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KALEAB BAYE YIMAM

Voies alimentaires d'amélioration de la biodisponibilité du fer et du zinc dans les aliments de complément consommés par les jeunes enfants en Ethiopie

Food-based strategies to enhance iron and zinc bioavailability of complementary foods consumed by young children in Ethiopia

Thèse dirigée par Jean-Pierre GUYOT et co-dirigée par Claire Mouquet-Rivier

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Jury :

Mme Ines EGLI	Chercheur senior, Swiss Federal Institute of Technology Zurich- ETH-Zurich	**Rapporteur**
Mr Jean François GRONGNET	Professeur, Agrocampus Ouest, Rennes	**Rapporteur**
Mme Marie-Josèphe AMIOT CARLIN	Professeur, Faculté de Médecine de la Timone, Marseille	**Examinateur**
Mme Valérie MICARD	Professeur, SupAgro, UMR IATE	**Examinateur**
Mr Jean-Pierre GUYOT	Directeur de recherche, UMR-Nutripass-IRD-Montpellier	**Directeur de thèse**

Dedication

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Table of Contents

Résumé en Français **xv**

1. Chapter one: Introduction **4**

2. Chapter two: Literature review **10**

2.1 Iron and zinc in human health **10**

2.2 Iron and zinc deficiencies and their consequences **10**

2.3 Etiology of iron and zinc deficiency **12**

2.3.1 Physiological factors 12

2.3.2 Disease-related excessive loss or impaired absorption 12

2.3.3 Inadequate intake/low bioavailability 13

2.4 Factors affecting iron and zinc bioavailability **16**

2.4.1 Subject factors 16

2.4.2 Dietary factors 16

2.5 Absorption enhancers **18**

2.5.1 Organic acids 18

2.5.2 Muscle proteins: the 'Meat factor' 19

2.6 Absorption inhibitors **19**

2.6.1 Milk, white egg or soybean proteins 19

2.6.2 Calcium 19

2.6.3 Phytates 20

2.6.3.1 Mineral-binding properties of phytates 22

2.6.3.2 Analysis of phytates 23

2.6.3.3 Enzymatic degradation of phytates 24

2.6.4 Polyphenols 26

2.6.4.1 Different classes of polyphenols 27

2.6.4.2 Analysis of phenolic compounds 31

2.6.4.3 Iron-binding properties 32

2.6.4.4 Enzymatic prevention of iron-binding by polyphenols 38

2.7 Dietary fibers- undisclosed factor **41**

2.7.1 Major components of dietary fiber 41

2.7.2 Classification of dietary fibers 42

2.7.3 Analysis of dietary fiber in foods 42

2.7.4 Effect of dietary fibers on mineral bioavailability 44

2.7.5 Plausible mechanism for mineral absorption inhibiting/enhancing effects of fibers 53

2.7.6 Enzymes targeting dietary fibers 53

2.8 Iron and zinc bioavailability assessment methods **56**

2.8.1 In vivo methods 56

2.8.2 In vitro methods 57

2.8.3 Mathematical models....58
2.9 Iron and zinc deficiency prevention and control strategies....59
2.9.1 Supplementation....60
2.9.2 Food fortification....60
2.9.3 Biofortification....61
2.9.4 Dietary diversification strategies....61
2.9.5 Dietary modification through household food processing....62
2.9.5.1 Thermal processing....62
2.9.5.2 Soaking....62
2.9.5.3 Decortication and milling....63
2.9.5.4 Fermentation....63
2.9.5.5 Germination....65
2.9.6 Complementary feeding strategies....65
2.10 Iron and zinc literature: the case of Ethiopia....66
2.10.1 Iron and zinc deficiencies in Ethiopia....66
2.10.2 Complementary feeding practices in Ethiopia....68
2.10.3 Mineral bioavailability estimation of Ethiopian staple foods....69
3. Chapter three: Materials and Methods....72
3.1 Study protocols....72
3.1.1 Food consumption survey....72
3.1.1.1 Location of the study....72
3.1.1.2 Study participants....73
3.1.1.3 Socio-demographic status and anthropometric measures....73
3.1.1.4 Dietary intake assessment....73
3.1.1.5 Compilation of local food composition database....74
3.1.1.6 Assessment of nutrient intake adequacy from complementary foods....74
3.1.1.7 Ethical approval....74
3.1.2 Household food processing observations and sample collection....75
3.1.3 Relative contribution of mineral absorption inhibitors on iron and zinc bioaccessibility 77
3.1.3.1 Dry heat sterilization of flours....78
3.1.3.2 Phytase (P) treatment....78
3.1.3.3 Xylanase+cellulase (X+C) co-treatment....78
3.1.3.4 Polyphenol oxidase (PPO) treatment....79
3.1.3.5 Phytase, xylanase, cellulase and polyphenol oxidase (P+X+C+PPO) treatment....79
3.2 Biochemical analysis....79
3.2.1 Dry matter....79
3.2.2 Changes in pH....80
3.2.3 Protein....80

3.2.4 Lipid content....80
3.2.5 Analysis of mono- and disaccharides and α-galactosides on fermented *injera* paste...80
3.2.6 Changes in water extractable mono and disaccharides after enzymatic treatments.....80
3.2.7 Analyses of lactic acid, acetic acid, mannitol and ethanol on fermented *injera* paste..81
3.2.8 Iron, zinc and calcium analysis....81
3.2.9 Mineral absorption inhibitors....81
3.2.9.1 Phytate (IP6) analysis....81
3.2.9.2 Fiber analysis....81
3.2.9.3 Polyphenols....82
3.2.9.4 Phytase activity....82
3.2.9.5 Prediction of iron absorption using algorithm....83
3.2.9.6 *In-vitro* iron and zinc bioaccessibility....83
3.3 Statistical analyses....85
3.3.1 Analyses of food consumption survey data....85
3.3.2 Analyses of biochemical analyses data....85
4. Chapter four: Results....88
4.1 Nutrient intake adequacy from complementary foods consumed by young children in North Wollo....88
4.1.1 Introduction....88
4.1.2 Nutrient intakes from complementary foods consumed by young children (aged 12-23 months) from North Wollo, northern Ethiopia: the need for agro-ecologically adapted intervention- *Public Health Nutrition, FirstView*, 1-10; doi:10.1017/S1368980012005277 89
4.1.3 Complementary results: access to health services, consumption frequency and access to staples....100
4.2 Fermentation kinetics and phytate hydrolysis of injera sourdough....104
4.2.1 Introduction....104
4.2.2 Influence of flour blend composition on fermentation kinetics and phytate hydrolysis of sourdough used to make *injera Food Chemistry, 138*(1), 430-436. 105
4.2.3 Complementary results: phytase activity of cereal grains used to prepare injera flours 113
4.3 Iron and zinc bioavailability estimation in Ethiopian staples....114
4.3.1 Introduction....114
4.3.2 Changes in mineral absorption inhibitors during traditional processing of Ethiopian injera and accompanying stews: implications for predicted iron bioavailability and bioaccessibility- Submitted to *Plant Foods for Human Nutrition*....115
4.3.3 Complementary results: Food composition results....136
4.4 Relative effects of mineral absorption inhibitors on iron bioaccessibility in injera flours....138
4.4.1 Introduction....138

4.4.2 Enzymatic degradation of phytate, polyphenols and dietary fibers in Ethiopian injera flours: Effect on iron bioaccessibility. Manuscript to be submitted to *Food Chemistry* 139

4.4.3 Complementary results: effect of phytase, carbohydrase and polyphenol oxidase treatment on total polyphenols and zinc bioaccessibility 157

5. Chapter five: General discussion 162

5.1 Complementary feeding practices 162

5.2 Potential of *injera* fermentation on phytic acid and polyphenol degradation 163

5.3 Estimation of iron and zinc bioavailability in *injeras* and stews 164

5.4 Relative importance of mineral absorption inhibitors in determining iron bioaccessibility 165

5.5 Highland Vs Lowland 165

5.6 Limitations of the study 166

6. Chapter six: Conclusions, recommendations and perpectives 170

6.1 Conclusions 170

6.2 Recommendations 171

6.3 Perspectives 172

References 174

Annex A- Consent form 196

Annex B- Questionnaires 197

Annex C- Chromatogram before and after phytase treatment 201

List of figures

Chapter 2

Fig. 2.1: Anemia in pre-school age children as public health concern

Fig. 2.2: Estimates of the prevalence of risk of zinc deficiency

Fig. 2.3: Desired contribution of complementary foods as a percentage of the requirements of a child aged 9-11 months consuming breast milk intake of average volume and composition

Fig. 2.4: Iron absorption depending on source and chemical forms

Fig. 2.5: Structure of phytic acid

Fig. 2.6: Hydrolysis of phytate by the action of phytase

Fig. 2.7: Structures of the 6 sub-classes of flavonoids

Fig. 2.8: The different classes of tannin

Fig. 2.9: Relative iron absorption from a bread and beverage meal relative to polyphenol contents of beverages

Fig. 2.10: Octahedral geometry of iron-polyphenol complexes

Fig. 2.11: Hydroxylation (a) and oxidation (b) reaction catalyzed by a polyphenol oxidase

Fig. 2.12: Mechanism of action of tannase

Fig 2.13: Geographical variation in the prevalence of IDA, ID and anemia in Ethiopian women of childbearing age

Chapter 3

Fig. 3.1: Map of Ethiopia showing the study sites *Doro Gibir* and *Debot* in North Wollo

Fig. 3.2: Flow diagram showing the protocol used for the field observation of injera processing and household sampling

Fig 3.3: Experimental design of enzymatic treatments applied to Teff-white Sorghum (TwS) and Wheat-red Sorghum (WrS) blends used to prepare Ethiopian *injera*

Chapter 4

Fig. 4.1: Effect of different enzymatic treatments on zinc bioaccessibility

Fig. 4.2: Changes in total polyphenols during enzymatic treatments

List of tables

Chapter 2

Table 2.1: Zinc and iron Required Nutrient Intakes (RNI) in mg/day for different life stages

Table 2.2: Content of phytic acid/ phytate in commonly consumed cereals and legumes

Table 2.3: Phytase activity of common grains and seeds

Table 2.4: Classification and structure of polyphenols

Table 2.5: Classification of polyphenols by iron-binding properties

Table 2.6: *In vitro* studies on the effect of fiber on mineral availability

Table 2.7: *In vivo* (animal/human) studies on the effect of fiber on mineral bioavailability

Table 2.8: Enzymes degrading non-starch polysaccharides and associated substances

Table 2.9: Examples of traditional fermented cereal-based foods and beverages consumed in tropical countries

Table 2.10: Major cereal-based traditional complementary foods

Chapter 4

Table 4.1: Birth order, access to health services of mothers and young children

Table 4.2: Legumes used in *Shiro* preparations in the highlands and lowlands of North Wollo, northern Ethiopia

Table 4.3: Cereals used for making *Injera* in the highland and lowlands

Table 4.4: Access of legumes and cereals

Table 4.5: Apparent phytase activity of raw cereals use in injera preparations

Table 4.6: Protein and fat content of prepared foods

Table 4.7: Polyphenol and fiber contents in cereal grains, *injeras* and accompanying stews

Table 4.8: Iron, zinc, calcium and mineral absorption inhibitors in raw legume seeds

Abbreviations/acronyms

ADF	Acid detergent fiber
α -GOS	α-galactosides
AOAC	Association of Official Analytical Chemists
ASF	Animal Source Food
BF	Breastfed
β-ODAP	β-Oxalyldiaminopropionic acid
BSA	Bovine Serum Albumin
BW	Barley-wheat
Caco-cells	Colon adenoma carcinoma cells
CMN	Carences en micronutriments
CSA	Central statistics Agency
DALY	Disability adjusted life years
DP	Degree of polymerization
DM	Dry matter
DMT 1	Divalent metal transporter 1
EDS	Ethiopian Demographic Survey
EDTA	Ethylenediaminetetraacetic acid
EGU	Endo-glucanase unit
EHNRU	Ethiopian Health and Nutrition Research Unit
ENI	Ethiopian Nutrition Institute
FAO	Food and Agriculture Organization
FOS	Fructo-oligosacharide
GC	Gas chromatography
HHDP	Hexahydrodiphenic acid
HPAIC	High performance anion exchange chromatography
HPLC	High performance liquid chromatography
IDA	Iron Deficiency Anemia
ID	Iron deficiency
IDF	Insoluble dietary fiber
IP6	Inositol hexa-phosphate
LAZ	Length for age Z- score
LSD	Least significant difference
MALDI	Matrix-assisted laser desorption/ionization
MND	Micronutrient deficiency
mRNA	Messenger ribonucleic Acid
MS	Mass Spectroscopy
NSP	Non-starch polysaccharide
OMS	Organisation Mondiale de la Santé
OPS	Organisation Panaméricaine de la Santé
PAHO	Pan American Health Organization
PDMS	Polydimethylsiloxane
PPO	Polyphenol oxidase
PU	phytase unit
TwS	Teff-white sorghum
RBV	Relative bioavailability value
RNI	Required Nutrient Intakes
RS	Resistant starch
WAZ	Weight for age Z- score
WE	Water extractable
WHO	World Health Organization

Résumé en Français

1. Introduction

La période d'alimentation complémentaire (6-23 mois) revêt une importance particulière car c'est le stade où les nourrissons et les jeunes enfants connaissent une croissance et un développement rapide. Durant cette période, les retards de croissance et les carences en micronutriments (CMN) sont très répandues, en partie à cause des besoins beaucoup plus élevés que les ingérés en énergie et en micronutriments (Shrimpton et al., 2001). Chez les enfants, ces carences sont associées à une faible croissance, un développement cognitif suboptimal et un mauvais état de santé (Black et al., 2008). L'impact du retard de croissance est souvent irréversible après l'âge de deux ans (Martorell et al., 1994). À cet égard, le rôle d'une alimentation complémentaire adéquate, à la fois en quantité et en qualité, est d'une grande importance.

Dans de nombreux pays en développement, les régimes alimentaires des jeunes enfants sont surtout basés sur les céréales et les légumineuses, avec peu ou pas d'aliments d'origine animale, de fruits et de légumes. Cette alimentation complémentaire monotone principalement à base de plantes est associée à des apports insuffisants en micronutriments en particulier en fer et en zinc car ces derniers se trouvent en très faibles concentrations dans le lait maternel (Gibson et al., 1998). l'insuffisance de ces ingérés est aggravée par la faible biodisponibilité du fer et du zinc dans ces aliments (Gibson et al., 2010). Cette faible biodisponibilité s'explique principalement par leurs teneurs élevées en constituants chélateurs des minéraux comme les phytates et les polyphénols (Lopez et al., 2002, Hurrell, 2010). Bien que les résultats soient moins clairs, l'effet négatif du calcium sur la biodisponibilité des minéraux a également été démontré. Le cas des fibres reste controversé, avec des études in vitro qui montrent des effets inhibiteurs tandis que les études in vivo (animal /humain) ne montrent aucun effet ou même dans certains cas des améliorations de l'absorption des minéraux en présence de fibres (Frölich, 1995).

Tenant compte des besoins très élevés en fer et en zinc au cours des deux premières années et de la faible capacité gastrique des jeunes enfants, répondre aux besoins est souvent problématique. Par conséquent, le fer et le zinc sont parmi les nutriments souvent décrits comme «problem nutrients» (OMS, 1998). Ainsi, pour que les enfants couvrent leurs besoins, les apports en fer et en zinc doivent être augmentés soit par une planification minutieuse de régimes diversifiés ou en ayant recours à la fortification des aliments, ou par amélioration de la biodisponibilité. Cette dernière voie a aussi l'avantage de réduire les besoins en ingérés. En outre, la qualité sanitaire des aliments complémentaires devrait être assurée afin d'éviter les pertes de minéraux dues à la diarrhée, et les pertes d'appétit ou les malabsorptions liées aux maladies.

Plusieurs études ont documenté l'effet bénéfique de la fermentation en regard des qualités nutritives et sanitaires des aliments (Svanberg et Lorri, 1997, Nout, 2009). La production d'acides organiques tels que les acides lactique et acétique réduit le pH et peut donc limiter la contamination par les pathogènes d'origine alimentaire. En outre, la fermentation peut activer plusieurs enzymes, parmi lesquelles des phytases, des polyphénoloxydases, des tannases, etc., qui peuvent hydrolyser des inhibiteurs d'absorption de minéraux (Greiner et Konietzny 2006, Towo et al., 2006). Toutefois, la mesure dans laquelle ces enzymes sont activées dépend de la cinétique de fermentation, qui, à son tour, dépend de la nature des matières premières utilisées (Hammes et al., 2005).

Malgré des améliorations récentes, la malnutrition des enfants reste un problème de santé publique en Ethiopie. Selon la dernière EDS (CSA / ICF, 2012), 44% des enfants de moins de cinq ans soufrant de retard de croissance, 10% d'émaciation et 29% d'insuffisance pondérale, l'Ethiopie a des taux de malnutrition parmi les plus élevés d'Afrique sub-saharienne. Bien que des mesures directes de la prévalence de la carence en zinc ne soient pas disponibles à l'heure actuelle, la forte prévalence de retard de croissance suggère qu'il s'agit d'un problème de santé publique. L'importance des carences en fer et en zinc est confirmée par les quelques études existantes sur les jeunes enfants (Adish et al., 1999; Gibson et al., 2009). Par conséquent, des interventions nutritionnelles visant à améliorer l'alimentation complémentaire et les statuts en micronutriments sont nécessaires. Cependant, il existe peu de données sur l'adéquation des apports énergétiques et nutritionnels des jeunes enfants et sur la biodisponibilité du fer et du zinc dans les aliments les plus fréquemment consommés, informations nécessaires à la conception d'une stratégie appropriée visant les aliments de complément.

Dans de nombreux pays en développement, les aliments de complément à base de céréales fermentées sont fréquents (Guyot, 2012). En Ethiopie, l'aliment le plus consommé par les jeunes enfants et les adultes est l'*injera,* qui est une sorte de crêpe fermentée souvent consommée accompagnée de sauces à base de légumineuses. Bien qu'il ait été déjà montré que la fermentation de l'*injera* à base de teff, ou de sorgho conduit à une dégradation au moins partielle du phytate, son effet sur les autres chélateurs de minéraux (i.e. polyphénols) demeure inconnue (Abebe et al., 2007). De plus, différents mélanges de céréales comprenant le teff, le sorgho, l'orge et le blé, sont utilisés dans la préparation de l'injera. La nature de ces mélanges peut influencer la cinétique de fermentation et donc la dégradation des phytates, des polyphénols, et des α-galactosides (α -GOS), mais peu de données sont disponibles à ce sujet. Ces pratiques de mélange peuvent ouvrir la possibilité d'intégrer des graines avec des activités enzymatiques endogènes d'intérêt (i.e. phytase) élevées, permettant ainsi l'optimisation du processus de fermentation en vue d'une plus grande diminution des teneurs en facteurs chélateurs des minéraux.

Les tentatives précédentes pour estimer la biodisponibilité des minéraux dans les aliments éthiopiens ont été basées sur les ratios molaires phytate:Fe et phytate:Zn (Abebe et al, 2007). Cependant l'utilisation de cette méthode d'estimation de la biodisponibilité du fer peut avoir des limites car les polyphénols, qui ne sont pas pris en compte dans ce calcul de ratios, peuvent être tout aussi importants que les phytates dans leur effet inhibiteur. Il a été en effet montré que la dégradation des phytates en présence de polyphénols ne permet pas d'améliorer la biodisponibilité du fer (Hurrell et al., 2003). Par ailleurs, l'effet des fibres natives sur la biodisponibilité des minéraux est méconnu car la plupart des études antérieures portaient sur l'effet de fibres ajoutées susceptibles d'avoir des caractéristiques différentes de celles des fibres alimentaires natives (Harris et Smith, 2006).

L'application d'enzymes exogènes peut donc être une approche efficace pour la détermination de l'effet relatif des facteurs chélateurs de minéraux, car elle permet de cibler et d'obtenir une plus grande diminution des teneurs en certains chélateurs (Matuschek et al., 2001; Lestienne et al., 2005 ; Wang et al., 2008). En outre, l'utilisation combinée des enzymes ciblant les composés chélatant le fer peut permettre de comprendre la mesure dans laquelle l'application de modifications alimentaires pourrait améliorer la biodisponibilité du fer et du zinc.

Par conséquent, les objectifs de cette thèse étaient les suivants:

1 - Caractériser les pratiques alimentaires des jeunes enfants (12-23 mois) dans Gobalafto district, au nord du Wollo, nord de l'Ethiopie.

2 - Calculer les ingérés en énergie et nutriments à partir des aliments de complément et évaluer leur adéquation par rapport aux recommandations de l'OMS.

3 - Caractériser les procédés de préparation de l'injera à base de différents mélanges de farines par des observations de terrain.

4 - Évaluer l'influence du type de mélange de farine sur la cinétique de fermentation et de ses implications possibles sur la dégradation des phytates et des polyphénols.

5- Estimer la biodisponibilité du fer et du zinc dans les différents types d'injeras et sauces à base de légumineuses prélevés dans les ménages Ethiopiens.

6 - évaluer la contribution respective des différents chélateurs phytates, fibres, et polyphénols à la réduction de la bioaccessibilité du fer et estimer la part de fer qui pourrait être libérée par leur dégradation.

2. Conception de l'étude et méthodes appliquées

Cette thèse s'est déroulée en trois grandes parties. La première a consisté en une enquête sur le terrain où la consommation des jeunes enfants (12-23 mois) a été évaluée à l'aide d'une étude transversale, utilisant deux rappels de 24h. La deuxième partie a comporté un suivi détaillé des préparations ménagères des aliments les plus fréquemment consommés, suivi par des caractérisations au laboratoire des échantillons recueillis dans les ménages. La troisième partie était une étude mécanistique qui avait pour but d'évaluer l'effet de la dégradation enzymatique des phytates, des polyphénols et des fibres sur la bioaccessibilité du fer.

Une enquête par rappel de 24h a été menée auprès des mères de 76 enfants de 12-23 mois (70 répétitions) en utilisant la technique décrite par Gibson (1999) adaptée et validée pour son utilisation dans les pays en développement. Tous les jours de la semaine ont été également représentés dans l'échantillon final. L'étude a été menée dans deux villages, l'un dans les hauts plateaux et l'autre dans les plaines du nord du Wollo, au nord de l'Ethiopie. Les aliments couramment consommés par les jeunes enfants ont été identifiés. Les apports en énergie et en certains nutriments à partir des aliments de compléments ont été calculés et leurs adéquations par rapport aux besoins estimés par l'OMS ont été évaluées.

L'enquête de consommation a identifié que l'injera, les sauces à base de légumineuses, et le pain sont les aliments les plus fréquemment consommés. Une attention particulière a été accordée à l'injera, puisque la fermentation peut entraîner des dégradations des inhibiteurs des facteurs chélatant les minéraux tels que les phytates, et pourrait donc potentiellement influencer la biodisponibilité des minéraux. Les observations ont montré que l'injera peut être préparé à partir de différents mélanges de céréales en fonction de la localisation géographique (hauts plateaux/plaines). Les mélanges de farine les plus fréquemment observés ont été standardisés et les préparations des différents injeras ont été suivies. Des échantillons ont été prélevés sur place, à différents intervalles de temps, afin de caractériser les cinétiques de fermentation des différents injeras. L'influence de la composition du mélange de farine sur la cinétique de fermentation et sur l'hydrolyse des phytates a été étudiée. À cette fin, les teneurs en mono-et di-saccharides (glucose, fructose, maltose et saccharose), en produits de fermentation (lactate, acétate, mannitol et éthanol), en α-galactosides (raffinose et stachyose) et en phytates ont été déterminées.

Puisque que l'injera est toujours consommée accompagnée de sauces à base de légumineuses, et étant donné que l'effet de la dégradation des phytates sur la biodisponibilité des minéraux est également dépendante de la quantité d'autres inhibiteurs de l'absorption comme les polyphénols,

l'influence des procédés de préparation traditionnels des sauces sur les fer, le zinc, le calcium, les phytates, les fibres (ADF) et les polyphénols chélateurs du fer a été évaluée. La biodisponibilité du fer et du zinc dans les différents injeras et sauces a été estimée en utilisant les rapports molaires (phytate:fer / phytate:zinc), la bioaccessibilité in vitro, et les prédictions d'absorption du fer par algorithme.

L'effet relatif des inhibiteurs potentiels de l'absorption du fer sur la bioaccessibilité du fer a également été évalué en utilisant une approche impliquant l'utilisation d'enzymes ciblant les phytates, les polyphénols et les fibres.

3. Résultats et discussion

Cette thèse avait pour objectif d'étudier jusqu'à quel point des stratégies alimentaires visant à modifier les procédés de transformation des aliments peuvent améliorer la biodisponibilité du fer et du zinc des aliments de complément consommés par les jeunes enfants dans le Nord du Wollo, au nord de l'Ethiopie.

3.1 Pratiques d'alimentation complémentaire

La croissance rapide au cours des deux premières années de vie nécessite un apport considérable en fer et en zinc (Dewey, 2001). Pour répondre à ces exigences, les aliments de compléments doivent contenir des quantités suffisantes de formes biodisponibles de ces nutriments. Les aliments de complément consommés par les jeunes enfants enquêtés dans le nord du Wollo étaient principalement à base de plantes, avec peu ou pas de produits d'origine animale, de fruits et de légumes-feuilles (article 1) Les aliments les plus fréquemment consommés étaient l'injera accompagnée de sauces à base de légumineuses, et le pain. Les deux sauces les plus consommés étaient la sauce à base de petit pois [1] cassé et le *shiro* préparé à base de gesse[2] et de pois de culture[3]. Les ingérés en Ca, vitamine C et vitamine A étaient insuffisants, alors que les ingérés en protéines étaient adéquats. Les ingérés en Zn n'arrivait à couvrir les besoins que quand une biodisponibilité moyenne (30%) est supposée, tandis que l'apport en fer est apparu suffisant, même dans le cas d'une faible biodisponibilité (5%). La plupart des aliments ont des teneurs en fer très élevées, mais une grande partie de ce fer est attribuable à une contamination par le sol. La faible densité nutritionnelle des aliments ainsi que des pratiques d'alimentation inadéquates pourraient avoir contribué aux apports sous-optimaux en énergie et certains nutriments. Une alimentation complémentaire inadéquate est souvent associée à des retards de croissance, ce qui pourrait expliquer en partie le

[1] *Pisum sativum*
[2] *Lathyrus sativus*
[3] *Vicia faba*

nombre élevé d'enfants soufrant de retard de croissance dans les villages enquêtés (Anderson et al., 2008, Gibson et al., 2009). Environ 33% des jeunes enfants, ~46% dans le village à basse altitude et 24% dans le village à haute altitude, souffraient de retard de croissance ($P< 0.05$).

Malgré la nature des aliments complémentaires principalement d'origine végétale et non fortifiés, l'apport en fer a pu couvrir les recommandations de l'OMS, même dans l'hypothèse d'une faible biodisponibilité (article 1). Ceci est en contraste avec les résultats de nombreux pays en développement (Gibson et al., 1998) et industrialisés (Friel et al., 2010), mais est en accord avec les études précédentes sur l'apport en fer en Ethiopie (Adish et al., 1999 ; Abebe et al., 2007). Toutefois, dans notre étude, une part importante du fer était probablement due à une contamination par le sol, très riche en fer en Ethiopie, et présentait une faible solubilité et bioaccessibilité (article 3). Malgré des rapports d'études antérieures sur la rareté des carences en fer en Éthiopie (Gebre-Medhin, 1976), les plus récentes études épidémiologiques indiquent que le problème de carence en fer est d'une ampleur telle qu'il peut être qualifié de problème de santé publique (Haidar et Pobocik, 2009). Ceci suggère que l'origine des carences en fer est plutôt due à une faible biodisponibilité et non pas à un faible apport. Étant donné que la plupart des enfants consomment régulièrement de *l'injera,* les possibilités d'amélioration de la biodisponibilité du fer et du zinc par l'activation des enzymes endogènes et ou microbiennes (i.e. phytases, polyphénoloxydases) méritaient d'être explorées. Cependant, l'ampleur de la dégradation dépend de plusieurs facteurs, y compris les matières premières utilisées et des paramètres de fermentation tels que le pH.

3.2 Potentiel de dégradation de phytates et de polyphénols lors de la fermentation de l'*injera*

La préparation de l'*injera* se fait généralement à partir de mélanges de céréales dont la composition varie d'un ménage à l'autre et en fonction de l'altitude. Les résultats d'enquête ont montré que dans le village d'altitude, les mélanges les plus utilisés étaient les mélanges orge-blé (BW) et blé-sorgho rouge (WrS), tandis que dans le village de basse altitude, le mélange Teff-sorgho blanc (TwS) était plus courant. L'observation des procédés de fabrication in situ a mis en évidence l'incorporation de malt d'orge au cours des procédés de fabrication des *injeras* BW et WrS, alors que le malt n'est pas utilisé dans le cas du mélange TwS. Les cinétiques de fermentation ainsi que l'hydrolyse des phytates ont été enregistrées directement auprès des ménages lors de la préparation d'*injera* à partir des trois types de mélanges. Une hydrolyse complète des phytates a été observée dans les mélanges BW et WrS alors que seulement 28% des phytates ont été hydrolysés dans le mélange TwS (article 2). Plusieurs hypothèses peuvent être formulées pour expliquer cette différence :

(i) une activité phytasique endogène variable d'un mélange à l'autre. En effet, par rapport à d'autres céréales, le blé et l'orge sont de bonnes sources de phytases endogènes (Egli et al, 2002, Reale et al,

2007), ainsi les mélanges de farine contenant ces deux céréales avaient des activités phytasiques supérieures (article 2).

(ii) l'apport d'une activité phytasique via l'incorporation de malt

(iii) ou la mise en place de conditions favorables à la croissance des levures susceptibles d'avoir des activités phytasiques élevées. En effet, certaines levures comme *Saccharomyces cerevisiae* sont connues pour leurs activités phytasiques (Cuves et Banerjee, 2004). .

Ces résultats suggèrent que l'incorporation de céréales ayant des activités phytasiques endogènes élevées, ou l'ajout de malt pourrait permettre une dégradation plus prononcée de l'acide phytique pendant la fermentation. Toutefois, lors de la préparation du malt, des mesures minimisant le risque de production de mycotoxines lors de la germination doivent être prises (Trèche et Mouquet-Rivier, 2008).

D'autre part, l'effet de la fermentation sur les polyphénols chélatant le fer n'était pas aussi marqué que celui sur les phytates (article 3). Une dégradation partielle a été observée lors de la fermentation de l'*injera* WrS*,* tandis qu'un tel effet n'a pas été observé pour les autres *injeras.* Cette différence pourrait être due à la localisation, la nature ou à la quantité des polyphénols contenus, ou encore à l'influence des procédés de transformation tels que le décorticage qui influencent l'accessibilité des polyphénols à la dégradation enzymatique. Ceci étant, malgré les diminutions observées des teneurs en polyphénols chélatant le fer, les teneurs résiduelles étaient encore assez élevées pour exercer un effet inhibiteur sur l'absorption du fer.

3.3 Estimation de la biodisponibilité du fer et du zinc dans les *injeras* et les sauces

La dégradation quasi-totale des phytates lors de la fermentation des *injeras* BW et WrS ou sous l'effet d'une phytase exogène ont permis une diminution importante des ratios molaires phytate:Fe et phytate:Zn, suggérant une biodisponibilité élevée. Toutefois, aucune différence significative sur la bioaccessibilité *in vitro* du fer n'a été observée. Cela suggère que les valeurs critiques des ratios molaires phytate:fer peuvent être moins adapté pour prédire la biodisponibilité des aliments contaminés par du fer extrinsèques (sol) ou aussi que les phytates ne sont pas les seuls inhibiteurs de l'absorption, et donc leur dégradation, en présence d'autres inhibiteurs ne suffit pas à obtenir une amélioration importante de la bioaccessibilité. Ceci est en accord à la fois avec les résultats antérieurs obtenus par des études de bioaccessibilité (Lestienne et al., 2005) et ceux obtenus par des études d'absorption sur l'homme (Petry et al., 2010), et est également confirmé par les valeurs d'absorption du fer prédites par l'algorithme de Hallberg et Hulthen (2000) (article 3).

Parmi les sauces d'accompagnement, de meilleures biodisponibilités en fer et zinc ont été estimées pour le *shiro*. Par conséquent, la promotion de la consommation du *shiro* plutôt que les sauces à base de pois cassé pourrait être avantageuse en termes de biodisponibilité des minéraux. Cependant,

dans les sites étudiés, le *shiro* a été préparé à partir de légumineuses comme les fèves (favisme) et les gesses (β-ODAP) avec des effets potentiellement toxiques. Les procédés comme le trempage et la torréfaction des graines, ou l'inclusion d'ingrédients aux propriétés antioxydantes connues (gingembre, l'ail, etc.) pendant la préparation du *shiro,* peut réduire les effets toxiques (Getahun et al., 2005). Toutefois, compte tenu de l'endémicité du neurolathyrisme dans le nord de l'Ethiopie (Haimanot et al., 2005), le faible poids des enfants et les multiples carences en micronutriments dont ces enfants peuvent être victimes, il serait préférable de remplacer les fèves et les gesses par d'autres légumineuses disponibles, comme les lentilles, les pois chiches et les pois cultivés.

3.4 Importance relative des phytates, des polyphenols et des fibres dans la bioaccessibilité du fer

Dans les mélanges de farine d'*injera* avec des teneurs en polyphénols élevées (WrS) et faibles (TwS), des traitements enzymatiques ciblant les phytates, les fibres (cellulose et hémicelluloses) et les polyphénols chélatant le fer, ont montré que la seule déphytinisation n'améliore pas de façon significative la bioaccessibilité du fer (article 4). Toutefois, dans les farines déphytinisées, l'hydrolyse des fibres alimentaires par la xylanase et la cellulase entraine une augmentation significative de la bioaccessibilité du fer. Ceci est en accord avec les études de bioaccessibilité in *vitro* menées par Lestienne et al. (2005). Cependant, les quelques études menées chez l'homme basées sur l'ajout de fibres exogènes dans le régime, n'ont pas mis en évidence d'effets inhibiteur des fibres sur l'absorption des minéraux, probablement parce que le fer piégé par des fibres peut être libérés par l'action de la microflore colique pour être absorbé au niveau du côlon (Nordgaard et Mortensen, 1995).

Dans les deux types de farines préalablement déphytinisées, l'oxydation des polyphénols par la polyphénoloxydase (PPO) a pu augmenter la fraction de fer soluble non-dialysable, mais une augmentation significative de la fraction dialysable n'a été observée que dans le mélange TwS. Cela suggère qu'une dégradation plus importante des groupements chélatant le fer dans les polyphénols peut être nécessaire pour augmenter significativement la fraction de fer dialysable. Bien que le traitement à la PPO empêche la fixation du fer par les groupes fonctionnels -OH des galloyls et catéchols, le fer peut toujours être pris au piège dans les grands complexes tanins-protéines qui peuvent limiter sa dialysabilité. Le traitement par les PPO après déphytinisation et traitement par une carbohydrase a pu augmenter la fraction dialysable dans la farine WrS, ainsi suggérant que les polyphénols associés aux parois cellulaires peuvent être en partie responsable de la faible bioaccessibilité du fer. Malgré ces améliorations, une majeur partie du fer reste sous forme insoluble après digestion in vitro, probablement parce qu'il s'agit de fer de contamination très faiblement biodisponible.

Sur la base des résultats actuels, on peut présumer que l'optimisation des procédés alimentaires visant une réduction simultanée des teneurs en polyphénols (galloyls/catéchols) et en phytates, ainsi qu'une hydrolyse des fibres permettrait d'améliorer la bioaccessibilité du fer, par comparaison avec une simple déphytinisation. Cependant, la consommation fréquente de thé et de café par les enfants juste avant et après les repas, et les faibles apports en vitamines A et C sont susceptibles d'entraver l'application de stratégies visant à améliorer la biodisponibilité du fer et du zinc, à moins que ces habitudes alimentaires soient changées.

3.5 Comparaisons entre hauts plateaux et plaines

Le taux de retard de croissance était beaucoup plus important dans les hauts plateaux que dans les plaines ($P < 0,05$), probablement en raison de la dureté des conditions physiques et socio-économique dans les régions montagneuses. Bien que *l'injera* soit l'aliment de base sur les deux sites d'enquête, les mélanges de céréales utilisés pour sa préparation se sont révélés différents. En outre, la densité en zinc du régime des enfants des hauts plateaux était supérieure à celle des plaines et devrait répondre aux besoins estimés à condition que le régime soit d'une biodisponibilité moyenne (article 1). Pour le fer, il n'y avait pas de différence dans le pourcentage de fer bioaccessible entre les injeras des hauts plateaux et celui des plaines. Toutefois, compte tenu de la forte teneur en fer (~ 3 fois) dans *l'injera* des basses terres, une plus grande quantité de fer bioaccessible devrait être consommée dans les plaines (article 3).

Les réponses des farines d'*injera* vis-à-vis des différents traitements enzymatiques étaient également différentes, ce qui suggère que les stratégies à adopter pour la diminution des chélateurs du fer dans les hauts plateaux et les plaines ne seront pas forcément les mêmes (article 4).

En outre, les comparaisons entre les deux sites ont révélé des différences marquées d'apports en vitamine C, ce qui pourrait par ailleurs avoir des répercussions sur la biodisponibilité des minéraux du régime.

4. Conclusion et recommandations

4.1 Conclusion

Cette thèse a permis de caractériser de façon détaillée les pratiques d'alimentation complémentaire des jeunes enfants dans le nord du Wollo, et d'évaluer l'adéquation des ingérés à partir des aliments de complément. L'influence de la fermentation de l'*injera* sur la dégradation des phytates et des polyphénols et ses implications sur la biodisponibilité du fer et du zinc a été étudiée. Enfin, l'amélioration relative de la bioaccessibilité du fer, suite à la dégradation des phytates, de l'hydrolyse des fibres, ou de l'oxydation des polyphénols a été évaluée.

Plusieurs pratiques d'alimentation ne sont pas en conformité avec les recommandations de l'OPS/OMS. Les différences agro-écologiques, principalement liées aux différences d'altitude apparaissent avoir un impact sur les apports nutritionnels. Ces différences pourraient donc se traduire par un effet sur la croissance des enfants.

Les profils de fermentation de l'*injera* des hauts plateaux et des plaines ont été différents, principalement en raison de différences dans la composition des mélanges de farine utilisés. La composition du mélange de farine –en influençant le degré d'hydrolyse des phytates a conduit à des injeras avec des teneurs en phytates très différentes. Toutefois, l'action combinée de malt, l'utilisation de mélanges de céréales ayant une activité phytasique endogène élevée (par exemple le blé, l'orge) et l'activité phytasique des levures peut probablement expliquer la dégradation complète en phytate dans les *injeras* contenant de l'orge et/ou du blé. Cela suggère la possibilité d'atteindre des niveaux élevés de dégradation des phytates au cours de la fermentation au sein des ménages.

Cependant, la dégradation complète des phytates dans l'injera n'a pas amélioré la bioaccessibilité du fer, ce qui suggère qu'en présence d'autres chélateurs tels que les polyphénols et les fibres, elle ne suffit pas. Cela met en évidence les limites de l'utilisation des rapports molaires phytate:fer dans l'estimation de la biodisponibilité des minéraux dans les aliments, surtout quand une grande partie du fer est extrinsèque (i.e. sols).

En utilisant différentes enzymes exogènes qui ciblent les phytates, les polyphénols chélatant le fer, et les fibres alimentaires (cellulose et hémicelluloses), le rôle relatif de chacun sur la bioaccessibilité du fer dans les farines d'*injera* a été évalué. L'hydrolyse des fibres ou l'oxydation des polyphénols conduit à une plus grande bioaccessibilité du fer que la seule déphytinisation. Toutefois, les importances relatives des chélateurs de minéraux ainsi que les réponses aux traitements enzymatiques sont apparues dépendantes de la matrice alimentaire.

4.2 Recommandations

Sur la base des résultats obtenus dans cette thèse, plusieurs recommandations susceptibles d'améliorer la qualité de l'alimentation complémentaire dans le nord du Wollo, au nord de l'Ethiopie, mais aussi dans d'autres contextes similaires peuvent être faites.

-Dans les deux villages (hauts plateaux et plaines) des actions devront être mises en place pour améliorer la diversité du régime alimentaire en y incluant des produits d'origine animale, des produits laitiers, des fruits et légumes riches en vitamine A et en vitamine C.

-Des interventions visant à promouvoir les pratiques d'alimentation complémentaire recommandées par l'OMS et prenant en compte les contextes agro-écologiques sont nécessaires.

-Des recommandations devraient être formulées pour décourager la consommation de gesse et de fèves, notamment par les jeunes enfants. Les aides-soignants doivent être informés des effets

toxiques potentiels associés à la consommation de gesse et de fèves, et la consommation d'autres légumineuses disponibles comme les pois chiches, les lentilles, et les pois de culture devrait être encouragée

-Bien que les boissons comme le thé et le café aient l'avantage d'être généralement plus sûres en termes de contamination microbienne, leur consommation devrait être découragée au moins juste avant et après les repas, compte tenu de leurs effets négatifs sur l'absorption des minéraux et l'appétit des enfants.

-L'ajout de malt ou l'inclusion de céréales à activité phytasique relativement élevée (blé, orge) peut permettre une dégradation accrue des phytates qui pourrait ultérieurement améliorer la biodisponibilité des minéraux, en particulier celle du zinc.

Chapter 1- *Introduction*

1. Chapter one: Introduction

The period of complementary feeding (6-23 months) is of particular importance as this is when infants and young children experience rapid growth and development. During this period, growth faltering and micronutrient deficiencies (MND) are highly prevalent partly because of the high nutrient needs relative to energy and micronutrients intakes (Shrimpton et al., 2001). In children, these deficiencies are associated with poor growth, impaired cognitive development and poor health status (Black et al., 2008). The overwhelming impact of growth faltering is usually irreversible after the age of two, thereby leaving a small window of opportunity for intervention (Martorell et al., 1994). In this regard, the role of adequate complementary feeding, both in quantity and quality, is of great importance.

In many developing countries, diets of young children are predominantly cereal and legume based, with little or no animal source foods (ASF), fruits, and vegetables. Such monotonous plant-based complementary diets are associated with suboptimal nutrient intakes, particularly for nutrients like iron and zinc which are found in very low concentrations in breast-milk (Gibson et al., 1998a). The low intake is further exacerbated by the low bioavailability of iron and zinc in cereal/legume-based diets (Gibson et al., 2010). Bioavailability refers to the proportion of the ingested nutrient that is actually absorbed at the level of the intestinal mucosa and thus can be used for normal body functions (Fairweather-Tait et al., 2007). The low bioavailability in cereal and legume based diets is mainly attributed to their high contents in mineral chelating constituents like phytic acid and polyphenols that form insoluble complexes in the lumen of the gastrointestinal tract (Lopez et al., 2002, Hurrell, 2010). Although findings are less consistent, calcium has also been demonstrated to have negative effects on mineral bioavailability. The case of fiber is much more controversial, as conflicting results were obtained with in-vitro studies showing inhibitory effects while animal and in-vivo studies showing either no effect or even in some cases mineral absorption enhancing properties (Frölich, 1995).

Given the very high iron and zinc intake requirements during the first two years and the low gastric capacity of young children, meeting requirements is often problematic. As a result, iron and zinc are among nutrients often described as “problem nutrients” (WHO, 1998). Therefore, for the children to meet their requirements, iron and zinc intakes should be increased either by careful planning of diversified diets or fortification, and, or bioavailability should be improved. Improving

bioavailability has also the advantage of lowering intake requirements. Furthermore, the sanitary quality of complementary foods should be assured to prevent mineral losses due to diarrhea and avoid disease-related loss of appetite or malabsorption.

Several studies have documented the beneficial effect of fermentation in improving both the nutrient and sanitary qualities of foods (Svanberg and Lorri, 1997, Nout, 2009). Production of organic acids such as lactic and acetic acid reduces pH and may thus limit contamination by food-borne pathogens. Furthermore, fermentation can activate several enzymes including phytases, polyphenol oxidases, tannases, etc., and may thus result in products with reduced mineral absorption inhibitors (Greiner and Konietzny, 2006, Towo et al., 2006). However, the extent to which such enzymes are activated depends on the fermentation kinetics, which in turn, depends on the raw materials used (Hammes et al., 2005).

Despite recent improvements, child malnutrition remains a public health concern in Ethiopia. With approximately 44% of children under five being stunted, 10% wasted, and 29% underweight relative to the WHO (2006) multicentre growth reference (CSA/ICF, 2012), Ethiopia has one of the highest malnutrition rates in Sub-Saharan Africa. Although direct measures of the prevalence of zinc deficiency are not available at the present, the high prevalence of stunting indicates that it is likely of public health concern. Although representative data on iron deficiency in young children is lacking, the few existing studies suggest that the prevalence can be high (Adish et al., 1999). Therefore, nutritional interventions with the aim of improving complementary feeding and micronutrient status are needed. However, little is known about the adequacy of energy and nutrient intakes of young children and the bioavailability of the frequently consumed foods, all of which are required for the design of appropriate complementary food-based strategies.

In many developing countries, cereal-based fermented complementary foods are common (Guyot, 2012). In Ethiopia, the most widely consumed food by young children and adults alike is *injera,* which is a thin, flat, traditional fermented pancake that is consumed accompanied most often by legume-based stews. Although teff, and sorghum based *injera* fermentation was previously shown to result in phytate degradation, its effect on other mineral absorption inhibitors like polyphenols remains unknown (Abebe et al., 2007b). Moreover, different cereal blends that may include teff, sorghum, barley and wheat are used in *injera* preparation. Such blends may influence the fermentation kinetics and the reduction of phytic acid (IP6), polyphenols, and α-galactosides (α-GOS), but little is known in this regard. Such blending practices open the opportunity to incorporate

grains with high endogenous enzymatic activities of interest (i.e. phytase), allowing better optimization of the fermentation process for greater decrease in mineral absorption inhibitors.

Previous attempts to estimate the bioavailability of minerals in Ethiopian staples were based on phytate: Fe and phytate: Zn molar ratios (Umeta et al., 2005, Abebe et al., 2007b). Although these molar ratios are thought to give an estimate of bioavailability, they may be limited in the estimation of iron bioavailability since polyphenols can be equally as important as phytate in their iron absorption inhibitory effect. Degradation of phytate in the presence of polyphenols was shown to not improve iron bioavailability (Hurrell et al., 2003). Furthermore, little is known regarding the effect of native dietary fiber on mineral bioavailability since much of the previous studies were on added fibers which are likely to have different characteristics than native dietary fibers (Harris and Smith, 2006).

The application of exogenous enzymes may be an effective approach for the determination of the relative effects of mineral absorption inhibitors as it permits better target and greater decrease of specific mineral absorption inhibitors (Lestienne et al., 2005a, Matuschek et al., 2001, Wang et al., 2008). Furthermore, the combined use of enzymes targeting fiber, phytate, and iron-binding phenolics may give an indication on the extent to which application of dietary modifications aiming to decrease mineral absorption inhibitors is likely to improve iron and zinc bioavailability. This is especially important in the context of rural communities in Ethiopia, where child malnutrition is high and other alternative approaches such as fortification are less practical given the absence of central processing units.

Therefore the objectives of this thesis were to:

1- Characterize the feeding practices of young children (12-23 months) in Gobalafto district, north Wollo.

2- Calculate energy and nutrient intakes from complementary foods and evaluate their adequacy to WHO recommendations.

3- Investigate the processing of injera made from different flour blends based on field observations.

4- Evaluate the influence of the type of flour blend on fermentation kinetics and its possible implications in phytic acid (IP6) and polyphenol degradation.

5- Estimate the bioavailability of iron and zinc in the different types of injeras and legume-based stews sampled in Ethiopian households.

6- Evaluate to what extent iron bioaccessibility can be improved by reducing the contents in the major mineral absorption inhibitors.

This PhD thesis is organized as follows:

Chapter two presents the literature review that covers a brief desciption of the roles of iron and zinc in human health, an overview of the prevalence and consequences of iron and zinc deficiencies, that is followed by a presentation of their etiological factors. The determinants of iron and zinc bioavailability as well as existing public health strategies for the control and prevention of deficiencies are discussed. Finally, a brief description of the relevant literature on iron and zinc nutrition and complementary feeding practices in Ethiopia is presented.

Chapter three gives a description of the experimental designs, and the materials and methods applied in the present studies.

Chapter four presents the findings of the different studies in the form of published papers, submitted manuscript, or manuscript prepared for publication. The chapter is organized as follows:

Section 4.1 (paper published in *Public Health Nutrition*, first view 2012) presents the results from the food consumption survey conducted on young children. It gives an overview of the complementary feeding practices and evaluates the adequacy of complementary foods consumed by young children in two villages of the North Wollo area to WHO recommendations.

Section 4.2 (paper published in *Food Chemistry*, 2013) presents the field-based characterization of the processing methods of the most frequently consumed *injera* types as well as results from investigations of the influence of flour blend composition on fermentation kinetics and phytic acid hydrolysis.

Section 4.3 (paper submitted for publication in Plant Foods for Human Nutrition) presents results on the estimation of bioavailability of Ethiopian staples by making use of three different methods: phytate:mineral molar ratio, absorption prediction algorithm and in vitro bioaccessibility measurements.

Section 4.4 (draft article) presents results on the relative contribution of phytate, dietary fiber, and iron-binding phenolics to the reduction of iron and zinc bioaccessibility in injera flours investigated through the application of exogenous enzymes.

Chapter five gives a general discussion on the findings of the studies.

Chapter six presents the summary of the results, conclusions and perspectives.

Chapter 2- *Literature review*

2. Chapter two: Literature review

2.1 Iron and zinc in human health

Iron and zinc are essential micronutrients for human health (Haas and Brownlie IV, 2001, Yeung et al., 2005). Both are required for various biological processes including growth, the immune system and psychomotor development (Prasad, 2008, Krebs, 2000). Iron is required for the formation of heme and the synthesis of hemoglobin and myoglobin which are involved in the transport and storage of oxygen. Hence, iron plays a key role in the maintenance of physical activity and work capacity (Haas and Brownlie IV, 2001). In addition, several enzymes such as cytochrome P450 are iron dependent, hence iron is also implicated in the synthesis of steroid hormones and bile acids, detoxification of foreign substances in the liver; and signal controlling in some neurotransmitters, such as the dopamine and serotonin systems in the brain (FAO/WHO, 2004).

Zinc is present in all body tissues and fluids and acts as a stabilizer of the structures of membranes and cellular components (Fraker et al., 2000). It thus plays a central role in cellular growth, differentiation, and metabolism (Beyersmann and Haase, 2001). A large number of enzymes (> 300) have zinc as a cofactor (McCall et al., 2000). Zinc is important for the maintenance of the immune system and is involved in the expression of the metallothionein gene, apoptosis and regulation of synaptic signaling (Andrews, 2000, Frederickson et al., 2005).

2.2 Iron and zinc deficiencies and their consequences

Iron and zinc deficiencies are among the most prevalent micronutrient deficiencies in the world (WHO, 2009). Iron deficiency (ID) affects both developing and developed countries (Ramakrishnan and Yip, 2002). At least 30% of the world's population is believed to be affected (Zimmermann and Hurrell, 2007). However, most country specific data, especially in developing countries are based on hemoglobin measures and thus report mostly figures of anemia only . Although a large share of anemia is due to iron deficiency, other causes such as deficiencies in vitamin B12 or folate, infections (i.e. malaria) and genetic abnormalities (i.e. thalassemia) may not be excluded and therefore care should be taken in interpreting the figures (Fig.2.1).

The magnitude of worldwide zinc deficiency is expected to be as high as that of iron; however, direct measures of the prevalence of zinc deficiency are scarce, mainly due to the absence of sensitive biomarkers for use in population (Gibson et al., 2008). Instead, stunting prevalence is used as a proxy of the risk of zinc deficiency (Davidsson et al., 2007, Gibson et al., 2008). According to

stunting prevalence of ≤20%, >20%–40%, and ≥ 40%, the risk of zinc deficiency is classified as low, moderate, and high, respectively (Fig. 2.2).

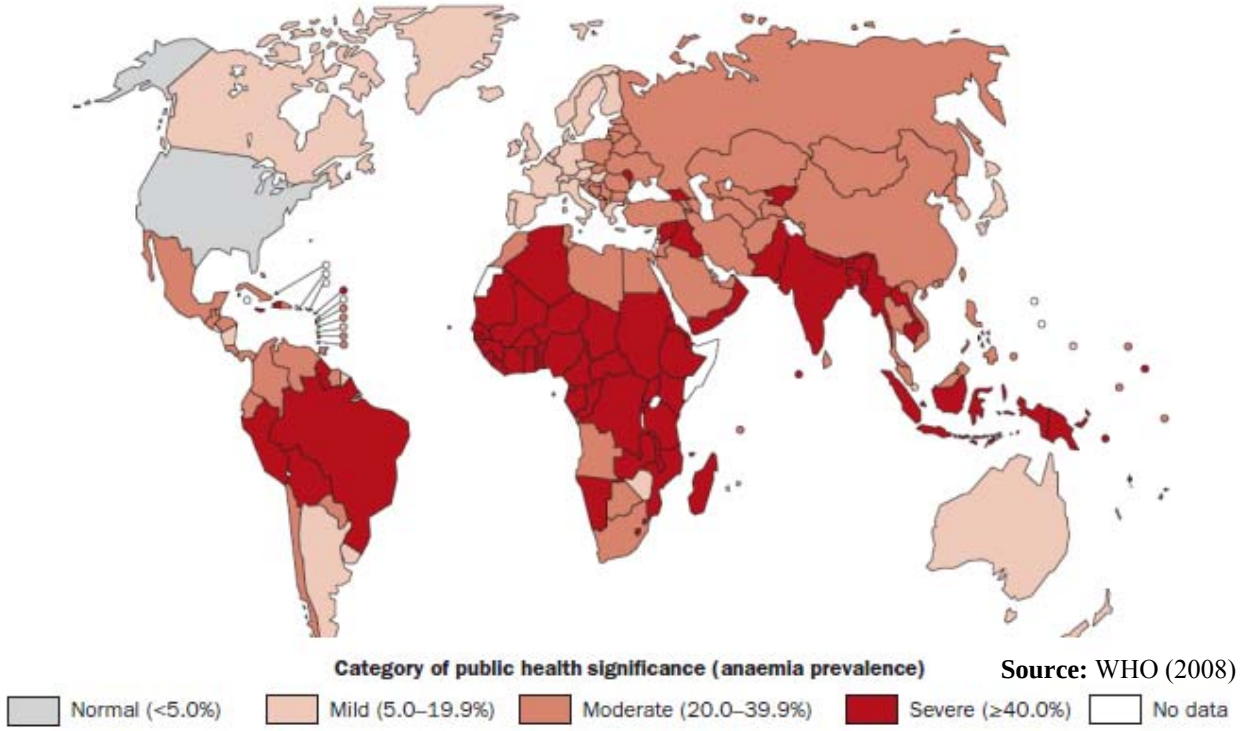


Fig. 2.1: Anemia in pre-school age children as public health concern

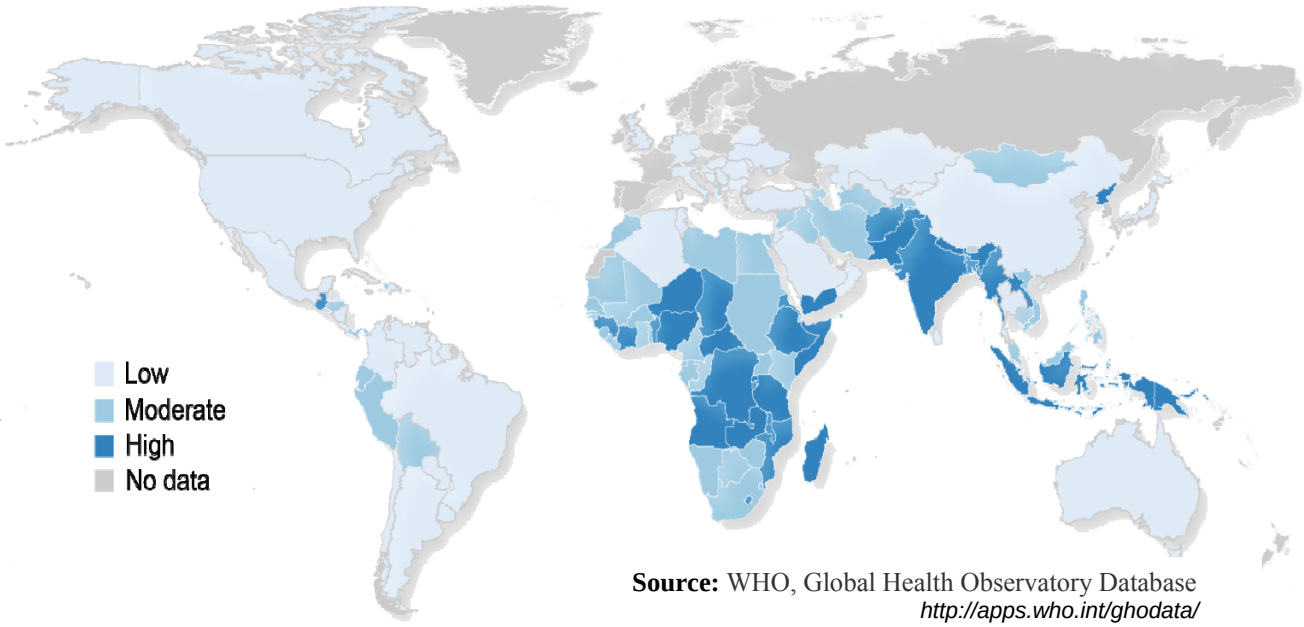


Fig. 2.2: Estimates of the prevalence of risk of zinc deficiency

Given the important role that iron and zinc play in human health, it is evident that deficiencies in them disturb key biological processes. Growth retardation, impaired mental and psychomotor development, child and maternal morbidity/mortality, decreased immunity and work performance, are some of the negative implications of iron and zinc deficiencies ;(Scrimshaw, 1984, Georgieff,

2011). Zinc deficiency has also been associated with anorexia, dermatitis, skeletal abnormalities, pneumonia, diarrhea, and alopecia, to name few (Nriagu, 2010).

Recent reviews suggest that iron and zinc deficiencies in early life (6-23 months) have persistent negative effects later in life (Beard, 2007, Black et al., 2008). Therefore, there is urgent need to prevent or correct deficiencies.

2.3 Etiology of iron and zinc deficiency

The etiological factors responsible for the development of iron and zinc deficiencies are physiological states that increase requirements, diseases that induce excessive loss or impair utilization, low intakes and, or low bioavailability (Hotz and Brown, 2004).

2.3.1 Physiological factors

In adults, physiological iron requirements are dependent on iron losses. In healthy adult men, body iron losses are ~1 mg/d (Green et al., 1968, Hallberg, 1981a). Under normal circumstances, requirements in men are adequately covered by dietary iron absorption (Cook, 1990, Hulten et al., 1995) but this can be more problematic for women of child bearing age who faces an additional loss of 0.5-1.5 mg Fe per day (Hallberg and Rossander-Hulten, 1991). As a result, women of childbearing age, both pregnant and non-pregnant are at high-risk of iron deficiency (Hallberg et al., 1996). Adolescent girls have additional iron needs as they need to sustain growth and at the same time compensate losses due to menstruation. During pregnancy, average daily Fe requirements can reach as high as 4-6 mg during the second and third trimester (Carpenter and Mahoney, 1992) since extra iron is needed for the growth of the fetus (Scholl, 2011).

Likewise, children require 0.5-1.5 mg iron in excess of their daily losses to satisfy the extra needs associated with their rapid growth (Stanner, 2003). Such high demand is difficult to cover and explains why iron deficiency anemia is widespread in these life stages.

2.3.2 Disease-related excessive loss or impaired absorption

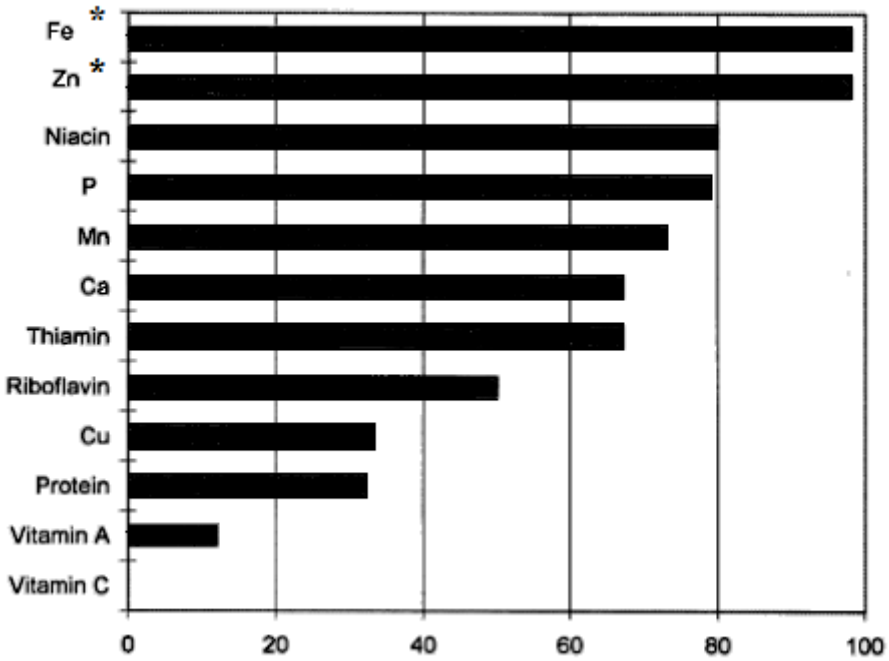
Several diseases have been implicated in the etiology of iron and zinc deficiencies. The mechanism by which they lead to deficiencies is mainly through increased losses or malabsorption. For instance, liver and kidney diseases, and alcoholism (Malyszko et al., 2006, Sullivan and Heaney, 1970) as well as increased catabolism associated with surgery, burns, diabetes mellitus and chronic bleeding (i.e. due to parasites such as hookworm) leads to iron and zinc deficiencies through increased losses (Nriagu, 2010, Stoltzfus et al., 1996). Infectious diseases such as malaria and *Helicobacter pylori* have been associated with iron deficiency. Malaria causes anemia due to hemolysis, increased clearance of both infected and uninfected red blood cells by the spleen, that leads to interruption of erythropoiesis (Nagel, 2002). On the other hand, *H. pylori* remove the acid

gastric barrier and thereby limit the absorption of dietary iron (DiGirolamo et al., 2007, Kurekci et al., 2005).

Chronic infection and obesity have also been found to induce expression of hepcidin, a peptide produced in the liver and adipose tissue, responsible for iron homeostasis by the regulation of absorption (Bekri et al., 2006, Yanoff et al., 2007). Diseases such as celiac disease, atrophic gastritis and postsurgical status (i.e gastrectomy) have also been associated with malabsorption (Ganz and Nemeth, 2006).

2.3.3 Inadequate intake/low bioavailability

The prevalence of iron and zinc deficiency is not evenly distributed within a population or throughout the world (Ramakrishnan, 2002). High prevalence is found in women and children in developing countries, where diets are predominantly plant-based and very little iron- and zinc-rich foods such as animal source foods (ASF) are consumed (Sandstead, 2000). Surprisingly, data on vegetarians in developed countries tend to indicate that despite lower iron and zinc absorption and thus lower iron stores, associated adverse health effects are not observed (Hunt, 2002). In some developed countries, iron and zinc intakes of vegetarians may be equivalent to omnivores, as plant-based foods are often fortified (Hunt, 2003). Furthermore, vegetarians in the developed countries have more diversified diets that those in developing countries. In line with this, the American dietetic association published its position paper stating that "well-planned vegetarian diets are appropriate for individuals during all stages of the life cycle, including pregnancy, lactation, infancy, and adolescence…" (Craig and Mangels, 2009). This suggests that not plant-based diets *per se*, but unplanned/undiversified plant-based diets may be the problem. Therefore, unplanned plant-based diets as often observed in developing countries may not contain enough iron and zinc to meet requirements, especially in physiological states where needs are very high (i.e. pregnancy, infancy). For instance, breastfed infants not receiving complementary foods rich in iron and zinc by 6 months of age can quickly become iron and zinc deficient (Lynch and Stoltzfus, 2003, Krebs and Hambidge, 2007). This is because breastmilk is poor in these nutrients (Dewey and Brown, 2003).



***assuming moderate bioavailability** **Source**: Gibson et al., 1998

Fig. 2.3: Desired contribution of complementary foods as a percentage of the requirements of a child aged 9-11 months consuming breast milk intake of average volume and composition

Total iron and zinc requirements and hence intake recommendations (table 2.1) depend on the type of the diet which determines mineral bioavailability (FAO/WHO, 2004). The term bioavailability refers to the proportion of mineral (i.e. iron, zinc) in the diet that is utilized for normal metabolic functions (Fairweather-Tait, 2002).

Table 2.1: Zinc and iron Required Nutrient Intakes (RNI) in mg/day for different life stages

Life stage	Assumed body Wt (kg)	High bioavailability		Moderate bioavailability		Low bioavailability		Very low bioavailability
		Fe	Zn	Fe	Zn	Fe	Zn	Fe
Infants/young children								
< 6 months	6		1.1		2.8		6.6	
7-12 months	9	6.2¶	0.8, 2.5	7.7¶	4.1	9.3¶	8.4	18.6
1-3 years	12	3.9	2.4	4.8	4.1	5.8	8.3	11.6
4-6 years	17	4.2	2.9	5.3	4.8	6.3	9.6	12.6
7-9 years	25	5.9	3.3	7.4	5.6	8.9	11.2	17.8
Adolescents								
Female	47		4.3		7.2		14.4	
11-14 years NM*		9.3		11.7		14.0		28.0
11-14 years		21.8		27.7		32.7		65.4
15-18 years		20.7		25.8		31.0		62.0
Male	49		5.1		8.6		17.1	
11-14 years		9.7		12.2		14.6		29.2
15-18 years		12.5		15.7		18.8		37.6
Adults								
Female (pre-menopause)	55	19.6	3.0	24.5	4.9	29.4	9.8	58.8
Post-menopause		7.5	3.0	9.4	4.9	11.3	9.8	22.6
Pregnant women‡								
First trimester			3.4		5.5		11.0	
Second trimester			4.2		7.0		14.0	
Third trimester			6.0		10.0		20.0	
Lactating women		10.0		12.5		15.0		30.0
0-3 months			5.8		9.5		19.0	
4-6 months			5.3		8.8		17.5	
6-12 months			4.3		7.2		14.4	
Male	65	9.1	4.2	11.4	7.0	13.7	14.0	27.4

*NM, non-menstruating; ¶highly variable; ‡iron absorption is affected by the stage of pregnancy **Source:** adapted from FAO/WHO, (2004)
Zinc bioavailability was estimated based on phytate: zinc molar ratios of <5 (high), 5-15(moderate),>15(low).
High, moderate, low and very low bioavailability estimated based on iron stores represent 15 %, 12%, 10% and 5%, respectively.

2.4 Factors affecting iron and zinc bioavailability

2.4.1 Subject factors

The bioavailability of minerals is affected by both subject/host and dietary factors. Host factors associated with iron and zinc bioavailability include mineral status, deficiencies in other nutrients such as vitamin A and riboflavin, infection/inflammation, and genetic disorders (Hurrell and Egli, 2010).

Vitamin A deficiency can lead to decreased erythropoiesis with less iron incorporated into red blood cells (Zimmermann et al., 2006, Roodenburg et al., 2000). Data on the effect of vitamin A deficiency on zinc absorption is limited. Significant decline in zinc absorption was observed in cases of severe vitamin A deficiency in animal studies (Christian and West, 1998, Sklan et al., 1987), but this has not been clearly demonstrated in humans and hence requires further study.

Infection and inflammation as discussed above (section 2.3.2) also decrease bioavailability through hypochlorhydria in the case of *H. pylori* and increased hepcidin expression in the case of inflammation or obesity.

Several genetic disorders such as hemochromatosis or iron overload have been related to impaired iron erythropoiesis. Both thalassemia homozygotes and heterozygotes for α-thalassemia 1 and β-thalassemia, are at risk for iron overload as adequate down-regulation with increased iron stores is lacking (Zimmermann et al., 2008, Hurrell, 2010, Hurrell and Egli, 2010).

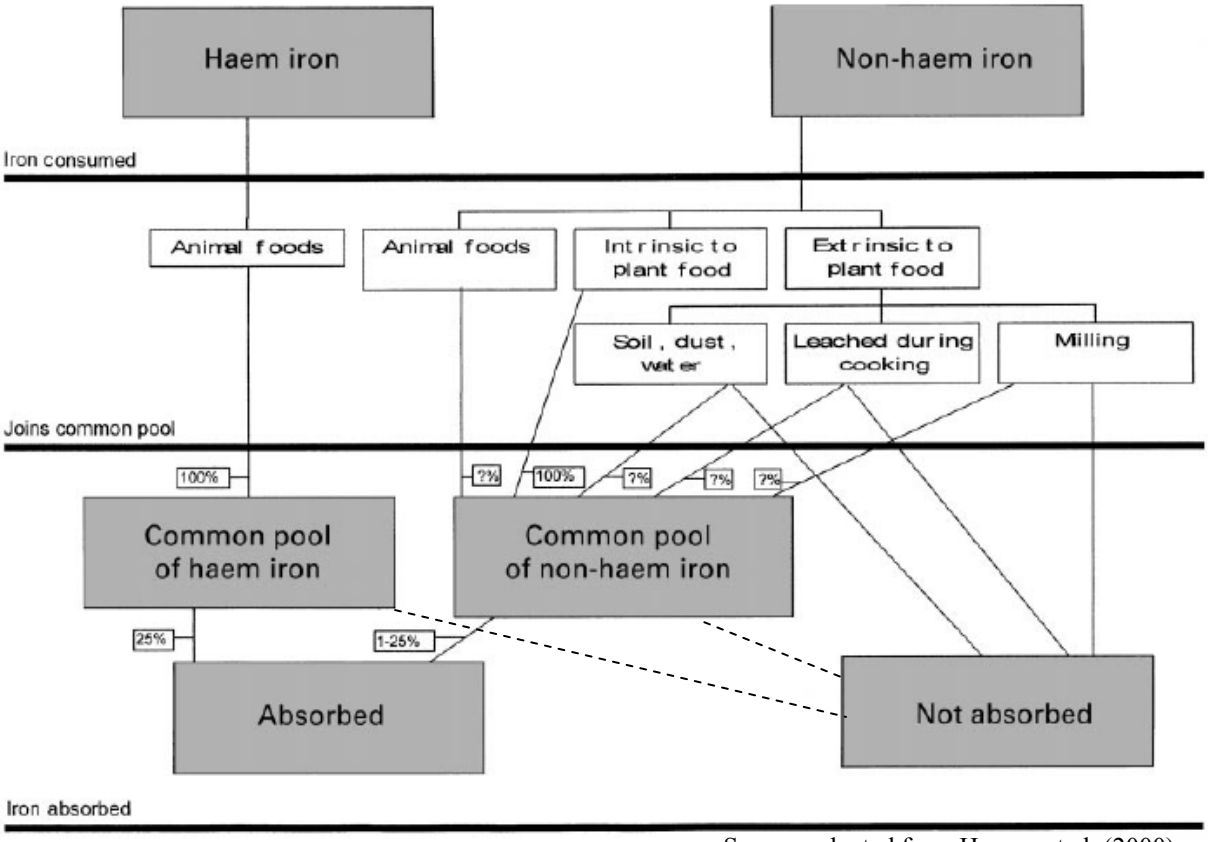
On the other hand, genetic disorders that may affect zinc absorption include acrodermatitis enteropathica, a disorder associated with mutations in the hZIP4 gene, a member of the SLC39 family, whose members encode membrane-bound putative zinc transporters (Ackland and Michalczyk, 2006).

2.4.2 Dietary factors

Dietary factors affecting iron and zinc bioavailability can be grouped into two: (i) the mineral form, and (ii) the presence of compounds influencing mineral absorption.

Dietary zinc is found in inorganic and organic forms, the latter being more bioavailable (Solomons et al., 1979). Similarly, dietary iron exists in different forms, heme, non-heme and ferritin/phytoferritin. These are absorbed to a varying extent and through distinct pathways (Fig. 2.4) (Cook and Monsen, 1976, Hallberg et al., 1979). Heme iron is found in foods of animal origin and is of high bioavailability with 15-35% being absorbed (Carpenter & Mahoni, 1992). On the other hand, non-heme iron, the form under which iron exists in plant-based foods, is much less bioavailable (<10%) as it is susceptible to the effects of absorption enhancers and inhibitors present in the food (Hurrell and Egli, 2010).

The third form of iron is ferritin, an iron storage protein present in all organisms. Iron bioavailability investigations from non-heme ferritin are relatively recent. Ferritin iron is naturally coated with a stable protein that makes it relatively stable to proteolysis, and to effects of other mineral absorption inhibitors such as phytates. The absorption of ferritin is through clathrin-dependent receptor endocytosis, mediated by a receptor that is still not yet identified (Theil, 2011). Despite earlier controversies on the extent of bioavailability of ferritin, recent studies suggest that this form of iron is well absorbed irrespective of the source (plant or animal ferritin) (Lönnerdal, 2009, Theil, 2011). However, more studies are needed to explain as to why iron deficiency is prevalent in predominantly legume consuming communities.



Source: adapted from Harvey et al. (2000)

Fig. 2.4: Iron absorption depending on source and chemical forms

In developing countries, iron from extrinsic sources (i.e. contamination by dust, milling equipments, etc.) can contribute to an important share of the total iron intake (Harvey et al., 2000). However, findings on the bioavailability of contaminant iron have not been consistent and in some instances, even decreases in iron bioavailability were reported due to extrinsic contamination (Hooda et al., 2004, Hooda et al., 2002). Such inconsistencies may partly be explained by the difference in bioavailability

between soils (Hallberg, 1981b), or the lack of appropriate methods of measuring bioavailability of contaminant iron, since the extent of exchangeability (fraction that joins the common non-heme pool) is not exactly known (Fig. 2.4).

Given that good dietary mineral bioavailability allows reduction in total mineral intake requirements (table 2.1), improving bioavailability can be as important as increasing intake. The following sections will therefore discuss in detail dietary absorption enhancers and inhibitors that influence iron and zinc bioavailability in plant-based foods.

2.5 Absorption enhancers

2.5.1 Organic acids

Among the various organic acids, ascorbic acid is the most efficient absorption enhancer of zinc and non-heme iron (Teucher et al., 2004). Several mechanisms for the enhancing properties of ascorbic acid exists, these are: promotion of acidic conditions in the stomach and intestine providing optimal conditions for iron and zinc absorption; chelation of ferric iron and zinc to maintain it in a stable and soluble complex at higher pH and thereby preventing formation of insoluble complexes with phytate and, or polyphenols; and finally reduction of ferric iron to its ferrous form prevents the precipitation of iron as ferric hydroxide (Teucher et al., 2004). However, to promote iron absorption in meals containing low to medium amounts of inhibitors, an ascorbic acid (AA): Fe molar ratio of 2:1 is needed, whereas ratios as high as 4:1 may be needed for those with high contents of inhibitors (Teucher et al., 2004).

In most developing countries, consumption of fruits and vegetables rich in vitamin C is minimal; hence the promotion of vitamin C-rich fruits may be a good strategy to improve the absorption of iron and zinc. However, the enhancing effect of ascorbic acid in such foods is believed to be offset by the inhibiting effect of the polyphenols they contain (Gillooly et al., 1983).

The addition of AA to foods may be beneficial, but is limited by the instability of AA during food processing and storage. However, several derivatives of AA, notably ascorbyl palmitate and erythorbic acid, have been shown to retain their absorption enhancing properties even after thermal processing (i.e. baking). Furthermore, despite the low vitamin C activity of erythorbic acid, it appears that it has more absorption enhancing properties than ascorbic acid (Fidler et al., 2004).

Other organic acids such as citric acid, lactic acid, etc., have also been shown to enhance absorption, but for them to be effective, their molar ratio to iron needs to be very high (>100) (Teucher et al., 2004).

2.5.2 Muscle proteins: the 'Meat factor'

Meat, poultry and fish are important enhancers of the absorption of non-heme iron and zinc (Etcheverry et al., 2006, Hunt et al., 1995). The enhancing properties of meat have been related to muscle proteins, however the exact mechanism by which absorption is enhanced is still unknown (Hurrell and Egli, 2010). It has been hypothesized that this would be due to the binding of iron by peptides of myosin (generated by pepsin degradation in the gut) to remain in solution (Storcksdieck et al., 2007); the reduction of ferric iron to ferrous iron by sulphydryl groups (i.e. cysteine) in meat (Mulvihill and Morrissey, 1998); or the induction of gastric juice production by meat proteins (Carpenter and Mahoney, 1992).

In a human absorption study, the L-α-glycerophosphocholine increased non-heme iron absorption from a vegetarian lasagna test meal significantly from 3.5 ± 2.9% to 4.9 ± 5.1% (P=0.023) (Armah et al., 2008). However, this finding was not confirmed in women consuming high phytate maize meal (Troesch et al., 2009). Until the exact mechanism by which meat enhances iron and zinc absorption, it is likely that the term "meat factor" will remain in the literature.

2.6 Absorption inhibitors

2.6.1 Milk, white egg or soybean proteins

Animal proteins in milk such as casein and whey (Hurrell et al., 1989), eggs, and albumin have been shown to have iron and zinc absorption inhibiting effect (Cook and Monsen, 1976, Hurrell et al., 1989). However the inhibitory effect on zinc was less pronounced than that on iron.

Similarly, plant proteins from soybean have been implicated in the inhibition of iron and zinc absorption (Hurrell et al., 1992, Davidsson et al., 1996). Despite concerns that the observed inhibitory effect was due to associated phytates and not the soy proteins (Lönnerdal, 2000), dephytinized soy protein isolates were found to have iron and zinc inhibitory effects as well (Hurrell et al., 1992, Davidsson et al., 1996).

2.6.2 Calcium

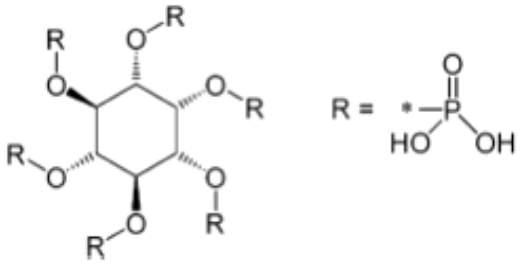
Calcium is unique in that it inhibits the absorption of both non-heme and heme iron (Hallberg et al., 1991). The inhibitory effect of calcium is dose dependent, but the quantity above which absorption is

inhibited is matrix dependent (Lynch, 2000), and seems to be exaggerated in single-meals (Hallberg et al., 1991). Moreover, the absorption inhibitory effect of calcium from supplements may be more pronounced than that from food (Hemalatha et al., 2009). The inhibitory effect of calcium on iron absorption was found to be at the uptake by enterocytes and not during transport as previously taught (Hallberg et al., 1992, Roughead et al., 2005).

On the other hand, calcium is not directly involved in the inhibition of zinc absorption (Lönnerdal et al., 1984, Spencer et al., 1984), but is involved indirectly by potentiating the negative effect of phytate through the formation of insoluble phytate-calcium-zinc complexes. For this reason, the use of [phytate][Ca]/[Zn] ratio has been recommended for the prediction of zinc bioavailability (Hemalatha et al., 2007). However, only calcium from supplement and not from conventional diet is expected to hamper zinc absorption (Hunt and Beiseigel, 2009, Hemalatha et al., 2009).

2.6.3 Phytates

Phytate is a generic name given for phytic acid, known as inositol hexakisphosphate (IP6) (Fig 2.5), and its salts, inositol penta to inositol monophosphate (IP5-IP1). Inositol phosphate consists of an inositol ring and at least one phosphate group. .



Source: (Kumar et al., 2010)

Fig. 2.5: Structure of phytic acid

Phytate is a common constituent of cereals and legumes (Table 2.2) and thus much of the phytate intake is from cereals and legumes (Schlemmer et al., 2009). Phytate is formed during maturation of plant seeds and grains (Kumar et al., 2010, Greiner and Konietzny, 2006). It is the primary form of storage of phosphorus in seeds and accounts for 60-90 % of the total phosphorus and can contribute as much as 1.5 % of seeds dry weight (Bohn et al., 2008, Loewus, 2002).

In cereals, phytate is located mainly in the germ and the aleurone layer (Reddy, 2002). However, abrasive decortication of pearl millet and sorghum grains has shown that the endosperm can also contain a non-negligible amount of phytate (Hama et al., 2011).

In legume seeds, phytate predominantly (up to 90%) occurs in the protein bodies of the endosperm or the cotyledon (Reddy, 2002). The seed coat contains little phytate, hence dehulling legumes seeds has been shown to increase phytate content (Deshpande et al., 1982).

Cultivation conditions such as season, soil profile, and cultivar can determine the phytate content in grains (Feil and Fossati, 1997). Phytic acid content in cereals/grains has been positively associated with soil phosphorous content and hence the application of fertilizers (nitrogen and phosphorus) is also likely to increase phytic acid content (Buerkert et al., 1998, Reddy et al., 1989).

Table 2.2: Content of phytic acid/ phytate in commonly consumed cereals and legumes

Grain/seed type		**Taxonomic names**	**Phytic acid/ phytate[a] (g/100g DM)**
		Cereals	
Maize		*Zea mays*	0.72–2.22
	Germ		6.39
Wheat		Triticum spp. (25 species)	0.39–1.35
	bran		2.1–7.3
	germ		1.14–3.91
Rice		*Oryza glaberrima/sativa*	0.06–1.08
	Bran		2.56–8.7
Barley		*Hordeum vulgare*	0.38–1.16
Sorghum		*Sorghum spp*. (~30 species)	0.57–3.35
Oat		*Avena sativa*	0.42–1.16
Rye		*Secale cereal*	0.54–1.46
Millet		*Pennisetum sp.*	0.18–1.67
Triticale		*Triticale secale*	0.50–1.89
Wild rice		*Zizania sp.*	2.20
		Legumes	
Kidney beans		*Phaseolus vulgaris*	
Haricot beans			
Pinto beans			0.61–2.38
Navy beans			
Black eye beans			
Broad beans		*Vicia faba*	0.51–1.77
Peas		*Pisum sativum var. arvense*	0.22–1.22
Cowpeas/ Black eyed peas		*Vigna unguiculata*	0.37–2.90
Soybean*			0.92–0.17
Chickpeas		*Cicer arietinum*	0.28–1.60
Lentils		*Lens culinaris*	0.27–1.51

[a] depending on the data published **Source:** data from table 2 & 3 compiled by Schlemmer et al., (2009)

* data from Greiner & Konietzny (2006).

2.6.3.1 Mineral-binding properties of phytates

The mechanism by which phytate inhibits mineral absorption is based on the formation of insoluble phytate-mineral or peptide-mineral-phytate complexes in the gastrointestinal tract (Weaver and Kannan, 2002). Furthermore, phytates form complexes with endogenously secreted minerals such as zinc (Sandström, 1997, Manary et al., 2002) and calcium (Morris and Ellis, 1985), making them unavailable for reabsorption into the body.

The formation and stability of insoluble complexes are dependent on many factors including pH, size and valence of the mineral, mineral/phytate ratio, food matrix, and the presence of enhancers and other absorption inhibitors (Crea et al., 2008).

Phytic acid has six strongly dissociated protons (pK's 1.1 to 2.1) and six weakly dissociated protons (pK's 4.6-10) (Weaver & Kannan, 2002), giving it a net charge of about -3 at pH 1.5 which quickly rises to -8 at a pH 7.5. This large negative charge is neutralized by the binding of phytate to positively charged proteins, amino acids and minerals (Selle, Ravindran, & Bryden, 2000). Such binding forms complexes that may only be soluble when pH< 3.5, and thus are largely insoluble at the physiological pH of the intestine (pH 4-7) (Crea et al., 2008).

The strength of phytic acid binding to different minerals increases with the increase in the atomic number of the mineral (Persson et al., 1998) and zinc-phytate complexes are more stable than iron-phytate ones.

$$Cu^{2+} > Zn^{2+} > Co^{2+} > Mn^{2+} > Fe^{3+} > Ca^{2+}$$

Impairment of iron and zinc absorption by phytate can start at very low concentrations (2-10 mg) (Hallberg et al., 1989), but this is dependent on the phytate to minerals ratio of foods. As a result, phytate:iron and phytate:zinc molar ratios are used to estimate bioavailability (Hunt, 2003).

Phytate: iron molar ratio of 1:1 or preferably 0.4:1 is needed to significantly improve iron absorption in cereal and, or legume-based meals that do not contain any enhancers of iron absorption. In meals containing ascorbic acid and, or muscle proteins, a ratio of 6:1 may be sufficient to improve absorption (Hurrell, 2004).

On the other hand, phytate: Zn molar ratios <5, between 5 and 15, and >15 have been associated with high, moderate and low zinc bioavailability, corresponding to around 50%, 30% and 15% of total zinc, respectively (Gibson, 2006).

2.6.3.2 Analysis of phytates

Precipitation methods & colorimetric methods

Precipitation methods are based on the precipitation of phytic acid as an insoluble salt, mostly as ferric phytate. Precipitation methods can be classified as direct and indirect methods. The direct method, first introduced by (McCance and Widdowson, 1935) relied on the estimation of precipitated ferric-phytate complexes based on colorimetric determination of phosphates.

Colorimetric methods are more rapid and simple than precipitation methods, and are often based on the reaction between ferric chloride and sulfosalicylic acid. The method was first developed by Young (1936), and was subsequently improved by Davids and Reid (1979). The improved version minimized the amount of sample and reagent needed for the assay. Several versions of this method exist (Latta and Eskin, 1980, Vaintraub and Lapteva, 1988). The method developed by Latta & Eskin (1980) is based on the monitoring of the pink color formed between sulfosalicylic acid and ferric iron. In the presence of phytate, ferric iron preferentially binds to phytate and this leads to the decrease in the intensity of the pink color. This decrease in intensity is measured colorimetrically to estimate the concentration in phytate.

Shortfall in the precipitation methods includes the varying extent of adsorption of iron (III) on the pecipitate. Both precipitation and colorimetric methods are unable to separate inositol hexaphosphate from lower inositol phosphates, and are limited by the possible interference of phosphates especially in foods with high phosphate content (Skoglund and Sandberg, 2002).

Ion exchange methods

Ion exchange methods were first introduced by (Harland and Oberleas, 1977). This allowed separation of phytic acid from the crude extract and avoided the loss of even minute quantities of phytate in the precipitation. Compared to precipitation methods, ion exchange methods allowed higher recovery of phytic acid (Ellis and Morris, 1982). The recovery was further improved by the addition of EDTA and adjustment of the sample extract's pH that prevented binding of phytic acid to proteins and minerals (Ellis and Morris, 1983).

A modified version of the original ion exchange method was later adopted as an AOAC method (AOAC 986.1) (Harland and Oberleas, 1986). However, this method has the shortfall of overestimating the phytic acid content due to its inability to separate lower inositol phosphates (Phillippy and Johnston, 1985). Furthermore, the accuracy of the method largely depended on the choice of the resin.

The method developed by (Phillippy and Bland, 1988) allowed the separation of the different inositol phosphates by the application of a step-gradient ion exchange method followed by precipitation.

HPLC and HPIC method

HPLC/HPIC methods are more sensitive and reproducible and thus are suitable for the measurement of lower amounts of phytate (Skoglund et al., 1997, Talamond et al., 2000). These methods can simultaneously separate and determine IP6 and the lower inositol phosphates. The only limitation is the need for more expensive instruments, standards, and the requirement for expertise to operate them.

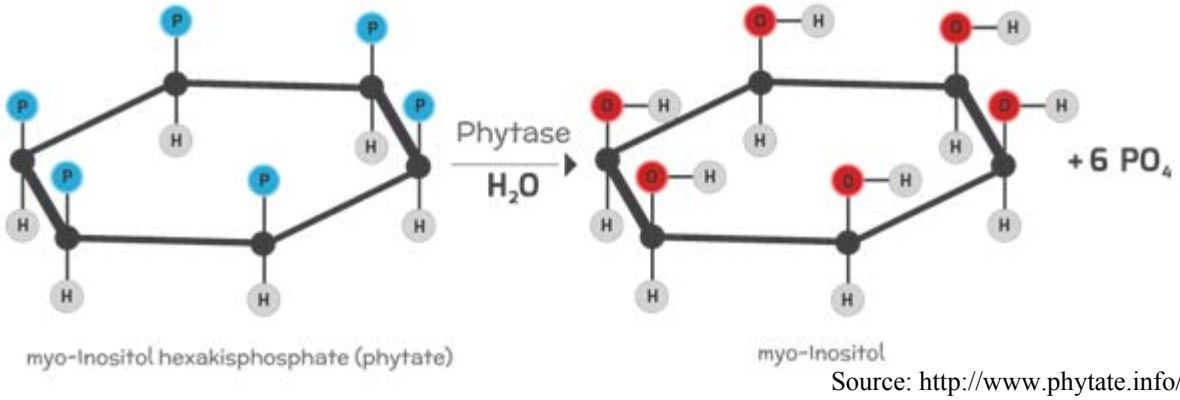
Other methods

Other methods for phytate analysis in foods include NMR spectroscopic methods (Mazzola et al., 1986), near-IR methods (Xu et al., 1992), inductively coupled plasma mass spectrometry (Muñoz and Valiente, 2003), and more recently synchronous fluorescence method (Chen et al., 2009).

2.6.3.3 Enzymatic degradation of phytates

Enzymatic hydrolysis of phytates by phytases, myo-inositol (1-6) hexakisphosphate phosphohydrolase, is achieved through sequential dephosphorylation to produce free myo-inositol along with six inorganic phosphates (Selle and Ravindran, 2007, Debnath et al., 2005). Depending on the site where hydrolysis is initiated and the pH of optimal activity, two types of phytase are recognized by the International Union of Pure and Applied Chemistry (IUPAC) and the International Union of Biochemistry (IUB).

The 3-phytase (EC 3.1.3.8; myo-inositol hexakisphosphate 3-phosphohydrolase) first liberate the phosphate at position C3 whereas the 6-phytase (EC 3.1.3.26, myo-inositol hexakisphosphate 6-phosphohydrolase) releases phosphate from position C6 of the myo-inositol hexaphosphate ring (Selle & Ravindran, 2007). Based on optimum pH, phytases can also be categorized into histidine acid phosphatases which have their optimum at pH ~5.0 and alkaline phytases with optimum pH of around 8.0 (Cao et al., 2007).



Source: http://www.phytate.info/phytases

Fig. 2.6: Hydrolysis of phytate by the action of phytase

Phytases can be from plant and or microbial (fungal and bacterial phytase) origin. Phytases can also be generated by the small intestinal mucosa and gut-associated microflora of pigs, but not humans (Hu et al., 1996). Many cereals and legumes have endogenous phytase activities (table 2.3). The phytase activities of a wide range of grains and seeds commonly used in complementary foods have been investigated (Egli et al., 2002), cereals as rye, triticale, wheat, and buckwheat have been shown to have high phytase activity.

Table 2.3: Phytase activity of common grains and seeds

Cereal Common name	Botanical name	Phytase[a] activity	Grains Common name	Botanical name	Phytase[a] activity
	Cereals			***Legumes***	
Barley	*Hordeum vulgare*	1.83 ± 0.05	Black eyed bean	*Vigna unguiculata*	0.39 ± 0.00
Maize	*Zea mays*	0.13 ± 0.00	Chickpea	*Cicer arietinum*	0.25 ± 0.02
Millet	*Pennisetum typhoides*	0.24 ± 0.01	Dwarf bean	*Phaseolus vulgaris nana*	0.28 ± 0.01
Oat	*Avena sativa*	0.14 ± 0.00	Lentil	*Lens culinaris*	0.26 ± 0.01
Rice	*Oryza sativa*	0.19 ± 0.00	Leucerne	*Medicago sativa*	0.81 ± 0.08
Rye	*Secale cereal*	6.92 ± 0.41	Lupin	*Lupines albus*	0.24 ± 0.02
Sorghum	*Sorghum sudanensis*	0.11 ± 0.00	Mugbean	*Phaseolus aureus/vigna radiate*	0.27 ± 0.00
Sweet maize	*Zea mays saccharafa*	0.38 ± 0.01	Pea	*Pisum sativum*	0.20 ± 0.01
Triticale	*Triticosecale*	4.82 ± 0.04	Soybean	*Glycine max*	0.34 ± 0.01
Wheat	*Triticum aestivum*	3.08 ± 0.17	White bean	*Phaseolus vulgaris*	0.23 ± 0.01
	Pseudocereals			***Oilseeds***	
Amaranth	*Amaranthaceae*	1.27 ± 0.02	Rapeseed	*Brassica napus oleifera*	0.30± 0.01
Buckwheat	*Fagopyrum esculentum*	2.90 ±0.17	Sunflower seed	*Heliathus annuus*	0.13 ± 0.01
Quinoa	*Chenopodium quinoa*	0.62 ± 0.02			

Source: adapted from Egli et al.(2002)

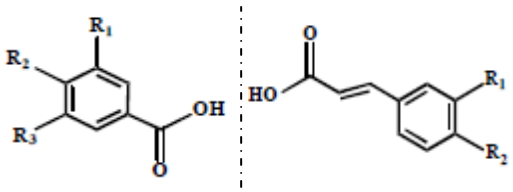
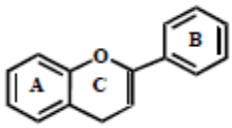
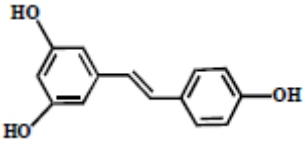
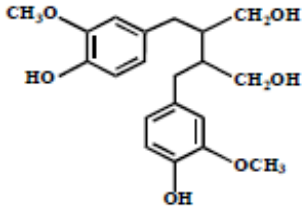
[a]phytase activity expressed as phytase unit (PU) /g DM, where 1 PU is equivalent to the enzymatic activity that liberates 1µmol inorganic phosphate per min; values are mean ± SD.

Several food processing techniques such as heat treatment, soaking, germination and fermentation can activate endogenous enzymes to a varying extent (Egli et al., 2002). A more complete degradation of phytates can also be achieved by the application of exogenous phytase during processing, or just before consumption. Such applications have been shown to significantly improve iron and zinc absorption (Troesch et al., 2009, Egli et al., 2004). However, in the presence of high amount of polyphenols, the effect of dephytinization on iron bioavailability seems to be limited (Hurrell et al., 2003, Petry et al., 2012b).

2.6.4 Polyphenols

Polyphenols are secondary metabolites of plants, generally involved in the defense against pathogens or ultraviolet radiation. Based on their structure, polyphenols are classified into phenolic acids, flavonoids, stilbenes, lignans and tannins.

Table 2.4: Classification and structure of polyphenols

Polyphenols	Examples	Common chemical structure
1. Phenolic acids	Gallic acid	
-hydroxybenzoic acids	Protocatechuic acid	
-hydroxycinnamic acids	Caffeic acid	
	Ferulic acid	
2. Flavonoids	Quercetin	
	Myricetin	
	Catechin	
	Cyanidin	
3. Stilbenes	Resveratrol	
	Picied	
	Astrigin	
4. Lignans	Secoisolariciresinol	
	Matairesinol	

Source: Pandey & Rizyi, 2009

2.6.4.1 Different classes of polyphenols

a) Phenolic acids

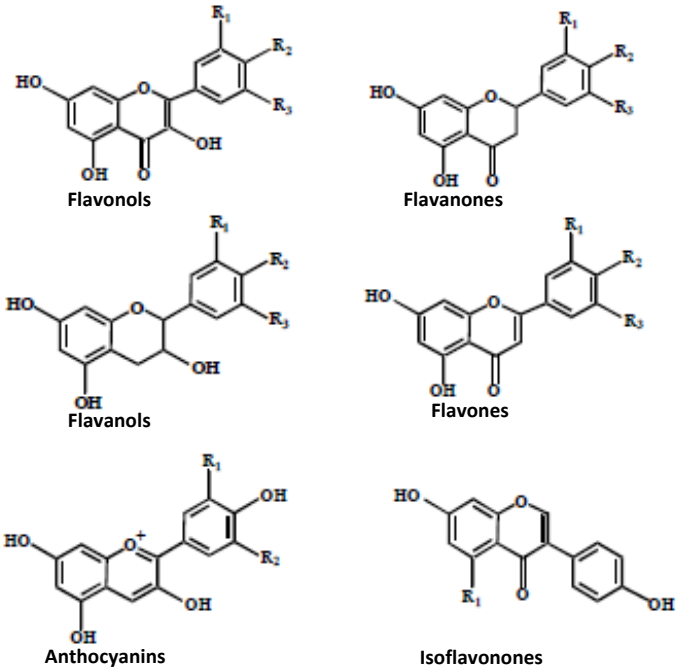
There are two classes of phenolic acids: hydroxybenzoic acids and hydroxycinnamic acids. *Hydroxybenzoic acids* are usually found esterified to *D*-glucose. Examples of hydroxybenzoic acids are gallic, p-hydroxybenzoic, vanillic, syringic, and protocatechuic acids. In foods, they are usually bound to complex structures like hydrolysable tannins or lignins. Hydrolysable tannins, themselves are either composed of esterified gallic acid units (gallotannin) or esterified hexahydroxydiphenic acid units (Haslam, 2007). Hydroxybenzoic acids are found in various cereals like sorghum, millet, barley, wheat and rice (Dykes and Rooney, 2007), and in legumes like beans, peas and lentils (Shahidi and Naczk, 2003).

Hydroxycinnamic acids are more common than the hydroxybenzoic acids. They have a C6–C3 structure and are mainly consisted of *p*-coumaric, caffeic, ferulic and sinapic acids. Hydroxycinammic acids are mainly found in bound forms as glycosylated derivatives of esters of quinic, shikimic and

tartaric acid (Manach et al., 2004). Caffeic acid represents 75-100% of the total hydroxycinnamic acid content of most fruits whereas ferulic acid is mainly found in the *trans*- form, esterified to arabinoxylans and hemicelluloses in the aleurone and pericarp of cereals (Manach et al., 2004). Several dimers of ferulic acid may form bridge structures between chains of hemicelluloses. Ferulic acid is the most abundant phenolic acid in cereals and represents about 90% of the total polyphenol content (Manach et al., 2004).

b) Flavonoids

There are six subclasses of flavonoids namely, flavonols, flavones, isoflavones, flavanones, anthocyanidins and flavanols (Cheynier, 2005, Manach et al., 2004).



Source: adapted from (Pandey and Rizvi, 2009)

Fig. 2.7: Structures of the 6 sub-classes of flavonoids

The *flavonols* are the most ubiquitous of the flavonoids and are mainly represented by quercetin and kaempferol (Manach et al., 2004). They are present in glycosylated forms, often associated with glucose or rhamnose, but may also be associated with galactose, arabinose, xylose and glucuronic acid (Manach et al., 2004). Flavonols are mainly located in the outer layer of plants, mainly because their biosynthesis depends on exposure to light. Onion, kale and broccoli, and to some extent tea and red wine are good sources of flavonols (Manach et al., 2004). In cereals, flavonols have been reported in

sorghum in the form of kaempferol, 3-rutinoside-7-glucuronide, dihydroflavonols taxifolin, and taxifolin 7-glucoside (Awika et al., 2005, Gujer et al., 1986).

Flavones mainly consist of glycosides of luteolin and apigenin and are much less common. Important sources of flavones include parsley and celery (Manach et al., 2004). In cereals, flavones have been reported in millet, sorghum, oat and wheat (Dykes and Rooney, 2006). In sorghum they are predominantly found in pigmented varieties (Dykes et al., 2009).

Flavanones are generally found glycosylated by a disaccharide at position 7 (El Gharras, 2009). Flavanones are mainly found in tomatoes and in some aromatic plants such as mint. In citrus fruits, the albedo contains significant amount of flavanones (Pandey and Rizvi, 2009). In cereals, the flavanones eriodictyol (Kambal and Bate-Smith, 1976) and eriodictyol 5-glucoside (Gujer et al., 1986) have been reported. Naringenin has also been reported in red sorghum (Gujer et al., 1986).

Isoflavones are almost exclusively found in legumes, with the highest concentration in soybean. Although not steroids, isoflavones have hydroxyl groups in position 7 and 4' which makes their structure resemble that of estrogens, explaining their pseudo-hormonal properties (Rietjens et al., 2012).

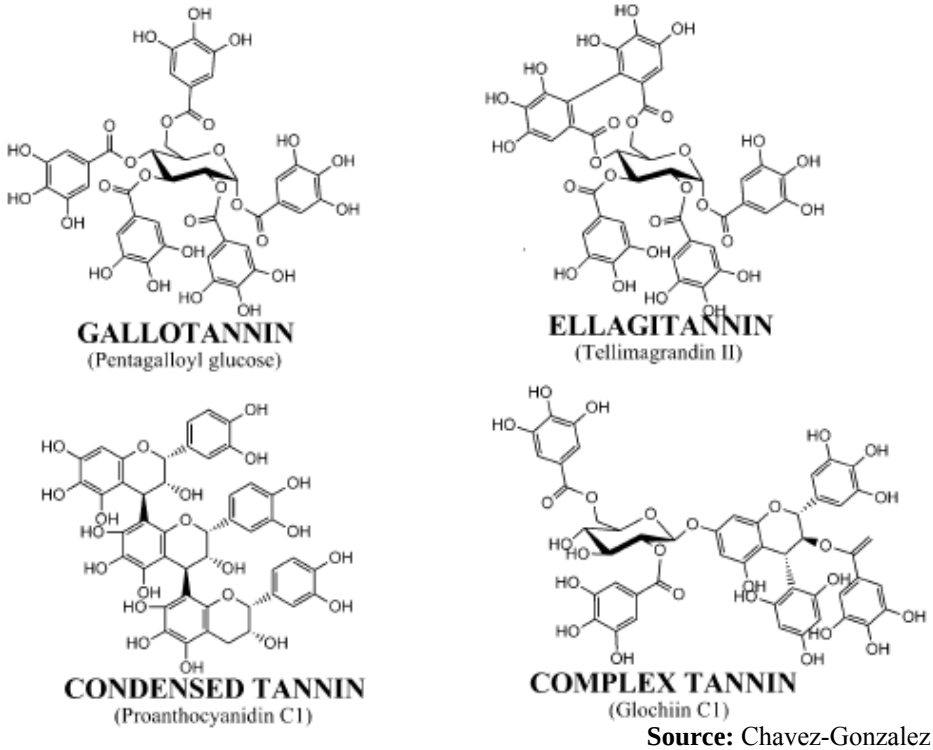
Flavanols exists both in the form of monomers as catechins (flavan-3-ols) and in the form of polymer as proanthocyanidins. Catechins are found in various fruits, red wine, and black tea but also in much greater quantity in green tea and chocolate (Arts et al., 2000).

Proanthocyanidins (condensed tannins) are dimers, oligomers, and polymers of catechins that are bound together by links between C4 and C8/C6. Proanthocyanidins are also important constituents of polyphenols in red pericarp sorghums and are found in the form of flavan-4-ol compounds, such as luteoforol and apiforol (Bröhan et al., 2012).

Anthocyanins are a group of intensely colored pigments responsible for the colors of many fruits, vegetables, flowers, leaves, roots and other storage organisms of plants. However, depending on the pH, they can also exist in non-colored forms (Lapidot et al., 1999). They are naturally found in the form of polyhydroxylated or methoxylated heterosides which derive from the flavylium ion or 2-phenylbenzopyrilium (Escribano-Bailón et al., 2004). The aglycon (anthocyanidin) is highly unstable and is susceptible to degradations due to light, pH, and oxidation states (Sun et al., 2011). However, glycosylation with glucose at position 3, and esterification with organic acids and polyphenols prevents its degradation (Escribano-Bailón et al., 2004).

Anthocyanins are found in red wine, certain leafy and root vegetables, but most abundantly in fruits (Oancea and Oprean, 2011). In recent years, their identification in various cereals such as sorghum, corn, wheat, etc. has spawn research interest (Escribano-Bailon et al., 2004).

Tannins are a unique group of phenolic metabolites with molecular weights between 500 and 30000 Da, and are widely distributed in almost all plant foods and beverages (Serrano et al., 2009). Traditionally, tannins have been classified into condensed and hydrolysable tannins, based on their hydrolytic properties in hot water, or response to tannase. However, this classification did not always take into account the structural diversity and associated hydrolytic properties of tannins, thus current classifications categorize tannins into four groups (Fig 2.8), namely: gallotannins, ellagitannins, condensed tannins, and complex tannins (Khanbabaee and van Ree, 2001).



Source: Chavez-Gonzalez et al., 2011

Fig. 2.8: The different classes of tannin

Gallotannins are the simplest tannins and are formed by units of galloyl or di-galloyl esterified to a core of glucose or other polyhydroxy alcohols. Ellagitannins are esters of hexahydrodiphenic acid (HHDP) which during hydrolysis, dehydrates and lactonizes spontaneously to form ellagic acid (Chávez-González et al., 2012). Condensed tannins are oligomeric and polymeric proanthocyanidins containing

flavan-3-ol (catechin) or flavan-3, 4-diol linked by C–C– bonds. The basic structure of complex tannins consists of a gallotannin or ellagitannin unit and one of catechin (Aguilera-Carbo et al., 2008).

c) Lignans

Lignans are a group of diphenolic compounds formed of 2 phenylpropane units that are present in the outer layers of grains. Flax seed is a rich source of lignans (Axelson et al., 1982). In wheat and triticale, lignans are mainly found in the bran layer. The major lignan in wheat bran is secoisolariciresinol diglycoside (SDG) and is converted to enterodiol and enterolactone by intestinal microbiota (Setchell et al., 1981). Lignans are also found in leguminous plants such as lentils and in vegetables such as garlic, asparagus, and carrots. In fruits such as pears and prunes, they exist as minor constituents (Thompson et al., 1991).

d) Stilbenes

Stilbenes are rarely found in human diets, and are found in low quantities in wine as resveratrol. Recently, resveratrol was discovered in hop plants used in the brewing industry to provide bitterness and aroma (Callemien et al., 2005). Stilbenes have also been found in red sorghum (Bro□han et al., 2011).

2.6.4.2 Analysis of phenolic compounds

Several methods exist for the determination of phenolic compounds in foods. The most common methods are described as follows.

Measurement of total polyphenols

Methods widely used for the measurement of total polyphenols are the Prussian Blue (Price et al., 1978) and the Folin Ciocalteu methods(Singleton and Rossi, 1965). The principle of these methods relies on the reducing power of phenolic hydroxyl groups. As a result, easily oxidizable non-phenolic compounds such as ascorbic acid can interfere with the assay.

Functional group methods

The vanillin method allows the measurement of all flavonoids (Price et al., 1978). On the other hand, the Butanol/HCl method allows the determination of condensed tannins (Porter et al., 1985). A specific method for the determination of iron-binding galloyl and catechol groups was also developed (Brune et

al., 1992). This method is based on the ability of ferric ammonium sulfate to form colored complexes with galloyl and catechol groups. The absorbance of the colored complexes is measured at 680 nm and 578 nm, corresponding to the absorption maxima of Fe-galloyl and Fe-catechol complexes, respectively.

Protein precipitation methods

These methods are based on the ability of tannins to bind and precipitate proteins, usually bovine serum albumin (BSA) dyed with Remazol Brilliant Blue (Asquith and Butler, 1985). The tannin and the dyed protein are mixed at a defined pH and ionic strength conditions and are allowed to precipitate. After centrifugation, the pellet is re-dissolved in an alkaline SDS buffer for the measurement of dyed protein content.

HPLC methods

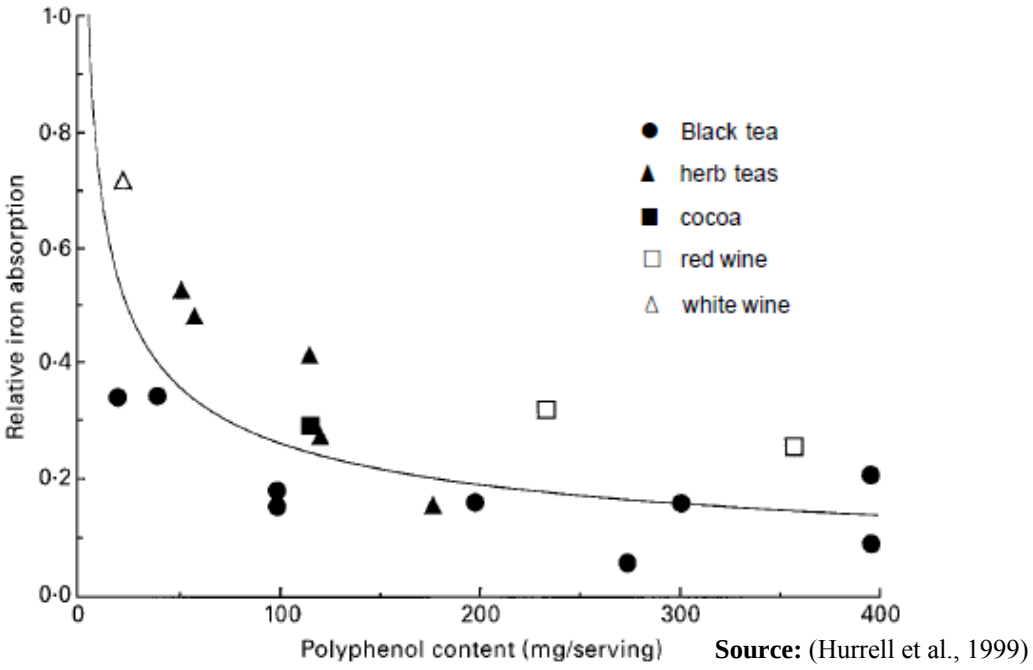
Several HPLC techniques exist for the measurement of polyphenols in foods. Simple phenolic compounds, hydrolyzable tannins, and condensed tannins up to 7-8 units may be measured. However, good separation of condensed tannins is difficult (Schofield et al., 2001). Both reversed and normal phase columns can be used (Cheynier et al., 1999).

Several detection methods including UV, electrochemical and fluorescence can be applied (Waterman and Mole, 1994, Lazarus et al., 1999). More precise and selective analysis techniques, such as diode array spectroscopy and mass spectrometry (MS, PDMS, MALDI, etc.) coupled or not to liquid chromatography, facilitate the detection and characterization of pigmented polyphenols (anthocyanins), making these methods of primary choice.

2.6.4.3 Iron-binding properties

Since the first observations of the inhibitory role of tea polyphenols on iron absorption (Disler et al., 1975), several studies have confirmed the inhibitory effects of polyphenols from different matrices (Hurrell et al., 1999, Cook et al., 1995, Tuntipopipat et al., 2006).

The inhibitory effect of polyphenols is attributed to the formation of insoluble complexes with iron in the lumen, hence making iron unavailable for absorption. This effect depends both on the quantity and type of polyphenol. Studies on different beverages (black tea, herb tea, cocoa, red and white wine) with varied polyphenol contents (Fig. 2.9) showed that the inhibitory effect was dose-dependent (Hurrell et al., 1999). Similarly, dose-dependent inhibition of iron absorption was also found for solid foods (Tuntawiroon et al., 1991).



Source: (Hurrell et al., 1999)

Fig. 2.9: Relative iron absorption from a bread and beverage meal relative to polyphenol contents of beverages

Relative iron absorption is defined as iron absorption (% dose) from a bread meal consumed together with a beverage relative to iron absorption in the same subject from a bread meal consumed with water

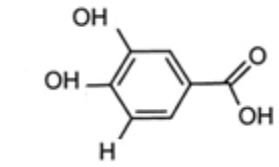
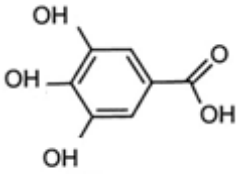
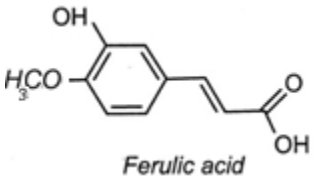
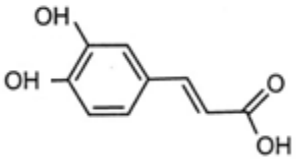
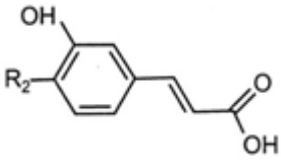
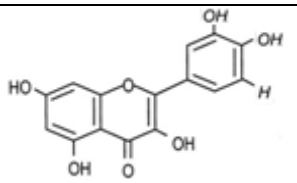
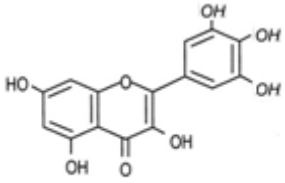
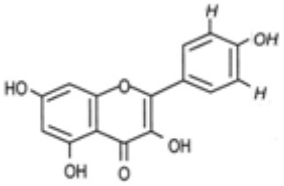
The extent of iron absorption inhibition by polyphenols depends on the meal composition, notably the content of absorption enhancers in the meal. Food constituents such as ascorbic acid and muscle proteins, known as absorption enhancers counteract the negative effect of polyphenols (Hallberg, 2000). For instance, 35 mg of polyphenol extract from green tea and rosemary inhibited iron absorption in a pasta/meat sauce meal by only 20-30%, compared to the 50-70% reduction observed in bread (Samman et al., 2001)**.**

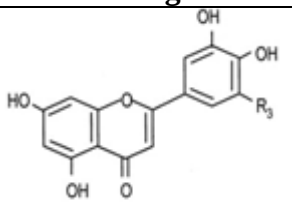
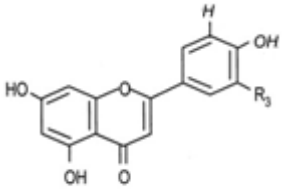
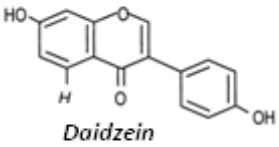
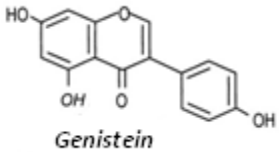
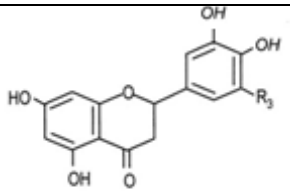
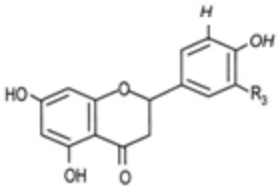
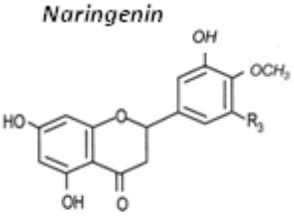
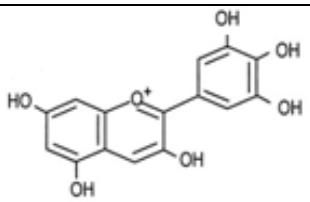
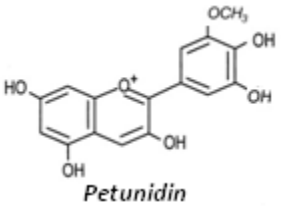
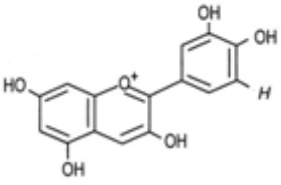
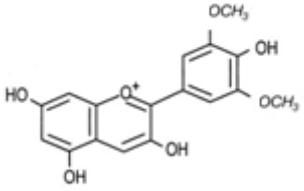
On the other hand, for comparable contents, polyphenols from black tea were found to be more inhibitory than those from herb tea and wine (Hurrell et al., 1999). More surprisingly, chilli had more iron absorption inhibitory effect than turmeric, despite a lower content of polyphenols in the former (Tuntipopipat et al., 2006).

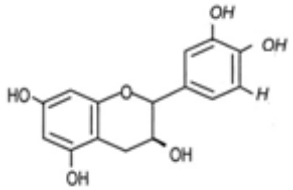
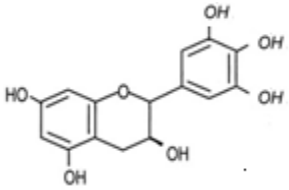
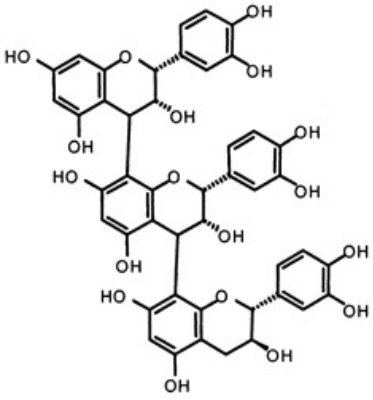
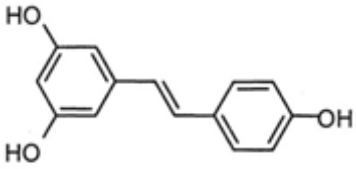
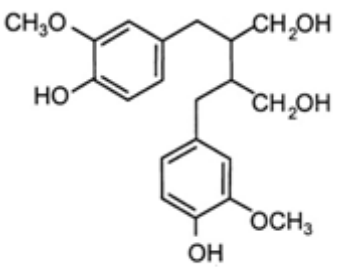
The iron absorption inhibiting effect of individual polyphenols, namely tannic, gallic, and chlorogenic acids, as well as catechin was studied by Brune et al. (Brune et al., 1989). For equimolar amounts of gallic and chlorogenic acids, the reduction in iron absorption was 52% and 33%, respectively. Gallic and tannic acids were found to be equally inhibitory per mole of galloyl groups, whereas catechin did

not show any inhibitory effect. This has led to the conclusion that iron binding properties of polyphenols are mainly due to the catechol (ortho-dihydroxy benzene) and galloyl (trihydroxy-benzene) functional groups (Brune et al., 1989).

Table 2.5: Classification of polyphenols by iron-binding properties*

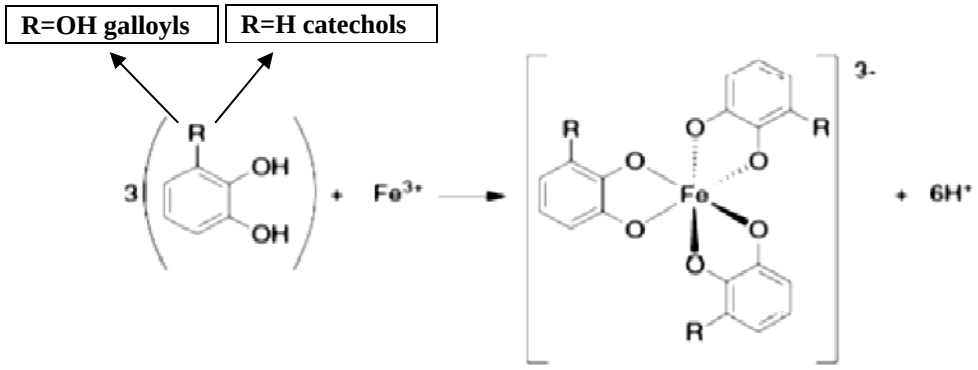
Polyphenol type	**Iron-binding**	**Not iron-binding**
Phenolic acids		
Hydroxybenzoic acids	 Protocatechuic acid  Gallic acid	 Ferulic acid
Hydroxycinammic acids	 Caffeic acid	 Coumaric acid
Flavonoids		
Flavonols	 Quercetin  Myricetin	 Kaempferol

Polyphenol type	**Iron-binding**	**Not iron-binding**
Flavones	 *Luteolin*	 *Apigenin*
Isoflavones		 *Daidzein*  *Genistein*
Flavanones	 *Eriodictyol*	 *Naringenin*  *Hesperetin*
Anthocyannidins	 *Delphinidin*  *Petunidin*	 *Pelargonidin*  *Malvidin*

Polyphenol type	**Iron-binding**	**Not iron-binding**
Flavanols	 *Catechins*  *Gallocatechin*  *Trimeric procyanidin*	
Stilbenes		
		 *Resveratrol*
Lignans		
		 *Secoisolariciresinol*

*Based on galloyl and catechol groups

The formation and stability of iron-polyphenol complexes is determined by several factors including, the valence of the iron, the pH, the presence of oxygen, etc. For catechols and galloyls to bind iron, they first need to be deprotonated. This easily takes place at or below physiological pH in the presence of iron. Deprotonated catechol (catecholate) and galloyl (gallate) groups form complexes with Fe^{2+}/Fe^{3+} often in a 3:1 coordination mode (Fig. 2.10) (Perron & Brumaghim, 2009). However, based on pH, complexation can follow different coordination modes.



Source: adapted from Perron & Brumaghim (2009)

Fig. 2.10: Octahedral geometry of iron-polyphenol complexes

The promotion of auto-oxidation of Fe^{2+} to Fe^{3+} by polyphenols may also play a negative role on iron absorption, because Fe^{3+} is much less soluble/ bioavailable than Fe^{2+}. In the presence of oxygen or other anions such as hydroxides, auto-oxidation of Fe^{2+} to Fe^{3+} is a slow process, but when in complexes with polyphenols (catechol/galloyl), the rate of autoxidation is increased due to the higher stability of Fe^{3+}-catecholate/gallate complexes (Perron et al., 2010, Kawabata et al., 1996). The rate of auto-oxidation of Fe^{2+}-gallate complexes is faster than that of Fe^{2+}-catecholate complexes (Perron et al., 2010), possibly explaining previous remarks that galloyls have higher iron-binding properties than catechols (Brune et al., 1989).

In recent years, polyphenols have also been shown to increase iron absorption. Polyphenol monomers such as grape seed extracts and epigallocatechin-3-gallate were shown to significantly increase apical iron uptake by caco-2 cell lines resulting in higher iron concentration in the enterocyte (Kim et al., 2008). This may be related to reduction of Fe^{3+} by polyphenols. Previous studies have shown that under acidic conditions (i.e. stomach), reduction of Fe^{3+} is common as a result of the oxidation of polyphenols into semiquinone, which once protonated, forms a neutral ligand which favors Fe^{2+} over Fe^{3+} for stability. The semiquinone is capable of reducing another equivalent of Fe^{3+} by being oxidized into a

quinone (Perron and Brumaghim, 2009). Such reducing effects have been studied for monomers like (−)-epigallocatechin-gallate ((−)-EGCg), (−)-epicatechin-gallate ((−)-ECG), quercetin and morin (Ryan and Hynes, 2007, Ryan and Hynes, 2008), gallic acid and gallic acid methyl esters, and catechins (Hynes and Ó Coinceanainn, 2001). However at higher pH, the complexation of iron with two to three polyphenol ligands along with the increase in the pH limits the Fe^{3+} reduction process. This may explain the low transfer of iron across the basolateral membrane of the enterocyte once uptaken by the apical membrane of caco-2-cells (Kim et al., 2008).

In support of this hypothesis, the reduction in the transport of iron by polyphenols was found to be dose dependent and was overcome by addition of ascorbic acid (Kim et al., 2011). However, evidences of the possible positive effect that polyphenols may have on iron absorption are scant, thus further studies are needed. However, given that polyphenols in foods are usually engaged in large complexes, their ability to be soluble enough to pass through the apical intestinal membrane is minimal. Nevertheless, the application of enzymes such as tannases in industrial food processing may lead to the breakdown of large polyphenol complexes into their monomers, whereas polyphenol oxidases may shift oxidation-reduction properties of the polyphenols.

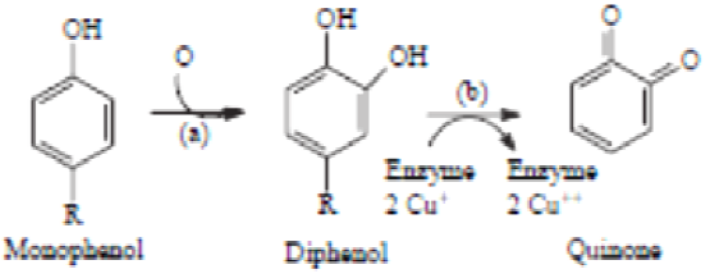
2.6.4.4 Enzymatic prevention of iron-binding by polyphenols

a) Polyphenol oxidase

Polyphenol oxidases (PPO) or tyrosinases are copper containing oxido-reductases. They are ubiquitous as they are found in animals, plants, bacteria and fungi (Mayer, 2006). Depending on their nomenclature, two classes of PPOs can be distinguished: monophenol oxidase (tyrosinase, EC 1.14.18.1) and catechol oxidase or *o*-diphenol:oxygen oxidoreductase (EC 1.10.3.2). PPO's are essentially localized in plastids such as chloroplasts and mitochondria (Marquès et al., 1995). During processes that damage the tissue to the point of rupturing plastids and vacuoles, PPOs can come in contact with their substrates (phenolic groups) mainly stored in the vacuoles. This leads to enzymatic browning due to oxidation of polyphenols (Queiroz et al., 2008). Since enzymatic browning affects the palatability and appearance of foods, PPO's have been regarded as critical enzymes in food technology, specifically in fruit and vegetable processing. As a result, much attention has been given to the understanding of the mechanism of oxidation with the aim of inactivating the enzyme or finding an inhibitor.

The active site of PPO consists of two copper atoms and the enzyme catalyzes two different reactions in the presence of molecular oxygen: the hydroxylation of monophenols (monophenolase activity) and

the oxidation of o-diphenols to o-quinones (diphenolase activity) (Fig. 2.11). This reaction is followed by non-enzymatic polymerization of the quinones giving rise to melanins, pigments of high molecular mass, and dark color (Peñalver et al., 2005, Espín et al., 1998).



Source: Quierroz et al., 2008

Fig. 2.11: Hydroxylation (a) and oxidation (b) reaction catalyzed by a polyphenol oxidase

Since plant foods contain a large variety of phenolic compounds, substrate's specificity may differ accordingly. However, the affinity of PPOs is usually higher for the ortho-dihydroxyphenols, but the enzyme can also oxidize mono- and tri-hydroxyphenols (Mayer & Harel, 1991).

Reducing agents such as ascorbic and citric acid have anti-browning properties (Iyengar and McEvily, 1992). Their effect may be related to the entrapment of intermediate *o*-quinone or to the lowering of pH below levels that inactivate PPOs. However, the large quantity of ascorbic acid needed to prevent browning is likely to negatively affect sensory properties (Komthong et al., 2007). Sulfites are more effective in preventing browning, but are associated with food safety concerns (Sapers, 1993).

Although many of the efforts have been on preventing enzymatic browning, PPOs can have a beneficial role in the processing of cocoa, coffee, wine, black tea, etc., where oxidation of polyphenols plays an important role in the characteristics color and taste of products. Furthermore, PPOs could have applications in designing foods with better iron bioavailability as the oxidation of polyphenols have been proven to decrease iron-binding properties and hence increase iron bioaccessibility (Matuschek and Svanberg, 2005, Matuschek et al., 2001). However, very few, and mostly in vitro studies have been conducted in this regard, and thus in vivo confirmation is needed.

b) Tannase

Tannase (EC. 3.1.1.20), tannin acyl hydrolase (TAH), catalyzes the hydrolysis of ester bonds present in gallotannins, ellagitannins, complex tannins, and gallic acid esters. The enzyme has important applications in food and pharmaceutical industries. Tannases are mainly used in the processing of ice

tea, a corn liquor, and production of gallic acid from plant sources with high tannin contents (Belmares et al., 2004). Gallic acid is in turn used in the production of antioxidants and is an intermediate molecule in the production of the antibiotic trimethoprim (Aguilar et al., 2007).

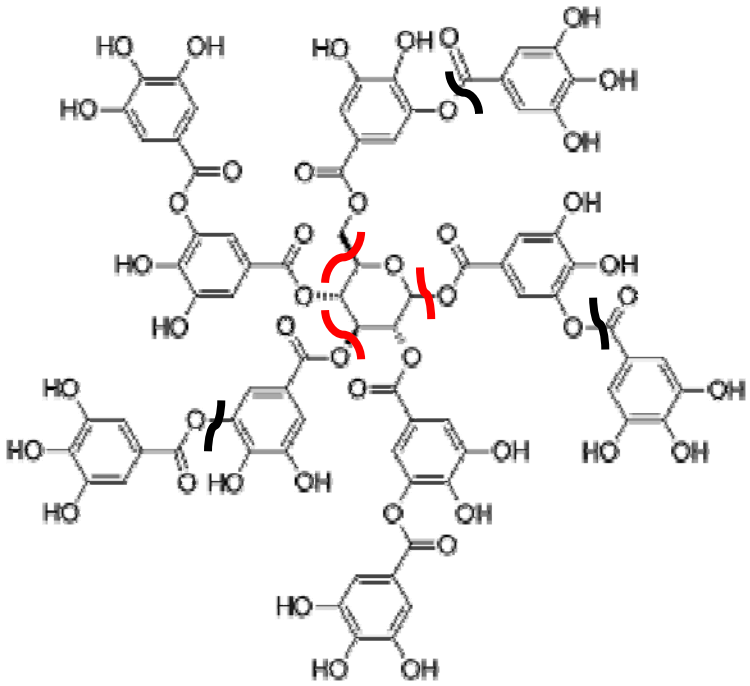


Fig. 2.12: Mechanism of action of tannase

Ester and depside bonds broken by tannase

Tannase acts on gallotannins, ellagitannins, and complex tannins by breaking only ester bonds. Since it does not break carbon–carbon bonds, it does not affect the condensed tannins (Haslam and Stangroom, 1966). Although tannase has a low affinity with condensed tannins, it may still have an effect on the level of condensation by breaking down depside (two or more monocyclic aromatic units linked by an ester bond) or ester bonds. However, comparison of monomers versus polymers has been found to have little effect on iron bioavailability (Brune et al., 1989). Nevertheless, a more recent study reported that application of tannase in tea increased antioxidant properties by increasing iron chelating properties (Lu and Chen, 2008). This is despite the little effect that tannase has on the functional groups of galloyls and catechols. This suggests that more studies are needed in this regard.

Moreover, given that most of the studies on the iron absorption inhibitory effect of polyphenols relied only on assayable/extractable polyphenols, the effect of non-extractable, fiber-associated, polyphenols that are predominant forms in cereals and legumes remains unknown and hence needs to be studied.

2.7 Dietary fibers- undisclosed factor

The term dietary fibers was originally coined by (Hipsley, 1953) and was referred as non-digestible constituents making up the plant cell wall. Since then, the term has seen several revisions (DeVries, 2003). The American Association of Cereal Chemists in 2000 defined dietary fibers as the edible parts of plant or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine (Anon, 2000). However, in recognition that most fibers are at least partially fermentable, it was later suggested to refer to them as partially/poorly and well fermented fibers (Tungland and Meyer, 2002). However, there is still debate regarding the most appropriate definition and classification of dietary fibers (Lunn and Buttriss, 2007, Saura-Calixto and Díaz-Rubio, 2007).

2.7.1 Major components of dietary fiber

The major components of dietary fibers as presently defined include non-starch polysaccharides (NSP), inulin and fructooligosaccharides, resistant starch and lignin. NSPs along with lignin and resistant starch are the major constituents of total dietary fibers.

Non-starch polysaccharides (NSPs)

The term NSP covers a large variety of polysaccharide molecules, excluding α-glucans (starch). These polysaccharides are typically long polymeric carbohydrate chains containing up to several hundred thousand monomeric units. In general, the monomers are hexoses and pentoses, e.g., galactose, glucose, arabinose, xylose and mannose (Choct, 1997) mostly linked through glycosidic linkage. NSP are key constituents of the cell wall of plants as they can account up to 90% of the cell wall of plants (Selvendran and Du Pont, 1980). NSPs are usually classified into three groups, namely cellulose, non-cellulosic polymers, and pectic polysaccharides (Bailey, 1973) (table 2.7). The most abundant plant cell wall NSPs are cellulose, hemicelluloses and pectins. The fructans, glucomannans, and galactomannans are not so abundant and serve as the storage polysaccharides within plants. Other constituents include mucilages, alginates, exudate gums, β-glucans and various modified polysaccharides (Asp et al., 1983).

Inulin & fructooligosaccharides (FOS)

Resistant oligosaccharides, such as the fructans are characterized as carbohydrates with a relatively low degree of polymerization (DP), as compared to the NSPs. FOS differs from fructo-polysaccharides

(inulin) only in chain length (Niness, 1999). Although a clear demarcation between oligosaccharides and polysaccharide has been found problematic, most authorities define oligosaccharide as a chain of monomeric units with a DP of 3- 10 (McNaught, 2003). The solubility of inulin depends on the degree of polymerization but both FOS and inulin are well fermented in the colon (Chawla and Patil, 2010).

Resistant starch (RS)

RS is defined as the sum of starch and products of starch degradation that is not broken down by human enzymes in the small intestine (Englyst and Cummings, 1990). RS is classified into four types (Englyst et al., 1992). RS1 relates to physically inaccessible starch as found in partly milled grains or chewed cereals, seeds, or legumes. RS2 are forms naturally found in uncooked products such as green banana uncooked potato and high amylase corn. RS3 is retrograded amylose formed during cooling of gelatinized starch formed during cooling of cooked foods whereas RS4 includes chemically modified, commercially produced resistant starches and are used in many baby food applications (Tungland and Meyer, 2002).

Lignin

Lignin is a phenylpropane polymer, and not a carbohydrate, that is covalently bound to the fibrous polysaccharides (cellulose) of plant cell walls. Lignin has a heterogeneous composition ranging from 1 or 2 units to many phenylpropanes that are cyclically linked. Lignins are important constituents in plants as they contribute to the rigidity of cell wall and thus confer resistance to microbial attacks (Benhamou and Nicole, 1999).

2.7.2 Classification of dietary fibers

Several systems have been used to classify the different components of dietary fibers.The most widely used are based on the solubility of dietary fibers in water at a defined pH, or their fermentability in an in vitro system using aqueous human alimentary enzymes (Tungland and Meyer, 2002). Generally, well fermentable fibers are soluble whereas poorly fermentable ones are not (Asp, 1996). Accordingly, dietary fibers like pectin, guar gum, gum arabic, inulin, polydextrose, and oligosaccharides are soluble/ well fermented, while cellulose, hemicelluloses, lignin and resistant starches are poorly soluble/fermentable (Chawla and Patil, 2010).

2.7.3 Analysis of dietary fiber in foods

Gravimetric analysis

The traditional way of fiber analysis is the gravimetric method, which involves chemical or enzymatic solubilization of dietary protein, starch and fat followed by weighing of the insoluble residues. The oldest version of this method was that of crude fiber (Horwitz, 1980). This method lacked accuracy due to the low recovery of fiber constituents. The refined version of this method is the detergent method which allows the separate quantification of acid detergent fibers (cellulose and lignin) and neutral detergent fibers (cellulose, hemicelluloses, and lignin) (Van Soest and McQueen, 1973). Although this method alleviated much of the caveats of crude fiber analysis, it still had the limitation of underestimating the total fiber content due to poor recovery of soluble components of fibers (i.e. pectins, mucilages, etc.).

Later, enzymatic methods, namely enzymatic gravimetric AOAC methods (Li, 1995), and enzymatic chemical methods (Englyst and Hudson, 1987) were developed. The enzymatic gravimetric (AOAC) procedures primarily measures NSP, lignin, and part of retrograded amylase-type RS, whereas the enzymatic chemical method does the same with the exception of RS and lignin. In both methods, protein and starch are digested enzymatically by using protease, heat stable α-amylase and amyloglucosidase (Champ et al., 2003).

None of the above methods are able to measure quantitatively all constituents of fibers (i.e. inulin, FOS, RS and lignin) and thus several methods are usually used in concert (Goñi et al., 1996, Dysseler and Hoffem, 1995, Theander and Westerlund, 1993).

However, more recently an inclusive method that quantifies fiber fractions as defined by Codex Alimentarius was developed (McCleary, 2007). This method (AOAC 2009.01) has been subjected to inter-laboratory evaluation through the AOAC (McCleary et al., 2010). Although the method increased the time required, it still takes less time than carrying out several assays separately and combining the results mathematically.

A modified method for measuring separately insoluble dietary fibers (IDF), dietary fibers soluble in water but precipitated in 78% aqueous ethanol (SDFP), and dietary fibers soluble in water and in 78% aqueous ethanol (SDFS) has also been developed (AOAC Method 2011.25) and was successfully evaluated more recently (McCleary et al., 2012).

Monomeric component analysis

In the monomeric component analysis, all starch is hydrolysed enzymatically and NSPs are measured as the sum of the constituent sugars released by acid hydrolysis (Englyst and Cummings, 1988). The individual sugars are quantified either by gas chromatography (GC) or by high-performance liquid

chromatography (HPLC) (Englyst et al., 1992). Another alternative is to use colorimetric procedure that measures NSPs as reducing sugars, but this gives a single value for total sugars (Englyst et al., 1994). The GC technique for dietary fiber analysis measures NSP as the sum of neutral sugars obtained by GC while uronic acids are measured separately using colorimetry after treatment with concentrated acid at high temperature (Mongeau et al., 2001). The HPLC method of dietary fiber analysis is very common and measures NSPs as the sum of neutral sugars and uronic acids, directly by electrochemical detection. However, values obtained by GC or HPLC are typically lower in comparison to those obtained by the gravimetric methods because of the exclusion of lignin and resistant starch during chromatographic assay. In recent years, rapid and non destructive methods for the determination of NSP using near IR spectroscopy have been developed (Salgó et al., 2009). Although methods using near IR give fast and inexpensive alternatives, they are limited by the comparative nature of the technique which requires multivariate calibration of sample spectra and reference (Næs et al., 1994).

2.7.4 Effect of dietary fibers on mineral bioavailability

a) In-vitro studies

Several in-vitro studies (table 2.6) have shown that semi-purified insoluble (cellulose, hemicelluloses and lignin) as well as soluble fibers (gums and pectin) have mineral-binding properties (Fernandez and Phillips, 1982a, Debon and Tester, 2001, Bosscher et al., 2003, Miyada et al., 2011, Ismail-Beigi et al., 1977). However, the mineral-binding effect was found to be dependent of the type of fiber. For instance, in the study of Ismail-Beigi et al., (1977), 42.7 % of the zinc in solution was bound by carboxymethylcellulose while only 14.5 % was bound by methylcellulose. Binding effect was also dependent on the amount (Fernandez & Phillips, 1982). In addition, pH and ionic strength determined the binding properties of several fibers, suggesting that ion exchange interactions, probably involving carboxyl and hydroxyl groups are partly responsible for the binding (Debon & Tester, 2001; Miyada et al., 2011). At acidic pH, the binding properties of gums is minimal, whereas pectin bound iron is released in solution to varying extents depending on the ionic strength of the solution (Miyada et al., 2011). Among insoluble fibers, lignin had more mineral binding capacity than cellulose and hemicelluloses (psyllium husk), probably because of the associated polyphenols. The mineral binding properties of both insoluble (cellulose, hemicelluloses and lignin) and soluble (pectin) dietary fibers were prevented by EDTA and citrates but not by ascorbic acid and cysteine (Fernandez & Phillips, 1982).

b) In vivo studies

Several animal and human studies (table 2.7) have failed to demonstrate the *in-vitro* observed negative effects of insoluble and soluble fibers (Catani et al., 2003, Cook et al., 1983, Turnlund et al., 1984, Fly et al., 1996, Van den Heuvel et al., 1998a). Although an earlier study on rats has shown that the addition of various gums at 10 % level decreased absorption of minerals (i.e. Fe, Zn and Ca), the study was limited by the fact that the content of other more potent (i.e. phytates) absorption inhibitors was unknown. A subsequent stable isotope study by Turnlund et al., (1985) showed that Zn absorption was not affected by α-cellulose but was markedly reduced by phytate. The studies of Fly et al., (1998) and Cook et al., (1983) have also shown that while hemicelluloses and lignin had no negative effect on the absorption of supplemental or added (extrinsic) iron, the iron intrinsic to these fibers was not available for absorption, suggesting that minerals trapped in insoluble fibers are less likely to be available for absorption.

On the other hand, mineral absorption enhancing properties were observed for some soluble dietary fibers such as pectins and fructooligosaccharides while no such effect was observed for insoluble ones (Greger, 1999, Sakai et al., 2000). This absorption enhancing properties of soluble fibers is not universal for all minerals and is dependent on specific characteristics of the soluble fiber in question. For instance, absorption enhancing properties were observed for pectins of low molecular weight and high degree of esterification while no effect was observed for those of high molecular weight and, or low degree of esterification (Kim et al., 1996).

c) Explaining disparities between in vitro and in vivo studies

Earlier in vitro/ in vivo studies lacked control over more potent absorption inhibitors and enhancers such as phytates, polyphenols, ascorbic acid, etc. (Cook et al., 1988, Van den Heuvel et al., 1998). Most of the animal studies were performed on rats and this may be limited given that iron absorption in rats, especially from low bioavailable sources, is better than in humans (Reddy and Cook 1991, 1994). The few existing human studies on the topic were conducted mostly on healthy young men, who most likely have adequate iron stores (Van den Heuvel et al., 1998). Differences in iron bioavailability among diets may be obscured when iron stores are adequate (Hulten et al., 1995).

On the other hand, comparison of the results is also made difficult because of the use of different types and amounts of fibers with varying physiological and physicochemical properties.

Notwithstanding these limitations, the existing literature suggests that both soluble and insoluble fibers may decrease gastrointestinal absorption due to mineral binding or physical entrapment of minerals. However, both physically and chemically bound minerals are likely to be available for absorption in the colon as a result of fiber fermentation. Indeed, a recent study comparing the effect of pectin on iron absorption in ileorectomized, caectomized, and normal rats showed that absorption in the small intestine was decreased while it was increased in the colon (Miyada et al., 2012). Iron absorption from the large intestine is less efficient compared with the duodenum, but could be significant in case of iron deficiency (Yeung et al., 2005).

Besides, mineral absorption enhancing effects were also reported for some soluble fibers like fructo-oligosaccharides, inulin and pectin (Sakai et al., 2000). However, the enhancing properties of inulin have been less consistent as recent human studies have failed to show improvements in iron absorption (van den Heuvel et al., 1998b, Coudray et al., 1997, Petry et al., 2012a). However, the effect of inulin might have been underestimated since the studies were either conducted on healthy young men (van den Heuvel et al., 1998b, Coudray et al., 1997) or compounds with potential absorption enhancing properties such as maltodextrins were used as placebo (Miyazato et al., 2010, Petry et al., 2012a).

Table 2.6: *In vitro* studies on the effect of fiber on mineral availability

Type of fiber studied	Source	Fiber category	Type of study	Effect on mineral	Remark	References
Cellulose	Purified			Zn binding	Dephytinized	(Ismail-Beigi et al., 1977)
	Rice	NSP	solubility	↑ Ca and Zn due to cellulase treatment but No effect on Fe		(Wang et al., 2008)
Hemicellulose	Wheat bran			Fe, Zn binding		(Mod et al., 1982)
				Ca, Mg binding		
Water soluble hemicellulose	Rice			↑ in Fe with xylanase treatment No effect on Zn		
Alkali soluble hemicellulose						(Lestienne et al., 2005a)
Hemicellulose	Pearl millet					
Pectin	Semi-purified			Minimal binding		(Fernandez and Phillips, 1982a)
High esterified pectin	purified					(Debon and Tester, 2001)
Low esterified pectin	Citrus					(Bosscher et al., 2003)

Type of fiber studied	Source	Fiber category	Type of study	Effect on mineral	Remark	References
Gums & mucilages	Purified	NSP	Binding in FeSO4 sol			(Fernandez & Phillips, 1982)
Psyllium						
Alginic acid			Dialysis	↓Ca while ↑Fe &Zn absorption		(Bosscher et al., 2001)
Locust-bean gum				↓Fe &Zn absorption		(Debon & Tester, 2001)
Guar gum						
Agar				No binding in acidic condition except for Fe3+		
K-karrageenan						
Gum xanthan						
Gum arabic						
Gum karaya			Dialysis			(Bosscher et al., 2003)
Gum tragacanth						
Guar gum						
Locust bean gum						
Lignin	Wheat bran	Lignin		Fe& Zn binding		(Ismail-Beigi et al.,1977)
Neutral lignin	Semi-purified		binding in $FeSO_4$ sol.	High iron binding	Counteracted by citrate and EDTA	(Fernandez & Phillips, 1982)
Acid lignin	Semi-purified					
Inulin	Purified	Inulin	Dialysis	30% ↑ Ca absorption 2.2% ↓ Fe		(Bosscher et al., 2003)
Resistant starch						
Modified starch+ maltodextrin	Rice starch	RS	Dialysis	3.8% ↑ in Fe ↓ Zn		(Bosscher et al., 2003)

Table 2.7: *In vivo* (animal/human) studies on the effect of fiber on mineral bioavailability

Type of fiber studied	Source	Fiber category	Type of study	Effect on mineral	Remark	References
			Animal studies			
Cellulose	Diet with 100g/kg cellulose	NSP	Rat hemoglobin regeneration	No effect on Fe	No effect for the control either, probably due to the use of elemental iron which is poorly bioavailable	(Catani et al., 2003)
Hemicellulose	Synthetic Psyllium	NSP	Chicks	No effect on Fe No effect on extrinsic Fe but ↓intrinsic Fe absorption		(Fly et al., 1996)
	Synthetic -Acidic xylan-oligosaccharide		Iron deficient adult female rats	Promoted recovery ↓hepatic hepcidin mRNA ↑Fe absorption ↓Fe excretion		(Kobayashi et al., 2011)

Type of fiber studied	Source	Fiber category	Type of study	Effect on mineral	Remark	References
Pectin (Different MW & degree of esterification)		NSP	Anemic rats-hemoglobin repletion	↑Hb regeneration efficiency ↑hematocrite ↑Serum Fe ↑transferrin saturation ↓Fe unsaturated and total Fe-binding capacity	Effect was dependent of MW and degree of esterification No improvement in bioavailability for high MW and low DE pectin + effect for low MW and high DE pectins	(Kim and Atallah, 1992, Kim et al., 1996)
				No effect on Fe absorption		
			Iron deficient rats	↑Hb regeneration	High degree of esterification but no information regarding MW	(Feltrin et al., 2009)
				↓ absorption in the SI ↑release of pectin bound Fe by microbial degradation , this ↑ bioavailability in the large intestine		(Miyada et al., 2011)
			Ileorectomised, caecectomized rats		Pectin bound Fe is utilized by rats	(Miyada et al., 2012)

Type of fiber studied	Source	Fiber category	Type of study	Effect on mineral	Remarks	References
Gum & mucilage Carrageenan	Synthetic 10% added		Growing rats	↓absorption of Fe, Zn, Ca, Cu, Co	Other absorption inhibitors not known	(Harmuth-Hoene and Schelenz, 1980)
Agar, agar				↓absorption of Fe, Zn, Ca, Cu, Co		
				↓ iron ↓ Zn		
Sodium alginate Carob bam gum Guar gum				↓ Zn		
FOS	Difructose anhydride III 30g FOS	FOS	Rats	↑in Fe absorption	DFA partially prevents tannic acid induced anemia whereas FOS had no effect	(Afsana et al., 2003)
Resistant starch	Corn (16 %)	RS	Piglets	↑ Fe and Ca absorption		(Morais et al., 1996)
	RS-II		Rats mineral retention	↑ Ca, Mg, Zn, Fe and Cu absorptions		(Lopez et al., 2001)
	Maltodextrins		Rats	↑ Ca, Mg, Zn, Fe absorption	Fe & Zn absorption not affected by cacectomy	(Miyazato et al., 2010)

Type of fiber studied		Source	Fiber category	Type of study	Effect on mineral	Remarks	References
				Human studies			
Cellulose		Wheat muffin+cellulose	NSP	Human multiple radioiron absorption (male +female)	No effect on Fe	Phytate, polyphenols not assessed	(Cook et al., 1983)
		Diet with α-cellulose		Human-(Young men) Zn stable isotope study	No effect on Zn Phytate was inhibitory	Phytate was controlled	(Turnlund et al., 1984)
Inulin	20 g/d			Human isotope study- women with low Fe status	No increase in Fe absorption	Limitation: use of maltodextrin as a placebo. ↑ in Fe absorption observed for resistant maltodextrins	(Petry et al., 2012)
				Chemical balance - healthy young men	↑Ca absorption No change in Fe, Zn, Mg		(Coudray et al., 1997)
	15 g/d			Double isotope- Healthy men	No effect		(Van den Heuvel et al, 1998)

2.7.5 Plausible mechanism for mineral absorption inhibiting/enhancing effects of fibers

Minerals need to be soluble and at the same time be engaged in molecular complexes small enough to be absorbed by the enterocytes in the small intestine. Components of fiber (i.e NSP, lignin, etc.) can form insoluble large complexes chelating minerals through the carboxyl group of uronic acid, the carboxyl and hydroxyl groups of phenolic compounds, and the surface hydroxyl of cellulose thereby decreasing mineral bioavailability (Torre et al., 1995). Besides, some soluble NSP's induce increase in digesta viscosity which can hinder contact with digestive enzymes and thus the availability of nutrients for absorption (Van der Klis et al., 1995, Guillon & Champ, 2000). Physical entrapment of minerals in fibers has also been suggested to be responsible for the decrease in absorption (Fly et al., 1996).

On the other hand, the enhancing effect of soluble/fermentable fibers could be related to fermentation products such as osmotically active sugars that may increase passive absorption (Greger, 1999) and, or the production of weak organic acids that may have absorption enhancing properties. The lowering of pH is also likely to result in the reduction of ferric to ferrous iron and thereby improve bioavailability as ferrous is more soluble than ferric iron. For instance much of the iron bound to pectin was found to be in the ferrous form, probably because of the effective reduction of ferric iron to ferrous iron by pectins (Miyada et al., 2011).

The fermentation of fibers in the colon often produces short chain fatty acids that can stimulate increase in epithelial cell proliferation, which increases the absorptive surface area that may in turn increase bioavailability (Bauer et al., 2006, Yeung et al., 2005). Little is known on the exact time needed for such physiological effects. However, the observation of beneficial effect on iron absorption of oligosaccharides in long term studies in contrast to short term ones suggests that temporal effects may be important (Sazawal et al., 2010).

Another possible mechanism by which soluble fibers may exert their absorption enhancing effect is by affecting the expression of mineral transport proteins. A recent study in rats with diet-induced iron deficiency anemia (IDA) has shown that synthetic xylanooligosaccharides (soluble fiber) promoted recovery from IDA and was shown to decrease the expression of divalent metal transporter 1 (DMT1) and ferroportin mRNA (Kobayashi et al., 2011).

2.7.6 Enzymes targeting dietary fibers

In recent years, the emergence of enzyme technologies has led to the commercialization and application of carbohydrases primarily in the feed but also in the food industry. In the food industry, a combination

of pectinase, cellulase, hemicellulase, collectively called macerating enzymes are for example used to facilitate extraction and clarification of fruit and vegetable juices (Grassin and Fauquembergue, 1996, Bhat, 2000). Other food applications include processing of beer, wine, bread and biscuits, and extraction of olive oil (Bhat, 2000). With progresses made in the past few years the substrate specificities and efficacy of carbohydrases have tremendously increased. This created a great opportunity for the development of better enzymatic methods for the determination of fiber in foods. The application of these enzymes also opens the opportunity for better estimation of the effect of native dietary fibers on mineral bioavailability *in situ* and hence can prevent previously encountered limitations associated with the use of isolated fibers. However, only few studies have made use of this opportunity (Matuschek et al., 2001, Wang et al., 2008, Lestienne et al., 2005a).

In addition, the application of carbohydrases along with, or subsequent to treatments with phytases, tannase and, or polyphenol oxidases may also minimize previously encountered confounding effect of more potent mineral absorption inhibitors like phytates and polyphenols. With appropriate design involving several of these enzymes, the relative effect of each mineral absorption inhibitor as well as their combined effect could be evaluated. Lestienne et al., (2005) observed that treatment of pearl millet and sorghum with xylanase and phytase solublilized more iron and zinc than phytase alone, hence confirming that hemicelluloses native to foods have indeed a negative effect on the absorption of these minerals in the small intestine. Similarly, Wang et al., (2008) has shown that treatment of rice with cellulase (1%) increased *in vitro* zinc and calcium availability, while having no effect on iron availability. However, given the variability in physicochemical properties of fibers between cereals and changes induced by different processing, more studies are needed. Several animal studies using exogenous enzymes (xylanase, cellulase, and phytase) exist, but most focused on evaluating the digestibility of feeds, or the growth performance of the animal (Cowieson and Bedford, 2009, Woyengo and Nyachoti, 2011), and hence future animal and human studies are needed in this regard.

Table 2.8: Enzymes degrading non-starch polysaccharides and associated substances

NSP and associated substances	Monomers	Linkage	Enzymes
Cellulose	Glucose	β -(1-4)	Cellulase
		Non-cellulosic polymers	
Arabinoxylans	Arabinose & xylose	β -(1-4) linked xylose units	Xylanase/ hemicellulase
Mixed-linked β-glucans	Glucose	β -(1-3) & β-(1-4)	Cellulase; β-glucanasexyloglucan-specific β-1,4-glucanase
Mannans	Mannose	β-(1-4)	β-D-mannosidase
Galactomannans	Galactose & mannans	β-(1-4) linked mannans with α-(1-6) linked galactosyl side groups	β-D-mannosidase
Glucomannans	Glucose & mannans	β-(1-4) linked mannans with interspersed glucose residues	β-D-mannosidase
Arabinans	Arabinose	α-(1-5)	Arabinan endo-1,5- α-L-arabinanase
		Pectic polysaccharides	
Galactans	Galactose	β-(1-4)	β-galactosidase
Arabinogalactan type I	Arabinose & galactose	β-(1-4) galactan backbone with 5–linked and terminal arabinose residues	Arabinogalactan endo-1,4-β-galactosidase
Arabinogalactan type II	Arabinose & galactose	β-(1-3,6) linked galactose polymers associated with 3-/5-arabinose residues	β-1- 3,6-galactosidase

Sources: (Sinha et al., 2011); Brenda comprehensive enzyme information system (http://www.brenda-enzymes.org)

2.8 Iron and zinc bioavailability assessment methods

The existing bioavailability methods can be categorized into three, in vivo methods, in vitro methods and methods based on algorithms.

2.8.1 In vivo methods

In vivo methods can be applied both on animal or human studies. Several methods have been developed across time, these include: balance studies, hemoglobin repletion tests, radio isotopes, stable isotopes, and human efficacy.

Balance studies

This is one of the first technique applied for the assessment of mineral bioavailability. The principle of this method relies in estimating the difference between oral input and fecal, urine output. The major limitation of this method is that it is costly and laborious. The method is also not suited for the assessment of zinc bioavailability because of intestinal zinc excretion (Sandstrom, 1997).Furthermore, this method has been criticized for being imprecise (Rossander et al., 1992).

Isotope studies

The use of isotopes has led to great developments in the understanding of mineral bioavailability in humans. Two groups of isotopes are recognized: the earlier radio isotopes techniques and the stable isotope techniques developed in the 1960's. In radio isotopic studies, absorption is assessed by calculating the difference between the administered radioactivity and the radioactivity measured either in blood, feces or in the total body. Thus, radio isotopes absorption can be assessed either by whole body counting, or by using the iron incorporation in erythrocytes. However, the use of radioisotopes may raise ethical concerns especially when used in studies involving infants, children and pregnant women as it exposes subjects to radiation (Turnlund, 2006). In these circumstances, the use of stable isotopes such as ^{54}Fe, ^{57}Fe and ^{58}Fe and ^{70}Zn is preferred. Furthermore, multiple stable isotopes of one mineral or multiple minerals can be administered either simultaneously or sequentially. However, stable isotope techniques have also their limitations. Depending on the natural abundance of the isotopes, the amount of isotope to be administered can exceed tracer levels and thus can in some cases cause risks of metabolic perturbation. In addition experiments can be much more expensive that when using radioisotopes (Turnlund, 2006).

Hemoglobin repletion tests in rats

This method involves the use of reference standard and test diets containing three or more levels of iron from the respective sources that are fed to anemic rats. The relative biological value (RBV) of iron in the test source is compared to that of the reference compound after 14 days.

Studies have shown that dietary inhibitors and enhancers of non-heme iron absorption had different effects in humans and in rats (Reddy and Cook, 1991). Consequently, rats are not considered as a suitable model for studying the effect of different dietary factors on human iron absorption.

Efficacy studies

Human efficacy studies measure the efficacy of an intervention (i.e. supplementation, fortification, etc.) in increasing the iron or zinc status in comparison to a control group. Efficacy trials are usually performed under strictly controlled conditions, usually using randomization, double-blinded placebo-control, and a strict compliance control. Although efficacy trials have the advantage of providing good information on the efficacy of a certain intervention in "real life" situation, it can be difficult to manage and can be very expensive.

However, given that in vivo studies require special skills, are laborious and expensive; easier and less expensive alternatives are needed for the routine screening of samples. This demand has been essentially met by the development of in vitro methods such as in-vitro solubility, dialysability, or caco-2-cells.

2.8.2 In vitro methods

HCl-extractability

This method consists of a simple hydrolysis of foods with HCl at 37°C, followed by filtration. This method does not correlate well with results from human studies and is limited by the fact that it does not consider physiologically relevant pH changes during digestion.

In vitro solubility/ dialysability

The development of in vitro solubility measurements partly filled the caveats associated with the use of HCl extractability by simulating the digestion process. This led to a two step digestion, the first corresponding to pepsin digestion for 1h at pH 1-2 followed by a second digestion with pancreatin-bile mixture for ~2h at intestinal pH (6-7) (Narasinga Rao and Prabhavathi, 1978). further developed the method by incorporating a dialysis step after

digestion and assuring gradual pH change. Several variants of this method, with few modifications, have been used to investigate the influence of mineral absorption inhibitors (Miller et al. 1981; Wolfgor et al., 2002, Greffeuille et al., 2011).

Caco-2-cells

Caco-2-cells are cell lines, derived from human colon adenocarcinoma, that exhibit many of the functional features of small intestinal cells (Pinto, 1983). This method is principally used for the assessment of the bioavailability of iron and not zinc. Caco-2-cells have been shown to express DMT1 in response to both changes in cell iron status (Tallkvist et al., 2000, Martini et al., 2002) and hepcidin (Yamaji et al., 2004). They also express brush border ferric reductase activity (Ekmekcioglu et al., 1996). Therefore, the use of caco-2-cell lines after in vitro simulated gastric and pancreatic digestion has the added advantage of integrating physiological aspects such as cellular uptake. However, in vitro dialysis and caco-2-cells are both limited in accurately reflecting the magnitudes of the effects of factors that influence absorption and thus should mainly be used for ranking meals and single food items in terms of predicted iron bioavailability (Lynch, 2005).

2.8.3 Mathematical models

Molar ratios

Given that phytate is considered as the major absorption inhibitor, phytate to mineral molar ratios are generally used to evaluate the bioavailability of foods. Davies and Olpin (1979) were the first to use molar ratios to estimate bioavailability of foods given to rats. Since then, the good correlations of phytate to mineral molar ratios with human absorption studies have led to the wide application of the method.

In predominantly cereal and legume based meals phytate to iron molar ratio should be less than 1 or preferably less than 0.4 to significantly improve iron absorption (Hurrell, 2004). In mixed-meals containing ascorbic acid or meat, phytate: iron molar ratio <0.6 may be sufficient (Hurrell, 2004). However, particular caution should be taken when using these critical values given that the effect of phytate was shown to be dependent of the presence of polyphenols (Hurrell et al., 2003).

On the other hand, based on absorption studies in humans, phytate: Zn molar ratios <5, between 5 and 15, and >15 have been associated with high, moderate and low zinc

bioavailability, corresponding to around 50%, 30% and 15% of total zinc, respectively (Gibson, 2006).

Given that calcium may exacerbate the effect of phytate on zinc absorption (Cossack and Prasad, 1983), the use of the [phytate x Ca]: Zn molar ratio has been suggested rather than the phytate: Zn ratio. The deleterious effect of calcium on zinc absorption is expected for foods with [phytate x Ca]: Zn values exceeding 200 (Hemalatha et al., 2007).

Algorithms

In an attempt to predict iron and zinc bioavailability, several algorithms were developed. The first iron algorithm was developed by Monsen et al. (1978) and was later adapted to diets from developing countries by Murphy et al. (1992). In this algorithm, intakes in non-heme iron, heme iron, ascorbic acid and proteins are needed. The algorithm can be corrected for the inhibitory effect of polyphenols from tea and coffee on non-heme iron absorption. However, this algorithm has several limitations. The algorithm assumed 40% of the total iron muscle proteins to be heme irrespective of its source. However, heme content of flesh foods may vary from 30-70% (Gibson and Ferguson, 2008). Moreover, the algorithm does not adjust for the effects of phytates and vegetable proteins (i.e. soybean protein) on non-heme iron absorption.

Later, Tseng et al. (1997) refined murphy's model to allow correction for the enhancing effects of meat, poultry, fish, and vitamin C and the inhibitory effects of both tea and phytates on non-heme iron absorption. Reddy et al. (2000) developed a model to predict iron absorption from typical western diets. The algorithm accounts for the amount of animal tissue, phytic acid and ascorbic acid and reported that calcium, non-heme iron, and polyphenols were not significant predictors of iron absorption.

The most comprehensive algorithm was the one developed by Hallberg and Hulthen (2000). This algorithm takes into account the effects of almost all known enhancing and inhibiting factors, as well as their interaction. It is also unique in that it adjusts the absorption for serum ferritin levels.

2.9 Iron and zinc deficiency prevention and control strategies

The main public health interventions for the control and prevention of micronutrient deficiencies so far adopted are dietary diversification, food fortification and medicinal supplementation. Other alternative strategies would be optimization of traditional household processing. The prevention and control of iron and zinc deficiencies is likely to be more

effective if children in the first two years of life are targeted. This is because past the age of two, negative effects of these deficiencies may be irreversible.

2.9.1 Supplementation

Supplementation involves in the provision of pills, liquid or injection which may be of pharmacologic doses to be taken daily or weekly or mega-doses at an interval of 4 to 24 months. The delivery of medicinal supplementation could be preventive (i.e. folic acid-iron supplement) or therapeutic in nature (Cavalli-Sforza et al., 2005). This strategy relies on the existence of functional heath care or delivery system. Preventive supplementation, notably zinc supplementation has been found to reduce episodes of diarrhea, acute respiratory illnesses and pneumonia (Brown et al., 2009). Moreover, low compliance and low participation during mega-dose supplementation are some of the limitations (Shrimpton and Schultink, 2002). In addition, mineral supplementation can increase the risk of adverse micronutrient interactions and can even be detrimental as for example in the case of iron supplementation in malaria endemic areas (Sazawal et al., 2006, Haider and Bhutta, 2009).

Although supplementation strategies have reduced morbidities and mortalities, weak compliance and reliance on limited external support for the supply of supplements make them unsustainable in developing countries. Food-based strategies including food production, dietary diversification and food fortification may be more sustainable approaches (Tontisirin et al., 2002).

2.9.2 Food fortification

Food fortification relies on adding micronutrients to a food item that is widely consumed by a targeted population (Allen et al., 2006). Food fortification strategies are advantageous in the fact they do not require change in agricultural practices. Requirements for behavioral changes are also minimal provided that they do not compromise organoleptic properties. They can also reach a large share of the population in a relatively short period of time at a reasonable cost (Horton, 2006).

Fortification can be targeted to specific group of a population (i.e. infants and young children) or can be universal. In both cases, the decision to fortify should be based on careful evaluation of nutritional status and dietary intake data. Once the extent of the problem is determined, a food vehicle that is widely consumed by the general or targeted population needs to be identified. The type of fortificant should then be carefully choosen by considering several factors including, the bioavailability of the compound, its reactivity with food

components (i.e. discoloration, undesired changes), it's stability under storage and food preparation condidtions, its compatibility with other nutrients and its cost.

In order to ascertain adequate fortification, vehicles should preferably be processed in central processing units. In developing countries, especially in rural areas, such central processing units are rare, hence making fortification strategies less viable. However, household food fortification strategies (i.e. mix-me) that involve the addition of multiple micronutrients to diets at the point of consumption, can be a good alternative, provided that fortification guidelines are strictly followed by household members (Zlotkin et al., 2004). This is particularly important because improper fortification with certain micronutrients (e.g. iron) can be toxic or have adverse effects on sensory properties of the food (Allen, 2008). The other challenge is to achieve a good compromise between good bioavailability of the fortificant and minimal adverse organoleptic effects. This is because fortificants with higher bioavailbility tend to cause organoleptic changes. However, the development of encapsulation technologies are likely to meet the challenge (Hurrell, 2002).

2.9.3 Biofortification

Biofortification is a relatively new approach that intends to increase the nutrient content of staple food crops by agricultural, agronomic, or genetic means (Bouis et al., 2011). The strategy is promising as early studies showed the possibilities of enhancing the micronutrient content of many staple crops. This strategy is currently under study by harvest plus in consortium with a large number of international research institutions (www.harvestplus.org). Under its research program, studies on the retention and bioavailability of minerals during processing of biofortified crops, their efficacy, and the adoption rate of the new biofortified varieties are ongoing. Future studies on the potential risk to human health may be needed. Furthermore, given that this strategy relies on staples, concerns of further simplifying the already staple dependent diets have been raised. Due attention should therefore be given to not undermine the conservation and use of biodiversity for addressing other human needs (Johns and Eyzaguirre, 2007).

2.9.4 Dietary diversification strategies

Dietary diversification strategies encourage the consumption of a variety of foods with the ultimate goal of complementing each diet to meet the overall nutrient and energy requirement for a healthy and productive life. A more diversified diet has been associated with better growth and micronutrient status (Moursi, 2008). This strategy relies on the prior

identification of locally available foods rich in micronutrients to then promote their consumption. This is a long-term approach that requires several changes in behavior, agricultural practices, and further requires functional nutrition education programs (Gibson and Anderson, 2009).

2.9.5 Dietary modification through household food processing

Several household food processing techniques are likely to influence the content, form and bioavailability of micronutrients in foods. Several household food processing techniques can thus be used to enhance the bioavailability of micronutrients by increasing the accessibility of micronutrients, decreasing absorption inhibitors such as phytates, or increase bioavailability enhancing compounds.

2.9.5.1 Thermal processing

Thermal processing can result in moderate losses in phytic acid (Erdman and Pneros-Schneier, 1994); however, the potential benefit on iron and zinc bioavailability can be offset by concomitant destruction of absorption enhancers such as vitamin C (Rao et al., 2006).

In contrast to the limited effect of thermal processing on phytic acid, reduction in polyphenol contents can be significant. For instance, up to 90 % reduction in polyphenols during extrusion cooking of beans has been reported (Alonso et al., 2000). However, whether such decrease includes iron-binding phenolics needs to be determined.

On the other hand, thermal treatments can change the ratio of soluble to insoluble fibers and lead to greater recovery of soluble fibers such as pectins, arabinoxylans and β-glucans (Guillon and Champ, 2000). Extrusion cooking of cereals has been shown to increase the content of soluble fibers; the extent of this effect is dependent on the type of cereal and extrusion conditions like shear forces (Gualberto et al., 1997). Since some soluble fibers have been shown to increase mineral absorption, an increase in mineral bioavailability is possible. However the few existing studies failed to show improvements in bioavailability (Fairweather-Tait et al., 1987).

2.9.5.2 Soaking

Soaking of cereal grains and legume seeds is often performed to facilitate removal of the outer layer during subsequent treatments such as decortications. Depending on soaking conditions (i.e. time) diffusion of minerals and vitamins as well as mineral absorption inhibitors such as phytates and polyphenols can be substantial (Hotz and Gibson, 2007,

Lestienne et al., 2005b). Furthermore, soaking can facilitate the activation of endogenous enzymes such as phytases and polyphenol oxidases (Egli et al., 2002, Matuschek et al., 2001). For example during traditional household soaking of unrefined maize, some 50 % reduction in phytate content was reported (Hotz and Gibson, 2001). The extent of phytate degradation is dependent on the type of cereal and can range from 4 % in sorghum to 28% in millet in whole grains (Lestienne et al, 2005) and over 90% in white rice flour. In contrast to cereals, the effect of soaking on whole legumes is modest, mainly due to the difference in the localization of phytates (Perlas and Gibson, 2002).

Similarly, soaking can result in loss of polyphenols, and the extent of which is dependent on soaking conditions (i.e. pH, temperature, duration of soaking) and on the type of cereals or legumes.

In contrast, little is known regarding the effect of soaking on dietary fibers. The soaking of different legumes has been associated with small increases in insoluble NDF and ADF (Vidal-Valverde et al., 2006). It can be presumed that losses of soluble fibers are likely to occur.

2.9.5.3 Decortication and milling

Decortication is used to remove the bran and/or germ of cereals. In cereals, decortication may be associated with substantial losses of polyphenols and phytates when they are localized in the outer aleurone layer (i.e. rice, sorghum and wheat) (Hotz and Gibson, 2007). However, this process may also result in substantial losses of minerals depending on the type of decortication (i.e., abrasive, mechanical, manual) (Hama et al., 2011). Substantial fiber losses are also likely to occur with the removal of bran (Lestienne et al., 2007). Therefore, to improve bioavailability, the process should be optimized for minimal mineral losses while ascertaining maximal reductions in mineral absorption inhibitors. To this end, many countries compensate the mineral losses by fortifying cereal flours.

2.9.5.4 Fermentation

The wide consumption of fermented foods in developing countries (table 2.9) opens the opportunity to improve the bioavailability of minerals. There are various mechanisms by which fermentation can influence mineral bioavailability (Bering et al., 2006). During fermentation, several organic acids (i.e. lactic acid, acetic acid, etc.) are produced (Tou et al., 2006). These organic acids can bind to minerals like iron and zinc to form soluble complexes less available for chelation by phytates and polyphenols (Hotz & Gibson, 2007) and thus

increase the bioavailability. Besides, the resulting acidification may further contribute to the improvement of bioavailability by reducing ferric iron to ferrous iron.

Table 2.9: Examples of traditional fermented cereal-based foods and beverages consumed in tropical countries

Product	Country	Cereal	Malt	Product use
Atole agrio	Mexico	Maize		Beverage
Banku	Ghana	Maize, cassava		Cooked dough
Ben-saalga, Koko	Burkina Faso, Ghana	Pearl millet		Gruel
Boza	Bulgaria, Romania, Turkey, Albania	Wheat, rye, millet, maize, etc.		Beverage
Bushera	Uganda	Sorghum, millet	Sorghum/millet malt	Beverage
Burong isda	Philippine	Rice (+ fish)		Food/ ingredient Beverage
Cauim	Brazil	Rice, corn, cassava (+cotton seeds)		Beverage
Dosa	India	Rice (+ black gram)		Pancake (fried)
Gowe (Sifanu)	Benin	Sorghum	Sorghum malt	Beverage
Hussuwa	Sudan	Sorghum	Sorghum malt	Cooked dough
Idli	India	Rice and black gram		Steamed cake
Injera	Ethiopia	Tef, sorghum, corn, finger millet, barley, wheat		Flat bread
Kenkey	Ghana	Maize		Cooked/steamed dough
Kisra	Arabian Gulf, Sudan, Iraq	Sorghum, pearl millet		Flat bread
Mahewu	South Africa, Zimbabwe	Maize	Sorghum, millet malt	Beverage
Mawe	Benin, Togo	Maize		Ready to use foods
Ogi	West Africa	Maize, millet, sorghum		Ready to use foods
Poto poto	Congo	Maize		Gruel
Pozol	Mexico, Guatemala	Maize		Beverage
Selroti	Darjeeling hills and Sikkim (India), Nepal, Bhutan	Rice		Confectionery
Steamed bread	China, Thailand	Wheat		Bread
Togwa	East Africa	Maize	Millet malt	Beverage

Source: (Guyot, 2010)

Fermentation can also result in degradations of phytic acid and polyphenols by the activation of endogenous and, or microbial enzymes (i.e. phytase, polyphenol oxidase, tannase, etc.). However, unlike for phytate, the effect of fermentation on polyphenols has been less consistent and in some instances, increases in polyphenols have also been observed (Sharma and Kapoor, 1996, Sripriya et al., 1997), possibly due to increase in assayable polyphenols

resulting from enzymatic release of non-extractable polyphenols. However, further studies are needed in this regard.

2.9.5.5 Germination

Germination or malting refers to the soaking of cereal grains or legumes in water until sprouting, followed by drying. During this process several hydrolase enzymes such as phytase are generated either through *de novo* synthesis or through activation of endogenous ones. As a result, phytate degradation is commonly observed during germination. The extent of degradation is however dependent on the species and variety of grains/seeds. Among cereals commonly used in the preparation of complementary foods, greater phytate degradation was obtained during germination of rice, millet and mungo beans (Egli et al., 2003). In addition, the effect of germination is dependent on factors such as pH, extent of germination, moisture content, temperature, solubility of phytate, and presence of certain inhibitors (Larsson and Sandberg, 1992). Germination has also been shown to result in reduction of tannins and other polyphenols, possibly through formation of complexes with proteins or activation of polyphenol oxidases (Matuschek et al., 2001, Demeke et al., 2001).

2.9.6 Complementary feeding strategies

The age of 6-23 months is the time where incidence of growth faltering, micronutrient deficiencies and infectious diseases reach a peak. If not corrected in time, malnutrition in this period can lead to permanent functional impairments (Martorell et al., 1994). Given that prevention of malnutrition during the first two years benefits health, education, and the economic status of a population, ensuring adequate complementary feeding is of great importance. It has been estimated that successful coverage of complementary feeding interventions could prevent a 5.5 million disability-adjusted life-years (DALYs) in countries where malnutrition burden is high (Bhutta et al., 2008).

Complementary foods in developing countries are often exclusively plant-based. Such plant-based complementary foods have been associated with deficits in micronutrients, especially, in iron, zinc and calcium (Gibson et al., 2010). Complementary foods should provide about 60% of the calcium, 85% of the zinc and almost 100% of the iron requirements of children 6 to 23 months, assuming average breast milk intake (Gibson et al., 1998b, FAO/WHO, 2004). Meeting such high needs, based on exclusively plant-based complementary foods is unlikely (Gibson et al., 1998). This is further exacerbated by the poor bioavailability of the minerals in

these diets, partly due to the high content of phytate and polyphenols. Therefore, improving the quality of complementary foods by increasing dietary diversity with animal-source foods and, or the use of fortified products is recommended (PAHO/WHO, 2003).

In most developing countries, the amount of food consumed is lower than the theoretical gastric capacity (30 g/ Kg body weight/day). This can partly be explained by the "laissez faire" style of feeding observed in many developing countries (Engle and Zeitlin, 1996, Bentley et al., 2011). This further makes meeting intake requirements difficult. Since feeding style is as important as the type of food given to the child (Pelto, 2000) promotion of responsive feeding practices together with appropriate nutrition education should be an important part of complementary feeding strategies.

2.10 Iron and zinc literature: the case of Ethiopia

Iron and zinc deficiencies are among the most prevalent micronutrient deficiencies in the world (WHO, 2009). Iron deficiency (ID) affects both developing and developed countries (Ramakrishnan and Yip, 2002). At least 30% of the world's population is believed to be affected (Zimmermann and Hurrell, 2007). Although exact measurement of zinc deficiency prevalence is not yet available, it is estimated to be as widespread as iron deficiency. However, the prevalence of iron and zinc deficiency is not evenly distributed within a population or throughout the world (Ramakrishnan, 2002). High prevalence is found in women and children in developing countries, where diets are predominantly plant-based and very little iron- and zinc-rich foods such as animal source foods (ASF) are consumed (Sandstead, 2000).

2.10.1 Iron and zinc deficiencies in Ethiopia

According to a WHO report on worldwide prevalence of anemia , the prevalence of anemia in Ethiopian children of less than five years of age and pregnant women was estimated to be 75.2% and 62.7%, respectively . Both fall under the category of severe public health problem (Unicef, 2004). In women of reproductive age, WHO figures estimated 52.3% of anemia while a study on a sub-sample (n=970) of a representative sample of women of reproductive age in Ethiopia reported 29.4 % of anemia, 32.1% of iron deficiency (ID), and a mean serum ferritin of 58 ± 41.1 g/L, hence categorizing the problem as moderate public health concern (Haidar and Pobocik, 2009).

Such inconsistencies between studies estimating the prevalence and severity of iron deficiency anemia in Ethiopia are common. While earlier studies in Ethiopia documented ID as rare and ascribed this to the consumption of iron-rich *teff* and hypoxia due to altitude (Gebre-Medhin, 1976, Gebre-Medhin and Birgegård, 1981), recent ones report that ID is of mild to moderate public health concern (Haidar & Rebecca, 2009). Such differences in time may be explained by the reduction of teff consumption due to the rising cost of the grain. It may also be due to changes in political situation such as the long civil wars that the country hosted. Most of the earlier studies are pre-war, while those documenting ID, IDA and anemia as a public health concern are post-war. Earlier studies were also limited in geographical scope and hence may not be representative of the country, especially since prevalence of ID, IDA and anemia has been shown to vary according to geographical location (Umeta et al., 2008) (Fig. 2.13).

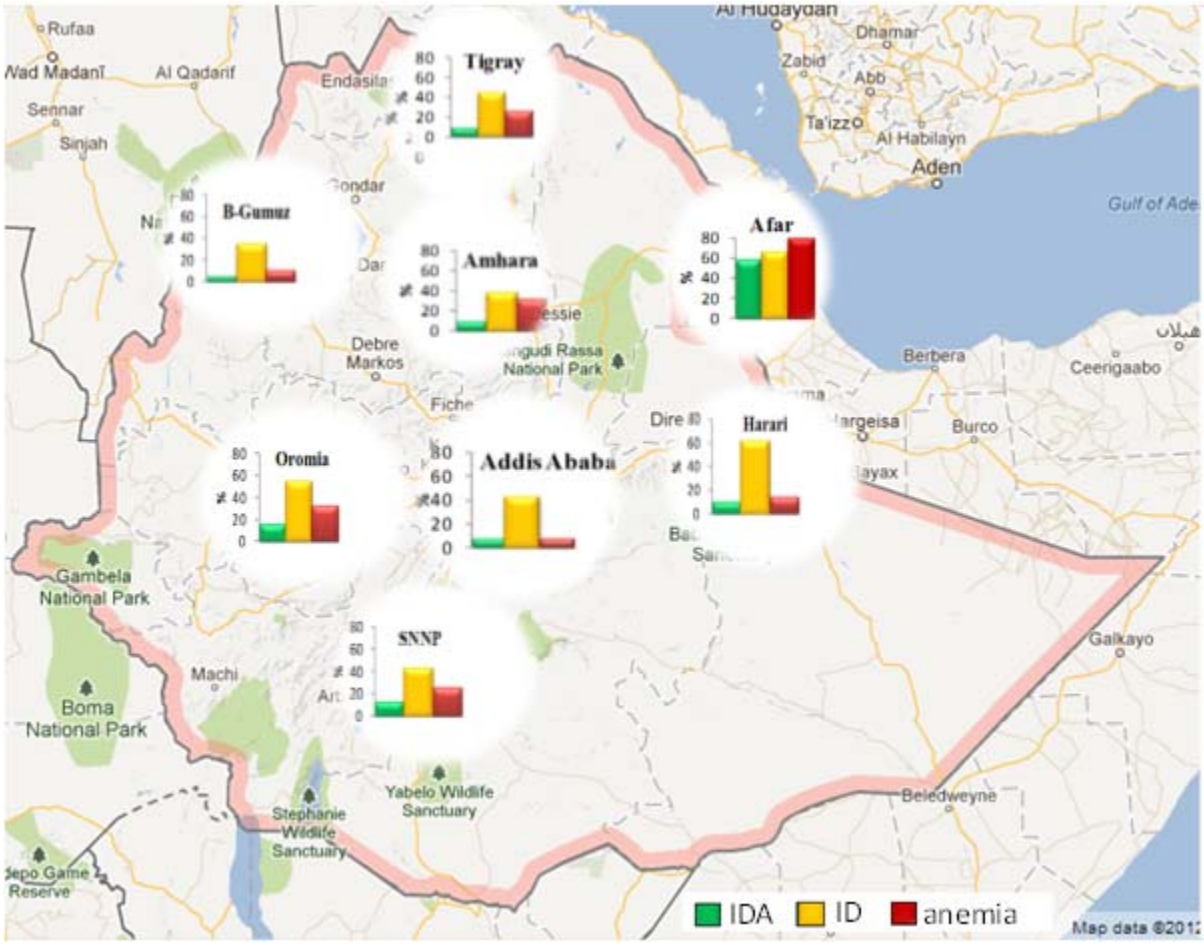


Fig 2.13: Geographical variation in the prevalence of IDA, ID and anemia in Ethiopian women of childbearing age (SNNP: South Nation and Nationalities and Peoples' regional state; B-gumuz: Benshagun gmumuz; ID: Iron deficiency; IDA: Iron deficiency anemia)

Source: data taken from Umeta et al., (2008)

On the other hand, coming up with a quantitative figure for the prevalence of zinc deficiency is difficult due to lack of accurate data. This is further exacerbated by the absence of accurate and feasible methodologies for the determination of zinc status in populations. A prevalence of stunting higher than 20% can be used as a proxy of zinc deficiency (Davidsson et al., 2007, Gibson et al., 2008). Given the high prevalence of stunting (~44%) in Ethiopia (CSA/ICF, 2012) zinc deficiency is likely to be a public health concern.

2.10.2 Complementary feeding practices in Ethiopia

Nutritious and safe complementary foods as recommended (Lutter and Dewey, 2003) are quasi-nonexistent in most parts of rural Ethiopia. Studies in different parts of the country reported that commonly consumed complementary foods are watery cereal-based gruels, bread (i.e. *dabo, kitta*), cow milk, eggs, and mostly adult diet which consists of *injera* (Selinus, 1970, Gebriel, 2006). However the regional variability in the type of complementary foods consumed is high and this is recognized as one of the major impediments for fortifying Ethiopian staples (Getahun et al., 2001). For instance, in contrast to previous findings, a more recent study in Sidama, Southern Ethiopia reported very low consumption of eggs and milk (Gibson et al., 2009).

Since much of the studies on complementary feeding practices in Ethiopia are dated, or are not representative of the country, complementary studies on current complementary feeding practices are needed. Table 2.10 summarizes the complementary foods commonly observed.

Table 2.10: Major cereal-based traditional complementary foods

Complementary foods	Raw Food Items Used*
Gruel	*Teff*, sorghum, barley, maize, wheat, emmerwheat[¶], and enset[¥]
Porridge	*Teff*, sorghum, barley, maize, wheat, emmerwheat and enset
Fetfet[1]	*Teff*, sorghum, barley, maize, wheat, broad beans, chick-peas, field peas, and lentil
Kitta[2]	*Teff*, sorghum, barley, maize, wheat, enset and chick peas
Dabo[3]	*Teff*, sorghum, barley, maize, wheat and emmerwheat

Source: (Alnwick et al., 1987)

*****varies from region to region depending on the availability of the crops; **[1]** *Injera* mixed with sauce made from legumes; **[2]** Unleavened bread; **[3]** Thick leavened bread; **[¶]** emmerwheat: *Triticum dicoccum*;**[¥]** enset: *Ensete ventricosum*

The few existing consumption surveys have shown that dietary iron intake meets, and in some instances exceeds, recommendations while dietary intake of zinc may be inadequate (Abebe et al., 2007a, Gibson, 2009).

According to Umeta et al., (2005) and Abebe et al. Abebe et al., (2007b), most of the foods consumed in Ethiopia contain high amounts of iron and zinc, but also have high contents of mineral absorption inhibitors such as phytates. This suggests that poor bioavailability but not inadequate intake may be behind reported iron and zinc deficiencies. However, further studies are required in this regard.

2.10.3 Mineral bioavailability estimation of Ethiopian staple foods

Most of the foods consumed in rural Ethiopia are believed to be inadequate sources of bioavailable iron and zinc as they have phytate:zinc and phytate:iron molar ratios exceeding 15 and 1, respectively (Umeta et al., 2005). However, lower phytate contents were observed in fermented products like *injera* (Umeta et al., 2005; Abebe et al., 2007). This may be due to the activation of endogenous phytase during fermentation. Based on mineral: phytate molar ratios, better iron and zinc bioavailability was estimated for teff *injera* relative to other foods. This was due to phytic acid degradation during injera fermentation. However, iron bioavailability estimates may be limited by the presence of high amount of contaminant iron, mostly from soil. Furthermore, the effect of other absorption inhibitors such as polyphenols remains unknown.

Therefore in the framework of this thesis, some of the questions related to complementary feeding practices, micronutrient intake adequacies, and the bioavailability of minerals in staples consumed in two agro-ecologically distinct (highland and lowland) villages in North Wollo, northern Ethiopia were evaluated. A special focus was given to *injera*, a staple that is strongly linked to the cultural identity of the Ethiopian people.

Chapter 3- *Materials & Methods*

3. Chapter three: Materials and Methods

The present thesis had three major parts. The first part was a field study where the food consumption of young children (12-23 months) was evaluated using a cross-sectional, two in-home 24h recalls. The second was a detailed follow-up of household preparations of the most frequently consumed foods followed by laboratory characterization of household collected samples. The third part was a mechanistic study evaluating the effect of enzymatic degradation of phytate, polyphenols and dietary fibers on iron bioaccessibility.

3.1 Study protocols

3.1.1 Food consumption survey

3.1.1.1 Location of the study

The study was conducted in Gobalafto district, Amhara region, Northern Wollo, Ethiopia.

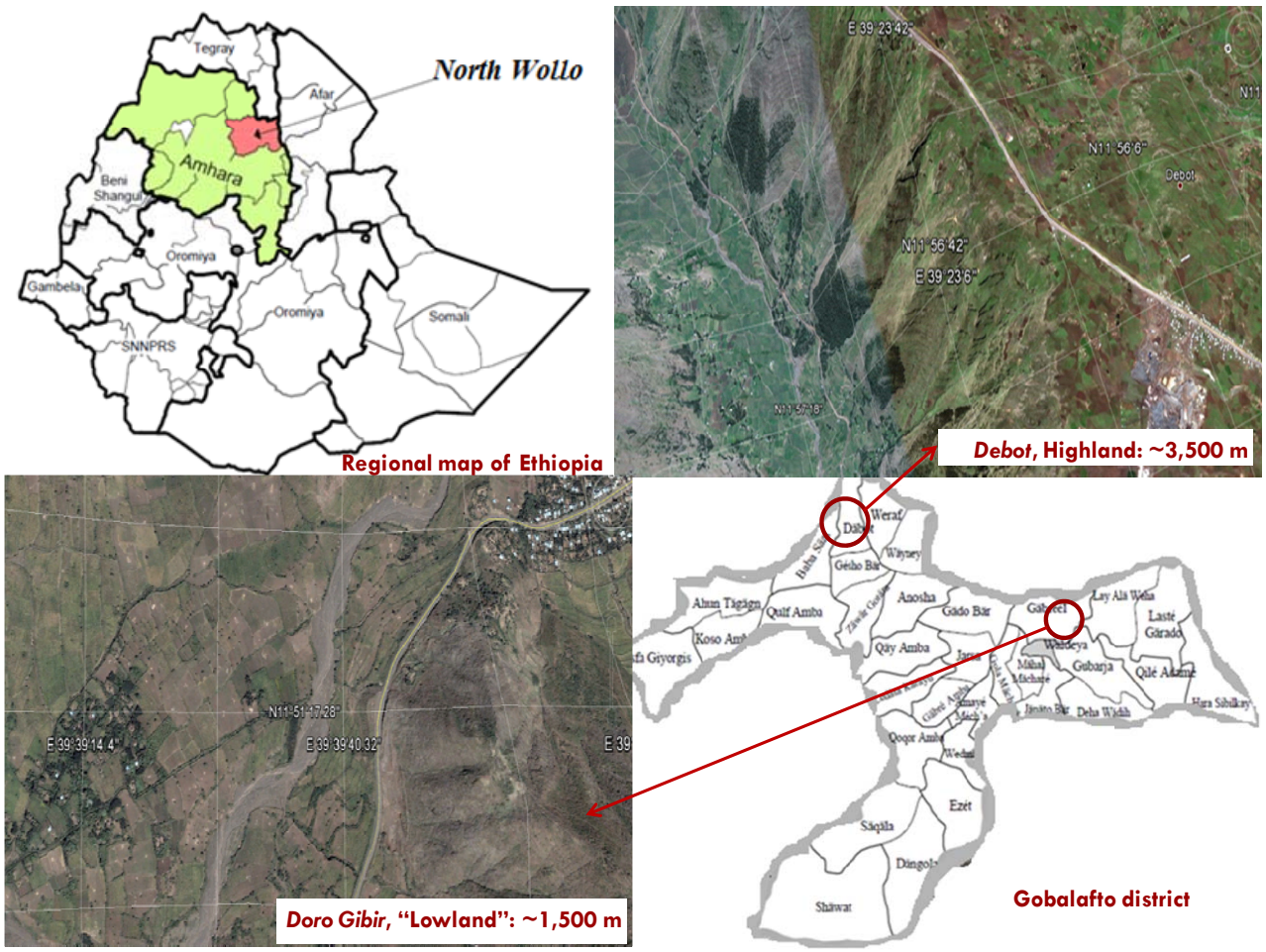


Fig. 3.1: Map of Ethiopia showing the study sites *Doro Gibir* and *Debot* in North Wollo

Two villages, *Debot,* in the highlands (~3,500 m), and *Doro-gibir* in the lowlands (~1,500 m) were surveyed. To be selected, the villages needed to be accessible enough for the collection of perishable samples.

3.1.1.2 Study participants

The study participants (*n=76*) were young children (aged 12-23 month) drawn from the databases of local health centers completed by a census conducted before the survey. For the lowland sample, 56 children were first identified among whom 40 were randomly selected; whereas for the highland, only 38 were identified and all were included in the study. Selection criteria to include a child in the study were for the child to reside permanently in the study area and to be apparently healthy. In the rare cases when several children in the same household fulfilled the inclusion criteria, one child was randomly selected.

3.1.1.3 Socio-demographic status and anthropometric measures

Socio-demographic characteristics of the subjects were assessed using a pre-tested questionnaire that included questions on livelihood activities, education level of parents, health and sanitary facilities, ownership of livestock, and the size of land owned (Annex B).

All anthropometric measurements were made by the same person to avoid inter-examiner errors. The length and weight of the children were measured in triplicate using standardized techniques with the subjects wearing light clothing and no shoes. *Z*-scores for length for-age (LAZ), weight-for-age (WAZ), weight-for-length (WLZ) were calculated using the WHO multicentre growth reference data (WHO, 2006) using the software ENA 2007. None of the children had unacceptably extreme anthropometric values (WHO, 1995). Stunting, underweight, and wasting were defined by Z-scores for LAZ, WAZ and WLZ <-2 standard deviations (SD) below the median values of the reference data, respectively.

3.1.1.4 Dietary intake assessment

An interactive quantitative 24h-recall was conducted with the caregivers of the children (*n=76*) using the multiple pass technique adapted and validated for use in developing countries (Gibson, 1999). In addition, a second day assessment was conducted (*n=70*). All days of the week were equally represented in the final sample. Experienced data collectors were locally recruited and trained in a classroom setting. This was followed by a pilot test on a group comparable to that of the actual study.

A day before intake was assessed (two days before the recall) plates and cups were provided to the caregivers who were instructed to not change the dietary pattern of the child on the

recall day. A demonstration was given on how the amount of each food consumed will be estimated. The interview was conducted in the participant's homes.

Portions were mostly estimated by direct weighing of salted replicas of actual foods prepared locally. The salted replicas consisted of *injera* (fermented flat, pancake), *shiro* (a legume-based spicy stew) and bread. Otherwise, graduated food models and common household measures were used. In most cases, siblings living in the household helped the caregiver in the recall by also following the child on the recall day.

3.1.1.5 Compilation of local food composition database

Whenever possible (i.e. for most cereal and legume-based foods), values for protein, calcium, iron and zinc contents were based on results of biochemical analyses conducted in our laboratory (see section 3.4 for details), otherwise data were compiled from Ethiopian food composition tables (Ågren, 1968, ENI, 1981, EHNRU, 1998) and published data (Umeta et al., 2005, Abebe et al., 2007b). Missing values were compiled from the USDA database (USDA, 2003), after adjustment of moisture content.

3.1.1.6 Assessment of nutrient intake adequacy from complementary foods

The median daily intakes from complementary foods of 12-23 month old children were compared to the estimated energy and selected nutrients needs (FAO/WHO, 2004, FAO/WHO/UNU, 2004), assuming average breast-milk intake and composition as proposed by WHO (1998) and Dewey and Brown (2003). The values of breast-milk intake used were: 346 kcal (1447 kJ) for energy, 5.8 g protein, 154 mg calcium, 0.2 mg iron, 0.7 mg zinc, 275 µg RE vitamin A, and 22 mg vitamin C. Nutrient densities (per 418 kJ or 100 kcal) were compared to desired values calculated by Dewey and Brown (2003). Median dietary diversity scores were calculated based on seven food groups as described in WHO (2008) and classified as low (0-2), medium (3-4) and high (>4) according to Arimond and Ruel (2004).

3.1.1.7 Ethical approval

Ethical approval was obtained from the Human Ethics Committees of Addis Ababa University and the Amhara Regional Health Bureau. Verbal informed consent was obtained from the mother or guardian of each child after the purpose and methods of the study had been explained in detail to them in the presence of local health community workers and *Kebele* (smallest administrative unit) administrators. All parents asked to participate in the study accepted, with the exception of those whose child was sick (*n=1*) or who had to

temporarily leave the village for funeral (*n 1*). All questionnaires were translated into Amharic before the survey.

The Food consumption survey was conducted from August to October 2010. Data on socio-demographic status, anthropometry, feeding practices, and dietary intakes were generated on 62 (30 in highland, 32 in lowland) breastfed (BF) and 14 (7 from each site) non-breastfed (NBF) children.

3.1.2 Household food processing observations and sample collection

The foods that were most frequently consumed by the young children were identified and their traditional preparations by women in both highlands and lowlands were observed. The type of cereals and legumes and the most common blend proportions used in the preparation of *injera* and *shiro* were determined. Accordingly, grains consumed in the lowland (teff, white sorghum, broad beans[4] and grass peas[5]), and those consumed in the highlands (barley, wheat, red sorghum, broad beans and grass peas), were purchased from local markets serving the two communities. Grains were purchased from the same batch in order to control variability due to grain varietal differences.

The processing of cereal grains into BW- and WrS- *injera* flours in the highlands and TwS-*injera* flour in the lowlands was conducted by women in the respective villages (fig. 3.2). Two groups of women (five in the lowlands and six in the highlands) together cleaned the grains, by removing dirt and inedible parts. The grains were then sun dried followed by manual decortication and winnowing, with the exception of *teff* that was not decorticated. After these preliminary steps, the cereals were mixed at a 1:1 ratio (w/w) to make teff-white sorghum (TwS) and barley-wheat (BW) blends and at a 4:1.5 ratio to make wheat-red sorghum (WrS) blends and were then milled in local community milling units that uses mechanical mills.

[4] *Vicia faba*

[5] *Lathyrus sativus*

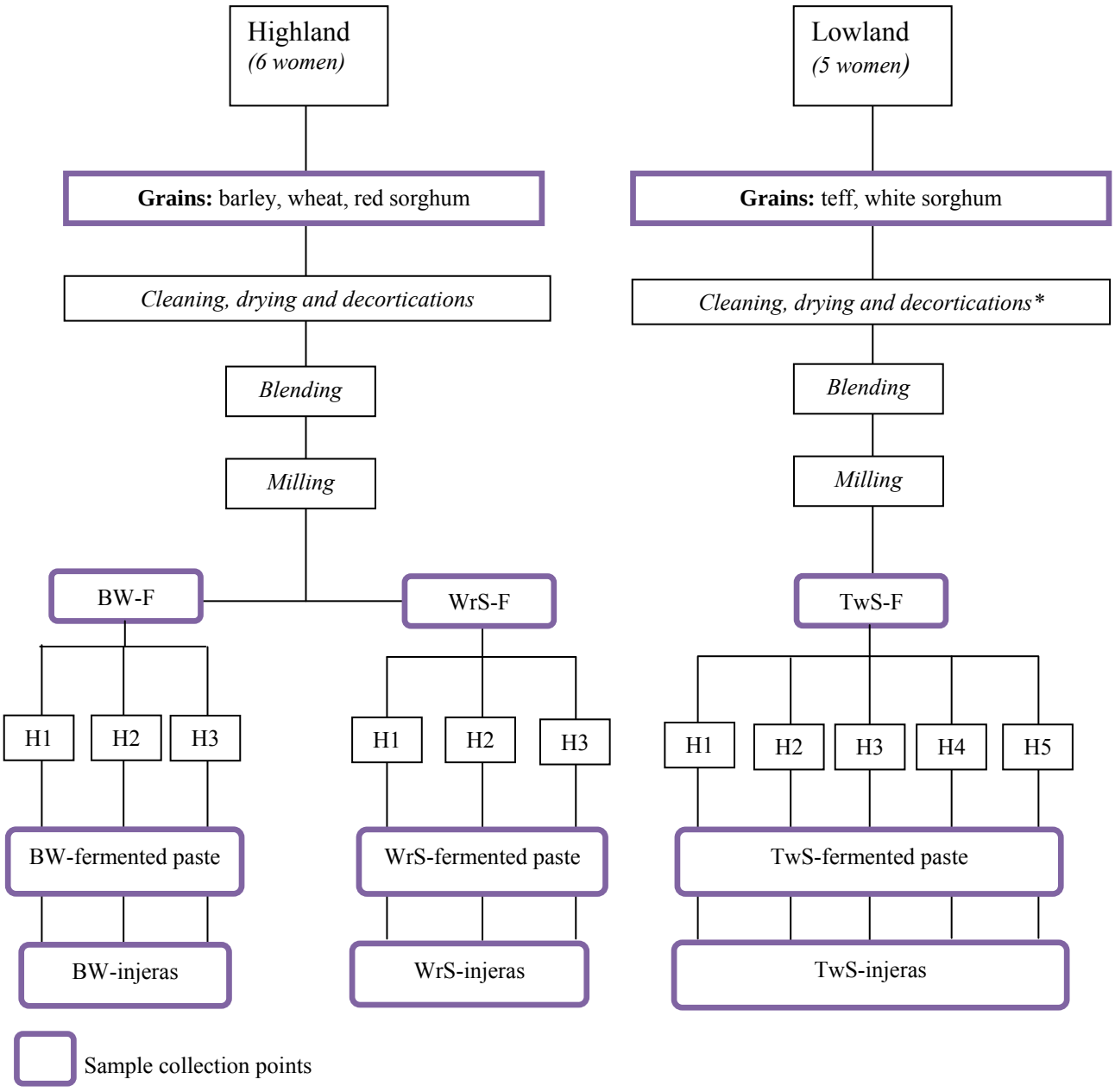


Fig. 3.2: Flow diagram showing the protocol used for the field observation of injera processing and household sampling

F: flour; fermented paste;* teff was not decorticated

The resulting TwS flour was subdivided into five equal parts and was distributed to five households to follow TwS *injera* sourdough fermentation. Likewise, BW and WrS injera sourdough fermentation were each followed in three households. The different households used the same flour but their own traditional starter culture (*ersho*) to trigger the fermentation.

To characterize the fermentation of *injera*, the following measurements were made in five households (n=5) for TwS-*injera* and three households (n=3) for each WrS- and BW-*injeras*: the duration of each step was monitored, the amounts of raw materials used (flour, water, barley malt and *ersho* starter) were weighed and parameters such pH and time were also recorded. Samples were collected at different intervals during the fermentation of the dough used to make *injera*. To avoid disturbing the households, samples were not collected during the night.

Similarly, *shiro (n=5)* and split field pea stew *(n=5)* preparations by local women were observed and samples were collected from the households. The raw materials used were also collected for analysis.

For all prepared foods, separate samples were collected for moisture content determination and biochemical analysis. The collected samples were transferred to a deep freeze (-20°C) at a local health center within two hours of collection. Samples were lyophilized before further analysis.

Household collected raw and processed foods were analyzed for minerals (Fe, Zn and Ca), phytates, fibers, and polyphenols. Phytase activity of raw cereals and injera flour blends were also measured. Fermented pastes were analyzed for α-gallactosides, mono- and di-saccharides, lactate, acetate, ethanol and mannitol.

3.1.3 Relative contribution of mineral absorption inhibitors on iron and zinc bioaccessibility

To better understand the role of phytate, fibers and polyphenols on mineral bioaccessibility, a mechanistic study was designed to successively degrade the different chelating agents by using specific exogenous enzymes. The experimental design is presented in fig. 3.3. Among the three injera flours, TwS from the lowlands and Wrs from the highlands were choosen for this study. The injera flours in the highlands (BW and WrS) had very similar characteristics and the same fermentation pattern, therefore only one was choosen.

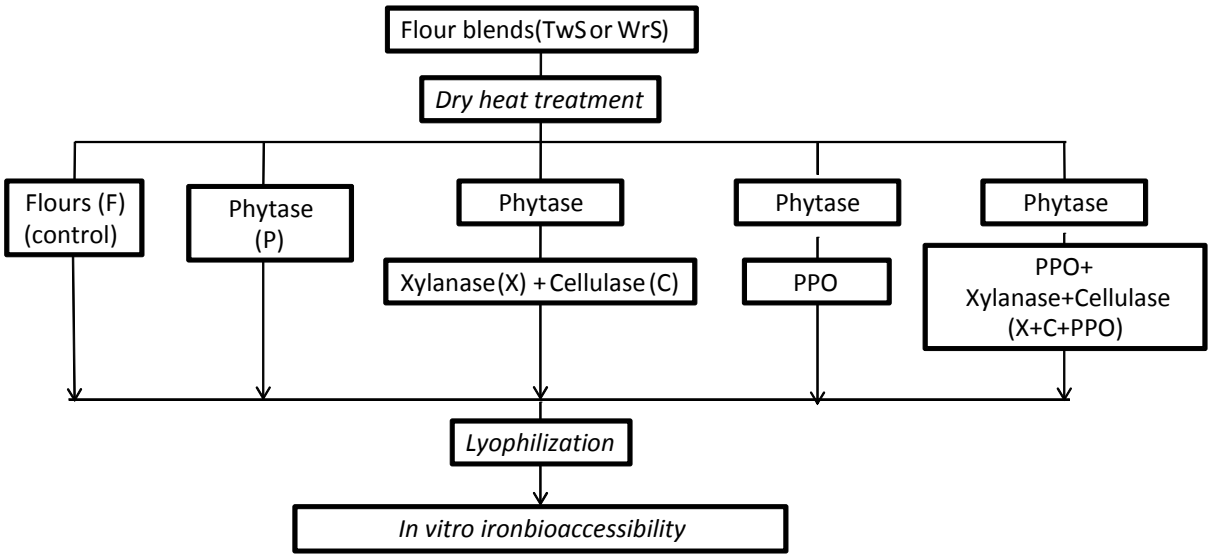


Fig 3.3: Experimental design of enzymatic treatments applied to Teff-white Sorghum (TwS) and Wheat-red Sorghum (WrS) blends used to prepare Ethiopian *injera.*

PPO: polyphenol oxidase

The enzymes used in the present studies were mushroom tyrosinase/polyphenol oxidase (Sigma EC 1.14.18.1, T7755, activity >1000 U/mg solid), xylanase from *Trichoderma viride* (Fluka, EC 3.2.1.8, 95595; 12000 u/l), cellulase from *Trichoderma reesei* (Celluclast 1.5 L, Novoenzyme- E.C. 3.2.1.4, activity 0.7 endo-glucanase unit (EGU)/mg**),** and phytase from *Aspergillus niger* (DSM, EC 3.1.3.8, 20 phytase unit (PU) /mg).

3.1.3.1 Dry heat sterilization of flours

To prevent fermentation and inactivate endogenous enzymes, dry heat treatment was performed by incubating flours at 190°C for 6 min, followed by immediate cooling in a box of ice.

3.1.3.2 Phytase (P) treatment

Cereal flours were suspended in 0.1 M acetate buffer (pH=5.6) in 1:3 (w/v) flour:buffer proportions. For every gram of flour, 0.009g (180 PU) of phytase was added, and the mixture was incubated in a shaking water bath at 35°C for 30 min. The duration of the incubation, the flour:buffer ratio and the amount of phytase to be added were those that allowed maximum degradation of phytate in the shortest incubation time possible as determined in preliminary assays.

3.1.3.3 Xylanase+cellulase (X+C) co-treatment

After flour dephytinization as described in section 3.6.2.2, 0.014g of xylanase (~50 U) and 8 µl of cellulase (6.8 EGU) were added per g of flour, and the mixture was incubated in a shaking water bath for 3 h at 35°C. For cellulose, the amount of enzyme to be added was based on the optimization of Wang et al. (2008).

3.1.3.4 Polyphenol oxidase (PPO) treatment

After dephytinization, NaOH was added till pH reached the optimum for PPO activity (pH=6.5) and 0.1 M MES (2-(*N*-morpholino) ethanesulfonic acid, Sigma- M8250) buffer was added to reach final flour: buffer ratio of 1:10 (w/v). The proportion of enzyme added (1 mg/g flour)was based on the works of Matuschek et al. (2001). The incubation was carried in a shaking water bath at 35°C for 16 h in the dark.

3.1.3.5 Phytase, xylanase, cellulase and polyphenol oxidase (P+X+C+PPO) treatment

The flours were sequentially treated in the order of P, X+C, and then PPO according to the above descriptions.

3.2 Biochemical analysis

Raw (grains), intermediary (*injera* and shiro flours), and final products (*injera* and stews) as well as *injera* flours subjected to different enzymatic treatments were subject to biochemical analyses. Protein, fat, mineral contents (Fe, Zn and Ca), mineral absorption inhibitors such as: polyphenols (total polyphenols, condensed tannins, iron-binding phenolics), fiber (ADF, NDF), and phytic acid (IP6), and α-galactoside (raffinose and stacchyose) contents were determined.

Fermentation kinetics measures, such as: pH change, change in mono- and disaccharides and production of lactic acid, acetic acid, mannitol and ethanol were also determined. Prior to biochemical analysis, grains and lyophilized samples were milled in a laboratory mill (IKA M20, Labortechnik, Staufen, Germany) to obtain a flour passing through a 500 µm sieve. Iron analysis was performed in both washed and non-washed grains. Washing of grains was carried under laboratory conditions using running de-mineralized water. Iron bioaccessibility measurements were conducted on *as eaten* (non-lyophilized) samples after thawing.

3.2.1 Dry matter

Dry matter (DM) contents were determined by oven drying at 105°C to constant weight.

3.2.2 Changes in pH

During fermentation, the pH of the slurry was recorded using a WTW 340i pH meter (Fisher Bioblock Scientific, Illkirch, France). The rate of change in pH (-dpH/dt) was calculated for each household observation as follows: -(dpH/dt) = pH(t+1) - pH(t) / (t+1)-t , where "t" stands for time (hours). The maximal value of -(dpH/dt) for each household observation was then averaged to give the maximal rate of change in pH (-(dpH/dt)max).

3.2.3 Protein

Protein content (N×6.25) was determined by the method of Kjeldahl (AFNOR, 1970) based on determination of nitrogen content.

3.2.4 Lipid content

Lipid content was determined using the semi-automatic 2055 Soxtec system (Foss, Nanterre, France), according to the AOAC (2006) approved procedures 2003.05 and 2003.06 (Thiex et al., 2003).

3.2.5 Analysis of mono- and disaccharides and α-galactosides on fermented *injera* paste

Mono- and disaccharides (glucose, fructose, maltose and sucrose) and α-galactosides (raffinose and stachyose) were extracted by diluting one gram of fermented paste in 2ml of milliQ water, the mixture was vortexed, then centrifuged at 4500 *g* for 10 min at 4°C. The supernatants were filtered through 0.20 μm pore size filters and were analysed by HPAEC (high performance anion-exchange chromatography) with a Dionex DX 500 apparatus connected to an amperometric detector Dionex Model ED 40 (Thermo Scientific, Courtaboeuf, France) using a Carbo PA1 column (Dionex S.A., Jouy en Josas, France) after appropriate dilution.

The following conditions were used: mobile phase (eluent) NaOH 90 mM, flow rate 1 mL/min, temperature 35°C, injection sample extract 25 µL (Haydersah et al., 2012). Results are expressed in mmol/kg of dough.

3.2.6 Changes in water extractable mono and disaccharides after enzymatic treatments

Glucose, cellobiose, arabinose, galactose, and xylose were extracted by diluting 80mg of lyophilized flour in 10 ml milliQ water, the mixture was vortexed, then centrifuged at 4500 g

for 20 min. The supernatants were filtered through 0.20 μm pore size filters and were analysed by HPAEC after appropriate dilution (80 ml of supernatant/ 50 ml milliQ H2O)
The following conditions were used: mobile phase (eluent) NaOH 150 mM, flow rate 1 mL/min, temperature 35°C, injection sample extract 25 µL. Results are expressed in g/100g DM of flour.

3.2.7 Analyses of lactic acid, acetic acid, mannitol and ethanol on fermented *injera* paste

Lactic acid, acetic acid, and ethanol were analyzed by HPLC using an Aminex HPX-87H, 300 × 7.8 mm column (Biorad, Yvry-sur-seine, France) connected to a refractive index detector (Model Waters 2410; Biorad, France) as previously described in Calderon et al. (2001).

3.2.8 Iron, zinc and calcium analysis

Iron, zinc and calcium were analyzed by flame atomic absorption spectrophotometry (AA800, Perkin Elmer, Les Ulis, France) after wet mineralization using an Ethos 1 microwave digester (Milestone, Sorisole, Italy) for 15 min at 200°C and with a maximum power of 1000 W.
Accuracy and precision of the analyses were checked by analysis of certified reference materials [BCR 191: brown bread and BCR 679: white cabbage].

3.2.9 Mineral absorption inhibitors

3.2.9.1 Phytate (IP6) analysis

After extraction from 0.2 g of sample in acid solution (10 ml of HCl 0.5 M) at 100°C for 6 min, the mixture was centrifuged (5000g, 4°C, 20 min). The supernatant was dried using a speedvac (Jouan- RC10-10, Saint Herblain, France). IP6 content was then determined by measuring myo-inositol hexaphosphate (IP6) content by HPAEC according to Lestienne et al. (2005b), using an AS-11 pre-column and column kit (Dionex, Sunnyvale, USA).

3.2.9.2 Fiber analysis

Neutral detergent fiber (NDF)

The neutral detergent fibre (NDF) content which corresponds approximately to cellulose, hemicellulose and lignin content was determined according to the gravimetric method of (Van Soest, 1963) using a Fibertec 1020 (Foss, Hillerod, Denmark).

Acid detergent fiber (ADF)

The acid detergent fibre (ADF) content, which corresponds approximately to cellulose and lignin content, was determined according to the gravimetric method of Van Soest (1963) using a Fibertec 1020 (Foss, Hillerod, Denmark).

3.2.9.3 Polyphenols

Total polyphenols

Total phenolics were determined following the method of Singleton and Rossi (1965). Briefly, phenolic compounds were extracted twice from 50 mg flour in 1.5 mL acetone/water 70/30 (v/v) acidified with 1% formic acid. Color formed during the reaction of extracted phenolic compounds with Folin-Ciocalteu's reagent (F9252; Sigma-Aldrich, Saint-Quentin-Fallavier, France) was measured spectrophotometrically (760 nm). Gallic acid (G7384; Sigma-Aldrich) was used as standard and the results are expressed in mg of gallic acid equivalent (GAE) per 100 g DM.

Condensed tannins

Condensed tannins (proanthocyanidins) were measured following the method of (Porter et al., 1986). Briefly, 6ml of Butanol/HCl (95/5 v/v) was added to 50 mg lyophilized sample. After homogenizing, 200µl of ferric solution was added and the mixture was heated at 95°C for 40 min. The color developed was measured at 550 nm against catechin standard curve using a spectrophotometer. The results were expressed in cyanidin equivalent per 100g DM.

Iron-binding polyphenols

Iron-binding polyphenols (galloyl and catechol groups) were analyzed using the method of (Brune et al., 1991). The amount of galloyl and catechol groups was determined using ferric ammonium sulfate (FAS) reagent after 16 h extraction of samples with 50% dimethylformamide in acetate buffer (pH 4.4). This method is based on FAS's ability to form colored complex with iron-binding galloyl and catechol groups. The absorbance of the colored complex was measured at 680 nm and 578 nm, corresponding to the absorption maxima of Fe-galloyl and Fe-catechol complexes, respectively. After subtracting food blank absorbance, the content of galloyl groups (expressed as tannic acid equivalents) and catechol groups (expressed as catechin equivalents) were calculated from standard curves for catechin and tannic acid at both wavelength.

3.2.9.4 Phytase activity

Inorganic phosphorus and phytates were removed from flours by ion exchange chromatography (Resin AG1-X8-Bio Rad) as described in (Konietzny et al., 1994). The resulting phytate free supernatant was then incubated in 2.5 mM sodium phytate solution at pH 5.6 and 55°C for 60 min, and liberated inorganic phosphate was determined using the spectrophotometric method described in (Heinonen and Lahti, 1981). Phytase activity was calculated as micromoles of inorganic phosphate liberated from sodium phytate per minute per gram (DM) of flour.

3.2.9.5 Prediction of iron absorption using algorithm

Prediction of iron absorption was calculated using (Hallberg and Hulthen, 2000) algorithm by making use of analyzed values of phytic acid-phosphorus (Phytate-P), calcium (Ca) and tannic acid (TA). Ascorbic acid (AA) values were taken from the Ethiopian food composition table. The following formula, as proposed by Beiseigel et al. (2007) was used:

$$\% \text{ absorption} = 22.1 \times f1 \times f2 \times f3 \times f4 \qquad (4)$$

Where,

The factor 22.1 corresponds to the mean absorption from a meal containing no components known to enhance or inhibit iron absorption, adjusted to 40% reference dose absorption

$$f1 = 10\,[-0.30 * \log (1+\text{phytate-P})] \qquad (5)$$

$$f2 = [1+0.01\ AA + \log \text{phytate-P} + 1] * 0.01 * 10\,(0.8875 * \log (AA+1)) \qquad (6)$$

$$f3 = 10\,[0.4515 - 0.715 * \log \text{tannic acid}] \qquad (7)$$

$$f4 = 0.4081 + [0.6059/(1+10^{-(2.022-\log(Ca+1))*2.919})] \qquad (8)$$

The algorithm uses base-10 logarithms; phytate-P, AA, TA and Ca were expressed in mg/ 40g meal, based on meal portion estimates from the results of the consumption survey.

Phytate contents were converted into phytate-P by multiplying by the factor 0.283. Factors *f*1, *f*2, *f*3, and *f*4 of this algorithm respectively adjust for the effects of phytic acid, AA, TA and Ca on iron absorption, and include interactions between these components. This algorithm was based on results from human absorption studies using stable isotope technique.

3.2.9.6 *In-vitro* iron and zinc bioaccessibility

Determination of titratable acidity and PIPES buffer molarity

The titratable acidity of the flour or the food (as eaten) to be tested for *in-vitro* bioaccessibility was determined as follows. The food/flour was first diluted to make a paste

of ~10% DM. For about 20g of diluted paste, α-amylase was added, and the mixture was incubated in water bath at 37°C for 5 min. The pH was adjusted to 2.0 by adding 6M HCl, then pepsin was added, and the mixture was incubated for 1h at 37°C. The volume of 1M KOH needed to adjust the pH to 6.5 was recorded and was used for the calculation of the molarity of PIPES (piperazine-N, N0-bis-[2-ethanesulfonic acid] sodium salt) buffer needed, as follows:

$$C_{PIPES} = (V_{KOH} + (f \times V_{KOH}) / (f \times V_{PIPES}) \quad (9)$$

Where,

C_{PIPES}= concentration of PIPES buffer (mol/l)

V_{KOH}= volume of 1M KOH needed to adjust pH to 6.5

$f = 10^{-pH\ desired}/10^{-pKa\ PIPES}$

V_{PIPES}=volume of PIPES to be introduced into the dialysis membrane (20 mL)

In-vitro digestion and dialysis

To determine iron bioaccessibility, enzymatic in vitro digestions were carried out in two stages, according to the procedure described in Greffeuille et al. (2011). Briefly, a 10% DM dispersion of ground sample in ultrapure water was brought to 37°C, 20 µL of bacterial α-amylase from *Bacillus licheniformis* (E.C. 3.2.1.1, Sigma A-3403-1MU) was added and the mixture was incubated at 37°C for 5 min. The pH was then brought to 2.0 with 1 M HCl, 1 mL of pepsin solution was added (Sigma, P-7000, 14,900 u/mL in 0.1 M HCl) and the mixture was incubated for 1 h at 37°C in a shaking water bath. Pepsin-digested samples were then transferred into separate tubes to which a dialysis bag (Spectra/por I dialysis tubing, MWCO 12–14 kDa) containing 20 mL of PIPES buffer (Sigma, P-3768) was introduced to mimic the gradual increase in pH during intestinal digestion. Tubes containing the sample and the dialysis bag were incubated at 37°C for 30 min to reach pH 7; a mix of pancreatin (from porcine pancreas, Sigma, P1750, 1.85 mg/mL) and bile extract solution (Sigma, B8631, 11 mg/mL in 0.1 M $NaHCO_3$) was added, and the resulting mixture was incubated for 2 h at 37°C in a shaking water bath. At the end of intestinal digestion, dialysis bags were removed and washed with pure water. The digested mixtures remaining in the tubes were centrifuged at 10,000g for 15 min at 4°C to separate the insoluble and soluble fractions, in the pellet and supernatant, respectively. The contents of dialysis bags (dialysates), the supernatants and the pellets were weighed and analyzed for iron content by atomic absorption spectrophotometry as described above.

Bioaccessible Fe and Zn corresponds to the percentage of dialysable Fe and Zn. Iron and zinc contents in the pellets and supernatants corresponded to the insoluble and soluble iron/zinc, respectively. Dialyzable, soluble and insoluble iron/zinc fractions were calculated on the basis of the total iron/zinc recovered at the end of the digestion, as follows:

$$\text{Dialyzable Fe/Zn \%} = CD\,(WD + WS)/\,(CDWD + CSWS + CIWI) \times 100 \quad (10)$$

$$\text{Soluble ND Fe/Zn \%} = WS\,(CS - CD)/\,(CDWD + CSWS + CIWI) \times 100 \quad (11)$$

$$\text{Insoluble Fe/Zn \%} = CIWI/\,(CDWD + CSWS + CIWI) \times 100 \quad (12)$$

Where: ND stands for non-dialyzable; CD, CS and CI are iron concentrations (µg/100g) in the dialysate, supernatant and pellet fractions and WD, WS and WI are the weights (g) of dialysates, supernatants and pellets. All samples were analyzed at least in quadruplicate.

3.3 Statistical analyses

3.3.1 Analyses of food consumption survey data

All continuous variables were checked for normality using the Kolmogorov–Smirnov test. Dietary intakes (per day) and nutrient densities (per 418 kJ or 100 kcal) were expressed as medians (inter-quartile range) because of non-normal distributions of some nutrients. Differences in the median energy and nutrient intakes between highland and lowland were examined using the non-parametric Mann-Whitney U (2-tailed) test. For categorical variables, Fisher exact's test (1-tailed) was used.

3.3.2 Analyses of biochemical analyses data

Average values of biochemical analyses performed at least in triplicate were analyzed using analysis of variance, followed by the Fisher's least significant difference (LSD) test to compare the means at the 5% significance level. Statistical analyses were performed using the SPSS statistical software package version 15 (SPSS Inc, Chicago, Illinois).

Chapter 4- *Results*

4. Chapter four: Results

4.1 Nutrient intake adequacy from complementary foods consumed by young children in North Wollo

4.1.1 Introduction

Ethiopia has one of the highest stunting prevalence in sub-Saharan Africa. Iron and zinc deficiencies are also likely to be highly prevalent. Given that growth faltering is usually irreversible after the age of two, interventions that work towards adequate complementary feeding practices may be needed. However, quantitative data on the adequacy of current feeding practices are lacking. The major foods consumed by young children need to be identified to better optimize food processing practices for improved iron and zinc bioavailability.

In this section (4.1), results from a food consumption survey conducted in North Wollo, northern Ethiopia are presented in the form of a published paper accompanied by supplementary results. The study was conducted in two villages, one in the highlands and the other in the lowlands to take into account altitude-related differences. The foods commonly consumed by young children in North Wollo were identified. Energy and selected nutrient intakes from complementary foods were calculated and their adequacy to WHO's estimated needs was evaluated. Based on the findings of the consumption survey, the possible influence of agro-ecology (altitude) on nutrient intakes has been highlighted and recommendations that can improve complementary feeding practices were made.

4.1.2 Nutrient intakes from complementary foods consumed by young children (aged 12-23 months) from North Wollo, northern Ethiopia: the need for agro-ecologically adapted intervention- *Public Health Nutrition, FirstView*, 1-10; doi:10.1017/S1368980012005277

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Nutrient intakes from complementary foods consumed by young children (aged 12–23 months) from North Wollo, northern Ethiopia: the need for agro-ecologically adapted interventions

Kaleab Baye[1,2,*], Jean-Pierre Guyot[2], Christèle Icard-Vernière[2] and Claire Mouquet-Rivier[2]

[1]Center for Food Science and Nutrition, Addis Ababa University, PO Box 150201, Addis Ababa, Ethiopia: [2]IRD UMR 204 «Prévention des Malnutritions et des Pathologies Associées» (Nutripass), IRD/UM1/UM2, Montpellier, France

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Abstract

Objective: To characterize current feeding practices and to evaluate the adequacy of energy and nutrient intakes of young children in subsistence farming rural households in North Wollo, Ethiopia.

Design: A cross-sectional study examining sociodemographic status, anthropometry, breast-feeding and complementary feeding practices using two in-home non-consecutive 24 h recalls.

Settings: Two rural villages in the highlands and lowlands of Gobalafto district, North Wollo.

Subjects: Seventy-six young children aged 12–23 months, thirty-nine from the lowlands and thirty-seven from the highlands.

Results: About 33 % of the children, ~46 % in the highlands and 24 % in the lowlands ($P = 0{\cdot}05$), were stunted. Complementary diets were low in animal products, fruits and vegetables. Cereals and legumes were the major sources of energy, protein, Ca, Fe, Zn and vitamin A. Legumes with potentially toxic components (grass pea, broad beans) and low nutrient-dense beverages such as tea were frequently consumed. Intakes of energy, Ca, Zn, vitamin A and vitamin C from complementary foods were below WHO recommendations assuming average breast-milk intakes. In contrast, Fe and protein intakes and densities met WHO recommendations. Although vitamin C intakes and densities were higher ($P < 0{\cdot}05$) for the lowlands, they remained far below WHO recommendations.

Conclusions: Interventions promoting the WHO guiding principles for complementary feeding practices and behaviours that take the agro-ecological contexts into account are needed here. Furthermore, specific recommendations should be formulated to discourage the consumption of grass pea, broad beans and low nutrient-dense beverages such as tea.

Keywords
Feeding practices
Micronutrients
Complementary foods
Altitude

The period of complementary feeding (6–23 months) is of particular importance as this is when infants and young children experience rapid growth and development. During this period, growth faltering and micronutrient deficiencies are highly prevalent because of children's high nutrient needs relative to their energy and micronutrient intakes[1]. Among micronutrient deficiencies, vitamin A, Fe and Zn deficiencies are the most prevalent[2]. In children, these deficiencies are associated with poor growth, impaired cognitive development and poor health status[3]. The overwhelming impact of growth faltering is usually irreversible after the age of 2 years, thereby leaving a small window of opportunity for intervention[4]. In this regard, the role of adequate complementary feeding, both in quantity and quality, is of great importance.

Despite recent improvements, child malnutrition remains a public health concern in Ethiopia. With ~44 % of children under the age of 5 years being stunted, 10 % being wasted and 29 % being underweight relative to the WHO 2006 multicentre growth reference[5,6], Ethiopia has one of the highest malnutrition rates in sub-Saharan Africa. In this region, like in many developing countries, complementary foods are largely made of unrefined cereals and legumes which may be inadequate in energy and growth-limiting micronutrients such as Fe and Zn because of poor bioavailability[7].

*Corresponding author: Email kaleabbaye@gmail.com

Studies on nutrient intakes of pregnant and lactating women in Ethiopia showed that Zn intakes but not Fe intakes were inadequate and that despite low consumption of animal-source foods (ASF), prevalence of Fe-deficiency anaemia was low for women in late pregnancy[8,9]. By 12 months of age, most young children already consume the same diet as adults. However, little is known about the adequacy of energy and nutrient intakes in children who consume an adult diet. Recently, the complementary feeding practices and energy and nutrient adequacy of complementary foods were evaluated in the southern part of Ethiopia where maize-based meals predominate[10]. However, such studies are also needed in the northern part of the country where food staples are quite different. Furthermore, in northern Ethiopia, there are considerable variations in altitude that influence the type of major food staples that can be grown and hence consumed in subsistence farming households.

There is therefore an urgent need for current quantitative data on feeding practices as well as on energy and nutrient adequacy of the diets of young children (aged 12–23 months) for the design of interventions aimed at improving the quality of complementary feeding. In the present cross-sectional study, data were generated on anthropometric indices, feeding practices and dietary intakes of young children from two agro-ecologically distinct villages (highland at altitude of ~3500 m/lowland at ~1500 m above sea level) in Gobalafto district, using a two non-consecutive 24 h recalls, to:

1. characterize the feeding practices of young children (aged 12–23 months) in Gobalafto district, North Wollo; and
2. calculate energy and nutrient intakes from complementary foods and evaluate their adequacy with respect to WHO recommendations.

Methods

Participants

The study was conducted in Gobalafto district, Amhara region, North Wollo, Ethiopia. The prevalence of stunted children (aged 6–59 months) in the Amhara region (52 %) is above the national average (44 %)[6]. The area is characterized by a rugged terrain with inhabited mountains reaching 3600 m above sea level and lowlands up to 1000 m above sea level. Two villages, Debot in the highlands (~3500 m) and Doro-gibir in the lowlands (~1500 m), were surveyed. To be selected, the villages needed to be accessible enough for the collection of perishable samples.

The subjects of the present study were 12–23-month-old children (*n* 76) drawn from the databases of local health centres completed by a census conducted before the survey. For the lowland sample, fifty-six children were first identified among whom forty were randomly selected; whereas for the highland, only thirty-eight were identified and all were included in the study. Selection criteria to include a child in the study were for the child to reside permanently in the study area and to be apparently healthy. In the rare cases when several children in the same household fulfilled the inclusion criteria, one child was randomly selected.

Ethical approval was obtained from the Human Ethics Committees of Addis Ababa University and the Amhara Regional Health Bureau. Verbal informed consent was obtained from the mother or guardian of each child after the purpose and methods of the study had been explained in detail to them in the presence of local health community workers and *kebele* (smallest administrative unit) administrators. All parents asked to participate in the study accepted, with the exception of those whose child was sick (*n* 1) or who had to leave the village temporarily for a funeral (*n* 1). All questionnaires were translated into Amharic before the survey.

The study was conducted from August to October 2010. Data on sociodemographic status, anthropometry, feeding practices and dietary intakes were generated on sixty-two (thirty in highlands, thirty-two in lowlands) breast-fed (BF) and fourteen (seven from each site) non-breast-fed (NBF) children.

Sociodemographic status and anthropometric measures

Sociodemographic characteristics of the participants were assessed using a pre-tested questionnaire that included questions on livelihood activities, education level of parents, health and sanitary facilities, ownership of livestock and the size of land owned.

All anthropometric measurements were made by the same person to avoid inter-examiner errors. The length and weight of the children were measured in triplicate using standardized techniques with the children wearing light clothing and no shoes. *Z*-scores for length-for-age (LAZ), weight-for-age (WAZ) and weight-for-length (WLZ) were calculated using the WHO multicentre growth reference data[5] using the software ENA 2007. None of the children had unacceptably extreme anthropometric values[11]. Stunting, underweight and wasting were defined respectively as LAZ, WAZ or WLZ <-2.

Dietary intake assessment

An interactive quantitative 24 h recall was conducted with the caregivers of the children (*n* 76) using the multiple-pass technique adapted and validated for use in developing countries[12]. In addition, a second day assessment was conducted (*n* 70). All days of the week were equally represented in the final sample. Experienced data collectors were locally recruited and trained in a classroom setting. This was followed by a pilot test on a group comparable to that of the actual study.

A day before intake was assessed (two days before the recall) plates and cups were provided to the caregivers who were instructed to not change the dietary pattern of the child on the recall day. A demonstration was given on how the amount of each food consumed would be estimated. The interview was conducted in the participants' homes.

Portions were mostly estimated by direct weighing of salted replicas of actual foods prepared locally. The salted replicas consisted of *injera* (fermented flat, pancake), *shiro* (a legume-based spicy stew) and bread. Otherwise, graduated food models and common household measures were used. In most cases, siblings living in the household helped the caregiver in the recall by also following the child on the recall day.

Collection of samples of complementary foods

The foods that were most frequently consumed by the young children were identified and their traditional preparations by women (n 10) in both highland and lowland areas were observed. Samples of *injera* made from teff–white sorghum (n 5), wheat–red sorghum (n 3) and barley–wheat (n 3) blends, *shiro* from grass pea and broad beans (n 5) and split field pea stews (n 5) were collected from households. The raw materials used were also collected for analysis. Separate samples were collected for moisture content determination and biochemical analysis. The collected samples were transferred to a deep freeze (−20°C) at a local health centre within 2 h of collection. Samples were freeze dried before further analysis.

Food composition analyses

DM content

DM contents were determined by oven drying at 105°C to constant weight.

Protein content

Protein content (N×6·25) was determined by the method of Kjeldahl[13] based on determination of N content.

Fe, Zn and Ca analyses

Fe, Zn and Ca were analysed by flame atomic absorption spectrophotometry (AA800; Perkin Elmer, Les Ulis, France) after wet mineralization using an Ethos 1 microwave digester (Milestone, Sorisole, Italy) for 15 min at 200°C and with a maximum power of 1000 W.

Accuracy and precision of the analyses were checked by analysis of certified reference materials (BCR 191: brown bread and BCR 679: white cabbage).

Compilation of local food composition database

Whenever possible (i.e. for most cereal- and legume-based foods), values for protein, Ca, Fe and Zn contents were based on results of biochemical analyses conducted in our laboratory; otherwise data were compiled from Ethiopian food composition tables[14–16] and published data[17,18]. Missing values were compiled from the US Department of Agriculture database[19], after adjustment of moisture content.

Assessment of nutrient intake adequacy from complementary foods

The median daily intakes from complementary foods of 12–23-month-old children were compared with estimated needs for energy and selected nutrients based on WHO/FAO[20–22] recommended intakes assuming average breast-milk intake and composition as proposed by WHO[23] and Dewey and Brown[24]. The mean daily nutrient intakes from breast milk, assuming an average intake of 549 g/d (~533 ml/d), were 1447 kJ energy, 5·8 g protein, 154 mg Ca, 0·2 mg Fe, 0·7 mg Zn, 275 μg RE (retinol equivalent) vitamin A and 22 mg vitamin C. Nutrient densities (per 418 kJ/100 kcal) were compared with desired values calculated by Dewey and Brown[24]. Median dietary diversity scores were calculated based on seven food groups as described in WHO[25] and classified as low (0–2), medium (3–4) and high (>4) according to Arimond and Ruel[26].

Statistical analyses

All continuous variables were checked for normality using the Kolmogorov–Smirnov test. Dietary intakes (per day) and nutrient densities (per 418 kJ/100 kcal) were expressed as medians and interquartile range because of non-normal distributions of some nutrients. Differences in the median energy and nutrient intakes between highlands and lowlands were examined using the non-parametric Mann–Whitney U test (two-tailed). For categorical variables, Fisher exact's test (one-tailed) was used. In all comparisons, differences were considered statistically significant when $P < 0·05$. Statistical analyses were performed using the SPSS statistical software package version 15.

Results

Sociodemographic characteristics and anthropometric status

The mothers surveyed had on average two or three children (Table 1). Most mothers were housewives, except for some who had some small trading activities. More mothers in the lowland village had a formal education than in the highland village ($P = 0·03$). About 79 % of the households owned private latrines. Fewer households in the highlands owned ≥1 ha of land than in the lowlands ($P = 0·001$). Thirty-four per cent of the households owned cows, and 55 % owned chickens. More households in the highlands owned chickens than in the lowlands ($P = 0·009$). More children were stunted in the highlands than in the lowlands ($P = 0·05$).

Feeding practices

Over 90 % of the children were fed complementary foods at least three times daily (Fig. 1); however, less than half

Table 1 Sociodemographic characteristics and nutritional status: young children (*n* 76) aged 12–23 months from a highland village and a lowland village in Gobalafto district, North Wollo, northern Ethiopia, August–October 2010

	All (*n* 76)		Highlands (*n* 32)		Lowlands (*n* 39)	
	n or Mean	% or SD	*n* or Mean	% or SD	*n* or Mean	% or SD
Sociodemographic characteristics						
Mean age of child (months)	18·3	4·4	19·2	3·7	17·4	4·8
Proportion of male children	43	57	22	60	21	54
Mean age of mother (years)	28·8	10·3	27·2	6·5	30·2	12·9
Mothers with some formal education*	26	34	10	27	16	41
Mean number of children†	2·3	1·5	2·7	1·6	2·0	1·3
Livelihood activities (housewives)	60	79	25	68	35	90
Owns a sanitary facility (latrine)*	60	79	25	68	35	90
Owns ≥1 ha of land*	27	36	5	14	22	56
Owns cows	26	34	14	38	12	36
Owns chickens*	42	55	28	76	14	36
Anthropometric indices						
Mean LAZ	–1·32	1·39	–1·59	1·45	–1·02	1·31
Mean WAZ	–1·26	1·11	–1·32	1·06	–1·19	1·15
Mean WLZ	–0·84	0·92	–0·73	0·47	–0·94	1·05
Stunted*	25	33	16	46	9	24
Underweight	21	28	11	31	10	27
Wasted	9	12	2	5	7	19

LAZ, length-for-age *Z*-score; WAZ, weight-for-age *Z*-score; WLZ, weight-for-length *Z*-score.
*Difference between highland and lowland sites was statistically significant according to Fisher's exact test (one-tailed): $P = 0{\cdot}03$ for mother's education; $P = 0{\cdot}02$ for owns a sanitary facility (latrine); $P = 0{\cdot}001$ for owns ≥1 ha of land; $P = 0{\cdot}009$ for own chickens; $P = 0{\cdot}05$ for stunted.
†Difference between highlands and lowlands was statistically significant according to Student's *t* test (two-tailed), equality of variances not assumed: $P = 0{\cdot}05$.

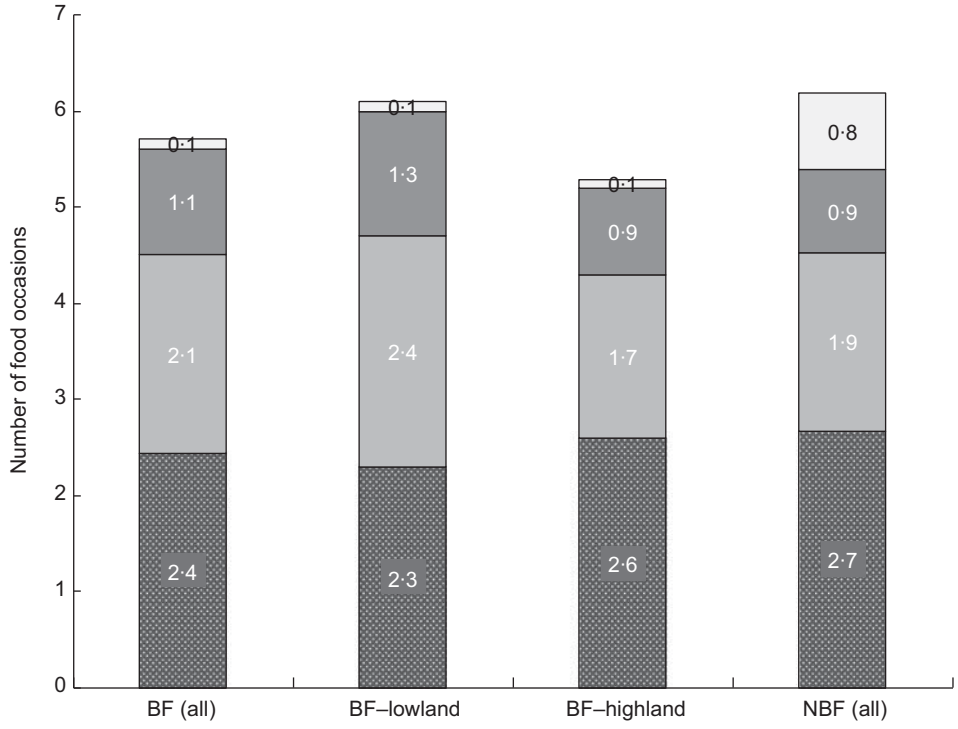

Fig. 1 Mean number of food occasions per type of food (□, gruel/porridges; ■, beverages; ▒, snacks; ▩, family foods) among breast-fed (BF) and non-breast-fed (NBF) young children (*n* 76) aged 12–23 months from a highland village (*n* 37) and a lowland village (*n* 39) in Gobalafto district, North Wollo, northern Ethiopia, August–October 2010

met the minimum number of food groups (≥3; Table 2). Many of the children's dietary diversity scores were in the low (0–2) to medium (3–4) range.

The diets of the young children were predominantly based on cereals and legumes (Tables 2 and 3). Consumption of ASF as well as fruits and vegetables was very low, although fruit consumption was higher in the lowlands ($P = 0{\cdot}01$). Fruits were the major contributor to vitamin C intake. Only 1% of the protein intake in the highlands *v.* 6% in the lowlands was provided by ASF. Moreover,

Table 2 Feeding practices according to breast-feeding status: young children (*n* 76) aged 12–23 months from a highland village and a lowland village in Gobalafto district, North Wollo, northern Ethiopia, August–October 2010

	BF children						NBF children	
	All (*n* 62)		Highlands (*n* 30)		Lowlands (*n* 32)		All (*n* 14)	
	n or Mean	% or SD	*n* or Mean	% or SD	*n* or Mean	% or SD	*n* or Mean	% or SD
Cereal products	62	100	30	100	32	100	14	100
Legumes and nuts	62	100	30	100	32	100	14	100
Animal-source foods								
Dairy	5	8	1	3	4	13	4	29
Eggs	3	5	0	0	3	8	0	0
Meat & poultry	2	3	0	0	2	5	0	0
Vitamin A-rich fruits & vegetables	11	18	3	10	8	25	0	0
Other fruits*	13	21	2	8	11	31	2	14
Tea	17	27	8	38	9	33	10	71
Coffee	2	3	2	8	0	0	1	7
Mean number of food groups (out of 7)	2·4	0·8	2·2	0·4	2·6	1·0	2·5	0·7
0–2	42	68	23	77	19	59	8	57
3–4	19	31	7	23	12	38	6	43
≥5	1	2	0	0	1	3	0	0
Fed minimum number of food groups or more†	20	32	7	23	13	41	6	43
Fed minimum number of solid/semi-solid foods‡	58	94	29	97	29	91	14	100
Fed according to IYCF practices§	20	32	7	23	13	41	4	29

BF, breast-fed; NBF, non-breast-fed; IYCF, infant and young child feeding.
*Statistically significant difference between highland and lowland sites according to Fisher's exact test (one-tailed): $P = 0{\cdot}01$.
†Minimum number of food groups: at least three daily for BF children and at least four daily for NBF children.
‡Minimum number of meals: three daily for BF children and four daily for NBF children.
§For BF children: need to be fed solids/semi-solids at least three times daily and be fed a minimum of three food groups[24]. For NBF children: need to drink at least two milk feedings and be fed at least four food groups (not including milk feeds) a minimum of four times daily[24].

Table 3 Percentage contribution (when ≥15 %) to energy and selected nutrient intakes from different food groups according to breast-feeding status: young children (*n* 76) aged 12–23 months from a highland village and a lowland village in Gobalafto district, North Wollo, northern Ethiopia, August–October 2010

	BF children			NBF children
	All (*n* 62)	Highlands (*n* 30)	Lowlands (*n* 32)	All (*n* 14)
Energy	Cereals (75) Legumes (17)	Cereals (73) Legumes (20)	Cereals (78) Legumes (15)	Cereals (71) Legumes (18)
Protein	Cereals (57) Legumes (28)	Cereals (55) Legumes (35)	Cereals (58) Legumes (21)	Cereals (60) Legumes (26)
Ca	Cereals (37) Legumes (20)	Cereals (38) Legumes (25)	Cereals (37) Dairy products (23) Legumes (16)	Cereals (34) Legumes (18) Dairy products (28)
Vitamin A	Legumes (38) Cereals (17) Root & tubers (15) Vegetables (16)	Legumes (26) Cereals (24) Root & tubers (24) Vegetables (16)	Legumes (50) Vegetables (19)	Legumes (55) Fruits (18) Root & tubers (17)
Fe	Cereals (61) Legumes (29)	Cereals (54) Legumes (37)	Cereals (68) Legumes (21)	Cereals (67) Legumes (25)
Zn	Cereals (62) Legumes (21)	Cereals (64) Legumes (25)	Cereals (60) Legumes (17)	Cereals (61) Legumes (20)
Vitamin C	Fruits (81)	Fruits (80)	Fruits (83)	Fruits (86)

BF, breast-fed; NBF, non-breast-fed.

the contribution of ASF to Fe intake was very low: 0 % in the highlands *v.* 6 % in the lowlands (data not shown).

Most children's complementary feeding practices were not in line with the recommended infant and young child feeding (IYCF) practices[25], as only few BF children consumed three or more food groups and were fed at least three times daily. Similarly, only a few NBF children were fed according to recommended IYCF practices[25].

A high proportion of the foods consumed by the children were family foods (Figs 1 and 2). The staple (family food) in both sites (like in the rest of Ethiopia) is *injera,* a flat fermented pancake that is consumed with stews, which was consumed by all children surveyed. However, the cereals used for the preparation of *injera* differed at the two sites. A mix of teff and sorghum was most commonly used in the lowlands, whereas mixes of barley and wheat or wheat and red sorghum were used in the highlands. *Injera* is consumed at all main meals, only the types of stews vary.

Shiro was the most commonly consumed stew, with about 81 % of the children having consumed it. This consists of roast, decorticated and ground legumes mixed with spices and then cooked. The most frequently

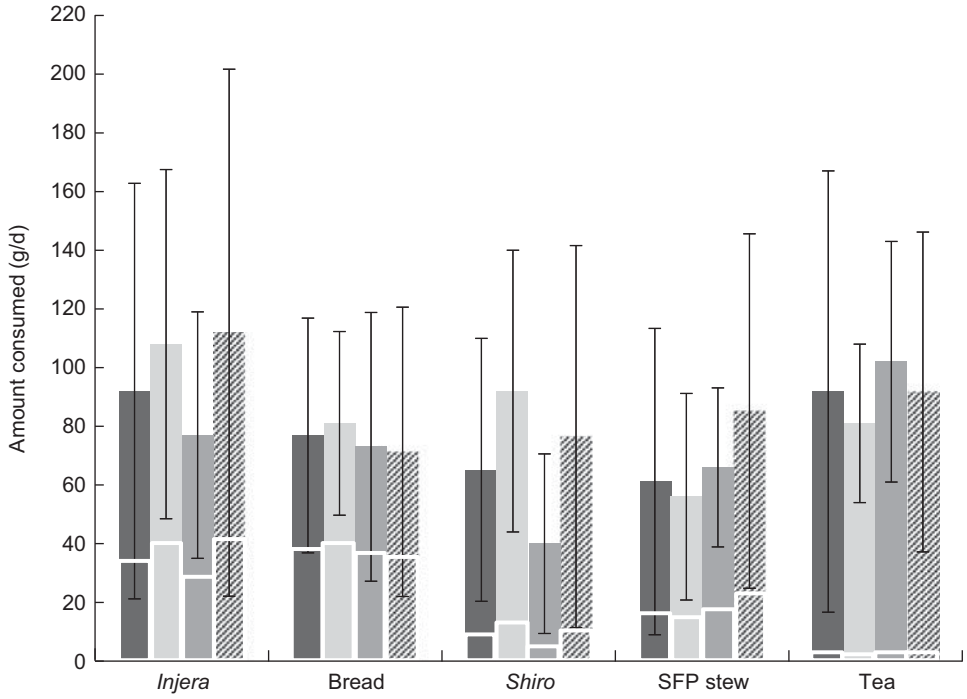

Fig. 2 Mean amounts of most frequently consumed foods per day, when eaten, among breast-fed (BF) and non-breast-fed (NBF) young children (*n* 76) aged 12–23 months from a highland village (*n* 37) and a lowland village (*n* 39) in Gobalafto district, North Wollo, northern Ethiopia, August–October 2010. ■, BF (all); □, BF–highlands; ▒, BF–lowlands; ▨, NBF (all). Values are means with their standard deviations represented by vertical bars; the white border represents the amount consumed (g DM) on dry weight basis (SFP stew, split field pea stew)

used legumes were grass pea (*Lathyrus sativus* L.) and broad beans (*Vicia faba* L.). After *shiro*, the next most frequently consumed stew (by 42 % of children) was made from split field peas (*Pisum sativum* L.). Very few meals such as gruels/porridges were specifically prepared for the children. More gruels/porridges were consumed by NBF than BF children. Mostly aluminium but also clay pots were used for cooking the foods. Snacks consisted of leavened bread, unleavened bread (*kitta*) or fruits.

Beverages consisted of tea, coffee, milk and in some instances *tella,* a local beer. The most frequently consumed beverage was tea. The children mostly drank tea with their breakfast, which was usually wheat bread. Otherwise, almost all of the children drank water after each meal. The amounts of food consumed per day and per type of food were low (Fig. 2), and appeared even lower, in particular for tea, when taking into account only the nutritious part of the food, i.e. expressed on a dry weight basis. Thus, it clearly appears that the main sources of nutrients in the young children's diet were cereals (*injera* + bread).

Adequacy of energy and selected nutrient intakes from complementary foods

Intakes of energy, Ca, vitamin A and vitamin C from complementary foods were far below estimated needs (Table 4). Zn intakes were also below the estimated needs when low bioavailability was assumed (15 %), which is highly probable as the diet is based predominantly on cereals and legumes. On the other hand, Fe and protein intakes met estimated needs. Fe intakes from complementary foods met estimated needs even under the assumption of low bioavailability (5 %). Consumption of Fe-fortified foods was non-existent. There were no significant differences in intakes between the highland and lowland villages except for vitamin C, of which more was consumed in the lowlands ($P < 0{\cdot}001$). Diets of BF and NBF children were similar; hence, the nutrients found to be suboptimal were the same. It also appeared that NBF children ate larger quantities and as a result had higher energy and nutrient intakes. When consumed, NBF children ate two times more gruel (amount) than BF children.

Ca, vitamin A and vitamin C densities were below the desired values (Table 5). Zn met desired values, but only when moderate bioavailability was assumed, whereas Fe met desired values even under the assumption of low bioavailability. Protein densities also met desired values. When comparing the two sites, vitamin C intakes and densities were seen to be significantly higher ($P < 0{\cdot}001$) in the lowlands, whereas protein ($P = 0{\cdot}04$) and Zn ($P = 0{\cdot}02$) densities were higher in the highlands.

Discussion

The present study evaluated the feeding practices and energy and nutrient intakes from complementary foods of

Table 4 Estimated daily median intakes (Q1, Q3) of energy and selected nutrients from non-breast-milk foods according to breast-feeding status in comparison with estimated needs: young children (*n* 76) aged 12–23 months from a highland village and a lowland village in Gobalafto district, North Wollo, northern Ethiopia, August–October 2010

	BF children (mean age: 18·0 (SD 4·4) months)							NBF children (mean age: 19·6 (SD 4·1) months)		
	All (*n* 62)		Lowlands (*n* 32)		Highlands (*n* 30)		Estimated needs†	All (*n* 14)		
Nutrient	Median	Q1, Q3	Median	Q1, Q3	Median	Q1, Q3		Median	Q1, Q3	RNI‡
Energy (kJ)	1598	1109, 2406	1669	837, 2284	1774	1343, 2485	2293	3548	1971, 4243	3740
Protein (g)	13·8	9·5, 20.2	12·8	6·1, 20·3	16·2	11·1, 20·9	5	33·4	13·8, 36·6	10·8
Ca (mg)	102	70, 168	96	51, 191	116	84, 172	346	215	146, 327	500·0
Fe (mg)	15·0	7·6, 23.4	13·0	5·5, 25·9	17·9	10·6, 24.1		28·9	12·9, 39·1	
L							11·4			11·6
M							5·6			5·8
Zn (mg)	3·3	2·0, 4.7	2·6	1·4, 3·8	3·8	2·3, 5.1		6·7	3·9, 8·1	
L							7·6			8·3
M							3·8			4·1
Vitamin A (μg RE)	53·6	16·6, 86·3	31·5	11·3, 89·5	56·0	41·4, 87·3	126	39·4	23·2, 133·6	400
Vitamin C* (mg)	0·8	0·2, 4·1	2·3	0·7, 7·3	0·4	0·0, 2.0	8	4·6	1·0, 23·0	30·0

Q1, 1st quartile; Q3, 3rd quartile; BF, breast-fed; NBF, non-breast-fed; RNI, Recommended Nutrient Intake; L, low bioavailability; M, medium bioavailability; RE, retinol equivalent.
*Statistically significant difference between highland and lowland sites according to the Mann-Whitney *U* test: $P < 0{\cdot}001$.
†For BF children, estimated needs from complementary foods are determined assuming average breast-milk intake and composition as proposed by WHO[23] and Dewey and Brown[24].
‡The RNI used are those of FAO/WHO/United Nations University[20], WHO[23] and WHO/FAO[22], for energy, protein, and vitamins and minerals, respectively.

BF and NBF young children (aged 12–23 months) in Gobalafto district, northern Ethiopia. Several feeding practices were not in accordance with WHO/Pan American Health Organization recommendations[27]. Shortfalls of Zn, Ca, vitamin C and vitamin A were observed, whereas Fe and protein intakes were adequate. Differences in the intakes and densities of certain nutrients were observed between the highlands and the lowlands.

Adequate nutrition in the first 2 years of life is critical for the child's development. In this regard, optimal breast-feeding and complementary feeding play a key role. In line with recently reported Demographic and Health Survey data, continued breast-feeding beyond the age of 1 year was practised by >80 % (62/76) of the surveyed households[6]. This is important, as breast milk is a good source of energy and essential fatty acids[28]. Although continued breast-feeding is associated with greater linear growth[29], a considerable proportion of the children studied (~33 %) were stunted.

Stunting may be due to intergenerational malnutrition, frequent infections and/or inadequate diet and feeding practices[30–32]. Although it was not possible to establish causal relationships in the present study, several features of the feeding practices of the children could be linked with poor growth.

Good sources of bioavailable nutrients like ASF were seldom consumed despite ownership of livestock. Earlier studies in Ethiopia showed that livestock is considered an asset and is therefore rarely consumed[10,33]. Dairy consumption – even in NBF children – was very low, perhaps lower than that reported for children in rural Sidama, southern Ethiopia[10]. Consumption of fruits and vegetables rich in provitamin A carotenoids also was very low despite recommendations to consume them daily[27]. This is in line with previous reports on Ethiopian complementary diets[7,10,26].

Frequent consumption of coffee and tea was observed. Such beverages contain phenolic compounds that inhibit the absorption of Ca and Fe. In addition to their appetite-suppressing effects, these beverages are of low nutrient density; thus when consumed especially with sugar, they may displace more nutritious foods[34]. This could partly explain the low energy intakes from complementary foods relative to WHO estimated needs. Nevertheless, since >90 % of the children were fed at least three times daily, better coverage of energy needs would have been expected. However, the quantity of foods consumed was small, probably due to the poor practice of responsive feeding and/or to malnutrition/infection-associated anorexia. Irrespective of the type of food consumed, the quantity did not exceed ~9 (SD 4) g/kg body weight per meal (calculation not shown), which is much lower than the theoretical gastric capacity of 30 g/kg body weight per meal.

In view of the low energy intake, the low dietary diversity and the low nutrient density of the foods consumed, the

Table 5 Median (Q1, Q3 quartile) nutrient densities of complementary foods in BF children in comparison with desired nutrient densities: young children (*n* 76) aged 12–23 months from a highland village and a lowland village in Gobalafto district, North Wollo, northern Ethiopia, August–October 2010

	All BF (*n* 62)		Lowlands (*n* 32)		Highlands (*n* 30)		
Nutrient density (per 418 kJ/100 kcal)	Median	Q1, Q3	Median	Q1, Q3	Median	Q1, Q3	Desired value†
Protein* (g)	3·4	3·1, 3·7	3·2	2·8, 3·6	3·5	3·2, 3·8	0·9
Ca (mg)	25·9	20·5, 34·2	24·9	20·3, 33·8	27·2	21·1, 34·3	63
Fe (mg)	3·8	2·6, 4·8	3·6	2·5, 4·5	3·9	2·6, 5·7	
L							2·1
M							1·0
Zn* (mg)	0·7	0·6, 0·9	0·7	0·6, 0·8	0·8	0·6, 0·9	
L							1·4
M							0·6
Vitamin A (μg RE)	12·8	6·3, 20·5	9·0	3·7, 18·3	14·6	8·0, 26·3	23
Vitamin C* (mg)	0·2	0·1, 1·4	0·5	0·2, 2·3	0·08	0·0, 0·3	1·5

Q1, 1st quartile; Q3, 3rd quartile; BF, breast-fed; L, low bioavailability; M, medium bioavailability; RE, retinol equivalent.
*Statistically significant difference between the highland and lowland sites according to the Mann–Whitney *U* test: $P = 0{\cdot}04$ for protein; $P = 0{\cdot}02$ for Zn; $P < 0{\cdot}001$ for vitamin C.
†Desired values were those calculated by Dewey and Brown[24].

high rate of stunting and the observed shortfalls of Zn, Ca, vitamin C and vitamin A are not surprising. Evidence for a close association between dietary diversity, stunting and micronutrient deficiencies in developing countries is already well documented[10,35–38]. Furthermore, considering the high proportion of stunted children, shortfalls may even be more pronounced if the children have to catch up on their growth[39].

However, a notable finding in the present study is that Fe was not a 'problem nutrient', as intakes and densities were above the estimated needs and desired values, even under the assumption of low bioavailability (5 %). Previous studies have shown that adult Fe intake in Ethiopia surpasses recommended values[8,40] and this was attributed to the high Fe contents of most cereals grown in the country[17,18]. However, a large proportion of this Fe was attributed to soil contamination[18,41]. Further investigations are required to evaluate the bioavailability of both intrinsic and contaminant Fe.

Of great concern is the wide consumption of legumes with toxic components like grass peas and broad beans. Grass pea (*Lathyrus sativus*) is associated with neurolathyrism, a neurodegenerative disorder, whereas broad bean (*V. faba* L.) is associated with favism, a haemolytic anaemia. Consumption of diets containing 30 % grass pea for 3 months or more is generally enough to trigger the onset of neurolathyrism[42]. However, consuming grass peas mixed with cereals rich in sulfur-containing amino acids can reduce the associated adverse effects[43]. So supplementing *shiro* with *injera* may help reduce the toxicity of grass pea[37], in addition to improving the quality and the content of proteins, as evidenced by the adequate protein intakes. Furthermore, processes such as soaking and roasting of grains, and the inclusion of spices with known antioxidant properties (ginger, garlic, etc.) in the preparation of *shiro*, may further reduce toxic effects[43]. However, considering the endemicity of neurolathyrism in the northern parts of Ethiopia[44], the low body weight of the children and the multiple micronutrient deficiencies these children may present, replacing grass peas and broad beans by other available legumes such as lentils, chickpeas and field peas may be preferable.

Despite the small sample of children surveyed at each site, comparison between the highlands and the lowlands revealed statistically significant differences in vitamin C intakes as well as Zn and protein densities of the diets. More stunted children were observed in the highlands ($P = 0{\cdot}05$). Possible explanations may be the harsh physical and socio-economic conditions in the highlands, the difference in staple cereals and the higher availability and thus higher consumption of fruits in the lowlands. This finding suggests that even more pronounced differences between the highlands and lowlands are likely, thus a more detailed study based on a larger sample from several highland and lowland sites is required. The small number of NBF children requires caution in interpreting the results.

The cross-sectional nature of the present study did not allow seasonal variation in food intakes to be considered. Although caregivers were instructed to not change their children's dietary pattern, this does not warrant the absence of deliberate changes. A further limitation is that breast-milk consumption was not quantified, thus values of average breast-milk intake reported in the literature were used to calculate the adequacy of energy and nutrient intakes.

Conclusions and recommendations

The present study provided an overview of the feeding pattern of young children in two rural villages (highlands/lowlands) in Gobalafto district, North Wollo, northern Ethiopia. The study provided evidence that, except for Fe and protein, energy and nutrient intakes were far below WHO recommendations.

In both highlands and lowlands, efforts to enhance dietary diversity by including ASF, dairy, and fruits and vegetables rich in vitamin A and vitamin C are needed.

However, promotion of consumption of fruits will first need selection and cultivation of varieties adapted to the agro-climatic conditions of the highlands. In the immediate future, including available green leafy vegetables like stinging nettles (*Urtica dioica* L.) in the complementary diets of the children in the highlands may help enrich the diets with vitamin C, provitamin A carotenoids and minerals[45].

Although beverages such as tea and coffee have the advantage of usually being safer in terms of microbial contamination, in view of their negative effects on mineral absorption and the appetite of the children, their consumption should be discouraged. Caregivers should also be informed of the potential toxicities associated with consumption of grass peas and broad beans, and the consumption of other available legumes like chickpeas, lentils and field peas should be encouraged. Further research on the effect of traditional processing on the toxic component of grass pea is needed. Future nutritional interventions may need to take into account potential agro-ecological influences on feeding practices and nutrient intakes.

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Public Health Nutrition

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4.1.3 Complementary results: access to health services, consumption frequency and access to staples

4.1.3.1 Complementary data on access to health services and sibling composition

Although 75% of them had their pregnancy followed by a health professional, the majority delivered at home. Given the high maternal mortality (671/100, 000) in Ethiopia (CSA/ICF, 2012), facilities for safer delivery, and education on the importance of having a health professional assisted delivery are needed.

Caregiver-reported child morbidity was high, with nearly half of the children having had diarrhea or fever, and one third having had cough in the two weeks prior the survey. The low health status of the children may be partially linked to their inadequate dietary intakes. Diseases such as diarrhea may further lead to losses of minerals or may impair absorption.

Table 4.1: Birth order, access to health services of mothers and young children

	(%)	Age category			
Number of brothers/sisters		12-23M	24-56 M	5-18 yrs	+18 yrs
0	42.1	-	-	-	-
1-2	34.2	5.2	18.4	31.5	7.9
3-4	19.7	0.0	0.0	13.1	1.3
>5	3.9	0.0	0.0	1.3	0.0
	Position of child			%	
	1st			34.2	
	2nd			25.0	
	3rd			11.8	
	4th			11.8	
	≥5			10.5	
	Pregnancy follow-up			75.0	
	Place of delivery				
	Health center			19.7	
	Home			80.3	
	Morbidity				
	Diarrhea			40.8	
	Fever			47.4	
	Cough			26.3	
	Complementary feeding education			76.3	
	Source of nutrition education				
	Health center			73.7	
	Family			2.6	

M: months; yrs: years

Surprisingly, ~74% of the household reported having had some sort of complementary feeding education through their health center. However, the diets of the children and the feeding practices were found inadequate, suggesting that either the education was poor in content or was not well accepted by the community. Future studies that determine the validity

of the contents of the nutrition training provided and the acceptability of key nutrition messages by the community are needed. This may be well captured using a knowledge, aptitude, and practice (KAP) methodology.

4.1.3.2 Frequency of cereal and legume consumption and raw material supply

Table 4.2 presents the frequency of consumption of different legumes in the villages surveyed. In both highlands and lowlands, *shiro* was mostly prepared from mixes of grass pea and broad beans. However, various mixes of legumes were observed. Using a retrospective question, it was possible to capture whether the choice of legumes and cereals was season-dependent.

Table 4.2: Legumes used in *Shiro* preparations in the highlands and lowlands of North Wollo, northern Ethiopia

	Highland		**Lowland**	
	Mostly consumed		**Mostly consumed**	
	during the survey†	**during the year¶**	**during the survey**	**during the year**
Legumes	Frequency* (%)	Frequency (%)	Frequency (%)	Frequency (%)
Grass pea (Lathyrus sativus)	7 / 37(18)	6 / 37 (16)	4/ 39 (10)	2 / 39 (5)
Broad beans (Vicia faba)	3 / 37(8)	2 / 37 (5)	1 / 39 (3)	3 / 39 (8)
Grass pea, broad beans	11 / 37(30)	8 / 37 (22)	12 / 39 (31)	11 / 39 (28)
Grass pea, field pea (Pisum sativum)	2 / 37 (5)	1 / 37(3)	1 / 39 (3)	1 / 39 (3)
Grass pea, chickpea (Cicer arietinum)	0 /37 (0)	0 / 37 (0)	4 / 39 (10)	2/ 39 (5)
Field pea, chickpea	0 /37 (0)	0 / 37 (0)	1 / 39 (3)	3 / 39 (8)
Field pea, broad beans	0 /37 (0)	0 / 37 (0)	3 / 39 (8)	3 / 39 (8)
Grass pea, broad beans, field pea	6 / 37(16)	3 / 37(8)	2 / 39 (5)	2 / 39 (5)
Grass pea, chickpea, broad beans	0 /37 (0)	0 / 37 (0)	1 / 39 (3)	2/ 39 (5)
Grass pea, field pea, chickpea	0 /37 (0)	0 / 37 (0)	1 / 39 (3)	0 / 39 (0)
Field pea, chickpea, broad beans	0 /37 (0)	0 / 37 (0)	2 / 39 (5)	2 / 39 (5)
Grass pea, broad beans, field pea, chickpea	2 / 37 (5)	6 / 37(16)	8 / 39 (21)	7 / 39 (18)
Broad beans, field pea, lentil, chickpea	1 / 37 (3)	2 / 37(5)	0 (0)	1 / 39 (2)

†legumes used at the time of 24 h recall survey
¶legumes mostly consumed during the year, based on caregivers' declaration
*% of consumers out of total respondents

The choice of legumes seemed to be more dependent on the socio-economic parameters than on seasonal effects. Families that are well-to-do tended to mix more variety of legumes while at the same time decreasing the proportion of grass pea. However, even the relatively well-to-do families did not abandon the use of grass pea, and justified this by the high water holding capacity of flour from grass pea that allows them to prepare more stews with less flour.

Cereal mixes for the preparation of injera was also very common, but the type of mixes frequently observed in the highlands and the lowlands were different (table 4.3). In the lowlands, mixtures of teff, sorghum and maize were mostly observed during the survey. However, information from caregivers revealed that blends of teff and sorghum were the most common, and that maize only finds its way in pre-harvest times (September-November) when stocks of teff and sorghum are depleted.

Table 4.3: Cereals used for making *Injera* in the highland and lowlands

	Mostly consumed	
	during the survey	**during the year**
	Highland	
Cereal type/mixes	*Frequency (%)	*Frequency (%)
Barley	1 / 37 (3)	22 / 37 (60)
Wheat, red sorghum	12 / 37 (32)	8 / 37 (22)
Barley , white sorghum	3 / 37 (8)	1 / 37 (3)
Barley, wheat	3 / 37 (8)	2 / 37 (5)
Barley, red sorghum	3 / 37 (8)	1 / 37 (3)
Barley, wheat, red sorghum	6 / 37 (16)	4 / 37 (11)
Barley, wheat, white sorghum,	3 / 37 (8)	2 / 37(5)
Barley, white sorghum, red sorghum	1 / 37 (3)	1 / 37 (3)
	Lowland	
Teff, sorghum	11 / 39 (28)	19 / 39 (49)
Teff, sorghum, maize	14 / 39 (36)	10 / 39 (26)
Teff, maize	7 / 39 (18)	4 / 39 (10)
Wheat, maize	1 / 39 (3)	1 / 39 (3)
Maize, sorghum	4 / 39 (10)	2 / 39 (5)
Maize	1 / 39 (3)	1 / 39 (3)
Teff, wheat, maize	1 / 39 (3)	2 / 39 (5)

*% of consumers out of total respondents

Type of cereal/legume mostly used at the time of the survey , throughout the year

In the highlands, injera was mostly made from blends of wheat and red sorghum. However, during the year, barley and barley-wheat mixes are important. Informal discussions as to why red sorghum was added despite not being produced locally (table 4.3) revealed that its

addition was believed to improve the digestibility of the corresponding injera. This justified the purchase of red sorghum, which can suggest that households are ready to spend extra money on foods for which they see noticeable health effects. In contrast, the consumption of grass pea even with the knowledge of the toxic effects indicates that the notion of toxicity in these communities may be limited to acute effects.
The choice of the cereals and legumes for the subsequent studies was based on consideration of the observed consumption during the 24h recall and throughout the year.

In an effort to investigate how legumes and cereals were accessed (table 4.4), it was observed that a good proportion of what was consumed was purchased. This is exceptional for farming rural households, but the fact that the survey was conducted in pre-harvest times (September-November) suggests that stocks of the food produced might have been depleted. This is also confirmed by the presence of food aid in the form of wheat and beans.

Table 4.4: Access of legumes and cereals

	% of household		
Source	**Own farm**	**Market**	**Aid**
Teff	46.9	53.1	0.0
White sorghum	29.4	64.7	5.9
Red sorghum	0.0	100.0	0.0
Maize	50.8	49.2	0.0
Barley	58.9	41.1	0.0
Wheat	23.6	29.2	47.2
Grass pea	2.9	97.1	0.0
Field pea	27.0	71.6	1.4
Broad bean	31.5	65.8	1.4
Chickpea	1.5	98.5	0.0
Lentil	17.6	82.4	0.0

4.2 Fermentation kinetics and phytate hydrolysis of injera sourdough

4.2.1 Introduction

The food consumption survey has identified that *injera*, legume-based stews, and bread were the foods most frequently consumed by young children. A special focus was given to *injera*, since fermentation can result in degradations of mineral absorption inhibitors like phytate, and thus potentially influence the mineral bioavailability. However, *injera* was observed to be prepared from different cereals depending on the location (highland/lowland). In the highlands, injera was most frequently prepared from barley-wheat (BW) and wheat-red sorghum (WrS) mixes, while in the lowlands mix of teff-white sorghum (TwS) was mostly used.

In this section (4.2), results from field observations of household fermentations and characterization of field collected *injeras* are reported. The most frequently observed flour blends were standardized and the *injera* preparations from these blends were observed. Household samples were collected at different time intervals so as to characterize the fermentation kinetics of the different *injeras*. The influence of flour blend composition on fermentation kinetics and hydrolysis of phytate was investigated, and possible ways to achieve greater phytate degradation have been put forward.

The results are presented in the form of a published paper, accompanied by supplementary results.

4.2.2 Influence of flour blend composition on fermentation kinetics and phytate hydrolysis of sourdough used to make ***injera*** *Food Chemistry, 138*(1), 430-436**.**

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Influence of flour blend composition on fermentation kinetics and phytate hydrolysis of sourdough used to make *injera*

Kaleab Baye [a,b], Claire Mouquet-Rivier [b], Christèle Icard-Vernière [b], Isabelle Rochette [b], Jean-Pierre Guyot [b,*]

[a] Center for Food Science and Nutrition, Addis Ababa University, P.O. Box 150201, Addis Ababa, Ethiopia
[b] IRD UMR 204 «Prévention des Malnutritions et des Pathologies Associées» (Nutripass), IRD/Montpellier2/Montpellier1, BP 64501, 34394 Montpellier Cedex 5, France

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ABSTRACT

The influence of cereal blends, teff–white sorghum (TwS), barley–wheat (BW) and wheat–red sorghum (WrS), on fermentation kinetics during traditional fermentation of dough to prepare *injera*, an Ethiopian traditional fermented pancake, was investigated in samples collected in households. Barley malt was used with BW and WrS flours. WrS- and BW-*injera* sourdough fermentations were characterised by a transient accumulation of glucose and maltose and a two-step fermentation process: lactic acid fermentation and alcoholic fermentation with ethanol as the main end product. Only transient accumulation of glucose was observed in TwS-*injera,* and equimolar concentrations of lactic acid and ethanol were produced simultaneously. Final α-galactoside concentrations were low in all sourdoughs. Phytic acid (IP6) was completely hydrolyzed in WrS and BW-*injeras* probably due to the combined action of endogenous malt and microbial phytases. Only 28% IP6 hydrolysis was observed in TwS *injera*. Ways to improve IP6 hydrolysis in TwS-*injera* need to be investigated.

1. Introduction

In many developing countries, most foods consumed by young children are cereal based. Cereal based foods contain high amounts of phytic acid (*myo*-inositol 1,2,3,4,5,6 hexakis [dihydrogen phosphate]), which strongly bind minerals like iron and zinc (Lopez, Leenhardt, Coudray, & Rémésy, 2002). Large amounts of these nutrients are required during early life due to accelerated growth (Dallman, 1992), consequently ensuring their bioavailability is critical.

Several studies have documented the beneficial effect of fermentation in improving both the nutrient and sanitary qualities of foods (Nout, 2009; Svanberg & Lorri, 1997). Production of low molecular weight organic acids, such as lactic and acetic acid, reduces pH and may thus limit contamination by foodborne pathogens (Nout & Motarjemi, 1997). Furthermore, fermentation can activate several endogenous enzymes including phytases and may thus result in products with reduced antinutritional factors (Greiner & Konietzny, 2006). The extent to which enzymes like phytases are activated depends on the fermentation kinetics, which in turn, depends on the raw materials used (Hammes et al., 2005).

Several cereal based traditional fermented foods exist in Africa including *kenkey* in Ghana, *togwa* in Tanzania, *mawè* in Benin and *ben-saalga* in Burkina Faso (Guyot, 2010; Nout, 2009). For practical reasons, the fermentation kinetics of traditional fermented foods have usually been characterised based on sample fermentation reproduced in the lab, and may therefore not satisfactorily reproduce fermentation conditions in the field (Tou et al., 2006).

In Ethiopia, the most widely consumed food by young children and adults alike is *injera,* which is a thin, flat, traditional fermented pancake. However, depending on the agro-ecology of the area concerned (highlands versus lowlands), different cereal blends are used to make *injera*. In North Wollo, located in northern Ethiopia, barley–wheat blends (BW) and wheat–red sorghum blends (WrS) are commonly used in the highlands whereas a blend of teff (*Eragrostis tef*) and white sorghum (TwS) is used in the lowlands. Current blending practices may be instrumental for nutrition interventions to help promote food-to-food fortification.

Only few investigations have been made on the traditional fermentation of cereal blends used for the preparation of *injera* (Gedamu, 2008; Yetneberk, Rooney, & Taylor, 2005). These studies mostly focused on the influence of cereal blends on the processing quality and acceptability of *injera* (Yetneberk et al., 2005). To what

* Corresponding author. Tel.: +33 467416285.
E-mail address: jean-pierre.guyot@ird.fr (J.-P. Guyot).

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extent such blends influence the fermentation kinetics and the reduction of constituents with antinutritional effects such as IP6 and α-galactosides remains unknown. In this connection, the present study investigated the processing of *injera* made from different flour blends based on field observations. The influence of the blend of flour on fermentation kinetics and its possible implications in phytic acid (IP6) hydrolysis was investigated.

2. Materials and methods

2.1. Raw materials

Households ($n = 76$) in two villages in North Wollo, northern Ethiopia, one in the highlands (~3500 m above sea level – a.s.l.) and the other in the lowlands (~1500 m a.s.l.), were surveyed to determine the type of cereals and the most common blend proportions used in the preparation of *injera* flours. Accordingly, grains consumed in the lowland (teff and white sorghum), and those consumed in the highlands (barley, wheat and red sorghum), were purchased from local markets serving the two communities. Grains were purchased from the same batch in order to control variability due to grain varietal differences. The processing of the grains into BW- and WrS-flours in the highlands and TwS-flour in the lowlands was conducted by women in the respective villages. Two groups of women (five in the lowlands and six in the highlands) together cleaned the grains, by removing dirt and inedible parts. The grains were then sun dried followed by manual decortication and winnowing, with the exception of *teff* that was not decorticated. After these preliminary steps, the cereals were mixed at a 1:1 ratio (w/w) to make teff–white sorghum (TwS) and barley–wheat (BW) blends and at a 4:1.5 ratio to make wheat–red sorghum (WrS) blends and were then milled in local community milling units that uses mechanical mills.

The resulting TwS flour was subdivided into five equal parts and was distributed to five households to follow TwS *injera* sourdough fermentation. Likewise, BW and WrS *injera* sourdough fermentation were each followed in three households. The different households used the same flour but their own traditional starter culture (*ersho*) to trigger the fermentation.

2.2. Observations and sampling in households

To describe the different processing steps and characterise the fermentation of *injera*, the following measurements were made in five households ($n = 5$) for TwS-*injera* and three households ($n = 3$) for each WrS- and BW-*injeras*: the length of each step was monitored, the raw materials used (flour, water, barley malt and *ersho* starter) were weighed and pH measured. Samples were collected at different intervals during the fermentation of the dough used to make *injera* and were kept at −20 °C until further analysis. To avoid disturbing the households, samples were not collected during the night.

2.3. Dry matter (DM) content

DM contents were determined by oven drying at 105 °C to constant weight.

2.4. Fermentation kinetics

2.4.1. Change in pH

During fermentation, the pH of the slurry was recorded using a WTW 340i pH meter (Fisher Bioblock Scientific, Illkirch, France). The rate of change in pH ($-\mathrm{dpH}/\mathrm{d}t$) was calculated for each household observation as follows: $-(\mathrm{dpH}/\mathrm{d}t) = \mathrm{pH}_{(t+1)} - \mathrm{pH}_{(t)}/(t_{+1}) - t$, where "$t$" stands for time (hours). The maximal value of $-(\mathrm{dpH}/\mathrm{d}t)$ for each household observation was then averaged to give the maximal rate of change in pH ($-(\mathrm{dpH}/\mathrm{d}t)_{\max}$).

2.4.2. Analysis of mono- and disaccharides and -galactosides

Mono- and disaccharides (glucose, fructose, maltose and sucrose) and α-galactosides (raffinose and stachyose) were extracted by diluting one gramme of fermented paste in 2 ml of milliQ water, the mixture was vortexed, then centrifuged at 4500*g* for 10 min at 4 °C. The supernatants were filtered through 0.20 μm pore size filters and were analysed by HPAEC (high performance anion-exchange chromatography) with a Dionex DX 500 apparatus connected to an amperometric detector Dionex Model ED 40 (Thermo Scientific, Courtaboeuf, France) using a Carbo PA1 column (Dionex S.A., Jouy en Josas, France) after appropriate dilution.

The following conditions were used: mobile phase (eluent) NaOH 90 mM, flow rate 1 ml/min, temperature 35 °C, injection sample extract 25 μl (Haydersah et al., 2012). Results are expressed in mmol/kg of dough.

2.4.3. Analysis of lactic and acetic acid, mannitol and ethanol

Lactic acid, acetic acid, and ethanol were analysed by HPLC using an Aminex HPX-87H, 300 × 7.8 mm column (Biorad, Yvry-sur-seine, France) connected to a refractive index detector (Model Waters 2410; Biorad, France) as previously described in Calderon, Loiseau, & Guyot (2003).

2.5. Analysis of phytate (IP6)

After extraction from 0.2 g of sample in acid solution (10 ml of HCl 0.5 M) at 100 °C for 6 min, IP6 content was determined by measuring myo-inositol hexaphosphate (IP6) content by HPAEC according to Lestienne, Icard-Vernière, Mouquet, Picq, and Trèche (2005), using an AS-11 pre-column and column kit (Dionex, Sunnyvale, USA).

2.6. Phytase activity in flours

Inorganic phosphorus and phytates were removed from flours by ion exchange chromatography as described in Konietzny, Greiner, and Jany (1994). The resulting phytate free supernatant was then incubated in 2.5 mM sodium phytate solution at pH 5.6 and 55 °C for 60 min, and liberated inorganic phosphate was determined using the spectrophotometric method described in Heinonen and Lahti (1981). Phytase activity was calculated as micromoles of inorganic phosphate liberated from sodium phytate per minute per gram (DM) of flour.

2.7. Statistical analyses

All values corresponding to the same type of *injera* (i.e., prepared from the same flour blend in different households) were averaged ($n = 5$ or $n = 3$ depending on *injera* type) and standard deviations are used to estimate the variation.

Data were submitted to analysis of variance (ANOVA), using the general model procedure of SPSS version 15. Statistical differences between means ($P < 0.05$) were tested by Duncan's multiple range test.

3. Results

3.1. Description of the processing of Injera

Injera preparation is a relatively lengthy process, mainly due to its extended fermentation period, which takes 2–3 days (Fig. 1).

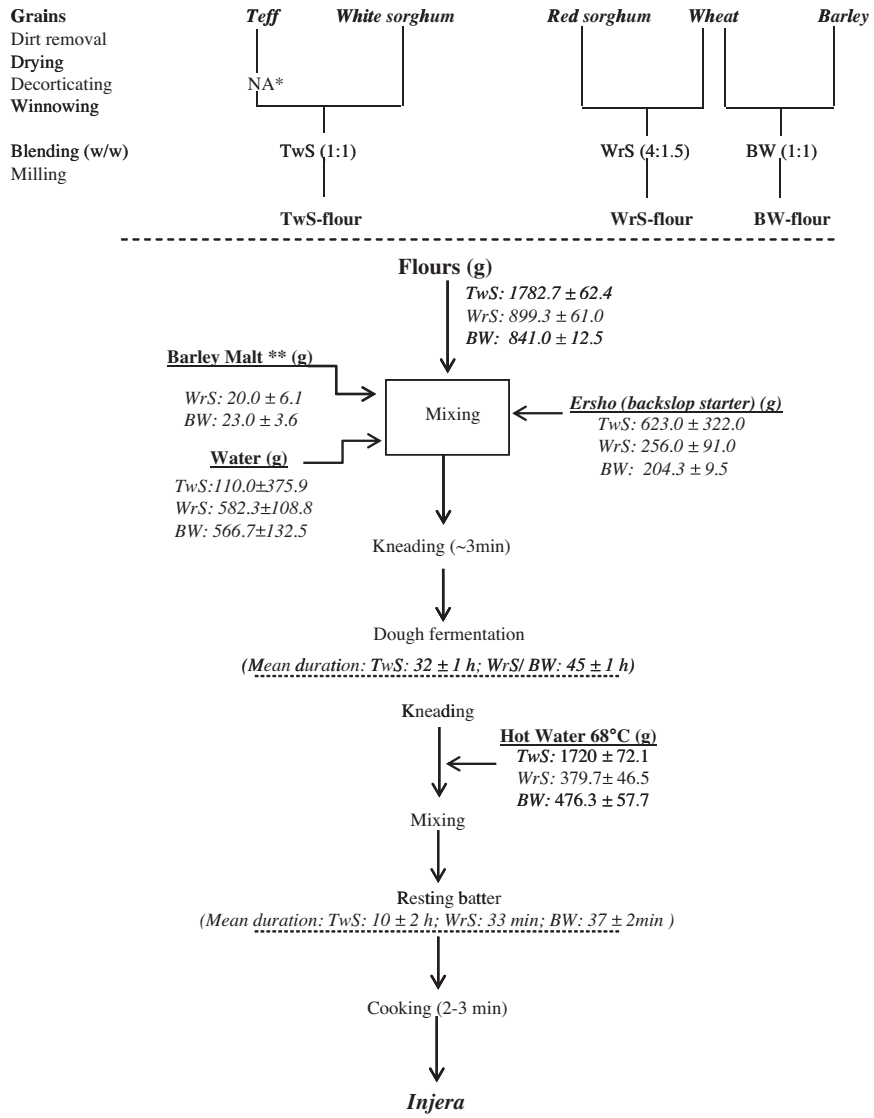

Fig. 1. Description of the processing of *injera.* TwS, teff–white sorghum; WrS, wheat–red sorghum; BW, barley–wheat NA*, not applicable; **, barley malt was not included in the preparation of teff–white sorghum *injera*. Values are mean ± standard deviations.

During household observations, the flours were first mixed with water and a small amount of barley malt (BM) was added in the preparation of BW- and WrS-*injera*s (Fig. 1). Fermentation was triggered by inoculating the resulting dough with *ersho*, a backslop starter obtained from previous fermentations. Processing is practically the same irrespective of the cereals used, apart from the length of the dough fermentation step, which took up to 45 h for WrS- and BW-*injera* but only 33 h for TwS-*injera*.

3.2. Fermentation kinetics

3.2.1. Changes in pH

The kinetics of the decrease in pH followed the same pattern in the three types of *injera*s (Fig. 2). However, in that of TwS, the mean pH ± SD at the start of the fermentation was lower (5.4 ± 0.4) than that of BW (6.0 ± 0.4) and WrS (6.3 ± 0.4).

During the first 10 h of fermentation, pH decreased on average from 5.4 to 4.0 in TwS-*injera*, and from, respectively, 6.0 to 4.4 and 6.3 to 4.5 in BW and WrS-*injera*s, with no time lag. The maximum rate of pH decrease was similar in the three fermentations but pH 4.5 was reached more rapidly in TwS-*injera* (Table 1). A second phase characterised by a slower rate of pH decrease that extended from 10 to 33 h in TwS and from 10 to 45 h in BW and WrS-*injera* was observed. The pH of the TwS-*injera* was lower throughout the fermentation period. The final pH of TwS-*injera* was 3.6, of WrS 4.0 and of BW-*injera*s 3.9.

3.2.2. Kinetics of substrate consumption and product formation

At the beginning of the fermentation of BW and WrS-*injera,* the dominant sugar was maltose followed by glucose (Fig. 3A). The concentration of maltose at the start of fermentation was 33.1 mmol/kg in BW and 32.2 mmol/kg in WrS-*injera*. An increase in maltose concentration was observed in BW and WrS in the first 2 h of fermentation, after which it started to decrease to reach final values of 4.4 and 0.6 mmol/kg, respectively. The initial glucose concentration was around 20 mmol/kg in both blends and followed a similar pattern to that of maltose.

In TwS-*injera*, glucose was the main sugar followed by maltose (Fig. 3A). The glucose concentration increased sharply from an initial value of 36–67 mmol/kg in the first 6 h of fermentation. This was followed by a decrease to reach a final concentration of ~4 mmol/kg.

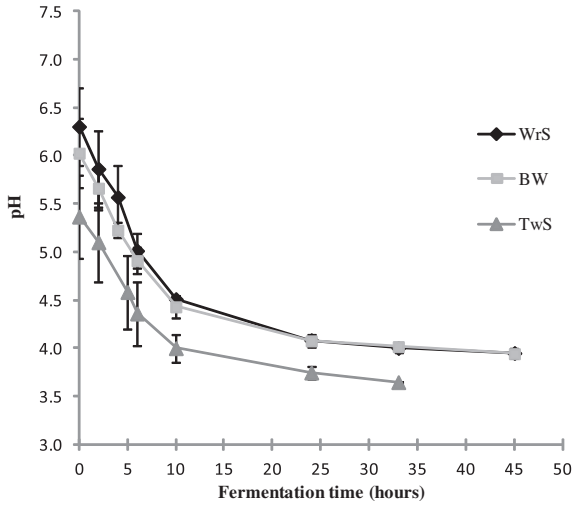

Fig. 2. Changes in pH during *injera* sourdough fermentation. TwS, teff–white sorghum; WrS, wheat–red sorghum; BW, barley–wheat. Error bars represent standard deviation of means.

Sucrose and fructose concentrations were relatively low in all three *injera*s and decreased to trace concentrations during the first 10 h of fermentation.

Trends in the production of lactate, acetate, ethanol and mannitol were similar in WrS- and BW-*injera*s. The concentrations of lactic acid, acetic acid and mannitol increased and levelled off after 10 h of fermentation in BW- and WrS-*injeras* (Fig. 3B). Lactic and acetic acid accumulation was consistent with the drop in pH observed during this period. However, ethanol was the main end product and its final concentration was nearly twice that of lactic acid by the end of dough fermentation (Fig. 3B).

TwS-*injera* displayed a distinctive pattern, with ethanol and lactic acid produced at equimolar concentrations and according to the same trend throughout fermentation (Fig. 3B). Very low concentrations of acetic acid and mannitol were detected.

3.3. Changes in components with antinutritional effects

3.3.1. Changes in α-galactosides

The initial concentration of raffinose was the highest in BW-*injera* (1.2 mmol/kg) followed by WrS-*injera* (0.6 mmol/kg) and TwS-*injera* (0.2 mmol/kg). A large proportion of raffinose was degraded in all *injera*s during the first 10 h of fermentation (Fig. 4). Stachyose was detected as traces in WrS- and BW-*injeras,* whereas in TwS-*injera,* it was found at a similar concentration (0.24 mmol/kg) to raffinose, but fermented more slowly (Fig. 4).

3.3.2. Kinetics of IP6 degradation

The pattern of IP6 degradation during dough fermentation differed with the type of *injera*. In *injera*s made from BW and WrS, IP6 degradation was already nearly complete after 10 h of fermentation. Whereas in *injera* made from TwS, only 28% of the IP6 was degraded, and degradation occurred at a very slow rate compared to the other types of *injera* (Fig. 4). It is worth noting that the level of IP6 at the beginning of fermentation was higher in TwS-*injera* than in the other two types. In BW- and WrS-*injeras,* degradation of IP6 mainly occurred between 2 and 6 h, corresponding to a pH of between 5.9 (at 2 h) and 4.9 (at 6 h), and in TwS-*injera* between 2 and 10 h, corresponding to pH of between 5.1 (at 2 h) and 4.0 (at 10 h).

3.4. Phytase activity

Significantly higher ($P < 0.05$) phytase activity was observed in BW- and WrS-flours than in TwS-flour (Table 2). Since barley malt was used to prepare BW- and WrS-*injeras*, phytase activity was also measured in this ingredient and in barley flour. Malted barley had higher ($P < 0.05$) phytase activity than raw barley. However, the effect of malting was highly variable as evidenced by the high standard deviations. Nevertheless, no significant difference was observed between barley malt and BW-*injera* flour.

4. Discussion

Due to agro-ecological conditions governing the cultivation of cereals in North Wollo (northern Ethiopia), *injera* is mainly made from barley and wheat in the highlands and from teff at lower altitudes. Different mixtures of these cereals with red or white sorghum are used in households. Barley malt is added to the wheat/barley or wheat/sorghum blends. A common characteristic among the different doughs is a rapid drop in pH likely due to backslopping (Nout, Rombouts, & Havelaar, 1989). The pH rapidly reached values below 4.5, promoting better hygienic conditions (Kingamkono, Sjogren, Svanberg, & Kaijser, 1994; Nout et al., 1989). Nevertheless, in the three types of sourdough, maximum rates of pH decrease $(-\mathrm{dpH/d}t)_{max}$ were nearly twice lower than those reported for the lactic acid fermentation of pearl millet (Songré-Ouattara et al., 2009) and rice/soybean slurries (Nguyen, Guyot, Icard-Vernière, Rochette, & Loiseau, 2007).

Different fermentation patterns occurred depending on the cereal flour blend. Maltose and glucose were the main fermentable sugars in the BW- and WrS-*injeras* with transient accumulation of maltose. In TwS-*injera,* glucose was the main sugar that accumulated transiently and no transient accumulation of maltose was observed. In BW- and WrS-*injeras*, fermentation patterns suggest a two-step fermentation process: lactic acid fermentation and alcoholic fermentation, suggesting combined action of lactic acid bacteria (LAB) and yeasts. The production of mannitol during the lactic acid fermentation step of BW- and WrS-*injeras* strongly suggests the presence of heterolactic LAB due to their ability to use free fructose or fructose bound in sucrose as electron acceptors to produce mannitol (Calderon et al., 2003; Vrancken, Rimaux, De Vuyst, & Leroy, 2008; Wisselink, Weusthuis, Eggink, Hugenholtz, & Grobben, 2002). The fact that lactic acid production stopped in the early stage of fermentation, whereas ethanol production continued to the end of fermentation, may be due to inhibition of LAB by ethanol and/or by efficient competition of yeasts for substrates. Indeed, like in traditional African brewing processes (Jespersen,

Table 1
Kinetic parameters of pH change during *injera* sourdough fermentation.

Sample type	Initial pH	$(-\mathrm{dpH/d}t)_{max}$	Time (h) to reach $(-\mathrm{dpH/d}t)_{max}$	Time (h) to reach pH 4.5	Final pH
TwS-*injera*	5.4 ± 0.4	0.22 ± 0.08	5.7 ± 0.6	5.0 ± 2.0	3.6 ± 0.01
WrS-*injera*	6.3 ± 0.4	0.28 ± 0.11	6.0 ± 0.0	10.0 ± 0.0	4.0 ± 0.02
BW-*injera*	6.0 ± 0.4	0.22 ± 0.08	4.7 ± 1.2	9.0 ± 1.0	3.9 ± 0.05

TwS, teff–white sorghum; WrS, wheat–red sorghum; BW, barley–wheat.
Data are expressed as mean ± standard deviations.

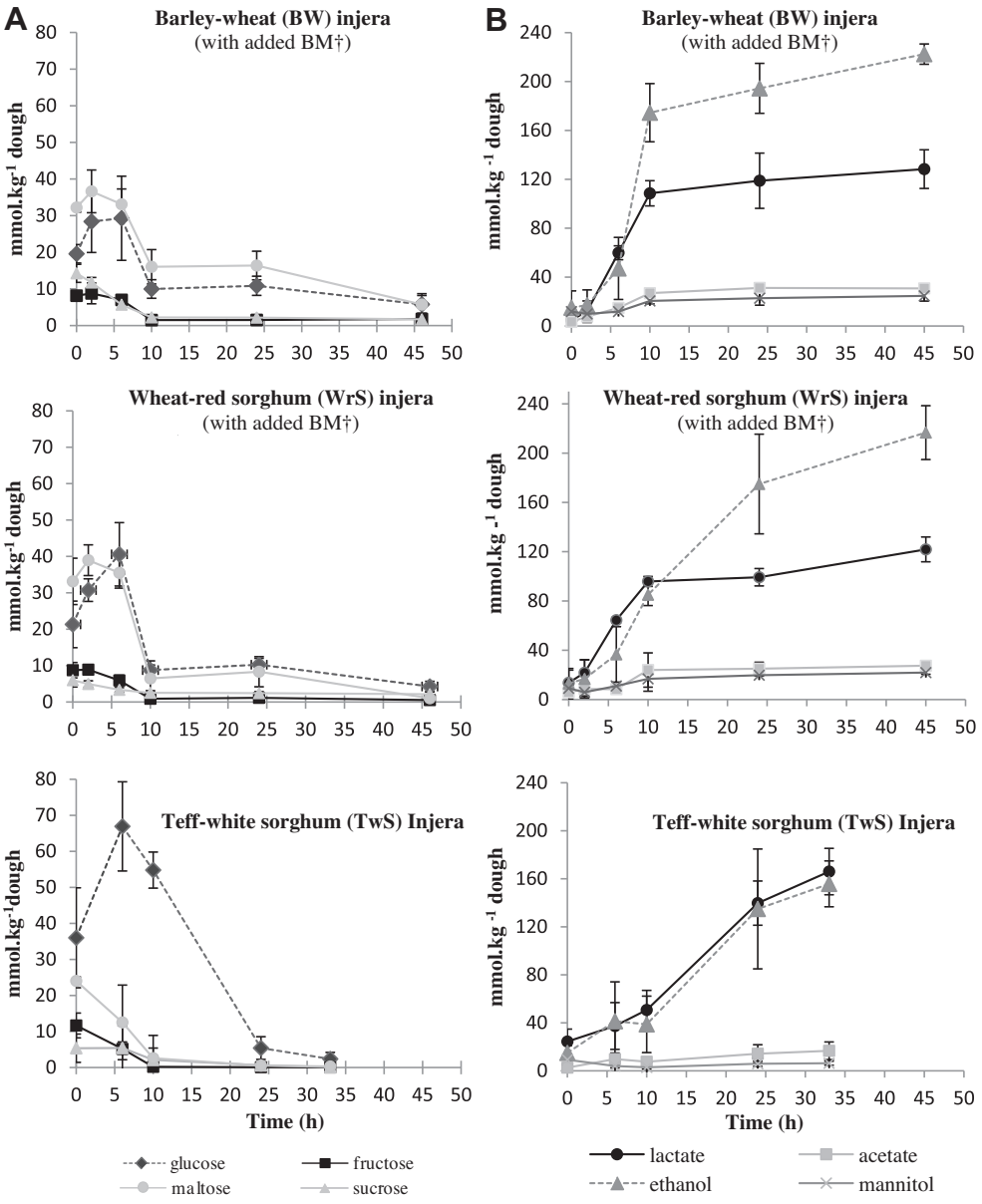

Fig. 3. Change in mono-, di-saccharide concentrations (A) and lactate, acetate, ethanol and mannitol concentrations (B) during fermentation of *injera* sourdough. BM†, barley malt; TwS, teff–white sorghum; WrS: wheat–red sorghum; BW, barley–wheat. Error bars represent the standard deviation of means.

2003), the addition of barley malt early in the process most probably triggered the formation of maltose and its consumption by yeasts to produce ethanol. In an attempt to modify the traditional African process to produce energy-dense fermented pearl millet gruels, Tou et al. (2007) showed that addition of barley malt changed the fermentation pattern by inducing maltose accumulation. However, contrary to what was observed in BW- and WrS-*injeras* fermentation, lactic acid production remained higher than that of ethanol. Such differences between BW- and WrS-*injeras* and pearl millet fermentation may not only be explained by the difference in cereals used, but also by the fermentation conditions, i.e., solid state fermentation and a lower rate of pH decrease for the *injera* sourdough and submerged fermentation with a more rapid drop in pH in pearl millet slurries that could affect diffusion of the substrates and hence their accessibility and rate of consumption by LAB or yeasts.

The fermentation pattern of the TwS-*injera* dough was more conventional since only one fermentation step was identified, and was comparable with other cereal fermentations in the absence of malt (Blandino, Al-Aseeri, Pandiella, Cantero, & Webb, 2003; Guyot, 2012). Simultaneous and equimolar production of both lactic acid and ethanol is typical of heterolactic fermentation and suggest the dominance of heterofermentative LAB.

In all types of *injera*, the higher lactic acid concentration than expected from consumption of free mono- and di-saccharides, suggests that starch may have provided extra carbon and energy due to the action of either malt amylases in the case of BW- and WrS-*injeras* or amylases produced by amylolytic lactic acid bacteria in the case of TwS-*injera,* as previously reported for the fermentations of other cereals (Diaz-Ruiz, Guyot, Ruiz-Teran, Morlon-Guyot, & Wacher, 2003; Guyot, 2012; Tou et al., 2007). Based on the fermentation patterns observed in the present study, LAB and yeasts and their relationships under the influence of malt addition and fermentation conditions require more detailed investigation.

Regarding raffinose and stachyose, which can cause gastric distress, their removal during fermentation of *injera* dough is consis-

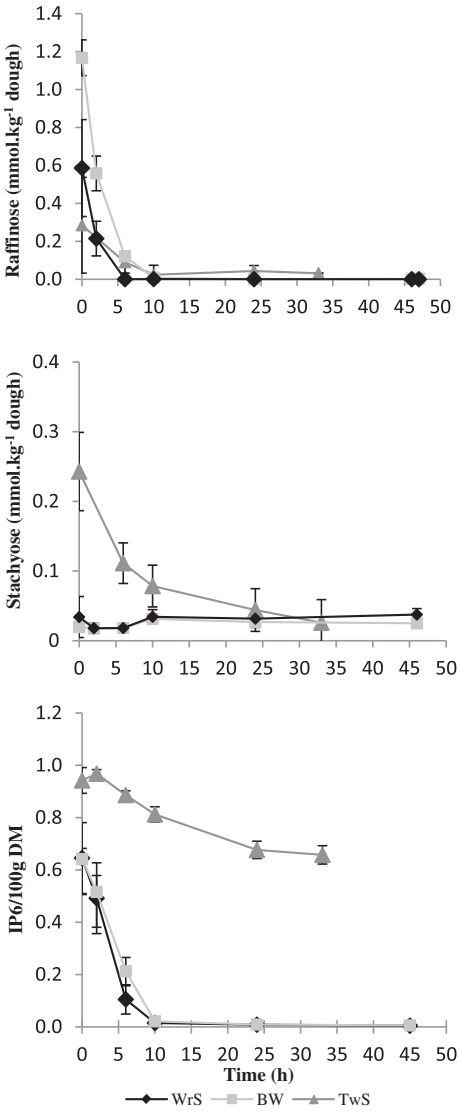

Fig. 4. Changes in α-galactosides and IP6 during *injera* sourdough fermentation. TwS, teff–white sorghum; WrS, wheat–red sorghum; BW, barley–wheat. Error bars represent the standard deviation of means.

Table 2
Phytase activities of flours used to prepare *injera*.

Sample type	Phytase activity (PU/g DM)
Barley	$0.36^{a} \pm 0.01$
Barley malt	$0.59^{c} \pm 0.15$
BW-flour	$0.49^{bc} \pm 0.01$
WrS-flour	$0.43^{b} \pm 0.07$
TwS-flour	$0.27^{a} \pm 0.00$

TwS, teff–white sorghum; WrS, wheat–red sorghum; BW, barley–wheat.
PU/g DM, phytase unit (PU) per gram dry matter.
Data are expressed as mean ± standard deviations.
Different superscripts represent statistically significant difference ($P < 0.05$).

tent with other works reporting fermentation of α-galactosides during natural fermentation of cereals by LAB (Tou et al., 2006; Songré-Ouattara et al., 2008). In the case of IP6, which reduces the bioavailability of minerals like iron, zinc, and calcium, its degradation is known to occur during lactic acid fermentation of cereals (De Angelis et al., 2003; Reale, Konietzny, Coppola, Sorrentino, & Greiner, 2007; Svanberg & Lorri, 1997). However, depending on the type of cereals and fermentation conditions, the efficiency of IP6 degradation can vary. Indeed, IP6 degradation was higher in BW and WrS-*injeras* than in pearl-millet fermentation (Tou et al., 2006), whereas in TwS-*injera,* phytate degradation was surprisingly low. Many factors are known to play a role in the rate and the extent to which IP6 is degraded, among which the endogenous phytase activities of the raw materials and the processing conditions like pH, which is known to modulate the activities of both plant and microbial phytases (Greiner & Konietzny, 2006). The higher endogenous phytase activity of flour blends containing barley and wheat is consistent with the results of previous studies showing higher activities for these cereals (Egli, Davidsson, Juillerat, Barclay, & Hurrell, 2002; Reale et al., 2007) and IP6 degradation under the range of optimal pH values observed for barley and wheat phytases (Greiner & Konietzny, 2006). Moreover, the addition of malt in BW- and WrS-*injeras* possibly helps create a favourable environment for yeasts with phytase activities, indeed some strains of yeast species like *Saccharomyces cerevisiae* are known to display phytase activity (Vats & Banerjee, 2004). The low efficiency of IP6 degradation during TwS-fermentation may be due to the low endogenous phytase content of the grains and to poor microbial activity. Based on the results obtained in BW- and WrS-*injeras*, degradation of phytate in TwS-*injera* could be promoted by the addition of malt.

5. Conclusion

Differences in the composition of flour blends used to prepare *injera* influenced fermentation patterns and hence the final composition of the sourdough. More striking was the difference in IP6 degradation patterns. Given the nearly complete degradation of phytate in BW and WrS-*injeras*, mineral bioavailability in these *injeras* is less likely to be hampered by IP6. Whether such differences in IP6 hydrolysis will result in products with different mineral bioavailability is currently under investigation. On the other hand, particular attention will have to be paid to identifying methods to improve IP6 degradation in TwS-*injera*.

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4.2.3 Complementary results: phytase activity of cereal grains used to prepare injera flours

Table 4.5 presents the phytase activity of the different cereal grains used in the preparation of injera flours. All grains had similar phytase activities except white sorghum that had a superior activity equivalent to that of barley malt. This is surprising as previous results have reported low phytase activity in sorghum (Egli et al., 2002). Varietal differences between the sorghum analyzed in the present study and previous ones may explain such differences. However, compared to WrS and BW, relatively lower phytase activity was observed in the blend containing white sorghum. This might mean that process parameters such as extent of decortication are likely to influence enzymatic activities. Phytases are usually found in the outer layer of cereal grains (Steiner et al., 2007) and thus with the removal of the outer layer due to decortication, significant amount of phytases can be lost, and this loss may vary depending on cereal type. Future studies may however need to be conducted to confirm such relationships.

Table 4.5: Apparent phytase activity of raw cereals use in injera preparations

	Phytase activity (PU/g DM)
Raw grains (whole)	
Teff	$0.35^{a} \pm 0.03$
Barley	$0.36^{a} \pm 0.01$
Barley malt	$0.59^{b} \pm 0.15$
Wheat	$0.34^{a} \pm 0.03$
Red sorghum	$0.25^{a} \pm 0.01$
White sorghum	$0.52^{b} \pm 0.06$

PU/g DM, phytase unit (PU) per gram dry matter; Data are expressed as mean ± standard deviations; Different superscripts represent statistically significant differences ($P < 0.05$).

4.3 Iron and zinc bioavailability estimation in Ethiopian staples

4.3.1 Introduction

The fermentation of BW- and WrS-injeras has resulted in >95% phytic acid degradation suggesting better iron and zinc bioavailability than in TwS injera for which only 28% degradation was observed. However, the effect of phytate degradation on mineral bioavailability is also dependent on the amount of other absorption inhibitors, notably polyphenols. Furthermore, *injera* is always consumed with legume-based stews and thus any positive effect consequent to phytate degradation may be offset if the stews contain significant amount of phytate.

In the present section (4.3), the influence of traditional household processing of *injeras* and legume-based stews on iron, zinc, calcium, phytate, fiber (ADF), and iron-binding polyphenol was evaluated. The iron and zinc bioavailability in the different *injeras* and stews was also estimated by using molar ratios (phytate:iron / phytate:zinc), in-vitro bioaccessibility, and iron absorption algorithm predictions. The possible limitations of using phytate:iron molar ratios in the estimation of iron bioavailability in case of high iron contamination have also been highlighted.

The study findings are presented in the form of a submitted manuscript, accompanied by supplementary results.

4.3.2 Changes in mineral absorption inhibitors during traditional processing of Ethiopian injera and accompanying stews: implications for predicted iron bioavailability and bioaccessibility- Submitted to *Plant Foods for Human Nutrition*

Changes in mineral absorption inhibitors during traditional processing of Ethiopian injera and accompanying stews: implications for predicted iron bioavailability and bioaccessibility

Running title: *Iron and zinc bioavailability predictions of Ethiopian staple foods*

Kaleab Baye[1,2], Claire Mouquet-Rivier[2*], Christèle Icard-Vernière[2], Christian Picq[2], Jean-Pierre Guyot[2]

[1] Center for Food Science and Nutrition, Addis Ababa University, P.O.Box:150201, Addis Ababa, Ethiopia

[2] IRD UMR 204 Nutripass, IRD/UM1/UM2, BP 64501, 34394 Montpellier Cedex 5, France

[*]Corresponding author: claire.mouquet@ird.fr ; phone +33467416295

Abstract

Possible changes in mineral bioavailability during processing of different types of *injera* and accompanying legume-based stews sampled in Ethiopian households were assessed using different methods: phytate:mineral molar ratio, absorption prediction algorithm and in vitro availability measurements.

Most foods analyzed were rich in iron, but most of the iron likely resulted from soil contamination. The highest iron, zinc and calcium contents were found in teff-white sorghum (TwS) *injera* and flour. The lowest IP6:Fe, and IP6:Zn molar ratios were found in barley-wheat (BW) and wheat-red sorghum (WrS) *injeras*. Although ideal IP6:Fe molar ratios (<0.4) were found in BW and WrS injeras, no significant difference in *in-vitro* iron bioaccessibility and algorithm predicted absorption was observed. In *injera*, IP6 degradation alone is unlikely to improve iron bioavailability, suggesting interactions with other absorption inhibitors. The use of IP6:Fe molar ratios to predict bioavailability may thus be less appropriate for iron contaminated foods.

Keywords: iron, zinc, calcium, phytic acid, bioaccessibility, algorithm, complementary food

1. Introduction

Mineral deficiencies, especially those of iron and zinc, are among the major causes of childhood morbidity and mortality in low income countries (WHO, 2009). The etiological factors responsible for the development of such deficiencies are a low mineral intake, physiological states that increase requirements, diseases that induce excessive loss or impair utilization, and low bioavailability (Hotz & Brown, 2004).

The bioavailability of a nutrient can be defined as the proportion of the ingested nutrient that can be used for normal body functions (Fairweather-Tait, Phillips, Wortley, Harvey, & Glahn, 2007). Zinc and iron are among the micronutrients with the lowest absorption ratios in the gastrointestinal tract. Depending on the form of the mineral and the food matrix, only 15 to 50% of zinc, and 2 to 35% of ingested iron are available for absorption (Hunt, 2003).

Plant-based diets contain large amounts of mineral absorption inhibitors like phytic acid (myo-inositol hexaphosphate, IP6) and its associated salts (phytates). Phytates form insoluble complexes with positively charged metal ions and hence reduce bioavailability of minerals (Lonnerdal, 2000). However, phytate degradation has repeatedly been reported in processes such as fermentation (Leenhardt, Levrat-Verny, Chanliaud, & Remesy, 2005). But to improve iron or zinc bioavailability, the extent of degradation needs to be greater than 90% (Hurrell, 2004).

In Ethiopia, like in most developing countries, diets of young children and adults alike are mainly plant based. The staple meal is *injera*, a traditional cereal-based fermented pancake, mostly consumed with legume-based stews like *shiro* stew (a broad bean or grass pea stew) or split field pea stew. *Injera* is prepared from fermented cereal dough, the resulting sourdough is then diluted in hot water to form a batter used to prepare the pancake. In north Wollo (northern Ethiopia), *injera* is traditionally prepared from different cereal mixtures: teff and white sorghum, wheat and red sorghum, and wheat and barley (Baye, Guyot, Icard-Vernière, Mouquet-Rivier et al, 2013[a]). Around 2% barley malt is added to wheat/red sorghum and wheat/barley flours. In wheat/red sorghum, and wheat/barley *injeras* IP6 degradation has been shown to be nearly complete whereas in *injera* made from teff and white sorghum, only 28% of IP6 was degraded (Baye, Mouquet-Rivier, Icard-Vernière, Rochette, & Guyot, 2013[b]), suggesting that mineral bioavailability could depend on the mixture of cereals used. Previous attempts to assess the bioavailability of minerals in Ethiopian staples were based on phytate:Fe and phytate:Zn molar ratios (Abebe, Bogale, Hambidge, Stoecker, Bailey, & Gibson, 2007; Umeta, West, & Fufa, 2005). However, given the large amounts of contaminant iron in most foods consumed in Ethiopia (Abebe et al.,

2007), and its poor solubility in comparison with that of the iron intrinsic to the food (Harvey, Dexter, & Darnton-Hill, 2000), whether the use of the phytate:iron molar ratio to estimate iron bioavailability is still valid is uncertain.

Furthermore, not only phytates but also polyphenols, and to some extent calcium and fibers have negative effects on mineral absorption (Hurrell & Egli, 2010; Lestienne, Caporiccio, Besancon, Rochette, & Treche, 2005a).

The objective of the present study was to estimate the bioavailability of iron and zinc in the different types of *injera*s and legume-based stews sampled in Ethiopian households. To this end, phytate:zinc and phytate:iron molar ratios, iron absorption algorithm and in vitro iron dialysability tests were used.

4. Materials and methods

2.1 Materials

Seventy-six households in two villages, one located in the highlands and one in the lowlands in north Wollo, northern Ethiopia, were surveyed to determine the type of cereals and legumes consumed and the blend proportions usually used in the preparation of staple foods (Baye et al., 2013[b]). Accordingly, the raw materials: teff, barley, wheat, red/white sorghum, grass peas, broad beans and split field peas, were purchased at local markets close to the villages surveyed and were given to local women to prepare according to local traditions but using standardized blend proportions.

2.2. *Processing and sampling*

The household preparations of *shiro* and split field pea stews (*n=5* each), and *injeras* made from blends of barley and wheat (BW) and wheat and red sorghum (WrS) in the village located in the highlands (*n=3* each) and teff and white sorghum (TwS) in the village located in the lowlands (*n=4*) were observed (Fig.1 & 2). Processing steps including time, temperature, pH, mass of materials used and products were recorded. Separate samples were collected for the determination of dry matter (DM) content and biochemical analysis. Raw (grains), intermediary (*injera* flour blends) and final products (*injera* and stews) were analyzed for contents in iron, zinc, calcium, acid detergent fiber (ADF), iron-binding phenolic compounds (galloyl and catechol groups), and phytic acid (IP6). In order to limit variability due to variation in the composition of raw materials, grains from the same batch were used.

The samples were sealed in polyethylene bags free of trace elements and transferred to a deep freeze (- 20°C) at a local health center within two hours of collection. All samples were transported in a pre-cooled (-20°C) ice box and were lyophilized before further analysis.

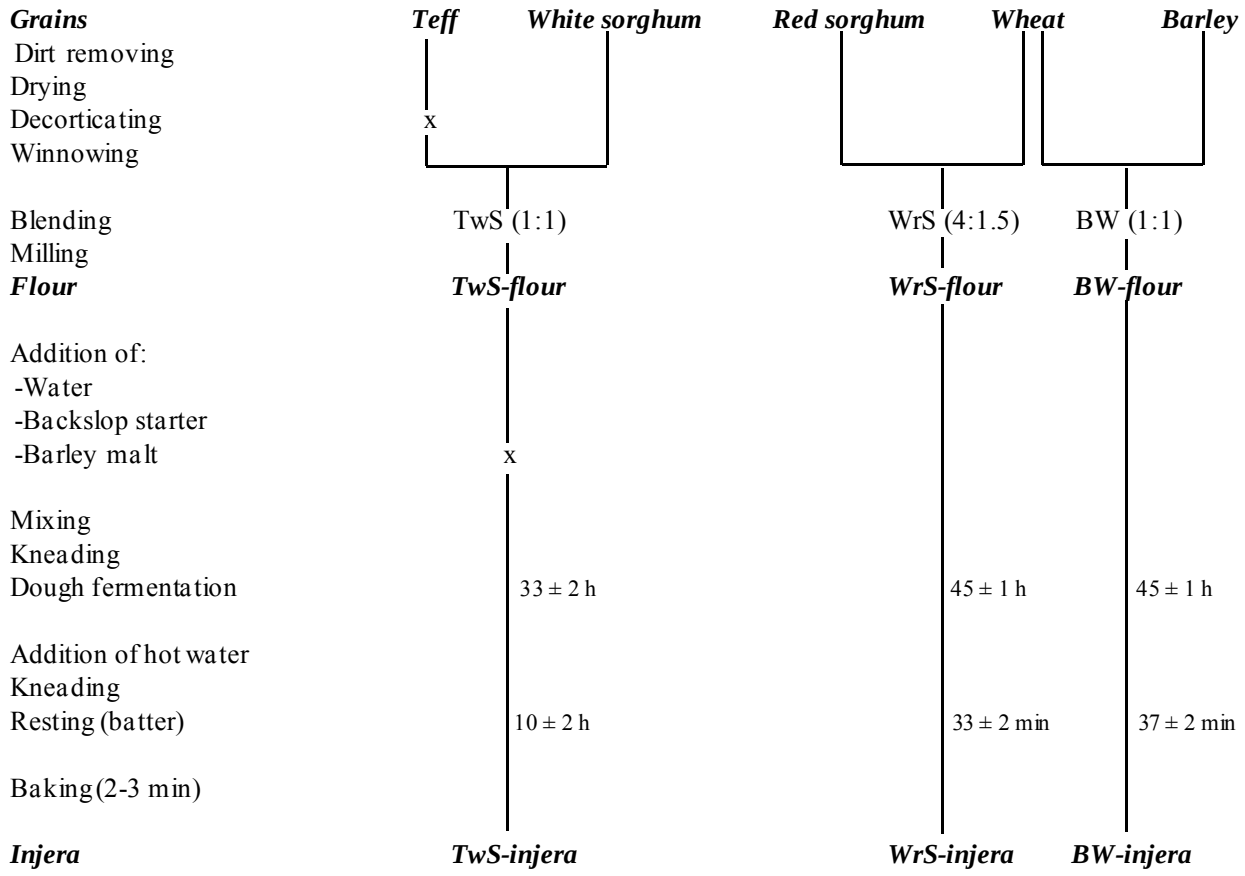

Fig.1: *Injera* processing flow diagrams

TwS, WrS, and BW stand for Teff-white sorghum, Wheat-red sorghum and Barley-wheat, respectively.
x indicates that the corresponding step was not included.
Products in bold and italics are those for which samples were collected and analysed

Prior to biochemical analysis, grains and lyophilized samples were milled in a laboratory mill (IKA M20, Labortechnik, Staufen, Germany) to obtain a flour that passed through a 500 µm sieve.

Iron analysis was performed using both washed and unwashed grains. Grains were washed using running distilled water under laboratory conditions.

Iron bioaccessibility measurements were conducted on *as eaten* (non-lyophilized) samples.

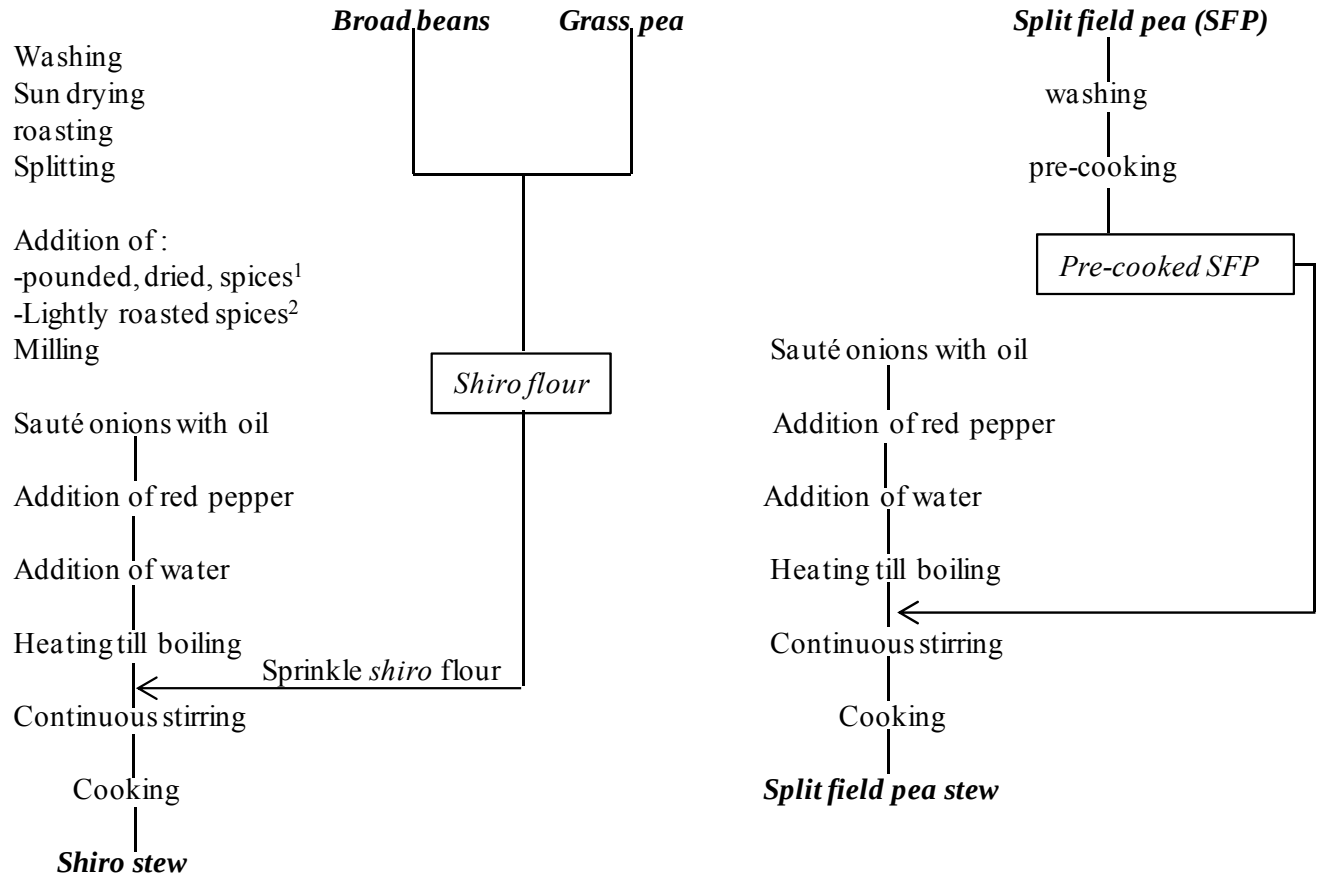

Fig.2: *Shiro* and split field pea stew processing flow diagrams

Products in bold and italics are those for which samples were collected and analysed
[1]Ginger (*Zingiber officinale),* Garlic (*Allium sativum),* Rue seeds *(Ruta chalepensis),* Red pepper (*Capsicum annuum)*
[2]Basil (*Ocimum basilicum),* False cardamom (Aframomum korarima), Ethiopian caraway *(Trachyspermum ammi),* Black pepper *(Piper nigrum)* Cloves *(Syzygium aromaticum),* Cinnamon *(Cinnamomum zeylanicum)*

2.3 Iron, zinc and calcium determination

Total iron, zinc and calcium were analyzed by flame atomic absorption spectrophotometry (AA800, Perkin Elmer, Les Ulis, France) after wet mineralization using an Ethos 1 microwave digester (Milestone, Sorisole, Italy) for 15 min at 200°C and with a maximum power of 1000 W as described in Hama, Icard-Verniere, Guyot, Picq, Diawara & Mouquet-Rivier (2011). Precautionary measures were taken to avoid adventitious contamination when handling the foodstuffs during sampling and analyses. The accuracy and precision of mineral analyses were checked by analysis of certified reference materials [BCR 191: brown bread and BCR 679: white cabbage].

2.4 Determination of mineral absorption inhibitors

Myo-inositol hexaphosphate (IP6) content was determined by high-performance anion-exchange chromatography using an AS-11 precolumn and column kit (Dionex, Sunnyvale, USA), after extraction of samples in acid solution (HCl 0.5 M) at 100°C for 6 min, according to the method described by Lestienne, Mouquet-Rivier, Icard-Verniere, Rochette & Treche (2005b). Results are expressed in mg IP6/100g DM.

Acid detergent fiber (ADF) content, which corresponds approximately to cellulose and lignin content, was determined using the gravimetric method of Van Soest (1963) with a Fibertec 1020 (Foss, Hillerod, Denmark).

Iron-binding polyphenols (galloyl and catechol groups) were analyzed using the method of Brune, Hallberg, & Skanberg (1991). The amount of galloyl and catechol groups was determined using ferric ammonium sulfate (FAS) reagent after 16 h extraction of samples with 50% dimethylformamide in acetate buffer (pH 4.4). This method is based on FAS's ability to form colored complexes with iron-binding galloyl and catechol groups. The absorbance of the colored complexes was measured at 680 nm and 578 nm, corresponding to the absorption maxima of Fe-galloyl and Fe-catechol complexes, respectively. After subtracting food blank absorbances, the concentration of galloyl groups (expressed as tannic acid equivalents) and catechol groups (expressed as catechin equivalents) was calculated from standard curves for catechin and tannic acid at the two wavelengths.

2.5 Determination of in vitro iron bioaccessibility

To determine iron bioaccessibility, enzymatic in vitro digestion was performed in two stages, according to the procedure described in Greffeuille, Kayode, Icard-Verniere, Gnimadi, Rochette & Mouquet-Rivier (2011). Briefly, a 10% DM dispersion of ground sample in ultrapure water was brought to 37°C, 20 µL of bacterial amylase (α-amylase from *Bacillus licheniformis*, Sigma A-3403-1MU) was added and the mixture was incubated at 37°C for 5 min. The pH was then brought to 2.0 with 1 M HCl, 1 mL of pepsin solution was added (Sigma, P-7000, 14,900 u/mL in 0.1 M HCl) and the mixture was incubated for 1 h at 37°C in a shaking water bath. Pepsin-digested samples were then transferred into separate tubes to which a dialysis bag (Spectra/por I dialysis tubing, MWCO 12–14 kDa) containing 20 mL of PIPES (piperazine-N,N0-bis-[2-ethanesulfonic acid] sodium salt) buffer (Sigma, P-3768) was introduced to mimic the gradual increase in pH during intestinal digestion. Tubes containing the sample and the dialysis bag were incubated at 37°C for 30 min to reach pH 7; a mix of

pancreatin (Sigma, P1750, 1.85 mg/mL) and bile extract solution (Sigma, B8631, 11 mg/mL in 0.1 M $NaHCO_3$) was added, and the resulting mixture was incubated for 2 h at 37°C in a shaking water bath. At the end of intestinal digestion, dialysis bags were removed and washed with pure water. The digested mixtures remaining in the tubes were centrifuged at 10,000g for 15 min at 4°C to separate the insoluble and soluble iron fractions, in the pellet and supernatant, respectively. The contents of the dialysis bags (dialysates), the supernatants and the pellets were weighed and analyzed for iron content by atomic absorption spectrophotometry as described above.

Bioaccessible Fe corresponds to the percentage of dialysable Fe. The iron contents of the pellets and supernatants corresponded to the insoluble and soluble iron, respectively. The dialyzable, soluble but non dialyzable (ND), and insoluble iron fractions were calculated on the basis of the total iron recovered at the end of the digestion, as follows:

$$\text{Dialyzable Fe \%} = C_D (W_D + W_S)/ (C_D W_D + C_S W_S + C_I W_I) \times 100 \quad (1)$$

$$\text{Soluble ND Fe \%} = W_S (C_S - C_D)/ (C_D W_D + C_S W_S + C_I W_I) \times 100 \quad (2)$$

$$\text{Insoluble Fe \%} = C_I W_I/ (C_D W_D + C_S W_S + C_I W_I) \times 100 \quad (3)$$

where C_D, C_S and C_I are iron concentrations (µg/100 g) in the dialysate, supernatant and pellet fractions and W_D, W_S and W_I are the weights (g) of dialysates, supernatants and pellets. All samples were analyzed at least in quadruplicate.

2.6 Prediction of iron absorption using algorithms

Iron absorption was predicted using Hallberg and Hulthen's (2000) algorithm by making use of analyzed values of phytic acid-phosphorus (Phytate-P), calcium (Ca) and tannic acid (TA). Ascorbic acid (AA) values were taken from the Ethiopian food composition table. The formula proposed by Beiseigel et al., (2007) was used:

$$\text{\% absorption} = 22.1 \times f_1 \times f_2 \times f_3 \times f_4 \quad (4)$$

Where the factor 22.1 corresponds to mean absorption from a meal containing no components known to enhance or inhibit iron absorption, adjusted to a 40% reference dose absorption

$$f_1 = 10^{[-0.30^* \log (1+\text{phytate-P})]} \quad (5)$$

$$f_2 = [1+0.01\ \text{AA} + \log \text{phytate-P} + 1]^*0.01^*10^{(0.8875^* \log (\text{AA}+1))} \quad (6)$$

$$f_3 = 10^{[0.4515-0.715^* \log \text{tannic acid}]} \quad (7)$$

$$f_4 = 0.4081+[0.6059/(1+10^{-(2.022-\log(\text{Ca}+1))^*2.919})] \quad (8)$$

The algorithm uses base-10 logarithms. Phytate-P, AA, TA and Ca were expressed in mg/meal. Phytate contents were converted into phytate-P by multiplying by the factor 0.283. Based on the results of a previous consumption survey among 12-23 month old children in

north Wollo (Baye et al., 2013[a]), an average meal portion of 40 g was used in the calculations (unpublished results). Factors f_1, f_2, f_3, and f_4 of this algorithm adjust for the effects of phytic acid, AA, TA and Ca respectively on iron absorption, and include interactions between these components.

2.7 Statistical analysis

Average values of biochemical analyses performed at least in triplicate were submitted to analysis of variance, followed by the Fisher's least significant difference (LSD) test, to compare the means at the 5% significance level, using the software SPSS version 15.

3. Results and discussions

3.1. Total iron, zinc and calcium contents

Among whole grains, *teff* contained the most iron followed by barley **(table 1)**. Wheat and red sorghum had the lowest iron contents. However, there was a significant reduction in iron contents in washed grains along with a simultaneous reduction in variability between replicates that resulted in similar iron contents between grains. The exception was *teff* which still had high and variable iron content (Table 1). This suggests that at least part of the iron content was due to soil contamination and that, even if the grains are washed before processing in the home (which is not always the case), this does not ensure that the extrinsic iron is completely removed particularly in the case of *teff*. In fact, *teff* has been associated with extreme soil contamination (Hallberg & Rassmussen, 1981) partly because it is traditionally threshed under the hooves of cattle (Bothwell et al., 1979). Although most cereal grains in Ethiopia are threshed in a similar way, the very small size of *teff* grains might mean it has more contact with the soil over a larger area.

Table 1: Iron, zinc and calcium contents of cereals, *injeras* and accompanying stews

	Mineral content (mg/100g DM)				
	DM	Iron		Zinc	Calcium
		Unwashed grains	Washed		
Raw grains (whole)					
Teff	88.8 ± 0.2	80.07 ± 17.44^{a}	31.61 ± 4.62^{a}	2.31 ± 0.17^{a}	78.84 ± 5.56^{a}
White sorghum	86.9 ± 0.2	14.14 ± 0.52^{c}	4.13 ± 0.28^{b}	1.36 ± 0.16^{b}	5.00 ± 0.01^{b}
Barley	86.2 ± 0.2	52.47 ± 3.64^{b}	6.09 ± 0.33^{b}	2.36 ± 0.07^{a}	10.69 ± 0.36^{c}
Wheat	88.8 ± 0.2	5.31 ± 0.23^{c}	3.65 ± 0.23^{b}	1.68 ± 0.10^{b}	15.15 ± 0.27^{d}
Red sorghum	86.5 ± 0.1	7.93 ± 0.55^{c}	3.53 ± 0.23^{b}	1.71 ± 0.21^{b}	5.82 ± 0.10^{b}
Flours					
TwS-F	90.9 ± 0.1	37.58 ± 0.64^{i}		1.67 ± 0.02	36.34 ± 0.70^{i}
BW-F	91.4 ± 0.1	8.79 ± 0.41^{j}		1.83 ± 0.08	11.56 ± 0.27^{j}
WrS-F	89.1 ± 0.2	12.39 ± 0.39^{k}		1.62 ± 0.10	11.69 ± 0.89^{j}
Injera (n*)					
TwS-*injera* (4)	39.9 ± 1.4	44.08 ± 2.59^{m}		2.60 ± 0.10	45.30 ± 1.15^{g}
BW-*injera* (3)	34.4 ± 2.6	15.36 ± 0.71^{n}		2.44 ± 0.07	14.15 ± 0.13^{h}
WrS-*injera* (3)	36.7 ± 1.4	16.22 ± 1.13^{n}		2.35 ± 0.07	13.78 ± 0.79^{h}
Stews					
Shiro (5)	14.6 ± 1.7	14.96 ± 1.31^{r}		3.05 ± 0.31	140 ± 9.13^{r}
Split field pea stew (5)	28.0 ± 1.9	8.38 ± 4.19^{s}		3.30 ± 0.54	93 ± 7.18^{s}

DM, TwS, WrS, and BW stand for dry matter, teff and white sorghum, wheat and red sorghum, and barley and wheat flour blends, respectively
Values are means ± standard error of mean. Different superscript letters for the same food type (raw, flours, *injera* and stews) in the same column indicate statistically significant differences (P <0.05).
*n=number of observations

Since cereal grains are seldom washed before being processed into flours, the flours used to prepare *injera* and the resulting *injeras* had similarly high and variable iron contents. The highest iron content was in TwS flour and *injera,* which had about three times more iron than WrS and BW. The iron content of the stews was also high, with the highest value in *shiro* (Table 1). Such results are not surprising since high variable iron content has already been reported in almost all cereal based foods analyzed in Ethiopia (EHNRU/FAO, 1998; Umeta et al., 2005; Abebe et al., 2007).

On the other hand, zinc contents were within the range commonly found in cereals. Teff and barley had higher zinc contents than white/red sorghum or wheat. The different flour blends and *injeras* had similar zinc contents. The stews were better sources of zinc than the *injeras* (Table 1).

Calcium content was highest in teff and lowest in white sorghum. The very high calcium content in teff compared with the other cereals has also already been reported (Abebe et al., 2007; Umeta et al., 2005).

TwS-flours and -*injeras* contained about three times more calcium than those of BW and WrS blends. Therefore, since white sorghum is relatively poor in iron, zinc and calcium, the addition of *teff* could be considered as fortification, and the resulting composite flour has better characteristics. The resulting stews were a better source of calcium than the *injeras*.

The relative consistency of the results for calcium and zinc unlike those for iron suggests that the contribution of soil contamination to these mineral contents was minimal. This was expected as the soil in the Wollo region is not calcareous and fertilizers containing zinc are not used by farmers.

In many developing countries including Ethiopia, contaminant iron substantially contributes to dietary iron intakes (Galan, Cherouvrier, Zohoun, Zohoun, Chauliac & Hercberg 1990). Reported intakes of Ethiopian children exceed recommendations (Baye et al, 2013[a]), suggesting that, provided the iron is bioavailable, the amount supplied by these foods is sufficient.

3.2. Effect of processing on mineral absorption inhibitors

The bioavailability of minerals in plant-based foods is mainly determined by the balance between absorption inhibitors and enhancers. IP6 and polyphenols are among the major absorption inhibitors. Although less consistent, absorption inhibiting effects have also been reported for calcium and fibers (Hurrell & Egli, 2010; Lestienne, Caporiccio, Besancon, Rochette, & Treche, 2005a).

Most of the grains analyzed contained high IP6 contents (> 1000 mg/100g DM) and ADF **(**Table 2). Like in the flours, IP6 and ADF contents in BW and WrS blends were of the same order of magnitude, but TwS-flour had higher IP6 and ADF contents, probably due to the inclusion of a wholegrain (teff) in this blend (Fig. 1). Higher IP6 and ADF contents for whole grains than decorticated ones are expected since both IP6 and ADF are concentrated in the bran of cereals (Hama et al., 2011). Nevertheless, substantial quantities of IP6 remain even after decortication because a non-negligible amount of IP6 is also located in the endosperm.

Consistent with the results of previous studies, the fermentation of flours into *injera* resulted in a significant decrease in IP6 (Abebe et al., 2007; Umeta et al., 2005). However, although near complete reduction of IP6 was observed in BW and WrS injeras, little reduction of IP6

was observed in TwS *injera* (Table 2). A closer look at the chromatograms revealed nearly complete hydrolysis of lower inositol phosphates as well.

Table 2 Mineral absorption inhibitors in cereals, *injeras* and accompanying stews

Food type	Content (per 100 g DM)			
	IP6 (mg)	**Fiber (ADF) (g)**	**Iron-binding phenolics**	
			Galloyl groups (mg TAE)	**Catechol groups (mg CE)**
Raw grains (whole)				
Teff	1544 ± 43^{a}	9.80 ± 0.70^{a}	210 ± 15^{a}	200 ± 9^{a}
White sorghum	1505 ± 19^{a}	8.00 ± 0.71^{a}	181 ± 24^{a}	125 ± 24^{b}
Barley	1284 ± 34^{b}	8.43 ± 0.12^{a}	194 ± 15^{a}	$176 \pm 12b^{a}$
Wheat	1242 ± 51^{b}	5.02 ± 0.36^{b}	164 ± 11^{a}	83 ± 9^{c}
Red sorghum	1044 ± 28^{c}	8.08 ± 0.84^{a}	832 ± 10^{b}	1914 ± 34^{d}
Cereal-based products				
TwS-Flour	1322 ± 54^{i}	6.95 ± 0.23	105 ± 20	111 ± 37
TwS-*injera*	652 ± 23^{k}	8.44 ± 0.26	130 ± 15	115 ± 21
BW-Flour	1113 ± 83^{j}	3.83 ± 0.03	116 ± 5	105 ± 13
BW-*injera*	48 ± 13^{l}	4.43 ± 0.09	108 ± 16	85 ± 15
WrS-Flour	1021 ± 20^{j}	4.23 ± 0.18	437 ± 33^{j}	452 ± 40^{j}
WrS-*injera*	33 ± 8^{l}	4.83 ± 0.33	307 ± 25^{l}	342 ± 17^{l}
Stews				
Shiro	391 ± 42^{s}	4.10 ± 0.34^{s}	130 ± 6	94 ± 25
Split field pea stew	621 ± 26^{r}	7.90 ± 0.70^{r}	103 ± 6	82 ± 3

TwS, WrS, and BW stand for teff and white sorghum, wheat and red sorghum and barley and wheat flour blends, respectively; TAE: Tannic acid equivalent; CE: Catechin equivalent; Values are means ± standard errors; different superscripts in the same column for the same food type (raw, cereal-based products and stews) indicate statistically significant differences ($p < 0.05$).

Processing of flours into *injera* resulted in a small statistically non-significant increase in ADF. Greffeuille et al. (2011) reported a similar increase in ADF and linked it to the probable structural changes that may occur during cooking.

Among grains, the largest quantities of iron-binding phenolic groups (galloyl and catechol groups) were found in red sorghum (Table 2). The amount of iron-binding phenolics was comparable to that reported by Towo et al. (2006) in tannin-rich red sorghum varieties.

Although the iron-binding galloyl and catechol groups were strongly reduced during processing of WrS-*injera*, the final content was still about three times higher than in the other types of *injera*. Svensson, Sekwati-Monang, Lutz, Schieber, & Ganzle (2010) showed that microbial fermentation of red sorghum reduced total polyphenol content and altered the

polyphenol profile. However, the difference in polyphenol reduction between fermented mixes of cereals may be due to different locations, and the nature and/or concentrations of polyphenols in the kernels. For instance, in sorghum, free phenolic acids are found in the outer layers of kernels, whereas bound phenolic acids are associated with the cell wall, and their relative content in the kernel differs depending on the variety of sorghum concerned (Dykes & Rooney, 2006).

For these reasons, processing, mainly decorticating and fermentation, are likely to affect the extent of enzymatic degradation during fermentation and consequently the availability of minerals in the *injera*. Nevertheless, in the present study, the amount of iron-binding phenolic groups remaining in the WrS *injera* was still high enough to potentially inhibit iron absorption.

3.3 Estimation of mineral bioavailability

3.3.1 Phytate:mineral molar ratios

As a result of the degradation of IP6 during fermentation (Table 2), IP6:mineral molar ratios in the *injeras* were significantly lower than in flours (fig. 3). Umeta et al. (2005) and Abebe et al. (2007) already reported decreases in IP6:mineral molar ratios in teff and sorghum *injera*, but not to values as low as those obtained for BW and WrS blends in this study.

Based on absorption studies in humans, IP6: Zn molar ratios <5, between 5 and 15, and >15 have been associated with high, moderate and low zinc bioavailability, corresponding to around 50%, 30% and 15% of total zinc, respectively (Gibson, 2006).

Based on these critical values, the IP6:zinc molar ratios in WrS and BW-*injeras* indicate high Zn bioavailability (fig. 3A), whereas low bioavailability is expected in TwS *injera* (IP6:Zn molar ratio =25). Among the stews analyzed, the zinc in *shiro* may be of moderate bioavailability (IP6:Zn molar ratio =13) whereas low bioavailability is predicted for split field pea (SFP) stew (IP6:Zn molar ratio =20).

Given the fact that calcium may exacerbate the effect of phytate on zinc absorption (Cossack and Prasad, 1983), the use of the [IP6 x Ca]:Zn molar ratio has been suggested rather than the IP6:Zn ratio. The deleterious effect of calcium on zinc absorption is expected for foods with [IP6 x Ca]:Zn values exceeding 200 (Hemalatha, Platel & Srinivasan, 2007). None of the foods analyzed in the present study had molar ratios greater than 200 (fig. 3B). This confirms previous reports stating that calcium contents in cereals and pulses are unlikely to impair zinc bioavailability (Hemalatha et al., 2007).

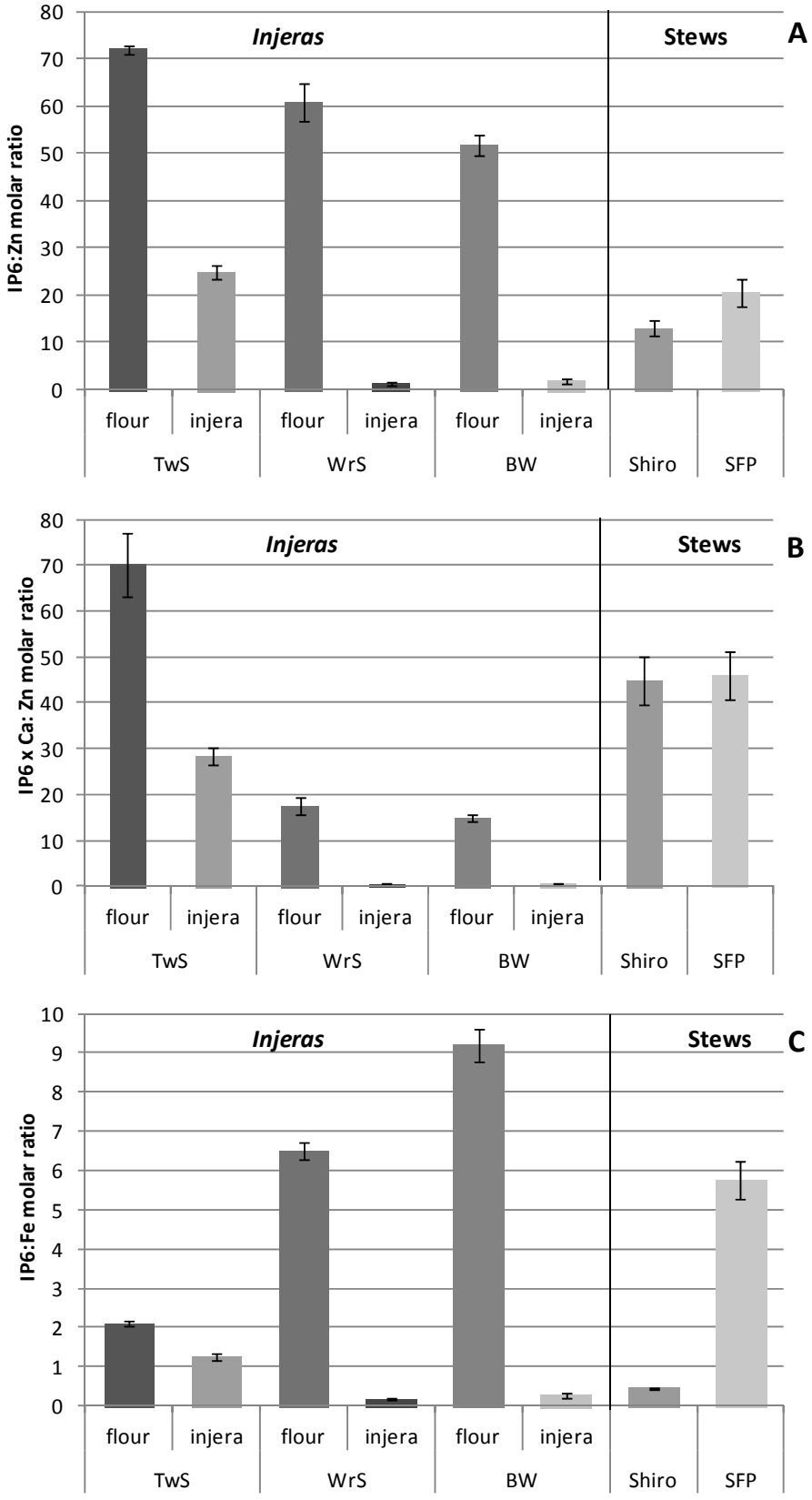

Fig. 3: Changes in IP6:Zn **(A)**, IP6xCa:Zn **(B)** and IP6: Fe **(C)** molar ratios in injeras and stews during processing.

Error bars represent mean ± SEM

On the other hand, to significantly improve iron absorption, the IP6:iron molar ratio in plant-based foods should be less than 1, and preferably less than 0.4 (Hurrell, 2004). As a result of fermentation, the IP6:Fe molar ratio was less than 0.4 in BW and WrS *injeras*, whereas it remained >1 in TwS *injera* (fig. 3C). The IP6:Fe molar ratio in *shiro* was lower than 1, whereas in SFP stews the ratio was high (near 6), suggesting that *shiro* could be a better source of bioavailable iron.

However, in cases where a large quantity of contaminant iron is present, IP6:Fe molar ratios may not be a good indicator of iron availability, as both contaminant and intrinsic iron are included in the calculation.

3.3.2 Predicting iron absorption using the Hallberg & Hulthén algorithm

Among existing absorption prediction algorithms, that of Hallberg & Hulthén (2000) is the most comprehensive, as it accounts for the effects of most absorption inhibitors and enhancers as well as their interactions. Moreover, fractional absorption is adjusted to absorption of 40% of a reference dose, which corresponds to a marginal serum ferritin level (12-15 µg/L). For these reasons, this algorithm was used in the present study (Table 3).

The biggest increase in predicted fractional absorption was found for the processing (fermentation) of WrS flours into *injera*, for which total IP6 degradation as well as a partial decrease in iron-binding phenolics content were observed. This suggests that both IP6 and iron binding phenolics may need to be significantly reduced to increase the absorption of iron.

For BW *injera,* the predicted fractional iron absorption was about twice higher than in other types of *injera*. This may be due to the low IP6 content and the relatively small amount of galloyl groups per meal in this *injera.* The highest fractional absorption was predicted for *shiro*, probably because of the low DM content and consequently small quantity of absorption inhibitors consumed per meal.

Table 3: Iron dialysability and iron absorption predicted by the Hallberg & Hulthén algorithm

Samples	Iron fraction				Algorithm calculation†
	Dialyzable		Soluble ND†	Insoluble	
	mg/100g DM	**%**	**%**	%	**%**
TwS- flour	0.87 ± 0.39^{c}	1.6 ± 0.6^{a}	2.6 ± 0.3^{ab}	95.8 ± 0.4^{a}	3.1
TwS-*injera*	0.77 ± 0.14^{bc}	1.0 ± 0.2^{a}	3.7 ± 0.2^{b}	95.2 ± 0.3^{a}	2.7
WrS- flour	0.25 ± 0.07^{ab}	1.5 ± 0.4^{a}	2.2 ± 0.4^{ab}	96.3 ± 0.6^{a}	2.5
WrS- *injera*	0.36 ± 0.06^{abc}	2.5 ± 0.4^{ab}	1.2 ± 0.8^{a}	96.5 ± 1.3^{a}	3.5
BW- flour	0.33 ± 0.07^{ab}	2.5 ± 0.5^{ab}	2.3 ± 1.1^{ab}	95.4 ± 1.7^{a}	6.4
BW- *injera*	0.17 ± 0.04^{a}	1.3 ± 0.3^{a}	1.7 ± 0.5^{a}	97.0 ± 0.5^{a}	6.7
Shiro	0.63 ± 0.06^{abc}	3.8 ± 0.7^{b}	8.7 ± 0.5^{c}	87.5 ± 0.8^{b}	8.9

TwS, WrS, and BW stand for teff and white sorghum, wheat and red sorghum and barley and wheat flour blends, respectively; †Soluble ND: soluble non-dialyzable; Values are means ± standard errors.
†predicted iron bioavailability using the Hallberg & Hulthèn's (2000) algorithm calculated for an average 40 g portion for young children (12-23 months). Different superscript letters in the same column indicate statistically significant differences, $p < 0.05$.

The iron absorption algorithm was developed based on food products that are not contaminated with extrinsic iron, thus, predicted fractional absorption may only apply for intrinsic iron. Although this method allows better prediction of the effect of processing on the fractional absorption of iron, it is limited by the fact that it requires the determination of the share of extrinsic and intrinsic iron in order to estimate the quantity of iron likely to be absorbed. Furthermore, little information is provided regarding the bioavailability of the extrinsic iron. Therefore, to evaluate the availability of both intrinsic and extrinsic iron in the present study, *in-vitro* iron dialysability measurements were performed.

3.3.3 In-vitro iron dialyzability

The percentages of dialyzable iron after simulated gastrointestinal digestion of *injeras* and *shiro* were low (1-4 %) (Table 3). This fraction represents the proportion of iron that can be released from the food matrix into the lumen of the gastrointestinal tract, and is then potentially available for absorption by enterocytes (i.e., bioaccessible). However, given the high iron content of these foods, particularly of TwS *injera* (Table 1), even such a low dialyzable fraction can represent a significant quantity of potentially bioavailable iron. The

soluble ND iron fraction in TwS *injera* was also higher than in the other *injeras,* suggesting that more iron is potentially absorbable if it is liberated from chelating agents (complexes).

In addition, the higher total amount of both dialyzable and soluble ND iron in TwS *injera* and flours than in BW and WrS (Table 3), suggests that either TwS flour and *injera* have higher intrinsic iron contents, and/ or that some of the extrinsic iron is dialyzable and thus bioaccessible. An earlier study reported that 2.5% of the contaminant iron in teff exchanged with added extrinsic radio-iron tracer and thus became potentially bioavailable (Hallberg & Bjorn-Rassmussen, 1981). A more recent study also showed that fermentation can increase the dialyzability (bioaccessibility) of contaminant iron (Greffeuille et al, 2011). However, since the bioavailability of contaminant iron depends on several factors including its source and the form in which it is present, this deserves further investigation. Nevertheless, irrespective of the type of *injera,* more than 95% of the iron was in insoluble form, probably because of the large proportion of iron present due to contamination.

Consistent with results from the iron absorption predictions, *shiro* had a significantly higher percentage of dialyzable iron than the *injeras*. The quantity of dialyzable iron was similar to that in TwS *injera* and significantly higher than that in BW and WrS *injera*. Furthermore, the soluble ND iron fraction was even higher than in TwS-*injera*, suggesting that more iron is likely to dialyze if liberated from soluble large complexes. In *shiro*, several condiments including onion and garlic are added during preparation (fig. 2). The high sulfur-containing amino acid content in onions and garlic has recently been shown to increase iron and zinc bioaccessibility (Gautam, Platel, & Srinivasan, 2010). Whether the higher bioaccessibility in *shiro* is due to the added onions and garlic needs to be investigated, especially since these ingredients are commonly used in the preparation of Ethiopian stews.

Despite the fact that IP6:Fe molar ratios in BW and WrS *injeras* were low enough to mean a significant improvement in iron bioavailability, no difference in iron dialysability was actually observed (Table 3). This may be because the critical IP6:Fe molar ratio needed to significantly improve iron absorption was determined during studies of foods not contaminated with extrinsic iron and thus may be less suitable for predicting bioavailability of iron contaminated foods. It could also be due to the fact that IP6 is not the only iron absorption inhibitor, thus its removal in the presence of other inhibitors like iron-binding phenolic groups may result in little improvement in absorption. This is consistent with the results of iron absorption predictions and findings reported in a recent human iron absorption study that showed that dephytinization in the presence of polyphenols did little to improve iron absorption (Petry, Egli, Zeder, Walczyk, & Hurrell, 2010).

Although direct quantitative comparisons cannot be made between results of the algorithm and *in vitro* dialysability, the algorithm predicted higher fractional absorption in all *injeras* and also in *shiro*. This suggests differences in bioavailability between intrinsic and extrinsic iron, the former being more bioavailable.

Conclusion

Most of the foods analyzed were rich in iron, but a high proportion of iron was associated with soil contamination. The low IP6:iron and IP6:zinc molar ratios in BW and WrS *injeras* and to some extent in *shiro* were within the range reported to facilitate iron absorption. However, *in vitro* iron dialyzability and iron absorption algorithm predictions did not reveal any significant improvement in bioaccessibility/bioavailability as a result of the fermentation step of *injera* processing. This was true despite the almost complete removal of IP6 and the partial degradation of iron-binding phenolic compounds (i.e. WrS *injera*).

Knowledge of the exact amount of contaminant iron and its bioavailability may thus be a pre-requisite for the correct use of IP6:iron molar ratios to predict bioavailability in foods containing contaminant iron.

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4.3.3 Complementary results: Food composition results

The protein and fat contents in TwS-and WrS-injeras were higher than in BW injera (table 4.6). This may be related to grain related differences. Not surprisingly, the legume-based stews had higher protein and fat contents than the injeras. The fat content in the stews was highly variable as the amount of oil added varied depending on the preference of the households.

Table 4.6: Protein and fat content of prepared foods

Food type *(n*)*	**Protein** g/100g DM	**Fat** g/100g DM
Injera		
TwS *injera (4)*	10.6 ± 0.2	3.0 ± 0.8
WrS *injera (3)*	11.2 ± 0.1	2.3 ± 0.2
BW *injera (3)*	8.7 ± 1.5	1.8 ± 0.1
Stews		
Shiro (5)	16.5 ± 3.6	28.2 ± 13.6
SFP stew *(5)*	19.3 ± 1.3	14.9 ± 7.4

*n= number of observations; TwS: Teff-white sorghum; WrS: Wheat-red sorghum; BW: Barley wheat; SFP: split field pea; values are mean ± standard deviations

Table 4.7: Polyphenol and fiber contents in cereal grains, *injeras* and accompanying stews

	Content /100g DM		
Food type	Tannins (mg CE)	Total polyphenols (mg GAE)	ADF Fibers (g)
Raw grains			
Barley	27 ± 0.7	310	8.43 ± 0.20
Wheat	17 ± 1.2	143	5.02 ± 0.63
Red sorghum	106 ± 3.5	1607	8.08 ±1.46
White sorghum	13 ± 1.2	81	5.02 ± 0.63
Teff	16 ± 1.4	140	9.80 ± 1.22
Flours			
BW-F	24 ± 2.2	246	3.83 ± 0.03
WrS-F	48 ± 2.2	521.8	4.23 ± 0.18
TwS-F	13 ± 1.6	132.9	6.95 ± 0.23
Injeras			
BW-*injera*	35 ±7.6	222 ± 91	4.43 ±0.09
WrS-*injera*	42 ±15.6	339 ± 134	4.83 ± 0.33
TwS-*injera*	19 ± 8.8	173 ± 33.9	8.44 ± 0.26

CE: cyanidin equivalent; GAE: gallic acid equivalent; F- flour

Red sorghum had markedly higher tannin and total polyphenol contents. The lowest polyphenol content was found in white sorghum. The blending of the cereals resulted in

injera flours with graded amount of polyphenols with TwS-flour having the lowest content, followed by BW-flour and WrS-flour. The fermentation of the flours into injera resulted in polyphenol contents with high inter-household variability as can be seen by the elevated standard deviations. This could suggest variable fate of polyphenols according to fermentation conditions, and thus requires further investigations.

Relative to broad bean and grass pea, split field pea had lower iron and calcium contents, but lower total polyphenol contents (table 4.8). The amount of polyphenols in grass pea and broad bean were considerably high.

Table 4.8: Iron, zinc, calcium and mineral absorption inhibitors in raw legume seeds

	Content /100g DM				
Legume seeds	**Fe (mg)**	**Zn (mg)**	**Ca (mg)**	**IP6 (g)**	**Total polyphenols (mg)**
Grass pea	6.93	2.37	90.63	0.32	473
Broad beans	6.19	3.92	68.01	1.08	706
Split field pea	4.83	2.05	16.27	0.69	80

4.4 Relative effects of mineral absorption inhibitors on iron bioaccessibility in injera flours

4.4.1 Introduction

In the previous section (4.3), the effect of fermentation on iron bioaccessibility was investigated. Despite nearly complete phytic acid degradations in the WrS and BW *injeras*, no significant improvement in iron bioaccessibility was observed. Several hypotheses that may explain these findings have been postulated. These included, the presence of contaminant iron that was mostly insoluble thus less bioavailable, the high content of polyphenols, or the presence of other absorption inhibitory factors (i.e. fibers).

In the present section (4.4), the relative effect of potential iron absorption inhibitors was evaluated by making use of a mechanistic approach that involved the application of exogenous enzymes targeting phytates, polyphenols, and fibers. The study gave an insight on the extent to which iron bioaccessibility can be improved by food-based approaches that rely on decreasing absorption inhibitors. The findings of this study may also give better guidance for future optimizations of household food processing that aim to improve iron and zinc bioaccessibility.

The results are presented in the form of a manuscript prepared for submission, accompanied by supplementary results.

4.4.2 Enzymatic degradation of phytate, polyphenols and dietary fibers in Ethiopian injera flours: Effect on iron bioaccessibility. Manuscript to be submitted to *Food Chemistry*

Enzymatic degradation of phytate, polyphenols and dietary fibers in Ethiopian injera flours: Effect on iron bioaccessibility.

Kaleab Baye[1,2] Jean-Pierre Guyot[2], Christèle Icard-Vernière[2], Isabelle Rochette[2], Claire Mouquet-Rivier[2]

[1] Center for Food Science and Nutrition, Addis Ababa University, P.O.Box:150201, Addis Ababa, Ethiopia

[2] IRD UMR 204 Nutripass, IRD/UM1/UM2, BP 64501, 34394 Montpellier Cedex 5, France

[*]Corresponding author: claire.mouquet@ird.fr ; phone +33467416295

Abstract

The effect of removing phytate (IP6), iron-binding polyphenols, and dietary fibers on fractional iron bioaccessibility of wheat-red sorghum (WrS) and teff-white sorghum (TwS) flour blends was evaluated by the application of exogenous enzymes.

Although treatment with phytases resulted in >90% reduction in IP6 and an IP6: Fe molar ratio <1, this did not improve iron bioaccessibility (P>0.05).

Treatment with phytase + xylanase + cellulase (P+X+C) increased iron bioaccessibility in both TwS and WrS flour blends, whereas phytase+polyphenol oxidase (P+PPO) treatment showed improvement only in TwS flour blend. The higher iron bioaccessibility for P+X+C+PPO treatment relative to P+PPO in WrS flour suggests that part of the polyphenols is bound to plant cell walls. Although responses to enzymatic treatments and iron bioaccessibility were matrix-dependent, the positive effect of dietary fiber hydrolysis with X+C was independent of the type of flour. Dietary fibers showed negative effect on iron bioaccessibility independent of phytates.

Keywords: xylanase, cellulase, phytase, polyphenol oxidase, iron bioaccessibility

1. Introduction

Iron deficiency affects about 2 billion people in the world, making it the most prevalent mineral deficiency (WHO, 2009). Although several causes exist, iron deficiency in developing countries has been associated with the predominantly plant-based nature of diets. Such diets contain relatively low amounts of bioavailable iron (Hambraeus, 1999).

The bioavailability of iron in plant-based foods is determined by the contents of absorption enhancers and inhibitors (Hurrell & Egli, 2010). Among absorption inhibitors, phytate and polyphenols have been found to be the most determinant factors. The effect of fiber on iron bioavailability has however remained controversial; while some studies have shown inhibitory effects, others have related this to associated phytates (Frölich, 1995).

Several food processing techniques, like fermentation and germination have been found to hydrolyze phytic acid and iron-binding phenolics (Matuschek et al., 2001). In several studies, dephytinization alone has been found not enough to significantly improve iron bioavailability, especially in the presence of large amounts of polyphenols (Lestienne, Caporiccio, Bresancon, Rochette, & Treche, 2005; Hurrell, Reddy, Juillerat, & Cook, 2003)

In Ethiopia, the most commonly consumed food by children and adults alike is *injera*, a fermented pancake prepared from different cereal blends (Baye et al., 2012). Teff-white sorghum, injera fermentation was shown to result in little phytate degradation (28%) and no changes in iron-binding polyphenols. In contrast, fermentation of wheat-red sorghum based *injera*, led to complete hydrolysis of phytate and partial removal of iron-binding phenolics. However, these degradations did not improve *in-vitro* iron bioaccessibility (article 3), suggesting that either more significant removal of iron-binding phenolics is needed and, or that other factors may be responsible of the low *in-vitro* iron bioaccessibility. The application of exogenous enzymes may be an effective approach for the determination of the relative effects of iron absorption inhibitors as it permits to better target and further degrade specific iron absorption inhibitors (Wang , Cheng, Ou, Lin, Liang, 2008; Lestienne et al., 2005; Matuschek et al., 2001). Although in those studies, the effect of the enzymatic treatments on iron bioaccessibility was reported, little or no information was provided on the extent of the degradation of targeted absorption inhibitors. This is unfortunate, since iron bioaccessibility does not only depend on the type but also on the amount of absorption inhibitors remaining in foods. Furthermore, the combined effect of enzymes targeting fiber, phytate and iron-binding phenolics on iron bioaccessibility has not been assessed.

The main objective of this study was to evaluate to what extent iron bioaccessibility can be improved by reducing or eliminating major mineral absorption inhibitors. To this end, the effect of several enzymatic treatments targeting phytates, iron-binding polyphenols and non-digestible carbohydrates was evaluated.

2. Materials and methods

2.1 Materials

Wheat, red sorghum, teff and white sorghum were purchased from local markets in North Wollo, Ethiopia. Flour blends commonly used to make *injera*, namely, Wheat-red sorghum (WrS) and Teff-white sorghum (TwS) were used in the current investigation (Baye et al., 2012).

The enzymes used in the present studies were mushroom tyrosinase/polyphenol oxidase (Sigma EC 1.14.18.1, T7755, activity >1000 U/mg solid), xylanase from *Trichoderma viride* (Fluka, EC 3.2.1.8, 95595; 12000 u/l), cellulase from *Trichoderma reesei* (Celluclast 1.5 L, Novoenzyme- E.C. 3.2.1.4, activity 0.7 Endo-glucanase unit (EGU)/mg)**,** and phytase from *Aspergillus niger* (DSM, EC 3.1.3.8, 20 Phytase unit (PU)/mg).

2.2 Experimental design

The bioaccessibility of iron was measured in TwS and WrS flours treated with phytase (P), phytase+xylanase+cellulase (P+X+C), phytase+polyphenol oxidase (P+PPO), or phytase +xylanase+cellulase+polyphenol oxidase (P+X+C+PPO) (fig.1).

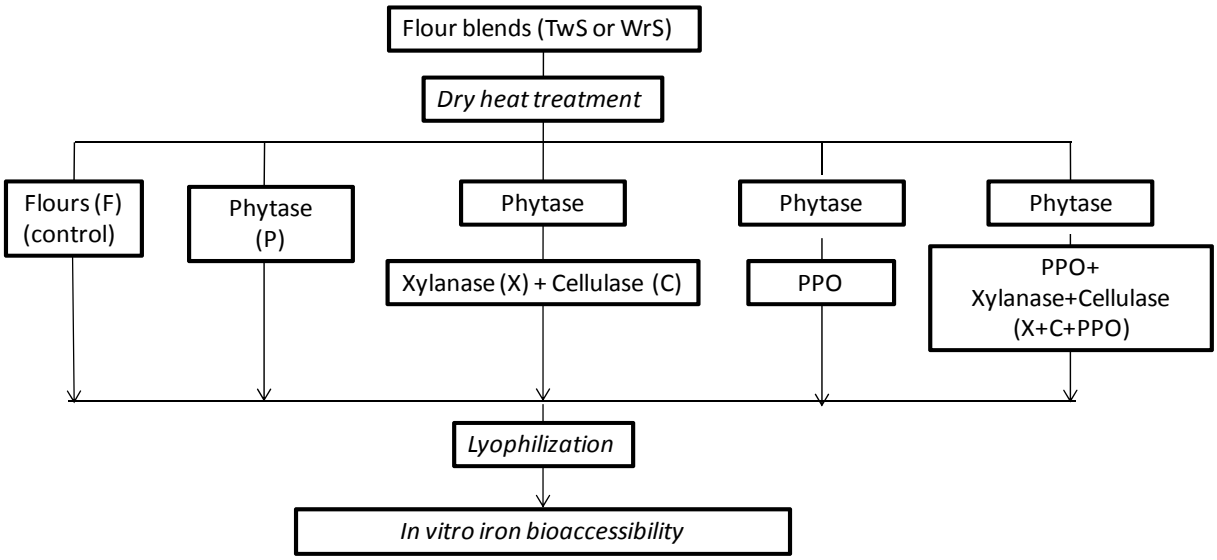

Fig. 1: Experimental design of enzymatic treatments applied to Teff-white Sorghum (TwS) and Wheat-red Sorghum blends used to prepare Ethiopian *injera*.

2.3 Enzymatic treatments

To prevent fermentation and inactivate endogenous enzymes, dry heat sterilization was performed prior to enzymatic treatments by incubating flours at 190℃ for 6 min (Darmady et al., 1961). After enzymatic treatments, flours were lyophilized prior to in-vitro bioaccessibility tests.

2.3.1 Phytase (P) treatment

Cereal flours were suspended in 0.1 M acetate buffer (pH 5.6) in 1:3 (w/v) flour:buffer proportions. For every gram of flour, 0.009g (180 PU) of phytase was added, and the mixture was incubated in a shaking water bath at 35°C for 30 min. The duration of the incubation, the flour: buffer ratio and the amount of phytase to be added were those that allowed total degradation of phytate in preliminary assays.

2.3.2 Cellulase+Xylanase (X+C) co-treatment

After flour dephytinization as described in section 2.3.1, 0.014g (~50 U) of xylanase and 8 µl of cellulase (6.8 EGU) were added, and the mixture was incubated in a shaking water bath for 3h at 35°C . For the treatment with cellulase, the amount of enzyme to be added was based on the optimization of Wang et al., (2008).

2.3.3 Polyphenol oxidase (PPO) treatment

After dephytinization, NaOH was added till pH reached the optimum for PPO activity (pH 6.5) and 0.1 M MES (2-(*N*-morpholino) ethanesulfonic acid-M8250) buffer was added to reach final flour:buffer ratio of 1:10 (w/v). The proportion of enzyme added was based on the works of Matuschek et al. (2001). The incubation was carried in a shaking water bath at 35°C for 16h in the dark.

2.3.4 Phytase, xylanase, cellulase and polyphenol oxidase (P+X+C+PPO) treatment

The flours were sequentially treated in the order of P, X+C, and then PPO according to the above descriptions.

2.4 Dry matter (DM) content

DM contents were determined by oven drying at 105°C to constant weight.

2.5 Iron determination

Total iron was analyzed by flame atomic absorption spectrophotometry (AA800, Perkin Elmer, Les Ulis, France) after wet mineralization using an Ethos 1 microwave digester (Milestone, Sorisole, Italy) for 15 min at 200℃ and with a maximum power of 1000 W. Precautionary measures were taken to avoid adventitious contamination when handling the foodstuffs during the sampling and analyses stages.

2.6 Determination of mineral absorption inhibitor contents

Myo-inositol hexaphosphate (IP6) content was determined by high-performance anion-exchange chromatography (Dionex, Sunnyvale, USA), after extraction of samples in acid solution (HCl 0.5 M) at 100°C for 6 min, according to the method described by Lestienne et al. (2005).

The neutral detergent fibre (NDF) content which corresponds approximately to cellulose, hemicellulose and lignin content was determined according to the gravimetric method of (Van Soest, 1963) using a Fibertec 1020 (Foss, Hillerod, Denmark).

Iron-binding polyphenols (galloyl and catechol groups) were analyzed using the method of Brune et al. (1991). The amount of galloyl and catechol groups was determined using ferric ammonium sulfate (FAS) reagent after 16 h extraction of samples with 50% dimethylformamide in acetate buffer (pH 4.4). This method is based on FAS's ability to form colored complex with iron-binding galloyl and catechol groups. The absorbance of the colored complex was measured at 680 nm and 578 nm, corresponding to the absorption maxima of Fe-galloyl and Fe-catechol complexes, respectively. After subtracting food blank absorbances, the content of galloyl groups (expressed as tannic acid equivalents) and catechol groups (expressed as catechin equivalents) were calculated from standard curves for tannic acid and catechin at both wavelengths.

2.7 Determination of water extractable sugars

Glucose, cellobiose, arabinose, galactose, and xylose were extracted by diluting 80mg of lyophilized flour in 10 ml milliQ water, the mixture was vortexed, then centrifuged at 4500 g for 20 min. The supernatants were filtered through 0.20 μm pore size filters and were analysed by HPAEC (high performance anion-exchange chromatography) with a Dionex DX 500 apparatus connected to an amperometric detector Dionex Model ED 40 (Thermo

Scientific, Courtaboeuf, France) using a Carbo PA1 column (Dionex S.A., Jouy en Josas, France) after appropriate dilution (80ml of supernatant/ 50 ml milliQ H2O).
The following conditions were used: mobile phase (eluent) NaOH 150 mM, flow rate 1 mL/min, temperature 35°C, injection sample extract 25 µL. Results are expressed in g/100g DM of flour.

2.8 *Determination of in vitro iron bioaccessibility*

To determine iron bioaccessibility, enzymatic in vitro digestions were carried out in two stages, following the procedure described in Greffeuille et al. (2011). Briefly, a 10% DM dispersion of ground samples in ultrapure water was brought to 37°C, 20 µL of bacterial amylase (α-amylase from *Bacillus licheniformis*, Sigma A-3403-1MU) was added and the mixture was incubated at 37°C for 5 min. The pH was then brought to 2.0 with 1M HCl, 1 mL of pepsin solution was added (Sigma, P-7000, 14,900 u/mL in 0.1 M HCl) and the mixture was incubated for 1 h at 37°C in a shaking water bath. Pepsin-digested samples were then transferred into separate tubes to which a dialysis bag (Spectra/por I dialysis tubing, MWCO 12–14 kDa) containing 20 mL of PIPES (piperazine-N,N 0-bis-[2-ethanesulfonic acid] sodium salt) buffer (Sigma, P-3768) was introduced to mimic the gradual increase in pH during intestinal digestion. The PIPES concentration needed to obtain a final pH of 7.0 after incubation at 37°C for 30 min was previously determined. A mix of pancreatin (Sigma, P1750, 1.85 mg/mL) and bile extract solution (Sigma, B8631, 11 mg/mL in 0.1 M $NaHCO_3$) was then added, and the resulting mixture was incubated for 2 h at 37°C in a shaking water bath. Dialysis bags were then removed and washed with pure water. The digestion mixtures remaining in the tubes were centrifuged at 10,000g for 15 min at 4°C, and the resulting supernatants were weighed and filtered on ashless filters (Whatman N°41). Iron contents in the pellets, supernatants and dialyzates corresponded to the insoluble, soluble non-dialyzable (ND) and dialysable fractions, respectively. These three iron fractions were calculated as follows:

$$\text{Dialyzable Fe \%} = C_D (W_D + W_S)/ (C_D W_D + C_S W_S + C_I W_I) \times 100 \quad (1)$$

$$\text{Soluble ND Fe \%} = W_S (C_S - C_D)/ (C_D W_D + C_S W_S + C_I W_I) \times 100 \quad (2)$$

$$\text{Insoluble Fe \%} = W_I (C_I)/ (C_D W_D + C_S W_S + C_I W_I) \times 100 \quad (3)$$

Where: C_D, C_S and C_I are iron concentrations (µg/100g) and W_D, W_S and W_I are the weights (g) of the dialysate, supernatant and pellet, respectively. All samples were analyzed in quadruplicate. Results are expressed as average values ± standard deviations (SD).

2.9 Statistical analysis

Average values of biochemical analyses performed at least in triplicate were submitted to analysis of variance, followed by the Fisher's least significant difference (LSD) test to compare the means at the 5% significance level, using the software SPSS version 15.

3. Results

3.1 Iron, IP6, and IP6:Fe molar ratio

The total iron content of untreated flours (F), in particular that of TwS (TwS-F) was high (Table 1). Nearly three times more iron was found in TwS than in WrS flour blend. There was no significant difference in iron contents as a result of the different enzymatic treatments ($P>0.05$).

The IP6 content of the flours was very high; however treatment with phytase for only 30 min allowed more than 90 % decrease in IP6. Upon subsequent incubations with the other enzymes (i.e. xylanase+ cellulase), the decrease in IP6 reached 100%. A closer look at the chromatograms revealed that no lower inositol phosphates were produced as a result of the hydrolysis (data not presented).

The IP6: Fe molar ratio in WrS-F was about three times higher than that of TwS-F, despite higher content of IP6 in the latter. Dephytinization of the flours resulted in an IP6: Fe molar ratio below the critical value for which iron bioavailability can be improved (<1) and subsequent enzymatic treatments with X+C and, or PPO resulted in molar ratios near zero.

Table 1: Effects of different enzymatic treatments on iron and IP6 contents and IP6: Fe molar ratios

	Fe mg/100g DM	**IP6 g/100g DM**	**IP6:Fe molar ratio**
Teff-white sorghum			
TwS-F	33.03 ± 3.95	1.32 ± 0.09[a]	2.1 ± 0.1[a]
TwS-P	36.28 ± 2.89	0.09 ± 0.01[b]	0.2 ± 0.0[b]
TwS-P+X+C	31.70 ± 1.86	0.04 ± 0.01[c]	0.1 ± 0.0[c]
TwS-P+PPO	33.62 ± 2.67	0.00 ± 0.00[d]	0.0 ± 0.0[d]
TwS-P+X+C+PPO	34.86 ± 2.33	0.00 ± 0.00[d]	0.0 ± 0.0[d]
Wheat-red Sorghum			
WrS-F	11.50 ± 1.06	1.02 ± 0.03[a]	6.5 ± 0.4[a]
WrS-P	11.59 ± 0.43	0.09 ± 0.01[b]	0.7 ± 0.1[b]
WrS-P+X+C	11.43 ± 0.53	0.00 ± 0.00[c]	0.0 ± 0.0[c]
WrS-P+PPO	11.12 ± 1.17	0.00 ± 0.00[c]	0.0 ± 0.0[c]
WrS-P+X+C+PPO	10.20 ± 0.97	0.00 ± 0.00[c]	0.0 ± 0.0[c]

F, flour; P, phytase; X, xylanase; C, cellulase; PPO, polyphenol oxidase; Different superscript letters within a column represent statistically significant difference (P <0.05)

3.2 Iron-binding polyphenols

The initial content in iron-binding polyphenols was about two times higher in WrS-F than in TwS-F (fig. 2). Overall, the different treatments resulted in samples containing 71-415 mg tannic acid equivalents (TAE)/100g DM and 80-469 mg catechin equivalents (CE)/100g DM. All enzymatic treatments, even those without PPO, had led to significant decreases in iron-binding phenolic groups. However, treatments comprising PPO had a more pronounced effect. Relative to untreated flours, the maximal decrease in iron-binding polyphenols was ~ 40% in TwS-P+X+C+PPO, and ~ 60% in WrS-P+PPO. Treatment of flours with P+X+C+PPO resulted in higher browning than P+PPO alone, while no browning was observed for the other treatments.

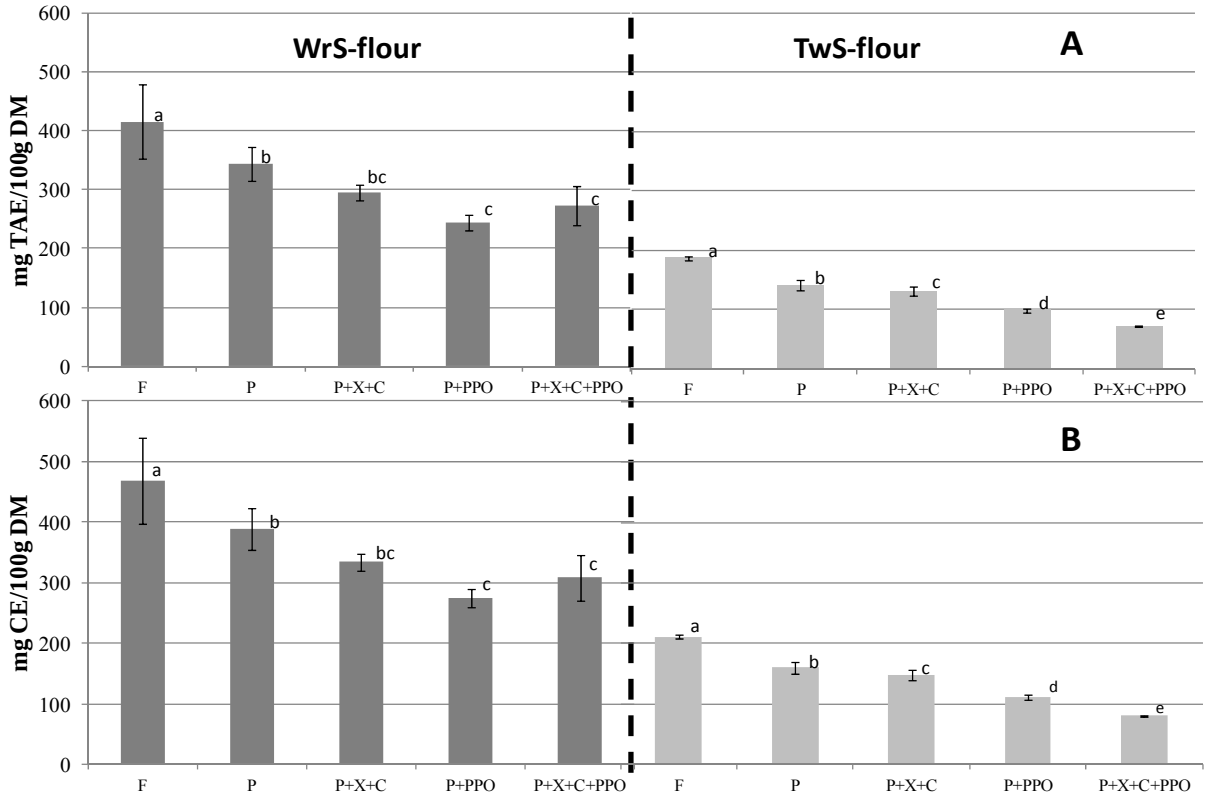

Fig. 2: Effects of enzymatic treatments on iron-binding galloyl (A) and catechol (B) groups in WrS and TwS flour blends

TAE, tannic acid equivalent; CE, catechin equivalent; WrS, wheat-red sorghum; TwS, teff-white sorghum; F-flour; P, phytase; X+C, xylanase+cellulase, PPO, polyphenol oxidase; Error bars represent standard deviations of means. Different superscript letters for the same food type represents statistically significant difference, p < 0.05.

3.3 NDF and water extractable sugar contents

Phytase treatment resulted in a small degradation of fiber in both TwS (~8 %) and WrS (~2 %) flours but reductions were only significant in the case of TwS (fig. 3). These degradations were associated with increases in water extractable (WE) galactose and glucose in WrS and in WE galactose, xylose and glucose in TwS (fig. 3, 4).

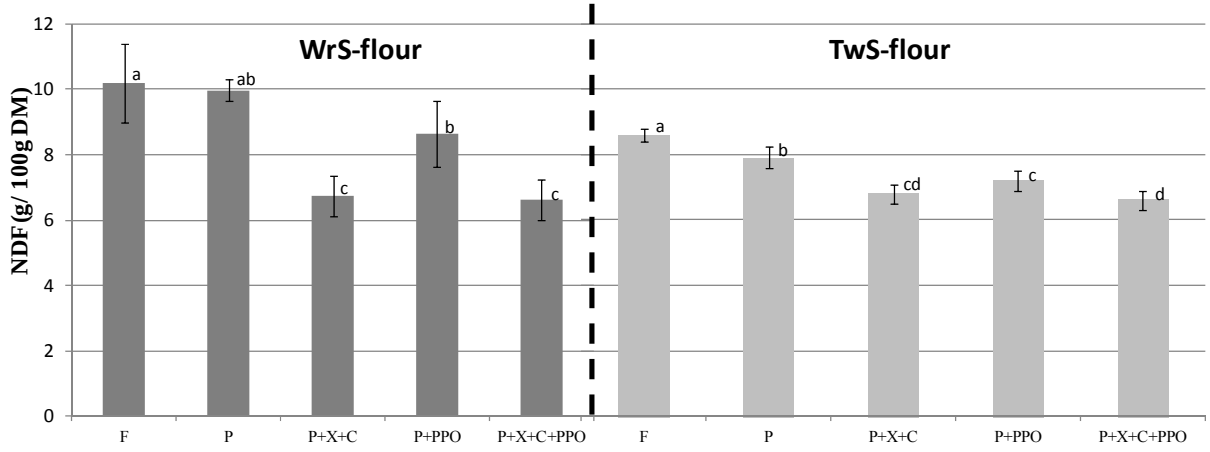

Fig. 3: Effect of different enzymatic treatments on the neutral detergent fiber (NDF) content of WrS and TwS flour blends

More important decreases in NDF contents were observed in both TwS (~ 21%) and WrS (~34 %) blends for X+C treatments ($P < 0.05$). A liquefaction was also observed for enzymatic treatments containing X+C. These degradations led to increases in all the analyzed WE sugars, and in line with NDF results, increases, especially in xylose were higher in the WrS than in the TwS blend (fig. 3, 4).

Treatments with P+PPO resulted in a small but significant decreases (~15-16%) in NDF ($P<0.05$) in both flours. Treatment with a cocktail of P+X+C+PPO resulted in decreases in NDF of about 35 % in WrS and 23 % in TwS blends relative to the initial flour.

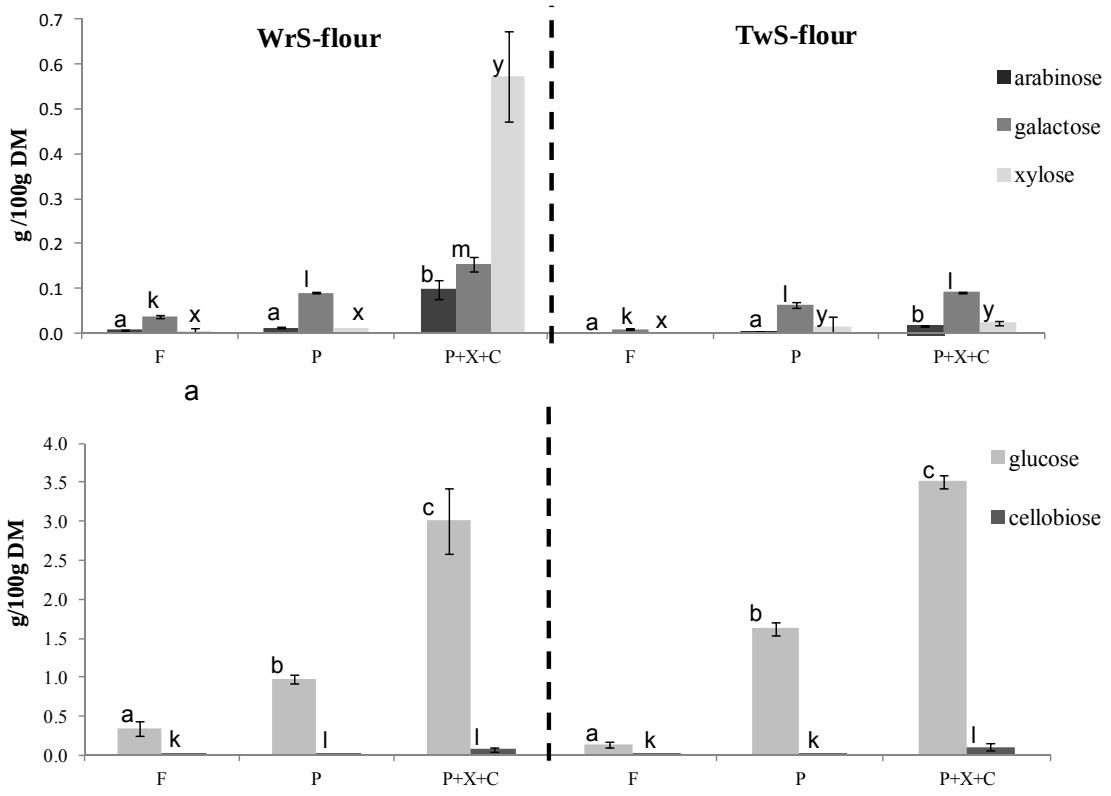

Fig. 4 Changes in water extractable sugar concentrations during enzymatic treatments

Different superscript letters for the same food type represents statistically significant difference, $p < 0.05$. Error bars represent standard deviations from mean

3.4 Iron bioaccessibility

Despite the different enzymatic treatments, that have led to substantial degradation of mineral absorption inhibitors, the fraction of dialyzable and soluble ND iron fractions did not exceed ~3.5 % and 7.5% in TwS and WrS blends, respectively. Despite the lower dialyzable iron fraction in TwS, the amount that dialyzed (~1.2 mg/100g DM) was higher than in the WrS blend (~0.9 mg/100g DM). Response in iron bioaccessibility consequent to enzymatic treatments (except phytase) was dependent of the types of flour blend (fig. 5).

In both flour blends, treatment with phytase alone had no effect on the dialyzable or the soluble ND fractions. In contrast, the dephytinization and treatment with X+C in both flours led to increases of ~69%. The highest fraction of dialyzable iron (%) was different depending on the type of blend (fig.5). The highest fractional iron dialyzability in WrS blend was obtained for treatments containing X+C (with or without PPO), whereas in TwS blend the highest dialyzability was for P+PPO treatment.

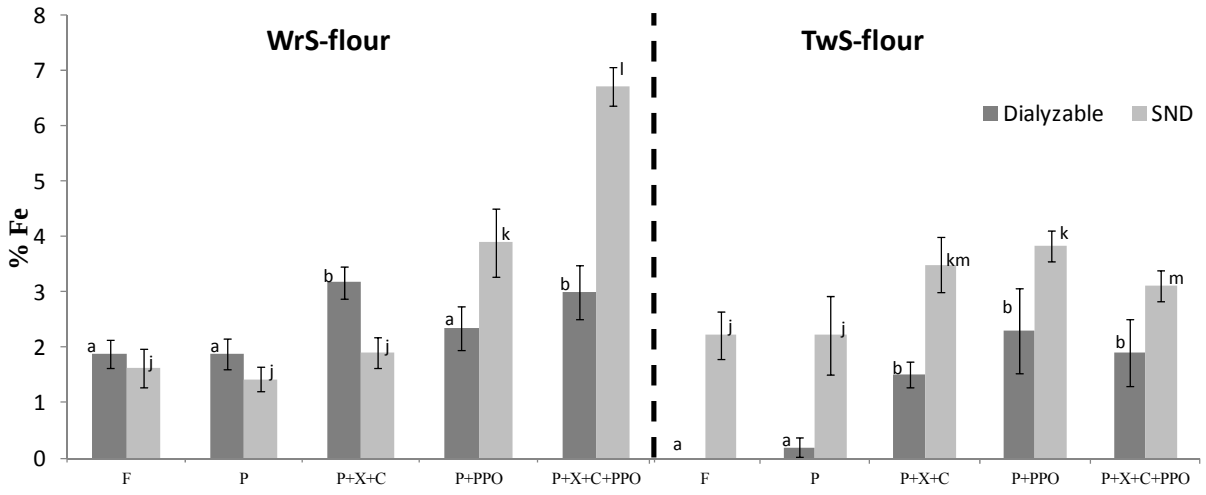

Fig. 5: Effect of different enzymatic treatments on soluble non-dialyzable and dialyzable iron fractions

WrS, wheat-red sorghum; TwS, teff-white sorghum; F- flour; P, phytase; X+C, xylanase+cellulase, PPO, polyphenol oxidase; Different superscript letters for the same food type represents statistically significant difference, $p < 0.05$.

The highest soluble ND fraction in WrS blend was obtained in the sample treated with P+X+C+PPO, followed by treatment with P+PPO. Relative to untreated flour (WrS-F), the WrS-P+X+C+PPO and WrS-P+PPO had 76 % and 58 % more soluble ND iron, respectively. Similar to TwS blends, the highest fraction of SND iron was obtained for treatments comprising X+C and, or PPO.

4. Discussion

In this study, the effect of different enzymatic treatments targeting phytate, iron-binding polyphenols and plant cell wall constituents (hemicelluloses + cellulose) on iron bioaccessibility was evaluated. The efficacy of the enzymatic treatments was also checked by measuring the decrease in the corresponding potential inhibitors, and/or the increase in their degradation products. Removal of phytates showed little improvements in iron bioaccessibility, whereas treatment of dephytinized flours with X+C and, or PPO improved iron bioaccessibility. However, the response to the different enzymatic treatments and hence the extent of improvement in iron bioaccessibility was dependent on the matrix.

Phytate is regarded as one of the major inhibitor of iron absorption. Consequently, many in-vitro and in-vivo studies have shown that substantial hydrolysis of phytates (>90%) by endogenous and, or exogenous phytases improves iron bioaccessibility/bioavailability (Frontela, Scarino, Ferruzza, Ros, & Martinez, 2009; Hurrell et al., 2003). However, the sole

removal of phytates in this study did not result in significant improvement in iron bioaccessibility. An earlier study on *injera* fermentation that used the same flour blends as in this study did not show any improvement in iron bioaccessibility despite the near complete removal of phytate (article 3). In the presence of high amount of polyphenols, removal of phytates had been reported to have little effect on iron bioaccessibility/bioavailability (Lestienne et al., 2005; Petry, Egli, Zeder, Walczyk, & Hurrell., 2010).

Phenolics bearing catechol and galloyl groups were found to be responsible for the iron-binding properties of polyphenols (Brune et al., 1989). The treatment of dephytinized flours with PPO in this study resulted in lower iron-binding galloyls and catechols. Evidence for reduction of iron-binding properties of polyphenols consequent to enzymatic oxidation of polyphenols by PPO already exists (Matuschek et al., 2001). Although to a lesser extent, treatments with phytase and, or X+C also resulted in lower iron-binding polyphenols. Such decreases in iron-binding polyphenols subsequent to phytase treatment were also previously documented (Matuschek et al., 2001). Several reasons may explain this finding. Although the dry heat pre-treatment was expected to inactivate endogenous PPO's, some residual PPO activity might have not been avoided. Furthermore, the treatment with X+C may disrupt plant cell wall integrity and thus lead to the release of endogenous PPO's trapped in the vacuoles and plastids of the grains. This might have resulted in greater contact of PPO's with polyphenols which would have been otherwise separated at the cellular level (Queiroz et al., 2008). This could have also been due to some residual side enzymatic activities of the added enzymes.

Treatment of dephytinized flours with PPO increased the soluble ND iron fraction in both WrS and TwS blends (fig 5); however, the increase in the dialyzable fraction was only statistically significant in the TwS blend. This could be due to the higher (> 3 times) content of iron-binding phenolic groups in WrS than in TwS, remaining after treatment with PPO. This may suggest that a greater decrease in iron-binding phenolic groups may be needed in order to significantly increase the dialyzable iron fraction. Elsewhere, this can also suggest that higher amount of condensed tannins which are less susceptible to PPO are present in the WrS blend (Nichols-Orians, 1991). Although treatment with PPO are likely to prevent the binding of iron by the -OH functional groups of galloyls and catechols, iron can still be trapped in large tannin-protein complexes which can limit its dialyzability. The significant increase in iron dialyzability of WrS flour treated with P+X+C+PPO further suggests that in

this blend, cell wall associated polyphenols may partly be responsible for the low bioaccessibility of iron (Saura-Calixto and Díaz-Rubio, 2007).

The NDF contents decreased significantly as a result of treatment of dephytinized flours with X+C. The hydrolysis of fibers has also resulted in higher contents of WE sugars (fig. 4). This was expected since hemicellulose and cellulose are substrates for these enzymes. Surprisingly, significant decreases in NDF contents were also observed for the dephytinized flours treated with PPO. This could be due to residual endogenous xylanase/cellulase activities and, or due to residual carbohydrase activities in the commercial PPO.
The decrease in NDF after enzymatic treatments containing X+C resulted in significant increase in both soluble ND and dialyzable iron fractions suggesting that certain types of fibers may have iron-binding properties. Although findings of several in-vitro studies reported such iron-binding properties (Debon and Tester, 2001, Fernandez and Phillips, 1982b) many in vivo studies failed to confirm this (Van den Heuvel et al., 1998a, Brune et al., 1992).
The reason behind this discrepancy may be the fact that iron trapped by fibers may be liberated for absorption in the colon, since part of this fiber can be hydrolyzed by the action of microorganisms in the colon (Nordgaard and Mortensen, 1995). In this connection, synthetic soluble fibers like partially hydrolyzed gums (de Cassia Freitas et al., 2006) and acidic xylooligosaccharide (Kobayashi et al., 2011) were shown to increase iron absorption and enhance serum iron levels when fed to rats with iron deficiency anemia. Similarly, the use of X+C in this study has shown that the hydrolysis of native fibers increase in-vitro iron bioaccessibility. The treatment with carbohydrase might have led to disruption of the plant cell wall integrity that allowed the release of trapped minerals, or the observed liquefaction might have allowed better contact with digestive enzymes in vitro(Guillon and Champ, 2000). Whether the partial hydrolysis of dietary fiber with X+C followed by further hydrolysis by the microbiota of the colon results in better iron bioavailability warrants a study of its own.

The two flour blends evaluated were quite different in many aspects. The TwS blend contained about three times more iron, probably because of the presence of iron from soil contamination. Given the low solubility of contaminant iron, the lower fractional iron dialyzability/ solubility of TwS relative to WrS is not surprising and is in agreement with a previous study (article 3). In addition, the NDF and iron-binding polyphenol contents in WrS

were higher than in TwS blend. Consequent to this inherent differences, the response to the different enzymatic treatments and hence the effect on both soluble ND and dialyzable iron fractions was different. For example treatment of dephytinized flours with PPO increased the soluble ND fraction but not the dialyzable fraction in WrS-blends whereas in the TwS blend the same treatment increased both the SND and the dialyzable fraction. Such differences in responses to enzymatic treatments may be related to the type and the size of polyphenols involved, the nature of the dietary fiber (degree of polymerization, cell wall thickness, etc.), as well as the nature of the association between polyphenols and fiber. Evidence of such food-matrix dependent variations in enzyme activities (Meng and Slominski, 2005) and iron bioavailability (Moretti et al., 2006) already exists. However, irrespective of the type of blend, treatment of dephytinized flours with X+C resulted in higher iron dialyzability ($P<0.05$).

5. Conclusion

The effect of the degradation of major iron absorption inhibitors - phytate, iron-binding phenolic groups and dietary fibers- on fractional iron bioaccessibility was evaluated. Phytate was not the only inhibitor in WrS and TwS flour blends. The hydrolysis of dietary fiber with X+C after dephytinization resulted in better iron bioaccessibility suggesting that the effect of fiber on iron bioaccessibility is not only due to associated phytates as previously thought. While the effect of PPO was matrix dependent, probably because of the different nature of the polyphenols (phenolic acid, flavonoids, etc.) and their degree of association with iron and plant cell walls, treatment with PPO after hydrolysis of phytate and fibers resulted in a better fractional iron bioaccessibility irrespective of the type of flour blend. However, the gain in fractional bioaccessibility was limited, indicating that large parts of the iron is in the insoluble form that is less sensitive to enzymatic treatments and probably not absorbable. The enzymatic approach used in this study may not allow the formulation of direct recommendations. It can however lead to better understanding of the possible interactions within the food matrix and give an estimate of the relative weight of specific mineral chelating agents on the bioaccessibility of iron. Such information can give better guidance for future optimizations of household food processing that aim to improve iron bioavailability.

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Highlights

- Flour blend composition influenced response to enzymatic treatments.
- > 90% removal of IP6 did not result in better fractional iron bioaccessibility.
- Dietary fiber hydrolysis increased fractional iron bioaccessibility.
- Effect of polyphenol oxidase treatment on dialyzable-Fe was food-matrix dependent.

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4.4.3 Complementary results: effect of phytase, carbohydrase and polyphenol oxidase treatment on total polyphenols and zinc bioaccessibility

4.4.3.1 Effect of phytase, carbohydrase and polyphenol oxidase treatments on zinc bioaccessibility

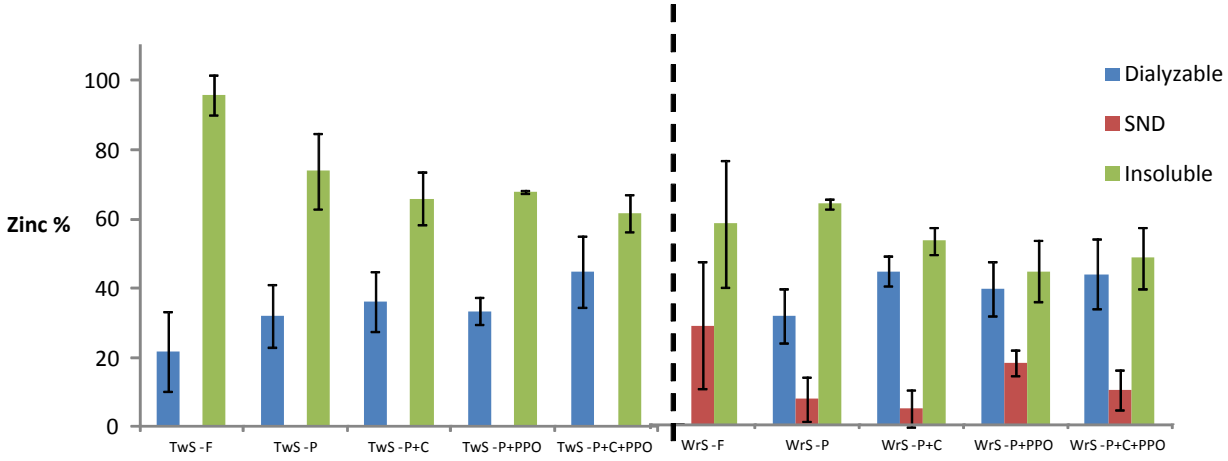

Fig. 4.1: Effect of different enzymatic treatments on zinc bioaccessibility

SND, soluble non-dialyzable

Very high variability in zinc bioaccessibility, especially in untreated flours, was observed (fig. 4.1). This can probably be due to the small zinc contents in these flours (1.6-1.7 mg/100 g DM) exacerbated by the possible adsorption of zinc on the tubes or the dialysis membrane during the in vitro experiments. Nevertheless, the effect of phytase on the bioaccessibility of zinc was noticeable as all the soluble zinc dialyzed. The data variability, coupled with the low zinc recovery, especially in untreated flours (30-70%) does not permit further interpretations of the results.

4.4.3.2 Effect of enzymatic treatments on total polyphenols

The treatment with carbohydrases resulted in higher content of assayable total polyphenols (fig. 4.10). This increase is most likely due to greater extractability of bound polyphenols. The increase was however only significant in the TwS injera, suggesting that polyphenols in this blend are mostly found bound to plant cell walls. Surprisingly, the treatment with polyphenol oxidase had no significant effect on the TwS injera, while significant decreases were observed in the WrS one.

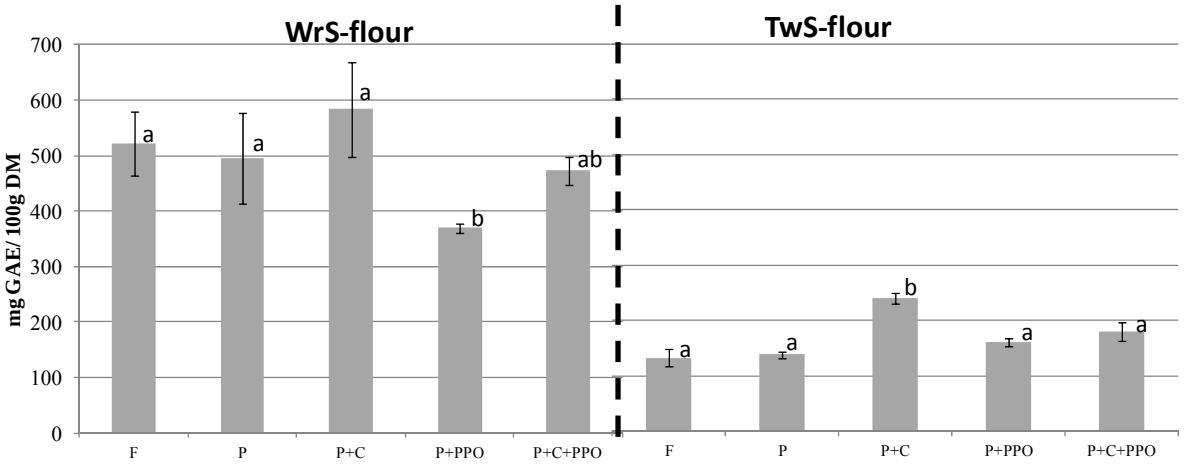

Fig. 4.2: Changes in total polyphenols during enzymatic treatments

GAE, Gallic acid equivalents
Different superscript letters for the same food type represents statistically significant difference, $p < 0.05$.
Error bars represent standard deviations from mean.

Chapter 5- *General discussion*

5. Chapter five: General discussion

The present thesis aimed at investigating dietary modification and household food processing strategies that may improve iron and zinc bioavailability of complementary foods consumed by young children in North Wollo, northern Ethiopia. The adequacy of energy and selected nutrients intakes from complementary foods were evaluated to WHO recommendations. The influence of traditional household processing of staple foods on mineral chelating agents was investigated and the implications for the bioavailability of iron and zinc estimated. Finally, the relative contribution of the major mineral absorption inhibitors in determining iron and zinc bioaccessibility was evaluated with the aim of designing food-based strategies that will eventually improve bioavailability.

5.1 Complementary feeding practices

The high growth rate in the first two years of life requires considerable iron and zinc intakes (Dewey, 2001). To meet these requirements, complementary foods need to have adequate amounts of bioavailable forms of these nutrients. The complementary diets of the children surveyed in north Wollo were mainly based on cereals and legumes, which are known to contain high amount of mineral absorption inhibitors. Little or no consumption of animal products, fruits and vegetables was observed (article 1). Such monotonous plant-based complementary diets correspond well to what is observed in many developing countries (Gibson et al., 1998b). Consequently, intakes of several nutrients were found to be suboptimal when compared to WHO recommendations from complementary foods. The low nutrient density of the foods along with inadequate feeding practices might have contributed to the suboptimal energy and nutrient intakes. Inadequate complementary feeding has been associated in many instances with stunting, which may partly explain the high number of stunted children in surveyed villages (Anderson et al., 2008, Gibson et al., 2009).

Despite the plant based nature of the foods consumed and the absence of fortified foods, iron intake was high and met WHO recommendations even under the assumption of low bioavailability (article 1). This is in contrast to findings from many developing (Gibson et al., 1998b) and developed countries (Friel et al., 2010), but is in agreement with previous findings on iron intake in Ethiopia (Abebe et al., 2007a, Adish et al., 1999). However, large

share of the iron was likely from soil contamination which exhibits poor solubility and bioaccessibility (article 3). Despite reports from earlier studies on the rarity of iron deficiency in Ethiopia (Gebre-Medhin, 1976), more recent epidemiologic studies indicate that iron deficiency is a public health concern (Haidar and Pobocik, 2009). This suggests that low iron bioavailability rather than low intake may be the problem. On the other hand, reported zinc deficiencies may be due to both low intake and low bioavailability (Abebe et al., 2007a). Given that most children regularly consume *injera,* a fermented product, possibilities for improving iron and zinc bioavailability through the activation of endogenous and, or microbial enzymes (i.e. phytases, polyphenol oxidases) exists. However, the extent of degradation is likely to depend on several factors including raw materials used and fermentation parameters like pH.

5.2 Potential of *injera* fermentation on phytic acid and polyphenol degradation

The fermentation kinetics, as well as phytate hydrolysis of *injeras* was influenced by the flour blend composition. Complete hydrolysis of phytate was observed in BW and WrS *injeras* while only 28% of the phytate was hydrolyzed in TwS *injera* (article 2). The difference in the hydrolysis of phytate can be explained by the difference in endogenous phytase activity of the flour blends, the possible additional phytase that might have been provided by malt and, or the setting of favorable conditions for yeasts with potential phytase activities. Indeed some yeasts like *Saccharomyces cerevisiae* are known to display phytase activity (Vats and Banerjee, 2004). Although not always, germination of cereals has often been associated with increases in phytase activity (Gibson and Ferguson, 1998, Egli et al., 2002). Wheat and barley, relative to other cereals, are known to be good sources of endogenous phytases (Egli et al., 2002, Reale et al., 2007); hence, flour blends containing these two cereals had higher phytase activities (article 2).

These results suggest that the incorporation of cereals with high endogenous phytase activities and, or the addition of malt is likely to result in higher phytic acid degradations during fermentation. However, cautionary measures that minimize the possible risk of production of mycotoxins during germination need to be taken (Trèche and Mouquet-Rivier, 2008).

On the other hand, the effect of fermentation on iron-binding polyphenols was not as remarkable as that on phytate (article 3). Partial degradation of iron-binding polyphenols was observed in WrS *injera*, while no such effect was observed for the other *injeras*. This

difference might have been due to different localizations, nature and/or contents of the polyphenols or to the influence of food processes such as decortication that influence the accessibility of polyphenols to enzymatic degradation. Despite the observed reductions in iron-binding polyphenols, the levels found were still high enough to potentially inhibit iron absorption.

5.3 Estimation of iron and zinc bioavailability in *injeras* and stews

The degradation of phytate as a result of BW and WrS *injera* fermentation and the application of exogenous phytase resulted in low phytate: Fe and low phytate: Zn molar ratios, suggesting high bioavailability. However, no significant difference in *in-vitro* iron bioaccessibility was observed. This suggested that critical phytate:Fe molar ratios may be less suited in predicting bioavailability of foods contaminated with extrinsic (i.e. soil) iron. It can also suggest that phytate is not the only iron absorption inhibitor, and thus its removal in the presence of other inhibitors may result in little improvement in absorption. This is in line with previous results from both *in-vitro* bioaccessibility (Lestienne et al., 2005a) and human absorption studies (Petry et al., 2010), and was further confirmed by the iron absorption prediction algorithm (article 3).

Among the accompanying stews, *shiro* had a better predicted iron and zinc bioavailability. Therefore, promoting *shiro* over split field pea stews may likely benefit mineral bioavailability. However, in the surveyed sites, *shiro* was prepared from legumes with potentially toxic effects such as broad beans (favism) and grass peas (β-ODAP). Processes such as soaking and roasting of grains, and the inclusion of ingredients with known antioxidant properties (ginger, garlic, etc.) in the preparation of *shiro*, may reduce toxic effects (Getahun et al., 2005). However, considering the endemicity of neurolathyrism in the northern parts of Ethiopia (Haimanot et al., 2005), the low body weight of the children and the multiple micronutrient deficiencies these children may present, replacing grass peas and broad beans by other available legumes such as lentils, chickpeas, and field peas may be preferable.

5.4 Relative importance of mineral absorption inhibitors in determining iron bioaccessibility

In injera flour blends with high (WrS) and low (TwS) polyphenol contents, enzymatic treatments targeting phytates, fibers (hemicelluloses/cellulose) and iron-binding polyphenols have shown dephytinization alone did not result in significant increases in iron bioaccessibility (article 4).. However, in dephytinized flours, hydrolysis of dietary fibers with xylanase and cellulase increased iron bioaccessibility. This is in line with previous findings from *in vitro* bioaccessible studies (Lestienne et al., 2005a). However, the few human studies with added fiber failed to show the absorption inhibitory effects of fibers, probably because iron trapped by insoluble fibers may be liberated for absorption in the colon by the action of colonic microflora (Nordgaard & Mortensen, 1995).

In both WrS- and TwS- dephytinized flours, oxidation of polyphenols with polyphenol oxidase increased the soluble ND iron fraction, but significant increases in the dialyzable fraction were only observed in the TwS blend. This suggested that greater decrease in iron-binding phenolic groups may be needed in order to significantly increase the dialyzable iron fraction. The difference in dialyzability can also be explained by the presence of higher amount of condensed tannins, less susceptible to PPO, in the WrS blend. Although treatment with PPO are likely to prevent the binding of iron by the -OH functional groups of galloyls and catechols, iron can still be trapped in large tannin-protein complexes which can limit its dialyzability. Treatment with PPO after phytase and carbohydrase treatment increased the dialyzable fraction in WrS flour suggesting that cell wall associated polyphenols may partly be responsible for the low bioaccessibility of iron.

Based on the present findings, it can be presumed that, if possible, optimization of food processes for the reduction of iron-binding polyphenols, phytates, and solubilization of dietary fibers is likely to have greater effect in improving iron bioaccessibility than dephytinization alone. However, the children's consumption of tea and coffee right after meals, and the low vitamin A and C intakes are likely to hamper strategies to improve bioavailability of iron and zinc, unless such dietary habits are changed.

5.5 Highland Vs Lowland

The differences between the highland and the lowlands were marked. Although *injera* was the staple in both sites, cereal blends used for its preparation were different and influenced the fermentation pattern resulting in a marked difference in the phytic acid and to some extent

in iron-binding polyphenol contents (article 2). Considering the fact that phytate is the major chelator of zinc, higher bioavailability is expected for *injeras* of the highlands for which complete phytate degradation and hence low phytate:zinc molar ratios were obtained. Besides, the zinc density of the diets of the children in the highlands was greater than that of the lowlands and is expected to meet estimated needs provided that the diet is of medium bioavailability (article 1). For iron, there was no difference in fractional (%) iron bioaccessibility between the highland and lowland *injeras*. However, considering the high content (~3 times) of iron in the lowland *injera*, higher amount of bioaccessible iron should be consumed in the lowlands (article 3).

The responses of the *injera* flour blends to different enzymatic treatments were also different, suggesting that strategies to be adopted for the decrease of iron chelators may differ for the highlands and lowlands (article 4).

In addition, comparison between the two sites revealed marked differences in vitamin C intakes which may further have implications on the bioavailability of the diets. More stunted children were observed in the highlands (P=0.05), possibly because of the harsh physical and socio-economic conditions in the highlands.

5.6 Limitations of the study

The food consumption survey provided an overview of the feeding pattern of young children in two rural villages (highland/lowland) in Gobalafto district, North Wollo, northern Ethiopia. However the consumption survey had several limitations that need to be taken into account when interpreting the results. The small number of the studied children might have underestimated the differences between the highland and lowland sites. The small number of NBF children requires caution in interpreting the results. The cross-sectional nature of the study did not allow seasonal variation in food intakes to be considered. Although caregivers were instructed to not change their children's dietary pattern, the absence of deliberate changes is not warranted. A further limitation is that breast-milk consumption was not quantified and instead values of average breast-milk intake reported in the literature were used to calculate the adequacy of energy and nutrient intakes.

The estimation of the bioavailability was limited by the fact that the share of intrinsic and extrinsic iron to the total iron was unknown. In vitro dialyzability tests were only valid for iron due to technical limitations faced with that of zinc, and hence the relative contribution of the mineral absorption inhibitors was only studied for iron. However, since phytate is the

major chelator of zinc, phytate:zinc molar ratios are expected to give good indications of zinc bioavailability since unlike iron, zinc is not of extrinsic origin.

The relative contribution of the mineral absorption inhibitors as evaluated with the application of several enzymes is likely to be influenced by processes such as decortications and milling which can determine the accessibility and thus the efficacy of the enzymatic treatments.

In addition, *in vitro* approaches to estimate bioavailability have obvious limitations but they have been employed as a useful tool particularly for screening samples before more elaborate and expensive human studies are performed. In the present studies, *in vitro* dialyzability tests have been complemented by other bioavailability measurement methods such as iron bioavailability prediction algorithms and phytate:mineral molar ratios, all of which have been found to correlate well with results from human absorption studies, except probably in cases where foods are contaminated with extrinsic iron.

Chapter 6- *Conclusion & perspectives*

6. Chapter six: Conclusions, recommendations and perpectives

6.1 Conclusions

In the present thesis the complementary feeding practices of young children in north Wollo were characterized, the adequacy of nutrient intakes from complementary foods were evaluated, the influence of *injera* fermentation on phytic acid and iron-binding polyphenols degradations and its implication for iron and zinc bioavailability was estimated. Finally, in a mechanistic study, the relative improvement in iron and zinc bioaccessibility consequent to degradation of phytate, solubilization of fiber and, or oxidation of polyphenols was evaluated.

Several feeding practices were not in accordance with WHO/PAHO recommendations. Shortfalls of calcium, vitamin C and vitamin A intakes were observed, whereas protein intakes were adequate. Zn intake was adequate only when moderate bioavailability was assumed, whereas iron intake was adequate even when low bioavailability was assumed. Most foods were found to have very high contents of iron, but most of it likely resulted from soil contamination. Agro-ecology, mainly altitude-related differences were shown to have an impact on nutrient intakes and also possibly on child growth.

Fermentation patterns of *injera* in the highland and lowlands were also shown to be different, mainly due to difference in flour blend composition. The flour blend composition influenced IP6 hydrolysis, which resulted in injeras with very different contents in IP6. The study could have suggested that degradation of IP6 in alcoholic (yeast) fermentation may be more effective than in lactic acid fermentation. However, the combined action of malt, the use of cereal blends with high endogenous phytase activity (i.e. wheat, barley) during *injera* fermentation and the possible phytase activity of yeast most likely explain the complete IP6 degradation observed in *injeras* containing barley and/or wheat. This suggested that extensive degradation in IP6 during household fermentation is a possibility.

However, the complete IP6 degradation in injera was shown to not improve iron bioaccessibility, suggesting that phytate degradation alone in the presence of other absorption inhibitors such as polyphenols and fibers is not enough. In line with this, the possible limitations of using phytate: iron molar ratios in estimating the bioavailability of foods,

especially when a large share of the iron is from extrinsic sources (i.e. soil) has been demonstrated.

Using a mechanistic approach that involved the application of exogenous enzymes that target IP6, iron-binding polyphenols, and dietary fiber (cellulose/hemicellulose), the relative role of each on iron bioaccessibility was evaluated. In injera flours, hydrolysis of dietary fiber or oxidation of polyphenols resulted in greater iron bioaccessibility than dephytinization alone. However, the relative importance of mineral absorption inhibitors as well as the response to enzymatic treatments was shown to be dependent of the food matrix.

6.2 Recommendations

Based on the findings of this thesis several recommendations that can improve the quality of complementary feeding in north Wollo, northern Ethiopia but also in other places with similar contexts can be made.

-In both highlands and lowlands, efforts to enhance dietary diversity by including ASF, dairy, and fruits and vegetables rich in vitamin A and vitamin C are needed.

-Interventions promoting the WHO guiding principles for complementary feeding practices and behaviors that take the agro-ecological contexts into account are needed.

-Specific recommendations should be formulated to discourage the consumption of grass pea and broad beans. Caregivers should be informed of the potential toxicities associated with consumption of grass pea and broad beans, and the consumption of other available legumes like chickpeas, lentils and field peas should be encouraged

-Although beverages such as tea and coffee have the advantage of usually being safer in terms of microbial contamination, in view of their negative effects on mineral absorption and the appetite of the children, their consumption should be discouraged at least right before and after meals.

-Addition of malt or inclusion of cereals with relatively high phytase activities (i.e. wheat, barley) may allow better IP6 degradations which would likely improve mineral bioavailability, especially that of zinc.

6.3 Perspectives

-Future studies on how grain types and food processing such as decortications and fermentation influence the degradation of mineral chelating agents may be important. More specifically, endogenous enzymatic activities (i.e. polyphenol oxidase) in widely consumed cereals should be characterized, and the influence of food processing conditions on these activities determined.

The microbiota of *injera* may also need to be studied using both culture-dependent and culture independent methods, in view of screening and quantifying the possible production of enzymes of interest. The potential benefits of hydrolyzing dietary fibers by xylanase and cellulase on mineral bioavailability may also need to be confirmed by making use of in vivo techniques. The acceptability of possible rheological and sensorial changes induced by enzymatic activites needs to be evaluated.

-The share of intrinsic and extrinsic iron to the total iron and the way extrinsic iron behaves with respect to known mineral absorption inhibitors and changes in physico-chemical conditions along the gastro-intestinal tract need to be evaluated.

-Studies on the effect of processes such as soaking, roasting, and addition of ingredients with known antioxidant properties (ginger, garlic, etc.) on the contents and toxic effects of grass pea toxin β-ODAP (β-N-oxalyl-L-α,β-diaminopropionic acid) are needed.

-Altitude/agro-ecology may have an important implication on the nutrition of children in subsistent farming rural households and thus needs to be studied in detail based on a larger sample representing different parts of the country.

-Given the possible micronutrient deficiencies that the children in North Wollo face, the role of other micronutrient deficiencies (i.e. vitamin A, B vitamins e.g. riboflavin) in determining the bioavailability of iron and zinc in the foods may need to be investigated.

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Annexes

Annex A- Consent form

FOOD SCIENCE AND NUTRITION PROGRAM
COLLEGE OF NATURAL SCIENCES
ADDIS ABABA UNIVERSITY

IRD
Institut de recherche pour le développement

Subject: informed consent form **Date :**

Dear Sir, Madame

We belong to the Food Science and Nutrition Program of the Addis Ababa University and Institute for development research. We work on the nutritional status of children between the ages of 12 and 23 months. Our goal is to evaluate the adequacy of iron and zinc intake, as well as energy intakes of your children. Your child has been selected to take part of our study. If you are willing to take part of our study, you will be asked questions regarding your child food consumption. Weight and height will also be recorded. We are therefore here to ask for your consent to take part of the study. You are totally free to accept or refuse to participate in the survey. If you decide to refuse, for whatever reason, there will not be any repercussions. If you accept, we guarantee you that confidentiality of all information collected will be assured. You are free to quit at any moment of the survey, without any prior notice or justification. This survey will not have any consequence neither to you nor to your surroundings.
Don't hesitate to ask us any questions regarding the objectives or the process of the investigation. If you are willing to proceed through the study, we are very glad to include you into our sample.

Household identification N°: |__||__||__||__||__|

Name of the mother or the guardian of the child:

Name of the household head:

Name of the person for which consent is asked:

Signature :

Annex B- Questionnaires

ID : |__||__||__||__||__|

Name of the interviewer:_____
Date of the interview: _____
Start of the interview: _____ end of the interview: _____

A. characteristics of the child

1. Birth date: |__||__|/|__||__|/|__||__||__||__|
2. Sex : 1=male 2=female |__|

3. Does the child have brothers and sisters? 1=yes 2=no |__|
   a. if yes, how many in total? |__|
   b. number of brothers and sisters in the following age groups
   < 12months|__| 12-23 months |__| 24-59 months |__| 5-18 years|__| + 18 yr |__|
   c. Position of the child: |__||__|
4. Who usually takes care of the child?
   1= mother **2**= grand-mother **3**= brother/sister **4**= father
   5= others, *specify*_____ |__|

B. Anthropometry (measure up to 3 equal or approximating to 100g or 1mm)

1. Weight

1st measure	2nd measure	3rd measure
|__|__| . |__| kg	|__|__| . |__| kg	|__|__| . |__| kg

2. Height

1st measure	2nd measure	3rd measure
|__|__| . |__| cm	|__|__| . |__| cm	|__|__| . |__| cm

C Access to health facilities

1. Did the mother, during her pregnancy, follow-up consultations at the health centre?
   1=yes 2=no 3=don't know |__|
2. Place where the mother gave birth:
   1= health centre 2=home 3=other, *specify* _____ |__|

3. Has the child ever visited the heath center since his/her birth? : 1=yes 2=no |__|

   a. If yes, frequency of the visits:
   1= >1 time/ month 2= 2 time/ month
   3= less than1 times/month 4=less than 1 time/6months |__|
4. If caregiver has documents providing birth weight of the child, please note: |__|,|__||__||__|

5. Morbidity:
   a. Did the child have had diarrhea in the past 2 weeks? 1=yes 2=no |__|
   b. Did the child have had cough in the past 2 weeks? 1=yes 2=no |__|

c. Did the child have had fever in the past 2 weeks? 1=yes 2=no |__|

6. Did the caregiver ever had advises on how and what to feed the child? 1=yes 2=no |__|

a. If yes, from whom ?

1=health centre 2=neighbors/friends 3=family, precise |__|

E. Characteristics of the caregiver

1. Relation to the child:

1=mother 2=grand-mother 3= sister 4= aunt 5=other (specify) _____ |__|

2. Age: |__||__|

3. Educational status:

a. Can caregiver read and write? 1=yes 2=no |__|

b. If yes, level of education?

1= Primary school 2= secondary school 3= higher education |__|

6. Religion:

1= Orthodox 2= Catholic 3=Muslim 4= protestant 5= other |__|

7. Caregivers' activities: _____

F. Characteristics of the household head

1. Livelihood strategy:

Farmer	1=yes	2=no	|__|
Pastoralist	1=yes	2=no	|__|
Civil servant	1=yes	2=no	|__|
Business man	1=yes	2=no	|__|
Student	1=yes	2=no	|__|
Other (*specify*)	1=yes	2=no	|__|

2. Is the household head the father of the child? 1=yes 2=no |__|

H. economic status

1. How much land do you have? _____

2. Do you have a radio? 1=yes 2=no |__|

3. Do you have a TV set? 1=yes 2= no |__|

4. Is the roof of the house made of corrugated iron sheet? 1=yes 2=no |__|

5. If you have stock, for how long does it last?

1= no stock 2=1 month 3= 1 to 3 month 4= 3 to 6 months 5= > 6months |__|

6. How many cow's and oxen's do you have? |__|

"I would like you to tell me what your child had to eat or drink after he/she woke up yesterday morning. Did he/she eat that food at home?
What did you have next and at what time?"
Proceed through the day, repeating these questions as necessary, and record each food or drink (including drinking water) consumed in column 3 of the 24-hour recall form (Table 5.1). Remember to probe for any snacks and drinks consumed between meals. Follow the example given in Table 5.1 to ensure you are recording the information correctly.

Interviewer Id no. Interview date Mon Tue wed thu fri sat sun			Location Sex Subject Id Age Subject name weight height			
Time	Place eaten	Food/drink	Description, cooking method	Amount	Weight equivalent(g)	Food code
Probe for sickness: ☐ Yes ☐ No If yes, did sickness affect appetite? ☐Yes ☐ No If yes, how? ☐Increase ☐decrease						
Was food intake unusual? ☐Yes ☐No If yes, how was it unusual?			Probe for tablets: ☐ Yes ☐ No ☐ Iron ☐vitamins ☐other supplements ☐anti-malarial			
Was it a feast day? ☐Yes ☐No Was it a market day? ☐Yes ☐No Was it a fasting day? ☐Yes ☐No						

Subjects name: sex:
Subject Id:
Interview date:
Day of the week food eaten:
Name of interviewer: **Name of mixed dish 1 - 5**
Amount of eaten by respondent (g or mL)
Wt empty pot Wt cooked mixed dish+pot:
Wt mixed dish Volume of cooked mixed dish: (mL)
Proportion of mixed dish consumed by respondent:

Ingredient	Description of ingredient and cooking method	Amount of raw ingredient in recipe	Weight of raw ingredient in recipe	Weight of raw ingredient consumed	Weight of cooked ingredient in recipe	Weight of cooked ingredient consumed

Annex C

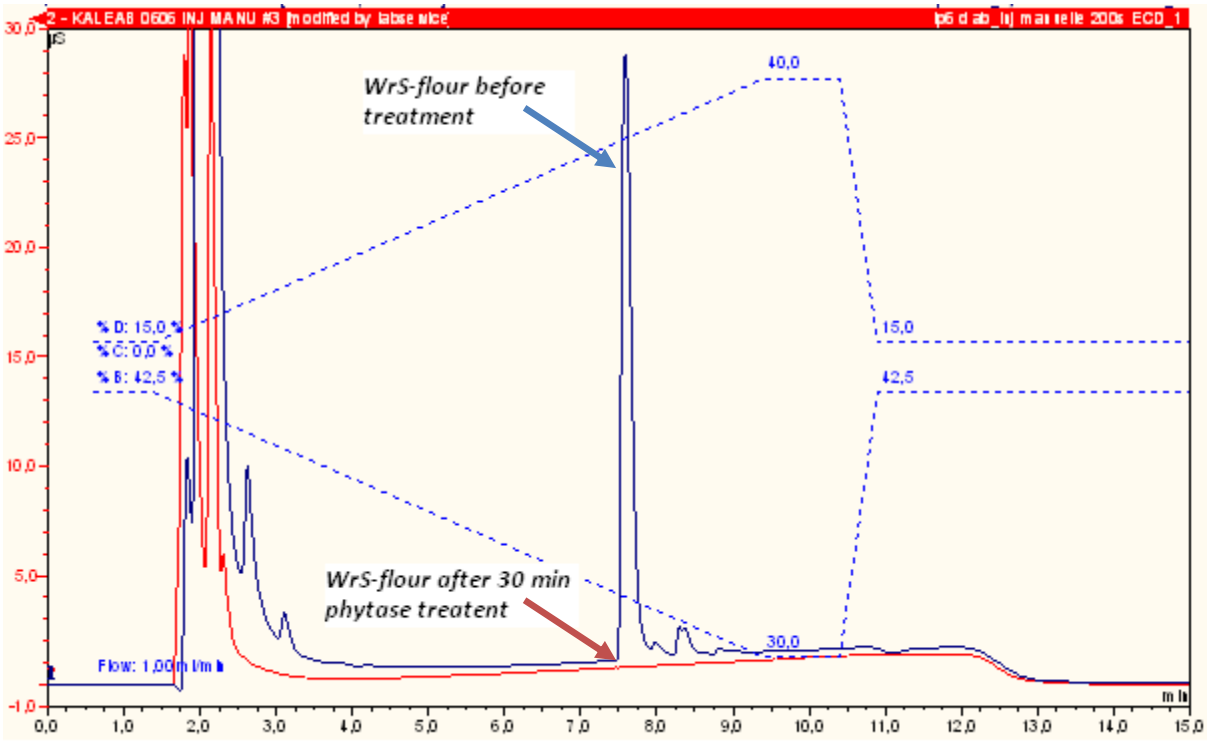

Fig. A-HPAIC chromatogram showing wheat-red sorghum flour before and after 30minute of phytase treatment

Voies alimentaires d'amélioration de la biodisponibilité du fer et du zinc dans les aliments de complément consommés par les jeunes enfants en Ethiopie

Résumé

Le retard de croissance et les carences en micronutriments sont largement répandus dans les pays en développement tels que l'Ethiopie et atteignent un pic pendant la période d'alimentation complémentaire. L'adéquation des pratiques d'alimentation complémentaire du jeune enfant a été évaluée par rappel de 24h auprès de 76 ménages dans la région nord du Wollo, au nord de l'Ethiopie. Plusieurs pratiques d'alimentation n'étaient pas en accord avec les recommandations internationales OPS/OMS. Les aliments les plus fréquemment consommés étaient l'*injera* –une sorte de galette à base de céréales fermentées- accompagnée de sauces à base de légumineuses, et le pain. Les procédés de transformation traditionnels de ces aliments ont été observés sur le terrain. Différents mélanges de céréales étaient utilisés pour la préparation de l'*injera.* Le type de mélange conditionne la cinétique de fermentation, qui à son tour, affecte l'hydrolyse de l'acide phytique. Même lorsque la dégradation de l'acide phytique était supérieure à 95%, la bioaccessibilité du fer et le pourcentage d'absorption calculé par algorithme n'étaient pas améliorés, alors que les ratios molaires phytate:Fe étaient optimaux (<0.4). D'autres inhibiteurs d'absorption semblent jouer un rôle important. A cet égard, les effets relatifs des phytates, polyphénols et fibres sur la bioaccessibilité du fer ont été évalués par une approche mécanistique utilisant des enzymes exogènes. Dans les farines utilisées pour la préparation de l'*injera*, la déphytinisation suivie de l'hydrolyse des fibres et/ou l'oxydation des polyphénols permet une augmentation de la bioaccessibilité du fer plus importante que la seule déphytinisation. La réponse aux traitements enzymatiques ainsi que l'importance relative des différents inhibiteurs d'absorption étaient dépendantes de la matrice alimentaire. L'optimisation des procédés traditionnels de transformation en vue de favoriser la dégradation des phytates, des polyphénols chélateurs de fer et des fibres pourrait améliorer significativement la biodisponibilité du fer dans les *injeras*. Des interventions visant à promouvoir les bonnes pratiques d'alimentation complémentaires recommandées par l'OMS sont nécessaires dans les régions enquêtées.

Food-based strategies to enhance iron and zinc bioavailability of complementary foods consumed by young children in Ethiopia

Abstract

Stunting and micronutrient deficiencies are widespread in developing countries like Ethiopia and reach their peak during the period of complementary feeding. The adequacy of complementary feeding practices of young children in north Wollo, northern Ethiopia was evaluated in 76 households using two 24h recalls. Several feeding practices were not in accordance with WHO/PAHO recommendations. The most frequently consumed foods were legume-based stews, bread, and *injera*- a fermented cereal-based pancake. Traditional processing of these foods was observed in-field. Different cereal blends were used in *injera* preparation and this influenced the fermentation kinetics which in turn affected phytic acid hydrolysis. Even when phytic acid degradation was >95%, iron bioaccessibility and algorithm predicted absorption were not improved, despite phytate:Fe molar ratios were ideal (<0.4). This suggested an important role of other absorption inhibitors. In this regard, the relative effect of phytate, polyphenols, and fibers on iron bioaccessibility was evaluated by making use of a mechanistic approach that involved the application of exogenous enzymes. In *injera* flours, dephytinization followed by hydrolysis of fibers and, or oxidation of polyphenols, resulted in higher iron bioaccessibility than dephytinization alone. The relative importance of mineral absorption inhibitors as well as the response to enzymatic treatments was dependent on the food matrix. If possible, optimization of household food processing for greater decrease in phytate, iron-binding polyphenols and fibers may be needed to significantly improve iron bioavailability in *injeras*. Interventions promoting the WHO guiding principles for complementary feeding practices and behaviors are recommended.

Keywords: Phytate, polyphenols, fibers, altitude, micronutrients, bioaccessibility

Discipline: Nutrition et Sciences des Aliments

Mots-clés: Phytate, polyphénols, fibres, altitude, micronutriments, bioaccessibilité

Intitulé du laboratoire : UMR 204, NUTRIPASS, IRD (Institut de Recherche pour le Dévelopement) Montpellier