PA/Fe is reduced to < 1 and preferably < 0.4 (Hurrell, 2004). According to World Health Organization (1996), 55% of Zn content of a food is expected to be absorbed (considered as highly available) if PA/Zn of the food is < 5; but it would be 35% (moderately available) if the ratio is 5-15 and only 15% (low availability) if > 15. Umeta et al. (2005) revealed that backslop fermented tef *injera* showed a 3-4 times lower molar ratios of PA/Zn (10.8), and PA/Fe (0.3) compared to unfermented tef *injera*. Molar ratios of PA/Zn and PA/Fe of traditionally fermented tef *injeras* in the range of 7-9 and 0.1-1.3, respectively in white and brown tef and it was speculated that those reductions of mole ratios could increase Fe and Zn bioavailability (Abebe et al., 2007). On the other hand, Baye et al. (2015) revealed destruction of more than 90% of the PA that led to a PA/Fe molar ratio of < 1 of *injera* made from mixture of tef and white sorghum but saw only little or no improvement in iron bioaccessibility. Catechol and galloyls containing groups of phenolic compound such as tannins, caffeic acid, gallic acid and catechin are also known to bind iron and reduce its bioavailability. Among the major catechol and galloyls groups containing PCs, catechin, rosmerinic acid, gallic acid and protocatechuic acid have been identified in tef (Kotaskova et al., 2016; Shumoy et al., 2017). Umeta et al. (2005) reported condensed tannin contents of tef made *injeras* in the range of 45-65 mg/100 g.

Table 1.3 Composition (g/100 g dm) of tef, common cereals and pseudocereals

Composition	Tef[1]*	Wheat[2]*	Rice[3]*	Maize[4]*	Sorghum[5]*	Oat[6]*	Quinoa[7]*	Buckwheat[8]*	Amaranth[9]*
Total carbohydrate	57-86	71-76	74-77	73-77	72-77	66-69	58-64	71-72	65-66
Crude protein	13-21	10-13	3-8	6-9	5-11	8-17	14-15	13-14	13-14
Crude fat	2-5	1-3	0.6-1	1-4	1-4	7-8	4-6	3-4	6-7
Total dietary fiber	4-12	2-13	4-5	2-13	5-10	5-11	7-17	3-14	7-16
Insoluble dietary fiber	4-7	2-10	3-4	10-12	7-8	5-7	10-14	0.7-7	8-9
Soluble dietary fiber	1-5	2-3	0.2-1	0.6-2	1-2	0.4-4	1-6	2-7	6-7

[1](Baye, 2014), (Collar and Angioloni, 2014), (Renzetti et al., 2008), (Forsido et al., 2013), (Abebe and Ronda, 2014) (USDA, 2010), (El-Alfy et al., 2012), (Wolter et al., 2013); [2](Rosell et al., 2016), (Baye, 2014), (Collar and Angioloni, 2014); [3](Baye, 2014), (Rosell et al., 2016), (Ramulu and Rao, 1997); [4](Ramulu and Rao, 1997), (Ingbian and Adegoke, 2007), (Baye, 2014), (Picolli et al., 2005); [5](Baye, 2014), (Rosell et al., 2016), (Ramulu and Rao, 1997), (Knudsen and Munck, 1985) ; [6](Arthur and B.L.D'Appolonia, 1979), (Baye, 2014), (Saturni et al., 2010); **[7]**(Baye, 2014), (Collar and Angioloni, 2014), (Repo-carrasco-valencia and Serna, 2011); [8](Rosell et al., 2016), (Baye, 2014), (Collar and Angioloni, 2014), (Skrabanja et al., 2004); [9](Rosell et al., 2016), (Collar and Angioloni, 2014), (Lucero et al., 2001).*(USDA, 2016).

1.5 Carbohydrates

As indicated in (Table 1.3) the total carbohydrate content of tef ranges from 57-86 g/100 g dm and showed close similarities with the other common cereals and pseudocereals. The relatively very low total carbohydrate content (57 g/100 g dm) reported by El-Alfy et al. (2012) in a red type of tef seems unrealistic. Abebe and Ronda (2014) have shown comparatively very high total carbohydrate content of different tef varieties in the range of 83-86 g/100 g. The total carbohydrate content of tef reported by El-Alfy et al. (2012) is quite suspicious in that the starch content which is only part of carbohydrate alone accounted as much as 74-79% (w/w) in different tef varieties (Abebe and Ronda, 2014; Marti et al., 2017). Similarly, Shumoy and Raes (2017) and Hager et al. (2012b) also revealed starch contents in the range of 66-76% (w/w) and 64% (w/w) dm in tef varieties grown in Ethiopia and in the Netherlands, respectively.

1.5.1 Starch composition and digestibility

Starch can be divided into linear polymers (amylose) and macromolecules of shorter chains with α-1-6 branch linkages (amylopectin). The proportion of amylose and amylopectin in a starch gives starch its typical functional properties during food processing. Amylose content of tef in the range of 20-31% was revealed (Bultosa et al., 2002). Starches containing an amylose-amylopectin ratio~20:80 are categorized as normal starches and those having a higher ratio are called high amylose starch (Tuano et al., 2015). High amylose starches require temperatures of up to 150°C in the presence of water to get fully gelatinized, which is not usually attainable under normal cooking and baking circumstances and thus results in foods with a lower digestibility. The linear chains of glucose in amylose are capable of forming a complex with fatty acids and become difficult for the access of hydrolytic enzymes during digestion while starches with higher proportion of amylopectin do not form glucose lipid complex which increases their vulnerability for easy access by the hydrolytic enzymes (Singh et al., 2013).

The degree of starch gelatinization is also highly dependent on the starch granule size, shear force and extent of processing temperature among others. The smaller is the starch granule size, the higher would be the surface area which inevitability increases the contact of the hydrolytic enzymes with the substrate (starch), finally resulting in a high starch digestibility. Surface area in this case is seen as relative to other cereals. For example, the total surface area of 10 g maize kernels with 10 g of tef kernels, the later will have higher surface are due to its smaller size. Starch

granule could be categorized as large (>25 μm), medium (10-25 μm), small (5-10 μm) and very small (5-10 μm) (El-Alfy et al., 2012) and based on this, tef starch is categorized as very small in diameter (Table 1.4). However, in addition to amylose/amylopectin proportion of native starch and starch granule size, other factors that determine the rate of enzymatic hydrolysis o starch includes, but not limited to, the feature of granular morphology that determines the mechanism by which the enzyme attacks the starch either by surface erosion or digestion via pore route, arrangement of crystalline and amorphous regions in the granule, size of blocklet that contains both amorphous and crystalline lamella, the structure of amylose and amylopectin which explains the distributions of branch (chain) lengths in both amylose and amylopectin and the crystalline types, 'A' or 'B'.

Table 1.4 Physicochemical properties of tef, common cereals and pseudocereals

Common cereals and Pseudocereals	Physicochemical properties		
	Granule Size (μm)	Amylose%	Gelatinization ([°C] (T_O,Tp,T_E))
Tef[1]	3-8	20-31	(68, 74, 80
Wheat[2]	10-35	3-70	(55, 61, 66)
Rice[3]	2-7	1-80	(71, 76, 87)
Maize[4]	3-20	0-84	(65, 73, 80)
Sorghum[5]	6-18	0-24	(64, 69, 73)
Oat[6]	3-10	20-25	(51, 56, 62)
Quinoa[7]	1-3	5-12	(52, 58, 64)
Buckwheat[8]	2-19	16-24	(59, 66, 72)

[1](Marti et al., 2017), (Wolter et al., 2013), (Abebe and Ronda, 2014), (Baye, 2014), (Bultosa et al., 2002), (Shumoy and Raes, 2017); [2](Feng et al., 2013), (Baye, 2014), (Wolter et al., 2013), (Bultosa et al., 2002); [3](Saturni et al., 2010), (Man et al., 2014), (Baye, 2014), (Salunkhe et al., 1983), (Regina et al., 2006), (Van Hung et al., 2006), (Van Hung et al., 2016), (Park and Shoemaker, 2007); [4](Wang et al., 2014), (Marti et al., 2017), (Cheetham and Tao, 1998); [5](Bultosa et al., 2002), (Baye, 2014), (Ang et al., 2008); [6](Wolter et al., 2013), (Baye, 2014), (Bultosa et al., 2002); [7](Wolter et al., 2013), (Baye, 2014), (Qian, 1999), (Dejmek et al., 2012); [8](Bultosa et al., 2002), (Baye, 2014), (Wolter et al., 2013), (Noda et al., 1998), (Neethirajan et al., 2012). Where: T_O: initial temperature; Tp: pasting temperature (the temperature at the beginning of gelatinization); T_E: final temperature.

In native starch of tef, the 'A' type of crystal accounts for about 37% (Bultosa et al., 2002) and this could mean that tef starch will have higher digestibility but it was proved that the α-amylase degradation of tef starch granules occurs by surface erosion, probably due to the absence of surface pores in the granules which obviously result in a slow starch digestion (Bultosa and Taylor, 2004b). Therefore, starch digestion is a function of all these properties.

Starch based on digestibility is classified as resistant starch (RS), slowly digestible starch (SDS) and rapidly digestible starch (RDS). Tef starch fractions of RS, SDS and RDS as reported in Soil and Crop Improvement BV (2007) - tef information map version accounted for 20%, 50% and 30% of the total starch, respectively. Moreover, RS, SDS and RDS, respectively in the range of 7-11%, 31-41% and 29-33% (Abebe et al., 2015) and 12-30%, 19-53% and 17-68% (Shumoy and Raes, 2017) were also reported in different tef varieties grown in Ethiopia.

An *in vitro* study on the native starches of tef flour indicated that only 4.7% of the starch was digested after incubation with α-amylase for 0.5 h (hour), while it increased up to 32.6% after 5 h (Bultosa and Taylor, 2004b). However, prediction of digestibility and/or GI merely based on flour's starch fraction content may not be adequate as the starch digestibility of a food product is largely dependent on the properties and contents of the starch fractions after cooking and /or at the point of consumption. The same flour may result in different contents of the starch fractions and their content could be highly affected by the way they were cooked (temperature, time, shear force, water content), the type and contents of other accompaniments. In addition to the contents of starch fractions, the rate of starch digestion or GI of a food product can also be determined by the contents of other macro and micro food constituents such as proteins, fat, and the interaction of SDS, RDS, phytic acid, phenolic compounds and their interaction.

Each of these food components and their interaction have different magnitude of impact on GI of a food product and it has been shown to follow a decreasing order of SDS > RS > fat > interaction between SDS and RDS > interaction between fat and RS > RDS (Meynier et al., 2015). When starch is heated in the presence of water, it gelatinizes and Lauro et al. (2000) revealed that gelatinization during food processing tremendously increased enzymatic *in vitro* starch hydrolysis.

Ostman (2003) also reported that gelatinization of starch increases the availability of the starch for enzymatic hydrolysis which eventually increases blood glucose levels. Gelatinization temperatures of onset (T_O), peak (T_P) and ends of gelatinization (T_E) as compared to common cereals and pseudocereals is shown in Table 1.4. Tef starch showed highest onset (T_O) and end of gelatinization (T_E) temperatures compared to wheat, quinoa, oat, buckwheat and sorghum. High gelatinization temperatures were positively correlated to a lower GI (Wolter et al., 2013). Indeed, it has been indicated that less gelatinized native starches are less susceptible to amylase hydrolysis (Bjorck et al., 1994). Gelatinization of starch is a change of form of the native starch into a rapidly digestible starch form that is easily accessible for enzymatic hydrolysis. Therefore,

during starchy food cooking, the starches get gelatinized and as a result, the RDS content increases while the SDS and RS contents of the native starches decrease. The formation of the RDS is highly dependent on the amount of water used for cooking among others factors as water is the key factor for starch granule to swell- melting of the crystal forms of starch and finally burst leaching the amylose contents. RDS content of different tef varieties showed an increase that ranged from 60- 85% and 3-69% when processed into traditional *injera* and porridges, respectively while a significant decrease both in the contents of SDS and RS was noticed- an indication of high GI food product (Shumoy and Raes, 2017).

The GI of foods is a term used to categorize foods according to the classification of the international table of GI which shows the glycemic response of foods whether foods eaten release glucose rapidly or in a slow and sustained fashion for a period of time. Few reports on the predicted GI of tef food products are by Wolter et al. (2013) who indicated that conventionally baked frozen tef bread had a GI of 74 which is lower than GI of breads from buckwheat (80), quinoa (95), and white wheat (100) but similar to those of oat (71) and sorghum (72). Predicted GI of 32, 45, and 67 of oat, tef and wheat based egg pastas, respectively were also reported (Hager et al., 2013). Furthermore, relatively higher GI that ranged from 79-99 and 94-137 for traditional fresh porridge and *injera*, respectively in seven tef varieties were also reported (Shumoy and Raes, 2017).

Foods are classified into three categories depending on their GI as: GI ≤ 55 low, GI (56-69) medium and GI ≥ 70 high if glucose is used as a reference material in calculating the area under curve (AUC) while, if fresh white wheat bread is used as a reference material, the standards of low GI, medium and high GI foods are defined as GI < 60, GI (60-85) and GI > 85 (Ferng et al., 2016). Based on this classification, as all of the GI specified here were calculated based on fresh white wheat bread, the second classification will be taken into consideration to compare the GI of the food products. Thus the GI of egg based tef pasta are categorized as low, the frozen tef breads (74) as medium, the fresh porridges (78-99) in the range of medium to high while those fresh *injera* (94-137) as high GI food products.

A possible explanation of the lower GI of the tef based bread and pasta food products as compared to the similarly prepared food products of other common cereals and pseudocereals could be attributed to the starch properties of tef and the presence of higher amounts of antinutritional factors such as PA and PCs in tef (Baye et al., 2014) which will be discussed more in detail further in this review. It is also known that the presence of catechin or tannic acid and/or

PA can reduce protein and starch digestibility. As explained above, tef is processed as whole meal and its high fiber content may reduce starch digestibility. The presence of fibers, the physical form of starch, cooking/gelatinization and natural amylase inhibitors (e.g. phytate, phenolic compounds) could have an effect on the starch hydrolysis rate, and thus influencing the GI of tef-based foods. The justification for the higher GI in both porridge and *injera* contrary to the lower GI of tef based bread and egg pasta could be attributed to the higher water content of the prior food products which was in the range of 71-73% and 59-66%, respectively in porridge and *injera* of the seven tef varieties (Shumoy and Raes, 2017). High water content significantly increases the rate of starch gelatinization which eventually increases the GI of the resulting food (Tester et al., 2004). Thus, GI of a food product is also dependent on the way the food was processed in addition to the nature of the starting material.

Total, soluble and insoluble dietary fiber contents of tef and other common cereals and pseudocereals are shown in Table 1.3. As the consumption mode of tef is as whole meal, it may significantly contribute to a higher dietary fiber intake which is highly associated with many health benefits. Human subjects with high intakes of dietary fiber showed considerably lower prevalence of developing coronary heart disease, diabetes, obesity, stroke, hypertension, duodenal ulcer, diverticulitis, constipation, hypercholesterolemia and certain gastrointestinal diseases (Fujii et al., 2013).

1.6 Protein

The crude protein content of tef ranges widely 13-21 g/100 g dm and is similar to the other common cereals and pseudocereal as shown in Table 1.3. However, the protein content (21 g/100 g) reported by El-Alfy et al. (2012) is exceptionally higher compared to the other reports of tef protein content. The reports of El-Alfy et al. (2012) must be nuanced as no other results match to it and it is unlikely that cereal crops could have this much protein unless they are genetically modified, however, this tef was not described as genetically modified. Indeed these authors also reported unusually very low total carbohydrate content (57%) of the same tef.

Nutritionally, tef could be a good source of dietary proteins due to its high contents of essential amino acids as presented in Table 1.5. Tef based food products could fulfill the daily protein requirement in that the essential amino acid contents of tef are at least equal to the FAO scoring patterns (Table 1.5). However, tef is known to contain both higher mineral (Table 1.2) and PA

(Baye et al., 2014) and the formation of a triple 'protein-mineral-phytate complex' is believed to inhibit enzymatic degradation, thereby decrease the digestibility and eventually the bioavailability of proteins. Literature on the effect of different food processing steps on the protein content and its digestibility is not available. The fact that tef is consumed as a whole meal and the location of both the minerals and PA in cereals is in the bran, makes it important to study the digestibility of tef protein and its nutritional significance to consumers.

Table 1.5 Amino acid profile of tef, common cereals and pseudocereals

AA (g/100 g protein)	Common cereals and pseudocereals							FAO[8]
	Tef[1*]	Wheat[2*]	Quinoa[3*]	BW[4*]	Oat[5*]	Rice[6*]	Maize[7*]	
Histidine	2-3	3-4	2-3	2-3	1-2	2-3	3-4	1.5-2.0
Threonine	4-5	3-4	3-6	3-4	3-4	3-4	3-4	2.3-3.1
Lysine	3-4	3- 4	2-6	5-6	3-4	3-4	2-3	4.5-5.7
Tyrosine	4-5	2-3	1-2	2-3	2-4	3-6	4-5	
Methionine	3-5	1-2	0.3-2	2-3	2-3	2-3	1-2	
Valine	5-7	4-5	1-4	5-5	5-6	5-6	4-5	3.9-4.3
Isoleucine	4-5	3-4	1-4	4-4	4-5	4-5	3-4	3-3.2
Leucine	8-10	7-8	1-6	6-7	7-8	8-9	12-13	5.9-6.6
Tryptophan	1-1	0.4-1	0.7-1	0.2-1	1-3	1-2	0.5-1	6-8.5
Phenylalanine	5-7	4-5	2-4	4-5	4-5	5-6	4-5	
Cysteine	2-3	1-2	0.1-1	2-3	2-3	2-3	1-2	
Serine	5-6	4-5	2-4	5-6	4-7	4-5	4-5	
Arginine	4-8	5-8	3-8	8-9	6-7	8-9	4-5	
Glycine	4-6	4-5	3-5	6-8	5-6	4-5	3-4	
Aspartic acid	6-8	5-6	4-8	9-11	8-9	9-11	5-7	
Glutamic acid	25-30	33-40	2-13	16-20	21-22	18-19	18-19	
Alanine	6-10	4-5	2-4	5-6	4-5	5-6	6-8	
Proline	5-8	12-16	2-6	4-5	5-6	3-6	9-11	

[1](Adebowale et al., 2011), (Bultosa and Taylor, 2004a); [2](Zhang et al., 2016), (Alijosius et al., 2016); [3](Escuredo et al., 2014), (Gewehr et al., 2017); [4](Yang et al., 2012), (Wei et al., 2003); [5](Labanowska et al., 2014), (Pomeranz et al., 1973); [6](Kalman, 2014), (Wei et al., 2003), (Mosse et al., 1988); [7](Peksa et al., 2016), (Al-Gaby, 1998) ;; *(USDA, 2016)- data recalculated to g/100 g protein. AA: amino acid, BW: Buckwheat, FAO: Food and Agriculture Organization. [8](FAO, 2007)- Amino acid scoring patterns, amino acid (g/100g protein) requirements/protein requirements for age group of 0.5 - >18 years (all age groups).

Seed storage proteins comprise a major part of the protein content of the seed and have an important role on the quality of the seed and they are classified based on their solubility traditionally known as Osborne solubility. Storage proteins are important because they determine the total protein content and have an effect on the nutritional quality of a food product and functional properties for food processing. Reports on Osborne solubility based

fraction of tef see storage protein are very few and they contradict to one another. The Osborne protein fractions decreased in the order of glutelins 44.6% > albumins 36.6% > prolamin 11.8% > globulins 6.7% (Ketema, 1997). Similarly, a deceasing order of glutelins 40% > albumin 36% > globulins 18% > prolamins 10% was reported (Bultosa and Taylor, 2004a). On the contrary, a decreasing order of prolamin 40% > glutelins 22% > (albumins + globulins) 11% was revealed (Adebowale et al., 2011). The differences in the content of the Osborne fraction could be attributed in part to the use of different extraction solvents. Tert-butanol 60% (v/v) with DTT as reducing agent was used to extract prolamin in the work of Adebowale et al. (2011) while only 60% ethanol was used by Ketema (1997). Based on these few and conflicting available results, it remained difficult to know the major storage protein of tef, solubility characterization and their nutritional implications, thus further studies are paramount to determine tef protein solubility characteristics. Also it has to be confirmed if the Osborne method is valid in gluten-free cereals protein fractionation.

1.7 Conclusions

Tef is claimed as gluten-free cereal and contains high nutritional components. Tef contains similar amounts of carbohydrate compared to common cereals and pseudocereals and its food products could result different GI (from low to high) depending on the way of processing. It contains fairly higher protein and mineral contents compared to other common cereals and pseudocereals. The consumption mode of tef is as whole meal and this could enable it as a significant source of high amount of dietary fiber, PCs and mineral. Tef contains the higher Ca and Fe compared to other common cereals and pseudocereals, however, it is not yet known whether these minerals in tef are intrinsic or due to soil contamination. Tef based foods, produced after traditional backslop fermentation showed major reduction of PA but no significant change in the *in vitro* iron and zinc bioaccessibility was observed.

CHAPTER 2: PHENOLIC CONTENT AND ANTIOXIDANT CAPACITY OF TEF

Part 2.1: Antioxidant Potentials and Phenolic Composition of Tef Varieties: An Indigenous Ethiopian Cereal

2.1.1 Abstract

In this study, it was aimed to profile and quantify the phenolic composition and antioxidant capacity of seven tef varieties. Soluble and bound phenolics ranged from 37 to 71 and 226 to 376 mg GAE/100 g dry basis (dm), while soluble and bound flavonoid contents varied between 36-64 and 113-258 mg CE/100 g dm, respectively. Protocatechuic, vanillic, syringic, *p*-coumaric, sinapic, ferulic and rosmarinic acids, catechin and naringenin were detected at least in three of the varieties studied. The dominant PCs were catechin followed by rosmarinic and ferulic acids in the soluble extracts, whereas ferulic followed by rosmarinic and *p*-coumaric acids were the dominant ones in the bound extract. Gallic, caffeic and salicylic acids were not detected in any of the varieties studied. The majority (>84%) of tef phenolics is found in bound form contributing to >84% of total DPPH and >80% of the total FRAP antioxidative capacity. These results clearly demonstrated the differences in phenolic profile among tef varieties. These results are relevant for developing healthy and nutritious tef-based food products.

2.1.2 Introduction

Tef is a cereal usually milled into whole flour to prepare whole meal that makes it as an important source of minerals and other bioactive components. It is already proven that most of the functional antioxidants such as phenolic acids, phytosterols, alkylresorcinols, lignans and folates of common cereals and pseudocereals are concentrated in the bran part of the seeds (Liukkonen et al., 2003; Mattila et al., 2005; Pihlava et al., 2015; Rosa-Sibakov et al., 2015). Thus, whole cereals naturally contain high amount of PCs which contribute to positive health benefits (Adom and Liu, 2002; Chandrasekara et al., 2012; Leoncini et al., 2012).

Despite the whole grain consumption mode of the tef grain and the likely positive health outcomes, the information on its phenolic content and the associated antioxidant capacity is far from complete. Boka et al. (2013), Forsido et al. (2013) and Salawu et al. (2014) studied the antioxidant capacity of tef, by taking samples of unknown tef varieties or tef flour. Several tef varieties are on the market, varying in color from white to brown (Bekabil et al., 2011), indicating possible differences in the phenolic profile among the varieties. Moreover, all these studies were reporting on the soluble phenolic fraction only, obtained by methanolic extraction of tef flour. Salawu et al. (2014) reported limited amounts of phenolic acids- *p*-coumaric, ferulic, *p*-hydroxybenzoic acid and of the flavonoid- apigenin that ranged from 0.87-59.75 mg/100 g of flour from soluble extracts of tef flour. However, it is well reviewed that most whole cereals such as rice, corn, wheat, sorghum, oats, millets and barley contain PCs largely in the bound form, contributing to more than 80% to their TPC (Acosta-Estrada et al., 2014). Therefore, the objective of this study was to investigate the profile and the content of individual PCs of the soluble and bound phenolic fractions and to evaluate their antioxidant capacity by using seven tef grain varieties, varying in color from white to brown.

2.1.3 Materials and methods

Chemicals and reagents: DPPH, TPTZ (2, 4, 6-tripyridyl-s-triazine), Trolox (6-hydroxyl-2,5,7,8-tetramethylchroman-2-carboxylic acid), catechin, naringenin, gallic acid, ferulic acid, *p*-coumaric acid, *o*-coumaric acid, vanillic acid, caffeic acid, syringic acid, sinapic acid, protocatechuic acid, salicylic acid and trifluoroacetic acid were purchased from Sigma–Aldrich Fine Chemicals (St. Louis, MO). HPLC grade methanol and water were purchased from VWR Chemicals (VWR international S.A.S., France). Analytical grade phenol reagent, aluminum chloride, sodium nitrite,

methanol, hydrochloric acid, sodium hydroxide, and sodium carbonate were purchased from Chem-Lab (Chem lab NV, Belgium).

Grain sample and preparation: Seven tef varieties *i.e.* Boset (DZ-Cr-409), Dega (DZ-01-2675), Quncho (DZ-Cr-387), Simada (DZ-Cr-285), Tsedey (DZ-Cr-37), Zagurey (local) and Zezew (local) were used in this study. All the tef varieties were originating from one location, grown in the same season and were the ones that were available at that region. The names outside the brackets are local names whereas the ones between brackets are the breed name which is specific to each variety. The first five (5) varieties are white whereas the last two (2) are brown. All the tef varieties, grown under similar agricultural conditions, were harvested in the main harvesting season (locally called *Meher*) in December 2013 and generously obtained (about 3 kg each) from Axum Agricultural Research Center (Tigray, Ethiopia). They were carefully cleaned manually and then milled by a local miller into flour using a disc attrition mill. They were sun dried while standing on the field (before harvest) and milled by disc attrition milling at a local tef miller, in the same way as tef is milled in Ethiopia. Some portions (about 1 kg) of each variety was pre-milled prior to each variety and discarded to prevent cross-contamination among the varieties. The flour passed through a sieve of mesh number 16 (sieve opening 1.19mm, Tylor test sieve, Mentor, OH, USA). Flour samples were packed in polythene pouches and stored at -20ºC until further analysis.

2.1.3.1 Extraction of soluble and bound phenolics

Extraction of soluble phenolics (Fig. 2.1.1) was based on the method described by Gonzales et al. (2014). Briefly, approximately 2 g of flour was placed in a 50 mL falcon tube and homogenized with 15 mL of 100% methanol at 3000 rpm using an Ultra-Turrax (IKA-T18D, Germany) for 45 s. The tubes were then placed on ice for 15 s. The mixture was centrifuged (Z 300 K, Hermle Labortechnik, GmbH, Germany) at 13000 g for 10 min at 4°C. The residue was re-extracted using 10 mL of 80% methanol following the same procedure. The supernatant was further filtered using filter paper with pore size of 5-13 μm (VWR; Leuven, Belgium) and the volume was corrected to 25 mL using 80% methanol. The phenolic content from these extracts will be further referred to as soluble phenolics. After removal of the supernatant, the residues were air dried overnight and stored at −20°C until further extraction for bound phenolics.

Alkaline hydrolysis of bound phenolic content of the residues (Fig. 2.1.1) was done following the optimized method of Gonzales et al. (2014). Briefly, 0.1 g of dried residue obtained after the methanolic extraction was hydrolyzed using 2 mL of 2M NaOH and sonicated (UP 400S, Hielscher, GmbH, Germany) at maximum amplitude (100%) for 30 min at 60°C in a screw-capped test tube previously flushed (dried) with nitrogen. The samples were then neutralized using 2M HCl. Then, 4 mL of methanol (100%) containing 0.1% formic acid was added as an extraction solvent followed by vortex mixing for 2 min. Then the tubes were centrifuged (Z300K, Hermle Labortechnik, GmbH, Germany) for 10 min at 10000 g and 4°C. Extraction was done twice and the supernatants were pooled and standardized to 20 mL using 80% methanol. The phenolic content from these extracts will be further indicated as bound phenolics.
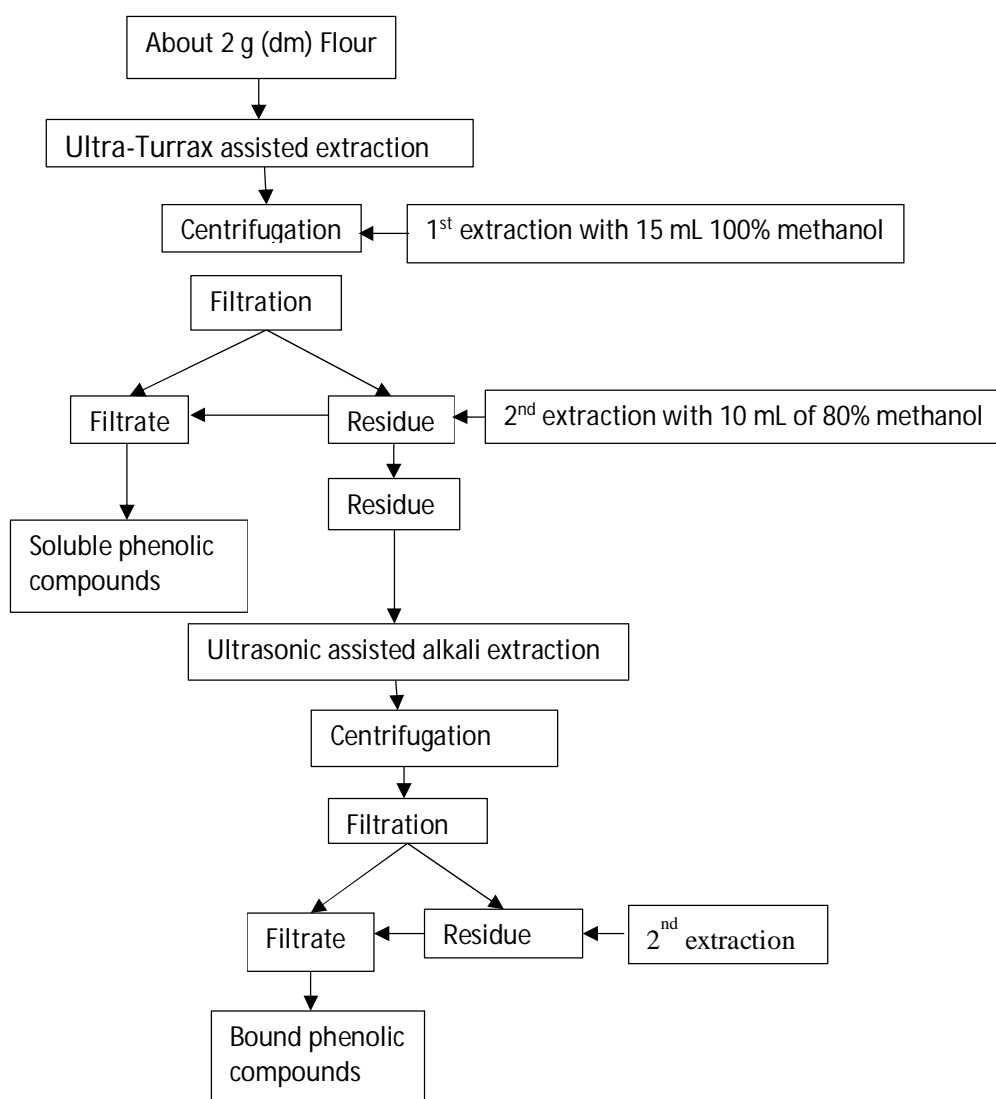
Figure 2.1. 1 Extraction process of soluble and bound phenolic compounds

2.1.3.2 Determination of total phenolic and flavonoid contents

The TPC of each extract was determined using the method described by Singleton et al. (1999). Briefly, 1 mL of each of bound and soluble extracts was mixed with 0.5 mL of 10 times diluted Folin-Ciocalteu reagent in a test tube, vortex mixed and were allowed to stand for 6 min. The reaction was neutralized by adding 1.5 mL of saturated sodium carbonate (20%), followed by the addition of 1 mL double distilled water and then thoroughly mixed. The contents were allowed to stand for 2h in dark at room temperature. The absorbance of the resulting blue color supernatant was measured at 760 nm using a spectrophotometer (Model 4001/4, Thermo Spectronic, USA) using methanol as a blank. Total phenolic content in each extract was determined using a standard curve prepared from gallic acid and the results were expressed as mg GAE/100 g flour dm).

Total flavonoid content (TFC) was determined according to the method described by Dewanto et al. (2002). Briefly, 75 µL of 5% $NaNO_2$ was mixed with 1 mL of the extracts and 1 mL of water, and thoroughly vortex mixed. After 6 min, 150 µL of a 10% $AlCl_3$ solution was added, and the mixture was allowed to stand for another 5 min. Then, 0.5 mL of 1M NaOH was added and the contents were allowed to stand for 15 min in dark at room temperature. The absorbance was measured at 510 nm using a spectrophotometer (Model 4001/4, Thermo Spectronic, USA) and methanol was used as a blank. The flavonoid content was determined using a standard curve prepared from catechin and the results were expressed as mg catechin equivalent (CE)/100 g flour dm).

2.1.3.3 Determination of antioxidant capacity

DPPH free-radical scavenging capacity: The method described by Kumaran and Karunakaran (2006) was used to measure the antiradical activity against the DPPH radical. The DPPH (0.1mM, 2 mL) solution in methanol was added to 100 µL extracts, vortex mixed for 10 s and left in the dark for 30 min at room temperature. The absorbance of the solution was measured at 517nm using a spectrophotometer (Model 4001/4, Thermo Spectronic, USA). The scavenging capacity of DPPH radical was calculated with respect to the Trolox standard curve and the results were expressed as µmol TE/100 g flour dm.

Ferric reducing antioxidant power (FRAP): The ferric reducing antioxidant power (FRAP) was estimated according to the procedure described by Benzie and Strain (1996). Stock solutions of

acetate buffer (300 mM, pH 3), 10 mM TPTZ solution in 40 mM HCl and 20 mM $FeCl_3.6H_2O$ solutions were prepared. The fresh working solution (FRAP) was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ solution, and 2.5 mL $FeCl_3.6H_2O$ solution. Bound and soluble flour extracts (100 µL) were allowed to react with 3 mL of the FRAP solution for 30 min in the dark at room temperature. The absorbance of the colored complex was measured at 593 nm using a spectrophotometer (Model 4001/4, Thermo Spectronic, USA). Methanolic solutions of known Fe^{2+} concentrations, ranging from 200 to 1000 µmol/L $FeSO_4.7H_2O$, were used for the preparation of a calibration curve. The FRAP antioxidant capacity was expressed in µmol Fe^{2+}/g flour dm.

2.1.3.4 Phenolic profiling

A method as outlined by Wen et al. (2005) was implemented for the determination of PCs. The separation of PCs was performed with an Agilent 1100 series HPLC system equipped with on-line degasser (Model 590, Alltech elite degassing system, USA), quatpump (G 1311A), Alltima^TM - Colomn18 5u (4.6 mm × 150 mm; GRACE, Deerfield, USA), photodiode array detector (DAD) (G 1315B, Agilent 1100 series). Instrument control and data analysis was carried out using Agilent HPLC Chemstation 10.1 edition through Windows 2000. The flow rate of the mobile phase was kept at 0.5 mL/min. Mobile phase A was HPLC grade water containing 0.02% trifluoroacetic acid (TFA), and phase B was HPLC grade methanol containing 0.02% TFA. The gradient conditions were: 0-5 min, 25% B; 5-10 min, 25-30% B; 10-16 min, 30-45% B; 16-18 min, 45% B; 18-25 min, 45-80% B; 25-30 min, 80% B; 30-40 min, 80-25% B. The temperature of the column was controlled at 25 °C. Injection volume was 10 μL. The detection wavelengths of DAD were set at four selected positions: 254, 275, 305, and 320 nm. Identification of the PCs was done by comparing retention times and spectra from the DAD detector with those of pure standards. *o*-Coumaric acid (5mg/L) was used as internal standard, and quantification was performed by external calibration curves for each identified phenolic compound.

2.1.3.5 Statistical analysis

All extracts were made in triplicate. Results are reported as mean ± standard deviation on a dry matter basis (dm). The differences of mean values among tef varieties were determined using one-way analysis of variance (ANOVA) followed by Tukey's Honest Significant Differences (HSD)

multiple rank test at $P < 0.05$ significance level. All statistical analyses were performed using SPSS version 22 (SPSS Inc., Chicago, IL, USA).

2.1.4 Results and discussion

The present study has dealt with the identification and quantification of PCs, as well as the determination of the antioxidative capacity of the extract of seven different tef flour varieties. The dry matter content of the flour from the different varieties ranged from 91.6-92.1g/100 g, with an average of 91.9 g/100 g and was not significantly different ($p > 0.05$).

2.1.4.1 Soluble and bound phenolic contents and their profiles

Soluble, bound and total phenolic content of the tef varieties are given in Table 2.1.1. The highest and lowest soluble PCs were 71 and 37 mg GAE/100 g dm in Zezew (brown) and Tsedey (white) varieties, respectively. There was a significant difference in soluble PCs among the varieties ($P<0.001$). Bound PCs of the varieties ranged from 226-376 mg GAE/100 g dm and showed significant difference among the varieties ($P<0.001$). TPC (bound + soluble) ranged from 263-441 mg GAE/100 g dm and decreased significantly in the order of Zezew > Zagurey > Dega > Boset > Quncho > Sidam > Tsedey ($P<0.001$).

Table 2.1.1 Soluble, bound and total phenolic and flavonoid contents of tef varieties

Variety	Phenolic content (mg GAE/100 g dm)			Flavonoid content (mg CE/100 g dm)		
	Soluble	Bound	Total	Soluble	Bound	Total
Boset	57.5 ± 4.0^{bc}	269 ± 5^{b}	327 ± 20^{bc}	41.8 ± 0.9^{b}	113 ± 11^{a}	155 ± 12^{a}
Dega	55.6 ± 3.9^{b}	296 ± 8^{c}	352 ± 12^{c}	53.6 ± 0.5^{c}	130 ± 13^{a}	184 ± 2^{b}
Quncho	43.8 ± 0.2^{a}	254 ± 7^{b}	298 ± 0^{ab}	41.0 ± 2.2^{b}	196 ± 6^{c}	237 ± 4^{c}
Simada	45.8 ± 3.2^{a}	230 ± 8^{a}	276 ± 5^{a}	42.1 ± 0.4^{b}	195 ± 11^{c}	237 ± 10^{c}
Tsedey	37.1 ± 3.0^{a}	226 ± 7^{a}	263 ± 10^{a}	35.5 ± 0.4^{a}	159 ± 9^{b}	195 ± 11^{b}
Zagurey	65.2 ± 2.6^{cd}	344 ± 9^{d}	409 ± 7^{d}	53.5 ± 3.4^{c}	197 ± 2^{c}	251 ± 1^{c}
Zezew	71.4 ± 2.4^{d}	376 ± 3^{e}	448 ± 13^{d}	63.6 ± 2.6^{d}	258 ± 7^{d}	322 ± 6^{d}
p-value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

[a,b,c,d] Values within column with different letters are significantly different ($p < 0.05$). (n=3).

The TPC are higher than reported TPC from other cereals such as corn, wheat, oat and rice (264.6, 136.0, 111.1 and 94.7 mg GAE/100 g dm respectively) (Adom and Liu 2002) and from different wheat varieties (133-174) mg GAE/100 g dm (Leoncini et al. 2012). Soluble phenolic contents of unknown tef varieties that ranged from 829-1147 (Boka et al., 2013) and 126 mg GAE/100 g dm by Forsido et al. (2013) are much higher compared to what is reported here. The discrepancies in the results may be explained by the difference of tef varieties, as well as by the extraction methods. The 'ultra-sonication' assisted soluble phenolic compound extraction method might have facilitated the release of bound phenolics that were esterified to cell wall structure such as cellulose, hemicellulose, pectin, lignin and structural proteins might have resulted in false higher total soluble phenolic compounds (Forsido et al., 2013). On the other hand, 'long extraction times up to 24 h' (Boka et al., 2013), that could release other food components like proteins and sugars which in turn can interfere with the Folin-Ciocalteu method leading to false higher result of soluble PCs (Box, 1983). The authors believe that further study of PCs involving more known tef varieties can help to have more reliable data regarding the soluble and bound phenolic contents of tef. The bound PCs were much higher than those reported for corn, wheat, oat, rice (228.5, 103.7, 81.0 and 59.0 mg GAE/100 g dm respectively) (Adom and Liu, 2002). The difference could be attributed in part to the difference in genetic make-up and the efficiency of extraction methods (Adom and Liu, 2002; Gonzales et al., 2014). Tef has high fiber content (Colla and Angioloni 2014) and the fact that bound PC are highly concentrated in the bran of grains linked to the cell wall of plant tissue (Pihlava et al., 2015; Rosa-Sibakov et al., 2015) could explain the higher amount of bound PCs of tef compared to other common cereals. The mean bound PCs of the varieties were 5.27 fold higher than the soluble PCs and the contributions of bound PCs to TPC ranged from 82-86%. This result is similar with the contribution of bound PCs in TPC of corn (85%), oats and wheat (75%) and rice (62%) (Adom and Liu 2002) and different Bolivian purple corn varieties (61-87%) (Montilla et al., 2011).

Soluble, bound and total flavonoid contents (FCs) of the tef varieties are shown in Table 2.1.1 The soluble FCs ranged from 36-64 mg CE/100 g dm and showed significant differences among the varieties ($P<0.001$). The bound FCs ranged from 113 (Boset) to 258 (Zezew) mg CE/100 g dm and were also significantly different among varieties ($P<0.001$). Similar to the bound PCs, bound FCs of the varieties were 2-5 fold higher than the soluble FCs and contributed for 68-83% to the total flavonoid content (TFC). TFC (bound + soluble) ranged from 154-321 mg CE/100 g dm and decreased significantly in the order of Zezew > Zagurey > Simada > Quncho > Tsedey > Dega >

Boset ($P<0.001$). Higher contributions of bound FCs to TFC in cereals such as wheat (93%), corn (91%), rice (65%) and oats (61%) has been reported (Adom and Liu 2002). Information on the TFC of tef is scarce in literature, and difficult to compare as standards used to express the TFC are not uniform. Boka et al. (2013) reported values for soluble FCs of unknown tef varieties between 103-213 mg CE/100 g dm. Unfortunately, the flavonoid content of the soluble extracts of this study didn't agree with that of Boka et al. (2013). The reason for this discrepancy could be ascribed by the use of different samples and extraction method as described in the above section. The longer extraction time (24 hours) could activate some enzymes to break the covalent bonds releasing some of the bound flavonoids and/or the flavonoids can also undergo undesirable reactions such as enzymatic oxidation that can interfere with the spectrophotometric method leading to false higher contents.

The content and distribution of individual PCs in the soluble and bound extracts of the seven tef varieties is given in Tables 2.1.2 and 2.1.3, respectively. All the tef varieties contained ferulic, rosmarinic and sinapic acids, catechin and naringenin but lacked gallic, caffeic and salicylic acids. Catechin followed by rosmarinic and ferulic acids were dominant in the soluble extracts, while ferulic acid followed by rosmarinic and *p*-coumaric acids were present in the highest amount in the bound extract. There was a significant difference in the individual PCs among varieties in both soluble and bound extracts. The bound phenolic extracts showed higher concentrations of all the detected individual PCs compared to the soluble ones, in agreement with the results observed for the TPC and TFC (Table 2.1.1). Literature showed that ferulic acid followed by *p*-coumaric acid were the most dominant phenolic acids in Bolivian purple corns and rice varieties (Montilla et al., 2011; Sompong et al., 2011).

However, *p*-coumaric acid was the third highest following salicylic and sinapic acids in black rice, finger and pearl millet varieties (Hithamani and Srinivasan, 2014; Sompong et al., 2011) revealing that the concentration of individual PCs can vary within varieties of the same and/or different cereals. Comparison with literature on the phenolic profile of tef is not possible, as to the best of our knowledge, it is not reported yet. Also comparison could be difficult as it is known that phenolic profiles and contents of common cereals and pseudocereals in general can differ depending on the location where they were grown, season and agricultural practices (Gasztonyi et al., 2011; Lu et al., 2015; Yafang et al., 2014). However, the varieties used in this study were all grown under similar conditions, making comparison between varieties relevant.

Table 2.1.2 Soluble phenolic profile of tef varieties (mg/kg dm)

Variety	Phenolic profile								
	Pro	Van	Sir	pC	Sin	Fr	Ros	Cat	Nar
Boset	Nd	2.30±0.01	1.10±0.01	7.00±0.02	4.80±0.02[b]	17.1±0.3[c]	28.7±0.7[bc]	63.4±0.5[c]	1.71±0.10[a]
Dega	2.00±.01	3.80±0.03	Nd	5.70±0.01	1.60±0.01[a]	8.72±0.09[a]	7.86±0.04[a]	30.1±0.2[a]	0.82±0.01[a]
Quncho	Nd	Nd	2.30±0.01	7.40±0.04	5.50±0.04[b]	24.0±0.3[d]	27.0±0.1[b]	51.8±0.1[bc]	1.50±0.10[a]
Simada	Nd	Nd	1.40±0.1	7.40±0.02	5.70±0.01[b]	18.8±0.2[cd]	37.9±0.1[c]	55.0±0.1[bc]	2.00±0.01[a]
Tsedey	Nd	Nd	2.40±0.01	Nd	1.50±0.10[a]	16.0±0.0[bc]	23.6±0.5[b]	55.8±0.3[bc]	0.84±0.02[a]
Zagurey	2.70±0.02	2.90±0.04	Nd	1.30±0.10	1.50±0.02[a]	11.1±0.1[ab]	27.7±0.4[bc]	56.2±0.2[bc]	3.81±0.08[b]
Zezew	2.30±0.01	2.50±0.01	Nd	1.80±0.10	3.00±0.20[d]	18.1±0.2[c]	6.10±0.10[a]	50.9±0.8[b]	6.42±0.05[c]
p-value					< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

[a,b,c] Values within a column with a different superscript are significantly different ($p < 0.05$). Pro, Protocatechuic acid; Van, Vanillic acid; Sir, Syringic acid; pC, *p*-Coumaric acid; Sin, Sinapic acid; Fr, Ferulic acid; Ros, Rosmarinic acid; Cat, Catechin; Nar, Naringenin. Nd, not detected. (n=3).

Table 2.1.3 Bound phenolic profile of tef varieties (mg/kg dm)

Variety	Phenolic profile								
	Pro	Van	Sir	pC	Sin	Fr	Ros	Cat	Nar
Boset	Nd	Nd	19.3±0.1[a]	68.0±0.4[b]	43.7±0.2[ab]	255±1[a]	241±1[a]	60.8±1.0[b]	34.2±0.2[a]
Dega	31.1±0.1	31.1±0.1	23.6±0.1[ab]	44.7±0.1[a]	34.1±0.1[a]	355±2[b]	251±1[a]	39.6±0.4[a]	23.6±0.1[a]
Quncho	Nd	Nd	35.9±1.0[b]	72.5±0.1[b]	49.3±0.8[b]	466±1[d]	357±7[c]	74.3±0.8[c]	166±2[b]
Simada	Nd	Nd	26.9±0.1[ab]	75.8±0.2[b]	42.4±0.2[ab]	411±1[c]	238±1[a]	73.2±0.3[c]	156±1[b]
Tsedey	Nd	Nd	20.6±0.2[ab]	52.1±0.2[a]	36.9±0.2[ab]	384±0[bc]	260±1[a]	58.5±0.1[b]	24.2±0.1[a]
Zagurey	55.9±0.1	42.6±0.1	26.4±0.1[ab]	65.2±0.6[b]	38.9±0.1[ab]	538±1[e]	312±2[b]	74.2±0.5[c]	32.0±0.1[a]
Zezew	47.6±0.5	Nd	21.6±0.3[ab]	45.1±0.3[a]	38.3±0.2[ab]	356±2[b]	260±1[a]	43.3±0.1[a]	28.2±0.1[a]
p-value			0.048	< 0.001	0.031	< 0.001	0.001	0.001	<0.001

[a,b,c,d,e] Values within column with different superscript letters are significantly different ($p < 0.05$). Pro, Protocatechuic acid; Van, Vanillic acid; Sir, Syringic acid; pC, *p*-Coumaric acid; Sin, Sinapic acid; Fr, Ferulic acid; Ros, Rosmarinic acid; Cat, Catechin; Nar, Naringenin. Nd, not detected. (n=3).

2.1.4.2 Free radical scavenging and reducing power tef phenolic extracts

DPPH radical scavenging capacity values of soluble and bound extracts of the seven studied tef varieties are given in Table 2.1.4. The highest and lowest DPPH value of soluble extracts was 6.5 and 3.0 µmol TE/g dm for Zezew and Boset varieties, respectively. There was a significant difference among DPPH values of the soluble extracts among the varieties (P<0.001). An IC_{50} DPPH value of 0.6, 0.8, 0.9 mg/mL was reported for unknown varieties of red, mixed (brown), and white tef varieties, respectively (Boka et al., 2013) while Forsido et al. (2013) revealed a 29 folds higher IC_{50} DPPH value (22.4 mg /mL) where tef variety and color was not described. The soluble extracts of tef varieties showed higher capacity to react and quench DPPH radicals compared to soluble extracts of wheat varieties (1-2 µmol TE/g) (Leoncini et al., 2012), but are within the range of those reported for several rice varieties (1.4-9.0 µmol TE/g dm (Zhang et al., 2015).

Bound phenolic extracts of varieties Dega and Tsedey showed the highest and lowest DPPH radical scavenging capacity (136 and 21 µmol TE/g dm respectively). There was a significant difference of DPPH values among the bound phenolic extracts of tef varieties (P < 0.05), due to the difference in the composition of individual PCs and their extent of reacting to the DPPH free radical assay among the varieties. DPPH radical scavenging capacity values of the bound phenolic extracts are very high as compared to varieties of whole wheat (6-8 µmole TE/g) (Leoncini et al., 2012) and rice (1.7-2.3 µmole TE/g) (Zhang et al., 2015).

Mean value of DPPH free radical scavenging capacity of bound phenolics of the varieties was 17.5 folds higher than that of soluble phenolics and contributed 94.6% to the total values of DPPH free radical quenching potential. In agreement to this result, Liyana-Pathirana and Shahidi (2006) revealed that the values of DPPH free radical scavenging capacity of bound phenolic extracts of white flour, whole flour and bran fractions of hard and soft wheat varieties contributed 63-87% to the total DPPH free radical scavenging capacity.

Similarly, Adom and Liu (2002) also disclosed that bound phenolics contributed 90% in wheat, 87% in corn, 71% in rice, and 58% in oats to the total antioxidant capacity assay. The DPPH free radical scavenging capacity of total (soluble + bound) extracts of tef varieties ranged from 25-142 µmol TE/g dm and decreased significantly in the order of: Dega > Sidam > Boset > Zezew > Zagurey > Quncho > Tsedey (P<0.001).

The FRAP of soluble and bound extracts of tef varieties are given Table 2.1.4. FRAP of soluble phenolics ranged from 6-16 µmol Fe^{2+}/g dm while those of the bound phenolics ranged from 36-63 µmol Fe^{2+}/g dm and significantly decreased in the order of: Zezew > Zagurey > Dega > Boset > Quncho > Simada > Tsedey. The FRAP of the bound phenolics was 5 times higher compared to the one obtained for the soluble phenolics. Soluble FRAP of 0.02 µmole TE/g was reported (Forsido et al., 2013) for unknown tef variety but it is difficult to compare with our results as the standards used are different.

Literature on FRAP of tef is lacking, but the result of the present study were higher compared to soluble and bound FRAP of whole wheat varieties (1.6-3.4) and (9.5-11) µmole Fe^{2+}/g), respectively but similar in that bound phenolic extracts of the whole wheat varieties contributed >80% to the total FRAP (Leoncini et al., 2012). Based on DPPH and FRAP results, and due to the fact that the consumption mode of tef is as whole meal, it could be suggested that tef is a better source of antioxidants compared to the widely used conventionally milled hard and soft white wheat flours and rice. The brown tef varieties contained higher TPC, TFC and FRAP compared to the white varieties though this trend was not reflected in the case of DPPH and the individual phenolic content. Zezew variety which is deep brown in color was found to contain the highest TPC, TFC, and FRAP contents followed by Zagurey which is light brown. This result was in agreement with work of Zhang et al. (2015), who revealed deep black rice contained the highest TPC and TFC than their counterpart light purple and white varieties.

Table 2.1.4 DPPH (µmol TE/g dm) and FRAP (µmol Fe^{2+}/g dm) capacities of tef varieties

Variety	DPPH			FRAP		
	Soluble	Bound	Total	Soluble	Bound	Total
Boset	2.88±0.04[a*]	99.9±0.3[de]	103±1[de]	7.03±0.07[ab]	47.8±1.2[b]	54.8±1.3[b]
Dega	5.73±0.06[d]	136±3[f]	142±3[f]	11.5±0.5[c]	50.2±2.6[b]	61.7±2.8[c]
Quncho	3.65±0.08[bc]	58.7±2.3[b]	62.4±2.4[b]	7.08±0.28[ab]	39.5±2.5[a]	46.6±2.7[a]
Simada	3.93±0.05[c]	106±5[e]	110±5[e]	7.37±0.06[b]	38.7±1.1[a]	46.1±0.9[a]
Tsedey	3.47±0.03[b]	21.1±0.4[a]	25.2±1.5[a]	6.22±0.15[a]	36.0±0.4[a]	42.2±0.5[a]
Zagurey	5.35±0.15[d]	87.8±5.1[c]	93.2±5.2[c]	12.3±0.5[c]	56.7±1.5[c]	69.0±1.9[d]
Zezew	6.49±0.17[e]	93.7±1.4[cd]	100±1[cd]	15.7±0.5[d]	63.4±1.7[d]	79.1±1.2[e]
p-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

[a,b,c,d,e,f] Values within column with different letters are significantly different ($p < 0.05$). (n=3).

The higher phenolic content and antioxidant capacity of brown colored varieties can be attributed to their seed color which in turn is affected by the higher content of anthocyanins. Anthocyanins are water soluble pigments that contribute to the purple, brown, black and red colors and they are the major component of flavonoid in cereals (Dykes and Rooney, 2006). The reason why the higher phenolic content is not reflected in the individual phenolic acids and flavonoids from HPLC is not clear. The sum of the phenolic acids and flavonoids content from HPLC is very low compared to the results from spectrophotometer. This could explain that there are other abundant individual phenolics but not determined in this study due to time and standard constraints. Therefore, it is difficult to make comparison between these two results without having the full profile of all the individual phenolics.

2.1.5 Correlations between measured parameters

The correlation of soluble and bound phenolic contents with the antioxidant assays is given in table 2.1.5. There was a strong correlation of the soluble PC and FC with their corresponding values of DPPH radical scavenging capacity ($r = 0.764$, $P<0.001$) and ($r = 0.969$, $P<0.001$), respectively. The soluble and bound fractions of PC and FC showed a strong correlation to their corresponding FRAP assays. This difference in the correlations between soluble and bound fraction with the antioxidative capacity measurements could be explained by the difference in composition and quantity of the major antioxidant components present in the soluble and bound phenolic extracts.

Table 2.1.5 Pearson's correlations among phenolic and antioxidant assays

		r			r			r
sPC[1]	sDPPH[2]	0.764**	bPC[1]	bDPPH	0.434	sFC[1]	sDPPH	0.969**
sPC	sFRAP[2]	0.944**	bPC	bFRAP	0.968**	sFC	sFRAP	0.950**
bFC[1]	bDPPH[2]	0.025	TPC[1]	TDPPH[2]	0.491*	TFC[1]	TDPPH	0,027
bFC	bFRAP[2]	0.464*	TPC	TFRAP[2]	0.981**	TFC	TFRAP	0.600*
sDPPH	sFRAP	0.956**	bDPPH	bFRAP	0.518*	TDPPH	TFRAP	0.527*

Significance **p < 0.01; *p < 0.05. [1]sPC, soluble phenolic content; bPC, bound phenolic content; sFC, soluble flavonoid content; bFC, bound flavonoid content; TPC, total phenolic content; TFC, total flavonoid content. [2]sDPPH, soluble DPPH; bDPPH, bound DPPH; sFRAP, soluble FRAP; FRAPb, bound FRAP.

The difference in the composition of individual PCs in the soluble and bound extracts may have distinct reactivity or quenching capacity in the DPPH free radical assay or in the FRAP assay. Also

other antioxidant compounds in the extracts could be present, which are different between bound and soluble fraction, and thus having a different impact on the DPPH or FRAP method. The FRAP of the different phenolic fractions were correlated with their corresponding DPPH radical scavenging capacity values. The positive and significant correlation between DPPH and FRAP assays is expected as both have similar mechanism of single electron transfer or redox reactions mechanism.

2.1.6 Conclusions

This study reported the total content of PCs, the antioxidant capacity and the phenolic profile of seven different tef varieties. Majority (>84%) of their phenolic content was found in bound form, contributing to >84 and 80%, respectively of the total DPPH and FRAP. Catechin followed by rosmarinic and ferulic acids in the soluble extracts whereas ferulic followed by rosmarinic and p-coumaric acids in the bound extract were the dominant PCs. The detailed phenolic profiles and the antioxidant capacity showed some differences among varieties, which could help further research towards the development of healthy based tef food products. The brown tef varieties showed higher TPC and antioxidant capacity compared to the white ones.

Part 2.2: Soluble and Bound Phenolic Contents and Antioxidant Capacity of Tef *Injera* as Affected by Traditional Fermentation

Part 2.2: Soluble and Bound Phenolic Contents and Antioxidant Capacity of Tef *Injera* as Affected by Traditional Fermentation

2.2.1 Abstract

Injera, a fermented pancake, is a major food in Ethiopia but there is limited information on its phenolic and antioxidant capacity. The aim of this study was to investigate the effect of fermentation on soluble and bound phenolic profiles and antioxidant capacity of 24, 72 and 120 h (hours) fermented *injera* from 4 tef varieties of brown and white color. The contribution of soluble phenolic extracts to the TPC ranged from 14-17% and 17-32%, before and after fermentation, respectively. Gallic, protocatechuic, vanillic, syringic, *p*-coumaric, salicylic, ferulic acid, catechin and naringenin were identified and quantified both in the fermented and unfermented *injeras* from Quncho and Zezew tef varieties. After fermenting for 72 h, the majority of the PCs increased in the range of log1.6-log3.3 in soluble extracts and decreased by log0.35-log2 in bound extracts in both varieties. FRAP of the soluble and bound phenolic extracts of *injera* increased by 54-138% and 30-40%, respectively. Total ABTS values, but not DPPH, increased with fermentation. Fermentation for 72 h showed the highest increase in total phenolic and antioxidant capacity. Brown seed colored varieties (Zagurey and Zezew) showed higher total phenolic and antioxidant capacity than the white varieties (Quncho and Tsedey).

2.2.2 Introduction

Total phenolic content (TPC) of seven pure tef varieties studied in part 2.1, ranged from 263-448 mg GAE/100 g dm, of which the bound phenolic content accounted to more than 84% of TPC. The major bound phenolic acids and flavonoids identified in tef include protocatechuic acid, vanillic acid, syringic acid, *p*-coumaric acid, sinapic acid, ferulic acid, rosmarinic acid, catechin and naringenin (Kotaskova et al., 2016; Shumoy and Raes, 2016). Adom and Liu (2002) and Montilla et al. (2011) also indicated that bound PCs represented 61-87 % of the total PCs in cereals such as oats, corn, rice and wheat. However, unlike to those reports, the contents of bound PCs of tef originated from Bolivia and the U.S.A. only represented 26-36% of the TPC (Kotaskova et al., 2016). A possible reason for this discrepancy could be due to the differences in extraction method of the soluble PCs, as Kotaskova et al. (2016) used an ultrasonic treatment to extract soluble PCs. Bound PCs in cereals are cross-linked to cell wall structural components of cellulose, hemicellulose, proteins, pectins and lignins which can survive the upper gastrointestinal digestion, and finally, reach the colon where they can be fermented by different microflora to exert their health benefits (Acosta-Estrada et al., 2014; Adom and Liu, 2002). Bound PCs could be particularly effective around the colon in preventing colon cancer while soluble PCs which are readily absorbable in the stomach and the small intestine could exert their beneficial health effect throughout the body (Chandrasekara and Shahidi, 2011; Liu, 2007).

Injera is made through fermentation and cooking processes which are known to enhance the release of bound PCs and increase the content of soluble PCs (Acosta-Estrada et al., 2014). The duration of *injera* dough fermentation differs from 24 h to more than one week, as the fermentation time is only subject to the individuals' preference of the resulting texture and flavor of *injera*.

It has been reported that *injera* dough fermentation is initiated by endogenous flora of yeast and lactic acid bacteria (LAB) originating from the flour; however, as fermentation progresses and the pH falls, yeasts may become the dominant flora (Umeta and Faulks, 1989). The difference in the profile of the microorganisms and concentration of the organic acids throughout the stages of fermentation could result in a varying content and profile of PCs. To date, literature on the distribution of soluble and bound PCs of *injera* from known tef varieties is scarce. Therefore, the objective of this study was to profile and quantify the soluble and bound PCs and to investigate the antioxidant capacity of *injera*s prepared from two brown and two white tef varieties using traditional backslop fermentation of 24, 72 and 120 h (hours).

2.2.3 Materials and methods

2.2.3.1 Chemicals and reagents

TPTZ (2,4,6-tripyridyl-s-triazine), DPPH, Trolox (6-hydroxyl-2,5,7,8-tetramethylchroman-2-carboxylic acid), ABTS, trifluoroacetic acid (TFA), gallic acid, protocatechuic acid, vanillic acid, syringic acid, *p*-coumaric acid, salicylic acid, ferulic acid, catechin, naringenin and Folin-Ciocalteu phenol reagent were purchased from Sigma–Aldrich (St. Louis, MO, USA). HPLC grade water and methanol were purchased from VWR International (Leuven, Belgium). Technical grades of aluminum chloride (100%), sodium nitrite (technical grade), methanol (> 98.5%), hydrochloric acid (37.2% w/w), potassium persulfate (100%), formic acid (98-100% w/w), iron(II)sulfate (> 99%), iron (III)chloride (99.3%), sodium hydroxide (97%), and sodium carbonate (> 99%) were purchased from VWR International (Leuven, Belgium).

2.2.3.2 Grain sample and *injera* preparation

Four tef varieties, namely, Quncho (DZ-Cr-387), Tsedey (DZ-Cr-37), Zagurey (local) and Zezew (local) were used in this study. The milling is done same as explained in part 2.1. The first two varieties are white whereas the latter are brown. The seven tef varieties as used in part 2.1 in general were chosen based on their yield and acceptance by the Ethiopian farmers. However, four out of them used in this part to make injera are also chosen based on their relatively higher acceptance on the basis of injera quality than the rest of the varieties. Tef *injera*s were prepared as presented in Fig. 2.2.1 following the procedure descibed by Urga and Narasimha (1997). Briefly, water, tef flour and backslop (a left over of previously fermented dough, a traditional method of preserving starter culture) were mixed in ratios of 11:6:1 (w/w/w) and fermented for 24, 72 and 120 h at 25°C followed by subsequent baking of the *injera* for about 3 minutes. Mixing of the dough was done manually using a glass rod stirrer and took 2-3 minutes. The dough was then fermented for 24, 72 and 120 h at 25°C followed by subsequent baking of the *injera* for about 3 minutes at about 180°C using a stainless steel teflon baking pan (Induction technology, France). The duration of the fermentation and baking were chosen based on the traditional practice of *injer*a making in Ethiopia. Fermentation can vary from one day to more than 5 days depending the preference of each household. There is no standard temperature and time of injera baking, but we baked the injera at a temperature of 180°C for 3 min. Unfermented *injeras* were prepared from each variety and used as controls. The pH of the dough was measured using

a digital pH meter (Model Consort C830, Belgium) at the start of fermentation and just before baking of each *injera*. Fermentation and baking process, for each variety, was performed in triplicate. All *injera* samples were stored at -20ºC until further analysis. The bound and soluble phenolic extracts of *injera* were executed as described in part 2.1.

Tef varieties

|

Milling

↓

Water ✚ Flour ✚ Backslop
11:6:1 (w:w:w)

Mixing

↓

Dough

| 0 h Fermentation at 25ºC | 24 h Fermentation at 25ºC | 72 h Fermentation at 25ºC | 120 h Fermentation at 25ºC |

Baking

↓
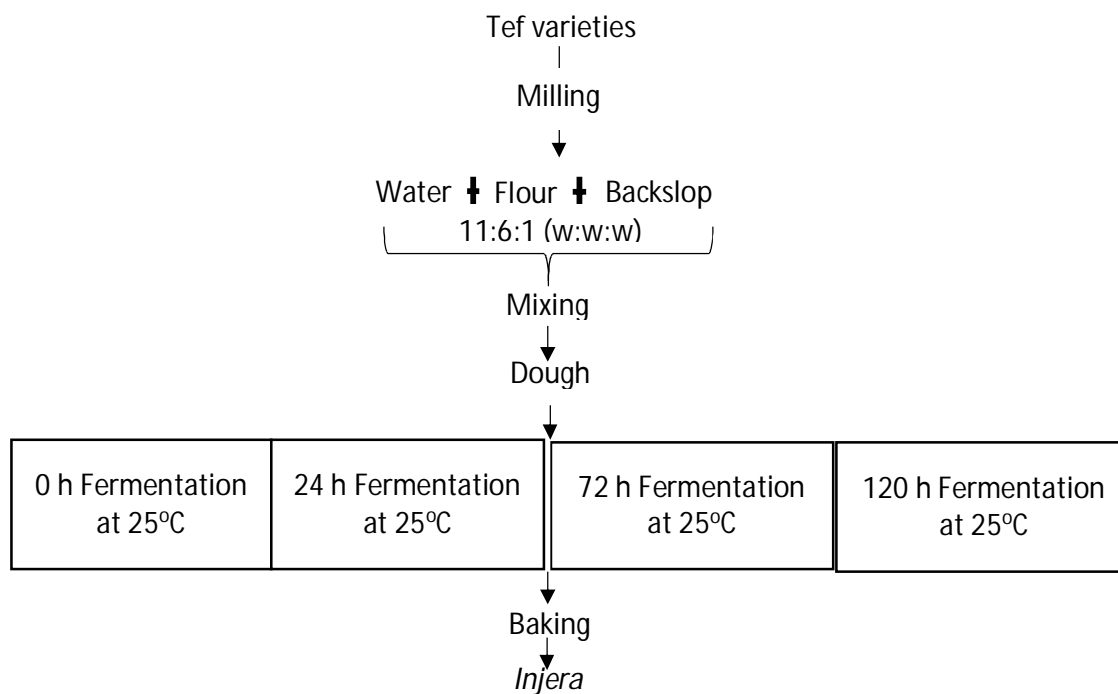
Injera

Figure 2.2.1 Tef *injera* preparation flowchart

2.2.3.3 Determination of ABTS radical scavenging capacity of tef phenolic extracts

ABTS radical scavenging capacity was determined following a method designed by Re et al. (1999). Briefly, a stock solution was prepared by dissolving ABTS in distilled water to a 7 mM concentration. ABTS radical cation was produced by reacting the ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand (12–16 h) in the dark at room temperature before use. A working solution of fresh ABTS radical cation was prepared by diluting the ABTS radical cation with 90% methanol to an absorbance of 0.70 ±0.02 at 734 nm and equilibrated at 30°C. Fresh ABTS radical cation solution (2 mL) was added into test tubes then, 20 µL sample extracts or trolox standard was pipetted, vortexed and incubated (5 min) in dark at 25°C. The absorbance was measured spectrophotometrically (Model 4001/4, Thermo Spectronic, Rochester, New York, USA) at 734 nm and methanol was as a blank. Trolox was used as a standard and the results were expressed as µmole TE/100 g *injera* dm. The TPC,

TFC, HPLC phenolic profiling, DPPH and FRAP of the phenolic extracts were performed as described in part 2.1.

2.2.4 Statistical analysis

All analyses were done in triplicate. Results are reported as mean ± standard deviation on a dry matter basis. The differences of mean values among tef varieties and fermentation times were determined using analysis of variance (ANOVA) followed by Tukey's Honest Significant Differences (HSD) multiple rank test at $P < 0.05$ significance level. All statistical analyses were performed using SPSS version 20 (SPSS Inc., Chicago, IL, USA).

2.2.5 Results and discussion

The effect of duration of fermentation and tef variety on the phenolic content, phenolic profile, and antioxidant capacity of soluble and bound phenolic extracts of *injera* are investigated in this part. The results will benefit tef consumers, processors and researchers to acquire data on the soluble and bound phenolic contents and antioxidant capacity and understand the health benefits of the backslop fermented and unfermented tef *injeras*.

2.2.5.1 Acidity of fermented tef dough

The pH (Table 2.2.1) of the backslop fermented tef dough decreased significantly from 5.75 to 3.40 as fermentation time progressed from 0-120 h.

Table 2.2.1 pH of unfermented and fermented tef dough used to prepare *injera*

FerT	Variety				p
	Quncho	Tsedey	Zagurey	Zezew	
0	5.75±0.08[bA]	5.63±0.04[cA]	5.63±0.05[cA]	5.61±0.06[cA]	0.073
24	3.51±0.04[aA]	3.55±0.00[bAB]	3.59±0.01[bB]	3.58±0.03[bB]	0.013
72	3.45±0.02[aB]	3.46±0.00[aB]	3.46±0.00[aB]	3.40±0.01[aA]	< 0.001
120	3.56±0.08[aA]	3.53±0.01[bA]	3.53±0.02[bA]	3.44±0.09[aA]	0.159
p	< 0.001	< 0.001	< 0.001	< 0.001	

[a,b,c] Values within a column with different superscripts are significantly different ($P < 0.05$). [A,B] Values across rows with different superscripts are significantly different ($P < 0.05$). FerT-fermentation time in hour, Q-Quncho, T-Tsedey, Za-Zagurey, Ze-Zezew. (n=3).

All the varieties showed a considerable pH decrease within the first 24 h of fermentation and then remained relatively stable afterwards. A similar pH drop during fermentation has been reported in tef dough by Umeta and Faulks (1989), and was explained by the dramatic increase of lactic acid and other organic acids such as acetic and propionic acid produced by LAB and yeast.

2.2.5.2 Effect of tef *injera* fermentation on soluble and bound phenolic contents

The soluble and bound phenolic contents of *injeras* of different tef varieties at different fermentation times are presented in Table 2.2.2. The soluble phenolic content differed significantly at different fermentation times within each variety, as well as at each fermentation time. Unfermented and fermented *injeras* of the two brown seed color varieties Zagurey and Zezew showed higher soluble phenolic contents than the white varieties.

Soluble PC of the varieties increased by 92-150% after fermentation and the highest increase was observed after 72 h of fermentation. A relatively lower increase of soluble phenolic content, between 15-38% as measured in mg GAE/g dm, was observed in buckwheat, wheat and rye after fermentation with LAB and yeast (Dordevic et al., 2010) and a very high increase, up to 14-22 folds of the soluble phenolic content was also seen in wheat after fermentation (Dey and Kuhad, 2014). The increase of the soluble PCs could be owing to the action of endogenous and microbial enzymes initiated during the fermentation which leads to the release of bound PCs. Indeed several yeast and LAB, which are also involved in tef fermentation, are capable of synthesizing enzymes like esterases, xylanases, and phenoloxidases that in turn are capable of breaking down ester linkages to release bound PCs in the form of soluble PCs (Ajila et al., 2011; Jamal et al., 2011; Oliveira et al., 2012). Unlike our expectations that there would be a decrease in bound phenolic content (Table 2.2.2) due to the increased soluble phenolic content after fermentation, an increase of bound phenolic content, ranging from 13-55%, was revealed as fermentation progressed from 0-120 h. This is in agreement with studies showing an increase of both soluble and bound phenolic contents after fermentation of lentils, soy bean, black cow gram and mottled cowpea, wheat, rye and whole barley (Anson et al., 2009; Gan et al., 2016; Hole et al., 2012). Many previous studies (Acosta-Estrada et al., 2014; Bhanja et al., 2009; Dvorakova et al., 2008) showed an increase in soluble phenolic content following fermentation.

Table 2.2.2 Phenolic and flavonoid content of soluble and bound phenolics of tef *injera*

FerT	Phenolic compounds mg (GAE)/100 g dm									
	Quncho	Tsedey	Zagurey	Zezew	p	Quncho	Tsedey	Zagurey	Zezew	p
	Soluble extracts					Bound extracts				
0	45.0 ± 2.7^{aAB}	38.3 ± 5.6^{aA}	52.8 ± 2.4^{aB}	56.1 ± 4.7^{aB}	0.011	226 ± 6^{aA}	227 ± 3^{aA}	307 ± 18^{bB}	309 ± 11^{aB}	0.002
24	87.1 ± 2.0^{bAB}	74.8 ± 9.9^{bA}	93.4 ± 7.6^{bAB}	105 ± 8^{bB}	0.014	265 ± 8^{aAB}	256 ± 14^{abA}	302 ± 11^{bB}	487 ± 22^{bC}	< 0.001
72	101 ± 5^{c}	102 ± 4^{c}	109 ± 8^{c}	103 ± 7^{b}	0.498	356 ± 39^{bB}	275 ± 19^{bA}	461 ± 21^{cC}	506 ± 10^{bC}	< 0.001
120	103 ± 4^{cAB}	100 ± 2^{cA}	118 ± 2^{cC}	110 ± 6^{bBC}	0.002	334 ± 22^{bB}	238 ± 10^{abA}	248 ± 4^{aAB}	427 ± 68^{bC}	0.002
p	< 0.001	< 0.001	< 0.001	< 0.001		0.003	0.023	< 0.001	0.001	
	Flavonoids mg (CE)/100 g dm									
	Soluble extracts					Bound extracts				
0	19.0 ± 2.4^{aA}	20.8 ± 0.3^{aA}	35.9 ± 1.1^{aB}	50.6 ± 3.3^{C}	< 0.001	243 ± 3^{cB}	165 ± 25^{cAB}	177 ± 24^{A}	239 ± 7^{bAB}	0.039
24	26.6 ± 2.8^{abA}	20.5 ± 1.0^{aA}	35.3 ± 0.7^{aB}	48.1 ± 3.1^{C}	< 0.001	114 ± 1^{bA}	87.6 ± 6.7^{aA}	153 ± 14^{B}	163 ± 5^{aB}	0.002
72	33.3 ± 2.2^{bA}	32.7 ± 1.5^{bA}	42.2 ± 2.2^{bB}	46.9 ± 3.1^{B}	0.001	87.8 ± 6.7^{aA}	121 ± 3^{abB}	147 ± 4^{C}	160 ± 8^{aC}	< 0.001
120	32.5 ± 2.5^{bA}	30.5 ± 3.1^{bA}	43.0 ± 0.9^{bB}	45.6 ± 2.1^{B}	0.006	117 ± 9^{bA}	117 ± 8^{abA}	135 ± 6^{A}	181 ± 3^{aB}	0.003
p	0.008	0.005	0.002	0.441		< 0.001	0.021	0.162	< 0.001	

[a,b,c] Values within a column with different superscripts are significantly different ($P < 0.05$). [A,B,C] Values within rows with different superscripts are significantly different ($P < 0.05$). FerT-fermentation time in hour, Q-Quncho, T-Tsedey, Za-Zagurey, Ze-Zezew, dm. (n = 3)

Table 2.2.3 Total (soluble + bound) phenolic[a] and flavonoids[b] content of tef *injera*

FerT	Total phenolic content				p	Total flavonoid content				p
	Quncho	Tsedey	Zagurey	Zezew		Quncho	Tsedey	Zagurey	Zezew	
0	271 ± 6^{aA}	265 ± 2^{aA}	360 ± 20^{aB}	365 ± 11^{aB}	< 0.001	262 ± 6^{cB}	186 ± 12^{bA}	213 ± 26^{aAB}	290 ± 10^{bB}	0.016
24	352 ± 10^{bA}	330 ± 9^{bA}	395 ± 4^{aB}	592 ± 21^{bC}	< 0.001	141 ± 5^{abAB}	108 ± 7^{aA}	188 ± 15^{aB}	211 ± 1^{aC}	< 0.001
72	457 ± 34^{cB}	377 ± 15^{cA}	569 ± 28^{bC}	608 ± 9^{bC}	< 0.001	121 ± 5^{aA}	154 ± 6^{abB}	189 ± 6^{aC}	207 ± 10^{aC}	0.001
120	437 ± 27^{cB}	338 ± 11^{bA}	366 ± 4^{aAB}	536 ± 74^{bC}	0.002	150 ± 6^{abA}	148 ± 11^{abA}	178 ± 1^{aA}	227 ± 3^{aB}	0.004
p	< 0.001	< 0.001	< 0.001	< 0.001		< 0.001	0.006	0.063	< 0.001	

[a,b,c] Values within a column with different superscripts are significantly different ($P < 0.05$). (n=3). (n=3). [A,B,C] Values within a row with different superscripts are significantly different ($P < 0.05$). [a](mg (GAE)/100 g dm), [b](mg (CE)/100 g dm).

Therefore, food processing steps such as fermentation, contribute to a better extraction efficiency of both soluble and bound PCs, resulting in a higher TPC after food processing compared to the raw material. The action of the endogenous and exogenous enzymes in the fermentation process may have improved the extractability of the bound PCs. The organic acids produced during the LAB fermentation could also have played a role in chemically rupturing the cell membranes leading to the release of extra bound PCs, which were extracted as soluble PCs in the fermented samples. This could be witnessed by the coincidence of the highest acidity of the 72 h fermented dough and the highest bound PC in their counterpart *injeras.*

The (TPC) (soluble + bound) (Table 2.2.3) significantly increased in each variety as fermentation progressed from 0-120 h. Also for each fermentation time, TPC was significantly different for all varieties ($P < 0.05$). The TPC ranged from 265-608 mg (GAE)/100 g dm and all the varieties showed the highest TPC in *injeras* baked after 72 h of fermentation. Each variety demonstrated an increase in TPC by 31-54% after fermentation. The contribution of soluble PC to TPC of unfermented *injeras* of the varieties ranged from 14-17%, while it increased to 17-32% after fermentation. These results clearly show the importance of fermentation in the overall enhancement of soluble phenolic contents, which could be related to a possible improved bioaccessibility of PCs. The two brown tef varieties revealed higher TPC in each fermentation time compared to the white varieties. These results are also in agreement with previous studies that proved that dark pigmented seeds of quinoa varieties, buckwheat, wheat germ, barley and rye, showed higher TPC than their light colored and white varieties (Dordevic et al., 2010; Tang et al., 2016).

The soluble flavonoid content (FC) of unfermented and fermented tef *injera* is given in Table 2.2.2. Significant differences among varieties at all the fermentation times are observed, obtaining a maximum increase of soluble FC after 72 h fermentation. The two brown varieties showed a higher content of soluble FC than the white tef varieties in unfermented and fermented samples, which is consistent with results of soluble phenolic content.

Bound FC (Table 2.2.2) showed significant differences among the fermentation times within each variety and across different varieties, except for variety Zagurey. As fermentation progressed from 0-120 h, bound FC decreased by 18-58% compared to the unfermented *injeras.* The decrease of the bound FC during fermentation could be due to the release of some of the bound flavonoids by microbial and endogenous enzymatic actions, which was

61

evidenced by the increase of the soluble flavonoids. Also, the increased acidic medium could cause cleavage of proanthocyanidins into flavan-3-ols, which thereafter could be oxidized to quinones (Beta et al., 2000; Porter et al., 1985).

Additionally, in the presence of water, flavonoid compounds can undergo self-polymerization and/or interact or bind with macromolecules such as proteins and polysaccharides making the flavonoids less assayable (Beta et al., 2000). Indeed, this could be the reason why there 20-40% decrease in the TFC (soluble + bound) (Table 2.2.3), following the fermentation.

2.2.5.3 Phenolic profiles of tef *injera*

Phenolic compounds such as gallic acid, protocatechuic acid, vanillic acid, syringic acid, *p*-coumaric acid, salicylic acid, ferulic acid, catechin and naringenin (Fig. 2.2.2) were identified and quantified from soluble and bound phenolic extracts of *injera* from two representative tef varieties (Quncho and Zezew). In the soluble extract (Fig. 2.2.2 A) the majority of the PCs showed a high percentage increase that ranged from log1.6-log3.3 and log2-log2.6, respectively in Quncho and Zezew varieties when compared between the unfermented and the 72 h fermented *injeras*. This was not the case for *p*-coumaric acid in Quncho, ferulic acid in both Quncho and Zezew and naringenin in Zezew. Gallic acid in Zezew, protocatechuic acid and catechin in both Quncho and Zezew varieties of the soluble extract were detected and quantified only in the fermented *injeras* whereas salicylic acid was not detected in the soluble extracts of unfermented and fermented *injeras* in both varieties.

Contrary to the soluble PCs, the bound PCs (Fig. 2.2.2 B) of both varieties showed a percentage decrease that ranged from log1.2-log1.8 and log0.35-log2, respectively in Quncho and Zezew except for salicylic and ferulic acid in Zezew and catechin in Quncho. Ferulic acid and catechin were dominant in the bound and soluble phenolic extracts, respectively. This was demonstrated in the flour samples of the same tef varieties as indicated in part 2.1. Dvorakova et al. (2008) also reported ferulic acid as a major phenolic acid in bound phenolic extracts of malted barley. Similarly in the study of Kotaskova et al. (2016), many of these PCs were identified in flours of white and brown tef varieties with ferulic acid as major compound in bound extracts but unlike to our study, *p*-coumaric acid was the major compound in the soluble extracts.
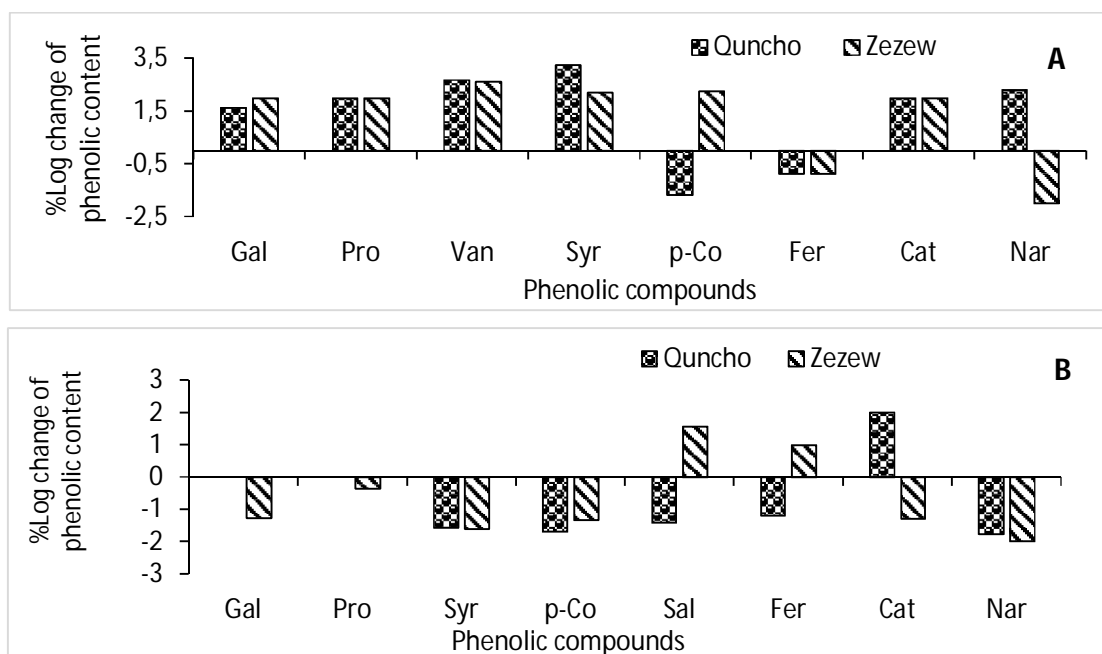
Figure 2.2.2 The percentage logarithmic change in phenolic contents
Soluble (A) and bound (B) injera extracts from Quncho and Zezew varieties fermented for 0 and 72 hours. Gal, Gallic acid; Pro, Protocatechuic acid; Van, Vanillic acid; Syr, Syringic acid; *p*-Co, *p*-Coumaric acid; Sal, Salicylic acid; Fer, Ferulic acid; Cat, Catechin; Nar, Naringenin.(n = 3).

2.2.5.4 Effect of tef *injera* fermentation on free radical reducing and scavenging capacity

FRAP is an electron transfer assay which is based on the reduction of Fe(III) to Fe(II) by antioxidants (Benzie and Strain, 1996). ABTS and DPPH are classified as mixed mode assays because their reaction mechanisms involve both electron and hydrogen atom transfer. Use of multiple assays that measure antioxidant capacity of a sample either directly by radical quenching and radical reducing mechanism (ABTS and DPPH) or indirectly via metal complexing (FRAP) has been recommended (Apak et al., 2016).

FRAP-values of soluble and bound phenolic extracts are given in Table 2.2.4. There was a dramatic increase (54-138%) of the FRAP of the soluble extracts of all varieties when fermentation progressed from 0-120 h. The FRAP of bound phenolic extracts significantly differed ($P < 0.05$) within each variety and among varieties at each fermentation time. FRAP of the bound extracts showed an increase that ranged between 30-40% after fermentation. The values of total FRAP of the combined soluble and bound phenolic extracts of the *injeras* (Fig. 2.2.3) noticeably increased following fermentation of the dough from 0-120 h.

Table 2.2.4 Antioxidant capacity of soluble and bound phenolic extracts of tef *injera*

FerT	Quncho	Tsedey	Zagurey	Zezew	p	Quncho	Tsedey	Zagurey	Zezew	p
	Soluble extracts					Bound extracts				
	FRAP µmole (Fe^{2+})/g dm *injera*									
0	3.25±0.50^{aA}	2.83±0.20^{aA}	5.11±0.50^{aB}	5.63±0.61^{aB}	< 0.001	13.7±1.0^{aA}	13.9±1.6^{aA}	22.3±1.2^{aB}	22.5±2.1^{aB}	< 0.001
24	6.86±0.08^{bA}	5.83±0.66^{bA}	10.7±1.3^{bB}	12.3±1.0^{bB}	< 0.001	17.0±0.5^{abA}	19.8±1.4^{bA}	28.3±2.0^{bB}	34.0±0.8^{bC}	< 0.001
72	6.74±0.13^{bA}	7.34±0.20^{cA}	10.6±1.3^{bB}	11.3±0.5^{bB}	< 0.001	19.4±1.6^{bA}	21.5±2.6^{bA}	25.6±0.3^{abAB}	30.2±4.1^{bB}	0.008
120	7.64±0.52^{bA}	7.01±0.46^{cA}	10.5±0.6^{bB}	12.3±0.6^{bC}	< 0.001	17.1±2.8^{abA}	17.0±1.2^{abA}	29.7±2.9^{bB}	29.0±2.3^{abB}	0.001
p	0.001	0.001	0.001	0.001		0.021	0.007	0.012	0.012	
	ABTS µmole (TE)/g dm *injera*									
0	2.59±0.17^{A}	2.95±0.29^{A}	4.55±0.11^{B}	4.14±0.51^{B}	< 0.001	12.3±3.1^{aA}	18.9±3.2^{abAB}	22.0±1.7^{aB}	24.7±3.8^{aB}	0.015
24	2.67±0.21^{A}	3.24±0.27^{A}	4.26±0.64^{B}	4.90±0.46^{B}	0.001	20.4±2.1^{bA}	21.5±4.7^{bA}	29.6±5.9^{bAB}	38.1±8.8^{bB}	0.020
72	3.43±0.33^{A}	3.52±0.21^{A}	4.30±0.34^{B}	4.41±0.18^{B}	0.003	18.4±0.2^{bA}	16.8±0.3^{abA}	17.5±1.5^{aA}	31.0±3.0^{abB}	< 0.001
120	4.07±0.43^{AB}	3.66±0.44^{A}	4.80±0.13^{B}	4.83±0.43^{B}	0.019	19.1±0.7^{bB}	12.9±1.8^{aA}	17.9±1.6^{aB}	23.6±1.7^{aC}	0.001
p	0.053	0.096	0.325	0.244		0.027	0.041	0.006	0.027	
	DPPH µmole (TE)/g dm *injera*									
0	3.65±0.08^{bA}	3.47±0.04^{cA}	5.35±0.17^{cB}	6.49±0.22^{cC}	< 0.001	18.5±3.5	24.9±2.3	27.3±4.7	27.2±0.2	0.099
24	2.39±0.41^{aA}	2.50±0.21^{aA}	3.30±0.55^{aAB}	4.02±0.38^{aB}	0.009	22.4±1.4^{AB}	21.5±0.9^{A}	28.8±0.8^{BC}	31.5±1.7^{C}	0.004
72	3.20±0.62^{abAB}	2.96±0.12^{bA}	3.90±0.18^{abBC}	4.40±0.09^{aC}	0.003	23.4±0.6^{B}	18.8±0.9^{A}	23.8±0.7^{B}	27.3±3.0^{B}	0.011
120	3.11±0.19^{abA}	2.53±0.15^{aA}	4.64±0.42^{bcB}	5.22±0.56^{bB}	0.001	20.9±1.7^{A}	21.4±1.0^{A}	24.8±0.6^{B}	22.7±1.5^{AB}	0.019
p	0.024	0.003	0.001	< 0.001		0.083	0.043	0.306	0.015	

[a,b,c] Values within a column with different superscripts are significantly different ($P < 0.05$). [A,B,C] Values across rows with different superscripts are significantly different ($P < 0.05$). FerT-fermentation time in hour, Q-Quncho, T-Tsedey, Za-Zagurey, Ze-Zezew. (n = 3).

The increase of FRAP in both the soluble and bound extracts was seen during the first 24 h of fermentation and remained relatively constant afterwards. This coincides with the major pH drop during the fermentation. The reason why there is variation in the FRAP of soluble and bound extract among the varieties and within one variety of different fermentation times could be explained by the variation in the concentration and proportion of simple and complex phenolic acids and flavonoids. The difference in the contents of particular Maillard reaction products such as sulfhydryl group from the possible reaction of glucose with cysteine during baking (Amarowicz, 2009) could also explain the variation of FRAP.

The type and amount of Maillard reaction products are dependent on the total protein, the profile and contents of amino acids of each variety. These results are in agreement with increased content of FRAP in barley fermented for 24 h using *L. rhamnosus* (Dordevic et al., 2010).

ABTS radical scavenging capacity of the soluble and bound phenolic extracts is depicted in Table 2.2.4. ABTS radical scavenging capacity of soluble phenolic extracts of all varieties did not change due to fermentation. However, fermentation time had a significant influence on the ABTS values among varieties ($P < 0.05$). In agreement to our findings, pizza baked from different fermented wheat varieties did not show any difference from the unfermented control (Moore et al., 2009). On the other hand, ABTS radical scavenging ability of the bound phenolic extracts exhibited an increase in the first 24 h of fermentation but diminished in subsequent fermentation times.

The combined (soluble + bound) phenolic extracts showed increased ABTS (Fig. 2.2.3) radical scavenging properties after fermentation. The varieties Quncho and Zezew showed a slight increase in ABTS radical scavenging properties when the fermentation progressed from 0-120 h except in the case of Zezew that showed a decrease after 72 h fermentation. Varieties Tsedey and Zagurey only exhibited an increase during the first 24 h fermentation and remained relatively constant afterwards.
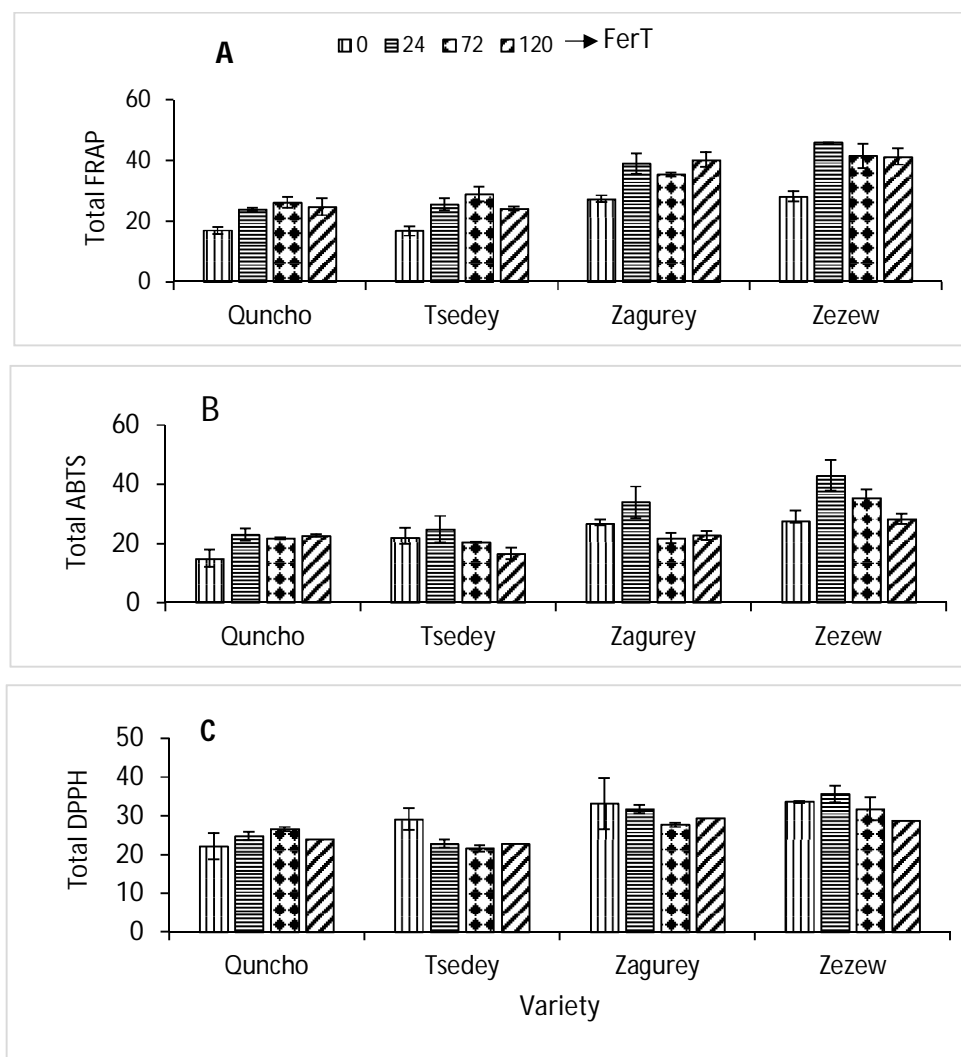
Figure 2.2.3 Total antioxidant capacities of the soluble and bound phenolic compounds
FRAP µmole (Fe^{2+})/g dm (A), ABTS µmole (TE)/g dm (B) and DPPH µmole (TE)/g dm (C). FerT-Fermentation time in hours. (n = 3).

DPPH free radical scavenging ability of soluble and bound phenolic extracts are presented in Table 2.2.4. There was significantly different ($P < 0.05$) DPPH radical scavenging capacity within each variety and among varieties during all the fermentation times. When fermented from 0-120 h, soluble phenolic extract showed a decrease of DPPH by 26-43% within the first 24 h fermentation and remained more or less constant till the end of the fermentation. DPPH radical scavenging capacity of the bound phenolic extracts did not show significant difference ($P < 0.05$) after fermentation. The DPPH radical scavenging values of the combined soluble and bound extracts (Fig. 2.2.3) showed a slight increase in Quncho when fermentation progressed from 0-120 h but it decreased in Tsedey and stayed constant in the case of Zagurey and Zezew varieties. The reason why FRAP showed an increase with increased fermentation time while

this was not the case for ABTS and DPPH could be attributed to the change in composition of individual phenolic compounds. Also during fermentation e.g. protein degradation occurs, and small peptides can also react with some of these antioxidants differently leading to differences among the antioxidant measurements.

The brown tef varieties showed superior FRAP, ABTS and DPPH radical scavenging capacity compared to their white counterparts in the soluble and bound extracts of both fermented and unfermented *injera* products. It was also reported that brown tef originating from U.S.A and Bolivia showed higher ABTS and DPPH radical quenching potential compared to their white counterparts (Kotaskova et al., 2016). Likewise, quinoa varieties of dark and red seed color exhibited higher FRAP than their counterpart white varieties (Tang et al., 2016).

2.2.6 Conclusions

The fermentation process significantly increased the total phenolic compounds, i.e. both soluble and bound ones. It also increased antioxidant capacity of FRAP and ABTS radical scavenging capacity of *injera* from all tef varieties but it decreased the DPPH radical scavenging and TFC. Fermentation process can increase the proportion of soluble PCs of tef *injera*. Individual PCs in the soluble extracts revealed a significant increase in *injeras* baked after 72 h fermentation whereas the majority of them showed a decrease in the bound extract. Seed color seems to play a crucial role in the phenolic contents in that brown tef varieties showed higher TPC, FRAP, DPPH and ABTS compared to their counterpart white varieties. This study clearly demonstrated that varieties grown in the same location had significantly different phenolic and antioxidant capacity. Therefore, it would be paramount to study the effect of growing location and seasonal difference on phenolic and antioxidant capacity of tef varieties.

Part 2.3: Effect of Fermentation on Bioaccessibility of Phenolic Compounds of Tef *Injera*

Part 2.3: Effect of Fermentation on Bioaccessibility of Phenolic Compounds of Tef *Injera*

2.3.1 Abstract

In this part, the focus was to investigate the effect of fermentation on the bioaccessibility of phenolic compounds of tef injera baked from 4 different tef varieties. A simulated static in vitro digestion of *injeras* at different fermentation times was performed in order to measure the dialyzable (D)/bioaccessible and soluble nondialyzable total phenolic content (TPC), total flavonoid content (TFC), and total antioxidants ABTS, DPPH and FRAP. The %D TPC, %SND TPC, %D TFC, %SND TFC of the D and SND phenolic extracts of the in vitro digested *injeras* were in the range of 2-3%, 5-10%, 1-3%, 4-9%, 18-51%, respectively. The %D ABTS, %SND ABTS, %D DPPH, %SND DPPH, %D FRAP, %SND FRAP contents of the D and SND extracts ranged from, 41 to 94%, 0.02 to 0.62%, 0.62 to 2%, and 8 to 16%. The TFC of the D and SND phenolic extracts decreased as the fermentation increased from 0 to 120 hours. Phenolic extracts from the D and SND fractions of the *in vitro* digested injera did not show uniform pattern in their TPC, DPPH and FRAP results while the ABTS-value increased with fermentation time.

2.3.2 Introduction

Despite the fact that the consumers of western and middle income countries are increasingly anxious to food safety, quality and health-related issues, a significant part of the population are still fighting modern age diseases such as obesity, osteoporosis, cancer, diabetes, allergies, stress and dental problems (Cencic and Chingwaru, 2010). An increased intake of refined carbohydrate and high energy dietary foods such as fat and protein concomitant with a decreasing intake of fiber have been reported as the major causes of the increased risk of many of the chronic diseases (Gross et al., 2004). Whole cereal based food products are recognized as healthy foods due to their crucial role in the prevention of these chronic diseases in part due to their possession of PCs that fight against physiological oxidative stress (Bjorck et al., 2012; Larsson et al., 2005). The association of whole cereal consumption and an overall health improvement is well documented and this led consumers to an evidence based perception of whole cereals as healthy. Unlike to the common cereals such as wheat, most of the ancient cereals and pseudocereals are processed into a whole meal. There is established evidence that PCs are localized in the bran part of cereals that makes these cereals an excellent source of PCs.

The PCs in cereals are found in two major forms –soluble and bound to cell wall material with the later accounts for majority of the TPC (Adom and Liu, 2002). The bioaccessibility of phenolic compounds during the simulated gasterointestinal digestion is dependent on the release of the PCs from the food matrix. To increase the bioaccessibility of PCs in the simulated gastrointestinal digestion, the phenolic compound should be released from the food matrix and be in the form of soluble PCs. Food processing methods such as fermentation are known to facilitate the release of bound PCs in the food matrix.

Tef [*Eragrostis tef* (Zucc). Trotter] is an ancient cereal currently gaining high acceptance in the global market due its gluten-free nature as well as high dietary fiber and minerals (Zhu, 2018). This cereal is processed as a whole meal and it reported to have high TPC and antioxidant capacity with the bound phenolic content accounting for more than 84% as shown in part 2.1. Up on fermentation of a traditional food product (*injera*) of this cereal, the soluble PCs increased and accounted to 17–32% of the TPC as shown in part 2.2. It was sugested that the increase in soluble PCs of *injera* could be a good precondition for the release or enhanced bioaccessibility of the PCs during the simulated gastrointestinal digestion as designated in part

2.2. Soluble phenolic coumpounds are known to be absorbed in the upper gastrointestinal tract where they impart their physiological use whereas the bound ones reach the large intestine and serve as a substrate for the indigenous beneficial complex fermenting microbial ecosystem that boosts the immune system. The increase in the soluble phenolic content in cereals is desirable in order to have a balance between soluble and bound PCs which have a different health benefit.

Although fermentation could increase the amount of soluble PCs in fermented food products, the physiological importance is still dependent on their absolute bioavailability which in turn is reliant on bioaccessibility in the gastrointestinal tract. Information regarding the bioaccessibility of PCs of tef products is not existing yet. *Injera* is one of the major traditional fermented tef food products and the increased soluble phenolic content of this food as mentioned above has prompted us to further dig into its gastrointestinal bioaccessibilty. Therefore, the focus of this study was to investigate the effect of fermentation on bioaccessibility of PCs of tef *injera* prepared from four tef varieties.

2.3.3 Materials and methods

2.3.3.1 Chemicals and reagents

TPTZ (2,4,6-tripyridyl-s-triazine), DPPH, Trolox (6-hydroxyl-2,5,7,8-tetramethylchroman-2-carboxylic acid), ABTS, gallic acid and Folin-Ciocalteu phenol reagent were purchased from Sigma–Aldrich (St. Louis, MO, USA). Technical grades of aluminum chloride, sodium nitrite (technical grade), methanol, hydrochloric acid, potassium persulfate, formic acid, iron (II) sulfate, iron (III) chloride, sodium hydroxide, and sodium carbonate were purchased from VWR International (Leuven, Belgium).

2.3.3.2 Grain sample and *injera* preparation

Grain sample were as explained in part 2.1 while *injera* preparation were carried out as described in part 2.2 section 2.2.3.3. Four tef varieties, namely, Quncho (DZ-Cr-387), Tsedey (DZ-Cr-37), Zagurey (local) and Zezew (local) as used in part 2.2 were used in this study.

2.3.3.3 Static *in vitro* digestion

The static *in vitro* digestion was carried out according to the **INFOGEST** standardized consensus model (Minekus et al., 2014) consisting of three phases *(vide infra).* **Oral phase**: Fresh (5 g) *injera* minced by a mixer (rondo 500 multifunction, SeB) was blended with 3.5 mL of simulated salivary fluid (SSF) electrolyte stock solution. The SSF is an electrolyte of pH 7 and is cocktail of KCl, KH_2PO_4, $NaHCO_3$, $MgCl_2(H_2O)_6$ and $(NH_4)_2CO_3$ at different concentrations made in bidistilled water. Prior to incubation for 2 min at 37°C in a shaking water bath, 0.5 mL α-amylase solution of 1500 units/mL made up in SSF solution, 25 µL of 0.3 M $CaCl_2$ solution and 975 µL of double-distilled water were added to this mixture. **Gastric phase**: The oral bolus (10 mL) was mixed with 7.5 mL of simulated gastric fluid (SGF) electrolyte stock solution. SGF electrolyte of pH 3 and is a cocktail of KCl, NaCl, KH_2PO_4, $NaHCO_3$, $MgCl_2(H_2O)_6$ and $(NH_4)_2CO_3$ at different concentrations made in bidistilled water. Afterwards, 1.6 mL of pepsin (25000 U/mL) made up in SGF, 5 µL of 0.3 M $CaCl_2$ solution and 695 µL of water were added to this mixture and the pH of the mixture was corrected to 3 using 0.2 mL of 1 M HCl. Finally, the mixture was incubated at 37°C in a shaking water bath. After 1.5 h of incubation, dialysis bags containing $NaHCO_3$ (5.5 mL, 0.5 M) and NaCl (5.5 mL, 0.9%) were put into the gastric chime according to (Wolfgor et al., 2002) and the incubation was continued for 30 min. **Intestinal phase:** After adding 11 mL of simulated intestinal fluid (SIF) electrolyte stock solution, 5.0 mL of pancreatin solution (800 units/mL) made up in SIF, 2.5 mL of fresh bile (160 mM), and 40 µL of 0.3 M $CaCl_2$ solution and 1.31 mL of water to this mixture, the pH of the mixture was corrected to a value of 7. Finally, the mixture was incubated for 2 h at 37°C in a shaking water bath. SIF is a cocktail of KCl, NaCl, KH_2PO_4, $NaHCO_3$, and $MgCl_2(H_2O)_6$ at different concentrations made in bidistilled water.

After the simulated intestinal digestion phase, the dialysis bags were taken out, rinsed and dried using a paper cloth. Then, the contents, which include dialyzed (D) PCs were transferred into falcon tubes. The remaining digestion solution was centrifuged at 4,000 x g, afterwards, the supernatants, containing SND PCs were separated from the pellet and stored in plastic recipients while the pellets were discarded. Both the D and SND contents were freeze dried and extracted (thrice) following the methanolic extraction method as described in part 2.1 and the supernatants were pooled and kept at-20°C for further analysis. The TPC, TFC, DPPH and FRAP of the D and SND phenolic extracts were carried out as described in part 2.1 while that

of ABTS from part 2.2. The data TPC, TFC, ABTS, DPPH, and FRAP of *injera* used for the calculation of %D and %SND were taken from part 2.2 as the same *injera* samples were also used in this study. The percentage TPC, TFC, ABTS, DPPH or FRAP contents of the D and SND extracts were calculated as follows:

$$A\ (\%) = \left(\frac{B}{C}\right) x\ 100$$

Where: **A** is either D or SND of TPC, TFC, ABTS, DPPH or FRAP; **B** is either of TPC in mg (GAE)/100 g dm, TFC in mg (CE)/100 g dm, ABTS in μmol (TE)/g dm, DPPH in μmol (TE)/g dm or FRAP in μmol (Fe^{2+})/g dm of contents of the D or SND extracts; **C** is either of TPC in mg (GAE)/100 g dm, TFC in mg (CE)/100 g dm, ABTS in μmol (TE)/g dm, DPPH in μmol (TE)/g dm or FRAP in μmol (Fe^{2+})/g dm contents of the sample (*injera*) extracts soluble and bound (combined).

Throughout the text, solubility refers to the sum of phenolic and antioxidants in D+SND while bioaccessibility refers to the D fraction. For better understanding, the definition of bioaccessibility and bioavailability is also given as follows: Bioaccessibility: *in vivo*: it is a fraction of nutrients or compounds potentially available in the gut lumen for absorption. But when *in vitro*: It is used to indicate the *in vitro* dialyzable (the food molecule that passes through the dialysis bag/membrane) fraction of food components. Bioavailability**:** *in vivo***:** It is a fraction of an ingested nutrient or compound that reaches the systemic circulation and may be utilized by the cells. Therefore, it includes: gastrointestinal digestion, absorption, metabolism, tissue distribution, and bioactivity.
The TPC, TFC, DPPH and FRAP of the D and SND phenolic extracts were determined as described in part 2.1 while the ABTS radical scavenging capacity was determined as designated in part 2.2.

2.3.3.4 Statistical analysis

To assess differences in %D and %SND among tef varieties and fermentation times, two-way analysis of variance (ANOVA) was performed. If the interaction between the main factors fermentation time x tef variety was significant ($p < 0.05$), one-way ANOVA was done to check for individual effects. Multiple comparison was done by Tukey's Honest Significant Differences (HSD) multiple rank test at $p < 0.05$. All statistical analyses were performed using SPSS version 24 (SPSS Inc., Chicago, IL, USA). All analyses were carried out in triplicate. Results were reported on dm basis.

2.3.4 Results and discussion

2.3.4.1 Total phenolic and flavonoid content of D and SND extracts

The %TPC and %TFC contents of the D and SND extracts of the *in vitro* digested fermented *injeras* of different tef varieties are given in Tables 2.3.1 and 2.3.2. Both the %D and %SND of TPC showed significant differences ($p < 0.05$) among *injeras* of different fermentation times within a variety as well as among different varieties of similar fermentation times. Unfermented *injeras* showed highest %TPC in D and SND fractions with all the tef varieties revealing a decreasing pattern of %TPC in D and SND fractions as fermentation time progressed from 0-120 h with the exception of Zagurey variety. The difference in the %TPC in D and SND fractions among *injeras* with different fermentation time within a variety is due to the difference in the TPC of the starting samples (*injera*).

The absolute TPC of D and SND fractions (values in bracket) are similar and/or fluctuate only slightly among *injeras* of different fermentation times within a variety. The %TPC in D and SND fraction of the *injera* from white tef varieties were higher than their corresponding *injera* from brown tef varieties.

The soluble %TPC of D+SND of the *injeras* of the four tef varieties ranged from 7-14%. In general, there are very limited studies on bioaccessibility of phenolic compound of cereals and tef in particular. A study that used an *in vitro* model without dialysis bags showed relatively comparable to a fairly higher solubility of 21%, 13%, and 30% for extruded brown rice, wheat and oats, respectively (Zeng et al., 2016). Another study also revealed extremely higher solubility of 58%, 45%, 62%, and 41%, respectively for breads of wheat, buckwheat, rye and oat (Angioloni and Collar, 2011). Although the %solubility of TPC in our study is lower compared to the breads in the latter study, the absolute soluble TPC which ranged from 36 to 46 (mg GAE/100 g dm), is at least equal to or higher than the absolute soluble TPC of the studied breads (26-40mg GAE/100 g dm) (Angioloni and Collar, 2011).

Table 2.3.1 Total phenolic contents of fractions of *in vitro* digested tef *injera*

FerT	Quncho	Tsedey	Zagurey	Zezew	p
	% Total phenolic content in D fraction				
0	3.81 ± 0.31^{cB} (10.3)	3.56 ± 0.18^{bB} (9.4)	2.81 ± 0.09^{bA} (10.1)	3.04 ± 0.21^{cA} (11.1)	0.002
24	3.32 ± 0.14^{bC} (11.7)	3.35 ± 0.24^{abC} (11.1)	2.76 ± 0.07^{bB} (10.9)	2.16 ± 0.11^{bA} (12.8)	< 0.001
72	2.63 ± 0.19^{aB} (12.0)	3.13 ± 0.07^{aC} (10.6)	1.99 ± 0.16^{aA} (11.3)	1.79 ± 0.04^{aA} (10.9)	< 0.001
120	2.61 ± 0.15^{aB} (11.4)	3.28 ± 0.02^{abC} (11.1)	3.40 ± 0.01^{cC} (12.4)	2.16 ± 0.14^{bA} (11.6)	< 0.001
p	< 0.001	0.05	< 0.001	< 0.001	
	% Total phenolic content in SND fraction				
0	9.80 ± 0.44^{Bb} (26.6)	10.2 ± 0.6^{bB} (27.1)	8.00 ± 0.74^{bcA} (28)	9.07 ± 0.71^{bAB} (33.1)	0.011
24	8.86 ± 0.79^{bC} (31.2)	8.49 ± 0.07^{aC} (28.0)	7.18 ± 0.17^{bB} (28.4)	5.55 ± 0.29^{aA} (32.9)	< 0.001
72	6.45 ± 0.61^{aB} (29.5)	7.83 ± 0.20^{aC} (26.4)	5.10 ± 0.29^{aA} (29.0)	5.10 ± 0.16^{aA} (31.0)	< 0.001
120	6.64 ± 0.31^{aA} (29.0)	8.35 ± 0.23^{aB} (28.2)	8.28 ± 0.19^{cB} (30.3)	5.95 ± 0.50^{aA} (31.9)	< 0.001
p	< 0.001	< 0.001	< 0.001	< 0.001	

[a,b,c] Values within column with different small superscript letters are significantly different ($p < 0.05$). [A,B,C] Values across rows with different capital superscript letters are significantly different ($p < 0.05$). p- p-value, FerT-fermentation time in hours. Values in brackets represent the actual D or SND phenolic and flavonoid contents, respectively in mg (GAE)/100 g dm and TFC mg (CE)/100 g dm. (n=3).

Table 2.3.2 Total flavonoids contents fractions of *in vitro* digested tef *injera*

FerT	Quncho	Tsedey	Zagurey	Zezew	p
	% Total flavonoid content in D fraction				
0	1.58±0.31[aA] (3.9)	2.97±0.21[cB] (5.5)	2.61±0.25[bB] (5.6)	2.37±0.08[bB] (6.9)	0.010
24	2.45±0.34[b] (3.5)	2.29±0.08[bc] (2.5)	2.53±0.15[b] (4.8)	2.63±0.35[b] (5.6)	0.494
72	2.32±0.07[bB] (2.8)	1.61±0.33[abA] (2.5)	1.62±0.18[aA] (3.1)	1.48±0.06[aA] (3.1)	0.013
120	1.53±0.09[aA] (2.3)	1.27±0.00[aA] (1.9)	1.53±0.20[aA] (2.7)	2.06±0.07[bB] (4.7)	0.010
p	0.001	0.003	0.004	0.002	
	% Total flavonoid content in SND fraction				
0	5.86±0.38[aAB] (15.4)	6.40±0.51[aB] (11.9)	6.60±0.39[cB] (14.1)	5.08±0.08[bA] (14.7)	0.007
24	6.58±0.27[bB] (9.3)	8.19±0.52[bC] (8.9)	5.76±0.22[bAB] (10.8)	4.81±0.47[bA] (10.2)	< 0.001
72	8.96±0.88[bC] (23.5)	6.32±0.52[aB] (9.7)	4.49±0.46[aA] (8.5)	4.60±0.45[abA] (9.5)	< 0.001
120	5.99±0.88[bB] (15.7)	6.43±0.35[aB] (9.5)	4.48±0.13[aA] (8.0)	3.83±0.21[aA] (8.7)	0.001
p	0.028	0.011	< 0.001	0.011	

[a,b,c] Values within column with different small superscript letters are significantly different ($p < 0.05$). [A,B,C] Values across rows with different capital superscript letters are significantly different ($p < 0.05$). p- p-value, FerT-fermentation time in hours. Values in brackets represent the actual D or SND phenolic and flavonoid contents, respectively in mg (GAE)/100 g dm and TFC mg (CE)/100 g dm. (n=3).

The %TFC in the D fraction of *injera* of all tef varieties showed a decreasing pattern when fermentation increased from 0 to 120 h with the highest %TFC in D fraction shown by unfermented *injera*, exceptions are the 24 h fermented injera of Quncho and Zezew which showed higher %TFC in D fraction than their 0 h *injera.* Similarly, the %TFC in SND fraction of brown tef varieties decreased as the fermentation increased from 0 to 120 h, while the white ones did not show any uniform pattern. Parallel to their relative TFC, expressed as %D and %SND, the absolute TFC of the D and SND phenolic extracts (expressed in mg CE/100g dm) of all the varieties showed a decreasing pattern as the fermentation time increased with the exception of Quncho variety. The total soluble TFC of the *injeras* of all the varieties ranged from 5-12%. In part 2.2, we demonstrated the increase of soluble phenolic content of *injera* following the fermentation from 0 to 120 h. Based on these results, it was suggested that the increase in the amount of soluble phenolic content could also improve their bioaccessibility. Indeed, it has been reported that fermentation and other bioprocessing techniques such as germination could improve the bioaccessibility of PCs (Angelino et al., 2017; Gabaza et al., 2016). Unlike to the expectation, the absolute TPC of the D and SND fraction of the *in vitro* digested extracts of *injeras* did not show any increase. This could be attributed to the arrays of reactions taking place within the simulated gastrointestinal digestion. The solubility and stability of phenolic compounds are high at low pH (gastric pH) and it decreases with the increase in pH (intestinal pH) (Pods et al., 2014), indicating that phenolic compounds could be degraded and change their form during their stay in the small intestine. In the process of simulated digestion, PCs and the other food components can also come into contact with each other which enable various interactions, possible chemical bonding, and entrapment of smaller molecules into porous structure of bigger molecules which could affect bioaccessibility of PCs. Dietary PCs undergo a series of interactions with co-existing molecules such as fiber, starch, protein, fat and minerals that interfere with their bioaccessibility and bioavailability (Dominguez-Avila et al., 2017).

Phenolic compounds are localized in the bran of grains while most of the fat content is in the germ part. During the simulated gastrointestinal digestion, the soluble PCs come into contact with the fats due to the size reduction and increase of surface area by mastication and action of the enzymes. In the process of simulated digestion, the fat content forms emulsions of small droplets which possess many hydrophobic heads which enables them to actively interact with the hydroxyl groups of PCs forming bigger complex aggregates which eventually reduces the

bioaccessibility and availability of both the fat and the PCs (Jakobek, 2014). The complexation of PCs and fats has been reported as beneficial, because the PCs captured by fat could be stable during the whole simulated digestion process and reach the lower parts of the gastrointestinal tract where they mainly give their beneficial antioxidant properties (Ortega et al., 2009).

The increased free phenolic compounds during the *injera* fermentation process concomitant with the increase in free amino acids due to the hydrolytic actions of enzymes in the course of *in vitro* digestion could have enhanced the interaction of PCs with free amino acids. It was shown that polyphenols bind to hydrophobic sites of amino acids, through hydrogen and covalent bonding and transformation of PCs into quinones which may further irreversibly react with nucleophilic groups on the protein molecule (Jakobek, 2014) leading to the decease of bioaccessibility of both the PCs and protein. It was shown that the structure and molecular weight of polyphenols play an important role in protein–polyphenol interactions in that the order of PCs binding to proteins increase as the number of OH groups on the polyphenol molecule increase (Frazier et al., 2010). This could largely contribute to the low solubility and bioaccessibility of the PCs as the flour tef varieties used in this study had tannin content that ranged from 65 to 302 mg CE/100 g dm flour as shown in chapter 3.

Furthermore, the brown varieties (Zezew and Zagurey) showed 2 to 5-folds higher tannin level compared to the white ones (Tsedey and Quncho) as shown in chapter 3 and this could explain why the D and SND of the *in vitro injera* extracts of the brown varieties showed a relatively lower % TPC and %TFC of the D and SND fractions compared to their corresponding *in vitro injera* extracts of the white varieties.

Although the fermentation of the *injera* was effective in releasing the bound PCs which is a precondition for bioaccessibility, the presence of fiber in the food matrix by itself could also play an entrapping role of the free PCs with the polysaccharides throughout the *in vitro* digestion and formation of bigger complexes could also inhibit the bioaccessibility of PCs. Bioaccessibility of PCs in whole wheat and white wheat breads was compared and it was shown that the TPC of the whole wheat bread was higher than the white wheat bread , conversely, higher bioaccessible proportion of the white wheat bread (4.9%) vs than the whole wheat bread (1.1%) revealed the bioaccessibility interference of the fiber matrix (Anson et al., 2009).

However, the phenolic compound entrapping role of dietary fiber is also beneficial because it serves as a carrier thereby enabling the phenolic compound to reach the colon where they undergo fermentation and encourage the growth of beneficial bacteria while inhibiting the growth of pathogenic bacteria (Saura-Calixto, 2011). The enzymatic activity and the metabolic activity of microorganisms of a fermenting food can help to improve the nutritive and bioactive properties of the food matrices which is beneficial for human health (Filannino et al., 2013). During fermentation, the activation of enzymes such as decarboxylases and reductases facilitate the metabolism of phenolic compounds into beneficial source of energy for heterofermentative microorganisms (Marco et al., 2017).

Another possibility why solubility and bioaccessibility of PCs tef *injera* did not increase despite the increased contents of soluble PCs as a result of fermentation could also be attributed to complexation of PCs with minerals. It was reported that iron can bind itself with galloyl or catechol bearing PCs forming larger complexes with PCs containing high number of hydroxyl groups such as catechin and tannins that showed the highest affinity (Khokhar and Apenten, 2003). Protocatechuic acid, vanillic acid, syringic acid, *p*-coumaric acid, sinapic acid, ferulic acid, rosmarinic acid, catechin and naringenin were detected in the sampled *injeras* with catechin as the major phenolic compound in the soluble extracts as shown in part 2.2. Due to fermentation, the PA content of the sampled *injera* decreased by more than 50% as indicated in part 2.2 suggesting the increase in free minerals, specifically iron. Therefore, the co-existence of the high affinity galloyl bearing PCs such as catechin and iron in free form throughout the *in vitro* digestion could have increased the rate and chance of the complexation between iron and PCs leading to a reduced bioaccessibility of the PCs and iron. The other reason why the increase in soluble PCs in the *injeras* was not reflected in the solubility and bioaccessibility could be attributed to the digestion method used. Although, the static *in vitro* digestion employed in this study is a consensus method, it should be noted that it has its own limitations in measuring bioaccessibility of the PCs. The facts that it is not dynamic like the real physiological digestion could cause a decrease in diffusion rate of the PCs into the dialysis bag as the digestion progress from simulated gastric phase to simulated intestinal phase due to difference in concentration gradient. This can be evidenced by the similarity of the actual TPC content in the D fraction of the *injeras* within a variety and across the varieties regardless of the initial content of soluble phenolic content of the *injera*. It has been already reported that static *in vitro* digestions do not provide the most accurate

simulation of the complex dynamic physiological processes occurring during *in vivo* conditions (Angelino et al., 2017).

2.3.4.2 Antioxidant capacity of D and SND phenolic extracts

The antioxidant capacity of phenolic compounds (PCs) in the D and SND fractions of the *in vitro* digested *injera* as measured by ABTS, DPPH and FRAP is given in Tables 2.3.3 & 2.3.4. The %ABTS radical scavenging capacity of PCs in the D and SND fractions of the *injera* increased as fermentation time increased, exception is variety Quncho. Similarly, in each variety, the actual ABTS radical scavenging capacity of the PCs of the D and SND fraction showed an increasing pattern as fermentation progressed from 0 to 120 h. The sum of the ABTS radical scavenging capacity of PCs in the D and SND fractions ranged from 17-27 µmol (TE)/g dm *injera* and these results are higher than the total ABTS radical scavenging capacity of the corresponding *injeras sampled*. Similarly, phenolic extracts from an *in vitro* gastric and intestinal supernatant of different fruits have showed higher ABTS radical scavenging capacity than the total ABTS radical scavenging capacity of their corresponding phenolic extracts of the fruits prior to *in vitro* digestion (Tagliazucchi et al., 2010). Relatively lower ABTS radical scavenging capacity of bioaccessible PCs was reported for raw and extruded brown rice, wheat and oats (Zeng et al., 2016). The complex changes occurring during the simulated gastrointestinal digestion could be the reason why the sum of PCs from the D and SND fractions showed higher ABTS radical scavenging capacity compared to their corresponding *injeras.*

Most of the PCs in plants are found as glycosylated forms or as esters or polymers, which could be hydrolyzed during the simulated gastrointestinal digestion due to the action of digestive enzymes and the acidic environment of the stomach as well as the alkaline environment of the intestine (Alminger et al., 2014; Tagliazucchi et al., 2010). These hydrolysis actions lead to numerous changes in the phenol structure such as hydroxylation, methylation, isoprenylation, dimerization, and glycosylation, as well as the formation of phenolic derivatives by partial degradation of the combined forms or by losing the moieties between phenols and sugars (Chen et al., 2016). These changes could result in new PCs of high ABTS radical scavenging capacity. The DPPH radical scavenging capacity of the PCs in the D and SND fractions of the *in vitro* digested *injeras* were very low.

Table 2.3.3 Total ABTS and DPPH capacities of fractions of *in vitro* digested tef *injera*

FerT	Quncho	Tsedey	Zagurey	Zezew	p
			%ABTS in D fraction		
0	34.2±0.4[B] (5.1)	25.8±1.9[aA] (5.7)	23.8±1.5[aA] (6.4)	21.6±1.9[bA] (6.3)	0.002
24	31.3±0.7[C] (7.2)	28.5±0.1[aC] (7.1)	22.5±2.0[aB] (7.7)	17.5±1.0[aA] (7.5)	< 0.001
72	35.2±2.2[B] (7.8)	42.6±2.0[bC] (8.5)	36.8±1.4[bB] (8.1)	21.9±0.7[bA] (7.7)	< 0.001
120	33.9±2.8[A] (7.8)	50.7±4.4[bB] (8.6)	37.2±2.5[bA] (8.6)	30.2±1.3[cA] (8.5)	0.002
p	0.319	0.002	< 0.001	< 0.001	
			%ABTS in SND fraction		
0	83.3±1.8[bB] (12.5)	54.6±4.6[aA] (12.0)	51.7±3.1[aA] (14.0)	45.0±3.8[A] (13)	< 0.001
24	72.4±0.1[aD] (16.7)	66.3±2.4[bC] (16.6)	48.1±0.6[aB] (16.4)	41.2±0.6[A] (17.7)	< 0.001
72	80.6±4.5[abB] (17.7)	85.5±2.9[bB] (17.1)	78.9±5.1[bB] (17.4)	43.5±0.5[A] (15.2)	< 0.001
120	74.7±3.4[abB] (17.2)	94.0±1.7[cC] (16.0)	79.5±3.5[bB] (18.3)	50.2±6.4[A] (14.1)	< 0.001
p	0.043	< 0.001	< 0.001	0.159	
			%DPPH in D fraction		
0	0.06±0.00 (0.01)	0.02±0.01[a] (0.01)	0.15±0.06[a] (0.05)	0.27±0.15 (0.10)	0.116
24	0.29±0.13 (0.07)	0.62±0.18[c] (0.15)	0.37±0.06[b] (0.12)	0.45±0.06 (0.16)	0.114
72	0.41±0.15 (0.11)	0.33±0.04[b] (0.07)	0.18±0.03[a] (0.05)	0.32±0.01 (0.10)	0.167
120	0.22±0.07[A] (0.05)	0.41±0.05[bcB] (0.10)	0.33±0.06[abAB] (0.10)	0.38±0.03[AB] (0.11)	0.047
p	0.108	0.005	0.018	0.291	

[a,b,c] Values within column with different small superscript letters are significantly different ($p < 0.05$). [A,B,C] Values across rows with different capital superscript letters are significantly different ($p < 0.05$). p- p-value, FerT-fermentation time in hours. Values in brackets represent the actual D or SND ABTS, DPPH and FRAP contents, respectively in µmol (TE)/g dm, µmol (TE)/g dm, µmol (Fe^{2+})/g dm. (n=3).

Table 2.3.4 Total DPPH and FRAP capacities of fractions of *in vitro* digested tef *injera*

FerT	Varieties				p
	Quncho	Tsedey	Zagurey	Zezew	
	%DPPH in SND fraction				
0	1.15 ± 0.16^{aB} (0.25)	0.76 ± 0.13^{aA} (0.21)	0.94 ± 0.04^{AB} (0.31)	0.64 ± 0.06^{A} (0.22)	0.013
24	2.06 ± 0.04^{bB} (0.52)	1.21 ± 0.12^{aA} (0.29)	1.11 ± 0.34^{A} (0.36)	0.67 ± 0.13^{A} (0.24)	0.002
72	1.00 ± 0.19^{a} (0.27)	1.28 ± 0.16^{b} (0.28)	1.24 ± 0.30 (0.35)	0.88 ± 0.02 (0.28)	0.215
120	1.06 ± 0.03^{aB} (0.27)	1.09 ± 0.15^{aB} (0.26)	0.80 ± 0.04^{A} (0.23)	0.62 ± 0.06^{A} (0.17)	0.004
p	< 0.001	0.044	0.358	0.057	
	%FRAP in D fraction				
0	3.57 ± 0.25^{abA} (0.61)	12.4 ± 0.1^{C} (2.1)	3.31 ± 0.24^{aA} (0.90)	6.56 ± 0.49^{B} (1.8)	< 0.001
24	4.28 ± 0.11^{bA} (1.00)	10.3 ± 0.4^{C} (2.7)	2.89 ± 0.39^{abA} (1.1)	6.13 ± 1.10^{B} (2.8)	< 0.001
72	3.34 ± 0.32^{aA} (0.87)	10.1 ± 1.8^{C} (2.9)	2.41 ± 0.26^{aA} (0.87)	6.00 ± 0.64^{B} (2.5)	< 0.001
120	3.20 ± 0.37^{aA} (0.80)	12.9 ± 0.7^{C} (3.1)	2.26 ± 0.20^{aA} (0.90)	6.29 ± 0.42^{B} (2.6)	< 0.001
p	0.024	0.053	0.006	0.787	
	%FRAP in SND fraction				
0	14.7 ± 0.1^{abB} (2.5)	13.1 ± 0.2^{bB} (2.2)	10.5 ± 1.8^{A} (2.8)	10.6 ± 0.8^{bA} (3.0)	0.005
24	15.5 ± 0.7^{bD} (3.7)	11.8 ± 0.9^{abC} (3.1)	9.73 ± 0.15^{B} (3.8)	7.65 ± 0.41^{aA} (3.5)	< 0.001
72	12.4 ± 0.7^{aC} (3.2)	8.91 ± 0.86^{aA} (2.6)	10.5 ± 0.0^{B} (3.8)	8.24 ± 0.31^{aA} (3.5)	< 0.001
120	12.5 ± 1.2^{a} (3.1)	11.2 ± 2.1^{ab} (2.7)	9.24 ± 0.27 (3.7)	9.31 ± 1.42^{ab} (3.8)	0.064
p	0.016	0.016	0.352	0.008	

[a,b] Values within column with different small superscript letters are significantly different ($p < 0.05$). [A,B,C] Values across rows with different capital superscript letters are significantly different ($p < 0.05$). p- p-value, FerT-fermentation time in hours. Values in brackets represent the actual D or SND ABTS, DPPH and FRAP contents, respectively in µmol (TE)/g dm, µmol (TE)/g dm, µmol (Fe^{2+})/g dm. (n=3).

The %DPPH radical scavenging capacity of the D fraction of the unfermented *injera* were lower than the fermented *injera* in that the %DPPH of the D of the fermented *injeras* exhibited 1.4-22-folds higher DPPH radical scavenging capacity. Unlike the PCs in the D fraction, the PCs in the SND fraction did not show any consistent pattern on the DPPH radical scavenging capacity following the fermentation. The DPPH radical scavenging capacity of the absolute total bioaccessible PCs in the D and SND fractions ranged from 0.18-0.52 µmole Trolox/g dm injera. The Pcs in both the D and SND fractions showed increase in FRAP as fermentation of the *injera* increased from 0 to 120 h, however the actual FRAP of PCs in the D and SND fractions seemed to vary only slightly within a variety, indicating that there was no any proportional increase in FRAP as fermentation increased from 0 to 120 h.

The PCs in both the D and SND fractions showed an increase in %FRAP as fermentation of the *injera* increased from 0 to 120 h, however the absolute FRAP of PCs in the D and SND fractions seemed to vary only slightly within a variety. The total %FRAP of the bioaccessible PCs of the different *injeras* across the varieties ranged from 10 to 28 and the absolute FRAP of the bioaccessible PCs ranged from 3-7 µmol (Fe^{2+})/g dm. Literature on the ABTS and DPPH radical scavenging and FRAP of bioaccessible PCs of tef or its food products is nonexistent. The total bioaccessible PCs of the D and SND fractions showed absolute ABTS and DPPH radical scavenging capacity that ranged from 17 to 27 µmole Trolox/g, 0.18 to 0.52 µmole Trolox/g dm *injera*, respectively.

The Pearson's correlation coefficients between the %TPC and %TFC of D and SND fraction with % ABTS, %DPPH and %FRAP of the D and SND fractions in general were very weak. The % TPC of D fraction showed a weak correlation (r = 0.345, p = 0.036) with % ABTS of D fraction while no correlated with %DPPH and %FRAP of the D fractions. Moreover, the %TPC in SND fraction only correlated with % FRAP of the SND fraction (r = 0.536, p < 0.001). The %TFC in D fraction only correlated with % ABTS of D fraction (r = -0.541, p = 0.002) while %TFC of SND fraction correlated with %DPPH and %FRAP of the SND fraction (r = 0.363, p = 0.038) and (r = 0.538, p < 0.001), respectively. The reason for the low correlations could be attributed to the difference in the composition of the phenolic extracts in the D and SND fractions that can lead to the difference in their potential of scavenging ABTS and DPPH radicals and FRAP. Another reason could be the contribution of the antioxidant capacity from Maillard reaction products (Yu and Beta, 2015) which obviously could follow a different reaction mechanisms with ABTS, DPPH and FRAP.

2.3.5 Conclusions

Tef injera fermentation increased the soluble phenolic contents, and thus an increase in bioaccessibility of phenolic compounds was hypothesized. However, the increase in soluble phenolic content of injera following the fermentation was not reflected in an increased bioaccessibility of phenolic compounds nor in an increased antioxidant capacity as measured by DPPH and FRAP in the D and SND fraction. Nonetheless, an increased ABTS radical scavenging capacity in the bioaccessible fraction was observed for fermented tef injera compared to non-fermented one.

CHAPTER 3: TRADITIONAL FERMENTATION OF TEF *INJERA*: IMPACT ON *IN VITRO* IRON AND ZINC DIALYSABILITY

CHAPTER 3: TRADITIONAL FERMENTATION OF TEF *INJERA*: IMPACT ON *IN VITRO* IRON AND ZINC DIALYSABILITY

3.1 Abstract

The aim of this study was to evaluate the *in vitro* bioaccessibility of Fe and Zn in a backslop fermented flat bread known as *injera*. The Ethiopian traditional fermentation reduced PA in the range of 49-66% in different tef varieties. Molar ratios of PA:Fe and PA:Zn decreased from 14 to 1 and from 63 to 19, respectively, after 120 h of fermentation. The total soluble fractions of Fe and Zn ranged between 11 and 38% and between 11 and 29%, respectively, after 120 h of fermentation. The bioaccessible Fe content of the white varieties ranged between 3 and 9% after 120 h fermentation while no effect was observed for the brown varieties. The bioaccessible Zn ranged between 2 and 11%, with only a clear effect of fermentation in one white variety. Consumption of tef could be a good source of Fe and Zn, but may not provide the absolute recommended daily Fe and Zn intakes.

3.2 Introduction

Iron and zinc deficiencies are highly prevalent in the world, *i.e.* ranked 9th and 11th, respectively, in the list of the major risk factors for global burden of disease and they predominantly occur in developing countries (Lachat et al., 2006; Raes et al., 2014). In Ethiopia, an estimated prevalence risk of about 14% and 81% was reported for Fe and Zn deficiency, respectively. These values are among the highest on the African continent (Joy et al., 2014). Deficiency of Fe principally causes anemia and diseases of the immune system, whereas that of Zn causes growth retardation, impaired cognitive and immune system development (Humer and Schedle, 2016).

Increasing the efficiency of the release of minerals during gastro-intestinal digestion (Raes et al., 2014) and artificial food fortification with micronutrient powders (Paganini et al., 2016) were suggested as potential strategies to improve the Fe and Zn status of individuals. Biofortification of staple crops is also known as one of the sustainable strategies to help combat iron and zinc deficiencies of malnourished rural populations in developing countries (Dhuique-mayer, 2017). However, due to the non-existence of governmental regulations to fortify major food sources in Ethiopia, dietary food remained as the sole source of Fe and Zn. Bioaccessibility of Fe in animal-based food products ranges between 15 and 35%, while it is only about 10% in plant-based food products (Zimmermann et al., 2005). Bioaccessibility of non-heme Fe and Zn in plant-based food is mainly inhibited by PA, PCs and calcium (Humer and Schedle, 2016). Different and/or combinations of food processing techniques, *e.g.,* sprouting, malting, fermentation and heat treatment, have been reported as effective strategies for elimination and/or degradation of many of the mineral inhibitors (Humer and Schedle, 2016; Platel and Srinivasan, 2016; Raes et al., 2014).

In Ethiopia, consumption of plant-based food complemented with almost no animal-based food prevails due to the poor economic background and religious–inspired dietary habits. About 44% of the Ethiopian population are orthodox religion followers (CSA (Central Statistic Agency), 2007). This religion strongly prohibits consumption of any animal based food products for roughly 215 days of the year which forces majority of the population to be exclusively dependent on cereals and legume based foods. Tef [*Eragrostis tef* (Zucc.) Trotter is processed into different forms of food, mainly as *injera*, porridge but also as gluten-free cake bread and pasta (Zhu, 2018). *Injera*, a fermented soft and porous pancake made of

different cereals, preferably from tef, occupies the traditional Ethiopian food staple. Different reports indicate a wide range of Fe levels (5-150 mg/100 g dm) and a moderate range of Zn levels (2-4 mg/100 g dm) in tef (Abebe et al., 2007; Baye et al., 2014; Mamo and Parsons, 1987). Although tef could be a good source of Fe due to its unusually high Fe levels, the co-existence of high contents of inhibitors, as investigated for other (pseudo)cereals, *e.g.,* PA, tannins and PCs, might impair its dialysability (Raes et al., 2014). The inhibitory effect of these anti-nutrients could even be exacerbated by consumption of tef as a whole grain.

Previous studies on the efficiency of traditional fermentation for improving the bioaccessibility of Fe and Zn in tef *injera* were merely based on PA:mineral molar ratios (Abebe et al., 2007; Umeta et al., 2005; Urga and Narasimha, 1997). However, this molar ratio method was claimed to be not reliable for predicting physiological bioaccessibility of minerals in both white and brown tef varieties, if other mineral-binding anti-nutrients are involved (Baye et al., 2014). White and brown tef varieties do not have different physical properties apart from their seed color (Bultosa, 2007), however, it has been revealed that brown tef varieties contained higher phenolic (part 2.1) and Fe content (Abebe et al., 2007) compared to white tef. Information on the effect of the traditional fermentation on the reduction of inhibitory compounds and on the possible improvement of the bioaccessibility of Fe and Zn in *injeras* made of pure white and brown tef varieties is limited. Hence, the objective of this study was the investigation of the effect of the Ethiopian traditional fermentation on the *in vitro* bioaccessibility of Fe and Zn in tef *injera* using known tef varieties of brown and white colored seed coats.

3.3 Materials and Methods

Chemicals and Reagents: α-amylase from porcine pancreas (Type VI-B, > 10 units/mg solid), pepsin from porcine gastric mucosa (3200-4500 units/mg protein), pancreatin from porcine pancreas (8xUSP, P7545**),** bile from porcine bile extract (P1001879903), dialysis membranes (MMCO 12400 Da, 99.99% retention, width 32 mm, height 30 m, D0530-100 FT), gallic acid, catechin, Folin-Ciocalteu reagent, 2,2-bipyridine, thioglycolic acid (TGA), PA sodium salt and vanillin were purchased from Sigma-Aldrich (Belgium). Technical grade CH_3OH, $FeCl_3$, NaOH, HCl, KCl, NaCl, KH_2PO_4, $NaHCO_3$, $CaCl_2(H_2O)_2$, $MgCl_2(H_2O)_6$, NH_4Cl and HNO_3 were acquired from VWR Chemicals (VWR international, Leuven, Belgium). ICP multi-element standard solution IV was purchased from Inorganic Ventures, the Netherlands.

Samples and sample preparation: The preparation of tef sample and fermented tef *injera* was done as described in part 2.2 section 2.2.3.3. *Injeras* from each of the 4 varieties as used in part 2.2 were prepared from three independent replicate fermentations. Prior to mineral and PA analysis, the *injeras* were oven-dried (105°C) 24 h, until a constant moisture level was obtained (Abebe et al., 2007). Subsequently, the *injeras* were ground into fine flour using a porcelain mortar and pestle until a 16 mesh (1.19 mm pore size) could be passed. Frozen *injeras* were used for total phenolic compound analysis while grinded dried *injeras* were used for phytic and mineral analysis. For *in vitro* digestion, fresh *injera* (sampled 1 hour after baking) were used. Part of the *injeras* was stored at -20°C, part was oven dried and another part was used fresh, depending on the type of analysis.

3.3.1 Determination of phytic acid

The PA content was determined spectrophotometrically (Reichwald and Hatzack, 2008). Samples of flours of dried ground *injeras* (0.1 g) were put into screw capped test tubes, followed by the addition of 1 M HCl (1 mL). Subsequently, the samples were incubated in a vigorously shaking water bath at 100°C for 45 min. After cooling to room temperature, the samples were centrifuged for 5 min at 13,000 x g. Supernatant aliquots (500 µL) were transferred to new tubes and diluted with 2 mL of deionized water. $FeCl_3$ (800 µL) was added to the diluted solution (400 µL) or standard and then this mixture was incubated at 100°C for 45 min in a vigorously shaking water bath. The samples were cooled in an ice bath for 15 min to allow the formation of an iron-phytate precipitate and subsequently centrifuged at 13000 x g for 10 min at 0 °C. Supernatant aliquots (600 µL) were transferred to cuvettes, followed by the addition of 800 µL of the complexing reagent (consisting of 1 g 2,2-bipyridine and 0.13 mL thioglycolic acid in 100 mL 0.2 M HCl). Finally, the absorbance was measured at 540 nm.

3.3.2 Determination of total phenolic

The extraction and analysis of both soluble and bound phenolic content was done as described in part 2.1 sections 2.1.3.1 and 2.1.3.2, respectively.

3.3.3 Determination of tannins

The tannin content was determined using the vanillin-HCl method according to Herald et al. (2014) and Price et al. (1978). Test tubes were filled with 1 mL of soluble or bound extract and

5 mL of vanillin reagent (50:50 mixture of 5% vanillin and 24% HCl w/v), followed by incubation (20 min, 30 $^{\circ}$C) in a water bath. A second set of control tubes containing 1 mL of extract was prepared using the same procedure but the vanillin reagent was replaced by 12% HCl. The absorbance for the solutions of both sets was measured at 500 nm. The final absorbance was corrected by subtracting the absorbance obtained for the sample control from that obtained for the corresponding vanillin-containing sample. The tannin contents of the soluble and bound extracts were combined and reported as tannin content. Catechin was used as a standard and the tannin content was expressed as mg CE/100 g dm.

3.3.4 Measurement of mineral content

Samples were dry ashed and solubilized in HNO_3 according to Ashoka et al. (2009) and the minerals were quantified *via* inductively coupled plasma-mass spectrometry (ICP-MS) (ThermoScientific, Germany) (Mataveli et al., 2010; Rodushkin et al., 1999).

3.3.5 Static *in vitro* digestion and bioaccessibility measurement

The static *in vitro* digestion was carried out according to the **INFOGEST** standardized consensus model (Minekus et al., 2014) as described in part 2.3 section 2.3.3.3.

3.3.5.1 Iron and zinc determination

After the simulated intestinal digestion phase, the dialysis bags were taken out, rinsed and dried using a paper cloth. Then, the contents, which include dialyzed (D) Fe and Zn, were transferred into falcon tubes. The remaining digestion solution was centrifuged at 4,000 x g. Afterwards, the supernatants, containing soluble but nondialysable (SND) Fe and Zn, and the pellets, containing insoluble (In) Fe and Zn, were collected separately. Prior to determination of Fe and Zn *via* ICP-MS (Mataveli et al., 2010; Rodushkin et al., 1999), the D, SND and In fractions were oven-dried, dry-ashed and solubilized using 1 M HNO_3. Finally, the D%, SND% and In% of Fe and Zn were calculated as follows:

$$\text{D\%} = \left(\frac{D}{D + SND + In} \right) x\ 100$$

$$\text{SND\%} = \left(\frac{SND}{D + SND + In} \right) x\ 100$$

$$\text{In\%} = \left(\frac{In}{D + SND + In} \right) x\ 100$$

Throughout the text, solubility refers to the sum of D+SND fractions while bioaccessibility refers to the D fraction.

3.3.6 Statistical analysis

To assess differences among tef varieties and fermentation times Two-way analysis of variance (ANOVA) was performed. As the interaction between the main factors fermentation time x tef variety was always significant ($p < 0.05$), the data were further subjected to one-way ANOVA. Multiple comparison was done by Tukey's Honest Significant Differences (HSD) multiple rank test at $p < 0.05$. All statistical analyses were performed using SPSS version 22 (SPSS Inc., Chicago, IL, USA). All analyses were carried out in triplicate. Results were reported on a dry matter basis.

3.4 Results and Discussion
3.4.1 Mineral and tannin contents of tef flour

Mineral and tannin contents of the four tef varieties studied in this research are provided in Table 3.1. Significant differences ($p < 0.05$) between the different varieties were also found for all minerals analyzed, except for Cu. In this study, Zezew showed the highest Fe and Ca contents of 30 and 188 mg/100 g, respectively. In agreement with our results, Hager et al. (2012b) reported similar contents of Fe, Zn, Mn, Cu, Na, K, Mg and Ca for tef harvested in the Netherlands. However, in this study, the Zezew variety, which has a deep dark brown seed color, showed up to 2.5- to 3.5-fold higher Fe levels compared to the other varieties. In contrast, Fe levels ranging between 36 and 150 mg/100 g dm, which is 4-5 fold higher than our results, were reported for tef harvested in Ethiopia (Abebe et al., 2007; Baye et al., 2014). Different studies have shown that brown tef varieties contain higher Fe levels than white ones (Abebe et al., 2007; Baye et al., 2014). This was also partially proven in our work because the Zezew variety, which has a deep brown seed coat color, showed up to 3.5-fold higher Fe level, while Zagurey, which has a brown seed coat color, showed Fe content similar to the white varieties.

Table 3.1 Ash, mineral (mg/100 g) and tannin (mg CE/100 g) dm contents of tef flour

Variety	Ash (g/100 g)	Fe	Zn	Ca	Mg	Mn	Na	K	Cu	Tannin
Quncho	2.21 ± 0.04^a	8.71 ± 0.17^a	2.19 ± 0.07^a	141 ± 4^b	187 ± 7^a	4.36 ± 0.10^b	5.41 ± 0.58^a	413 ± 14^a	0.70 ± 0.01	107 ± 6^b
Tsedey	2.21 ± 0.08^a	11.0 ± 0.2^b	2.29 ± 0.05^a	142 ± 4^b	191 ± 5^a	5.79 ± 0.03^c	7.93 ± 1.03^b	414 ± 14^a	0.70 ± 0.14	65 ± 5.0^a
Zagurey	2.43 ± 0.03^a	12.1 ± 0.1^b	2.49 ± 0.05^b	128 ± 2^a	201 ± 2^{ab}	4.02 ± 0.05^a	6.92 ± 1.12^{ab}	473 ± 7.1^b	0.71 ± 0.28	222 ± 1^c
Zezew	3.85 ± 0.02^b	30.3 ± 2.0^c	2.46 ± 0.05^b	188 ± 9^c	214 ± 11^b	4.19 ± 0.09^b	9.12 ± 1.41^b	417 ± 17^a	0.76 ± 0.20	302 ± 13^d
P	< 0.001	< 0.001	< 0.001	< 0.001	0.008	< 0.001	0.01	0.001	0.97	< 0.001
Tef*	2.37	7.36	3.63	180	184	9.24	12	427	0.81	

[a,b,c,d] Values within a column with different letters are significantly different ($p < 0.05$). (n=3). Tef*: USDA data of mineral composition of unknown tef variety; used here for the purpose of comparison.

Since the varieties studied in this research were grown under the same conditions, the difference in the mineral contents can only be attributed to the difference in genotype. A relationship was observed between the color of the seed coat beans and the Fe content, *i.e.* colored seed coat beans contain higher Fe levels compared to white seed ones (Moraghan et al., 2002). This relationship was attributed to the presence of higher levels of tannin in the colored beans and the ability of tannins to complex Fe.

In this study, all tef varieties showed tannin levels that ranged between 65 and 302 mg CE/100 g dm flour, but the brown varieties (Zezew and Zagurey) showed 2 to 4.6-fold higher tannin level compared to the white ones (Tsedey and Quncho). Tannins content of injera made of tef was reported elsewhere in the range of 16-65 CE mg/100 g dm (Baye, 2014; Umeta et al., 2005). Also Parker et al. (1989) suggested the existence of tannins in red tef which they associated with the presence of pigmented osmophilic material detected only in developing brown tef grain (in our study, the name 'brown tef' is used instead of 'red tef). The fact that in our study the white tef varieties showed lower tannins than the brown ones and the dark brown variety exhibited the highest levels is in agreement with the suggestion of (Parker et al., 1989) and tef indeed contains tannin. However Bultosa and Taylor (2004) contend that they did not detect any significant level of tannins in any of the white or brown tef samples they studied (methods and varieties not disclosed).

On the other hand, the results of our study are in agreement with the observation by Moraghan et al. (2002) in that Zezew, which contained up to 3-fold higher tannin levels compared to the white varieties, also contained a higher (3.5-fold) Fe level. Previous studies have shown that lactic acid produced by the fermenting lactic acid bacteria (LAB), known as the dominating micro-organisms in the traditional fermentation process is responsible for the pH drop (Fischer et al., 2014). It has been suggested that acid production, during fermentation may be the major mechanism of LAB to improve mineral bioaccessibility (Poutanen et al., 2009). Solubility of non-heme iron is highly affected by pH and also by the redox potential of the environment (Ndlid, 2003). Acidity tends to increase ionization as well as favor the ferrous state, which has greater solubility at intestinal pH than does the ferric state (Clydesdale, 1982). Thus, the low pH in the fermented food matrix will help to reduce the ferric iron into ferrous form which then increases its bioaccessibility because the later can form a soluble complex with weak mineral chelators such as ascorbic acid, amino acids and monosaccharides throughout the simulated gastrointestinal digestion tract. Ascorbic acid with its reducing and

chelating properties, is the most efficient enhancer of non-heme iron absorption when its stability in the food vehicle is ensured (Teucher et al., 2004). Mineral enhancers can be added as pure compounds or as food-to-food fortification approach with local food matrices rich in mineral enhancers.

3.4.2 Impact of fermentation of tef on phytic acid and total phenolic contents

The pH values of the doughs used to prepare tef *injera* are presented in Fig. 3.1A. The pH significantly changed during the fermentation process. All varieties showed a substantial drop in pH from 5.8 (prior to fermentation) to 3.4 (after 72 h of fermentation). The PA contents of *injeras* baked after 24, 72 and 120 h of fermentation are shown in Fig. 3.1B. The PA content of the unfermented *injeras* of all varieties ranged between 1205 and 1552 mg/100 g dm. After fermentation, the PA content was reduced by 49-66%. Together with a substantial pH drop, all the varieties showed a drastic drop in PA content within the first 24 h of fermentation, followed by a further slight decrease in PA content, and finally reached their lowest PA levels after 72 h of fermentation, except for Quncho which showed a significant reduction in PA content observed after 120 h. Different cereals including oats, rye and wheat exhibited maximal endogenous phytase activity in the pH range of 5-6 (Konietzny and Greiner, 2002) which could explain why all the tef varieties studied here also showed the largest drop in PA content within the first 24 h fermentation in which the pH at the start of the fermentation was in the range of 5.6-5.8 as shown in Fig. 3.1A. During fermentation of cereals, endogenous phytase are reported to play the major role in decreasing PA while the importance of lactic acid bacteria was only to reduce the pH and create favorable conditions with limited phytase activity (Reale et al., 2007). Therefore, the LAB fermentation is mainly used to create an optimum pH for the phytase enzyme to act on PA degradation. This fact could explain the slight degradation of the PA as the acidity further increased or as the pH deviates from its optimum range of phytase activity, during the 24 to 120 h course of the fermentation. Studies on the backslop fermentation of tef dough showed different magnitudes of PA degradation in the range of 42%-80% (Abebe et al., 2007; Fischer et al., 2014; Urga and Narasimha, 1997).

In addition to the creation of optimum pH for the phytase enzyme to act on PA, the lactic acid production could also induce a PA hydrolysis effect (Clydesdale and Camire, 1983). This could explain why most of the tef varieties attained their lowest PA contents at 72 h which

corresponds to the lowest pH. However, the reason why the PA showed an increasing tendency in line with the increasing tendency of pH when the fermentation further progressed to 120 h could be attributed to a phosphorylation process.

The difference in the potential of backslop fermentation to reduce the PA content could be associated with the difference in microbiota and the endogenous phytase activity, owing to the differences in source of the materials, particle size of the flour, variety, harvest season, duration and temperature of fermentation. Endogenous flour phytase activity dominates the activity of sourdough microflora phytase during fermentation of cereals (Poutanen et al., 2009).
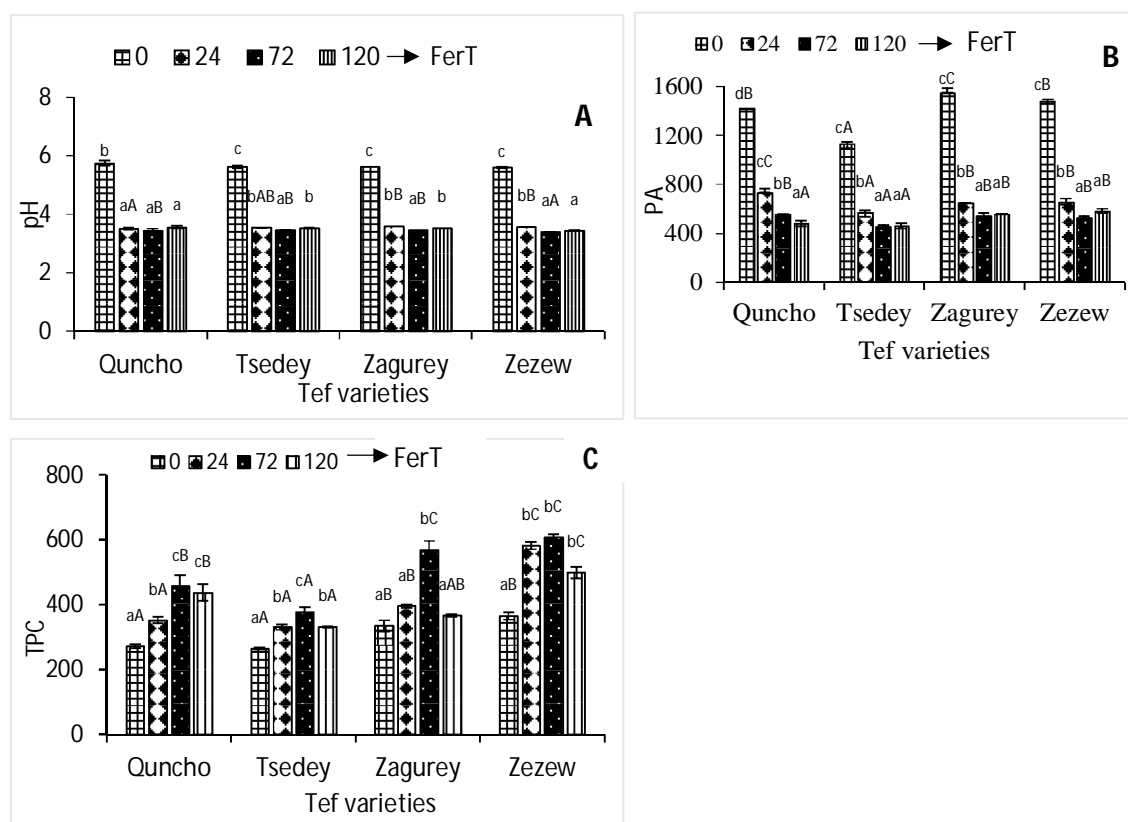
Figure 3.1 pH of dough, phytic acid and total phenolic contents of injera

(A) pH of unfermented and fermented tef dough, (B) PA content (mg 100 g^{-1} dm *injeras*) and (C) TPC (mg GAE)/100 g dm *injeras* made from unfermented and fermented dough of four tef varieties. The error bars indicate the standard deviation of the means. (n=3). [a,b,c] Values within same variety with different small superscript letters are significantly different (p < 0.05). [A,B,C] Bars of same color with different capital superscript letters are significantly different (p < 0.05). FerT-fermentation time in hour.

Thus, the difference in the extent of PA destruction could largely be attributed to the difference in variety as endogenous phytase activity could be dependent on the variety of the cereal.

In cereals, minerals and PCs are mainly localized in the bran of a seed (Raes et al., 2014). The majority of these PCs exists in bound form (part 2.1) and exert a mineral inhibitory effect. The TPC of the unfermented and fermented *injeras* of the different tef varieties studied is shown in Fig. 3.1C. Although the TPC content in all varieties increased significantly by 42%-70% after fermentation, the proportion of the bound phenolic content in the same *injeras* as used in this study decreased from 83% to 68% (part 2.2) leading to a reduced inhibitory effect on the mineral bioaccessibility. As expected, a decreased content in galloyl- or catechol-bearing PCs, *e.g.* gallic acid and protocatechuic acid was observed in the bound fraction of the PCs in *injera* after 120 h of fermentation (part 2.2). Thus, the decrease in the mineral co-existing bound PCs could improve the mineral solubility and consequently, increase the bioaccessibility of the minerals in the small intestine.

The PA:Fe and PA:Zn molar ratios, which are frequently used to predict mineral bioaccessibility, are presented in Fig. 3.2A and 3.2B, respectively. All varieties showed a 3- to 4-fold decrease in the PA:Fe molar ratio after 120 h of fermentation. In concordance with the PA:Fe molar ratio, also a ~3-fold decrease in the PA:Zn molar ratio was observed for all varieties. Many researchers have tried to make associations between the exact amount of PA left after fermentation (or any other process) and the bioaccessibility of Fe and Zn. The adverse effect of PA on Fe bioaccessibility seems only to be eliminated by decreasing the PA content to a level below 100 mg/100 g dm (Greffeuille et al., 2011). Moreover, Hurrell (2004) suggested a degradation of more than 90% of the phytate content and/or even a complete dephytinization to reduce the PA:Fe molar ratio to a value < 1 or preferably < 0.4 for enhancing the Fe bioaccessibility. Based on the WHO (1996) recommendations, PA:Zn molar ratios with a value < 5, with a value between 5 and 15 and with a value > 15 would result in a bioaccessibility of 55%, 35% and 15% for Zn, respectively. Based on these recommended molar ratio predictions, the traditional fermentation used in this study was not able to reduce the PA content below the suggested levels to significantly increase the bioaccessibility of Fe and Zn.

3.4.3 Effect of fermentation on Fe and Zn i*n vitro* bioaccessibility

The *in vitro* bioaccessible Fe and Zn contents of *injera* fermented for 0, 24, 72 and 120 h are displayed in Table 3.2. The %bioaccessible Fe of the two white varieties increased as fermentation progressed, *i.e.* for *injeras* made of Quncho from 3 to 9% after 120 h fermentation and Tsedey from 3.5 to 6% within the first 24 h of fermentation. For both brown varieties, no effect of fermentation on the %bioaccessible Fe was observed. However, the %bioaccessible Fe of the *injeras* made of Zagurey (7-9%) was considerably higher than those made of Zezew (2-2.9%).

The %bioaccessible Zn content of the *injeras* made of Quncho and Zagurey increased as a function of the fermentation time, while for the other varieties, no clear effect of fermentation was observed. Literature on Fe and Zn bioaccessibility in tef food products is limited. Baye et al. (2014) reported a 51-96% destruction of PA (resulting in a remaining PA content of 652-33 mg/100 g dm) in *injeras*, made of a 1:1 mixture of tef and sorghum, after 42-46 h of fermentation, resulting in 1-2.5% bioaccessible Fe. These results indicate that even major destruction of PA could not be sufficient for improving the bioaccessibility of Fe and Zn.
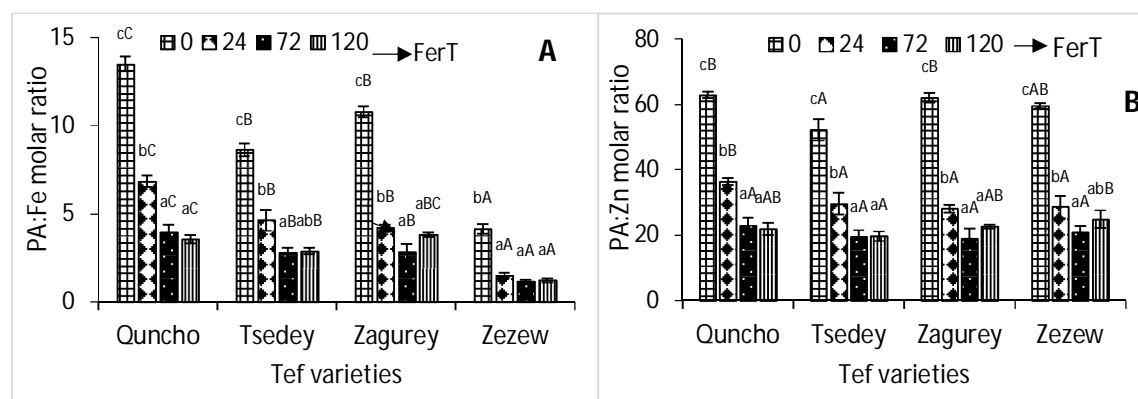
Figure 3.2 Phytic acid to iron and zinc molar ratios

(**A**) PA:Fe molar ratios and (**B**) PA:Zn molar ratios of *injeras* made from unfermented and fermented dough of four tef varieties. The error bars indicate the standard deviation of the means. (n=3). [a,b,c] Values within same variety with different superscript of small letters are significantly different ($p < 0.05$). [A,B,C] Values of bars of same color of different varieties with different superscript of capital letters are significantly different ($p < 0.05$). FerT-fermentation time in hour.

This can also clearly be observed in the *injeras* made of the Zezew variety which showed a comparable reduction in PA level after fermentation to that of other varieties but, still, showed a very low %bioaccessible Fe. This leads to the hypothesis that not only PA, but also other inhibitors, *e.g.,* PCs and tannins, could have a pronounced effect on the binding of Fe

and Zn. Our results are in agreement with this hypothesis because Zezew also contained the highest tannin and TPC contents. A decreased bioaccessibility of Fe and Zn in tannin- and phenolic-containing cereals has been reported in previous studies (Lestienne et al., 2005; Towo et al., 2006).

Calcium, which is present in tef in very high concentrations (Table 3.1), can potentially also exert an inhibitory effect on the bioaccessibility of Fe with increased severity at higher concentrations (Hallberg et al., 1991). This can be explained in that Zezew variety, which showed the lowest proportional bioaccessible Fe level in the soluble fraction in the *injeras* (Table 3.2), also showed the highest level of Ca . According to Hallberg et al. (1991), two mechanism have been proposed by which Ca inhibits iron absorption: 1) an indirect effect *i.e.* by inhibiting the enzymatic degradation of the phytic acid because it forms insoluble Ca-phytate complex. 2) by direct inhibition on Fe absorption in which this mechanism is not known but theoretically, calcium could, for example, affect the balance between intraluminal ligands, influence gastrointestinal transit time, decrease iron uptake by receptor competition, or interfere with the transfer of Fe through the mucosal cells.

Although the varieties Quncho, Tsedey and Zagurey have relatively lower Fe levels and 2 to 3-fold higher PA:Fe molar ratios (Fig. 3.1B) compared to Zezew, they showed a 3 to 4-fold higher bioaccessibility of Fe compared to Zezew. This result further proves the work of Baye et al. (2014) which reported that in the presence of other mineral binding antinutrients like galloyl containing PCs, the use of PA:mineral molar ratio for prediction of Fe or Zn bioaccessibility could be misleading and it should be treated with caution.

The proportions of SND and insoluble (In) Fe and Zn in the *in vitro* digested *injeras* are given in Table 3.2. During fermentation, the %SND Fe of *injeras* of Quncho variety tended to increase (p = 0.081), while those of Zezew decreased. Tsedey and Zagurey did not show a clear trend of increase or decrease. For the *injeras* made of Tsedey and Zagurey, an increase in the %SND Zn was established during fermentation, but for the *injeras* made of Quncho and Zezew no uniform increasing or decreasing trend could be established. No clear effect on %insoluble Fe was observed after fermentation among the varieties, while for Zn a decreasing trend was observed after 72-120 h of fermentation.

The total solubility (calculated as D% + SND%) in *injeras* of Quncho, Tsedey, Zagurey and Zezew was in the range of 11-36, 28-33, 25-38 and 11-19%, and of 13-29, 11-24, 9-25 and 15-29% for Fe and Zn, respectively, when fermented from 0 to 120 h. Similarly, an increase of 53 and 62%

of Fe and Zn solubility in white and brown fermented tef *injeras*, respectively was revealed (Urga and Narasimha, 1997).

Higher *in vitro* Fe and Zn total solubility of *injeras* of the same variety at different fermentation times did not necessarily show a higher bioaccessible Fe and Zn content (Table 3.2). Although the total solubility of Fe was relatively higher compared to that of Zn in all *injeras* of all varieties, the proportion of bioaccessible Zn (22-50%) was higher than that of Fe (9-33%). This phenomenon suggests not only that higher solubility does not always mean a higher bioaccessibility, but also proves that Fe and Zn seem to be associated with different compounds or undergo different interactions, influencing the proportion of the bioaccessible fraction in the total soluble part. It was revealed that PCs show a higher affinity or stronger chelating effect towards Fe than towards Zn cations (Olivares et al., 2013; Sreenivasulu et al., 2010).

The recommended daily Fe intake range in mg/day are (i) 0.58-0.93 for infants and children, (ii) 1.4-3.27 for female adolescents, (iii) 1.46-1.88 for male adolescents, (iv) 1.13 for female adults and (v) 1.37 for male adults (FAO and WHO, 2001).

Healthy adults are assumed to consume about 500 g dm of a mixed diet daily (World Health Organization, 1996). The estimated frequency of injera consumption in Ethiopia is about three meals a day, each meal including an *injera* of about 100 g (with an average of 35 g dm/100 g *injera*).

Considering Fe content in the range of 8.7-30 mg/100 g dm for all varieties (Table 3.1), a person consuming the above diet, will get soluble and bioaccessible Fe in the range of 1.6-7 and 0.28-1.2 mg Fe/day, respectively. These results indicate that either the amount of tef consumption should be increased or each meal should be complemented with other Fe-rich food sources to fulfil the daily requirements. Biofortification of food staples has also been recommended as a more sustainable approach to increase the iron content in plant foods, by increasing seed ferritin, the natural iron store, (Bouis, 1996; Theil et al., 1997). Increasing iron content/intake, however, will not be successful in eliminating iron deficiency anemia unless the diet is also low in iron absorption inhibitors or contains enhancers of iron absorption (Lucca et al., 2002). This is also observed in our result i.e. Zezew variety contained about 3 fold higher iron content (Table 3.1) than the other varieties but still it showed a low dialysability (Table 3.2).

Table 3.2 Iron and zinc contents of fractions of *in vitro* digested tef *injera*

FerT	Quncho	Tsedey	Zagurey	Zezew	p	Quncho	Tsedey	Zagurey	Zezew	p
	Dialyzable (D) % Fe *injera* dm					Dialyzable (D) % Zn *injera* dm				
0	2.88±0.39[aAB]	3.53±0.23[aB]	7.47±0.20[C]	2.06±0.03[A]	0.001	3.54±0.17[aB]	4.24±0.09[B]	2.33±0.55[aA]	9.06±0.07[C]	0.001
24	4.30±0.31[aAB]	6.26±1.14[bAB]	7.13±0.28[B]	2.90±1.30[A]	0.032	3.49±0.59[aA]	5.04±1.26[A]	4.81±0.25[aA]	7.30±0.55[B]	0.004
72	7.44±0.99[bB]	5.59±0.70[abAB]	7.21±1.33[B]	2.91±0.73[A]	0.014	5.25±0.18[a]	7.59±3.49	10.9±2.6[b]	11.0±2.5	0.07
120	8.84±0.59[bB]	4.51±0.75[aA]	9.18±1.82[B]	2.59±0.33[A]	0.005	8.23±1.2[bAB]	5.55±1.53[A]	11.2±1.2[bB]	9.44±0.25[AB]	0.018
p	0.001	0.028	0.332	0.771		0.002	0.430	0.002	0.107	
	soluble nondialyzable (SND) % Fe *injera* dm					soluble nondialyzable (SND) % Zn *injera* dm				
0	14.5±4.4	27.0±5.4	27.3±2.0	21.0±1.8[b]	0.07	18.6±4.4[bB]	6.60±1.08[aA]	6.66±0.69[aA]	10.1±2.5[aA]	0.010
24	25.0±6.5[AB]	19.1±9.8[AB]	31.1±1.6[B]	10.6±0.6[aA]	0.04	9.18±1.24[a]	11.9±2.2[b]	8.59±11.09[a]	8.10±0.25[a]	0.121
72	28.4±2.9	22.9±8.0	18.2±2.4	16.4±0.2[b]	0.08	17.5±0.6[bC]	12.6±0.6[bAB]	14.2±21.9[bB]	11.1±0.6[aA]	0.001
120	22.9±4.3	25.2±1.4	28.9±10.5	8.06±2.39[a]	0.08	21.2±0.2[bB]	18.6±1.4[cAB]	14.3±0.25[bA]	19.4±3.7[bAB]	0.039
p	0.081	0.704	0.146	0.004		0.001	0.001	0.006	0.010	
	Insoluble (In) % Fe *injera* dm				p	Insoluble (In) % Zn *injera* dm				p
0	82.4±4.0[bB]	69.4±6[AB]	65.2±2.2[A]	77.0±2.0[aB]	0.037	77.7±4.0[bA]	88.4±0.6[bB]	91.0±1.2[dB]	80.8±2.6[abA]	0.005
24	70.8±6.8[abA]	74.7±9[AB]	62.8±0.1[A]	87.1±2.7[bB]	0.014	87.7±1.8[c]	83.1±3.6[b]	86.5±0.9[c]	84.6±0.5[b]	0.193
72	64.2±2.0[a]	71.5±7	74.5±3.7	81.3±2.2[a]	0.063	77.2±0.8[bA]	83.2±1.9[bB]	75.0±0.7[aA]	777.9±2.9[abA]	0.024
120	68.3±3.7[aA]	70.3±0.4[A]	61.9±8.7[A]	89.3±3.3[bB]	0.025	71.3±1.4[aA]	75.2±0.1[aAB]	80.3±1.1[bB]	71.9±5.1[aA]	0.041
p	0.022	0.848	0.095	0.005		0.001	0.003	0.001	0.045	

[a,b,c] Values within a column with different superscripts of small letters are significantly different ($p < 0.05$). [A,B,C] Values across rows with different superscripts of capital letters are significantly different ($p < 0.05$). p: p-value. FerT: duration of fermentation in hours. (n=3).

Therefore, when biofortification is foreseen to alleviate Fe and Zn deficiency, not only increasing the content of Fe and Zn but also decreasing the inhibitors is important, e.g. by the addition of exogenous phytase to improve bioaccessibility of the minerals. An introduction of ferritin gene from *Phaseolus vulgaris and* thermo-tolerant phytase from *Aspergillus fumigatus* into rice grains has been shown to have great potential for increased iron bioaccessibility (Lucca et al., 2002). In addition to biofortification, focusing on processing techniques that increase the accessibility of minerals is also necessary. The recommended daily intakes of Zn in mg/day (FAO and WHO, 2001) are (i) 0.4-1.65 for infants and children, (ii) 2.15 for female adolescents, (iii) 2.55 for male adolescents, (iv) 1.5 for female adults and (v) 2.1 for male adults. Assuming the same diet as stipulated for Fe, a person including 105 g dm *injera* in his diet per day would get total soluble and bioaccessible Zn in the range of 0.28-0.73 and 0.06-0.29 mg/day, respectively. Thus, the absolute recommended daily Zn intakes are not fulfilled by consuming only three *injeras* a day. Hence, the tef diet needs to be complemented with other Zn-rich food sources to boost the daily Zn intake of the consumers.

3.4.4. Conclusions

Tef varieties studied in this research showed significantly different mineral, total phenolic and tannin contents. The brown tef varieties (Zagurey and Zezew) showed higher tannin, total phenolic, Fe and Zn contents compared to the white ones (Tsedey and Quncho). The Ethiopian traditional tef *injera* fermentation reduced the existing PA by more than 50% and slightly improved the total solubility and bioaccessibility of Fe and Zn. Phenolic compounds, Ca and tannin could also play a role in inhibiting the bioaccessibility of Fe and Zn in tef *injera*. Fermentation seems to favor the solubility and bioaccessibility of Fe and Zn, but the effect was dependent on the tef variety. Although brown tef varieties contain substantially higher Fe levels, the concomitant inhibitors may result in a lower bioaccessibility of Fe and therefore, result in the same or even a lower daily Fe intake than for white tef varieties with lower Fe levels. Thus, tef can be a potential source of Fe and Zn but it may not fulfil the recommended daily intakes of Fe and Zn. Reduction of PA alone via fermentation does not guarantee improvement of Fe or Zn bioaccessibility in the presence of other mineral binding PCs. Therefore, to further enhance the bioaccessibility of Fe and Zn, fortification with mineral enhancers such as ascorbic acid could be important.

CHAPTER 4: TEF STARCH *IN VITRO* DIGESTIBILITY AND ESTIMATED GLYCEMIC INDEX

Part 4.1 *In Vitro* Starch Hydrolysis and Estimated Glycemic Index: Tef Porridge and *Injera*

4.1.1 Abstract

The aim of this study was to investigate the *in vitro* starch digestibility of *injera* and porridge from seven tef varieties and to estimate their glycemic index. The total starch, free glucose, apparent amylose, resistant, slowly digestible and rapidly digestible starches of the varieties ranged between 66-76, 1.8-2.4 g/100 g flour dm, 29-31%, 17-68, 19-53, 12-30 g/100 g starch dm, respectively. After processing into *injera* and porridge, the rapidly digestible starch content increased by 60-85% and 3-69%, respectively. The estimated glycemic index of porridge and *injera* of the varieties ranged 79-99 and 94-137 when estimation is based on model of Goni et al. (1997) whereas from 69-100 and 94-161, respectively based on Granfeldt et al. (1992). Fresh tef porridge and *injera* can be classified as medium- high eGI foods, not to be considered as a proper food ingredient for diabetic patients and people in weight gain control.

4.1.2 Introduction

Frequent consumption of high glycemic index (GI) carbohydrate foods is increasingly associated with higher risk of obesity, coronary heart disease, type 2 diabetes, cancer and other chronic syndromes. Glycemic index of a particular meal determines the rate of blood glucose rise (Cassidy et al., 1994; Deckere et al., 1995; Jenkins et al., 1987). Type 2 diabetics prevalence of Ethiopia adjusted to its national population was 4.4% (about 4.14 million) in 2013 and is projected to be 5.1% (about 7.75 million) by 2035 (Guariguata et al., 2014).

There is no single solution to suppress the increase of postprandial blood glucose level and the associated health disorders, however, incorporating organic acids in a meal, slow and low heat cooking process, replacing portions of carbohydrates by proteins, use of whole flour breads and fruits and vegetables has been recommended. Adherence to low GI food and/or limited amount intake of high GI foods has been also reported as a major mitigation strategy to control the increase of blood glucose level in people with diabetes type 2 and to those of engaged in body weight management (Karl et al., 2015).

Based on digestibility, starches are categorized as rapidly digestible starch (RDS)-starches hydrolyzed within the first 20 min of simulated digestion, slowly digestible starch (SDS)-starches digested within the following 100 min after RDS, and resistant starch (RS)-starches not digested within the 120 min of *in vitro* digestion (Englyst et al., 1992). RDS causes a rapid increase in blood glucose level after ingestion, whereas SDS releases glucose slowly and consistently over an extended time. RS which resists enzymatic hydrolysis, is fermented in the large intestine releasing short chain fatty acids which are considered as beneficial (Lehmann and Robin, 2007). The rate of digestion of a typical starchy food is influenced by its botanical origin, which consequently determines the structure and shape of starch granules and the amylose content (Gallant et al., 1992), physicochemical structure of the starch such as crystallinity, chain length and chain distribution, molecular weight and weight distribution (Tian et al., 2016), thermal processing and moisture content, which determine the extent of starch gelatinization (Sasaki et al., 2016) and the presence of dietary fiber that changes the microstructure of foods and limits its water availability, and thus restricting starch gelatinization (Cleary and Brennan, 2006; Holm and Bjorck, 1992).

Tef [*Eragrostis tef* (Zucc.) Trotter] is reported to have amylose content that ranged from 20-32% (Bultosa et al., 2002; Hager et al., 2012b) depicting a wide variability of normal to high

amylose starch. High amylose starches require temperatures of up to 150°C in the presence of water to fully gelatinize, which is not indeed attainable under normal cooking and baking circumstances and thus could result in foods with a lower GI (Van Amelsvoort and Weststrate, 1992). Soil and Crop Improvement (SCBV, 2007-01)- tef information map version stated that the total starch content of tef is 60 g/100 g, of which the RDS, SDS and RS accounted for 20, 50 and 30 g/100 g dm starch, respectively. Abebe et al. (2015) also showed that RS, SDS and RDS contents were in the range of 7-11, 31-41 and 29-33 g/100 g dm tef flour, respectively. Glycemic index GI of 74 and 45 for bread and egg pasta respectively from unknown tef varieties were reported by Wolter et al. (2013) and Hager et al. (2013), respectively.

Even though, there are many tef varieties differing in their seed color from milky-white to almost dark-brown, there is no study on the properties of GI of the common tef food products such as *injera* (pancake) and porridge. *Injera* and porridge are among the major food products of tef and are staples of millions mainly in Ethiopia and frequently used in Ethiopian restaurants in major cities around the world. Therefore, the aim of this study was to investigate the *in vitro* starch digestibility and estimate the GI of fresh *injera* and porridge prepared from seven tef varieties which vary in color from brown to white.

4.1.3 Materials and methods

Sample and preparation: The same tef varieties used part 2.1 were used in this study. The first five are white whereas the last two are brown seed color varieties. They were sun dried while standing on the field (before harvest) and milled by disc attrition milling at a local tef miller, in the same way as tef is normally milled for the preparation of *injera* and porridge in Ethiopia. Some portions (about 1 kg) of each variety was pre-milled prior to each variety and discarded to prevent cross-contamination among the varieties. The flour moisture contents ranged from 7.9-8.4 g/100 g flour, with an average of 8.1 g/100 g flour and were not significantly different among varieties ($p > 0.05$). The distribution of the flour particle size of the tef varieties was measured using a test sieve shaker (Endecott, LTD, London SW, England). This was in the range of 100% < 850 µm, 99-100% < 425 µm, 96-99% < 300 µm, 78-85% < 212 µm, 66-77% < 150 µm.

Fermented tef *injeras* were prepared following the procedure descibed by Urga and Narasimha (1997). Each of the varieties were backslop fermented for 42 h at 25°C and subsequently baked at 180°C for about 3 min as shown in Fig. 2.2. 1. Stiff tef porridge was

prepared following the traditional method. Briefly, tef flour and water were mixed in a ratio of 2:5 and cooked for 8 min at about 180ºC. The process of both *injera* and porridge making was based on the traditional practices in Ethiopia. The food products were sampled as eaten (fresh, when the temperature of the food was about 40ºC). Three independent preparations were made for each product from each variety.

4.1.3.1 Determination free glucose (FG) of tef flour, porridge and *injera*

The FG content was measured according to Englyst et al. (1992) using an assay kit GOPOD (glucose oxidase/peroxidase)-format K-GLUC 09/14 (Megazyme International Ireland Ltd) and was calculated as:

$$\%glucose = \frac{At \ X \ Vt \ X \ C \ X \ D}{As \ X \ Wt} x100$$

Where

At: absorbance of test solutions, **Vt**: total volume of test solutions (Vt = 25.2 plus 1 mL per gram wet weight of samples used), **C**: concentration (C = 0.394 mg glucose/mL) of standard, which may be corrected for moisture content, **D**: dilution factor = 18.

4.1.3.2 Determination of total starch and starch digestibility of flour, porridge and *injera*

Total Starch (TS): The TS content was measured according to Englyst et al. (1992) using an assay kit GOPOD-format K-GLUC 09/14 (Megazyme International Ireland Ltd), calculated and expressed as: TS = (TG − FG) × 0.9 (g starch)/100 g flour dm.

Where %TG is calculated as follows:

$$\%TG = \frac{At \ X \ Vt \ X \ C \ X \ D}{As \ X \ Wt} x100$$

Where

At: absorbance of test solutions, **Vt**: total volume of test solutions (Vt = 35.5 plus 1 mL per gram wet weight of samples used), **C**: concentration (C = 17.6 mg glucose/mL) of standard, which may be corrected for moisture content, **D**: dilution factor = 1.

Vt= 35.5 plus 1mLpergram wet weight of sample used, C=17.6 and D=1.

TG: total glucose; 0.9: glucose to starch conversion factor

Rapidly Digestible Starch (RDS): The RDS content was measured based on an *in vitro* starch digestibility procedure (Englyst et al., 1992) using an assay kit GOPOD-format K-GLUC 09/14

(Megazyme International Ireland Ltd), calculated and expressed as: RDS = (G20 − FG) × 0.9 (g RDS)/100 g starch dm.

Where; G20: glucose content after 20 minutes of simulated digestion, 0.9: glucose to starch conversion factor.

Slowly Digestible Starch (SDS): The SDS content was measured based on an *in vitro* starch digestibility procedure (Englyst et al., 1992) using an assay kit GOPOD-format K-GLUC 09/14 (Megazyme International Ireland Ltd), calculated and expressed as: SDS = (G120 − G20) × 0.9 as (g SDS)/100 g starch dm.

Where; G120: glucose content after 120 minutes of simulated digestion

G20: glucose content after 20 minutes of simulated digestion

0.9: glucose to starch conversion factor

Resistant Starch (RS): The RS content was determined based on an *in vitro* starch digestibility procedure (Englyst et al., 1992), calculated and expressed as: RS = TS − (SDS + RDS) (g RS)/100 g starch dm.

4.1.3.3 Determination of apparent amylose content tef flour

The amylose content of the starch of each tef flour was determined by the Megazyme kit K-AMYL (Megazyme International Ireland Ltd). The amylose content was calculated as:

$$\text{Amylose } (\%) \left(\frac{w}{w}\right) = \frac{\text{Absorbance (Con A supernatant)}}{\text{Absorbance (total starch aliquot)}} \text{ X } \frac{6.15}{9.2} \text{ X100/1}$$

Where: 6.15 and 9.2 are dilution factors for the Con A and total starch extracts, respectively.

Con A: Concanavalin A

4.1.3.4 Measurement of i*n vitro* glycemic index of tef porridge and *injera*

The rate of *in vitro* starch hydrolysis was analyzed following the method recommended by Goni et al. (1997). Briefly, 50 mg porridge/*injera* portion was weighed into a 50 mL screw caped test tube and HCl–KCl buffer (10 mL, pH 1.5) was added and samples were homogenized (40 sec, 2000 rpm) using an Ultra Turrax homogenizer (T18D, Germany). Then, 0.2 mL of solution containing 1 mg of pepsin from porcine gastric mucosa (P6887, Sigma Aldrich, St. Louis, MO, USA) made in 10 mL HCl–KCl buffer (pH 1.5) was added to each sample tube, followed by incubation (60 min, 40°C) in a shaking water bath. The volume was raised to 25 mL by adding 15 mL of tris–maleate buffer (pH 6.9). To start the starch hydrolysis, another 5 mL of tris–maleate buffer containing 2.6 IU of α-amylase from porcine pancreas (P7545, Sigma

Aldrich, St. Louis, MO, USA) was added to each sample. The sample containing flasks were placed in a shaking water bath at 37°C with moderate agitation and aliquots (0.1 mL) were taken from each flask every 30 min from 0-3 h. The α-amylase was inactivated immediately by placing the tubes containing the aliquots in a boiling water for 5 min. Then, 1 mL of sodium–acetate buffer (0.4 M, pH 4.75) and 30 μL of amyloglucosidase from *Aspergillus niger* (Megazyme International Ireland Ltd) were added. To hydrolyze digested starch into glucose, samples were incubated for 45 min at 60°C. Glucose concentration was measured using glucose oxidase–peroxidase kit GOPOD reagent enzymes R-GLC4 07/13 (Megazyme International Ireland Ltd). The rate of simulated starch digestion was expressed as a percentage of the total starch hydrolyzed at different times (30, 60, 90, 120, 150, and 180 min). Each analysis was performed in triplicates.

A non-linear model established by Goni et al. (1997) was applied to describe the kinetics of starch hydrolysis. The first order equation has the form: $C = C\infty\left(1 - e^{-kt}\right)$ where C corresponds to the percentage of starch hydrolyzed at time t, $C\infty$ is the equilibrium percentage of starch hydrolyzed after 180 min, k is the kinetic constant and t is the time (min). The parameters $C\infty$ and k were estimated for each variety and each treatment based on the data obtained from the *in vitro* hydrolysis procedure.

The area under the hydrolysis curve (AUC) was calculated using the equation

$$AUC = C\infty(t\infty - to) - (C\infty/k)[1 - \exp[-k(t\infty - to)]\,]$$

where $C\infty$ corresponds to the equilibrium percentage of starch hydrolyzed after 180 min, $t\infty$ is the final time (180 min), to is the initial time (0 min) and k is the kinetic constant.

The hydrolysis index (HI) was calculated as AUC of a sample as percentage of the corresponding AUC of fresh white bread (Goni et al., 1997; Granfeldt et al., 1992). The conventionally baked white bread used had a dry matter content of 62 g/100 g. The total starch content was 78 g/100 g dm and crumb of the bread was used for sampling as per the method. The eGI was calculated according to equations suggested by:

Goni et al. (1997): $eGI_G = (0.549 \times HI) + 39.71$, and

Granfeldt et al. (1992): $eGI_{Gr} = (0.862 \times HI) + 8.198$

4.1.3.5 Statistical analysis

All analyses were done in triplicate. Results are reported as mean ± standard deviation on a dry matter basis. The differences of mean values among tef varieties was determined using analysis of variance (ANOVA) followed by Tukey's Honest Significant Differences (HSD) multiple rank test at $p < 0.05$ significance level. All statistical analyses were performed using SPSS version 20 (SPSS Inc., Chicago, IL, USA).

4.1.4 Results and discussion

4.1.4.1 *In vitro* starch digestibility of flour, porridge and *injera*

The total starch, apparent amylose, free glucose and starch fraction of the flour of different tef varieties are given in Table 4.1.1. The TS content of the tef varieties ranged from 66-76 g/100 g dm, with a significant different TS content between Zezew and Tsedey variety ($p < 0.05$). Similarly a TS content of 72-76 g/100 g dm was reported in tef (Abebe and Ronda, 2014; Giuberti et al., 2016) whereas slightly lower contents in the range of 58-60 g/100 g dm were reported by Hager et al. (2012) and Soil and Crop Improvent (SCBV, 2007-01). The highest and lowest FG contents were 1.76 and 2.4 g/100 g dm, with Zezew having a significantly lower TG content compared to Boset, Simada, Tsedey and Zagurey varieties ($p < 0.05$). The apparent amylose content of the varieties ranged from 29-31%. In agreement with these results, Bultosa et al. (2002) reported a range of 25-32% in five tef varieties, while Hager et al. (2012) reported a lower amylose content of 20%. The difference in the TS and amylose contents of tef varieties from different sources could be attributed to at least the difference in genotype, harvesting season and growing geographical location. Tef samples used in our study and by Giuberti et al. (2016) were grown in Ethiopia and the U.S.A, respectively whereas the studied samples of Hager et al. (2012) were grown in The Netherlands. Nhan and Copeland (2014) reported that growing location and harvesting season of five Australian wheat varieties were significantly affected in their total starch and amylose content. They also showed a strong positive correlation between starch content and prevailing number of clear and warm days. The higher altitude, and warm temperature weather condition prevailing throughout the year in Ethiopia compared to the low altitude and cold temperature weather in The Netherlands maybe attributed to the difference in TS and amylose contents of tef.

Table 4.1.1 Free glucose and starch properties of tef varieties

Variety	Free glucose (g/100 g dm flour)	Apparent amylose (%)[A]	Total starch (g/100 g dm flour)	Starch fraction (g/100 g dm starch)		
				RDS[a]	SDS[b]	RS[c]
Bosset	2.32±0.17[b]	28.7±3.0	67.9±0.98[ab]	26.9±5.4[cd]	40.9±5.9[bc]	32.2±0.47[b]
Dega	2.08±0.10[ab]	30.3±1.6	67.4±0.54[ab]	29.7±5.3[d]	53.4±8.6[c]	16.9±3.3[a]
Quncho	2.11±0.01[ab]	30.6±1.4	70.5±1.0[ab]	24.3±2.6[bcd]	42.0±5.6[bc]	33.7±3.1[b]
Simada	2.30±0.08[b]	29.0±2.1	67.4±0.84[ab]	17.8±0.21[abc]	19.3±1.1[a]	62.9±0.86[c]
Tsedey	2.40±0.17[b]	30.6±1.4	76.3±6.8[b]	12.2±1.8[a]	19.2±5.2[a]	68.4±6.9[c]
Zagurey	2.18±0.06[b]	30.0±1.5	69.0±0.40[ab]	12.0±0.45[a]	22.8±0.33[a]	65.1±0.11[c]
Zezew	1.76±0.16[a]	30.0±1.9	66.0±1.1[a]	14.9±1.4[ab]	27.5±5.6[ab]	57.6±4.2[c]
p-value	0.004	0.789	0.042	0.003	<0.001	<0.001

[a,b,c] Values within column with different superscript letters are significantly different ($p < 0.05$). [A] Apparent amylose content in acetate buffer measured spectrophotometrically after removal of free and bound lipids. (n=3). [a]Rapidly digestible starch, [b]Slowly digestible starch, [c]Resistant starch.

The RS, SDS and RDS g/100 g dm of the flour of the different tef varieties are given in Table 4.1.1. RS, SDS, RDS ranged widely from 17-68, 19-53 and 12-30 g/100 g dm, respectively and were significantly different among varieties (p < 0.05). Soil and Crop Improvement (SCBV, 2007-01) revealed RS, SDS and RDS of flour as 30, 50 and 20 g/100 g, respectively. Abebe et al. (2015) also showed RS, SDS and RDS fractions in the range of 7-11 g/100 g, 31-41 g/100 g and 29-33 g/100 g dm of flour in five tef varieties. The starch fraction in the report of Abebe et al. (2015) is relatively smaller both from ours and from that of SCBV and this could be due to the difference in the calculation, in that the prior was based on the total flour sampled instead of the total starch content.

After cooking the flour into porridge and *injera* (Table 4.1.2), RDS increased by 60-85% and 3-69%, respectively. When cooked into porridge, all the varieties showed a decrease in SDS and RS by 32-76% and 60-91%, respectively but no uniform decrease or increase was seen in the case of *injera*. The dramatic increase of the RDS in porridge and injera shows that a significant gelatinization took place during the cooking process. This increase of RDS could be a good predictor that these food products would result in high GI. Similarly, Roopa and Premavalli (2008) indicated a 63% increase in RDS while a decrease of SDS and RS, respectively by 40 and 30% after cooking of finger millet flour. When native starch is heated in the presence of water, it absorbs water and starts to swell or gelatinize and this causes the weakening of bonds between starch and protein making it easy for the hydrolytic enzymes to act on. So, during cooking or baking the amount of RS and SDS starch decrease while the RDS starches increase. Although fat, protein and antinutritional factors like tannins, PA and PCs could have a decreasing effect on starch digestibility, SDS, RS and RDS composition of the starch are the major determinants in the rate of its digestibility and the resulting glycemic index (Meynier et al., 2015).

Table 4.1.2 Free glucose (FG) and starch fractions of tef food products

Variety	Porridge				Injera			
	FG^A	RDS^{aB}	SDS^{bB}	RS^{cB}	FG	RDS^{aB}	SDS^{bB}	RS^{cB}
Bosset	3.91±0.18^c	67.5±4.0	19.7±3.3	12.9±0.7^{bc}	2.34±0.24^{ab}	27.6±2.1^{ab}	36.1±2.9^{ab}	36.4±4.9^b
Dega	3.89±0.34^c	75.6±5.9	21.7±4.6	2.64±1.3^a	2.57±0.01^b	26.5±1.2^a	39.4±0.4^{ab}	34.1±0.9^b
Quncho	3.66±0.14^c	65.9±12	25.5±8.7	8.59±3.0^{abc}	3.62±0.07^c	32.4±0.9^{bc}	39.8±0.5^{ab}	27.9±0.4^{ab}
Simada	1.25±0.03^a	74.1±0.4	12.2±2.5	13.8±2.1^c	2.98±0.40^b	27.4±1.5^{ab}	38.0±3.1^{ab}	34.6±3.8^b
Tsedey	1.09±0.09^a	79.9±3.4	13.1±4.9	7.01±1.5^{abc}	2.54±0.24^b	35.1±0.9^{cd}	43.1±1.5^b	21.7±2.4^a
Zagurey	1.38±0.20^a	70.8±11	23.2±8.8	6.03±2.0^{ab}	1.85±0.06^a	38.5±3.1^d	35.0±0.1^a	26.5±2.9^{ab}
Zezew	2.22±0.25^b	85.7±2.1	6.58±0.80	7.71±1.3^{abc}	4.53±0.08^d	32.3±0.4^{bc}	38.5±0.5^{ab}	29.3±0.9^{ab}
p	0.001	0.171	0.083	0.005	0.002	0.001	0.046	0.009

a,b,c Values within column with different superscript letters are significantly different ($p < 0.05$). ^{A}g glucose/100 g flour dm, ^{B}g starch/100 g starch. p: p value (n=3).^{a}Rapidly digestible starch, ^{b}Slowly digestible starch, ^{c}Resistant starch.

4.1.4.2 *In vitro* starch digestibility and glycemic index of tef porridge and *injera*

The *in vitro* starch digestibility of porridge and *injera* prepared from different tef varieties is summarized in Fig. 4.1.1. In the case of porridge, the total starch content hydrolyzed during the 180 min of *in vitro* digestion ranged from 68-98% with descending order of Zezew (98%) > Dega (97%) > Bosset (90%) > Simada (86%) > Zagurey (76%) > Quncho (73%) > Tsedey (68%) whereas that of *injera* ranged from 91-100% with decreasing order of Zezew (100%) > Zagurey and Quncho (98%) >Dega (97%) > Tsedey (94%) > Simada (93%) > Bosset (91%). The order of *in vitro* starch hydrolysis for the different varieties was different in the porridge samples compared to the *injera* samples.
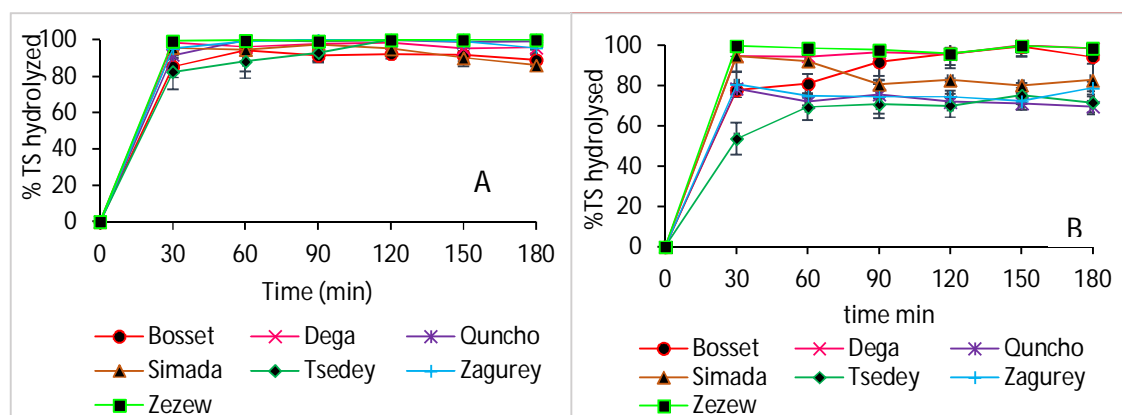
Figure 4.1.1 *In vitro* starch hydrolysis of tef food products
(A) *injera* and (B) porridge of different tef varieties. Error bars indicate the standard deviation. (n=3).

The eGI values of porridges and *injera* are shown in table 4.1.3. The eGI of both *injera* and porridge samples were calculated based on the correlation equations between HI and GI outlined by Goni et al. (1997) and Granfeldt et al. (1992), respectively taking white bread as reference food. The equations of these authors are considered as best correlation between *in vitro* and *in vivo* and are used interchangeably but obviously results in different eGI. The model of Goni et al. (1997) exaggerated eGI for food with very low HI whereas it underestimates the eGI of higher HI and the reverse is true for the model of Granfeldt et al. (1992). The correlation model designed by Granfeldt et al. (1992) always resulted in lower eGI for our products than the model of Goni et al. (1997) if the HI was less than 100, whereas if HI was greater than 100 the opposite was true. The difference in the eGI of both models gets bigger as the HI gets far from 100. Therefore, we found it worthy to use both models to overcome future

inconsistencies of reports from similar products and aiming at giving complete information for readers.

The eGI of porridge and *injera* varied significantly ($p < 0.05$) among the varieties and ranged from 79-99 and 94-137 when estimated based on model of Goni et al. (1997) (eGI$_G$) whereas from 69-100 and 94-161, respectively based on the model of Granfeldt et al. (1992) (eGI$_{Gr}$). The highest eGI$_G$ and eGI$_{Gr}$ was shown by Dega variety while the lowest is exhibited by Simada variety. Hager et al. (2013) reported comparatively lower eGI$_{Gr}$ of 45 for pasta made of tef and Wolter et al. (2013) an eGI of 74 for tef bread. The lower moisture content of tef bread and pasta than the porridge and *injera* (Table 4.1.3) may explain why their eGI is lower than the latter food products. The difference in the eGI of the varieties in both porridge and *injera* could be attributed to the variation of the proportion of SDS, RS and RDS, as well as in the content of other macronutrients such as fat, fiber and protein. Each of these food components and their interactions have a distinct impact on the eGI of a food product. The impact of starch fractions, macronutrients and interactions among these constituents on a food product's eGI showed a decreasing order of SDS > fiber > fat > interaction between SDS and RDS > interaction between fat and fiber > RDS (Meynier et al., 2015).

Table 4.1.3 Estimated glycemic indexes and dry matter (g/100 g) of tef food products

Var	*Injera*			Porridge		
	Dry matter	eGI$_G$	eGI$_{Gr}$	Dry matter	eGI$_G$	eGI$_{Gr}$
Bo	33.8±0.7[a]	104±0.2[ab]	109±0.3[ab]	27.8±0.5[ab]	98.5±1[b]	100±2[b]
De	34.5±1.2[ab]	137±0.6[c]	161±0.9[c]	28.1±0.2[ab]	94.0±0.7[ab]	93.4±1.0[ab]
Qu	34.8±0.6[ab]	119±5.2[bc]	133±8[bc]	28.8±0.6[ab]	78.7±6[a]	69.4±9[a]
Si	35.60±0.01[ab]	94.3±11.1[a]	94.0±17[a]	27.6±1[ab]	84.4±5[ab]	78.4±9[ab]
Te	41.7±1.0[c]	95.6±6.9[a]	96.0±11[a]	29.1±0.4[b]	88.1±5[ab]	84.2±7[ab]
Za	40.6±0.8[c]	115±0.5[b]	127±0.8[b]	27.2±0.4[a]	87.7±6[ab]	83.6±10[ab]
Ze	36.7±0.4[b]	123±4.9[bc]	139±8[bc]	27.8±0.3[ab]	94.2±0.5[ab]	93.8±0.8[ab]
p	0.001	0.001	0.001	0.018	0.029	0.029

eGI$_G$= (0.549 × HI) + 39.71, eGI$_{Gr}$= (0.862 × HI) + 8.198. [a,b,c] Values within column with different superscript letters are significantly different ($p < 0.05$). Var, tef Variety; B, Bosset; D, Dega; Q, Quncho; S, Simada; T, Tsedey; Za, Zagurey; Ze, Zezew; p, p- value. (n=3).

The relation of consumption of a starchy food and the rise in blood glucose level is better predicted by GI than by its proportion of SDS, RDS and RS (Foster-Powell et al., 2002). Therefore, interpretation of the health impact of starchy foods depending on merely the starch fractions could be misleading as it does not take into account the effect of the other macronutrients.

The eGI from *injera* were in general higher than their counterpart porridges and the decreasing order of the varieties was also not similar in both food products. The reason why varieties with higher eGI in porridge did not necessarily show high eGI in the case of *injera* or vice versa could be ascribed by the difference in the effect of the process that specifically imposes to each of the constituents. Roopa and Premavalli (2008) has indicated big differences in finger millet starch gelatinization that ranged from 20-100%, in food processing methods such as cooking, baking, autoclaving, roasting and puffing. In their result, higher starch gelatinization did not necessarily result higher starch digestibility.

Since white bread is used as reference in the *in vitro* study (GI white bread=100), the standards of low GI, medium and high GI foods are defined as GI < 60, GI (60-85) and GI > 85 (Ferng et al., 2016). So, based on this classification, only porridge from Quncho and Simada demonstrated medium eGI when eGI_G model is used but porridge from Quncho, Simada, Tsedey and Zagurey resulted in medium level if eGI_{Gr} is used.

For *injera*, independent of the variety, all samples are classified as high GI foods. The statistical variations among varieties in both *injera* and porridge do not explain the eGI level according to the classification of the international table of GI in that statistically different GI values could be categorized in the same cluster as low, medium or high.

It has been widely reported that cereals with high amylose content have lower susceptibility to α-amylase and amyloglucosidase starch hydrolysis, and thus leading to a lower eGI (Noda et al., 2002). In this study, this was not confirmed as the apparent amylose content is not the only determining factor of the rate of starch hydrolysis. In agreement to these results, it was reported that cooked high amylose rice varieties also showed higher eGI values as compared to those of having lower amylose (Frei et al., 2003). In addition to amylose/amylopectin proportion of native starch, other factors that determine its rate of enzymatic hydrolysis includes, the feature of granular morphology (Shrestha et al., 2012), arrangement of crystalline and amorphous regions in the granule, size of blocklet (Tang et al., 2006), the structure of amylose and amylopectin which explains the distributions of branch (chain)

lengths in both amylose and amylopectin (Shrestha et al., 2012), and the crystalline types ,'A' or 'B' (Buleon et al., 1998) also have large effects on the rate and extent of enzymatic hydrolysis.

Literature about the physicochemical properties of tef starch granule is limited but possible reasons why the *injera* and porridge specifically scored high eGI are listed. The flour used to make these products were milled by disc attrition which leads to a higher starch damage and this in turn causes rapid starch digestibility, eventually resulting in a higher eGI. Indeed, the particle size of flour of the tef varieties was very fine in that 66-77% of the total flour of each variety was able to pass through 150 μm. Abebe et al. (2015) has reported that flour from different tef varieties, grown in Ethiopia, milled by disc attrition showed high starch damage and consequently resulted in a high *in vitro* starch digestion as compared to corresponding flour milled by Cyclotech Sample mill indicating that the mill type can have significant effect on starch digestibility. The dry matter content of porridge and *injera* as shown in Table 4.1.3 ranged from 27-29% and 34-41%, respectively. The fact that both food products contained high moisture content, might have contributed to the higher eGI.

It was revealed that gelatinization would not be restricted if there is enough water during heating regardless of the amylose content (Tester et al., 2004). This suggests that extensive gelatinization during the porridge and *injera* cooking has occurred. It has been also reported that the extent of starch digestion of brown rice showed a significant increase after addition of the amount of cooking water (Sasaki et al., 2016). The soft texture of porridge and *injera* could also be a cause for the higher susceptibility of hydrolytic digestion. Bjorck et al. (1994) reported higher eGI for soft textured pasta porridge compared to a cooked firm pasta containing the same moisture content. The size of the tef starch granule is reported to be in the range of 2-6 μm and categorized as very small and the 'A' type of native starch crystallinity accounts for about 37% (Bultosa et al., 2002). Both these characteristics could also contribute to the higher eGI of both the studied tef products. The smaller the size of starch granule, the higher would be the surface area which inevitability increases the contact of the hydrolytic enzymes with the substrate (starch), finally resulting in a high starch digestibility (Tester et al., 2004). It has also been reported that most 'A' type crystal native starches are more sensitive to enzymatic hydrolysis than 'B' type native starches (Srichuwong et al., 2005a, 2005b). The flour particle size distribution of the varieties was in a narrow range suggesting that the effect due to particle size difference is insignificant and it was also

reported that particle size difference of flour wheat, ranging from 850-37 μm, did not show any difference in GI (Behall et al., 1999).

The possible justifications why *injera* from all the varieties showed higher eGI than the corresponding porridges are: During fermentation, endogenous and microbial α-amylases could rather facilitate degradation of starch. Indeed, natural fermentation of sorghum has caused a significant decrease in resistant starch while increasing the *in vitro* starch digestibility (Elkhalifa et al., 2004) which inevitability will increase the GI. The excessive lactic acid produced during the fermentation could actually promote weakening and disruption of protein-starch network leading to easily swelling of the starch and thus resulting in a high GI. Fermentation also causes destruction of PA which indirectly could increase the rate starch digestibility as starch digestibility is inversely related to PA content (Thompson and Yoon, 1984). Indeed, in fermented tef *injeras*, up to 50% reduction of PA was observed as indicated in part 2.2. On the other hand, extent of starch gelatinization may not be the only determining factor of resulting GI. Hurdle of factors such as how the hydrolysis enzymes interact with the starches that gelatinized in different ways depending of the processing, how the fiber and protein interact with the gelatinized starch and their effect on digestion, the presence of starch digestion inhibitors such as phenolic compounds and how these inhibitors were affected during the different processes/gelatinization could be other factors determining the ultimate starch digestion in addition to the extent of the gelatinization.

4.1.5 Conclusions

While many previous studies showed positive correlation of high amylose content of native starch and low eGI in the corresponding food products, this study revealed that all tef varieties with high apparent amylose content resulted in medium-high eGI in porridge and high eGI in *injera*. Tef varieties with high RS and SDS of flour and food products did not necessarily exhibited lower eGI than those with lower RS and SDS, revealing that the starch fraction by itself is not always a good predictor of GI. Although confirmation using *in vivo* data from the same varieties is required, fresh porridge and *injera* prepared from tef may not be a good alternative for those of diabetic patients and individuals in weight gain control.

Part 4.2: Effect of Sourdough addition and Storage Time on *In Vitro* Starch Digestibility and Estimated Glycemic Index of Tef Bread

Part 4.2: Effect of Sourdough addition and Storage Time on *In Vitro* Starch Digestibility and Estimated Glycemic Index of Tef Bread

4.2.1 Abstract

Effect of sourdough amount and storage time on starch digestibility and estimated glycemic index (eGI) of tef bread was investigated. The rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS) of 0-30% sourdough fresh tef breads ranged from 49 to 58, 16 to 29 and 20 to 26 g/100 g starch, respectively. Storage of tef breads up to 5 days decreased the RDS by more than 2-folds while SDS and RS increased by 2 and 3 folds, respectively. The eGI for fresh and stored breads had ranged from 39 to 89. Addition of sourdough increased the eGI of fresh breads while no uniform pattern was seen in the stored breads. As the storage time increased, all the breads showed a decrease in eGI. *In vivo* study is necessary to further investigate the effect of sourdough on GI of tef bread.

Redrafted from:

4.2.2 Introduction

A study revealed that the world prevalence of diabetes among adults (aged 20–79 years) will increase to 8.8% in the year 2035 affecting as much as 592million adults compared to 8.3% (382 million adults) in the year 2013 (Guariguata et al., 2014). It is an established fact that a long term frequent intake of carbohydrates with a high glycemic index produces greater insulin resistance than the intake of low glycemic-index carbohydrates. In diabetic patients, it has been reported that replacing high-glycemic-index carbohydrates with low-glycemic-index forms would improve glycemic control and, among persons treated with insulin, will reduce hypoglycemic episodes (Willett et al., 2002). Prospective randomized controlled studies by the diabetes prevention program (DPP) USA (Ratner and Face, 2006), Finnish Diabetes Prevention (DPS) (Lindstrom et al., 2003), Da Qing IGT and Diabetes China (Li et al., 2008) and Malmo in Sweden (Eriksson and Lindgiirde, 1991) all showed that lifestyle modification involving diet and enhanced physical activity effectively helps to delay or avert the progression of impaired glucose tolerance which otherwise leads to development of type 2 diabetes mellitus. Owing to this, there is a global shift of consumers from refined white flours to a minimally refined flour or whole meal as consumption of high fiber containing flours are increasingly associated with a lower risk of weight gain, cardiovascular disease and other chronic diseases (Kim et al., 2016; Patel et al., 2017; Virkamaki et al., 2001).

Tef [*Eragrostis tef* (Zucc.) Trotter] is processed into a whole meal or whole flour resulting in high amounts of fiber (Collar and Angioloni, 2014). This cereal is becoming popular among consumers in western countries as it is increasingly considered as a healthy and nutritious food. In Ethiopia where tef is highly cultivated, this cereal is used to produce traditional food products mainly *injera* (a fermented flat bread) and thick porridge. As reported in part 4.1, the freshly prepared *injera* and porridge from different tef varieties exhibited a high eGI in the range of 94-137 and 79-99, respectively. Furthermore, Wolter et al. (2013) showed an eGI of 74 for a frozen conventional tef bread. Tef bread (100% tef) or mixed with wheat flour is becoming more and more popular among Western consumers. However, there is scarcity of information on GI of 100% conventional tef bread even though this information is very crucial for consumers and dieticians. The manufacture of bread without gluten causes a major technological problems for bakers. Indeed, gluten-free breads available on the market are often of poor quality, showing low volume, poor color and crumbling crumb and mostly with

low protein and high fat contents (Segura and Rosell, 2011). It has been shown that sourdough could improve the sensory and physical qualities, including higher specific volume as well as lower crumb hardness of gluten-free breads (Rinaldi et al., 2017; Ua-Arak et al., 2017). It has also been reported that inclusion of sourdough to gluten-free bread plays a crucial role in the extension of shelf-life (Scarnato et al., 2017). As tef contains high protein content (chapter 5), it could be a good alternative to manufacture a high protein gluten-free bread. However, literature regarding tef and the effect of sourdough on the resulting physical quality and protein and starch digestibility of tef bread is scarce. Despite breads in general be it at home or in supermarkets could stay for variable storage times, information pertaining to the freshness level, particularly of tef bread and associated GI is lacking. Therefore, this study was designed to investigate the effect of sourdough addition (10, 20 and 30%) and storage time (1, 2 and 5 days) on *in vitro* starch digestibility and glycemic index of tef bread.

4.2.3 Materials and methods

4.2.3.1 Bread preparation

Flour: In this study, unknown varieties of white (mixture of white, undefined varieties) and brown (mixture of brown, undefined varieties) were used as these are commercially available as such on the Ethiopian market. From both flours, a batch of 5 kg each was purchased at a market in Mekelle, Ethiopia. The mixtures were carefully cleaned manually and then milled at a local miller (Mekelle, Ethiopia), packed in polythene pouches, brought to Belgium and stored at -20ºC until further analysis. **Sourdough:** It was prepared using a commercial starter *Lactobacillus fermentum* (Florapan LA4K; kindly provided by Lallemand, France) according to Novotni et al. (2012). Briefly, 1% (based on flour weight) LA4K starter, tef flour and 62.6% water (based on dough weight) were mixed manually and fermented in a fermentation cabinet (30ºC, 85% relative humidity (RH)) for 19.5 hours until the pH of the sourdoughs reached 3.9–4.1. The titratable acidity of the sourdough was measured by potentiometric titration using 0.1 M NaOH until a target pH of 8.5 was reached and expressed in mL of 0.1M NaOH/g of sourdough as described in Wolter et al. (2014a). **Tef Bread:** Breads were baked following the procedure of Hager et al. (2012a) with some modifications. Preliminary baking experiments were performed to be able to produce a bread of optimum quality in terms of volume and texture. It was clear that water content and inclusion of Hydroxypropyl methylcellulose (HPMC) had a significant role in determining the bread volume and texture. At the same water

level, breads with or without HPMC showed significantly different volumes. Breads with HPMC showed higher bread volume and a better texture, pointing out that the use of HPMC as an ingredient in gluten-free breads could help to have a better bread quality.

Tef bread dough was prepared by mixing tef flour, sourdough in different proportions (0, 10, 20, and 30%), 3% yeast, 2% HPMC, 2% salt, 2% sugar and 139% water, all based on dry matter flour weight. The purpose of HPMC addition is to enhance the gas holding capacity/strength of the gluten free tef dough. The dough was then immediately divided and put into baking pans and allowed to ferment or proof for 45 min in a fermentation cabinet (30°C, 85% RH) followed by baking (190°C, 45 min) in a preheated baking oven (MIWE condo, Arnstein, Germany). The 2% HPMC, 139% water amount and 45 min of fermentation or proofing were optimized from our preliminary baking tests. For each bread type, sampling was done when fresh (after 2 h of baking). The rest of the breads were stored in a closed plastic bag at room temperature for up to 5 days. **White wheat bread:** White wheat breads (triplicate), used as reference were baked using the straight-dough method as described in the AACC (2000) method No. 10-10B. Briefly, white wheat flour was mixed with 0.15% malt, 1.5% salt, 1% yeast and 60.9% water all on flour weight basis. The dough was prepared in a mixer for 7 min, rested for 10 min, divided, rounded and fermented (30°C, 30 min, 85% RH). The fermented dough was punched, sheeted and rolled and placed into a lightly oiled baking pan. The loafs were proofed (30°C, 65 min, 85% RH) followed by baking at 230°C for 30 min. in a preheated baking oven (MIWE condo, Arnstein, Germany).

4.2.3.2 Physicochemical properties tef flour

Flour particle size distribution was measured by a laser diffraction particle size distribution analyzer (model) based on the manual instruction of the instrument.

The **Falling number (FN):** FN was determined according to AACC (1999) method No. 56-81b using 7 g flour sample and 25 mL distilled water.

Rheological analysis: Pasting properties of tef flour were determined using a rheometer (Modular compact rheometer series, Anton Paar, MCR 102). Flour (3 g, 14% moisture basis) was mixed with 25 g distilled water in the RVA canister. A programmed heating and cooling cycle was used, the samples were held at 50°C for 1 min, heated to 95°C in 7.5 min, held at 95°C for 5 min before cooling to 50°C in 7.5 min and holding at 50°C for 2 min. The pasting profiles (Fig. 4.2.1) such as temperature (PTemp), peak temperature (Peak Temp), peak time (PTime), initial

viscosity (IV), peak viscosity (PV), holding strength viscosity (HSV), final viscosity (FV), breakdown (BD), setback (SB) were recorded. The viscosity was expressed in mPa.s.
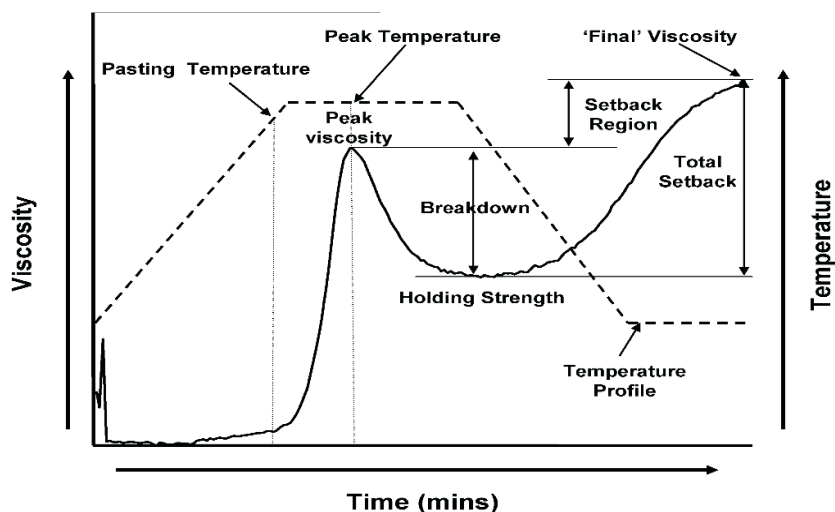
Figure 4.2.1 A typical pasting profile showing the commonly measured parameters
Adapted from Saunders (2010).

Protein content of tef flour: Total dietary protein content was analyzed by the Kjeldahl method (AOAC, 1995). To calculate the protein content (g/100 g dm) from the obtained N-content, a conversion factor of nitrogen to protein of 5.4 was used.

4.2.3.3 Bread physical features

Volume: The volume of breads was measured using a 3D Volscan Profiler (Stable Micro Systems Volscan Profiler 600, UK) following the manual of the instrument.

Bread Texture Analysis: Crumb texture (Hardness, Springiness, Cohesiveness, Chewiness and Resilience) was measured on uniform slices of 25-mm thickness according to Matos and Rosell (2013) using a texture analyzer (Stable Micro Systems Texture Analyzer). Three slices from the center of each bread (around 25 mm thickness) were used for texture evaluation. The texture analyzer was equipped with 5 kg load cell and SMS p/25 cylindrical probe (25 mm diameter). The settings used were pre-test speed 3 mm/sec, test speed 1.70 mm/sec, post-test speed 10 mm/sec, distance 11 mm and time 5 sec. Measurements were performed on four slices of from each bread.

4.2.3.4 Free glucose, starch digestibility fraction and amylose contents

The measurement of free glucose, amylose and starch digestibility fractions content were measured as described in part 4.1, sections 4.1.3.1, 4.1.3.2 and 4.1.3.3, respectively.

4.2.3.5 *In vitro* glycemic index measurement tef bread

The *in vitro* glycemic index was measured as described in part 4.1 section 4.1.3.4.

4.2.3.6 Statistical analysis

To assess differences among tef varieties and fermentation times Two-way analysis of variance (ANOVA) was performed. In case if ANOVA showed significant ($p < 0.05$) interaction between the main, data were further subjected to one-way ANOVA. Multiple mean comparison was then done by Tukey's Honest Significant Differences (HSD) multiple rank test at $p < 0.05$. All statistical analyses were performed using SPSS version 24 (SPSS Inc., Chicago, IL, USA). All analyses were carried out in triplicate and results were reported on dm.

4.2.4 Results and Discussion

4.2.4.1 Characterization of tef flour and sourdough

Particle size distribution: The average particle size distribution (Fig. 4.2.1) of both the brown (A) and white (B) tef flours were similar in that the traditional disc attrition milling resulted in fine milling *i.e.* as much as 60% of flour from both tef types had particle size of below 150 μm, 300 μm < 90% and 600 μm < 100%.
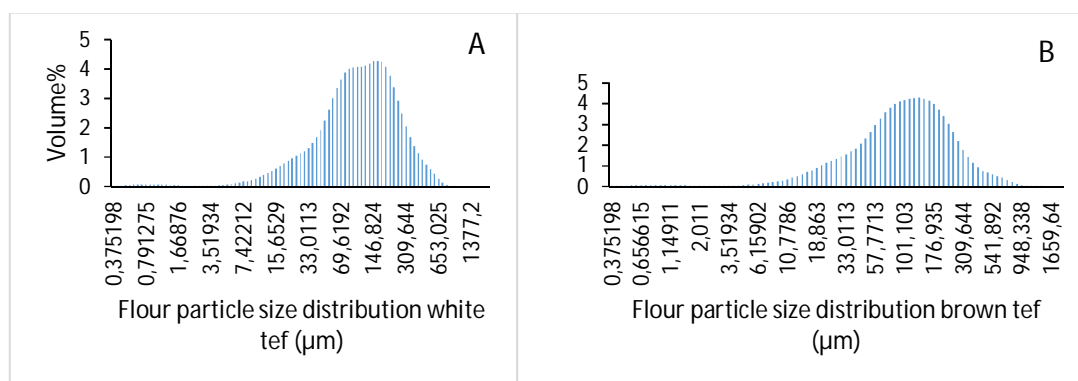

Figure 4.2.2 Particle size distribution of white and brown tef flours

The Falling Number (Table 4.2.1) of the white and brown types were 360 and 368 sec., respectively. FN is mostly used to grade wheat grain *i.e.* wheats with FN < 200 are graded as

low quality or with severe sprout damage, 300>FN>200, moderately sprout damaged and FN>300 no sprout damage and/or sound cereal (Kweon, 2010). Based on the FN wheat classification, our tef samples could be graded as sound or without any pre/postharvest sprout damage. With this, we could be confident that tef's characteristics related to its rheology, *in vitro* starch digestibility and glycemic index are triggered due to its inherent properties.

The total dietary protein of the brown and white tef flours are given in Table 4.2.1 and there was no significant difference (p < 0.05). In food processing, pasting property is mainly used to predict the processing parameters such as cooking time and temperature, thickening ability, temperature-pressure-shear induced viscosity breakdowns, gelling and retrogradation tendencies. The different pasting parameters of tef flour are given in Table 4.2.1. A significant difference in all the viscosity parameters were observed between white and brown tef (Table 4.2.1).

Compared to the pasting properties of other tef varieties reported by Bultosa et al. (2002), the values of pasting temperature, peak viscosity, holding viscosity, and final viscosity of our study are lower while, peak times is higher and breakdown and setback viscosities showed similarity. The titratable acidity of the sourdough of the brown and white tef types were 2.3 and 2.1 mL of 0.1M NaOH/g sourdough, respectively with both showing equal pH of 3.9. The titratable acidity content in this study is lower than other tef sourdough in previous reports (Novotni et al., 2012; Wolter et al., 2014a). The difference in the titratable acidity could be attributed to the difference source of tef, the starter used and the water to flour proportioned used to make the sourdough.

Obligate heterofermentative strains *Weissella cibaria* and facultative heterofermentative *Lactobacillus plantarum* were used in previous reports (Novotni et al., 2012; Wolter et al., 2014a) while we used a starter which was a mixture of lactic acid bacteria and yeast. Also the proportion of water to water to flour was in 1:1 ratio in the prior study while we used a rate of 62.6% water on dough weight basis.

Table 4.2.1 Falling Number, protein (g/100 g dm flour), pasting properties of tef flour

Tef	FN	Protein	Tef flour pasting properties								
			PTem	PeakT	PT	IV	PV	HV	FV	BD	SV
Brown	368±6	11 ± 0.6	66±0.3	92±0	9.4±0	17±0.3[b]	1371±27[a]	741±8[a]	1586±15[a]	630±19[a]	845±6[a]
White	360±1	9.0 ± 1.1	65±0.6	92±0	9.4±0	16±0.1[a]	1942±30[b]	949±4[b]	2057±10[b]	960±11[b]	1100±4[b]
p	0.192	0.107	0.125	0.312	-	0.019	0.002	0.001	0.001	0.002	< 0.001

FN: falling number in seconds, PTem: Pasting temperature (^{o}C), PeakT: Peak temperature (^{o}C), PT: Peak time (min), IV: Initial viscosity (mPa.s), PV: Peak viscosity (mPa.s), HV: Holding viscosity (mPa.s), FV: Final viscosity (mPa.s), BD: Breakdown viscosity (mPa.s), SV: Setback viscosity (mPa.s). (n=3).

4.2.4.2 Bread quality features

Volume: The specific volume of tef breads containing different sourdough proportions are shown in Table 4.2.2. As there was no significant difference in volume and texture of the breads using brown or white tef flour (Fig. 4.2.3), values are presented as mean values over the two tef types. The volumes of the 0-30% sourdough breads narrowly ranged from 1.8-1.9 mL/g.

Figure 4.2.3 Visual appearance tef bread slices
(A) 0%, (B) 10%, (C) 20% and (D) 30% sourdough of tef bread.

The specific volumes of breads in this study are much higher than previous reports of conventional tef breads with specific volumes in the range of 1.3-1.6 mL/g (Hager et al., 2012b; Marti et al., 2017). Indeed the specific volumes of our breads are higher or at least similar compared to the specific volumes of other gluten-free bread (maize, buckwheat quinoa,

sorghum and rice) and whole wheat with specific volumes ranging from 1.33 to 1.85 but lower than oat breads (2.4) mL/g (Hager et al., 2012a). The higher specific volumes of the tef breads in our study could be attributed to the difference in the formulations of the ingredients in that the breads in our study contained HPMC and higher water levels. In our study, the addition of different proportions of sourdough did not affect the specific volume. Contrarily, it has been reported that addition of sourdough, without however HPMC, to gluten-free breads showed improvement in specific volume (Axel et al., 2015; Moroni et al., 2009). Nonetheless, when HPMC is used concomitantly with sourdough, it may have a masking effect on the volume of gluten-free bread.

The metabolites produced by the lactic acid bacteria could improve the deformation capability of the dough during proofing and baking; however, in the presence of HPMC, the effect of the metabolite could be reduced/masked as HPMC can better improve the strength of the dough resulting in a larger loaf volume. The major problem in gluten-free bread baking is dough development and gas holding capacity during the leavening phase. HPMC plays a key role in the gas retention capacity as it increases the viscosity and stabilizes the gas bubbles at the liquid interface and finally resulting in bigger loaf volume breads (Mariotti et al., 2013).

Texture: The hardness of breads prepared from both the brown and white tef are displayed in Table 4.2.2. The hardness of the breads was in the range of 7.7-10.5 N and showed a slightly decreasing order as proportion of the sourdough increased from 0 to 30%. Tef bread which contained HPMC and xanthan hydrocolloids resulted in a relatively harder texture (24 N) compared to our breads (Hager and Arendt, 2013). A white wheat bread which is always used as a standard bread showed a hardness of 8.8 N (Hager et al., 2012a) which explains that our breads have an acceptable hardness. The springiness, cohesiveness, chewiness and resilience of the breads ranged from 0.87 to 0.93, 0.56 to 0.58, 3.83 to 5.56(J) and 0.27 to 0.30, respectively. Springiness and chewiness of the breads significantly decreased ($p < 0.05$) with increased proportion of sourdough while cohesiveness and resilience did not show any significant difference. There is scarce of literature on springiness, cohesiveness, chewiness and resilience of tef breads that contain HPMC and sourdough. However, tef breads without any hydrocolloid showed similar springiness (0.942) but higher chewiness (31.9 N) compared to our breads (Hager et al., 2012a).

Table 4.2.2 Specific volume (n=6) and texture (n = 8) of sourdough tef breads

SD%	SV (mL/g)	Hardness (N)	Springiness	Cohesiveness	Chewiness (J)	Resilience
0%	1.9±0.02[b]	10.4±105[c]	0.92±0.03[b]	0.57±0.03	527±49bc	0.27±0.02
10%	1.9±0.01[ab]	10.5±26[c]	0.93±0.03[b]	0.57±0.02	567±31c	0.27±0.01
20%	1.8±0.05[a]	9.2±64[b]	0.87±0.02[a]	0.58±0.04	492±63b	0.30±0.03
30%	1.9±0.01[b]	7.7±46[a]	0.87±0.01[a]	0.56±0.03	391±32a	0.28±0.02
p	0.003	<0.001	<0.001	0.543	<0.001	0.125

SD: sourdough, J: joules, N: newton, [b] Values within a column with different small superscript letters are significantly different ($p < 0.05$

4.2.4.3 Free glucose and starch fractions of tef flour and bread

The free glucose, total starch, rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS) contents of brown and white tef flours are given in Table 4.2.3. Both the brown and white tef showed similar apparent amylose content (24%). This result is slightly higher than the apparent amylose content of other tef varieties which ranged from 20-22% (Abebe and Ronda, 2015; Hager et al., 2012b) but lower than the contents of different tef varieties which ranged from 29-32% (Bultosa et al., 2002; Shumoy and Raes, 2017). The average RDS, SDS and RS contents of the flours were 26, 33 and 41 (g/100 g dm), respectively. The RDS and SDS contents of this study are in agreement while the RS is relatively higher than previous reports (Abebe et al., 2015; Shumoy and Raes, 2017).

The digestibility of the different starch fractions (RDS, SDS and RS) of 0%, 10%, 20% and 30% sourdough tef breads stored for 0, 1, 2 and 5 day are shown in Table 4.2.4. There was no clear influence of sourdough proportion on the RDS and SDS. However, RS showed a clear increasing pattern as the amount of the added sourdough increased. Similarly, fermentation of starchy slurries of breadfruit and sweet potato by amylolytic *Lactobacillus plantarum* and *Lactobacillus fermentum* caused an increase in RS due to the formation of limit dextrins by the action of α-amylase on the amylopectin part of the starch (Haydersah et al., 2012). Moreover, contrary to our results, a decrease in RS was revealed in sourdough added frozen tef breads (Wolter et al., 2014a).

The contradiction could be attributed to the difference in the starter cultures used and duration of fermentation. Obligate heterofermentative strain *Weissella cibaria* and facultative heterofermentative *Lactobacillus plantarum* were used in the latter while a mixture of lactic acid bacteria and yeast was used in our case. Furthermore, unlike to the latter study which used shorter fermentation time (30 min), in our study a longer fermentation time (45 min) was used. The extra time could have enabled the α-amylase to act on the amylopectin resulting in more limit dextrins which in turn increase the RS proportion.

Table 4.2.3 Free glucose and starch properties of brown and white tef flours

Types	Free glucose*	Amylose (%)	Total starch*	Starch fraction (g/100 g dm starch)		
				RDSᵃ	SDSᵇ	RSᶜ
Brown	0.5 ± 0.09	23.9 ± 0.62	74.6 ± 2.60	26.1 ± 0.16	32.4 ± 2.53	41.5 ± 2.64
White	0.5 ± 0.02	23.8 ± 0.48	76.7 ± 1.96	26.3 ± 0.68	33.1 ± 3.40	40.6 ± 3.33
p	0.507	0.895	0.319	0.668	0.785	0.732

*(g/100 g dm flour), p: p value, (n = 3). ᵃRapidly digestible starch, ᵇSlowly digestible starch, ᶜResistant starch.

Table 4.2.4 Starch fractions and free glucose contents of sourdough added tef breads of different storage time (day)

Storage	Sourdough %				p	Sourdough %				p
	0	10	20	30		0	10	20	30	
	RDSᵃ (g/100 g dm starch)					SDSᵇ (g/100 g dm starch)				
0	52±3.4ᵇᴬ	49±2.4ᶜᴬ	51±1.6ᵈᴬ	58±1.6ᵈᴮ	<0.001	29±2.6ᵃᴮ	29±1.9ᵃᴮ	28±2.1ᵃᴮ	16±1.5ᵃᴬ	<0.001
1	40±2.3ᵃᴬᴮ	44±2.7ᵇᴮ	40±4.5ᶜᴬᴮ	35±0.7ᵇᴬ	0.001	39±2.9ᵇᴮ	33±2.3ᵇᴬ	33±0.76ᵇᴬ	35±1.8ᵇᴬ	0.008
2	39±2.1ᵃᴮ	38±1.9ᵃᴬᴮ	35±1.6ᵇᴬ	38±0.97ᶜᴬᴮ	0.015	37±3.0ᵇᴬᴮ	33±1.9ᵇᴬ	40±0.21ᶜᴮ	33±2.1ᵇᴬ	0.006
5	35±3.3ᵃᴮ	37±1.3ᵃᴮ	28±2.7ᵃᴬ	26±1.0ᵃᴬ	<0.001	35±1.9ᵇᴬᴮ	32±2.1ᵇᴬ	38±2.0ᶜᴮᶜ	39±3.1ᶜᶜ	0.002
p	<0.001	<0.001	<0.001	<0.001		<0.001	<0.001	<0.001	<0.001	
	RSᶜ (g/100 g dm starch)					Free glucose (g/100 g dm flour)				
0	20±1.3ᵃᴬ	22±1.2ᵃᴬ	23±2.6ᵃᴬᴮ	26±2.0ᵃᴮ	0.005	1.2±0.11ᵃᴬ	1.4±0.38ᴬ	1.8±0.18ᴮ	1.4±0.22ᵃᴬ	0.002
1	23±2.0ᵃᵇᴬ	25±1.7ᵇᴬᴮ	30±4.8ᵇᶜᴮ	30±2.5ᵇᴮ	0.005	1.7±0.26ᵇᴬ	1.6±0.26ᴬ	1.7±0.20ᴬ	2.7±0.07ᶜᴮ	<0.001
2	24±1.5ᵇᴬ	29±1.0ᶜᴮᶜ	27±2.7ᵃᵇᴮ	31±1.5ᵇᶜ	<0.001	2.0±0.10ᶜᴮ	1.5±0.28ᴬ	1.7±0.16ᴬᴮ	1.9±0.41ᵇᴮ	0.020
5	30±1.8ᶜ	31±2.3ᶜ	33±1.7ᶜ	33±3.2ᵇ	0.132	1.6±0.06ᵇ	1.6±0.36	1.5±0.34	1.7±0.11ᵃᵇ	0.634
p	<0.001	<0.001	<0.001	0.004		<0.001	0.794	0.142	<0.001	

ᵃ,ᵇ,ᶜ,ᵈ Values within a column with different small superscript letters are significantly different (p < 0.05). ᴬ,ᴮ,ᶜ Values across rows with different capital superscript letters are significantly different (p < 0.05). p: p-value. (n=6). ᵃRapidly digestible starch, ᵇSlowly digestible starch, ᶜResistant starch.

The RDS fractions of the sourdough breads clearly demonstrated a decreasing order as the age of the breads get older, while SDS and RS contents showed a uniformly increasing pattern as the breads get older (Table 4.2.4). All the breads showed significant difference ($p<0.001$) in RDS at all the storage days with the highest and lowest contents exhibited by the fresh and 5 days old breads, respectively independent of the sourdough proportion. The highest and lowest SDS and RS contents of all the breads were exhibited after 5 and 0 storage days, respectively. In agreement to our results, different rice varieties, cooked and stored for 1 to 7 days at 4°C showed a significant decrease in the RDS content while their SDS and RS contents significantly increased (Rachel et al., 2015a, 2015b). Unlike to the breads in our study that exhibited low RDS and relatively high SDS and RS, corn and potato based low moisture commercial gluten-free bread revealed significantly higher RDS and very low SDS and RS contents in the range of 75-93, 2-21 and 1-3 g/100 g dm, respectively (Segura and Rosell, 2011) indicating an almost complete gelatinization process obviously leading to a higher GI.

The RDS content of the fresh breads in our study (Table 4.2.4) showed nearly a 2-fold increase as compared to the RDS of the flours and/or the native starches (Table 4.2.3). However, it can be concluded that tef breads retain high amount of non-gelatinized starches (native starches) after baking compared to the corn and potato based gluten-free bread in which RDS accounts for up to 93 g/100 g of the starch (Segura and Rosell, 2011). Native starch is considered as a mixture of linear and highly branched polymers that assemble together to form an ordered granular architecture and the changes that it undergoes during heating and cooling are major determinants of its functional properties for processing and digestion. The increase in the RDS content following the baking process could be principally attributed to the heating process. When starch is heated in excess water, its granules undergo an irreversible phase transition, known as gelatinization, in which the native starch is disrupted and loses its structure. Starch gelatinization has been broadly defined as the collapse (disruption) of molecular orders (breaking of hydrogen bonds) within the starch granule manifested in irreversible changes in properties such as water uptake, granular swelling, crystallite melting, unwinding of double helices, loss of birefringence, starch solubilization and viscosity development (Wang and Copeland, 2013). The increase of the RDS contents of the freshly baked breads (Table 4.2.4) were accompanied by a nearly 2-fold decrease of RS as compared to their corresponding flour RS contents.

During ageing of the breads from day 0 till day 5, a reverse process called crystallization/ retrogradation could have taken place. This can be evidenced by the successive decrease of RDS while SDS and RS increased almost becoming closer to their flours' nature. It is a fact that storage of starch gels at temperatures from 4 to 30°C induces crystallization of amylopectin with the fastest crystallization occurring at 4°C (Wang et al., 2015). It was also reported that once the bread is cooled down, the retrogradation of starch begins immediately turning the starch into a more ordered state, where both amylose and amylopectin form double helical associations (Jacobson et al., 1997; Klucinec and Thompson, 1999). This process is well demonstrated by all bread types in our study. As the age of the breads increased from 0 to 5 days, the proportion of the starch fraction exhibited a closer similarity to their corresponding flour starch fraction proportions.

The added sourdough did not show any clear pattern variation in the rate of retrogradation process. The decrease of RDS and increase of SDS and RS of the breads during storage was also accompanied by loss of water mainly in breads of no or lower sourdough containing breads. The dry matter contents of the breads used in this study ranged from 44% in fresh to 53% in the 5 days old breads (results shown in brackets in Table 4.2.5). The free glucose (FG) content of the tef breads is displayed in Table 4.2.4. The FG content of the breads did not show any uniform trend owing to the difference in sourdough proportion and storage days. Nonetheless, it showed 2-5 fold increase compared to its flour FG contents (Table 4.2.3). The increase in FG could be explained by the enhanced starch hydrolysis action of α-amylase and the yeast during the fermentation process.

4.2.4.4 Estimated glycemic index (eGI)

The estimated glycemic index (eGI) of the brown and white tef breads is given in Table 4.2.5. The eGI was calculated by using both models of Goni et al. (1997) (eGI$_G$) and Granfeldt et al. (1992) (eGI$_{Gr}$) as these models are used interchangeably but could result in different eGI as indicated in part 4.1. Increasing the proportion of the sourdough has increased the eGI of the fresh breads while there was no uniform pattern in the eGI of the stored breads. In line to our results, sourdough breads of quinoa and buckwheat showed higher eGI in comparison to their corresponding control breads while tef and sorghum sourdough breads showed lower eGI than the control breads (Wolter et al., 2014a).

Table 4.2.5 Estimated glycemic index (eGI) of sourdough added tef breads of different storage ages in days

	Sourdough proportion%					Sourdough proportion%				
	0	10	20	30		0	10	20	30	
ST	eGI_G				p	eGI_{Gr}				p
0	75 ± 9^b (45)	83 ± 7^b (44)	85 ± 3^c (44)	89 ± 1^c (44)	0.006	72 ± 2.4^{cA}	82 ± 11^{cAB}	77 ± 0.88^{cAB}	86 ± 1.7^{bB}	0.026
1	72 ± 5^b (46)	70 ± 6^a (45)	74 ± 3^b (46)	74 ± 9^b (45)	0.784	58 ± 8.5^b	67 ± 10^b	62 ± 4.5^b	54 ± 11.5^a	0.283
2	66 ± 2^{abA} (48)	69 ± 3^{aAB} (47)	74 ± 3^{bB} (48)	73 ± 5^{bB} (45)	0.025	51 ± 2.2^{bA}	55 ± 4.3^{bAB}	62 ± 5.3^{bB}	60 ± 4.01^{aAB}	0.034
5	57 ± 3^{aA} (53)	67 ± 2^{aB} (48)	63 ± 3^{aAB} (51)	62 ± 7^{aAB} (45)	0.009	39 ± 3.8^{aA}	50 ± 2.3^{aBC}	45 ± 3.9^{aAB}	52 ± 1.0^{aC}	0.001
p	0.002	0.002	< 0.001	<0.001		<0.001	0.001	<0.001	<0.001	

eGIG = (0.549 X HI) + 39:71, eGIGr = (0.862 X HI) + 8.198. [a,b,c] Values within a column with different small superscript letters are significantly different (p < 0.05). [A,B] Values across rows with different capital superscript letters are significantly different (p < 0.05). p: p-value. (n=6). ST, storage time in days. Values in brackets () are the dry matter contents of the breads.

Sourdough fermentation resulted in a soft bread crumb (Wolter et al., 2014b) and a softer bread structure was reported to increase starch hydrolysis (Fardet et al., 2006). Indeed, as is seen in Table 4.2.2, the hardness of the breads was reduced when the amount of the added sourdough increased which could explain the increased eGI.

The highest and lowest eGI were recorded for fresh and the 5 days old breads in all the breads regardless of the amount of sourdough as seen in Table 4.2.5. So far, high eGI in the range of 94-137 and 79-99, respectively for freshly prepared *injera* (fermented flat bread) and thick porridge were reported as shown in part 4.1. The reason why tef *injera* and porridge exhibited much higher eGI than the tef breads could be attributed to the difference in processing, ingredients and mainly moisture contents of the products. The moisture content of the fresh *injera* and porridge which ranged from 71-73% and 59-66%, respectively (part 4.1) was very high compared to that of 47-56% in tef bread. It has been well evidenced that at high water content (water/starch >1.5 or higher) and high temperature reaching 50–80°C, starches undergo almost a complete gelatinization which is a necessary precondition for starch hydrolysis and/or digestion leading to higher GI (Wang and Copeland, 2013).

Formulation of gluten-free bread involves high water levels and this could impose disadvantages, such as resulting in a higher GI and shorter shelf-life (Novotni et al., 2012). In fact, it was reported that higher levels of water during bread processing lowered the RS in bread (Dewettinck et al., 2008) and the amount of RS in breads is always inversely related with GI. However, the tef breads (fresh and aged) containing high water content in our study showed comparatively lower eGI compared to the low moisture containing (26-42%) corn and potato sourdough breads with eGI in the range of 83-96. This indicates that there is difference in the nature of raw material and therefore, tef could be a potential cereal to prepare low GI specialty food products even at higher water levels, compared to the water level of standard white bread, which is the case in our breads.

If fresh white flour wheat bread is used as a reference to calculate the HI, (HI of white wheat bread = 100), food products can be classified, respectively as low GI, medium and high GI if GI < 60, GI (60–85) and GI > 85 (Ferng et al., 2016). Based on this classification, the fresh tef breads showed a medium eGI except for breads that contained 30% sourdough. Interestingly, after one day of room temperature storage, the eGI of all the breads fell into the lower medium category of GI. This study reports for the first time on the *in vitro* eGI of 100% conventionally prepared tef breads as eaten. So far, Wolter et al. (2013), had reported an eGI

of 74 for a 100% conventional tef bread, however the breads were frozen for undefined period of time before eGI was analyzed, which makes it difficult to practically interpret this result since breads are not consumed as frozen.

Our study clearly showed that the same bread prepared from a particular cereal could have significantly different GI depending on how fresh or old it was during sampling. Breads in the contemporary bakery and/or supermarkets can be found from freshly prepared to up to many days old. Thus, when reporting eGI of food products, it could be worthy to indicate the duration and the temperature at which the samples were stored in order to meet the personalized demand of a diversity of consumers. As bread is one of the most versatile food products in the world, the results of our study could have importance to help consumers in choosing which type of breads they should consume depending on their personalized requirements. Fresh breads are generally considered as having the best quality in terms of their textural and organoleptic properties. Nonetheless from a nutritional and/or health point of view, specifically GI, breads of 1 or 2 days old could also be more important for diabetic people or for those of who are in body weight management. To that end, breads of 5 days old could be consumed if the safety is not compromised.

Even though, GI is known as the best indicator of blood glucose release of starchy food products, digestibility based starch fractions could also be a good indicator if complemented with the GI results (Haydersah et al., 2012). Pearson's correlation analysis was performed and the RDS content of all the breads was strongly correlated to their corresponding eGI ($r = 0.79$, $p < 0.001$), while the SDS and RS were negatively correlated ($r = - 0.67$, $p < 0.001$) and ($r = -0.52$, $p < 0.001$), respectively. Therefore, it can be said that the effect of the contents of starch fractions of tef bread on the resulting eGI is dependent on RDS >SDS > RS in a decreasing order. The eGI of the breads also showed a strong negative correlation with the age of the breads ($r = -0.76$, $p < 0.001$) while it exhibited a weak positive correlation with the added sourdough proportion ($r = 0.32$, $p = 0.05$). The other interesting result was the correlation of the aging duration of the breads with their starch fractions. The RDS, SDS and RS contents strongly inversely correlated with the duration of bread storages ($r = 0.-79$, $p < 0.001$), ($r = 0.50$, $p < 0.001$) and ($r = 0.72$, $p = 0.05$), respectively. Moreover, unlike to RSD and SDS which did not show any correlation with the proportion of the added sourdough, the RS showed meaningful positive correlation ($r = 0.48$, $p < 0.001$).

4.2.5 Conclusions

Addition of sourdough slightly increased the RS contents, without however, affecting the RDS and SDS contents. The increase in the amount of added sourdough has increased the eGI of fresh tef breads. Fresh tef breads resulted in medium eGI, however, after 1 or 2 days of room temperature storage they fell into a lower medium category of eGI. Consumption of breads after 1 or 2 days storage could be a good option instead of eating freshly breads to attain a lower GI. The practicality of these results are guaranteed as breads in bakeries and/or supermarkets are normally sold from fresh to up to 4 days old. The effect of sourdough addition on shelf life and organoleptic properties of tef bread is worth of study.

CHAPTER 5: TEF PROTEIN: SOLUBILITY CHARACTERIZATION, *IN VITRO* DIGESTIBILITY AND ITS SUITABILITY AS A GLUTEN-FREE INGREDIENT

CHAPTER 5: TEF PROTEIN: SOLUBILITY CHARACTERIZATION, *IN VITRO* DIGESTIBILITY AND ITS SUITABILITY AS A GLUTEN-FREE INGREDIENT

5.1 Abstract

In this study, total protein content, SDS-PAGE characterization of total protein and Osborne fractions, *in vitro* digestibility and immunogenicity of tef protein of seven different varieties were investigated. The total protein content of the tef varieties ranged from 8.5-9.4 g/100 g dm. The major bands of SDS-PAGE of total proteins of all the tef varieties were observed between 14.4-66.2 kDa. Major bands of SDS-PAGE of molecular weight distribution of albumin, globulin, prolamin and glutelin proteins appeared between molecular weights markers of 14.4-97.4, 14.4-66.2, 14.4-45 and 14.4-66.2 kDa, respectively. *In vitro* protein digestibility of the flour and *injera* ranged from 71-72% and 73-75%, respectively. The gluten content of the tef varieties ranged from 7.4-14.5 mg/kg, proving that tef is a gluten-free cereal and therefore, is suitable for gluten free food formulation.

5.2 Introduction

Gluten is defined as a protein which comprises gliadin and glutenin (1:1) proteins and is a storage protein in wheat, barley and rye (Rosell et al., 2016). In the gluten intolerance context, the European commission defined gluten as "a protein fraction from wheat, rye, barley, oats or their crossbred varieties and derivatives thereof, to which some persons are intolerant and which is insoluble in water and 0.5 M sodium chloride solution" (European Commission, 2009). Celiac disease (CD) is a chronic intolerance to gluten-containing foods (Martin-Fernandez et al., 2016; Penas et al., 2014) and one of the most common lifelong disorders affecting 0.5–1% of the general population of developed countries (Catassi and Fasano, 2008). It is also claimed to be mostly prevalent in Europe and countries to which Europeans have emigrated, including North America, South America and Australia (Anderson et al., 2013; Wieser and Koehler, 2008). The prevalence of celiac disease in developing countries in general is unknown yet probably due to the nonexistence of data.

Currently, lifelong exclusion of gluten containing food is the only solution to those gluten intolerant segments of population. The European Commision (2009) regulation "concerning the composition and labelling of foodstuffs suitable for people intolerant to gluten" stated that foods containing less than 20 mg/kg may be labeled as 'gluten-free', if between 20 and 100 mg/kg as 'low gluten' and those with a value greater than 100 mg/kg as gluten containing foods. Technological removal of gluten from wheat, rye, oats and barley, and the extra processing costs incurred to protect contamination and then complying with the labeling rules makes the process of gluten-free food products in this way very expensive. There is thus a need for other naturally gluten-free cereals, as e.g. buckwheat, oat, sorghum, rice are well-known. However, sometimes people also react against some of their proteins e.g. as known for oat (Arentz-Hansen et al., 2004) or for buckwheat (Krkookova and Mrazova, 2005).

Although tef is considered as a gluten-free cereal (Hopman et al., 2008; Spaenij-Dekking et al., 2005), there is no study on its gluten content which is important to confirm its compliance with the European food regulation directive. Moreover, the availability of other protein allergens in tef is not also studied. In addition, there are also only few but conflicting reports of the Osborne solubility properties of tef protein as indicated by Adebowale et al. (2011) and Mulugeta 1978 cited in Ketema (1997). The knowledge on the content of the Osborne fraction of tef protein is necessary, because of their nutritional and functional significance of tef food

products. Tef could be a potential source of protein considering its high total protein content and its balanced amino acid composition (Gebremariam et al., 2012), however, there is no literature on the protein digestibility of its food products. Information concerning its digestibility is nutritionally significant to make technological improvements during processing and preparation of food. Therefore, the aim of this study is to investigate the gluten content and *in vitro* protein digestibility and Osborne protein solubility of seven pure tef varieties grown in Ethiopia.

5.3 Materials and Methods

Sample and preparation: Seven tef varieties, Boset (DZ-Cr-409), Dega (DZ-01-2675), Quncho (DZ-Cr-387), Simada (DZ-Cr-285), Tsedey (DZ-Cr-37), Zagurey (local) and Zezew (local) were used for this study. The first five are white whereas the last two are brown varieties. These tef varieties were obtained from Axum Agricultural Research Center (Tigray, Ethiopia). All the tef varieties were originating from one location and were the ones that were available at that region. They were milled using a disc attrition mill at a local tef miller in Ethiopia, in the same way as tef for *injera* making is milled. Some portions (about 1 kg) of each variety was pre-milled prior to each variety and discarded to avoid mixing among one other. The dry matter contents of the flour ranged from 92.1-91.6 g/100 g, with an average of 91.9 g/100 g and there were no significant differences among varieties (p > 0.05). The particle size distribution of the flours as measured by a test sieve shaker (Endecott, LTD, London SW, England) was distributed as 100% < 850 μm, 99-100% < 425 μm, 96-99% < 300 μm, 78-85% < 212 μm, 66-77% < 150 μm. Fermented tef *injeras* were prepared following the procedure as descibed in part 2.2. Briefly, water, flour and previously fermented backslop were mixed in a 11:6:1 (w:w:w) ratio and fermented for 72 h followed by 3 minutes baking at about 180°C. The same conventional tef breads as prepared in section 4.2.3.1 have also been used here for *in vitro* protein digestibility study.

5.3.1 Total dietary protein

Total dietary protein content was analyzed by the Kjeldahl method (AOAC, 1995). To calculate the protein content (g/100 g dm) from the obtained N-content, a conversion factor of nitrogen to protein of 5.4 was used.

5.3.2 Osborne protein fractionation

The solvent solubility extraction was done according classical protein fractionation procedure of Osborne as modified by Chen and Bushuk, (1970). Flour samples (10 g dm) were extracted with 40 mL NaCl (0.5 M) by stirring with a magnetic stirrer for 2 h at 4°C. Each suspension was centrifuged for 30 min at 1860 x g and the supernatant was decanted. This was followed by a second similar extraction of the residue for 1 h. The residue was extracted for the third time with distilled water (40 mL) for 30 min to remove residual salt. The three supernatants (containing Albumin and globulin) were combined and dialyzed against cold distilled water for 48 h, followed by centrifugation (1860 x g, 4°C). The residue remained after extraction with salt solution and water was then extracted similarly with two portions of ethanol (70%, 40 mL each), stirring for 2 h for the first and 1 h for second extraction step and was centrifuged for 30 min at 1860 x g and the supernatant (containing prolamin) was decanted. The remaining residue was further extracted with two portions of acetic acid solution (0.05 M, 40 mL) for 2 and 1 h in the first and second extraction, respectively and was centrifuged for 30 min at 1860 x g and the supernatant (containing glutelin) was decanted. Rotary evaporator was used to remove the ethanol from the supernatant. All the fractions were freeze-dried and stored at -20°C until further analysis of the protein content using Kjeldahl method. The results for the different fractions (albumin, globulin, prolamin, glutelin) were expressed as a proportion of the total protein content.

5.3.3 Determination of i*n vitro* protein digestibility

The *in vitro* protein digestibility (IVPD) was performed by a multi-enzyme system, as described by Hsu et al. (1977). Briefly, a multi-enzyme mixture was prepared consisting of trypsin (1.6 mg/mL) from porcine pancreas 13,000-20,000U/mg, α-chymotrypsin (3.1 mg/mL) from bovine pancreas ≥ 40U/mg, and protease (1.3 mg/mL) from *Streptomyces griseus* ≥ 3.5 U/mg (Sigma Aldrich; St. Louis, MO, USA) in distilled water. Flour, injera or bread suspension of 5.4 mg protein/mL (corresponding to 1 mg N/mL) was prepared in a total volume of 50 mL distilled water and was shaken in a water bath (1h, 37°C). The pH of both the suspension and enzyme mixture was adjusted to 8 using sodium hydroxide (0.1 M). The multi-enzyme mixture (5 mL) was added to the suspension and pH of the suspension was measured at start of the simulated digestion. The sample was then incubated (37°C, 10 minutes) in a shaking water bath and pH of the sample was measured at the end of 10 minutes incubation.

The IVPD was calculated as IVPD% = 65. 66 + 18.1 ΔpH_{10min}

Where ΔpH is the pH difference of the initial pH and the pH after 10 minutes simulated digestion.

5.3.4 Determination of gluten content

The prolamin fraction of the tef and control samples were extracted by Cocktail patented solution (R7006) (R-Biopharm AG, Darmstadt, Germany). Cocktail patented solution (2.5 mL) was added into 0.25 g of flour sample, vortex mixed and incubated (50°C, 40 min). After cooling (22°C), ethanol (7.5 mL, 80% (v/v)) was added and gently shaken (1 h) and centrifuged (2500 x g, 10 min, 22°C). The clear supernatant was diluted (1:12.5 (v/v)) with sample diluent and 0.1 mL of this aliquot was used per well in the assay. The RIDASCREEN Gliadin (R7001) (R-Biopharm AG, Darmstadt, Germany) 96 well plate kit comprising the R5-antibody was used for a direct sandwich Enzyme-Linked Immunosorbent Assay (ELISA) according to the manufacturer's instructions. Each determination was referred to an appropriate standard curve (done by a cubic spline function) that was run simultaneously with the samples. The gluten concentration was expressed in mg/kg following the calculation instructions in the kit. Commercial gluten-free blend of patented composition (Brat mix, MixB, Schar, Belgium), and wheat flour (purchased from Colruyt group supermarket, Belgium) were used as a negative and positive control, respectively. The linearity of the method was: R^2 =0.95. Repeatability: the coefficient of variation (CV%) (n=8) which was given for the absorbance of each of the concentration of the standards (5-80.00 ng/mL) ranged from 2.6 to 6.7. The limit of detection (LOD) and limit of quantification (LOQ) were 0.5 mg/kg gliadin and 2.5 mg/kg gliadin, respectively.

5.3.5 SDS-PAGE molecular distribution

The extraction was done according to Moroni et al. (2010). Briefly, 40 mg flour was solubilized in 2 mL of extraction buffer containing 5 M urea, 2% (w/v) SDS, 2 M thiourea and 50 mM DTT (v/v) in Tris–HCl buffer (0.1 M, pH 8.8). The suspensions were incubated (22°C, 16 h) under stirring at 200 rpm, centrifuged (13000 x g, 30 min, 22°C) and supernatants were collected. The SDS-PAGE was performed according the mini-protean precast gels instruction manual (Bio-Rad, Bulgaria). Briefly, the supernatants were mixed with XT sample buffer (Bio-Rad XT Sample Buffer 4x) (Bio-Rad, Bulgaria) to a concentration of 1 µg protein/µL buffer. Then,

samples (20 µL) were loaded into 12% mini-protean (7.2 cm x 8.6 cm) gel (Criterion XT Precast Gel 12% Bis-Tris) (Bio-Rad, Bulgaria). The electrophoresis run in an 800 mL running buffer (Bio-Rad XT MOPS running buffer 20x) (Bio-Rad, Bulgaria) (140 V, room temperature) for approximately 90 min. The gels were then dyed with Coomassie Brilliant Blue G-250 (Bio-Rad G250) (Bio-Rad, Bulgaria) for 30 min at room temperature. A pre-stained molecular weight marker ranging from 6.5 to 200 kDa (broad range, Bio-Rad) (Bio-Rad, Bulgaria) was used to run in parallel to the samples. Commercial wheat flour (purchased from Colruyt group supermarket, Belgium) used as a reference was also run in the gel. Logarithmic curve of the relative migration distance of the molecular weight markers was used to calculate the molecular weight of the proteins in the SDS-PAGE.

5.3.6 Statistical analysis

All analyses were done in triplicate. Results are reported as mean ± standard deviation and calculated based on a dry matter basis (dm). The difference among mean values was determined using one way ANOVA followed by Tukey's Honest Significant Differences (HSD) multiple rank test at $p < 0.05$ significance level. All statistical analyses were performed using SPSS version 24 (SPSS Inc., Chicago, IL, USA).

5.4 Results and Discussion

5.4.1 Total dietary protein content and Osborne protein fractionation

The total protein content (Table 5.1) of the tef varieties varied from 8.5-9.4 g/100 g dm flour. Total protein contents in the range of 10-11.6 g/100 g were reported for different tef varieties (Adebowale et al., 2011; Bultosa, 2007), using a 6.25 nitrogen to protein conversion factor unlike to the 5.4 used in our case. The difference in the conversion factor could be the reason for the slight variation in the results. The nitrogen to protein conversion factor for cereals ranged from 5.3 to 5.8, while 5.4 is considered as the most appropriate and accepted value for cereals (Mariotti et al., 2008). From a nutritional point of view, tef could be seen as a good protein source as its total protein content is either fairly higher or equal to the other common cereals (Gebremariam et al., 2012). Although not analyzed in our study, tef also contains high amounts of some essential amino acids such as lysine, methionine and valine (Gebremariam et al., 2012), which are limited in many cereals.

Figure 5.1 shows the SDS–PAGE protein molecular weight distribution profile of whole tef, gluten-free and white wheat flour *protein extracts*. The major protein bands of all the tef varieties were observed between a molecular weight of 14.4-66.2 kDa. Similarly, major bands of tef protein extracts were seen in the region of 17-60 kDa (Moroni et al., 2010). Unlike the tef varieties which have diverse low molecular weight (LMW) and high molecular weight (HMW) proteins, the wheat used as a comparison showed its major protein bands between 66.2-116.2 kDa representing proteins with HMW.
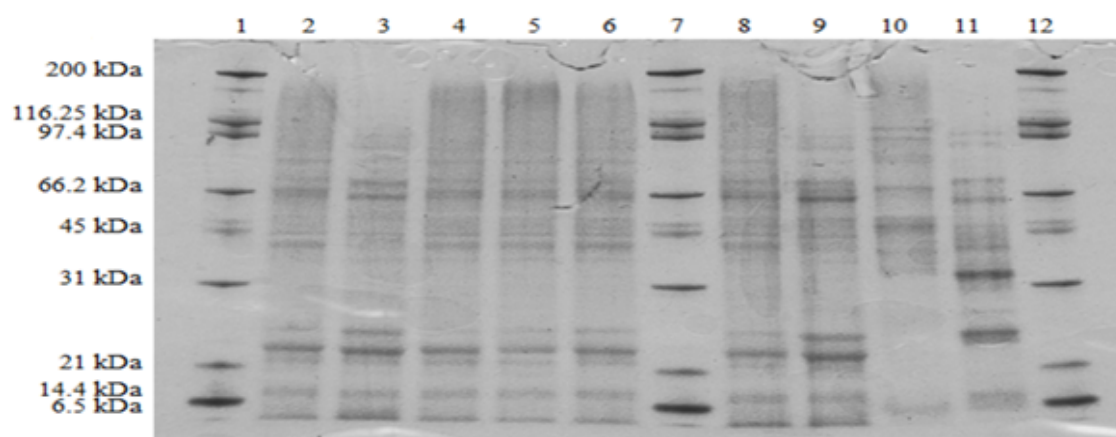
Figure 5.1 SDS–PAGE of whole tef, gluten-free and white wheat flour protein extracts
(1,7 and 12: Molecular weight marker; 2: Boset; 3: Dega; 4: Quncho; 5: Simada; 6: Tsedey; 8: Zagurey; 9: Zezew; 10: Wheat; 11: Gluten-free flour).

The Osborne solubility fractions of different tef varieties are shown in Table 5.1. The contents of storage proteins: albumin, globulin, prolamin and glutelin ranged from 5.4 to 8.7, 9.6 to 13, 2 to 2.5 and 0.3 to 0.6 g/100 g dm, respectively among the varieties. The total recovery of total protein content after the Osborne fractionation was very low, ranging from 18 to 25% among the different varieties. These recoveries are low, especially when compared to the used reference (commercial white wheat flour), having a total protein recovery of 85.7%. Albumin and globulin contributed to 86-90% of the recovered total proteins in tef whereas the major contribution (78%) of wheat proteins is from prolamin and glutelin.

Table 5.1 Protein[a], gluten[b] and storage protein of tef, wheat and gluten-free flours

Varieties	Protein	Gluten	Tef storage proteins (g/100 g total protein)				
			Albumin	Globulin	Prolamin	Glutelin	Recovery%
Boset	9.4 ± 0.1^f	14.3 ± 0.2^b	6.1 ± 0.9^a (25.3)	9.6 ± 1.0^a (29.0)	2.1 ± 0.2 (12.1)	0.59 ± 0.23^b (3.03)	18 ± 2^a
Dega	8.7 ± 0.1^b	8.3 ± 0.9^a	8.7 ± 0.4^b (24.2)	12.9 ± 0.8^b (48.1)	2.5 ± 0.3 (9.63)	0.42 ± 0.12^{ab} (2.09)	25 ± 2^b
Quncho	8.9 ± 0.01^c	7.6 ± 1.1^a	5.4 ± 0.5^a (12.5)	11.3 ± 1.9^{ab} (28.5)	2.1 ± 0.8 (12.1)	0.27 ± 0.01^a (0.59)	19 ± 0.1^a
Simada	8.8 ± 0.01^{bc}	7.4 ± 1.5^a	6.2 ± 0.8^a (25.3)	11.8 ± 0.6^{ab} (34.4)	2.3 ± 0.2 (8.60)	0.25 ± 0.14^a (1.04)	21 ± 2^{ab}
Tsedey	9.1 ± 0.1^d	8.7 ± 0.7^a	6.4 ± 0.5^{ab} (15.1)	9.6 ± 1.2^a (28.8)	2.1 ± 0.3 (7.03)	0.34 ± 0.01^{ab} (0.75)	18 ± 2^a
Zagurey	8.5 ± 0.1^a	12.6 ± 2^b	7.3 ± 1.5^{ab} (22.8)	10.8 ± 1.1^{ab} (26.2)	2.1 ± 0.3 (11.1)	0.51 ± 0.01^{ab} (2.20)	21 ± 3^{ab}
Zezew	9.3 ± 0.01^e	14.5 ± 1.1^b	7.2 ± 0.5^{ab} (25.0)	11.9 ± 0.6^{ab} (48.2)	2.0 ± 0.7 (8.64)	0.27 ± 0.10^a (1.87)	22 ± 1^{ab}
p-value	0.021	< 0.001	0.010	0.019	0.784	0.004	0.026
Wheat	9.32±0.17	> 100	14.43 ± 1.1 (29.0)	4.07 ± 0.2 (90.8)	28.63 ± 4 (83.2)	38.57 ± 0.5 (22.4)	85.70 ± 6
GF		< 4					

[a,b,c,d,e,f] Values within a column with different superscripts are significantly different (p < 0.05). Values in brackets indicate the purity of the storage protein extracts (g storage protein/100 g freeze dried storage protein extract). (n=3). [a](g/100 g dm flour), [b](mg/kg dm flour).

There are only a few but conflicting reports regarding Osborne solubility fractions of tef proteins. Adebowale et al. (2011) has reported 10-12% albumin + globulin, 38-43% prolamin and 20-24% glutelin in three tef varieties. On the other hand, Mulugeta 1978, as cited in Ketema (1997) has reported 37% albumin, 7% globulin, 12% prolamin and 45% glutelin as fractions present in tef.

Both of these reports considerably disagree to each other and to our results. This could be attributed to the use of different solvents. Tert-butanol 60% (v/v) with DTT as reducing agent was used to extract prolamin in the work of Adebowale et al. (2011) while 60% and 70% of ethanol was used (Mulugeta, 1978) as cited in Ketema (1997) and in our study, respectively. Osborne fractions from proteins of different cereals will have a different amino acid composition which certainly affects the efficiency of the extracting solvents used. This is evidenced in our study that the 70% (v/v) ethanol and 0.02 M acetic acid were fairly efficient in extracting relatively very high prolamin and glutelin, respectively from wheat while this amount was very low in the case of tef samples.

Higher efficiency of prolamin extraction in tef was attributed to the use of tert-butanol solvent assisted by DTT (Adebowale et al., 2011) a less polar solvent than ethanol. The fact that ethanol is used in our experiment and was able to extract high amount of prolamin from wheat but not from tef, may indicate that tef prolamin is less polar than that of wheat prolamin. However, there is no evidence that prolamin in gluten-free cereals or in tef has less polar properties than those in gluten containing cereals. Therefore, the reason why ethanol (70%) is efficient in extracting wheat prolamin but failed to extract tef prolamin is unclear. It has been stated that the content of the Osborne fractions are not clear-cut and varies considerably and depends on genotype and growing conditions of the starting materials and experimental conditions leading to different and sometimes contradictory results (Koehler and Wieser, 2013). This could be consolidated by significantly varied Osborne solubility within the same and among different cereals as reviewed by Janssen et al. (2016).

Globulin showed higher recovery rate compared to the other three storage protein which can be justified as follows: The tef flour was milled as whole which makes it to be rich in aleurone layer and germ, besides it was very fine in that 66-77% of the flour was able to pass through a sieve of 150 µm pore size. Both these factors could lead to a higher solubility of globulins and its overall higher proportion in the tef protein.

The SDS-PAGE of Osborne fractions of two white (Quncho and Tsedey), two brown (Zagurey and Zezew) tef varieties and a white wheat flour are shown in Fig. 5.2A, 5.2B and 5.2C, respectively. There was a visible difference among the lanes of the extracts of the white wheat flour protein and each of the Osborne fractions of tef varieties. There was also a clear difference among the lanes of the four Osborne fractions within a variety and among the tef varieties.
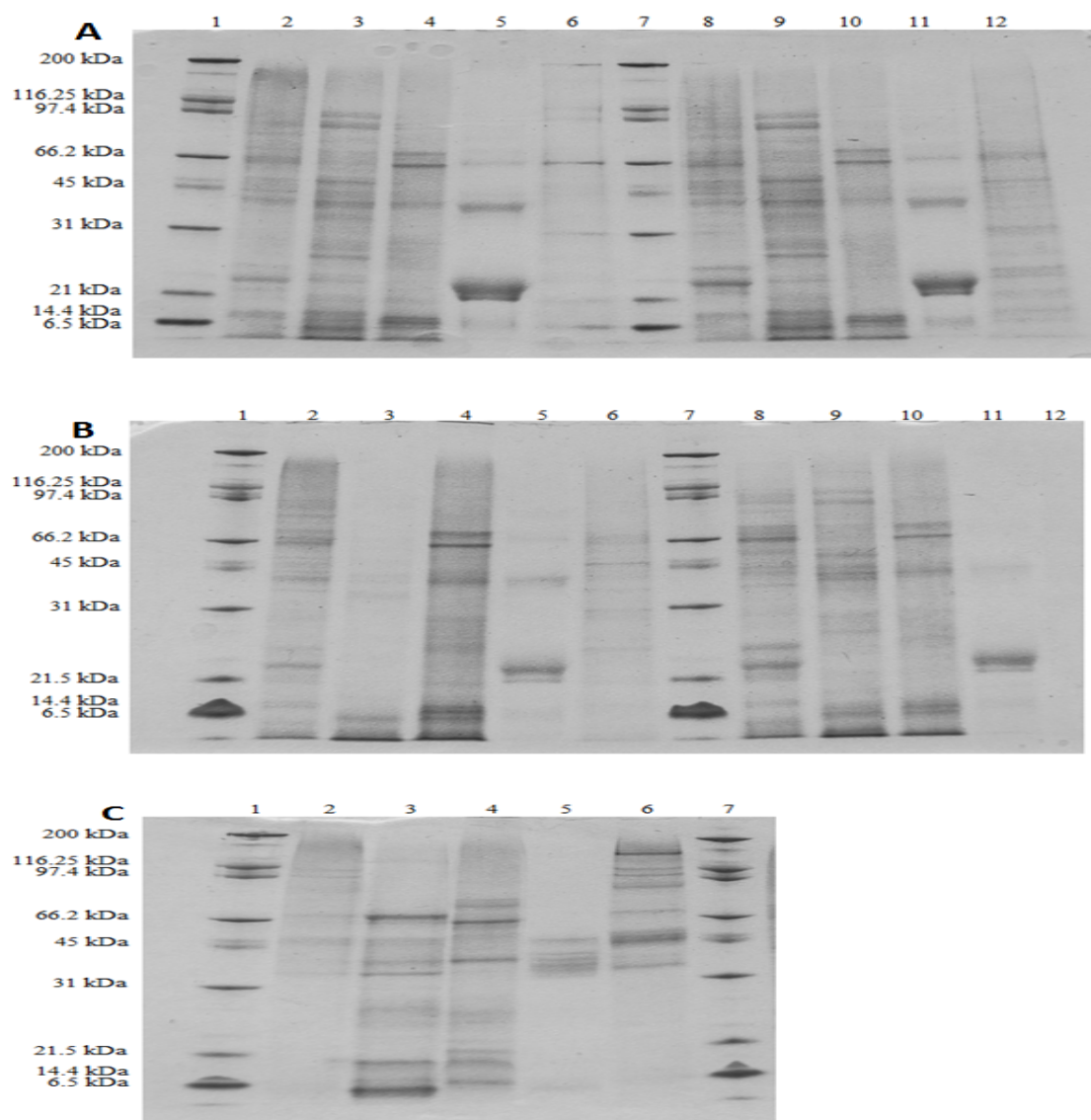
Figure 5.2 SDS-PAGE of storage proteins of tef and wheat
A: SDS-PAGE Osborne fractions Quncho and Tsedey (1,7: Molecular weight marker; 2: Quncho whole flour; 3: Quncho albumin; 4: Quncho globulin; 5: Quncho prolamin; 6: Quncho glutelin; 8: Tsedey whole flour; 9: Tsedey albumin; 10: Tsedey globulin; 11: Tsedey prolamin; 12: Tsedey glutelin), **B**: SDS-PAGE Osborne fractions Zagurey and Zezew (1,7: Molecular weight marker; 2: Zagurey whole flour; 3: Zagurey albumin; 4: Zagurey globulin; 5: Zagurey prolamin; 6: Zagurey glutelin; 8: Zezew whole flour; 9: Zezew albumin; 10: Zezew globulin; 11: Zezew prolamin; 12: Zezew glutelin), **C**: SDS-PAGE Osborne fractions wheat (1,7: Molecular weight marker; 2: Wheat whole flour; 3: Wheat albumin; 4: Wheat globulin; 5: Wheat prolamin; 6: Wheat glutelin).

The major bands of each of the corresponding Osborne fractions showed a close similarity across all the tef varieties. Albumin fractions of each of the tef varieties showed bands with the highest molecular weight range between 14.4-97.4 kDa. Next to the albumin fraction, globulin showed the highest range of major bands between 14.4-66.2 kDa in all the varieties with an approximate MW of 15.4, 15.8, 16.2, 33 46 and 51.3 kDa. The major bands of prolamin fractions are situated between 16.4 and 46.3 kDa in all the tef varieties. Unlike to the other fractions, glutelin showed less major bands and even with no visible band appeared in the case of Zezew variety. The SDS-PAGE of a white wheat flour whole protein extract and Osborne fractions are used for comparison.

There is scarcity of literature on the SDS-PAGE molecular characterization of total protein and Osborne fractions of tef. The literature so far only focused on prolamin fraction and showed that major proteins of prolamins in different tef varieties have approximate MW of 20.3 and 22.8 kDa (Adebowale et al., 2011) and 22.5 and 25.0 kDa (Tatham et al., 1996). These results are in close agreement with some of the major bands of the prolamin fraction in our study.

5.4.2 Gluten content

The gluten content of seven tef varieties are presented in Table 5.1. All the tef varieties showed an absolute gluten content of less than 20 mg/kg dm flour. The white wheat and gluten-free flours used as positive and negative controls, respectively showed gluten contents of >100 mg/kg and <20 mg/kg. Thus, according to the European Commission (2009), regulation number 41/2009, tef could be referred as a gluten-free cereal and its food products could be safe for consumption by gluten intolerant people.

Different gluten intolerant people have diverse tolerance level of gluten and due to this, knowledge of the exact gluten content of individual food materials could be indispensable. Although tef has been considered as a gluten-free cereal and it is already included in the list of gluten-free foods of 'Celiac Diseases Foundation' and 'Celiac Support Association', there was no study showing the exact content of gluten and if there exists varietal difference. Oat was considered as a gluten-free cereal until researchers had proved that there exists difference on the potential of immunogenicity of oat varieties (Rashid et al., 2007; Silano et al., 2014) and screening of the available oat cultivars was needed to classify them and limit the safe amount of daily oat intake among people with celiac diseases of different age groups.

Therefore, our report of gluten content involving different tef varieties will be valuable in the screening of this cereal for the convenience of celiac diseases concerned associations, policy makers and individuals. So far, tef has been proven to be gluten-free and it is becoming the newly raising ancient cereal among celiac disease patients in particular and the western consumers   in general (Hopman et al., 2008; Spaenij-Dekking et al., 2005). One of the limitations of our study is that it only incorporated seven pure varieties even though there are plenty of improved and landrace varieties majorly in Ethiopia and Eritrea as well as in western countries such as the Netherlands, and in the United States. Therefore, a screening study that incorporates as many as the available varieties to prove if all of them qualify as "gluten-free" and are safe for gluten intolerant population is necessary.

5.4.3 *In vitro* protein digestibility of tef flour, *injera* and bread

The *in vitro* protein digestibility (IVPD) of tef flour and its food products *-injera* and conventional bread are given in Fig. 5.3. The IVPD of the tef flour, ranging from 71-72% are relatively higher than other *gluten-free* cereals such as finger millet (48%) (Antony and Chandra, 1998), sorghum and maize (59-67%) (Duodu et al., 2002), pearl millet (62%) (Chowdhury and Punia, 1997) and oat (58%) (Li and Xu, 2015).

All the tef varieties showed an increased IVPD when processed into fermented *injera* while no significant change was seen in the case of conventional breads. Antinutrients such as PA, tannins and PCs are known to interfere with protein digestibility by complexing with proteins and to inhibit the hydrolytic enzymes (Antony and Chandra, 1998). These same tef varieties were found to contain high amounts of PA (1129-1552) and TPC (271-365) in mg/100 g dm flour (part 2.1). Reduction of PA by 49-66% and a decrease of the proportion of bound PCs from 83% to 68% (chapter 3) has been demonstrated in all the tef varieties after fermentation and baked into *injera*. The improved protein digestibility in the case of *injera* could thus be attributed to the fermentation process. It has been reported that the reduction of these antinutrients by fermentation and malting and/or germination significantly increased IVPD of finger millets, amaranths and quinoa (Hejazi et al., 2014; Hejazi and Orsat, 2016; Rizzello et al., 2016). The higher combined proportion of albumin and globulin fractions in all the tef varieties (Table 5.1) could be attributed to the fairly higher IVPD of tef. Unlike to the longer duration of fermentation in *injera*, the fermentation of the conventional bread was rather

158

short (45 min) and due to this, not much reduction of phytic acid is expected resulting higher protein digestibility interference.

As this is the only report on IVPD of tef flour, *injera* and bread, investigation on the effect of processing parameters (heat, water content and time) on different tef based food products could be necessary to optimize the process and maximize protein digestibility.
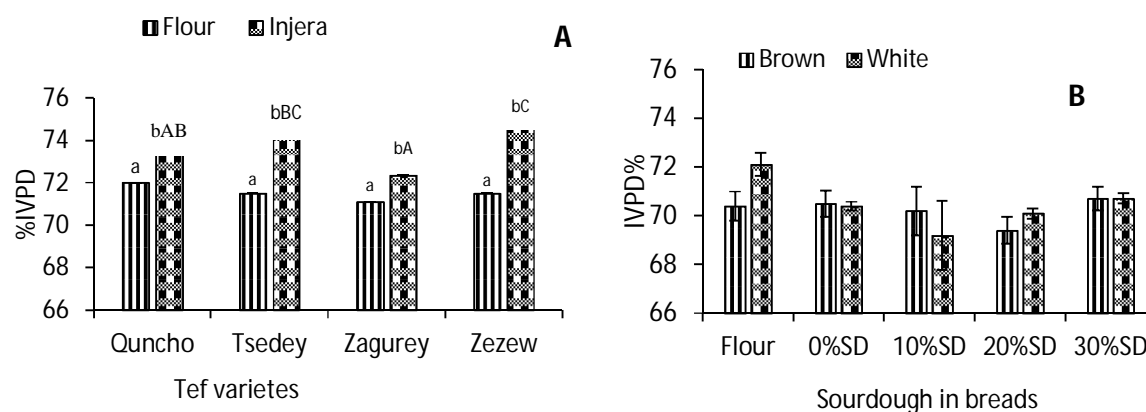

Figure 5.3 In vitro protein digestibility tef flour and its food products

(A) injera and tef flour (n=2) and (B)conventional tef bread. [a,b] Bars within same variety with different letters are significantly different ($p < 0.05$). [A,B,C] Bars of same color with different letters are significantly different ($p < 0.05$).

5.4.4 Possible allergens in tef protein

Table 5.2 shows the possible allergens that could be present in tef flour and its food product-*injera.* The possible presence was estimated based on the counter comparison of molecular weights of tef proteins in the SDS-PAGE (Fig. 5.1, Fig. 5.2A, 2B & 5.2C) with protein of other cereals from literature. Investigation for existence of other allergens in tef is worth of further study as several of the allergen proteins with similar molecular weight to that estimated from tef proteins using the SDS-PAGE have shown allergen properties. From Table 5.2, it can be inferred that the SDS-PAGE analysis showed that the major bands of the possible allergens disappeared when tef was processed into *injera.* Several research groups have exploited different food processing techniques including fermentation and heat treatment to eliminate food allergenicity (Aviles et al., 2013; Varga et al., 2011; Verhoeckx et al., 2015). Our study reports for the first time on the possible presence of allergens in tef, however, further immunological studies are necessary to confirm if all or part of the compounds listed as possible allergens are actually present in tef and cause allergenic reactions.

Table 5.2 List of protein allergens confirmed in other cereals in literature[Δ] and their possible presence in tef and its fermented food product-[*]injera

Molecular weight (kDa) broad range marker	Allergen	Estimated molecular weight (kDa)	Confirmed in	Tef flour	Tef *injera*
66.2 -97.4	HMW glutelin	88	Wheat		
	Starch synthase	85-91	Wheat		
45 -66.2	Endochitinase	67	Wheat	x	
	Purothionin	66	Wheat		
	Lipid transfer protein	66	Wheat		
	Germin-like protein	65	Wheat		
	Omega-5 gliadin	65	Wheat	x	
	Beta-amylase	60	Wheat	x	
	Purple acid phosphatase	60	Wheat	x	
	Globulin-like protein	50	Maize	x	x
	Alpha-amylase	47.8	Barley	x	x
	LMW glutenin GluB3-23	46	Wheat		x
31 – 45	Serpin	43	Wheat		
	Peroxidase	38.8	Wheat	x	x
	Omega2_Gliadin	38	Wheat		
	Glyceraldehyde-3-phosphate-dehydrogenase	37	Wheat	x	x
	Peroxidase	36	Wheat	x	x
	Starch synthase	36	Wheat	x	x
	Grasses group 43	35.6	Wheat	x	x
	Endosperm transfer cell-specific protein	35.5	Wheat	x	x
	Glutenin subunit	34.6	Maize	x	x
	Elongation factor 1	33.6	Wheat	x	
	Dehydrin	33.4	Wheat	x	
	Thioredoxin	33.2	Wheat	x	
	Gamma-hordein 3	33.1	Barley	x	
	Alpha-bèta-gliadin	33	Wheat	x	
	Gamma-gliadin	32.6	Wheat		
	Grasses group 42	31.5	Wheat		
21.5 – 31	Glutenin subunit	30	Wheat	x	
	Thaumatin-like protein	29.6	Wheat	x	
	Chitinase	29-30	Maize, Wheat	x	
	Thiol reductase homologue	27	Wheat	x	
	Triosephosphate-isomerase	27	Wheat	x	
	Glutenin	25	Oat	x	
	Peroxiredoxine	23-24	Barley, Maize,	x	
	13S/11S Globulin	23-24	Rye, Wheat	x	
	Proteasome subunit	23.1	Buckwheat, Oat	x	
	Gliadin	23	Maize		
	Peroxidase 1	23	Wheat	x	

Table 5.2 continued

Range (kDa)	Allergen	Molecular weight (kDa)	Cereal	Tef flour	Tef *injera*
14.4-21.5	Agglutinin isolectin 1	21.2	Wheat		
	NFKB 1-like protein	20.2	Wheat	x	
	Alpha-amylase/subtilisin inhibitor	19.6	Wheat	x	x
	7S Vicilin	19	Buckwheat	x	x
	2S albumin	15	Buckwheat	x	
	Alpha-amylase inhibitor	15-16.4	Barley, Rye,	x	x
	Trypsin inhibitor	15.8-16.1	Wheat	x	x
	Purothionin	15	Barley, Maize,	x	
	Leucine-rich repeat protein	15	Wheat	x	
	Xylanase inhibitor	15	Barley, Rye	x	
	Glutenin subunit	14	Wheat		
	Thioredoxin	13-14	Wheat	x	x
	Profilin	14	Wheat	x	x
		14	Maize	x	x
			Wheat		
			Wheat		
6.5-14.4	Alpha-amylase/trypsin inhibitor	13.9	Wheat		
	Ribosomal inactivating protein	13	Wheat		
	Alpha-amylase inhibitor 0.19				
	Alpha-purothionin	13.3	Wheat		
	Dehydrin	12	Wheat		
	Alpha-amylse/trypsin inhibitor	12	Wheat		
	Lipid transfer protein	10	Wheat		
	Serine protease inhibitor				
	Peroxidase	9	Barley,Wheat		
		9	Wheat		
		9	Wheat		

*The possible presence of protein in tef and its *injera* was estimated by counter comparing of the molecular weight of proteins identified in Fig. 5.1 and Fig. 5.2 **A** and **B** with molecular weight of protein allergens found other cereal found from literature. x denotes the possible presence of allergen. Δ (Golde et al., 1970; Hurkman et al., 2009; Shutov et al., 2003; Takumi et al., 2000; K. Verhoeckx et al., 2015; Zilic et al., 2011).

5.5 Conclusions

The total protein content of tef is comparable to other common cereals. The Osborne protein solubility method may not be directly applied to tef protein fractionation as its recovery was very low in tef while very high in wheat. All the tef varieties showed similar SDS-PAGE molecular weight distribution but showed difference from that of wheat. The SDS-PAGE molecular distribution of albumin, globulin, prolamin and glutelin fractions were significantly different among one another. Tef protein is fairly digestible and showed a significant increase when cooked into *injera* and demonstrating tef could be a good source of dietary protein. Proteins of all the tef varieties did not show any gluten immunogenicity and thus tef could be considered as a safe ingredient in gluten-free food formulations.

CHAPTER 6: GENERAL DISCUSSION, CONCLUSIONS AND FUTURE PERSPECTIVES

CHAPTER 6: GENERAL DISCUSSION, CONCLUSIONS AND FUTURE PERSPECTIVES

There is a massive shift of the contemporary consumers from highly processed gluten containing cereals into whole meal of ancient gluten-free cereals as the latter are increasingly associated with high nutrition and health benefits. Tef is one of the ancient cereals which has been used as human food only in Ethiopia until very recently. The global interest of this cereal has been considerably increased, without however, much evidence on its particular compositional and nutritional benefits. Data on the compositional and nutritional properties of this cereal in terms of phenolic compound and antioxidant capacity, mineral bioaccessibility and bioavailability, starch digestion and glycemic index and protein characterization and possible immunogenicity are of high interest to consumers and all other parties involved in processing or production of this cereal.

Thus, the objective of this PhD dissertation was to study the compositional and nutritional properties of tef. This PhD study followed a broad research approach focusing on phenolic content and antioxidant properties, mineral bioaccessibility, starch digestibility and GI as well as protein characterization and digestibility of tef and its food products aiming at opening doors for more research on these topics. For the purpose of this PhD, seven pure and widely used tef varieties of brown and white seed color which are grown in Ethiopia were mainly used. Nonetheless, unknown varieties of white and brown tef types, as they are commercially available on the Ethiopian market, were also used. The major limitation of this PhD is the use of a static *in vitro* study which only slightly simulates the real physiological gastrointestinal digestion. Therefore, the data on this study only give a way for the necessity of further *in vivo* studies in order to have a complete understanding of the compositional and nutritional properties of this cereal and its food products.

6.1 Reflections on the Methods Used in This Study
6.1.1 Phenolic analysis

Upon critical observation of the current literature, there is inconsistency in the proportion of soluble and bound phenolic contents of cereal crops, mainly due to the differences in extraction methods used. In fact, there is a huge discrepancy of the soluble and bound phenolic extraction methods in the current literature (Adom and Liu, 2002; Chandrasekara and Shahidi, 2010; Kotaskova et al., 2016; Massaretto et al., 2011; Pihlava et al., 2015), in that

ultrasonication, and magnetic stirring are being mostly used to assist the solvent and hydrolysis extraction methods of the soluble and bound PCs, respectively. Ultrasonication-assisted solvent extraction of soluble PCs could lead to a false higher soluble but lower bound phenolic content. Indeed, ultrasonication could misleadingly increase the proportion of soluble PCs by releasing naturally bound PCs due its mechanical force and thus breaking linkages of bound PCs with food macromolecules (Gonzales et al., 2014). Moreover, use of solvent-assisted ultraturrax extraction, as done in our experiments, could extract as much as soluble PCs available but not bound phenolics. Due to the short application time of the Ultraturrax treatment (40 sec), compared to 30 min ultrasonic treatment, damage of cell structure is limited. The pretreatments through which the samples gone through like fermentation (as can be seen in part 2.2) could also have a big impact on the extractability of the phenolic compounds. Therefore, it is worthwhile to standardize both the soluble and bound extraction methods for different food matrices, and in particular for cereal crops, so as to clearly understand the real proportion of the PCs in food materials.

Although quantification of the extracted phenolic compounds is preferably done by chromatographic methods, spectrophotometric method is frequently used. This is mainly due to lack of standards of the different phenolic compounds to use in chromatographic methods, and the fact that these analysis are very time consuming. In this respect, spectrophotometric method is used to have an estimation of the total phenolic/flavonoid content and the antioxidant capacity of the extracts. These methods are very useful as screening methods, and to make comparison between different samples/varieties or processing treatments. However conclusions should be drawn carefully as these results cannot be linked directly to an increased or decreased amount of one particular phenolic compound.

Besides, there is inconsistency in the use of standards in the analysis of TPC, TFC and antioxidant capacity methods (ABTS, DPPH and FRAP) which makes comparison of data among different studies difficult. The base for the choice of standards during phenolic content and antioxidant capacity analysis is not clear in the current literature (Bouayed et al., 2011; Chandrasekara and Shahidi, 2010; Min et al., 2012; Nipornram et al., 2018; Pang et al., 2018; Shen et al., 2009). Generally, the fundamentals on how and why to use a particular standard for TPC or TFC, and antioxidant assays of ABTS, DPPH and FRAP analyses need to be justified. Also important to take into account are possible interfering compounds in the food matrix/extracts in these spectrophotometric methods.

HCl–vanillin method is one of the most frequently cited method for measuring tannin content, is well established and carries validity (Herald et al., 2014). However, the HCl–vanillin assay is not without major drawbacks including: non-tannin phenolic compounds react with vanillin and thus it is not specific and using catechin as a standard may overestimate the level of tannins (Earp et al., 1981; May and Burns, 1971). It also needs a skilled personnel for repeatability but despite all these drawbacks it remains a method of choice for determining tannin content in cereals.

6.1.2 *In vitro* methods

In vivo digestion and absorption of food is a spatiotemporal and dynamic process involving complex enzymatic and transport reactions. Reproduction of all these biochemical and physiological events in a single *in vitro* model still remains difficult. Simulated digestion methods try to mimic physiological conditions *in vivo*, considering the presence of an array of digestive enzymes and their concentrations, pH, digestion time, and salt concentrations, among other factors. *In vitro* digestions have the advantage over *in vivo* methods in terms of low cost and most importantly at short time and no ethical clearance is needed. However, any *in vitro* method does not match the accuracy level achieved by actually studying a food digestion *in vivo*. Most importantly, it is not possible to simulate influx of endogenous compounds to the digestive tract and their subsequent digestion and absorption, replicate the effect of antinutritional factors and interactions between the host, the food and the bacteria present in the digestive tract (Coles et al., 2005). *In vitro* digestion in general could be broadly classified into two main categories as dynamic and static. The dynamic *in vitro* digestion models such as TNO-model (Verhoeckx et al., 2015) use advanced computerized technology which helps to simulate the dynamic features of digestion such as transport of digested meals, variable enzyme concentrations and pH changes over time as much as possible. On the other hand, simulated static *in vitro* digestions mimic *in vivo* digestions with constant ratios of meal to enzymes, salt, bile acids etc. at each step of the digestion. Static *in vitro* models of human digestion have been used to address questions of digestibility and bioaccessibility and/or matrix release of macronutrients (proteins, carbohydrates, lipids), and micronutrients (minerals, trace elements and secondary plant compounds including carotenoids, and

phenolic compounds) (Bouayed et al., 2011; Hasjim et al., 2010; Kopf-Bolanz et al., 2012; Tavares et al., 2013). Throughout our study, we have used 4 types of simulated static *in vitro* digestion models, viz. *in vitro* digestion for digestibility based classification of starch fractions (Englyst et al., 1992), starch hydrolysis procedure to measure glycemic index (Goni et al., 1997), the INFOGEST standardized model to measure the phenolic and mineral bioaccessibility (Minekus et al., 2014) and the pH-drop *in vitro* method to measure protein digestibility (Hsu et al., 1977). The INFOGEST consensus static *in vitro* model used in the bioaccessibility measurement of mineral and phenolic compounds is not validated to be used for digestibility of macro molecules such as protein and starch. Analysing free glucose and amino acids to determine the digestibility of macro-nutrients is not appropriate, since the pancreatic digestion is not complete and needs an additional step with brush border enzymes such as amylo-glucosidase or peptidase to complete starch and protein digestion, respectively (Minekus et al., 2014). The additional step mentioned herewith is not given yet in the protocol, therefore, we used the traditional *in vitro* protein and starch digestibility specific methods.

Significant variations in the use of *in vitro* digestion parameters between the individual models have been reported impeding the possibility to compare results across studies and to deduce general findings (Williams et al., 2012). This type of disagreements could only be avoided by using uniformly agreed methods. However, difference can still arise despite the use of similar *in vitro* models, due to differences in sampling techniques following the end of *in vitro* digestion as it will be detailed below. We tried to point out the weaknesses/differences of sampling techniques following the end of an *in vitro* digestion procedure using the INFOGEST standardized model- an internationally agreed static *in vitro* digestion model (Minekus et al., 2014) which we used in the determination of phenolic (chapter 2.3) and mineral (chapter 3) bioaccessibility measurements. In the INFOGEST standardized model as is used in chapter 2.3, at the end of the gastrointestinal digestion, the digested food was divided into three parts: 1) Liquid sample containing the dialyzed (D) phenolic content, 2) liquid sample containing the soluble nondialyzable (SND) phenolic content and 3) pellet that contained the bound phenolic contents. The current literature shows that the liquid samples containing the D and SND phenolic compounds are directly used in further analysis as if they were phenolic extracts. In our case, we freeze-dried the liquid samples and performed a solid-liquid extraction by using 80% methanol as a solvent, similar as done for the cereal samples as such.

In a typical *in vitro* digestion model for mineral bioaccessibility, consisting dialysis bag, the final digestion will generate three fractions i.e. 1) a fraction which contain dialyzed minerals, 2) fraction containing soluble but nondialyzable minerals, and 3) the pellet fraction that contains the insoluble minerals as described in chapter 3. In the literature, there is no uniform description on how to pretreat the fractions before mineral contents are analysed. Clear methods on how to deal with the analysis of the digestion fractions is necessary in order to get reproducible data and have a better prediction of the physiological digestion. Overall, the difference in handling the samples at the last stages of the *in vitro* digestion could lead to large differences in intra and inter laboratories.

Related to the methods of analyzing the starch fraction and GI analysis, what is commonly lacking or at least not clearly indicated in the literature is the freshness level of the food products used during the experiments. As starch properties in terms of digestibility and GI is highly dependent on the state and freshness level of the food products, the sampling techniques and the storage conditions of the food matrices should be clearly indicated.

In the pH drop *in vitro* method of protein digestibility, cleavage of the proteins by the cocktail of enzymes at alkaline pH, leads to the release of peptides, amino acids and more importantly to the release of protons resulting in a drop of pH (Moyano et al., 2014; Tinus et al., 2012). However, the formula (%IVPD = 65.66 + 18.1 $\Delta pH_{10\ min}$) used to calculate IVPD needs to be viewed critically. First, this equilibrium will give a IVPD equal to 66%, even if no protein digestion occurs. However, the drop in pH results from the release of amino acids and peptides as proteins are digested in that the release of amino acids during proteolysis is not expected to be linear or of zero order. Second, mathematically, IVPD can have a value that is greater than 100% when $pH_{10\ min}$ is <6.1, or $\Delta pH_{10\ min}$ >1.9 (Tinus et al., 2012). The pH-drop method can and already was criticized because of its simplicity compared with the complex processes taking place *in vivo*. Also, food components with a buffering capacity can influence the pH-drop. Although the pH-drop method is criticized, this method was chosen because it still is the most used technique worldwide due to its simplicity and the relatively low cost. Very complex gastrointestinal models which include computer-controlled dynamic models simulating several physiological features of stomach and intestine (pH changes, peristaltic movements, and transit rates, biliary and pancreatic secretions) could better simulate the complex *in vivo* digestion of proteins.

Overall, the major disadvantage of *in vitro* digestion models is their inability of measuring absorption of food components. This drawback can be tentatively partially solved by using dialysis membranes as used in our study or dynamic models but even then they still remain *in vitro*. Unlike to the *in vivo* digestion in which the absorbed food components are constantly taken up by their target tissues, in the case of static *in vitro* that uses dialysis membrane, there will be a gradual build of absorbed food components in the dialysis membrane leading into an equilibrium state. Therefore, the absorption of food components into the dialysis membrane will not be same as throughout the digestion time which could be considered as the limitation of dialysis membrane.

6.1.3 Osborne solubility based storage protein classification

Traditionally, seed storage proteins have been classified on the basis of their solubility characteristics. This solubility classification, as originally developed for wheat proteins, seems not to be valid for all cereal types, example tef. There is limited literature in tef regarding its proportion of storage proteins, however, the results in those papers contradict to one another. In our experiments even the total recovery was very low indicating that the solvents used were not efficient in extracting the storage proteins. Advanced laboratory techniques such as amino acid sequencing and mass spectrometry can be used to better quantify the storage proteins. However, these techniques necessitate the accurate annotation, classification, characterization and decoding of the biological function of the amino acid sequences. Application of machine learning algorithms for classification of seed storage proteins needs amino acid or dipeptide compositions or physicochemical properties of the protein or different combinations of these three features as an input to be able to classify storage proteins (Radhika and Rao, 2015). This type of analysis could lead to a better classification of the storage proteins than the traditional Osborne solubility based classification.

6.2 Phenolic Content and Antioxidant Capacity of Tef Varieties

Fruits and vegetables were considered as the major sources of phenolic compounds (PCs) in the human diet until recently. Research interest on the phenolic compound content and antioxidant capacity of cereals has considerably increased in recent years. It became clear that

cereals also contain a significant amount of PCs and have a huge antioxidant capacity to mitigate chronic diseases (Bjorck et al., 2012; Larsson et al., 2005). Much of the current literature dealing with cereal phenolic contents, only the soluble phenolic fraction is reported as TPC without considering the bound phenolic content (Boka et al., 2013; Chen et al., 2018; Li et al., 2015; Singh, 2012). Nevertheless, Adom and Liu (2002) argued that in cereals, bound PCs account for the majority of the TPC and therefore, reports on TPC which only considered soluble extracts would be a huge underestimation of the real TPC and antioxidant capacity of cereals. The findings of part 2.1 of this PhD, supports the argument of Adom and Liu (2002) in that more than ¾ of the TPC was found in the bound form in all the tef varieties. Knowledge on the real proportion of soluble and bound phenolic content of a cereal is indispensable to optimize the food processing. Knowledge on the real proportion of soluble and bound phenolic content of a cereal is indispensable to optimize the food processing. One important factor that determines the bioavailability of PCs is their bioaccessibility, which in turn is governed by the amount of phenolic compounds released from the food matrix and solubilized during digestion, and then become potentially available for absorption (Alminger et al., 2014). The release of the PCs from food matrices during digestion is also dependent on how the PCs exist in the food *i.e.* in soluble or bound form. The soluble form of PCs are known to be bioavailable in the upper (stomach and small intestine) gastrointestinal tract. On the other hand, the majority of the bound or unreleased PCs reach the large intestine where they will be used as a substrate for the fermenting beneficial intestinal microbial ecosystem and exert their array of health benefits (Bjorck et al., 2012; Price et al., 2008). The colonic fermentation of food matrices containing PCs releases bioactive phenolic metabolites which help in the mitigation of cancers, type 2 diabetics, and cardiovascular diseases (Anson et al., 2011; Ansona et al., 2009). Both the soluble and bound phenolic fractions play their own independent health role in different ways, and therefore, knowledge on their proportion in a particular food material is important in order to optimize the processing and use the foods according to individual needs. In part 2.1, it was also clearly shown that varieties with brown seed color had a higher TPC which was also reflected in their antioxidant capacity. Tef can be found in different colors from milky white to dark brown, with different seed size, field maturity and the ability to grow in a wide range of agro-climatic conditions. Further studies on the effect of gene vs environment variability in relation to their phenolic content could be important if this cereal has to be integrated with the current nutrition sensitive agriculture.

171

6.3 Fermentation and Bioaccessibility of Phenolic Compounds

Fermentation is known to increase the soluble phenolic content of the food matrix due to enzymatic and microbial actions, causing the release of bound PCs. Fermentation of tef for the production of *injera* increased the proportion of soluble PCs and their corresponding antioxidant capacity as indicated in part 2.2. Surprisingly, the fermentation process has increased the TPC by increasing both the soluble and bound phenolic content. The reason why there is a difference on the TPC of raw and a corresponding processed foods remains unclear. However, there are few studies showing that new PCs could be formed as a result of complex enzyme mediated reactions during the fermentation. The formation of new PCs following lactic acid fermentation of pomegranate juices was reported (Valero-Cases et al., 2017). Indeed, it was also evidenced in our study (part 2.2) that gallic acid, protocatechuic acid and catechin were detected only in the *injera* samples, unlike to their corresponding flour samples. In order to fully understand why fermentation increases the TPC, it could be interesting to identify microorganisms which specifically have the capacity to release some PCs or to convert some PCs into others.

The health promoting significance of PCs entirely depends on their release during digestion and then absorption in the gut (Parada and Aguilera, 2007). Therefore, their bioaccessibility and bioavailability are worth of investigation. Unlike the increase of soluble phenolic contents in the *injera* (part 2.3), the *in vitro* bioaccessible TPC, the FRAP and DPPH radical scavenging capacity did not increase. The major reason for this could be an interaction of the PCs with macromolecules of the food matrix throughout the simulated gastrointestinal digestion system. The interaction and formation of phenolic compound-protein complexes are one of the most important issues in plant nutrition. This complex formation is considered as a double edge sword affecting both the nutritional values of PCs by masking their antioxidant capacity and influencing the structure of proteins which may cause their precipitation or decrease their susceptibility to digestion (Czubinski and Dwiecki, 2017; Gonzales et al., 2015). Changes in the nature of PCs at different pH may affect their stability and solubility (Stojadinovic et al., 2013) signifying the dependency of the formation of phenolic compound-protein complexes on the type of food matrices in which PCs occur. Phenolic compounds can also form complexes with carbohydrates leading to not only reduced bioaccessibility of the PCs but also causes a

reduced digestibility of carbohydrates (Jakobek, 2015). Somehow, the later interaction could be desirable if low GI food product is needed. The direct interaction of the free PCs with hydrolytic enzymes (e.g. amylase, protease and lipase) throughout the digestive tract could also negatively influence the digestibility of the food products.

The interpretation of the bioaccessible TPC and their antioxidant capacity in terms of physiological use is difficult. Whether the antioxidant capacity of PCs is composition or content dependent is not yet clear, which makes it difficult again to give meaningful health benefits of the major individual PCs and flavonoids in a particular food product. It is advised that future studies focus on the health benefits of PCs from the viewpoint of what matters *i.e.* composition or content of PCs.

6.4 Antinutrients are not all Bad

Ever since, overnutrition became the same burden as that of undernutrition in the contemporary society, the previously disadvantageous features of antinutrients are becoming beneficial from health perspectives in one way or another. For example, the presence of PA, PCs and fibers in a plant-based food product were once seen undesirable as antinutrients. However, currently the presence of such compounds is also seen as beneficial. The presence of fiber in a meal is seen as healthy and necessary, the interference of PA in carbohydrate digestibility is becoming desirable as consumers are preferring lower calorie foods. The effect of PA on GI, as observed in part 4.1, *i.e.* fermented *injera* which has a higher dry matter content than porridge (not fermented) showed higher eGI. The traditional fermentation process of tef i*njera* eliminates more than 50% of PA as shown in part 2.2, which could indirectly increase starch digestibility, resulting in a higher eGI. This is of course not desirable for diabetic and people on diet control, implying the benefit of the PA when low GI food product is needed.

The advantage of PCs as an antioxidant is overweighing their negative image as antinutrients on mineral bioaccessibility/bioavailability and carbohydrate digestibility. In general, the advantage and disadvantage of the presence of a particular antinutrient in a food product is dependent on the intended use of that typical food and the targeted nutrient/health benefits that the consumers are looking for. The current nutrition trend is focusing on individual needs, or personalized nutrition, leading to an increased diversity of food be it through processing or increasing the sources of plant foods.

From mineral bioavailability perspective, antinutrients are still bad because minerals, specifically Fe and Zn deficiency, are considered as a persisted global burden (Hemery et al., 2018; Nair and Augustine, 2018), particularly in developing countries (Gebreegziabher and Stoecker, 2017; Harika et al., 2017). Tef contains high Fe and Zn content, but their co-existence with mineral chelators such as PA and PCs, could limit their bioaccessibility. The formation of a larger complex of phenolic compound-Fe is a double edge sword that plays a negative role by inhibiting the bioaccessibility of both phenolic compounds and the iron. After fermentation, significant amount of Fe is set free from PA, however, due to the parallel increase of soluble phenolic content in the food matrix, it seems that Fe would be exposed to interact with the soluble phenolic compounds to form bigger complexes thereby reducing the bioaccessibility of iron. On the other hand, depending on the type of phenolic compounds, it is possible that some phenolic compounds could facilitate the bioaccessibility of Fe, by weakly chelating the Fe in the gastrointestinal digestion and make it accessible for absorption in the intestinal phase. Some soluble phenolic compounds can also compete with other potent Fe chelators then reduce the chance of the formation of big insoluble Fe complexes. Hence, the balance of advantage and disadvantage of soluble phenolic compounds in terms of Fe bioaccessibility may depend on the composition and quantity of the phenolic compounds that form soluble and insoluble complexes.

Chapter 3 detailed the effect of fermentation of tef *injera* on Fe and Zn bioaccessibility in different tef varieties. Only a moderate improvement in the bioaccessibility of Fe and Zn was seen despite the high Fe and Zn contents and more than 50% destruction of PA. However, even a complete destruction of PA may not result in an increase of bioaccessibility of Fe and Zn (Baye et al., 2014). Indeed the PA/mineral mole ratio prediction of mineral bioaccessibility is only little resistant to close scrutinization, specifically in the presence of PCs containing galloyl and catechol groups, compounds also well-known as mineral chelators (Baye et al., 2014; Brune et al., 1991; Gabaza et al., 2017). Therefore, only the elimination of PA may not guarantee an increase in mineral bioaccessibility.

To overcome the inhibition of PCs, it could be important to look into the possibilities of addition of mineral bioavailability enhancers such as ascorbic acid (Cercamondi et al., 2014). It would also be important to prepare or consume tef in mixtures with other foods which have mineral bioaccessibility enhancing properties. In Ethiopia, a plant called *moringa* (*Moringa oleifera*) which contains high amounts of micronutrients including vitamin C (Gopalakrishnan

et al., 2016) has been used as a food by particular ethnic groups but now it is becoming popular throughout the country. The optimum enrichment of tef with such plant could be an ideal way to improve the bioaccessibility of iron.

As tef contains the highest Fe, Ca and fairly high Zn contents compared to other cereals, its potential as a source of these minerals should be further studied in terms of bioavailability through manipulations of processes, addition of enhancers or mixing with spices or foods which have a mineral bioavailability enhancing property. The issue of whether the high Fe content of tef is intrinsic or coming from contamination is not yet solved. Considering the importance of Fe in nutrition and the burden of its higher prevalence of deficiency particularly in developing countries, further verification studies on this regard is indispensable.

6.5 Glycemic Index of Tef Food Products

Nutritionally, cereals are known as an excellent source of carbohydrates. However, an immense amount of scientific data have already shown that there is a direct relationship between the frequent consumption of easily digestible, refined or noncomplex carbohydrates and a high prevalence of diabetes, obesity, cardiovascular disease and related health complications (Akhoundan et al., 2016; Azadbakht et al., 2016; Kim et al., 2016; Luna Lopez et al., 2014). To reduce the prevalence of such chronic diseases, dietary interventions *i.e.* shift of consumption towards whole grain which contains high amounts of slowly digestible complex carbohydrates, and plant-based foods in general, has been widely recommended (Goff et al., 2005; Kim et al., 2016; Patel et al., 2017; Virkamaki et al., 2001). As shown in chapters 1, 2 and 3, tef whole flour has high fiber, phenolic and PA contents. Dietary fiber, PCs and PA interfere in the gastrointestinal starch digestion in such a way that it reduces its digestibility and affects the resulting glycemic index which could be considered as desirable when a lower GI food is needed. Dietary fiber can entrap starch granules and restrict the availability of water during gelatinization and then limiting the accessibility of starch granules to digestive enzymes, which results in the lowering of the GI (Kyung et al., 2014). Phenolic compounds and phytates could also be considered as amylase inhibitors which ultimately decrease the GI of starchy food products (Hoover and Zhou, 2003).

The measurement of GI has proven to be a more useful nutritional concept than the chemical classification of carbohydrates as simple or complex, as sugars or starches, or as available or

unavailable, allowing new insights into the relation between the physiological effects of carbohydrate-rich foods and health outcomes (Foster-Powell et al., 2002). The GI category of tef based food products is missing in the international GI table (last updated in 2008) containing more than 2400 food items (Atkinson et al., 2008). As tef is the main staple cereal particularly in Ethiopia, the eGI of the tef based food products in our study will have a practical importance.

Chapter 4 discussed the digestibility and GI level of fresh *injera* and porridge (part 4.1) and a conventional bread (part 4.2). In part 4.1, we revealed that the GI of porridge or *injera* is at least medium or high according to the international GI table. However, these results could only serve as a base for further *in vivo* studies due to two main reasons: 1) the study was *in vitro*, 2) the sampling did not take into account the confounding factor coming from the accompaniment such as butter (fat), meat and vegetables or complex mixture of all. For example, porridge is consumed as fresh and is served with spiced butter. At least the effect of butter used as an accompaniment should be investigated as its presence could significantly affect the starch digestibility and resulting glycemic response. It is already known that adding fat to carbohydrates reduces glycemic responses by delaying gastric emptying and stimulating insulin secretion (Moghaddam et al., 2006).

The static *in vitro* digestion is not able to see such effects on eGI as the time of the static *in vitro* is predetermined regardless of the completion of the digestion in the gastrointestinal system. As a limitation, *in vitro* digestion is not able to measure the eGI of composite foods, in fact, it was already reported that GI of mixed meals is more strongly correlated with fat and protein content, than with carbohydrate content alone (Brand-Miller and Wolever, 2005). Moreover, the high eGI value resulting from the *in vitro* digested *injera* could largely deviate from *in vivo* GI of similar *injera*, as the organic acid (mainly lactic, acetic and propionic acids) (Umeta and Faulks, 1989) in *injera* could actually slow down the gastric emptying (Liljeberg and Bjorck, 1996, 1998) resulting in a lower GI. Therefore, follow up of *in vivo* studies on *injera* and porridge is indispensable to establish concrete conclusions whether these food products are suitable for diabetic people.

Moreover, unlike porridge always consumed as fresh, *injera* could be consumed as fresh or after 3-4 days storage. The results of this study only investigates GI of freshly baked *injera* which otherwise will change on cooling of the *injera* due to the starch retrogradation process.

Therefore, future studies should also investigate the fate of GI of *injera* at different freshness level and also the effect of other food accompaniments with which *injera* is principally served. In part 4.2, effect of sourdough and storage time on eGI of conventional tef breads was investigated. We discovered that replacement of the dough with up to 30% sourdough increased the resulting eGI. The addition of sourdough increased the softness of the breads. It has been reported that food structure might have an impact on starch hydrolysis in that the increase in cell volume and/or crumb porosity renders the starch more accessible to hydrolytic enzymes and finally increases the rate of starch hydrolysis resulting high GI (Fardet et al., 2006; Hager et al., 2013).

The storage of the breads for more than one day induced a significant decrease in eGI signifying that the breads could have undergone a tremendous retrogradation during the room temperature storage. It is already established that food products with high water level exhibit high retrogradation (Carini et al., 2017; Li et al., 2017; Zeleznak and Hoseney, 1996), increasing the resistant and slowly digestible starches or decreasing the rapidly digestible starch (Li et al., 2017), also as evidenced in part 4.2. Practically, both *injera* and bread are consumed at different freshness levels (fresh to after 4 days of storage), however, the safety of these foods after storage should be critically taken care of. Both food products contain high moisture level (as shown in parts 4.1 and 4.2), which could facilitate proliferation of microorganisms during room temperature storage.

If storage time has to be used as a strategy to manipulate the GI of food products, maximum care should be taken not to compromise their safety and organoleptic properties. Therefore, it is worthy that future studies investigate the optimum storage time to get the lowest possible GI, without however, sacrificing their safety and much of the organoleptic properties.

When reporting GI of food products, it is worthy to clearly indicate the freshness level of the food products at the point of sampling. Most importantly, the GI of food products is better be measured as eaten for the ease of practical application, otherwise measuring GI of a frozen conventional tef bread (Wolter et al., 2014a), without however, emphasizing the need for freezing the breads could be misleading to the users of the data as breads actually are consumed either as fresh or after 1-4 days of room temperature storage. Therefore, reporting GI of a typical food product should be complemented mainly with its water level, processing conditions and freshness level. Most importantly, this type of reporting would be vital for

traditional food products in which the way of processing is subjective unlike to conventional food products which roughly have a common way of processing.

6.5.1 Amylose content of cereals and resulting GI

It has been already proven that cereals with high amylose content result in products with a low GI due to their unique ability to retain native starches during heat processing and resilience to enzymatic digestion (Fredriksson et al., 1998; Kim and White, 2012; Klucinec and Thompson, 1999; Sandstedt et al., 1962; Van Amelsvoort and Weststrate, 1992; Van Hung et al., 2016). Passing through the same process, a food product from a waxy starch cereal will have higher GI than that of produced from a high amylose starch cereal. However, this does not mean that a high amylose content cereal always results in a low GI food product. GI is rather highly dependent on the type of process it passes (Kumar and Prabhasankar, 2014; Nayak et al., 2014) in that the GI of different foods prepared from the same cereal could be different. This have been confirmed in part 4.1 where *injera* and porridge showed different GI although they were prepared from the same raw material.

In the current literature, it is well documented that the resulting RS, SDS and RDS of a processed food product is affected by the nature of the type of starch and the processing treatments, however, research questions like what happens to the resulting GI, if the RDS is replaced by the same amount of RS or SDS? Which starch fraction interacts most with the rest of macro- and micro food composition in a way to reduce GI is yet not answered. Research findings to these questions will help the current efforts of artificial modification of GI of food products. Overall, the chance of getting cereals that result in a lower GI regardless of the processing steps they pass is very low, therefore, process manipulation is the key factor for investigation to achieve a desirable GI.

6.6 Tef Protein

It has been widely reported that higher consumption of animal protein sources is associated with an increased risk of diseases such as cancer, type 2 diabetes and cardiovascular diseases (Moller et al., 2017; Song et al., 2016). Although animal-based foods are considered as potential sources of dietary proteins in terms of quantity and quality (digestibility and amino acid composition), plant derived proteins are becoming more important from a long-term

health outcome perspective. In the contemporary consumers, plant-based food products are increasingly preferred and dietary guidelines suggest moving towards a more plant-based diet for protein sources (USDA and HHS, 2015). Cereals account for more than 50% of the global food protein supply (Pedrazzini et al., 2016). Protein quantity of cereals may not be an issue, taking into account the big portions of cereal based foods consumed per day, nonetheless, the digestibility of cereal proteins is rather low compared to animal-based proteins (Millward, 1999) and this remains an issue, particularly for those of exclusively dependent on plant-based diets.

Tef contains comparable amounts of protein as those of common cereals and pseudocereals as shown in chapter 5, while its digestibility is slightly higher compared to other gluten-free cereals. It was revealed that the total Osborne protein solubility recovery was very low in tef compared to wheat samples used as a reference in our study prompting a future need of optimization of this method for gluten-free cereals. Tef was also proved to be in compliance with the European commission food regulation directive to be labelled as a gluten-free cereal. However, this study also showed the possible presence of other protein allergens in tef by SDS-PAGE analysis.

Important to mention is also that the SDS-PAGE analysis showed that the major bands of the possible allergens disappeared when tef was processed into *injera i.e.* after the combined fermentation and baking process. Several research groups have exploited different food processing techniques including fermentation and heating to eliminate food allergenicity (Aviles et al., 2013; Besler et al., 2001; Urisu et al., 1997; Varga et al., 2011; K. Verhoeckx et al., 2015; Yamada et al., 2005). Our study reports for the first time on the possible presence of allergens in tef. However, the analysis is only of biochemical meaning in that further immunological studies are necessary to confirm if all or part of the compounds listed as possible allergens are actually present in tef and cause allergenic reactions.

6.6.1 Nitrogen-protein default conversion factor: 6.25 or 5.4 for cereals?

Protein content in food products is calculated by multiplying the nitrogen content by a default (6.25) nitrogen-protein conversion factor. In the previously available literature, 6.25 has been used as default conversion factor to determine protein contents of many cereals (Adebowale et al., 2011; Escuredo et al., 2014; Peksa et al., 2016). However, it has been criticized that if 6.25 is used irrespective of the foodstuff, the resulting protein content tells little about the

real dietary proteins (Mariotti et al., 2008). The 6.25 conversion factors assumes the nitrogen content of proteins to be 16%. Nevertheless, it has been pointed out that this conversion factor could be prone to up to 15–20% error in the actual protein content of foods because the nitrogen content of amino acids is different in that different foods have different composition of amino acids (Mariotti et al., 2008). All nitrogenous compounds in foodstuffs do not only comprise protein or amino acids, but also include numerous molecules such as nucleic acids, amines, urea, ammonia, nitrates, nitrites, phospholipids, nitrogenous glycosides (Mariotti et al., 2008). After a comprehensive review over the use of conversion nitrogen-protein factors, Mariotti et al. (2008) found out that the specific nitrogen-protein conversion factors used for different cereals ranged from 5.3 to 5.8 and they recommended 5.4 to be as an agreed default conversion factor for cereals. The 5.4 factor has a particular importance and takes into account only the nitrogen in the amino acids and use it when protein basically means amino acids which is important from a nutritional viewpoint. We used the 5.4 conversion factor for the first time in tef. Although our results are slightly lower than generally reported, our estimations are closer to the real tef dietary protein content when protein in fact means amino acids.

6.7 Is Tef a Healthy and Nutritious Cereal?

Nutritionally, cereals are traditionally known as source of carbohydrate as this accounts for more than ¾ of the total mass of cereals. From GI angle, it is not possible to say tef is healthy or not, because GI of a meal is only slightly reliant on the nature of the raw materials used, rather it is mainly dependent on the way it was processed (milling, heat, water level, fermentation etc.), and duration of consumption after heat treatment to mention some. The main limiting nutrients of cereals in general are the content and quality (in respect to digestibility and amino acid composition) of their protein as well as content and bioavailability of their minerals. When evaluated from this viewpoint, tef could be one of the best cereals to depend on, because it contains high total dietary protein with high digestibility (chapter 5) and attractive essential amino acid profile with even high lysine content which is the most limiting amino acid in many of the cereals (chapter 1). Tef contains the highest Fe, Ca and fairly higher Zn content among the cereals (chapters 1 & 3) and could be one of the potential sources of these minerals. It is also clear that tef is a gluten-free cereal (chapter 5) making it a

suitable ingredient for gluten-free food products particularly important for celiac diseases patients. The health imparting properties of a cereal is also seen from a viewpoint of fiber content *i.e.* soluble, insoluble and total dietary fiber contents, bioactive components such as PCs, vitamin B complex and vitamin E as well as enzymes. The small size of tef kernel results in a high surface area and this in turn gives it a unique property of a relatively high total, soluble and insoluble dietary fiber (chapter 1) which fundamentally plays a crucial role in the gut and digestive health, in particular for preventing colorectal cancer (Shaw et al., 2017) and an overall boost of the immune system (Schley and Field, 2002). However, the high fiber content could also be a disadvantage for fiber intolerant people. Unfermented tef food products may contain high antinutrients such as phytic acid mainly concentrated in the fiber may also hamper the starch and protein digestibility but also chelates dietary minerals. Such disadvantages from consumption of foods of high fiber, mainly affect communities who mainly base on cereals and plants based protein sources complimented with less or non-animal based foods.

This cereal also has a high phenolic content and antioxidant capacity which in general makes it an attractive healthy food source, as these bioactive compounds fight against chronic disease causing free radicals (Cardoso et al., 2017). Cereals are also rich in enzymes particularly proteases, amylases, lipases, and oxidoreductases, however, the contents of these enzymes is not yet investigated in tef, and this prompts the need for future study. Comparing the white and brown tef types in terms of nutrition and health significances, further studies are needed that incorporates as much as many varieties and taking into account the gene vs environmental interactions which include geographical location, soil type and other agricultural inputs. So far, the brown varieties seem to outrival in Fe, phenolic content and antioxidant capacity (chapters 1, 2 & 3). Overall, it can be concluded that tef is a gluten free cereal packed with food composition of nutritional and health significance.

6.8 What is Next for Tef?

As of now, information regarding tef's nutritional and health benefits including what is contributed from this study could be a fairly good source for consumers and processors. In the current literature, the technological application of tef and its process suitability is limited, compared to its growing global acceptance. So far, there have been some works on the

suitability of tef as malt for gluten-free beer production (Di Ghionno et al., 2017; Gebremariam et al., 2013). Also some of the food products that could be produced from tef alone or in mixture with wheat include sourdough/non-sourdough breads, extruded products, cookies, weaning food, lactic acid beverages, fat replacer etc. (Zhu, 2018), however, more optimization studies and possible use of this cereal to produce other types of modern foods is worth of study.

Generalization

This PhD study has clearly showed that, tef contains high Fe, Zn and Ca, phenolic content and antioxidant capacity. It also showed that tef is a gluten-free with a high digestible dietary protein but may also contain other protein allergens. Finally, this study showed that GI is highly affected by processing and freshness level of the food product in question.

References

References

AACC, 1999. Determination of Falling Number:, in: Approved Method of the American Association of Cerial Chemists. American Association of Cereial Chemists,Inc, St.Paul,MN, pp. 1–4.

Abebe, W., Collar, C., Ronda, F., 2015. Impact of variety type and particle size distribution on starch enzymatic hydrolysis and functional properties of tef flours. Carbohydr. Polym. 115, 260–268.

Abebe, W., Ronda, F., 2015. Flowability, moisture sorption and thermal properties of tef [Eragrostis tef (Zucc.) Trotter] grain flours. J. Cereal Sci. 63, 14–20.

Abebe, W., Ronda, F., 2014. Rheological and textural properties of tef [Eragrostis tef (Zucc.) Trotter] grain flour gels. J. Cereal Sci. 60, 122–130.

Abebe, Y., Bogale, A., Hambidge, K.M., Stoecker, B.J., Bailey, K., Gibson, R.S., 2007. Phytate, zinc, iron and calcium content of selected raw and prepared foods consumed in rural Sidama, Southern Ethiopia, and implications for bioavailability. J. Food Compos. Anal. 20, 161–168.

Acosta-Estrada, B., Gutierrez-Uribe, J., Serna-Saldivar, S.O., 2014. Bound phenolics in foods, a review. Food Chem. 152, 46–55.

Adebowale, A.R.A., Emmambux, M.N., Beukes, M., Taylor, J.R.N., 2011. Fractionation and characterization of teff proteins. J. Cereal Sci. 54, 380–386.

Adom, K.K., Liu, R.H., 2002. Antioxidant activity of grains. J. Agric. Food Chem. 50, 6182–6187.

Ajila, C.M., Brar, S.K., Verma, M., Tyagi, R.D., Valero, J.R., 2011. Solid-state fermentation of apple pomace using Phanerocheate chrysosporium - liberation and extraction of phenolic antioxidants. Food Chem. 126, 1071–1080.

Akhoundan, M., Shadman, Z., Jandaghi, P., Aboeerad, M., Larijani, B., Jamshidi, Z., Ardalani, H., Nikoo, M.K., 2016. The association of bread and rice with metabolic factors in type 2 diabetic patients. PLoS One 11, 1–12.

Al-Gaby, A.M.A., 1998. Amino acid composition and biological effects of supplementing broad bean and corn proteins with Nigella sativa (black cumin) cake protein. Nahrung 42, 290–294.

Alaunyte, I., Stojceska, V., Plunkett, A., Ainsworth, P., Derbyshire, E., 2012. Improving the quality of nutrient-rich teff (Eragrostis tef) breads by combination of enzymes in straight dough and sourdough breadmaking. J. Cereal Sci. 55, 22–30.

Alijosius, S., Svirmickas, G.J., Bliznikas, S., Gruzauskas, R., Sasytė, V., Raceviciute-stupeliene, A., 2016. Grain chemical composition of different varieties of winter cereals. Zemdirbyste-Agriculture 3, 273–280.

Almgard G., 1963. High content of iron in tef and some other crop species in Ethiopia - a result of contamination. Lantbrukshogskolans Ann. 29, 215–220.

Alminger, M., Aura, A.M., Bohn, T., Dufour, C., El, S.N., Gomes, A., Karakaya, S., Martínez-Cuesta, M.C., Mcdougall, G.J., Requena, T., Santos, C.N., 2014. In vitro models for studying secondary plant metabolite digestion and bioaccessibility. Compr. Rev. Food Sci. Food Saf. 13, 413–436.

Amarowicz, R., 2009. Antioxidant activity of maillard reaction products. Eur. J. Lipid Sci. Technol. 111, 109–111.

Andersen, R., Winge, T., 2012. The access and benefit-sharing agreement on teff genetic resources, Journal of Sustainable Development in Africa. Fridtjof Nansen Institute (FNI), Norway.

Anderson, R.P., Henry, M.J., Taylor, R., Duncan, E.L., Danoy, P., Costa, M.J., Addison, K., Tyedin, J.A., Kotowicz, M.A., Knight, R.E., Pollock, W., Nicholson, G.C., Toh, B., Brown, M.A., Pasco, J.A., 2013. A novel serogenetic approach determines the community prevalence of celiac disease and informs improved diagnostic pathways. BMC Med. 188, 1–13.

Ang, Y.I.S., Ean, S.C.B., Eib, P.A.U.L.A.S., Edersen, J.E.F.F.P., Hi, Y.O.N.G.H.S., 2008. Structure and functional properties of sorghum starches differing in amylose content. J. Agric. Food Chem. 58, 6680–6685.

Angelino, D., Cossu, M., Marti, A., Zanoletti, M., Chiavaroli, L., Brighenti, F., Del Rio, D., Martini, D., 2017. Bioaccessibility and bioavailability of phenolic compounds in bread: a review. Food Funct. 8, 2368–2393.

Angioloni, A., Collar, C., 2011. Polyphenol composition and "in vitro" antiradical activity of single and multigrain breads. J. Cereal Sci. 53, 90–96.

Anson, N.M., Aura, A.-M., Selinheimo, E., Mattila, I., Poutanen, K., Berg, R. van den, Havenaar, R., Bast, A., Haenen, G.R.M.M., 2011. Bioprocessing of Wheat Bran in Whole Wheat Bread Increases the Bioavailability of Phenolic Acids in Men and Exerts Antiinflammatory Effects ex Vivo. J. Nutr. 141, 137–143.

Anson, N.M., Selinheimo, E., Havenaar, R., Aura, A.M., Mattila, I., Lehtinen, P., Bast, A., Poutanen, K., Haenen, G.R.M.M., 2009. Bioprocessing of wheat bran improves in vitro bioaccessibility and colonic metabolism of phenolic compounds. J. Agric. Food Chem. 57, 6148–6155.

Ansona, N.M., Berg, R. van den, Havenaar, R., Bast, A., Haenenb, G.R.M.M., 2009. Bioavailability of ferulic acid is determined by its bioaccessibility. J. Cereal Sci. 49, 296–300.

Antony, U., Chandra, T.S., 1998. Antinutrient reduction and enhancement in protein, starch, and mineral availability in fermented flour of finger millet (Eleusine coracana). J. Agric. Food Chem. 46, 2578–2582.

AOAC, 1995. AOAC Association of Official Analytical Chemists- Method 46-11.02. Crude protein- improved Kjeldahl method. 16th ed.

Apak, R., Ozyurek, M., Guclu, K., Capanoglu, E., 2016. Antioxidant activity/capacity measurement. 1. Classification, physicochemical principles, mechanisms, and electron transfer (ET)-based assays. J. Agric. Food Chem. 64, 997–1027.

Areda, A., Ketema, S., J., I., Davis, R.H., 1993. The iron content of tef [Eragrostis tef (Zucc.) Trotter] and its controversy. Ethiop. J. Sci. 16, 5–13.

Arentz-Hansen, H., Fleckenstein, B., Molberg, O., Scott, H., Koning, F., Jung, G., Roepstorff, P., Lundin, K.E.A., Sollid, L.M., 2004. The molecular basis for oat intolerance in patients with celiac disease. PLoS Med. 1, 084–095.

Arthur, L.A.M., B.L.D'Appolonia, 1979. Comparison of oat and wheat carbohydrates.II. starch. Cereal Chem. 56, 458–461.

Ashoka, S., Peake, B.M., Bremner, G., Hageman, K.J., Reid, M.R., 2009. Comparison of digestion methods for ICP-MS determination of trace elements in fish tissues. Anal. Chim. Acta 653, 191–199.

Atkinson, F., Foster-Powell, K., Brand-Miller, J.C., 2008. International tables of glycemic index and glycemic load values. Diabetes Care 31, 2281–2283.

Aviles, B., Klotz, C., Smith, T., Williams, R., Ponder, M., 2013. Survival of Salmonella enterica Serotype Tennessee during simulated gastric passage is improved by low water activity and high fat content. J. Food Prot. 76, 333–337.

Awadalkareem, A.M., A.I.Mustafa, Tinay, A.H. El, 2008. Protein, mineral content and amino

acid profile of sorghum flour as influenced by soybean protein concentrate supplementation. Pakistan J. Nutr. 7, 475–479.

Axel, C., Rocker, B., Brosnan, B., Zannini, E., Furey, A., Coffey, A., Arendt, E.K., 2015. Application of Lactobacillus amylovorus DSM19280 in gluten-free sourdough bread to improve the microbial shelf life. Food Microbiol. 47, 36–44.

Azadbakht, L., Izadi, V., Ehsani, S., Esmaillzadeh, A., 2016. Effects of the dietary approaches to stop hypertension (DASH) eating plan on the metabolic side effects of corticosteroid medications. J. Am. Coll. Nutr. 35, 285–290.

Baye, K., 2014. Teff: Nutrient composition and health benefits (No. 67), Ethiopia Strategy Support Program. Addis Ababa, Ethiopia.

Baye, K., Guyot, J.P., Icard-Verniere, C., Rochette, I., Mouquet-Rivier, C., 2015. Enzymatic degradation of phytate, polyphenols and dietary fibers in Ethiopian injera flours: Effect on iron bioaccessibility. Food Chem. 174, 60–67.

Baye, K., Mouquet-Rivier, C., Icard-Verniere, C., Picq, C., Guyot, J.P., 2014. Changes in mineral absorption inhibitors consequent to fermentation of Ethiopian injera: Implications for predicted iron bioavailability and bioaccessibility. Int. J. Food Sci. Technol. 49, 174–180.

Behall, K.M., Scholfield, D.J., Hallfrisch, J., 1999. The effect of particle size of whole-grain flour on plasma glucose, insulin, glucagon and thyroid-stimulating hormone in humans. J. Am. Coll. Nutr. 18, 591–7.

Bekabil, F., Behute, B., Simons, R., Tareke, B., 2011. Strengthening the tef value chain in Ethiopia. Addis Ababa, Ethiopia.

Beloshapka, A.N., Buff, P.R., Jr, G.C.F., Swanson, K.S., 2016. Compositional analysis of whole grains, processed grains, grain co-products, and other carbohydrate sources with applicability to pet animal nutrition. Foods 23, 1–16.

Benzie, I.F., Strain, J.J., 1996. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Anal. Biochem. 239, 70–76.

Besler, M., Steinhart, H., Paschke, A., 2001. Stability of food allergens and allergenicity of processed foods. J. Chromatogr. B 756, 207–228.

Beta, T., Rooney, L.W., Marovatsanga, L.T., Taylor, J.R.N., 2000. Effect of chemical treatments on polyphenols and malt quality in sorghum. J. Cereal Sci. 31, 295–302.

Bhanja, T., Kumari, A., Banerjee, R., 2009. Enrichment of phenolics and free radical scavenging property of wheat koji prepared with two filamentous fungi. Bioresour. Technol. 100, 2861–2866.

Bhati, K.K., Aggarwal, S., Sharma, S., Mantri, S., Singh, S.P., Bhalla, S., Kaur, J., Tiwari, S., Roy, J.K., Tuli, R., Pandey, A.K., 2014. Differential expression of structural genes for the late phase of phytic acid biosynthesis in developing seeds of wheat (Triticum aestivum L.). Plant Sci. 224, 74–85.

Bjorck, I., Granfeldt, Y., Asp, N.A., Liljeberg, H., Tovar, J., 1994. Food properties affecting the digestion and absorption of carbohydrates. Diabetologia 59, 1676–1684.

Bjorck, I., Ostman, E., Kristensen, M., Mateo Anson, N., Price, R.K., Haenen, G.R.M.M., Havenaar, R., Bach Knudsen, K.E., Frid, A., Mykkanen, H., Welch, R.W., Riccardi, G., 2012. Cereal grains for nutrition and health benefits: Overview of results from in vitro, animal and human studies in the healthgrain project. Trends Food Sci. Technol. 25, 87–100.

Boka, B., Woldegiorgis, A.Z., Haki, G.D., 2013. Antioxidant properties of Ethiopian traditional bread (injera) as affected by processing techniques and tef grain (Eragrostis tef (zucc .)) varieties. Can. Chem. Trans. 1, 7–24.

Bouayed, J., Hoffmann, L., Bohn, T., 2011. Total phenolics, flavonoids, anthocyanins and

antioxidant activity following simulated gastro-intestinal digestion and dialysis of apple varieties: Bioaccessibility and potential uptake. Food Chem. 128, 14–21.

Bouis, H.E., 1996. Enrichment of Food Staples Through Plant Breeding : A New Strategy for Fighting Micronutrient Malnutrition. Nutr. Rev. 54, 131– 137.

Box, J.D., 1983. Investigation of the Folin-Ciocalteu phenol reagent for the determination of polyphenolic substances in natural waters. Water Res. 17, 511–525.

Brand-Miller, J.C., 2003. Glycemic load and chronic disease. Nutr. Rev. 61, 49–55.

Brand-Miller, J.C., Wolever, T.M.S., 2005. The use of glycaemic index tables to predict glycaemic index of breakfast meals. Br. J. Nutr. 94, 133.

Brewer, L.R., Cai, L., Shi, Y.C., 2012. Mechanism and enzymatic contribution to in vitro test method of digestion for maize starches differing in amylose content. J. Agric. Food Chem. 60, 4379–4387.

Brune, M., Hallberg, L., Skanberg, A.N.N.B., 1991. Determination of iron binding phenolic groups in foods. J. Food Sci. 56, 128–131.

Buleon, A., Colonna, P., Planchot, V., Ball, S., 1998. Starch granules: Structure and biosynthesis. Int. J. Biol. Macromol. 23, 85–112.

Bultosa, G., 2007. Physicochemical characteristics of grain and flour in 13 tef [Eragrostis tef (Zucc .) Trotter] grain varieties. J. Appl. Sci. Res. 3, 2042–2050.

Bultosa, G., Hall, A.N., Taylor, J.R.N., 2002. Physico-chemical characterization of grain tef [Eragrostis tef (Zucc.) Trotter] starch. Starch/Stärke 54, 461–468.

Bultosa, G., Taylor, J.R.N., 2004a. Teff overview: The world of food grains, in: Wrigley, C., Corke, H., Seetharaman, K., Faubion, J. (Eds.), Encyclopedia of Food Grains. Elsevier, Sydney, pp. 209–220.

Bultosa, G., Taylor, J.R.N., 2004b. Paste and gel properties and in vitro digestibility of tef [Eragrostis tef (Zucc). Trotter] starch. Starch/Staerke 56, 20–28.

Cardoso, L. de M., Pinheiro, S.S., Martino, H.S.D., Pinheiro-Sant'Ana, H.M., 2017. Sorghum (Sorghum bicolor L.): Nutrients, bioactive compounds, and potential impact on human health. Crit. Rev. Food Sci. Nutr. 57, 372–390.

Carini, E., Curti, E., Fattori, F., Paciulli, M., Vittadini, E., 2017. Staling of gluten-free breads: physico-chemical properties and 1H NMR mobility. Eur. Food Res. Technol. 243, 867–877.

Cassidy, A., Bingham, S. a, Cummings, J.H., 1994. Starch intake and colorectal cancer risk: an international comparison. Br. J. Cancer 69, 937–942.

Catassi, C., Fasano, A., 2008. Celiac disease. Curr. Opin. Gastroenterol. 24, 687–691.

Cencic, A., Chingwaru, W., 2010. The role of functional foods, nutraceuticals, and food supplements in intestinal health. Nutrients 2, 611–625.

Cercamondi, C.I., Gli, I.M., Zeder, C., Hurrell, R.F., 2014. Sodium iron EDTA and ascorbic acid, but not polyphenol oxidase treatment, counteract the strong inhibitory effect of polyphenols from brown sorghum on the absorption of fortification iron in young women. Br. J. Nutr. 111, 481–489.

Chandrasekara, A., Naczk, M., Shahidi, F., 2012. Effect of processing on the antioxidant activity of millet grains. Food Chem. 133, 1–9.

Chandrasekara, A., Shahidi, F., 2011. Bioactivities and antiradical properties of millet grains and hulls. J. Agric. Food Chem. 59, 9563–9571.

Chandrasekara, A., Shahidi, F., 2010. Content of insoluble bound phenolics in millets and their contribution to antioxidant capacity. J. Agric. Food Chem. 58, 6706–6714.

Cheetham, N.W.H., Tao, L., 1998. Variation in crystalline type with amylose content in maize starch granules : an X-ray powder diffraction study. Carbohydr. Polym. 36, 277–284.

Chen, C., Wang, L., Wang, R., Luo, X., Li, Y., Li, J., Li, Y., Chen, Z., 2018. Phenolic contents, cellular antioxidant activity and antiproliferative capacity of different varieties of oats. Food Chem. 239, 260–267.

Chen, C.H., Bushuk, W., 1970. Nature of proteins in triticale and its parental species I. Solubility characteristics and amino acid composition of endosperm protein. Can. J. Plant Sci. 50, 9–14.

Chen, G.L., Chen, S.G., Chen, F., Xie, Y.Q., Han, M. Di, Luo, C.X., Zhao, Y.Y., Gao, Y.Q., 2016. Nutraceutical potential and antioxidant benefits of selected fruit seeds subjected to an in vitro digestion. J. Funct. Foods 20, 317–331.

Chowdhury, S., Punia, D., 1997. Nutrient and antinutrient composition of pearl millet grains as affected by milling and baking. Nahrung - Food 41, 105–107.

Cleary, L., Brennan, C., 2006. The influence of a (1-3)(1-4)ß-D-glucan rich fraction from barley on the physico-chemical properties and in vitro reducing sugars release of durum wheat pasta. Int. J. Food Sci. Technol. 41, 910–918.

Clydesdale, F.M., 1982. The Effects of Physicochemical Properties of Food on the Chemical Status of Iron, in: Nutritional BioaVailability of Iron. American Chemical Society, Washington, pp. 55–84.

Clydesdale, F.M., Camire, A.L., 1983. Effect of PH and heat on the binding of iron, calcium, magnesium, and zinc and the loss of phytic acid in soy flour. J. Food Sci. 48, 1272–1283.

Coles, L.T., Moughan, P.J., Darragh, A.J., 2005. In vitro digestion and fermentation methods, including gas production techniques, as applied to nutritive evaluation of foods in the hindgut of humans and other simple-stomached animals. Anim. Feed Sci. Technol. 124, 421–444.

Collar, C., Angioloni, A., 2014. Pseudocereals and teff in complex breadmaking matrices: Impact on lipid dynamics. J. Cereal Sci. 59, 145–154.

CSA (Central Statistic Agency), 2007. Population and housing census of Ethiopia. Cent. Stat. agency.

Czubinski, J., Dwiecki, K., 2017. A review of methods used for investigation of protein–phenolic compound interactions. Int. J. Food Sci. Technol. 52, 573–585.

Deckere, E.A.M. DE, Kloots, W.J., van Amelsvoort, J.M., 1995. Both raw and retrograded starch decrease serum triacylglycerol concentration and fat accretion in the rat. Br. J. Nutr. 73, 287–98.

Dejmek, P., Rayner, M., Timgren, A., Sj, M., 2012. Quinoa starch granules: a candidate for stabilising food-grade pickering emulsions. J. Sci. Food Agric. 1–7.

Dekking, L.S., Kooy-Winkelaar, Y., Koning, F., 2005. The Ethiopian cereal tef in celiac disease. N. Engl. J. Med. 353, 1748–1749.

Demeke, T., Marcantonio, D., 2013. Analysis of incentives and disincentives for teff in Ethiopia. Rome, Italy.

Deshpande, S., Cheryan, M., 1984. Effects of phytic acid, divalent cations, and their interactions on α-amylase activity. J. Food Sci. 49.

Dewanto, V., Xianzhong, W., Adom, K.K., Liu, R.H., 2002. Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity. J. Agric. Food Chem. 50, 3010–3014.

Dewettinck, K., Van Bockstaele, F., Kuhne, B., Van de Walle, D., Courtens, T.M., Gellynck, X., 2008. Nutritional value of bread: Influence of processing, food interaction and consumer perception. J. Cereal Sci. 48, 243–257.

Dey, T.B., Kuhad, R.C., 2014. Upgrading the antioxidant potential of cereals by their fungal

fermentation under solid-state cultivation conditions. Lett. Appl. Microbiol. 59, 493–499.

Dhuique-mayer, C., 2017. Factors influencing micronutrient bioavailability in biofortified crops. Ann. N. Y. Acad. Sci. 1390, 74–87.

Di Ghionno, L., Sileoni, V., Marconi, O., De Francesco, G., Perretti, G., 2017. Comparative study on quality attributes of gluten-free beer from malted and unmalted teff [Eragrostis tef (zucc.) trotter]. LWT - Food Sci. Technol. 84, 746–752.

Dominguez-Avila, J.A., Wall-Medrano, A., Velderrain-Rodriguez, G.R., Chen, C.-Y.O., Salazar-Lopez, N.J., Robles-Sanchez, M., Gonzalez-Aguilar, G.A., 2017. Gastrointestinal interactions, absorption, splanchnic metabolism and pharmacokinetics of orally ingested phenolic compounds. Food Funct. 8, 15–38.

Dordevic, T.M., Siler-Marinkovic, S.S., Dimitrijevic-Brankovic, S.I., 2010. Effect of fermentation on antioxidant properties of some cereals and pseudo cereals. Food Chem. 119, 957–963.

Duodu, K.G., Nunes, A., Delgadillo, I., Parker, M.L., Mills, E.N.C., Belton, P.S., Taylor, J.R.N., 2002. Effect of grain structure and cooking on sorghum and maize in vitro protein digestibility. J. Cereal Sci. 35, 161–174.

Dvorakova, M., Guido, L.F., Dostalek, P., Skulilova, Z., Moreira, M.M., Barros, A., 2008. Antioxidant properties of free, soluble ester and insoluble-bound phenolic compounds in different barley varieties and corresponding malts. Inst. Brew. Distill. 114, 27–33.

Dykes, L., Rooney, L.W., 2006. Sorghum and millet phenols and antioxidants. J. Cereal Sci.

Earp, C.F., Akingbala, J.O., Ring, S.H., Rooney, L.W., 1981. Evaluation of several methods to determine tannins in sorghums with varying kernel characteristics. Cereal Chem. 58, 234–238.

Edwardson, S., 1996. Buckwheat: pseudocereal and nutraceutical, in: Janick, J. (Ed.), Progress in New Crops. ASHS Press, Alexandria, VA., pp. 195–207.

El-Alfy, T.S., Ezzat, S.M., Sleem, A.A., 2012. Chemical and biological study of the seeds of Eragrostis tef (Zucc.) Trotter. Nat. Prod. Res. 26, 619–629.

Elkhalifa, A.E.O., Schiffler, B., Bernhard, R., 2004. Effect of fermentation on the starch digestibility , resistant starch and some physicochemical properties of sorghum flour. Nahrung/Food 48, 91–94.

Endalew, B., 2017. Teff production and marketing in Ethiopia. RADIX Int. J. Res. Soc. Sci. 6, 1–17.

Englyst, H.N., Kingman, S.M., Cummings, J.H., 1992. Classification and measurment of nutrittionally important starch fractions. Eur. J. Cinical Nutr. 46, 33–50.

Eriksson, K.-F., Lindgiirde, E., 1991. Prevention of Type 2 (non-insulin-dependent) diabetes mellitus by diet and physical exercise: The 6-year Malmo feasibility study. Diabetologia 34, 891–898.

Escuredo, O., Inmaculada, G.M.M., Wells Moncada, G., Fischer, S., Hernandez Hierro, J.M., 2014. Amino acid profile of the quinoa (Chenopodium quinoa Willd.) using near infrared spectroscopy and chemometric techniques. J. Cereal Sci. 60, 67–74.

Ethiopian Agricultural Transformation Agency, 2013. Working strategy for strengthening Ethiopian's tef value chain.

European Commission, 2009. Commission regulation (EC) No. 41/2009 of 20 January 2009 concerning the composition and labeling of foodstuffs suitable for people intolerant to gluten. Off. J. Eur. Union 52, 3–5.

FAO, 2007. Protein and amino acid requirements in nutrition. Geneva.

FAO, WHO, 2001. Human Vitamin and Mineral Requirements, Joint: Food and Agriculture Organization of the United Nations, World Health Organization. Bangkok, Thailand.

Fardet, A., Leenhardt, F., Lioger, D., Scalbert, A., Remesy, C., 2006. Parameters controlling the glycaemic response to breads. Nutr. Res. Rev. 19, 18–25.

Feng, N., He, Z., Zhang, Y., Xia, X., Zhang, Y., 2013. QTL mapping of starch granule size in common wheat using recombinant inbred lines derived from a PH82-2/Neixiang 188 cross. Crop J. 1, 166–171.

Ferng, L.H., Liou, C.M., Yeh, R., Chen, S.H., 2016. Physicochemical property and glycemic response of chiffon cakes with different rice flours. Food Hydrocoll. 53, 172–179.

Filannino, P., Bai, Y., Di, R., Gobbetti, M., Michael, G.G., 2013. Metabolism of phenolic compounds by Lactobacillus spp . during fermentation of cherry juice and broccoli puree. Food Microbiol. 46, 272–279.

Fischer, M.M., Egli, I.M., Aeberli, I., Hurrell, R.F., Meile, L., 2014. Phytic acid degrading lactic acid bacteria in tef-injera fermentation. Int. J. Food Microbiol. 190, 54–60.

Food and Agricultural Organization (FAO) and World Health Organization (WHO), 1989. Protein quality evaluation. Bethesda, Md.

Forsido, S.F., Rupasinghe, H.P.V., Astatkie, T., 2013. Antioxidant capacity, total phenolics and nutritional content in selected ethiopian staple food ingredients. Int. J. Food Sci. Nutr. 64, 915–20.

Foster-Powell, K., Holt, S., Band-Miller, J., 2002. International table of glycemic index and glycemic load. Am. J. Clin. Nutr. 76, 5–56.

Frazier, R.A., Deaville, E.R., Green, R.J., Stringano, E., Willoughby, I., Plant, J., Mueller-Harvey, I., 2010. Interactions of tea tannins and condensed tannins with proteins. J. Pharm. Biomed. Anal. 51, 490–495.

Fredriksson, H., Silverio, J., Andersson, R., Eliasson, A.-C., Åman, P., 1998. The influence of amylose and amylopectin characteristics on gelatinization and retrogradation properties of different starches. Carbohydr. Polym. 35, 119–134.

Frei, M., Siddhuraju, P., Becker, K., 2003. Studies on the in vitro starch digestibility and the glycemic index of six different indigenous rice cultivars from the Philippines. Food Chem. 83, 395–402.

Fujii, H., Iwase, M., Ohkuma, T., Ogata-Kaizu, S., Ide, H., Kikuchi, Y., Idewaki, Y., Joudai, T., Hirakawa, Y., Uchida, K., Sasaki, S., Nakamura, U., Kitazono, T., 2013. Impact of dietary fiber intake on glycemic control, cardiovascular risk factors and chronic kidney disease in Japanese patients with type 2 diabetes mellitus: the Fukuoka Diabetes Registry. Nutr. J. 12, 159.

Fuwa, H., Nakajima, M., Hamada, A., Glover, D. V., 1977. Comparative susceptibility to amylases of starches from different plant species and several single endosperm mutants and their double-mutant combinations with opaque-2 inbred Oh43 maize. Cereal Chem. 54, 230–237.

Gabaza, M., Muchuweti, M., Vandamme, P., Raes, K., 2017. Can fermentation be used as a sustainable strategy to reduce iron and zinc binders in traditional African fermented cereal porridges or gruels? Food Rev. 33, 561–586.

Gabaza, M., Shumoy, H., Muchuweti, M., Vandamme, P., Raes, K., 2016. Effect of fermentation and cooking on soluble and bound phenolic profiles of finger millet sour porridge. J. Agric. Food Chem. 64, 7615–7621.

Gallant, D.J., Bouchet, B., Buleon, A., Perez, S., 1992. Physical characteristics of starch granules and susceptibility to enzymatic degradation. Eur. J. Clin. Nutr. Nutr. 46, 3–6.

Gan, R.-Y., Shah, N.P., Wang, M.-F., Lui, W.-Y., Corke, H., 2016. Fermentation alters antioxidant capacity and polyphenol distribution in selected edible legumes. Int. J. Food Sci. Technol.

51, 875–884.

Gasztonyi, M.N., Farkas, R.T., Berki, M., Petroczi, I.M., Daood, H.G., 2011. Content of phenols in wheat as affected by varietal and agricultural factors. J. Food Compos. Anal. 24, 785–789.

Gebreegziabher, T., Stoecker, B.J., 2017. Iron deficiency was not the major cause of anemia in rural women of reproductive age in Sidama zone , southern Ethiopia : A cross- sectional study. PLoS One 137, 1–13.

Gebremariam, M.M., Zarnkow, M., Becker, T., 2013. Effect of teff (Eragrostis tef) variety and storage on malt quality attributes. J. Inst. Brew. 119, 64–70.

Gebremariam, M.M., Zarnkow, M., Becker, T., 2012. Teff (Eragrostis tef) as a raw material for malting, brewing and manufacturing of gluten-free foods and beverages: a review. J. Food Sci. Technol. 51, 2881–2895.

Gewehr, M.F., Danelli, D., Melo, L.M. De, Flores, S.H., Jong, E.V. De, 2017. Nutritional and technological evaluation of bread made with quinoa flakes (Chenopodium quinoa willd). J. Food Process. Preserv. 41, 1–8.

Giuberti, G., Gallo, A., Fiorentini, L., Fortunati, P., Masoero, F., 2016. In vitro starch digestibility and quality attributes of gluten free "tagliatelle" prepared with teff flour and increasing levels of a new developed bean cultivar. Starch/Staerke 68, 374–378.

Goff, L.M., Bell, J.D., So, P.-W., Dornhorst, A., Frost, G.S., 2005. Veganism and its relationship with insulin resistance and intramyocellular lipid. Eur. J. Cinical Nutr. 59, 291–298.

Golde, D.W., Mcginnjss, M.G., Holland, P. V, 1970. Serum agglutinins to commercially prepared albumin. Am. J. Clin. Pathol. 55, 655–658.

Goni, I., Garcia-Alonso, A., Saura-Calixto, F., 1997. A starch hydrolysis procedure to estimate glycemic index. Nutr. Res. 17, 427–437.

Gonzales, G.B., Smagghe, G., Grootaert, C., Zotti, M., Raes, K., Van Camp, J., 2015. Flavonoid interactions during digestion, absorption, distribution and metabolism: a sequential structure–activity/property relationship-based approach in the study of bioavailability and bioactivity. Drug Metab. Rev. 47, 175–190.

Gonzales, G.B., Smagghe, G., Raes, K., Van Camp, J., 2014. Combined alkaline hydrolysis and ultrasound-assisted extraction for the release of nonextractable phenolics from cauliflower (Brassica oleracea var. botrytis) waste. J. Agric. Food Chem. 62, 3371–3376.

Gopalakrishnan, L., Doriya, K., Kumar, D.S., 2016. Moringa oleifera: A review on nutritive importance and its medicinal application. Food Sci. Hum. Wellness 5, 49–56.

Granfeldt, Y., Bjorck, I., Drews, A., Tovar, J., 1992. An in vitro procedure based on chewing to predict metabolic response to starch in cereal and legume products. Eur. J. Clin. Nutr. 46, 649–660.

Greffeuille, V., Polycarpe Kayodé, A.P., Icard-Vernire, C., Gnimadi, M., Rochette, I., Mouquet-Rivier, C., 2011. Changes in iron, zinc and chelating agents during traditional African processing of maize: Effect of iron contamination on bioaccessibility. Food Chem. 126, 1800–1807.

Gross, L.S., Li, L., Ford, E.S., Liu, S., 2004. Increased consumption of refined carbohydrates and the epidemic of type 2 diabetes mellitus in the United States: an ecological assessment. Am. J. Clin. Nutr. 70, 466–473.

Guariguata, L., Whiting, D.R., Hambleton, I., Beagley, J., Linnenkamp, U., Shaw, J.E., 2014. Global estimates of diabetes prevalence for 2013 and projections for 2035. Diabetes Res. Clin. Pract. 103, 137–149.

Hager, A.S., Arendt, E.K., 2013. Influence of hydroxypropylmethylcellulose (HPMC), xanthan

gum and their combination on loaf specific volume, crumb hardness and crumb grain characteristics of gluten-free breads based on rice, maize, teff and buckwheat. Food Hydrocoll. 32, 195–203.

Hager, A.S., Czerny, M., Bez, J., Zannini, E., Arendt, E.K., 2013. Starch properties, in vitro digestibility and sensory evaluation of fresh egg pasta produced from oat, teff and wheat flour. J. Cereal Sci. 58, 156–163.

Hager, A.S., Wolter, A., Czerny, M., Bez, J., Zannini, E., Arendt, E.K., Czerny, M., 2012a. Investigation of product quality, sensory profile and ultrastructure of breads made from a range of commercial gluten-free flours compared to their wheat counterparts. Eur. Food Res. Technol. 235, 333–344.

Hager, A.S., Wolter, A., Jacob, F., Zannini, E., Arendt, E.K., 2012b. Nutritional properties and ultra-structure of commercial gluten free flours from different botanical sources compared to wheat flours. J. Cereal Sci. 56, 239–247.

Hallberg, L., Brune, M., Erlandsson, M., Sandberg, A.-S., Rossander-Hulten, L., 1991. Calcium: Effect of different amounts on nonheme-iron and heme-iron arsorption in humans. Am. J. Clin. Nutr. 53, 112–119.

Harika, R., Faber, M., Samuel, F., Mulugeta, A., Kimiywe, J., Eilander, A., 2017. Are low intakes and deficiencies in iron, vitamin A, zinc, and iodine of public health concern in Ethiopian, Kenyan, Nigerian, and South African children and adolescents. Food Nutr. Bull. 38, 405–427.

Hasjim, J., Lavau, G.C., Gidley, M.J., Gilbert, R.G., 2010. In vivo and in vitro starch digestion: Are current in vitro techniques adequate? Biomacromolecules 11, 3600–3608.

Haydersah, J., Chevallier, I., Rochette, I., Mouquet-Rivier, C., Picq, C., Marianne-Pépin, T., Icard-Vernière, C., Guyot, J.P., 2012. Fermentation by amylolytic lactic acid bacteria and consequences for starch digestibility of plantain, breadfruit, and sweet potato flours. J. Food Sci. 77, 466–472.

Hejazi, S.N., Orsat, V., 2016. Malting process optimization for protein digestibility enhancement in finger millet grain. J. Food Sci. Technol. 53, 1–10.

Hejazi, S.N., Orsat, V., Azadi, B., Kubow, S., 2014. Improvement of the in vitro protein digestibility of amaranth grain through optimization of the malting process. J. Cereal Sci. 68, 59–65.

Hemery, Y.M., Laillou, A., Fontan, L., Jallier, V., Moench-Pfanner, R., Berger, J., Avallone, S., 2018. Storage conditions and packaging greatly affects the stability of fortified wheat flour: Influence on vitamin A, iron, zinc, and oxidation. Food Chem. 240, 43–50.

Herald, T.J., Gadgil, P., Perumal, R., Bean, S.R., Wilson, J.D., 2014. High-throughput micro-plate HCl-vanillin assay for screening tannin content in sorghum grain. J. Sci. Food Agric. 94, 2133–2136.

Heshe, G.G., Haki, G.D., Woldegiorgis, A.Z., 2015. Effect of conventional milling on the nutritional value and antioxidant capacity of wheat types common in Ethiopia and a recovery attempt with bran supplementation in bread. Food Sci. Nutr. 4, 534–543.

Hithamani, G., Srinivasan, K., 2014. Effect of domestic processing on the polyphenol content and bioaccessibility in finger millet (Eleusine coracana) and pearl millet (Pennisetum glaucum). Food Chem. 164, 55–62.

Hofvander, Y., 1968. Hematological investigations in Ethiopia with special references to a high iron intake. Acta Med. Scand. 494, 11–74.

Hole, A.S., Rud, I., Grimmer, S., Sigl, S., Narvhus, J., Sahlstrom, S., 2012. Improved bioavailability of dietary phenolic acids in whole grain barley and oat groat following

fermentation with probiotic Lactobacillus acidophilus, Lactobacillus johnsonii, and Lactobacillus reuteri. J. Agric. Food Chem. 6369–6375.

Holm, J., Bjorck, I., 1992. Bioavailability of starch in various wheat-based bread products: Evaluation of metabolic responses in healthy subjects and rate and extent of in vitro starch digestion. Am. J. Clin. Nutr. 55, 420–429.

Hoover, R., Zhou, Y., 2003. In vitro and in vivo hydrolysis of legume starches by α-amylase and resistant starch formation in legumes - a review. Carbohydr. Polym. 54, 401–417.

Hopman, E., Dekking, L., Blokland, M., Wuisman, M., Zuijderduin, W., Koning, F., Schweizer, J., 2008. Tef in the diet of celiac patients in The Netherlands. Scand. J. Gastroenterol. 43, 277–282.

Hsu, H.W., Satterlee, D.L., Miller, G.A., 1977. A multienzyme technique for estimating protein digestibility. J. Food Sci. 42, 1269–1273.

Humer, E., Schedle, K., 2016. Fermentation of food and feed: A technology for efficient utilization of macro and trace elements in monogastrics. J. Trace Elem. Med. Biol. 37, 69–77.

Hurkman, W.J., Vensel, W.H., Tanaka, C.K., Whitehand, L., Altenbach, S.B., 2009. Effect of high temperature on albumin and globulin accumulation in the endosperm proteome of the developing wheat grain. J. Cereal Sci. 49, 12–23.

Hurrell, R., 2004. Phytic acid degradation as a means of improving iron absorption. Int. J. Vitam. Nutr. Res. 76, 445–452.

Ingbian, E.K., Adegoke, G.O., 2007. Proximate compositions, pasting and rheological properties of mumu - A roasted maize meal. Int. J. Food Sci. Technol. 42, 762–767.

Jacobson, M.R., Obanni, M., Bemiller, J.N., 1997. Retrogradation of starches from different botanical sources. Cereal Chem. 74, 511–518.

Jakobek, L., 2015. Interactions of polyphenols with carbohydrates, lipids and proteins. Food Chem. 175, 556–567.

Jakobek, L., 2014. Interactions of polyphenols with carbohydrates, lipids and proteins. Food Chem. 175, 556–567.

Jamal, P., Idris, Z.M., Alam, M.Z., 2011. Effects of physicochemical parameters on the production of phenolic acids from palm oil mill effluent under liquid-state fermentation by Aspergillus niger IBS-103ZA. Food Chem. 124, 1595–1602.

James, L.E.A., 2009. Chapter 1 - Quinoa (Chenopodium quinoa Willd.): composition, chemistry, nutritional, and functional properties. Adv. Food Nutr. Res. 58, 1–31.

Janssen, F., Pauly, A., Rombouts, I., Jansens, K.J.A., Deleu, L.J., Delcour, J.A., 2016. Proteins of amaranth (Amaranthus spp.), buckwheat (Fagopyrum spp.), and quinoa (Chenopodium spp.): a food science and technology perspective. Compr. Rev. 1–20.

Jenkins, D.J., Wolever, T.M., Collier, G.R., Ocana, A., Rao, A. V, Buckley, G., Lam, Y., Mayer, A., Thompson, L.U., 1987. Metabolic of a low-glycemic-index. Am. J. Clin. Nutr. 46, 968–975.

Jiang, G., Liu, Q., 2002. Characterization of residues from partially hydrolyzed potato and high amylose corn starches by pancreatic α-amylase. Starch/Staerke 54, 527–533.

Joy, E.J.M., Ander, E.L., Young, S.D., Black, C.R., Watts, M.J., Chilimba, A.D.C., Chilima, B., Siyame, E.W.P., Kalimbira, A.A., Hurst, R., Fairweather-Tait, S.J., Stein, A.J., Gibson, R.S., White, P.J., Broadley, M.R., 2014. Dietary mineral supplies in Africa. Physiol. Plant. 151, 208–229.

Kalman, D.S., 2014. Amino acid composition of an organic brown rice protein concentrate and isolate compared to soy and whey concentrates and isolates. Foods 3, 394–402.

Karl, J.P., Roberts, S.B., Schaefer, E.J., Gleason, J.A., Fuss, P., Rasmussen, H., Saltzman, E., Das,

S.K., 2015. Effects of carbohydrate quantity and glycemic index on resting metabolic rate and body composition during weight loss. Obesity 23, 2190–2198.

Ketema, S., 1997. Tef. Eragrostis tef (Zucc.) Trotter., 1st ed, Promoting the conservation and use of underutilized and neglected crops. 12. International Plant Genetic Resources Institute, Rome, Italy.

Khokhar, S., Apenten, R.K., 2003. Iron binding characteristics of phenolic compounds: Some tentative structure-activity relations. Food Chem. 81, 133–140.

Kim, H.J., White, P.J., 2012. In vitro digestion rate and estimated glycemic index of oat flours from typical and high beta-glucan oat lines. J. Agric. Food Chem. 60, 5237–5242.

Kim, M., Song, G., Kang, M., Yoo, H.J., Jeong, T.-S., Lee, S.-H., Lee, J.H., 2016. Replacing carbohydrate with protein and fat in prediabetes or type-2 diabetes: greater effect on metabolites in PBMC than plasma. Nutr. Metab. 13, 1–11.

Klucinec, J.D., Thompson, D.B., 1999. Amylose and amylopectin interact in retrogradation of dispersed high- amylose starches. Cereal Chem. 76, 282–291.

Knudsen, K.E.B., Munck, L., 1985. Dietary fibre contents and compositions of sorghum and sorghum-based foods. J. Cereal Sci. 3, 153–164.

Koehler, P., Wieser, H., 2013. Chemistry of cereal grains, in: Handbook on Sourdough Biotechnology. pp. 11–45.

Konietzny, U., Greiner, R., 2002. Molecular and catalytic properties of phytate-degrading enzymes (phytases). Int. J. Food Sci. Technol. 37, 791–812.

Kopf-Bolanz, K.A., Schwander, F., Gijs, M., Vergeres, G., Portmann, R., Egger, L., 2012. Validation of an in vitro digestive system for studying macronutrient decomposition in humans. J. Nutr. 142, 245–250.

Kotaskova, E., Sumczynski, D., Mlcek, J., Valasek, P., 2016. Determination of free and bound phenolics using HPLC-DAD , antioxidant activity and in vitro digestibility of Eragrostis tef. J. Food Compos. Anal. 46, 15–21.

Krkookova, B., Mrazova, Z., 2005. Prophylactic components of buckwheat. Food Res. Int. 38, 561–568.

Kumar, S.B., Prabhasankar, P., 2014. Low glycemic index ingredients and modified starches in wheat based food processing: A review. Trends Food Sci. Technol. 35, 32–41.

Kumaran, A., Karunakaran, J.R., 2006. Antioxidant and free radical scavenging activity of an aqueous extract of Coleus aromaticus. Food Chem. 97, 109–114.

Kweon, M., 2010. Falling number in wheat [WWW Document]. Int. Organ. URL https://www.ars.usda.gov/ (accessed 9.6.17).

Kyung, I., Young, I., Gyu, H., 2014. International journal of biological macromolecules in vitro starch digestion and cake quality: Impact of the ratio of soluble and insoluble dietary fiber. Int. J. Biol. Macromol. 63, 98–103.

Labanowska, M., Kurdziel, M., Filek, M., Weselucha-Birczynsk, A., 2014. The impact of biochemical composition and nature of paramagnetic species in grains on stress tolerance of oat cultivars. J. Plant Physiol. 199, 52–66.

Lachat, C.K., Van Camp, J.H., Mamiro, P.S., Wayua, F.O., Opsomer, A.S., Roberfroid, D.A., Kolsteren, P.W., 2006. Processing of complementary food does not increase hair zinc levels and growth of infants in Kilosa district, rural Tanzania. Br J Nutr 95, 174–180.

Larsson, S.C., Giovannucci, E., Bergkvist, L., Wolk, A., 2005. Whole grain consumption and risk of colorectal cancer: a population-based cohort of 60,000 women. Br. J. Cancer 92, 1803–1807.

Lauro, M., Poutanen, K., Forssell, P., 2000. Effect of partial gelatinization and lipid addition on

alpha-amylolysis of barley starch granules. Cereal Chem. 77, 595–601.

Lehmann, U., Robin, F., 2007. Slowly digestible starch - its structure and health implications: a review. Trends Food Sci. Technol. 18, 346–355.

Leoncini, E., Prata, C., Malaguti, M., Marotti, I., Segura-Carretero, A., Catizone, P., Dinelli, G., Hrelia, S., 2012. Phytochemical profile and nutraceutical value of old and modern common wheat cultivars. PLoS One 7, 1–13.

Lestienne, I., Besancon, P., Caporiccio, B., Lullien-Pellerin, V., Treche, S., 2005. Iron and zinc in vitro availability in pearl millet flours (Pennisetum glaucum) with varying phytate, tannin, and fiber contents. J. Agric. Food Chem. 53, 3240–3247.

Li, G., Zhang, P., Ping, Wang, J., Gregg, E.W., Yang, W., Gong, Q., Li, H., Li, H., Jiang, Y., An, Y., Shuai, Y., Zhang, B., Zhang, J., Theodore J Thompson, R.B.G., Roglic, G., Hu, Y., Bennett, P.H., 2008. The long-term effect of lifestyle interventions to prevent diabetes in the China Da Qing Diabetes Prevention Study: a 20-year follow-up study. Lancet 371, 1783–1789.

Li, Q., Xu, J., 2015. Changes in nutritive value and in vitro digestibility of proteins. Eur. J. Food Sci. Technol. 3, 49–57.

Li, Y., Liu, S., Liu, X., Tang, X., Zhang, J., 2017. The impact of heat-moisture treatment on physicochemical properties and retrogradation behavior of sweet potato starch. Int. J. Food Eng. 13, 1–9.

Li, Y., Ma, D., Sun, D., Wang, C., Zhang, J., Xie, Y., Guo, T., 2015. Total phenolic, flavonoid content, and antioxidant activity of flour, noodles, and steamed bread made from different colored wheat grains by three milling methods. Crop J. 3, 328–334.

Liljeberg, H., Bjorck, I., 1998. Delayed gastric emptying rate may explain improved glycaemia in healthy subjects to a starchy meal with added vinegar. Eur. J. Clin. Nutr. 52, 368–371.

Liljeberg, H., Bjorck, I., 1996. Delayed gastric emptying rate as a potential mechanism for lowered glycemia after eating sourdough bread: studies in humans and rats using test products with added organic acids or an organic salt. Am. J. Clin. Nutr. 64, 886–893.

Lindstrom, J., Louheranta, A., Mannelin, M., Rastas, M., Salminen, V., Eriksson, J., Uusitupa, M., Tuomilehto, J., 2003. The Finnish Diabetes Prevention Study (DPS): Lifestyle intervention and 3-year results on diet and physical activity. Diabetes Care 26, 3230–3236.

Liu, R.H., 2007. Whole grain phytochemicals and health. J. Cereal Sci. 46, 207–219.

Liukkonen, K.-H., Katina, K., Wilhelmsson, A., Myllymäki, O., Lampi, A.-M., Kariluoto, S., Piironen, V., Heinonen, S.-M., Nurmi, T., Adlercreutz, H., Peltoketo, A., Pihlava, J.-M., Hietaniemi, V., Poutanen, K., 2003. Process-induced changes on bioactive compounds in whole grain rye., in: The Proceedings of the Nutrition Society. pp. 117–122.

Liyana-Pathirana, C.M., Shahidi, F., 2006. Importance of insoluble-bound phenolics to antioxidant properties of wheat. J. Agric. Food Chem. 54, 1256–1264.

Lu, Y., Lv, J., Hao, J., Niu, Y., Whent, M., Costa, J., Yu, L. (Lucy), 2015. Genotype, environment, and their interactions on the phytochemical compositions and radical scavenging properties of soft winter wheat bran. LWT - Food Sci. Technol. 60, 277–283.

Lucca, P., Hurrell, R., Ingo Potrykus, 2002. Fighting iron deficiency anemia with iron-rich rice fighting iron. J. Am. Coll. Nutr. 21, 184–190.

Lucero, H., Masciarelli, R., Tosi, E.A., Re, E., 2001. Dietary Fiber obtained from amaranth (Amaranthus cruentus) grain by differential milling. Food Chem. 73, 441–443.

Luna Lopez, V., Lopez Medina, J. a, Vazquez Gutierrez, M., Fernandez Soto, M.L., 2014. Carbohydrate: current role in diabetes mellitus and metabolic disease. Nutr. Hosp. 30, 1020–1031.

Mamo, T., Parsons, J.., 1987. Iron nutrition of Eragrostis tef (teff). Trop. Agric. 64, 313–317.

Man, J., Lin, L., Wang, Z., Wang, Y., Liu, Q., Wei, C., 2014. Different structures of heterogeneous starch granules from high- amylose rice. J. Agric. Food Chem. 62, 11254–11263.

Marco, M.L., Heeney, D., Binda, S., Cifelli, C.J., Cotter, P.D., Foligne, B., Ganzle, M., Kort, R., Pasin, G., Pihlanto, A., Smid, E.J., Hutkins, R., 2017. Health benefits of fermented food: microbiota and beyond. Curr. Opin. Biotechnol. 44, 94–102.

Mariotti, F., Tome, D., Mirand, P.P., 2008. Converting nitrogen into protein-Beyond 6.25 and Jones' factors. Crit. Rev. Food Sci. Nutr. 48, 177–84.

Mariotti, M., Pagani, M.A., Lucisano, M., 2013. The role of buckwheat and HPMC on the breadmaking properties of some commercial gluten-free bread mixtures. Food Hydrocoll. 30, 393–400.

Marti, A., Marengo, M., Bonomi, F., Casiraghi, M.C., Franzetti, L., Pagani, M.A., Iametti, S., 2017. Molecular features of fermented teff flour relate to its suitability for the production of enriched gluten-free bread. LWT - Food Sci. Technol. 78, 296–302.

Martin-Fernandez, B., De-Los-Santos-Alvarez, N., Martin-Clemente, J.P., Lobo-Castanon, M.J., Lopez-Ruiz, B., 2016. Challenging genosensors in food samples: The case of gluten determination in highly processed samples. Talanta 146, 490–495.

Massaretto, I.L., Madureira Alves, M.F., Mussi de Mira, N.V., Carmona, A.K., Lanfer Marquez, U.M., 2011. Phenolic compounds in raw and cooked rice (Oryza sativa L.) and their inhibitory effect on the activity of angiotensin I-converting enzyme. J. Cereal Sci. 54, 236–240.

Mataveli, L.R.V., Pohl, P., Mounicou, S., Arruda, M.A.Z., Szpunar, J., 2010. A comparative study of element concentrations and binding in transgenic and non-transgenic soybean seeds. Metallomics 2, 800–805.

Matos, M.E., Rosell, C.M., 2013. Quality indicators of rice-based gluten-free bread-like products: relationships between dough rheology and quality characteristics. Food Bioprocess Technol. 6, 2331–2341.

Mattila, P., Pihlava, J.M., Hellström, J., 2005. Contents of phenolic acids, alkyl- and alkenylresorcinols, and avenanthramides in commercial grain products. J. Agric. Food Chem. 53, 8290–8295.

May, P., Burns, E., 1971. Method for estimation of tannin in grain sorghum. Agron. J. 63, 511–512.

Mellado-Ortega, E., Hornero-Mendez, D., 2015. Carotenoids in cereals: an ancient resource with present and future applications. Phytochem. Rev. 14, 873–890.

Mengesha, M.H., 1966. Chemical composition of teff (Eragrostis tef) compared with that of wheat, barley and grain sorghum. Econ. Bot. 20, 268–273.

Meynier, A., Goux, A., Atkinson, F., Brack, O., Vinoy, S., 2015. Postprandial glycaemic response: how is it influenced by characteristics of cereal products? Br. J. Nutr. 113, 1931–1939.

Millward, D.J., 1999. Meat or wheat for the next millennium? The nutritional value of plant-based diets in relation to human amino acid and protein requirements. Proc. Nutr. Soc. 58, 249–260.

Min, B., Gu, L., McClung, A.M., Bergman, C.J., Chen, M.H., 2012. Free and bound total phenolic concentrations, antioxidant capacities, and profiles of proanthocyanidins and anthocyanins in whole grain rice (Oryza sativa L.) of different bran colours. Food Chem. 133, 715–722.

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., Carrière, F., Boutrou, R., Corredig, M., Dupont, D., Dufour, C., Egger, L., Golding, M., Karakaya, S., Kirkhus, B.,

Le Feunteun, S., Lesmes, U., Macierzanka, A., Mackie, A., Marze, S., McClements, D.J., Ménard, O., Recio, I., Santos, C.N., Singh, R.P., Vegarud, G.E., Wickham, M.S.J., Weitschies, W., Brodkorb, A., 2014. A standardised static in vitro digestion method suitable for food - an international consensus. Food Funct. 5, 1113–24.

Moghaddam, E., Vogt, J.A., Wolever, T.M.S., 2006. The effects of fat and protein on glycemic responses in nondiabetic humans vary with waist circumference, fasting plasma insulin, and dietary fiber intake. J. Nutr 136, 2506–2511.

Moller, G., Sluik, D., Ritz, C., Mikkilä, V., Raitakari, O.T., Hutri-Kähönen, N., Dragsted, L.O., Larsen, T.M., Poppitt, S.D., Silvestre, M.P., Feskens, E.J.M., Brand-Miller, J., Raben, A., 2017. A protein diet score, including plant and animal protein, investigating the association with HbA1c and eGFR-The PREVIEW project. Nutrients 9, 1–14.

Montilla, E.C., Hillebrand, S., Antezana, A., Winterhalter, P., 2011. Soluble and bound phenolic compounds in different Bolivian purple corn (Zea mays L.) cultivars. J. Agric. Food Chem. 59, 7068–7074.

Moore, J., Luther, M., Cheng, Z., Yu, L., 2009. Effects of baking conditions, dough fermentation, and bran particle size on antioxidant properties of whole-wheat pizza crusts. J. Agric. Food Chem. 57, 832–839.

Moraghan, J.T., Padilla, J., Etchevers, J.D., Grafton, K., Acosta-Gallegos, J.A., 2002. Iron accumulation in seed of common bean. Pant Soil 246, 175–183.

Moroni, A. V., Dal Bello, F., Arendt, E.K., 2009. Sourdough in gluten-free bread-making: An ancient technology to solve a novel issue? Food Microbiol. 26, 676–684.

Moroni, A. V., Iametti, S., Bonomi, F., Arendt, E.K., Dal Bello, F., 2010. Solubility of proteins from non-gluten cereals: A comparative study on combinations of solubilising agents. Food Chem. 121, 1225–1230.

Mosse, J., Huet, J.-C., Baudet, J., 1988. The Amino acid composition of rice grain as a function of nitrogen content as compared with other cereals : A reappraisal of rice chemical scores. Joournal Cereal Sci. 8, 165–175.

Moyano, F.J., Saenz, M.A. de R., Diaz, M., J., T.A.G., 2014. Application of in vitro digestibility methods in aquaculture : constraints and perspectives. Rev. Aquac. 6, 1–20.

Nair, K.M., Augustine, L.F., 2018. Food synergies for improving bioavailability of micronutrients from plant foods. Food Chem. 238, 180–185.

Nayak, B., De J. Berrios, J., Tang, J., 2014. Impact of food processing on the glycemic index (GI) of potato products. Food Res. Int. 56, 35–46.

Ndlid, T.H.A., 2003. Combined Impact of pH and Organic Acids on Iron Uptake by Caco-2 Cells. J. Agric. Food Chem. 51, 7820–7824.

Neethirajan, S., Tsukamoto, K., Kanahara, H., Sugiyama, S., 2012. Ultrastructural analysis of buckwheat starch components using atomic force microscopy. J. Food Sci. 7, 2–7.

Nhan, M.T., Copeland, L., 2014. location diffrence on starch.pdf. Cereal Chem. 91, 587–594.

Nipornram, S., Tochampa, W., Rattanatraiwong, P., Singanusong, R., 2018. Optimization of low power ultrasound-assisted extraction of phenolic compounds from mandarin (Citrus reticulata Blanco cv. Sainampueng) peel. Food Chem. 241, 338–345.

Noda, T., Kimura, T., Otani, M., Ideta, O., Shimada, T., Saito, A., Suda, I., 2002. Physicochemical properties of amylose-free starch from transgenic sweet potato. Carbohydr. Polym. 49, 253–260.

Noda, T., Takahata, Y., Sato, T., Suda, I., Morishita, T., 1998. Relationships between chain length distribution of amylopectin and gelatinization properties within the same botanical origin for sweet potato and buckwheat. Carbohydr. Polym. 37, 153–158.

Noda, T., Takigawa, S., Matsuura-Endo, C., Kim, S.-J., Hashimoto, N., Yamauchi, H., Hanashiro, I., Takeda, Y., 2005. Physicochemical properties and amylopectin structures of large, small, and extremely small potato starch granules. Carbohydr. Polym. 60, 245–251.

Novotni, D., Cukelj, N., Smerdel, B., Curic, D., 2012. Glycemic index and firming kinetics of partially baked frozen gluten-free bread with sourdough. J. Cereal Sci. 55, 120–125.

Olivares, M., Castro, C., Pizarro, F., Lopez De Romana, D., 2013. Effect of increasing levels of zinc fortificant on the iron absorption of bread co-fortified with iron and zinc consumed with a black tea. Biol. Trace Elem. Res. 154, 321–325.

Oliveira, S., Cipolatti, E.P., Furlong, E.B., Soares, L.D.S., 2012. Phenolic compounds and antioxidant activity in fermented rice (Oryza sativa) bran. Cienci. Tecnol. Aliment. Campinas. 32, 531–537.

Omoba, O.S., Taylor, J.R.N., de Kock, H.L., 2015. Sensory and nutritive profiles of biscuits from whole grain sorghum and pearl millet plus soya flour with and without sourdough fermentation. Int. J. Food Sci. Technol. 50, 2554–2561.

Ortega, N., Reguant, J., Romero, M.P., Macia, A., Motilva, M.J., 2009. Effect of fat content on the digestibility and bioaccessibility of cocoa polyphenol by an in vitro digestion model. J. Agric. Food Chem. 57, 5743–5749.

Ostman, E., 2003. Fermentation as a means of optimizing the glycaemic index - food mechanisms and metabolic merits with emphasis on lactic acid in cereal products. Lund University.

Paganini, D., Uyoga, M.A., Zimmermann, M.B., 2016. Iron fortification of foods for infants and children in low-income countries: Effects on the gut microbiome, gut inflammation, and diarrhea. Nutrients 8, 494–502.

Pang, Y., Ahmed, S., Xu, Y., Beta, T., Zhu, Z., Shao, Y., Bao, J., 2018. Bound phenolic compounds and antioxidant properties of whole grain and bran of white, red and black rice. Food Chem. 240, 212–221.

Parada, J., Aguilera, J.M., 2012. Effect of native crystalline structure of isolated potato starch on gelatinization behavior and consequently on glycemic response. Food Res. Int. 45, 238–243.

Parada, J., Aguilera, J.M., 2007. Food microstructure affects the bioavailability of several nutrients. J. Food Sci. 72, 21–32.

Park, I., Shoemaker, C.F., 2007. Gelatinization and pasting properties of waxy and non-waxy rice starches. Starch - Stärke 59, 388–396.

Parker, M.L., Umeta, M., Faulks, R.M., 1989. The contribution of flour components to the structure of injera, an Ethiopian fermented bread made from tef (Eragrostis tef). J. Cereal Sci. 10, 93–104.

Patel, H., Chandra, S., Alexander, S., Soble, J., Williams, K.A., 2017. Plant-based nutrition: an essential component of cardiovascular disease prevention and management. Curr. Cardiol. Rep. 104, 1–10.

Pedrazzini, E., Mainieri, D., Marrano, C.A., Vitale, A., 2016. Where do Protein Bodies of Cereal Seeds Come From? Front. Plant Sci. 7, 1–7.

Peksa, A., Kita, A., Jariene, E., Danilcenko, H., Gryszkin, A., Figiel, A., Kulaitiene, J., Cerniauskiene, J., Aniolowska, M., 2016. Amino acid improving and physical qualities of extruded corn snacks using flours made from Jerusalem artichoke (Helianthus tuberosus), amaranth (Amaranthus cruentus I.) and pumpkin (Cucurbita maxima I.). J. Food Qual. 39, 580–589.

Penas, E., Uberti, F., di Lorenzo, C., Ballabio, C., Brandolini, A., Restani, P., 2014. Biochemical

and immunochemical evidences supporting the inclusion of quinoa (Chenopodium quinoa Willd.) as a gluten-free ingredient. Plant Foods Hum. Nutr. 69, 297–303.

Picolli, L., Lourdes, M. De, Ciocca, S., 2005. Total, insoluble and soluble dietary fiber values measured by enzymatic – gravimetric method in cereal grains. J. Food Compos. Anal. 18, 113–120.

Pihlava, J.-M., Nordlund, E., Heiniö, R.-L., Hietaniemi, V., Lehtinen, P., Poutanen, K., 2015. Phenolic compounds in wholegrain rye and its fractions. J. Food Compos. Anal. 38, 89–97.

Platel, K., Srinivasan, K., 2016. Bioavailability of micronutrients from plant foods: an update. Crit. Rev. Food Sci. Nutr. 56, 1608–1619.

Pods, A., Redzynia, M.B., B, M.K., 2014. Matrix effects on the stability and antioxidant activity of red cabbage anthocyanins under simulated gastrointestinal digestion. BioMed ical Res. Int. 1–11.

Pomeranz, Y., Youngs, V.L., G.S.Robbins, 1973. Protein content and amino acid composition of oat species and tissues. Cereal Chem. 702–707.

Porter, L.J., Hrstich, L.N., Chan, B.G., 1985. The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin. Phytochemistry 25, 223–230.

Poutanen, K., Flander, L., Katina, K., 2009. Sourdough and cereal fermentation in a nutritional perspective. Food Microbiol. 26, 693–699.

Price, M.L., Scoyoc, S. Van, Butler, L.G., 1978. A critical evaluation of the vanillin reaction as an assay for tannin in sorghum grain. J. Agric. Food Chem. 26, 1214–1218.

Price, R.K., Welch, R.W., Lee-Manion, A.M., Bradbury, I., Strain, J.J., 2008. Total phenolics and antioxidant potential in plasma and urine of humans after consumption of wheat bran. Cereal Chem. 85, 152–157.

Qian, J., 1999. Characterization of amaranthus cruentus and chenopodium quinoa starch. Starch/Stärke 51, 116–120.

Rachel, H., Lu, S., Chang, Y.H., Chiang, W., 2015a. Effects of cooking, retrogradation and drying on starch digestibility in instant rice making. J. Cereal Sci. 65, 154–161.

Rachel, H., Lu, S., Chang, Y.H., Chiang, W., 2015b. Effects of added water and retrogradation on starch digestibility of cooked rice flours with different amylose content. J. Cereal Sci. 61, 1–7.

Radhika, V., Rao, V.S.H., 2015. Computational approaches for the classification of seed storage proteins. J. Food Sci. Technol. 52, 4246–4255.

Raes, K., Knockaert, D., Struijs, K., Van Camp, J., 2014. Role of processing on bioaccessibility of minerals: Influence of localization of minerals and anti-nutritional factors in the plant. Trends Food Sci. Technol. 37, 32–41.

Ramulu, P., Rao, P.U., 1997. Effect of processing on dietary fiber content of cereals and pulses. Plant Foods Hum. Nutr. 50, 249–257.

Rashid, M., Butzner, D., Burrows, V., Zarkadas, M., Case, S., Molloy, M., Warren, R., Pulido, O., Switzer, C., 2007. Consumption of pure oats by individuals with celiac disease: A position statement by the Canadian Celiac Association. Can. J. Gastroenterol. 21, 649–651.

Ratner, R.E., Face, M., 2006. An update on the diabetes prevention program. Endocr. Pract. 12, 1–5.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., Rice-Evans, C., 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic. Biol. Med. 26, 1231–1237.

Reale, A., Konietzny, U., Coppola, R., Sorrentino, E., Greiner, R., 2007. The importance of lactic

acid bacteria for phytate degradation during cereal dough fermentation. J. Agric. Food Chem. 55, 2993–2997.

Regina, A., Bird, A., Topping, D., Bowden, S., Freeman, J., Barsby, T., Kosar-hashemi, B., Li, Z., Rahman, S., Morell, M., 2006. High-amylose wheat generated by RNA interference improves indices of large-bowel health in rats. Proc. Natl. Acad. Sci. U. S. A. 103, 3546–3551.

Reichwald, K., Hatzack, F., 2008. Application of a modified Haug and Lantzsch method for the rapid and accurate photometrical phytate determination in soybean, wheat, and maize meals. J. Agric. Food Chem. 56, 2888–2891.

Renzetti, S., Dal Bello, F., Arendt, E.K., 2008. Microstructure, fundamental rheology and baking characteristics of batters and breads from different gluten-free flours treated with a microbial transglutaminase. J. Cereal Sci. 48, 33–45.

Repo-carrasco-valencia, R.A., Serna, L.A., 2011. Quinoa (Chenopodium quinoa, Willd .) as a source of dietary fiber and other functional components. Cienc. e Tecnol. Aliment. 31, 225–230.

Rinaldi, M., Paciulli, M., Caligiani, A., Scazzina, F., Chiavaro, E., 2017. Sourdough fermentation and chestnut flour in gluten-free bread: A shelf-life evaluation. Food Chem. 224, 144–152.

Rizzello, C.G., Lorusso, A., Montemurro, M., Gobbetti, M., 2016. Use of sourdough made with quinoa (Chenopodium quinoa) flour and autochthonous selected lactic acid bacteria for enhancing the nutritional, textural and sensory features of white bread. Food Microbiol. 56, 1–13.

Rodushkin, I., Odman, F., Branth, S., 1999. Multielement analysis of whole blood by high resolution inductively coupled plasma mass spectrometry. Fresenius. J. Anal. Chem. 364, 338–346.

Roopa, S., Premavalli, K.S., 2008. Effect of processing on starch fractions in different varieties of finger millet. Food Chem. 106, 875–882.

Rosa-Sibakov, N., Poutanen, K., Micard, V., 2015. How does wheat grain, bran and aleurone structure impact their nutritional and technological properties? Trends Food Sci. Technol. 41, 118–134.

Rosell, C.M., Barro, F., Sousa, C., Mena, M.C., 2016. Cereals for developing gluten-free products and analytical tools for gluten detection. J. Cereal Sci. 59, 354–364.

Salawu, S.O., Bester, M.J., Duodu, K.G., 2014. Phenolic composition and bioactive properties of cell wall preparations and whole grains of selected cereals and legumes. J. Food Biochem. 38, 62–72.

Salunkhe, D.K., Jadhav, S.J., Kadam, S.S., Chavan, J.K., Luh, B.S., 1983. Chemical, biochemical, and biological significance of polyphenols in cereals and legumes. Crit. Rev. Food Sci. Nutr. 17, 277–305.

Sandstedt, R.M., Strahan, D., Ueda, S., Abbot, R.C., 1962. The digestibility of high-amylose corn starches compared to that of other starches. The apparent effect of the ae gene on susceptibility to amylase action. Cereal Chem.

Sasaki, T., Okunishi, T., Sotome, I., Hiroshi, O., 2016. Effects of milling and cooking conditions of rice on in vitro starch digestibility and blood glucose response. Cereal Chem. 93, 242–247.

Saturni, L., Ferretti, G., Bacchetti, T., 2010. The gluten-free diet: Safety and nutritional quality. Nutrients 2, 16–34.

Saunders, J., 2010. Physicochemical properties of wheat starches and their relationship to

liquefaction and fermentative bioethanol by. Winnipeg, Manitoba, Canada.

Saura-Calixto, F., 2011. Dietary fiber as a carrier of dietary antioxidants: An essential physiological function. J. Agric. Food Chem. 59, 43–49.

Scarnato, L., Montanari, C., Serrazanetti, D.I., Aloisi, I., Balestra, F., Del Duca, S., Lanciotti, R., 2017. New bread formulation with improved rheological properties and longer shelf-life by the combined use of transglutaminase and sourdough. LWT - Food Sci. Technol. 81, 101–110.

Schlemmer, U., Frolich, W., Prieto, R.M., Grases, F., 2009. Phytate in foods and significance for humans: Food sources, intake, processing, bioavailability, protective role and analysis. Mol. Nutr. Food Res. 53, 330–375.

Schley, P.D., Field, C.J., 2002. The immune-enhancing effects of dietary fibres and prebiotics. Br. J. Nutr. 87, 221–230.

Segura, M.E.M., Rosell, C.M., 2011. Chemical composition and starch digestibility of different gluten-free breads. Plant Foods Hum. Nutr. 66, 224–230.

Shaw, E., Warkentin, M.T., McGregor, S.E., Town, S., Hilsden, R.J., Brenner, D.R., 2017. Intake of dietary fibre and lifetime non-steroidal anti-inflammatory drug (NSAID) use and the incidence of colorectal polyps in a population screened for colorectal cancer. J. Epidemiol. Community Health 71, 961–969.

Shen, Y., Jin, L., Xiao, P., Lu, Y., Bao, J., 2009. Total phenolics, flavonoids, antioxidant capacity in rice grain and their relations to grain color, size and weight. J. Cereal Sci. 49, 106–111.

Shrestha, A.K., Blazek, J., Flanagan, B.M., Dhital, S., Larroque, O., Morell, M.K., Gilbert, E.P., Gidley, M.J., 2012. Molecular, mesoscopic and microscopic structure evolution during amylase digestion of maize starch granules. Carbohydr. Polym. 90, 23–33.

Shumoy, H., Gabaza, M., Vandevelde, J., Raes, K., 2017. Soluble and bound phenolic contents and antioxidant capacity of tef injera as affected by traditional fermentation. J. Food Compos. Anal. 58, 52–59.

Shumoy, H., Raes, K., 2017. In vitro starch hydrolysis and estimated glycemic index of tef porridge and injera. Food Chem. 229, 381–387.

Shumoy, H., Raes, K., 2016. Antioxidant potentials and phenolic composition of tef varieties: An indigenous ethiopian cereal. Cereal Chem. 93, 465–470.

Shutov, A.D., Baumlein, H., Blattner, F.R., Muntz, K., 2003. Storage and mobilization as antagonistic functional constraints on seed storage globulin evolution. J. Exp. Bot. 54, 1645–1654.

Silano, M., Penas Pozo, E., Uberti, F., Manferdelli, S., Del Pinto, T., Felli, C., Budelli, A., Vincentini, O., Restani, P., 2014. Diversity of oat varieties in eliciting the early inflammatory events in celiac disease. Eur. J. Nutr. 53, 1177–1186.

Singh, J., Kaur, L., Singh, H., 2013. Food microstructure and starch digestion, in: Henry, J. (Ed.), Advances in Food and Nutrition Research. Elsevier Inc., Palmerston North, pp. 137–179.

Singh, R., 2012. Total phenolic, flavonoids and tannin contents in different extracts of Artemisia absinthium. J. Intercult. Ethnopharmacol. 1, 101–104.

Singleton, V.L., Orthofer, R., Lamuela-Raventos, R.M., 1999. Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. Methods Enzymol. 299, 152–178.

Skrabanja, V., Kreft, I., Golob, T., Modic, M., Ikeda, S., Ikeda, K., Kreft, S., Bonafaccia, G., Knapp, M., Kosmelj, K., 2004. Nutrient content in buckwheat milling fractions. Cereal Chem. 81, 172–176.

Soil & Crop Improvement BV, 2007. Eragrain tef [WWW Document]. Backgr. Inf. map Eragrain-

teff.

Sompong, R., Siebenhandl-Ehn, S., Linsberger-Martin, G., Berghofer, E., 2011. Physicochemical and antioxidative properties of red and black rice varieties from Thailand, China and Sri Lanka. Food Chem. 124, 132–140.

Song, M., Fung, T.T., Hu, F.B., Willett, W.C., Longo, V.D., Chan, A.T., Giovannucci, E.L., 2016. Association of animal and plant protein intake with all-cause and cause-specific mortality. JAMA Intern. Med. 176, 1453–1463.

Spaenij-Dekking, L., Kooy-Winkelaar, Y., Koning, F., 2005. The Ethiopian cereal tef in celiac disease. N. Engl. J. Med. 353, 1748–1749.

Sreenivasulu, K., Raghu, P., Nair, K.M., 2010. Polyphenol-rich beverages enhance zinc uptake and metallothionein expression in caco-2 cells. J. Food Sci. 75, 123–128.

Srichuwong, S., Naoto, I., Takashi, M., Hisamatsu, M., 2005a. Structure of lintnerized starch is related to X-ray diffraction pattern and susceptibility to acid and enzyme hydrolysis of starch granules. Int. J. Biol. Macromol. 37, 115–121.

Srichuwong, S., Sunarti, T.C., Mishima, T., Isono, N., Hisamatsu, M., 2005b. Starches from different botanical sources I: Contribution of amylopectin fine structure to thermal properties and enzyme digestibility. Carbohydr. Polym. 60, 529–538.

Stojadinovic, M., Radosavljevic, J., Ognjenovic, J., Vesic, J., Prodic, I., Stanic-Vucinic, D., Cirkovic Velickovic, T., 2013. Binding affinity between dietary polyphenols and ß-lactoglobulin negatively correlates with the protein susceptibility to digestion and total antioxidant activity of complexes formed. Food Chem. 136, 1263–1271.

Syahariza, Z.A., Sar, S., Hasjim, J., Tizzotti, M.J., Gilbert, R.G., 2013. The importance of amylose and amylopectin fine structures for starch digestibility in cooked rice grains. Food Chem. 136, 742–749.

Tagliazucchi, D., Verzelloni, E., Bertolini, D., Conte, A., 2010. In vitro bio-accessibility and antioxidant activity of grape polyphenols. Food Chem. 120, 599–606.

Takumi, K., Udaka, J., Kimoto, M., 2000. Structural and immunochemical homologies between foxtail millet glutelin 60 kDa and starch granule-bound starch synthase proteins from rice, barley, corn and wheat grains. J. Nutr. Sci. Vitaminol. 46, 109–112.

Tang, H., Mitsunaga, T., Kawamura, Y., 2006. Molecular arrangement in blocklets and starch granule architecture. Carbohydr. Polym. 63, 555–560.

Tang, Y., Zhang, B., Li, X., Chen, P.X., Zhang, H., Liu, R., Tsao, R., 2016. Bound phenolics of quinoa seeds released by acid, alkaline, and enzymatic treatments and their antioxidant and α-glucosidase and pancreatic lipase inhibitory effects. J. Agric. Food Chem. 64, 1712–1719.

Tatham, A.S., Fido, R.J., Moore, C.M., D. D. Kasarda, Kuzmicky, D.D., Shewry, P.R., Keen, J.N., 1996. Characterisation of the major prolamins of tef (Eragrostis tef) and finger millet (Eleusine coracana). J. Cereal Sci. 24, 65–71.

Tavares, L., Figueira, I., McDougall, G.J., Vieira, H.L.A., Stewart, D., Alves, P.M., Ferreira, R.B., Santos, C.N., 2013. Neuroprotective effects of digested polyphenols from wild blackberry species. Eur. J. Nutr. 52, 225–236.

Tefera, H., Belay, G., Sorrells, M.E., 2001. Narrowing the rift: tef research and development, International workshop on Tef genetics and improvement. Ethiopian Agricultural Research Organization, Addis Ababa.

Tester, R.F., Karkalas, J., Qi, X., 2004. Starch structure and digestibility enzyme-substrate relationship. World Poult. Sci. J. 60, 186–195.

Tester, R.F., Qi, X., Karkalas, J., 2006. Hydrolysis of native starches with amylases. Anim. Feed

Sci. Technol. 130, 39–54.

Teucher, B., Olivares, M., Cori, H., 2004. Enhancers of iron absorption : Ascorbic acid and other organic acids. Int. J. Vitam. Nutr. Res. 74, 403–419.

Theil, E.C., Burton, J.W., J.L. Beard, 1997. A sustainable solution for dietary iron deficiency through plant biotechnology and breeding to increase seed ferritin control. Eur. J. Clin. Nutr. 51, 28–31.

Thompson, L.U., Yoon, J.H., 1984. Starch digestibility as affected by polyphenols and phytic acid. J. Food Sci. 49, 1228–1229.

Tian, J., Chen, S., Wu, C., Chen, J., Du, X., Chen, J., Liu, D., Ye, X., 2016. Effects of preparation methods on potato microstructure and digestibility: an in vitro study. Food Chem. 211, 564–569.

Tinus, T., Damour, M., Riel, V. Van, Sopade, P.A., 2012. Particle size-starch – protein digestibility relationships in cowpea (Vigna unguiculata). J. Food Eng. 113, 254–264.

Towo, E., Matuschek, E., Svanberg, U., 2006. Fermentation and enzyme treatment of tannin sorghum gruels: Effects on phenolic compounds, phytate and in vitro accessible iron. Food Chem. 94, 369–376.

Tuano, A.P.P., Perez, L.M., Padolina, T.F., Juliano, B.O., 2015. Survey of grain quality of Philippine farmers ' specialty rices. Philipp. Agric. Sci. 98, 446–456.

Ua-Arak, T., Jakob, F., Vogel, R.F., 2017. Influence of levan-producing acetic acid bacteria on buckwheat-sourdough breads. Food Microbiol. 65, 95–104.

Umeta, M., Faulks, R.M., 1989. Lactic acid and volatile (C2-C6) fatty acid production in the fermentation and baking of tef (Eragrostis tef). J. Cereal Sci. 9, 91–95.

Umeta, M., Faulks, R.M., 1988. The effect of fermentation on the carbohydrates in tef (Eragrostis tef). Food Chem. 27, 181–189.

Umeta, M., West, C.E., Fufa, H., 2005. Content of zinc, iron, calcium and their absorption inhibitors in foods commonly consumed in Ethiopia. J. Food Compos. Anal. 18, 803–817.

Urga, K., Narasimha, H., 1997. Effect of natural fermentation on the HCl-extractability of minerals from tef (Eragrostis tef). Bull. Chem. Soc. Ethiop. 11, 3–10.

Urisu, A., Ando, H., Morita, Y., Wada, E., 1997. Allergenic activity of heated and ovomucoid-depleted egg white. J. Allergy Clin. Immunol. 100, 171–176.

USDA, 2016. USDA National nutrient database for standard reference [WWW Document]. Natl. Nutr. Database Release 28. URL https://ndb.nal.usda.gov/ndb/search/list%0A (accessed 2.8.17).

USDA, 2010. USDA national nutrient database for standard reference [WWW Document]. Natl. Nutr. Database. URL http://ndb.nal.usda.gov/ (accessed 9.25.16).

USDA, HHS, 2015. U.S. Department of health and human services and U.S. department of agriculture. 2015 – 2020 dietary guidelines for Americans (8th edition) [WWW Document]. URL https://health.gov/dietaryguidelines/2015/guidelines/ (accessed 10.9.17).

Valero-Cases, E., Nuncio-Jáuregui, N., Frutos, M.J., 2017. Influence of fermentation with different lactic acid bacteria and in vitro digestion on the biotransformation of phenolic compounds in fermented pomegranate juices. J. Agric. Food Chem. 65, 6488–6496.

Van Amelsvoort, J.M.M., Weststrate, J.A., 1992. Amylose-amylopectin ratio in a meal affects postprandial variables in male volunteers. Am. J. Clin. Nutr. 55, 712–718.

Van Hung, P., Chau, H.T., Phi, N.T.L., 2016. In vitro digestibility and in vivo glucose response of native and physically modified rice starches varying amylose contents. Food Chem. 191, 74–80.

Van Hung, P., Maeda, T., Morita, N., 2006. Waxy and high-amylose wheat starches and flours-characteristics, functionality and application. Trends Food Sci. Technol. 17, 448–456.

Varga, E.M., Kollmann, D., Zach, M., Bohle, B., 2011. Anaphylaxis to buckwheat in an atopic child: A risk factor for severe allergy to nuts and seeds? Int. Arch. Allergy Immunol. 156, 112–116.

Verhoeckx, K., Issers, Y.M., Joseph Baumert, L., Faludi, R., Feys, M., Flanagan, S., Herouet-Guicheney, C., Holzhauser, H., Shimojo, R., Bolt, N. van der, Wichers, H., Kimber, I., 2015. Food processing and allergenicity. Food Chem. Toxicol. 80, 223–240.

Verhoeckx, Cotter, P., Lopez-Exposito, I., Kleiveland, C., Lea, T., Mackie, A., Requena, T., Swiatecka, D., Wichers, H., 2015. The impact of food bioactives on health, 1st ed, INFOGEST. Springer, Switzerland.

Virkamaki, A., Korsheninnikova, E., Seppälä-Lindroos, A., Vehkavaara, S., Goto, T., Halavaara, J., Häkkinen, A.M., Yki-Järvinen, H., 2001. Intramyocellular lipid is associated with resistance to in vivo insulin actions on glucose uptake, antilipolysis, and early insulin signaling pathways in human skeletal muscle. Diabetes 50, 2337–2343.

Wang, S., Copeland, L., 2013. Molecular disassembly of starch granules during gelatinization and its effect on starch digestibility: a review. Food Funct. 4, 1564.

Wang, S., Li, C., Copeland, L., Niu, Q., Wang, S., 2015. Starch retrogradation: a comprehensive review. Compr. Rev. Food Sci. Food Saf. 14, 568–585.

Wang, S., Wang, J., Yu, J., Wang, S., 2014. A comparative study of annealing of waxy, normal and high-amylose maize starches: The role of amylose molecules. Food Chem. 164, 332–338.

Wei, Y.M., Hu, X.Z., Zhang, G.Q., Ouyang, S.H., 2003. Studies on the amino acid and mineral content of buckwheat protein fractions. Nahrung - Food 47, 114–116.

Wen, D., Li, C., Di, H., Liao, Y., Liu, H., 2005. A universal HPLC method for the determination of phenolic acids in compound herbal medicines. J. Agric. Food Chem. 53, 6624–6629.

Wieser, H., Koehler, P., 2008. The biochemical basis of celiac disease. Cereal Chem. J. 85, 1–13.

Willett, W., Manson, J., Liu, S., Nutr, A.J.C., 2002. Glycemic index, glycemic load, and risk of type 2 diabetes. Am. Soc. Clin. Nutr. 76, 274–280.

Williams, H.D., Anby, M.U., Sassene, P., Kleberg, K., Goma, J.B., Calderone, M., Jannin, V., Igonin, A., Partheil, A., Marchaud, D., Jule, E., Vertommen, J., Maio, M., Blundell, R., Benameur, H., Mu, A., Pouton, C.W., Porter, C.J.H., 2012. Formulations during in Vitro Digestion. Mol. Pharm. 9, 3286–3300.

Wolever, T.M., Jenkins, D.J., Jenkins, a L., Josse, R.G., 1991. The glycemic index: methodology and clinical implications. Am. J. Clin. Nutr. 54, 846–854.

Wolfgor, R., Drago, S.R., Rodriguez, V., Pellegrino, N.R., Valencia, M.E., 2002. In vitro measurement of available iron in fortified foods. Food Res. Int. 35, 85–90.

Wolter, A., Hager, A.-S.S., Zannini, E., Arendt, E.K., 2014a. Influence of sourdough on in vitro starch digestibility and predicted glycemic indices of gluten-free breads. Food Funct. 5, 564–572.

Wolter, A., Hager, A.S., Zannini, E., Arendt, E.K., 2013. In vitro starch digestibility and predicted glycaemic indexes of buckwheat, oat, quinoa, sorghum, teff and commercial gluten-free bread. J. Cereal Sci. 58, 431–436.

Wolter, A., Hager, A.S., Zannini, E., Galle, S., Gänzle, M.G., Waters, D.M., Arendt, E.K., 2014b. Evaluation of exopolysaccharide producing Weissella cibaria MG1 strain for the production of sourdough from various flours. Food Microbiol. 37, 44–50.

World Health Organization, 1996. Trace elements in human nutrition and health, Library Cataloguing in Publication Data. WHO Library Cataloguing in Publication Data, Geneva.

Yafang, S., Tang, F., Huang, Y., Xu, F., Chen, Y., Tong, C., Chen, H., Bao, J., 2014. Analysis of genotype X environment interactions for polyphenols and antioxidant capacity of rice by association mapping. J. Agric. Food Chem. 62, 5361–5368.

Yamada, C., Izumi, H., Hirano, J., Mizukuchi, A., Kise, M., Matsuda, T., Kato, Y., 2005. Degradation of soluble proteins including some allergens in brown rice grains by endogenous proteolytic activity during germination and heat-processing. Biosci. Biotechnol. Biochem. 69, 1877–1883.

Yang, D., Shin, J.A., Gan, L.J., Zhu, X.M., Hong, S.T., Sung, C.K., Cho, J.W., Ku, J.H., Lee, K.T., 2012. Comparison of nutritional compounds in premature green and mature yellow whole wheat in Korea. Cereal Chem. 89, 284–289.

Yohannes, T., Melak, F., Siraj, K., 2013. Preparation and physicochemical analysis of some Ethiopian traditional alcoholic beverages. African J. Food Sci. 7, 399–403.

Yu, L., Beta, T., 2015. Identification and antioxidant properties of phenolic compounds during production of bread from purple wheat grains. Molecules 20, 15525–15549.

Zeleznak, K.J., Hoseney, R.C., 1996. The role of Water in the retrogradation of wheat starch gels and bread crumb. Cereal Chem. 63, 407–411.

Zeng, Z., Liu, C., Luo, S., Chen, J., Gong, E., 2016. The profile and bioaccessibility of phenolic compounds in cereals influenced by improved extrusion cooking treatment. PLoS One 11, 1–11.

Zhang, H., Shao, Y., Bao, J., Beta, T., 2015. Phenolic compounds and antioxidant properties of breeding lines between the white and black rice. Food Chem. 172, 630–639.

Zhang, M., Ma, D., Wang, C., Zhao, H., Zhu, Y., Guo, T., 2016. Responses of amino acid composition to nitrogen application in high- and low-protein wheat cultivars at two planting environments. Crop Sci. 56, 1277–1287.

Zhu, F., 2018. Chemical composition and food uses of teff (Eragrostis tef). Food Chem. 239, 402–415.

Zilic, S., Barac, M., Pesic, M., Dodig, D., Ignjatovic-Micic, D., 2011. Characterization of proteins from grain of different bread and durum wheat genotypes. Int. J. Mol. Sci. 12, 5878–5894.

Zimmermann, M.B., Chaouki, N., Hurrell, R.F., 2005. Iron deficiency due to consumption of a habitual diet low in bioavailable iron: A longitudinal cohort study in Moroccan children. Am. J. Clin. Nutr. 81, 115–121.

Curriculum Vitae

Habtu Shumoy was born on 26th of July 1984 in a remote village called Kilto in Raya Azebo district, Ethiopia. He was graduated in Bachelor of Education in Chemistry 2006 from Bahirdar University, Ethiopia. He obtained his Master of Science in Food Science and Technology in 2010 from Haramaya University. In his master thesis, he studied the effect of pre-harvest sprouting on quality of wheat bread. On the 1st of July 2014, he obtained a three year fellowship from CARIBU (Strand 1/ Lot L18) project of an Erasmus Mundus Action 2 partnership program of the European Union and started his PhD at the research group of Food Microbiology and Biotechnology, department of Food Technology, Safety and Health, Faculty of Bioscience Engineering, Ghent University under the supervision of **Prof. dr. ir. Katleen Raes**. The focus of his PhD study was the nutrition and health benefits of tef and its food products. Articles from his study are published in reputable international journals.

Personal information

First name(s) / Surname	Habtu/Shumoy Abraha
Address	Elfde Julilaan 30, Kortrijk 8500, Belgium
Telephone	+32483 604818
E-mail	habtu.shumoy@ugent.be
Nationality	Ethiopian
Date of birth	July 26 1984
Gender	Male

Educational background

July 1, 2014 up to now	Studying PhD at Ghent University, Belgium
Dissertation	COMPOSITIONAL AND NUTRITIONAL PROPERTIES OF TEF AND TEF-BASED FOOD PRODUCTS
Promotor	Prof. dr. ir. Katleen Raes
2010	MSc. In Food Science and Technology**,** Haramaya University, Ethiopia
Thesis	Effect of variety, degree of sprouting and blending ratio on wheat (*Triticum aestivum*) bread quality
Promotor	Geremew Bultosa (Prof)
2006	**(BED)** Bachelor of Education major in Chemistry and minor Mathematics**,** Bahirdar University, Ethiopia

Work experience

March 2011-July 2014	Lecturer (teaching courses such as: grain science and technology and food chemistry) and researcher at Mekelle University, Tigray, Ethiopia
July 2006-Sept 2008	Teacher (chemistry), Dire Dawa senior secondary school, Dire Dawa, Ethiopia

Publications

1. Shumoy H. Pattyn S., Raes K. 2018. Tef rotein: Solubility characterization, *in vitro* digestibility and its suitability as a gluten-free ingredient. LWT-Food Science and Technology: 89, 697–703. Doi: 10.1016/j.lwt.2017.11.053.
2. **Shumoy** H., Raes K, 2017. Tef: The rising ancient cereal: what do we know about its nutritional and health benefits? Plant Foods and Human Nutrition. Doi: 10.1007/s11130-017-0641-2.
3. **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. 102:93–100. Doi: 10.1016/j.foodres.2017.09.092.
4. **Shumoy** H. & Raes K., 2017. *In vitro* starch hydrolysis and estimated glycemic index of tef porridge and *injera*. Food Chemistry, 229:381–387. Doi: 10.1016/j.foodchem.2017.02.060.
5. **Shumoy** H., Gabaza M., Vandevelde J. & Raes K., 2017. Soluble and bound phenolic contents and antioxidant capacity of tef *injera* as affected by traditional fermentation. Journal of Food Composition and Analysis. 58:52–59. Doi: 10.1016/j.jfca.2017.01.004.
6. **Shumoy** H. & Raes K., 2016. Antioxidant Potentials and Phenolic Composition of Tef Varieties: An Indigenous Ethiopian Cereal. Cereal Chemistry. 93(5):465–470. Doi: 10.1094/CCHEM-10-15-0210-R.
7. Gabaza M., **Shumoy** H., Muchuweti M., Vandamme P., Raes K., 2017. Iron and zinc bioaccessibility of fermented maize, sorghum and millets from five locations in Zimbabwe. Food Research International. DOI: 10.1016/j.foodres.2017.10.047.
8. Gabaza M., **Shumoy** H., Muchuweti M., Raes K, 2017. Traditional fermentation and cooking of finger millet: Implications on mineral binders and subsequent *in vitro* mineral bioaccessibility. Journal of Food Composition and Analysis. DOI: 10.1016/j.jfca.2017.05.011.
9. Gabaza M., **Shumoy** H., Muchuweti M., Vandamme P., Raes K, 2016. Effect of Fermentation and Cooking on Soluble and Bound Phenolic Profiles of Finger Millet Sour Porridge. Journal of Agriculture and Food Chemistry. 64:7615–7621. DOI: 10.1021/acs.jafc.6b03090.

Articles submitted for publication

1. **Shumoy** H., Gabaza M, Vandevelde J., Raes K. Effect of fermentation on bioaccessibility and bioavailability of phenolic compounds of tef *injera*. LWT-Food Science and Technology.
2. **Shumoy** H., Bockstaele F.V., Devecioglu D., Raes K. Effect of sourdough and storage time on *in vitro* starch digestibility and estimated glycemic index of tef bread. Submitted to Food Chemistry.
3. Gabaza M., **Shumoy** H., Muchuweti M., Vandamme P., Raes K. Baobab and mopane worm as potential functional ingredients to improve the iron and zinc bioaccessibility of fermented cereals. Innovative Food Science and Emerging Technologies.
4. Gabaza M., **Shumoy** H., Muchuweti M., Vandamme P., Raes K. Enzymatic degradation of mineral binders in cereals: impact on iron and zinc bioaccessibility. Journal of Cereal Science.

Tutorship of master thesis

1 **Julie Vandevelde**, 2016. *In vitro* digestion and bioaccessibility of minerals and phenolic compounds of tef *injera*. Gent University, Successfully defended.
2 **Margo Callens**, 2016. *In vitro* zetmeelverteerbaarheid en glycemische index van tef producten. Ghent University, Successfully defended.
3 **Sieglinde Pattyn**, 2017 Tef [*Eragrostis tef* (Zucc.)] protein characterization and its suitability as a gluten-free cereal. Gent University, Successfully defended.
4 **Dilara Devecioglu**, 2017. Effect of sourdough on tef bread texture and *in vitro* protein and starch digestibility. Gent University, Successfully defended.

Honors

2014 PhD grant from Erasmus Mundus Action 2, European Union, Europe

2013 Vlir-Uos fellowship, Belgium Government, for 3 months and 2 weeks International training program

2009 Research grant from ESDP of Italian government project at Haramaya University, MSc thesis research fund

Scientific Presentations

Poster presentation: *In vitro* starch hydrolysis and estimated glycemic index of tef porridge and *injera*. 1st food chemistry conference. 30 october-1 November, 2016. Amsterdam, Netherlands.

Poster presentation: Soluble and bound phenolic profile and antioxidant properties of tef *injera* as effected by fermentation and variety. 10th symposium of the Ghent Africa Platform – GAPSYM10**.** December 8-9/2016 Gent, Belgium.

Poster presentations: Tef (*Eragrostis tef*) whole consumption mode and antinutritional concerns. 12th European nutritional conference. October 20-23, 2015, Berlin, Germany.

Poster presentation: Mineral content and antinutritional components in tef, an indigenous Ethiopian cereal. 5th annual meeting Belgian Nutrition Society. April 3, 2015, Brussels, Belgium.

Oral presentation: Effect of variety, degree of sprouting and blending ratio on wheat (*Triticum aestivum*) bread quality, African Regional Workshop on: "The Right to Adequate Food and Nutrition: October 9, 2010. Addis Ababa, Ethiopia.

Additional training

Plunge into your Own Business Plan, Gent University, Belgium, 2017

Introductory Statistics, Basics of Statistical Inference. Gent University, Belgium, 2016.

Introduction to R statistical Software. Gent University, Belgium, 2016

Future Food, Potsdam University, Germany, 2015.

Food safety, Quality Assurance Systems and Risk Analysis. Ghent University, Belgium. 2013.